Question 4

Type II product.
(1) We have final product regulations for placing the cord blood into our open search system:

Sterility tests are performed with (i) the Oxoid Signal Blood Culture System (Oxoid, Cambridge, UK) using 0·5 ml of the final product mixed with DMSO, and with BacT/ALERT, (ii) BPA and (iii) BPN culture bottles (bioMérieux, Lyon, France) using 10 ml for each from the removed RBC fraction.

The TNC using an automated counter, the CD34 positive cell count using 7AAD/CD45/CD34 staining and the CFU-GM count (MethoCult H4034; Stemcell Technologies, BC, Canada) are posted for each cord blood unit.

For identification, the ABO and Rh typing, forward blood typing only, and HLA allele typing (Luminex 200; Luminex, Austin, TX, USA) are performed.

(2) Before releasing the unit, we carry out the following tests using a test sample:

(i) TNC with an automated counter, (ii) total cell viability with ethidium bromide and acridine orange (EB/AO) fluorescence counting, (iii) CD34+ cell counting with 7AAD/CD34/CD45 staining, and (iv) stem cell culture assay using MethoCult H4034.

The viability of the total nucleated cells should be 70% or more. The recovery rate of CD34 positive cells compared with before freezing should be 60% or more.

Question 5a

Type II product. The cell count recovery should be 80% or more. The viability of the total nucleated cells should be 70% or more. The recovery rate of CD34 positive cells compared with before freezing should be 60% or more. The recovery rate of the total CFU compared with the total CFU before freezing should be 50% or more, though the CFU assay result is used only for quality control, and not for the release decision.

The recovery tests for 10 057 units had mean ± SD of 99·8 ± 7·9% for the TNC, 82·2 ± 6·0% for the viability, 79·8 ± 19·5% for the CD34 positive cells and 76·0 ± 23·0% for the CFU.

Question 5b

Type II product. When the viability or the recovery rate of CD34 or CFU does not meet the criteria, we do the test again with the segment sample attached to the cryobag.

In the cases when the TNC recovery and cell viability is satisfactory but one of the other criteria is not met, and the prefreezing test result looks to have a possible overcounting, we suggest to use the cord blood unit. Otherwise, we search for other units and suggest to the transplantation centre to change the donor cord blood unit.

Question 6a

Type II product. (As above for Question 5a), the viability of the TNC should be 70% or more. The recovery rate of CD34 positive cells compared with before freezing should be 60% or more. The recovery rate of the total CFU compared with the total CFU before freezing should be 50% or more, though the CFU assay result is used only for quality control, and not for the release decision.

Question 6b

Type II product. Not applicable.

Reference

1 Keeney M, Chin-Yee I, Weir K, *et al.*: Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. International Society of Hematotherapy and Graft Engineering. *Cytometry* 1998; 34:61–70

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Question 1

National Health Service Blood and Transplant (NHSBT) is a national special health authority for England and North Wales in the UK, taking responsibility for the supply of blood, tissues, blood-related stem cells and their products and organs for the National Health Service. The information provided will cover the cellular therapies facilitated by the NHSBT Stem Cell and Immunotherapy (SCI) Department which encompasses (i) the NHS Cord Blood Bank with its six cord blood collection sites at Watford General, Barnet, Northwick Park, Luton and Dunstable, St George's, and University College NHS Hospitals which are in or close to London and has a collection hub at Colindale in London and a processing and banking facility in Bristol, and (ii) seven SCI laboratories which receive, process, manufacture, manipulate, bank and distribute haemopoietic stem cells and related cellular products for therapeutic use for approximately 50% of haemopoietic stem cell transplants in the UK. The latter laboratories are based in Birmingham, Bristol, Leeds, Liverpool (previously Manchester), Oxford, Sheffield and Southampton and are an integral part of NHSBT. Each provides a regional hub for the routine provision of such cellular products for transplantation. For example, Birmingham SCI serves five transplant units and

a population of ca. 2 million patients in the West Midlands. As well as the routine transplant services, the SCI laboratories have established themselves as a link between R&D programmes within NHSBT and academic centres of excellence within Universities and NHS teaching hospitals for the provision of cell therapeutics. The products listed are a snapshot of recent or ongoing clinical applications involving specific NHSBT SCI laboratories or departments.

Type I products include:

(1) chimeric T cells for treating haemopoietic and other cancers.
(2) dendritic cell therapies for treating cancers [1].

Type II products include:

(1) harvests of unrelated and related sibling allogeneic umbilical cord blood units (HPC, Cord Blood) are collected, tested, as necessary processed for volume reduction and removal of plasma and red cells and cryopreserved at <−150°C prior to selection for transplantation [2].
(2) related and unrelated allogeneic bone marrow harvests (HPC, Marrow) are processed, red cell depleted and cryopreserved at <−150°C and/or reinfused into patients [3, 4].
(3) autologous or related and unrelated allogeneic peripheral blood harvests mobilized with G-CSF with or without Plerixafor (HPC, Apheresis) are processed and cryopreserved at <−150°C and/or reinfused into patients [3, 4].
(4) allogeneic mobilized peripheral blood harvests (HPC, Apheresis) may also be T/B (CD3/CD19) cell depleted [4].
(5) allogeneic or autologous mobilized peripheral blood (HPC, Apheresis) harvests may also be CD34/CD133 selected for the treatment of haematological, cardiovascular or liver diseases [4].
(6) allogeneic donor therapeutic T cells (T-CT) and CMV-specific T cells may be selected using magnetic bead technology and reinfused into patients to treat or preempt CMV infections [5].
(7) CD25 depletion of peripheral blood cells for treating patients with cancers [6].

Question 2

Background to compliance with regulatory requirements within NHSBT SCI Departments. The regulatory requirements for cellular therapies have increased exponentially in the past few years in England, where the regulation of Type I and II products is split between different competent authorities. Routine stem cell and immunotherapy processing and minimal manipulation of cell therapy products (Type II products) as carried out in NHSBT SCI Departments are regulated by the Human Tissue Authority (HTA) and, as a legal requirement, each NHSBT SCI laboratory is now licensed by the HTA. Additionally, new scientific progress in cellular and molecular biotechnology has led to the development of advanced therapies (AT), such as gene therapy, somatic cell therapy and tissue engineering. New cellular therapies or advanced therapy medicinal products (ATMPs) offer opportunities for groundbreaking new treatments for many diseases/injuries and depend on more extensive manipulation of cell therapy products, here referred to as Type I products. Under the current EC framework, ATMPs are defined as products which have the properties to treat or prevent a disease or which may be administered to restore, correct or modify physiological functions via a pharmacological, immunological or metabolic action. Their evaluation requires specific expertise beyond that needed for routine NHSBT SCI service provision and conventional medicines, and the legislation is designed to ensure that this expertise is available to generate these products. This has more recently led to the requirement for adherence to regulations for ATMPs via the Medicines and Health Products Regulatory Agency (MHRA) licensing in England. The existence of different competent authorities regulating cell therapy products for human application has led to the development of a UK Stem Cell Toolkit for therapeutic cell manufacture and research (http://www.sc-toolkit.ac.uk/home.cfm).

HTA licensing of cellular products. The NHSBT SCI departments are licensed by the Human Tissue Authority (HTA) as the competent authority in England for handling human cells intended for human application. This is a legal obligation that has been enacted into criminal law in England. The principal HTA guidelines based on EU Directives and legislation and covering the activities of the NHSBT SCI Departments are listed.[1] Under HTA Directions 001/2006, donor selection criteria are based on an analysis of risks related to the specific cell or tissue application and where risks are identified for living donors by, for example, medical and behavioural history review, biological testing and physical examination. Donors are excluded based on a risk assessment where there is evidence of risks for transmissible diseases and where donor travel and exposure and the prevalence of local infectious disease are considered. The HTA provides regulatory alerts and advice on donor selection to licensed establishments handling human cells intended for human application (http://www.hta.gov.uk/licensingandinspections/regulatoryalerts.cfm). One recent example relates to donors visiting West Nile Fever endemic areas where, in relation to this, the HTA endorsed the 'Change Notification by the Joint UKBTS/NIBSC Professional Advisory Committee (JPAC) to the UK Blood

Transfusion and Tissue Transplantation Services Donor Selection Guidelines'.

Regulatory requirements for advanced therapy medicinal products (ATMPs) – Type I products. Where a cell therapy product is substantially manipulated (e.g. by gene therapy, tissue engineering or for somatic cell therapy), it is classed as an advanced therapy medicinal product (ATMP) for human use and categorized as a biological medicinal product in Annex I to Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use and regulations and Directives listed.[2] In addition, where appropriate and taking into account the specificities of ATMPs, additional requirements have been set out as follows:

(1) specific requirements for gene therapy medicinal products;
(2) specific requirements for somatic therapy medicinal products;
(3) specific requirements for tissue engineered products;
(4) specific requirements for advanced therapy medicinal products containing devices.

Legally in England, the MHRA is the competent authority for licensing establishments for the manufacture of investigational ATMPs or Type I cellular therapy products currently produced in NHSBT. It should be noted that ATMPs are regulated by the HTA in terms of donation, procurement and testing of human tissues and cells, while the MHRA regulate the manufacture, storage and distribution of the Type I CT product. While all NHSBT facilities are HTA licensed, the first MHRA licence for ATMPs involving an NHSBT SCI Department was granted in 2011 to the NHSBT Advanced Therapy Unit (ATU) in Liverpool, England.

FACT-Netcord, JACIE and FDA accreditation for Type II CT products. For routine NHSBT SCI minimally manipulated processing of cells for transplantaion (Type II CT products), all NHSBT SCI Departments are voluntarily FACT-Netcord (for unrelated cord blood banking) or JACIE accredited. These require adherence to standards, which must also be compliant with individual country's legal requirements.[3] The World Marrow Donor Association (WMDA) first developed standards specifically for unrelated donors. These WMDA standards can be found on the WMDA website (http://www.worldmarrow.org).[4] Unrelated cord blood donations exported by NHSBT to the USA also fall under the U.S. Department of Health and Human Services Food and Drug Administration (FDA) requirements. In October 2009, Center for Biologics Evaluation and Research produced final guidance for cord blood bank applications for licensure of minimally manipulated, unrelated allogeneic placental/umbilical cord blood, for specified indications entitled 'Minimally Manipulated, Allogeneic Placental/Umbilical Blood Intended for Hematopoietic Reconstitution for Specified Indications' (http://www.fda.gov/cber/guidelines.htm).[5] At this time, there are no public cord blood banks licensed by the FDA, and cord blood donations can only be used for transplantation in the USA through an Investigational New Drug (IND) exemption granted by the FDA to USA transplant centres and the NMDP. The NHS Cord Blood Bank has been approved by the NMDP to export via their IND exemption.

Other guidelines. Other guidelines covering NHSBT SCI Departments are (i) Guidance on the Microbiological Safety of Human Organs, Tissues and Cells used in Transplantation - advisory committee on the microbiology safety of blood and tissues for transplantation (SaBTO - Department of Health, England), (ii) Guidelines for the Blood Transfusion and Tissue Transplantation Services in the UK (Red Book ESD/PTI/001) and its associated supplements containing donor selection guidelines (http://www.transfusionguidelines.org.uk), and (iii) UK Blood Transfusion Services and NIBSC Tissue Donor Selection Guidelines Cord Blood.

Donors Release 08–21 June 2011 [UKBTS and NIBSC TDSG-CB 2003 (1 June 2007) Release 08 (21 June 2011)] produced by the Joint UKBTS/NIBSC Professional Advisory Committee (JPAC) of the UKBTS and NIBSC.

Donor selection criteria. For both Type I and II Products manufactured in NHSBT SCI Departments, the following apply as minimal requirements for donors:

Health history screening.
As detailed in the HTA (Quality and Safety for Human Application) Regulations 2007, HTA Directions, extensively in the UKBTS and NIBSC TDSG-CB 2003 (1 June 2007) Release 08 (21st June 2011) and in the SaBTO guidance, selection criteria for donors are based on an analysis of risks related to the specific cell or tissue application. All cell therapy products are tested for bacterial contamination from the environment during manufacture and storage.

Allogeneic and autologous donors are selected on the basis of their health and medical history and their physical status, with additional information obtained from healthcare specialists where possible. An interview is provided by a trained and qualified healthcare professional with the donor to assess medical, behavioural and travel history and with a donor questionnaire. This is then documented by the NHSBT SCI Department.

Exclusion criteria will differ for autologous and allogeneic donors depending on their health status and treatment needs. For allogeneic living donors (see specified guidelines for full details), exclusion or assessment

criteria include history of disease of unknown aetiology, history of malignant disease, risk of transmission of spongiform encephalopathies and other transmissible diseases, travel history outside the UK, systemic infection which is not controlled at the time of donation, any treatment received before donation, any history of blood, blood components, blood products, tissue or organ receipt, previous or current immunosuppression, plasma dilution, infections such as HIV, acute and chronic HBV, HCV, HTLV I and II or children aged less than 18 months born to mothers with HIV, HBV, HBC or HTLV, a history of chronic, systemic autoimmune disease which could be detrimental to the cells/tissues to be retrieved, exposure to substances that may endanger health, recent history of vaccination with a live attenuated virus, and transplantation with xenografts. Other specific exclusion criteria may also be added depending the cells being donated and could, for example, in some instances include pregnancy, breastfeeding and the potential for transmission of inherited conditions.

Infectious disease testing.
For donors of autologous cells that are stored or cultured, the same minimum biological testing requirements apply as for allogeneic living donors, although positive test results may not prevent storage, processing or re-implantation of the cell therapy product.

The following biological tests are carried out as a minimum requirement for the NHSBT SCI Department in accordance with HTA Directions and Guidance on the Microbiological Safety of Blood, Tissues and Organs (SaBTO) (http://www.dh.gov.uk):

(1) for autologous and allogeneic Type I and II products (except for cord blood) within 30 days prior to donation, HIV 1 and 2 using anti-HIV-1,2 testing, HTLV-I and II antibody testing, Hepatitis B virus (HBV) using HBsAg and anti-HBc testing, Hepatitis C virus (HCV) using anti-HCV antibody testing and Syphilis by serology using CE-marked kits according to HTA Directions;
(2) for both the above products, it is the NHSBT SCI Department's policy that testing is repeated on the day of collection or up to 7 days post-donation and that additionally NAT testing for HIV RNA, HBV DNA and HCV RNA is also performed on this sample;
(3) for cord blood donations, the above biological tests listed in (i) are carried out as a minimum requirement on the donor mother's blood sample on the day of birth or up to 7 days after birth, together with nucleic acid testing (NAT) for HIV and HCV (RNA) and HBV (DNA). Prior to release of a cord blood unit for transplantation, samples stored from the cord blood donation are also tested for the infectious disease markers using both the serological testing described above and NAT (HIV and HCV RNA; HBV and CMV DNA);
(4) additional infectious disease testing may be done depending on donor history or the donated cells or the cell therapy application, including malaria, toxoplasma, CMV, EBV, *Trypanosome cruzi*. For cord blood donations, additional tests that are routinely carried out on the day of birth or within 7 days of birth include CMV IgM and IgG antibody testing, with *Trypanosome cruzi* and malaria tested if indicated by maternal travel history.

Identity checking.
For each living donor, donation records contain as a minimum the age, sex, donor identification including first name, family name and date of birth. Where a mother and child are involved in the donation, the name and date of birth of the mother and the name (if known) and date of birth of the child are included. For unrelated donors of haemopoietic progenitor cells and related products used in the routine haemopoietic stem cell transplant setting and where these are collected by a bone marrow donor registry, the donor's name is not made available to the NHSBT SCI Departments, but this is replaced by an alternative identifier that is a unique donor number.

In addition, the NHSBT SCI Departments use the ISBT-128 bar coding system with unique bar code numbers for each donor and their donations to ensure traceability. Thus, for donations from within the UK, all donations are tracked using ISBT128 bar coded identifier labels, together with at least three forms of donor identity viz. date of birth, name or unique donor identifier, and NHS number.

At the time of procurement, the primary cell container/cryobag contains labels specifying the donation identification or code and the type of cells procured, together with the date and if possible the time of donation. For autologous donations, the label also states 'for autologous use only'.

Question 3
The assays performed to characterize the quality of the initial collection for both Type I and II products overlap considerably as they are primarily focussed on safety of the patient and the potency of the collection to meet the desired therapeutic benefit. As such the following relates to both Type I and II products derived from similar sources but, although general principles apply, specific examples are biased heavily towards HPC Type II products which are our main cell therapy products (see also Questions 4–6).

Identity checking. On receipt of a therapeutic cell donation, the first critical check is to confirm the identity of the donation and the intended recipient. This is done by

checking the bag label and accompanying paperwork against donor and recipient details as presented on the referral form provided by and signed by the transplant physician. Any discrepancies or anomalies are referred back to the transplant centre. As described above, the primary identification used for HPC donations, our main cell therapy products, is a unique ISBT 128 donation number assigned at the time of donation. For autologous and related donors, the donor name, date of birth and hospital number are also used, whereas for unrelated donors, where the donor's name should not be disclosed to the recipient, the donor number assigned by the registry can be used as an alternative to the donor name.

The assay most commonly used to confirm identity of the HPC donation is ABO Rh blood grouping. This test is required for JACIE accreditation. However, the test is limited as an identity check because some blood groups occur at high frequency in all populations, and where autologous donors are heavily transfused with compatible blood of a different group, anomalous results can occur and identity of a donation cannot be confirmed. Additionally, CE-marked blood grouping methods may not be validated or approved by the manufacturers for use with HPC, Apheresis, HPC, Cord Blood or HPC, Marrow products. As a consequence, ABO Rh grouping to confirm identity is performed on peripheral blood samples taken from the donor at the same time as the donation itself.

The short tandem repeat (STR) assay provides a more robust test of identify and is used to confirm identity of unrelated donor HPC, Cord Blood donations held by the NHS Cord Blood Bank. Here, the sample used to determine the HLA data for matching is compared with a line segment taken from the cord blood donation itself. If the two samples have identical STR patterns over the 16 loci tested, identity is confirmed. Alternatively, the HLA type can be repeated on the line segment taken from the bag and identity confirmed if the HLA type is identical to that previously recorded for the donation. This test is not used routinely to confirm identity of other therapeutic collections.

Initial cell quantification and purity. It is important to establish the quantities of the active cell populations in initial cell collections as this provides a baseline for assessing recovery of cells after processing and predicts cell doses likely to be achieved in the final product at release to the clinic. Where initial cell counts are poor, it may be necessary for further collections to be requested. Purity is not usually considered relevant in initial collections but is essential in assessing enriched products prior to release for use in the recipient. Donations are assessed on receipt in the NHSBT SCI laboratory using a sample taken from the tubing line attached to the donation. A validated procedure is required to take a 0·5–1 ml sample to ensure it is representative of the donation and that there is no exposure to the environment or risk of bacterial or fungal contamination. The sample is tested initially using a haematology analyser to determine the WBC count, haematocrit (HCT) and platelet count. Some haematology analysers identify all nucleated cells as white blood cells, and where nucleated Red Blood Cells (nRBC) are present, such as in HPC, Cord Blood and HPC, Marrow donations, this may be problematic. The quantity of active cells expressing specific markers, such as CD45, CD34, CD133, CD3, CD19, CD25 or CD56, is determined by flow cytometry. For HPC donations, a single platform method based on the ISHAGE gating strategy is used where CD34+ HPC are defined by their forward and side scatter characteristics, high CD34 and low CD45 expression. The CD45 cell count should be similar to the WBC result from the haematology analyser although it may be necessary to subtract nRBC that may be present in HPC, Marrow or HPC, Cord Blood donations.

The RBC content is also important where an ABO incompatibility is identified between the recipient and the donor, or where there is the possibility of RBC overload in the recipient. The volume of RBC in the graft is determined from the haematocrit (HCT) which is calculated from the mean cell volume (MCV) and the RBC count by the haematology analyser. The relatively high WBC concentration in HPC, Apheresis collections is sufficient to elevate the MCV and RBC count reported by some haematology analysers causing the RBC content to be significantly overestimated. The recipient ABO antibody titre is also determined for ABO incompatible grafts to assist in assessing the transfusion risk should RBC not be reduced to a safe level. The removal of RBC is significantly affected by the presence of storage artefacts, such as RBC crenation. To reduce this, incompatible HPC, marrow grafts should be shipped and stored refrigerated to preserve RBC structure [3].

Potency and viability. For haemopoietic stem cell transplants, the potency represents the ability of the HPC donation to engraft after transplantation. In most instances, this is directly related to the HPC content and cell viability. For all HPC donations, CD34+ and/or CD133+ cells are determined by single platform flow cytometry using the ISHAGE gating strategy with the results expressed as a dose per kg recipient weight. For a durable engraftment, a viable CD34+ cell dose of $>2 \times 10^6$ per kg recipient weight is recommended for HPC, Apheresis collections. The minimum CD34+ cell dose required to achieve engraftment for HPC, Marrow donations is not known due in part to the difficulties in reliably assessing CD34+ cells in bone marrow. Although CD34+ cells are not useful in assessing potency of HPC, Marrow, counting CD34 bright cells by flow cytometry

before and after processing can be helpful in assessing recovery of CD34+ cells and ensuring potency of the graft is maintained after volume or RBC reduction. Other cell markers, such as CD3 or CD19, are also determined by flow cytometry as and when required to assess risk of GvHD or EBV-induced lymphoproliferative disease in recipients. Recommendations for CD34 content of HPC, Cord Blood are described in [2].

Viability is determined using a vital dye, 7-aminoactinomycin-D (7-AAD), incorporated into the flow cytometry assay. The dye is able to penetrate the membrane of non-viable cells specifically staining the DNA which is then visible in the flow cytometry assay. A fresh HPC donation would be expected to have a CD34+ cell viability exceeding 95%. In our facility, CD34 viability below 95% would be investigated further, and in some cases, potency assessed using a colony forming unit (CFU) assay. The CFU assay measures the ability of the cells to proliferate and differentiate.

Sterility. In our facilities, sterility of donations is assessed using the BacT/Alert system. Ideally, 5% of the HPC collection should be used for sterility testing. Where cell doses are suboptimal, this may be reduced to ensure potency of the graft is preserved. In exceptional circumstances, where cell doses are very poor grafts may be released without sterility testing under a medical concession, depending on patient need. Mycoplasma testing if this is done is as described below.

Question 4

Type I products. A major quality release assay for Type I products is that of apoptosis and viability. This is more often done by flow cytometry with both Annexin V and 7-AAD used in combination, allowing detection of early apoptotic cells. This distinction is particularly important in cultured cells as 7-AAD alone can give a falsely low indication of cell viability being reliant on a loss of cell membrane integrity.

Type I products are often cultured for varying lengths of time and this adds to the risk of bacterial contamination. The presence of pyrogens such as endotoxins from the walls of Gram negative bacteria can result in severe patient reactions. Where possible, a test for pyrogens such as the limulus test is recommended. However, the results may not always be available prior to issue. There are three methods for the limulus test: the gel clot test based on gel formation, the turbimetric test based on development of turbidity after cleavage of an endogenous substrate and the chromogenic technique based on the development of colour after cleavage of a synthetic peptide–chromogen complex. In some cases, a Gram stain can be done to look for the presence of bacteria. However, this type of test should be done with caution as the level of bacteria may be below required detection limits especially if the contamination is at a very early stage.

A second contaminant that may develop in cultures is that of mycoplasma which can be present with no obvious change in the host culture, even when the concentration of mycoplasma exceeds that of the host cells by 10–100-fold. The quickest pharmacopoeia method for detecting mycoplasma contamination is that of co-culturing indicator cells such as Vero cells with supernatant from the test culture. After 3–5 days, the Vero cells are harvested, cultured on cover slips then stained with Hoechst stain and examined under fluorescence microscopy. Quicker and potentially more reliable PCR assays are becoming available but at present are not fully recognized as a pharmacopoeia technique.

Type II products. The general principles when developing quality control and release criteria are centred around potency and safety. The product should maintain the potency to achieve the therapeutic benefit intended and be safe for the recipient to receive. For example, HPCs defined as CD34+, CD133+ cells must be at a dose sufficient to achieve a durable engraftment. Equally, the product must be safe to infuse where risks of disease transmission and side effects, such as GvHD and ABO-mediated transfusion reactions, are minimized. Testing requirements for Type II products are outlined below.

Generic release criteria applied to all Type II HPC cellular products.

In all cases, HPC donations must achieve the following:

(1) Contain sufficient viable active cells as requested by the transplant centre.
(2) Sterile as determined minimally using the BacTAlert or similar system.
(3) Negative for all relevant virology markers and syphilis.
(4) ABO blood group compatible or contain <10 ml of incompatible RBC.

Additional release criteria for CD34 enriched or CD3/CD19 depleted allografts.

(1) The CD3 dose should be reduced sufficiently to minimize the risk of GvHD in the recipient. The transplant centre will often state a CD3 dose on the referral/prescription that is used as part of the release criteria. Where the CD3 dose is below the specified dose additional donor CD3+ T cells may be added to achieve the required dose.
(2) Purity should be >90% CD34+ cells. Contaminating cells in CD34 enriched products are usually B

lymphocytes and monocytes. If purity is below 90%, there is an increased risk of EBV-induced lymphoproliferative disease, and the donation is issued based on clinical need as a medical concession. Where CD3/CD19 cells are depleted, purity is not specified in the release criteria.

(3) The recovery of CD34+ cells after processing should be sufficient to maintain potency of the graft. Where CD34+ cell recovery is poor, the processing records would be reviewed to establish the likely root cause and where the transplant dose is below that requested by the transplant centre re-processing to recover more cells may be attempted.

Additional release criteria for cryopreserved HPC grafts.

Cryopreservation is a critical process where further assessment is restricted by sample availability. Line segments and cryovial samples are prepared and stored for each HPC bag although these are not considered representative of the final clinical product due to the differing thermodynamics of the tubing and cryovials compared with the clinical grade cryostorage bag. As such, poor viability identified in the line segment or cryovials may not necessarily identify poor viability in the HPC product itself, although this is usually inferred. To reflect these limitations, release criteria are based primarily on pre-cryopreservation data and satisfactory processing. JACIE recommends that viability post-cryopreservation is assessed, although for well-established validated procedures it may not be necessary to test every donation. Bags are not necessarily discarded on the basis of these results unless a reason for poor viability can be established. Where the procedure is not well established or extensively validated, every donation is tested prior to release to confirm viability post-cryopreservation. There are procedures established to define actions in the event of a significant warming event during storage. These actions include viability assessment by 7-AAD dye exclusion and CFU assessment of some or all affected donations.

Additional release criteria for cryopreserved HPC, cord blood.

HPC, Cord Blood donations stored by the NHS Cord Blood Bank are rigorously tested prior to release for transplantation. This is required as there is limited opportunity to assess the donor's suitability for use as a cord blood donor. The donation may be stored for many years before it is used for a transplant. To reduce the risk of disease transmission from donor to recipient the donor mother, within 7 days of donating HPC, Cord Blood is required to complete health and lifestyle questionnaires and is tested for markers of blood borne diseases mandated at the time of donation. Samples are stored from the mother and the HPC, Cord Blood donation for future use if testing requirements change while the donation is in storage. To further reduce the risk of disease transmission HPC, cord blood donors are screened for haemoglobinopathies. To reduce risks of a transfusion reaction, the donations are also tested to confirm the ABO Rh blood group. To allow matching with potential transplant recipients, the donations are HLA typed to intermediate resolution for HLA-A, B and C loci and to high resolution for DRB1. In addition, approximately 12 weeks after donating the mother is contacted to confirm the health of the infant donor.

When an HPC, Cord Blood donation is identified for a potential recipient, further tests are required prior to release to the transplant centre as follows:

(1) There is a complete review of all paper records including the processing records and freezing curve for the HPC, Cord Blood donation.
(2) A line segment taken from the bag is thawed and tested to confirm viability by dye exclusion with 7-AAD and CFU assays.
(3) Remaining blood from the line segment is used to confirm identity of the HPC, cord blood donation using STR analysis.
(4) The donor's general practitioner (GP) is contacted to confirm the donors continuing health and suitability for transplantation of their HPC, Cord Blood donation.
(5) The HLA type is confirmed and/or tested to higher resolution as required by the transplant centre.
(6) The HLA type of the donor's mother's is determined to confirm the family relationship with the HPC, Cord Blood donors.
(7) Additional mandatory markers for blood borne diseases not required at the time of donation.
(8) Stored samples from the HPC, Cord Blood donation are tested for all mandatory markers of blood borne diseases.

Question 5a

Type I products. For Type I cell therapy products, the release criteria can be multifold and are dependent on the type of cell therapy. For investigational medicinal products, the release criteria will have been specified in the clinical trials authorization (CTA) submission to the MHRA. The CTA will specify the target dose of cells to be given, and this will increase with each cohort in a dose escalation study. Once the cells have been harvested, they will be tested for viability and expression of relevant antigen. For T cell cultures, a viability of >50% is often set with both Annexin V and 7-AAD dual staining by flow cytometry.

Where cell expansion is involved, measurement of the cell number at an early stage in the cell culture is a good indicator that the cells are healthy and expanding well. For our T cell cultures, we looked at the expansion between days 5 and 8, during which time we would expect the cells to have expanded by at least 2·5-fold. For cell therapies involving genetic modification, it is important to know that at least a certain proportion of the cell population is expressing the transgene. Due to the nature and variability in obtaining high levels of transduction, a low acceptance level is set. For example, in the chimeric receptor trial, the acceptance level was set at more than 20% CD3 positive cells expressing the transgene. Along with transgene expression, it is also important that the therapeutic cells are capable of functioning; thus, for the chimeric receptor T cells, the antigen-specific upregulation of CD25 was measured with a cut off of >50%.

One of the most crucial release criteria, however, is that of sterility. With cultured products, it is not possible to obtain sterility results on the final day of harvest when the cells are administered immediately. In this case, a Gram stain negative result is acceptable with the recipient often given broad spectrum antibiotics. A bacteriology test such as BacT Alert is also undertaken but the result will take a few days to be known. Alongside a negative Gram stain result, the cells must also be negative for mycoplasma testing. In addition to the above, the donor cells will have been tested for markers of transmissible diseases which for allogeneic donors must be negative. Autologous marker positive cells can be returned to the donor although in certain trials marker positivity is often an exclusion criterion for entry into the trial.

Type II products. For Type II non-engineered cells where a selection process has been undertaken, there are different criteria dependent on whether the source material is apheresis derived or from bone marrow. Where the transplant physician has specified a cell dose on the prescription referral documentation, then this would form part of the release criteria. Where the dose is not specified as a general rule, for CD34 selection from an apheresis collection, the aim is for a purity of >90% CD34+ cells with >50% CD34 recovery and a > 4 log reduction in CD3 count. For HPC products undergoing a CD3 depletion, the target is to achieve >4 log reduction. Where a combination of CD3 and CD19 depletions is undertaken, the aim is to reduce CD3 by >3 logs and CD19 by >2 logs as a minimum. For marrow collections, volume reduction using a Cobe Spectra should result in a volume reduction to 20% of the start volume with >50% CD34+ cell recovery. For CD34 selection on a marrow collection, the collection volume is first reduced, then the recovery criteria are the same as that for apheresis collections.

Question 5b

Type I products. For Type I products used for clinical trials, the results are discussed with the Sponsor of the trial for a decision on how to proceed. In the majority of cases, the cells would be rejected as they would be outside the specification agreed within the clinical trials authorization. However, if the clinician incharge of the patient wished to transplant the cells in the best interests of the patient, then the cells would be issued under a concession following a written request. In these circumstances, the patient would then be excluded from the trial.

Type II products. For Type II products, the results would be discussed with the transplant consultant and cells issued under concession by the NHSBT SCI Department if the transplant unit makes the decision to proceed.

Question 6a

Type I products. For Type I assays, it is more often not possible to undertake potency testing of the final product due to the necessity to transplant the cells within a short time frame from culture harvest.

Type II products. Specific potency assays are not performed for HPC transplants or CD34/CD133 selected HPCs as these are well established cell therapies, and the gold standard for a haemopoietic stem cell potency is long-term engraftment after transplantation in patients. Surrogate assays are described below. For other products such as CMV-specific T cells, the purity of such cells is assessed by using CMV-specific tetramers and flow cytometry [5].

Question 6b

Type I products. For type I products, the product validation phase can involve analysis for the presence of activation markers such as CD25 which is upregulated following interaction of antigen with specific receptors on T cells. This indicates that the cells are responsive to the target antigen and thus have the potential to mount a response upon transplant into the patient. This responsiveness can also be tested by the use of cytotoxicity assays in which target cells that express the nominal antigen can be co-cultured with the cell therapy product and cell killing of the target cell determined either by a chromogenic assay or dye exclusion or flow cytometry. Other cell therapy products such as dendritic cells can be tested for their ability to stimulate reactive cell responses in immune cells such as T and B cells. These can be measured by the production of cytokines measured by ELISA or ELISpot assay. Where appropriate, such

analyses as sensecence testing, karyoptye, chromosomal integration, toxicity testing, etc., may be required by the regulatory authorities as part of the CTA for Type I products especially where cells are cultured or genetically modified.

Type II products. Potency is in part based on the number/dose of active cells and their viability as measured by dye exclusion using 7-AAD. CMV-specific T cell analyses are described (see [5] and references therein). For HPC collections, CFU assays are still a useful measure of the ability of progenitor cells to proliferate and differentiate and hence provide an additional viability measurement of progenitor cells within the graft. In our establishment, a durable engraftment in the patient should be achieved with defined minimum cell doses. For autologous HPC, Apheresis collections, the minimum dose required to achieve a durable engraftment is 1×10^6 per kg CD34+ cells. For allogeneic grafts, a minimum dose around 2×10^6 per kg is usually considered sufficient although higher doses may be advised if an HLA mismatched donor is used. There is no minimum CD34+ cell dose required to achieve engraftment for HPC, Marrow donations but as a rule, we collect 15–20 ml of bone marrow per kg recipient weight. Viability of CD34+ cells by dye exclusion should exceed 95% in fresh grafts and 80% in cryopreserved grafts. It is a requirement for JACIE and FACT-Netcord accreditation to monitor engraftment in transplant patients. This information provides the most accurate measure of potency and overall performance of the transplant programme.

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End Notes

Type I products: Engineered cells or tissues and Type II products: Non-engineered cells or tissues.

1. (i) Human Tissue Act 2004 and the Human Tissue Act 2004 (ethical approval, exceptions from licensing and supply of information about transplantation) Regulations 2006, (ii) HTA Directions 003/2010, (iii) consent in accordance with the Human Tissue Act 2004, the Human Tissues (Quality and Safety for Human Application) Regulations 2007 and the HTA Codes of Practice (http://www.hta.gov.uk) including HTA Code of Conduct: Consent and HTA Code of Practice: Donation of organs, tissues and cells, (iv) EU Directive 2004/23/EC of the European Parliament and Council on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells, (v) Directive 2006/17/EC implementing Directive 2004/23/EC of the European Parliament and Council as regards certain technical requirements for the donation, procurement and testing of human tissues and cells, (vi) Directive 2006/86/EC implementing Directive 2004/23/EC of the European Parliament and of the Council as regards traceability requirements, notification of serious adverse reactions and events and certain technical requirements for the coding, processing, preservation, storage and distribution of human tissues and cells.
2. (i) Directive 2001/83/EC: rules which are applicable to the various types of combinations of medicinal products and devices; (ii) Directive 2004/23/EC of the European Parliament and of the Council: standards of quality and safety for donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells for the manufacturing and distribution of ATMPs; (iii) All biotechnology medicinal products in the UK are now regulated at European Community level under the flagship of the European Medicines Agency (EMA), as established by Regulation (EC) No 726/2004 of the European Parliament, which has set out a centralized authorization procedure, evaluating the quality, safety and efficacy of each produc; (iv) Advanced therapies in Europe are regulated by Regulation (EC) No 1394/2007 applicable from 30th December 2008, which defines ATMPs and how ATMPs are authorized, supervised, and monitored to ensure quality, safety and efficacy (www.ema.europa.eu; advancedtherapies@ema.europa.eu; http://www.ema.europa.eu/SME/SME application.htm; http://www.ema.europa.eu/htms/human/advanced_therapies/certification.htm). This provides precise legal definitions, as well as the specific technical requirements for quality, preclinical and clinical data needed in order to demonstrate the quality, safety and efficacy of the product, ensures free movement of ATMPs within Europe, facilitates access to the EU market and fosters the competitiveness of European companies, guaranteeing the highest level of health protection for patients; (v) Commission Directive 2009/120/EC amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal

products for human use as regards ATMPs was formally adopted by the Commission on 14th September 2009.

3. Standards developed by FACT (the Foundation for Accreditation of Cell Therapy) in the USA and by JACIE (the Joint Accreditation Committee of ISCT (Europe) and the EBMT) or by Netcord (FACT-Netcord International Standards for Cord Blood Collection, Processing, Testing, Banking, Selection and Release. 4th edition) in Europe contain requirements detailing selection, evaluation and management of donors (http://www.factwebsite.org).
4. FACT-NetCord is also involved with the WMDA and the American Association of Blood Banks in the Alliance for the Harmonisation of Cellular Therapy Accreditation, which aims to harmonize international standards.
5. Use is intended for unrelated transplants for haematological malignancies, certain lysosomal storage and peroxisomal enzyme deficiency disorders, Hurler syndrome (MPS I), Krabbe disease (Globoid Leukodystrophy), X-linked adrenoleukodystropy, primary immunodeficiency diseases, bone marrow failure and thalassaemia.

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Question 1

We prepare Type II products only. The starting material is derived from apheresis and/or whole-blood collections.

Question 2

For autologous products, the donor/patient is selected by the clinician/hospital that has contracted with us to perform the collection. A brief physical exam and health history is performed before each collection to determine whether the donor/patient can tolerate the apheresis procedure cardiovascularly and that the haematocrit and platelet count are sufficient to proceed without a transfusion. The donor/patient is also examined for signs of an underlying infection to protect the quality of the collected product. The referring clinician determines whether a particular WBC or TNC is required before each collection procedure. Infectious disease testing may be required by protocol or by state statute. The donor/patient's identity is checked against the patient chart or enrolment form prepared by the referring clinician. A government issued picture ID must be presented. If there is a discrepancy between the name stated on the picture ID (even an initial versus the full middle name), the referring clinician/customer is contacted to confirm the identity of the donor/patient and agree to the collection proceeding.

Allogeneic donors are subjected to the same process for autologous donors, except that they are also tested for infectious diseases. For directed donations, the patient's physician may determine whether the donor will be accepted even with positive viral markers.

If the starting material is derived from a whole-blood collection, the allogeneic donor must meet the same criteria as that for a volunteer blood donor. The unit is also tested for the mandatory viral markers before release to further manufacturing. These tests include: HBsAg, anti-HCV, HCV RNA, anti-HIV-1/2, HIV-1 RNA, anti-HTLV-I/II, WNV RNA and a serologic test for syphilis.

Question 3

Depending upon the material, we take samples for sterility and initial cell count before further manipulation or freezing. Sterility is monitored using the BacT/Alert system (bioMérieux, Inc., Durham, NC, USA) CD 34 content is determined by flow cytometry.

Question 4

BacT Alert readings are used to determine whether sterility has been maintained. CD 34 content (if applicable) is determined by flow cytometry.

Question 5a
Yes.

Question 5b
If the thresholds of release are not met, the product is deemed to be 'non-conforming'. The ordering physician or manufacturing customer is notified, and the decision to distribute or destroy the product is made by that party.

Question 6a
No.

Question 6b
We do not perform any other assays other than those described in Question 4.

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Risk factor analysis of vasovagal reaction from blood donation

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ABSTRACT

Background: Vasovagal reaction (VVR) is the most frequent side effect at blood collection sites.
Aims: To protect donors, factors contributing to VVR were analysed.
Materials and methods: Complications following whole blood and apheresis donations have been recorded and accumulated by the Japanese Red Cross Tokyo Blood Centre. A dataset of 43,948 donors who had no complications was prepared as a control by randomly selecting days in each season in the 2006 and 2007 fiscal years. Factors contributing to 4924 VVR incidents in the 2006 and 2007 fiscal years were analysed by univariate and multivariate logistic regression.
Results: The age, weight, body mass index (BMI), predonation systolic and diastolic pressure, and circulating blood volume were lower, and the pulse was higher, for the VVR group compared to the control group ($p < 0.0001$). The VVR group had more female donors, less sleep, and more time since a meal than the control. In multivariate analysis, significant risk factors for 400 ml whole blood donors, which are the majority of donors, were an age <50 years, being female, a BMI <25, pulse ⩾90/min, sleep duration <8 h, the time after eating ⩾4 h, a first time donation and circulating blood volume of <4.3 l. Sleep duration of <6 h was shown to be a VVR risk as much as a first time donation.
Conclusion: From our analysis, the amount of sleep obtained the previous night should be considered at the reception of donors.

1. Introduction

The blood supply depends entirely on volunteers. Blood collection, though, sometimes causes adverse reactions. The most frequent adverse reaction with blood collection is a vasovagal reaction (VVR), a systemic prefaint reaction, and it is usually mild and transient. A severe VVR including loss of consciousness or syncope can cause injury, from bruising to bone fractures, losing teeth or a brain injury. The incidence of VVR from blood collection in a Japanese Red Cross (JRC) blood centre had been reported to be between 0.83% and 4.17% depending on the sex and donation type [1]. The high risk groups for VVR are generally reported to be young, female and first-time donors [2–6].

The JRC blood centres receive around 5 million donations of whole blood (WB) or apheresis components each year, providing 100% of the Japanese blood supply. The JRC Tokyo Blood Centre receives around 11% of all the donations in Japan. Since we experienced a fatal injury associated with syncope after a donation in 2005, we began multiple analyses that included setting up a database for

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donors who did not experience adverse reactions. This report describes the risks of VVR, using the donor complication data gathered for two fiscal years, compared with information on donors without adverse reactions randomly collected during the same period as a control.

2. Materials and methods

2.1. Collection site procedures

In Japan we have 200 ml WB (WB200), 400 ml WB (WB400), apheresis platelet and plasma collection. Donors who are 16 or 17 years old had been able to make only WB200 donations until April 2011, at which time we began to accept 17 year old male donors for WB400. The WB200 group has predominantly consisted of young, first-time donors or of low weight donors. All donations are allogeneic, from healthy individuals.

Platelets and plasma are collected with one of three apheresis systems, the Trima (Gambro BCT, Lakewood, CO), TerusysS (Terumo, Tokyo, Japan) or Haemonetics CCS (Haemonetics, Braintree, MA). The circulating blood volume (BV, l) is calculated according to Ogawa et al. [7]:

$$BV = 0.168H^3 + 0.050W + 0.444 \text{ for an adult male, and}$$

$$BV = 0.250H^3 + 0.0625W - 0.662 \text{ for an adult female,}$$

where H is the height (m) and W is the weight (kg). The collecting volume of plasmapheresis is between 300 and 600 ml, depending on the body weight, and the volume for platelet collection is 400 ml or less.

Adverse reactions which occur at the collection sites are managed by the collection staff, including physicians. Severe cases are sent to hospital for consultation and treatment. All donors are instructed to contact the blood centre if they experience problems or have concerns about their health after making the donation. We keep a systematic record of events that occur at the time of the donation, or that are reported later, including reports when donors receive outside medical care.

2.2. VVR group

Donor adverse reactions were recorded in our central data base system. When a donor presented symptoms including weakness, pallor, yawning, cold sweat, nausea, vomiting, fainting, convulsion and incontinence, with or without lower systolic blood pressure (BP) or lower pulse rate compared to those recorded at reception, the nurse recorded the complication as VVR, and more information about the timing, place and accompanying fainting was requested to add into the records.

From April 1, 2006 to March 31, 2008, during which time we accepted 1,119,716 donations, we recorded 13,320 donor adverse reactions (1.09%). Selecting records with the primary or secondary complication being VVR, and with sufficient information, we compiled a VVR group of 4924.

2.3. Control group

Days were selected randomly for each season over two years, and for donors who had been accepted on those days, BP, pulse rate before and after the donation, sleep duration, and time since eating were entered into a data base from the paper form which had been recorded at reception. The data was later combined with other information from the central donor data system, such as age, sex, number of donations, donation volume, donation site and time. By eliminating the donations with adverse reactions from the data, we prepared a control dataset of 43,948 donations.

2.4. Statistical analysis

Descriptive statistical analysis was performed to assess donor baseline characteristics, BMI, predonation pulse, predonation systolic and diastolic BP, sleep duration, time since eating, and type of donation. The group was dichotomised for BMI, pulse, BP, hours since a meal and sleep duration at reported or clinically relevant figures. The 2-sided chi-square test was used for categorical variables, and the 2-sided Wilcoxon rank sum test was used for continuous variables.

Furthermore, VVR rates for different subgroups were compared by calculating odds ratios (ORs) and 95% confidence intervals (CIs). A multivariate logistic regression analysis was performed to identify demographic variables that were independently associated with VVR after donations using SAS software (Version 9.1.3, SAS Institute, Inc., Cary, NC). In the multivariate logistic analyses, ORs were adjusted for age group, BMI group, pulse group and systolic BP group.

3. Results

3.1. VVR incidents

The location data was obtained for 4908 of the 4924 VVR incidents studied: 87.1% of the VVR occurred in the collection room, followed by 10.6% in the refreshment area, 1.2% outside of the collection sites, 0.8% on the way from the collection room to the refreshment area, and 0.4% in the restroom.

With the timing data obtained for 4818 incidents, 2621 (54.4%) of the VVR occurred during the collection, followed by 28.6% after the collection and 9.8% while in the refreshment area. There were 67 incidents of VVR which occurred after blood was collected and the donor left the site. Other VVR incidents associated with our procedure for a haemoglobin test, requiring venipuncture, included 4.0% after the haemoglobin test and before the blood collection, 1.6% during the haemoglobin test and 0.3% before the test.

The percentages of the VVR group whose donation site was a mobile bus, temporary donation site (mobile setup) or donation room were 34.1%, 5.0% and 60.9%, respectively, while the donation site ratio of the control group was 13.7%, 9.3% and 77.0%, respectively ($p < 0.0001$).

3.2. Characteristics of donors

The characteristics of the VVR and the control groups were significantly different ($p < 0.0001$) in: age (mean 28.3 vs. 34.9 years), height (163.9 vs. 166.4 cm), weight (58.2 vs. 62.8 kg), BMI (21.6 vs. 22.6), predonation pulse (77.7 vs. 76.1 beats/min), predonation systolic BP (116.6 vs. 119.5 mmHg), predonation diastolic BP (69.2 vs. 71.7 mm Hg) and circulating BV (4.04 vs. 4.33 l). The mean of the circulating BV was 4.30 l. The VVR group consisted of significantly ($p < 0.0001$) more female donors (50.5% vs. 36.9%), first time donors (38.2 vs. 11.2%), and donors who had more than 4 h since their last meal (43.1% vs. 36.0%). The VVR group also had more donors who had less sleep the night before ($p < 0.0001$), as shown in Fig. 1.

When the VVR group was divided according to the timing data, into 'VVR before or during collection' and 'VVR after donation' subgroups, the first subgroup was significantly younger (age 27.7 vs. 29.2 years old), shorter (163.4 cm vs. 164.6 cm), lighter (57.3 kg vs. 59.6 kg) and had a higher ratio of female donors compared to the 'VVR after donation' subgroup. The 'VVR after donation' group was also significantly younger, shorter, lighter, and had a higher ratio of female donors compared to the control group. The donor characteristics of the timing subgroups were influenced by the ratio of female donors with VVR from apheresis. With apheresis donation, 82% of the VVR occurred before or during the collection, and 76% of the VVR from apheresis were female donors. WB400 donors were the majority (76%) of the 'VVR after donation' subgroup, while they were 44% of the 'VVR before and during collection' subgroup, less than the apheresis donors (49%). The sleep duration, time from eating and first time donor ratio were not significantly different between the two subgroups.

3.3. Subgroup analysis by sex

For male donors, the VVR group was significantly younger (mean 28.2 years old vs. 36.6 years old), shorter (170.4 cm vs. 171.0 cm), lighter (63.8 kg vs. 68.3 kg), had a lower BMI (22.0 vs. 23.3), and a lower circulating BV (4.5 l vs. 4.7 l) ($p < 0.0001$). The VVR group also had significantly lower predonation systolic BP (121.9 mmHg vs. 124.2 mmHg) and diastolic BP (71.5 mmHg vs. 74.5 mmHg) ($p < 0.0001$).

For female donors, the VVR group was significantly younger (mean 28.3 years old vs. 31.9 years old), shorter (157.5 cm vs. 158.6 cm), lighter (52.7 kg vs. 53.5 kg), and had a lower circulating BV (3.6 l vs. 3.7 l) ($p < 0.0001$). The VVR group also had a significantly higher pulse (79.2 vs. 76.3 beats/min) ($p < 0.0001$).

In the multivariate analysis, the female subgroup showed a higher risk for ages 18 and 19 (OR 3.1, 95%CI 2.5–3.8) and for those aged in their twenties (OR 1.7, 95%CI 1.4–2.0), compared to those aged ⩾50. The male subgroup had a high OR in every age group <50 years, especially for ages 18 and 19 (OR 19.6, 95%CI 14.9–25.8). The male subgroup also showed an association with BMI with a higher OR for a BMI <25 compared to those of ⩾25. In both subgroups a predonation pulse ⩾90/min showed a high OR, at 1.6 (95%CI 1.4–1.8) for females and 1.2 (95%CI 1.1–1.4) for males, compared to those with <90/min. First time donors showed a high risk, with an OR of 2.3 (95%CI 2.0–2.5) for females and an OR of 5.9 (95%CI 5.3–6.4) for males, compared to other donors. Sleep of less than 6 h was associated with a high OR in both subgroups compared to those with ⩾8 h, at 3.6 (95%CI 3.2–4.1) for females and 5.1 (95%CI 4.5–5.9) for males. A time from the last meal of ⩾4 h was a risk for VVR in both subgroups, with an OR of 1.24 (95%CI 1.13–1.35) for females and 1.64 (95%CI 1.48–1.82) for males. The risk was significantly lower with WB200 than WB400 donations in both subgroups, while apheresis had shown a higher risk for females with an OR of 4.42 (95%CI 3.9–5.1), but lower for males with an OR of 0.7 (95%CI 0.5–1.0). A diastolic BP <70 mmHg was shown as a low risk factor in both subgroups.

3.4. Subgroup analysis by donation types

The characteristics of donation type subgroups are shown in Table 1. In every donation type, the VVR group was younger, lighter, had a smaller BMI, smaller circulating BV, and had more donors with less than 6 h sleep. Only for the 200WB donations were there more male donors in the VVR group.

The multivariate analyses for each subgroup, adjusted for age, sex, BMI, pulse and systolic pressure, are shown in Table 2. A younger age, shorter sleep duration, first time donation and circulating BV <4.30 l were the common risk factors for all three subgroups. Also, a predonation diastolic BP lower than 70 mmHg was a significant low risk factor for VVR for every subgroup.

For the WB200 donations, the age groups of 16 and 17 year olds, 18 and 19 year olds and of those in their twenties and thirties showed a high adjusted OR compared to those aged ⩾50 years. A sleep duration <8 h was shown to be a risk factor, especially when it was <6 h.

For WB400 donations, which make up the majority of our collections, a younger age compared to the age group of ⩾50 years showed significantly higher risk. Other significant high risk factors were being female, a BMI <25, a predonation pulse ⩾90 beats/min, sleep duration <8 h, especially when it is <6 h, time since eating ⩾4 h, a first-time donation and a circulating BV <4.30 l. A predonation systolic BP lower than 100 mmHg and a diastolic BP lower than 70 mmHg were significant low risk factors for VVR.

For apheresis donations, the significant factors for high risk of VVR were ages 18 and 19 years, being female, a BMI <25, a predonation pulse ⩾90 beats/min, sleep duration <8 h, especially when it is <6 h, time after eating ⩾4 h, a first-time donation and a circulating BV <4.30 l.

3.5. Risk analysis of syncope/fall

There were 75 syncope/fall incidents in the dataset. The majority, 74 incidents, were a consequence of VVR and the other case had a syncope following the side effect of haematoma. When they were divided by the timing data, 21 cases occurred before and during collection, and 53 cases had the syncope/fall incidents after the donation. The incident rate as a proportion of VVR was shown to

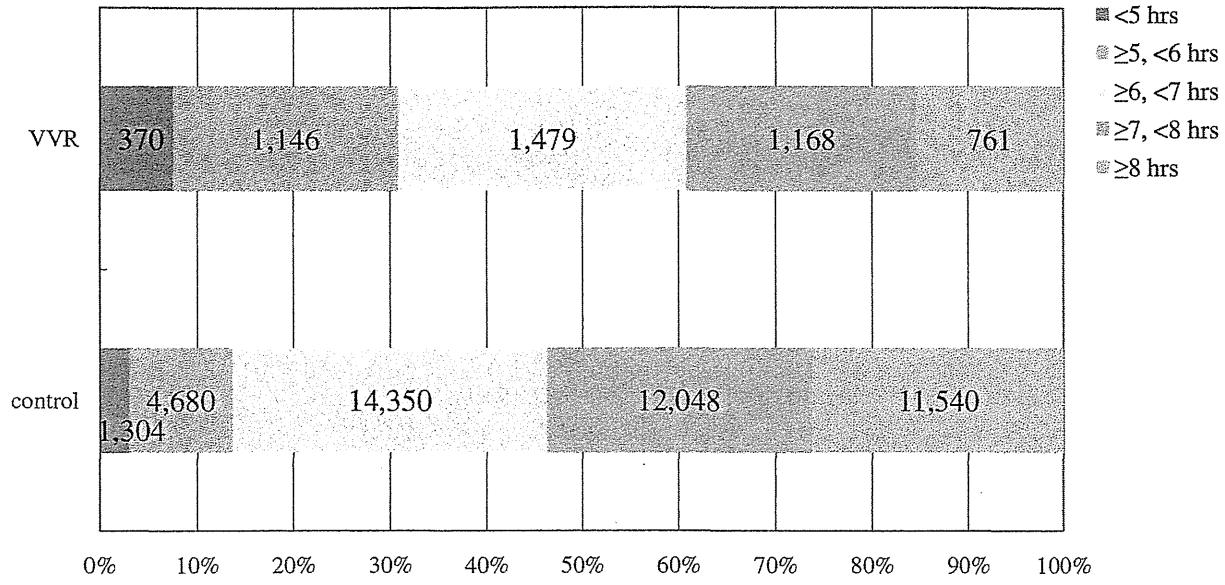

Fig. 1. Sleep duration for the VVR and control groups. The VVR group had significantly less sleep compared to the control group ($p < 0.0001$).

Table 1
Characteristics of the VVR and control group for donation type subgroups.

Variables	200 mL Whole blood		400 mL Whole blood		Apheresis	
	VVR (mean ± SD)	Control	VVR (mean ± SD)	Control	VVR (mean ± SD)	Control
	$n = 344$	$n = 5789$	$n = 2668$	$n = 24{,}602$	$n = 1637$	$n = 13{,}557$
Age (years old)	23.8 ± 7.5***	31.1 ± 12.5	28.0 ± 9.5***	36.1 ± 12.0	30.6 ± 11.0***	34.2 ± 11.2
Height (cm)	158.5 ± 6.9	158.4 ± 6.6	166.9 ± 7.7***	168.6 ± 7.5	159.7 ± 7.7***	166.0 ± 8.3
Weight (kg)	49.4 ± 6.7***	50.8 ± 7.6	61.6 ± 8.5***	66.2 ± 10.4	54.6 ± 8.9***	61.8 ± 11.2
BMI	19.6 ± 2.0***	20.2 ± 2.3	22.1 ± 2.6***	23.2 ± 3.0	21.4 ± 2.6***	22.3 ± 3.0
Predonation pulse (/min)	76.9 ± 11.4	76.3 ± 11.2	76.6 ± 12.3**	75.8 ± 11.6	80.0 ± 11.4***	76.5 ± 11.6
Predonation systolic pressure (mmHg)	110.1 ± 12.8	111.3 ± 14.2	120.0 ± 13.6***	123.3 ± 15.8	112.6 ± 14.0***	116.0 ± 14.7
Predonation diastolic pressure (mmHg)	67.2 ± 10.2	66.5 ± 10.5	71.0 ± 10.4***	74.0 ± 12.1	67.2 ± 10.3***	69.6 ± 11.5
Circulating blood volume (l)	3.34 ± 0.49**	3.51 ± 0.53	4.28 ± 0.53***	4.55 ± 0.61	3.76 ± 0.61***	4.27 ± 0.71
	(n)		(n)		(n)	
Sex Female/Male	280/64***	5115/674	847/1821***	5657/18,945	1249/388***	5445/8112
Sleep dilation (hours)						
< 6	127***	801	942***	3882	375***	1301
⩾ 6, < 7	84	1891	831	8206	483	4253
⩾ 7, < 8	83	1490	536	6510	471	4048
⩾ 8	50	1600	359	5990	308	3950
Time after eating (hours)						
<4/⩾4	148/196	2341/3441	1106/1562***	9377/15,192	753/884***	4058/9473
Donation status						
First-time/other	207/137***	1354/4435	1295/1373***	3353/21,249	178/1459***	233/13,324

** $p < 0.01$.
*** $p < 0.001$.

be higher after the donation (2.8% vs. 0.7%), suggesting the donors received closer care when the VVR symptom was noticed earlier. The multivariate analysis with the control group showed a trend similar to the VVR risk analysis, as the majority of syncope cases followed VVR. The risk of syncope was higher for the age group of 20–29 years with an OR of 3.7 (95%CI 1.1–12.3), being female with an OR of 1.9 (95%CI 1.2–3.1), sleep duration <6 h with an OR of 4.2 (95%CI 2.1–8.2), first time donors with an OR of 2.8 (95%CI 1.7–4.7), and circulating BV <4.3 l with an OR of 3.4 (95%CI 1.6–7.0). Donations other than WB200 also had a higher risk compared to WB200.

4. Discussion

As the only organization to collect blood for transfusion in Japan, the Japanese Red Cross has been trying to prevent adverse reactions from blood donation, as well as the side

Table 2
Donation subgroup analyses of VVR.

	Adjusted OR[a]	(95% CI)
200 ml Whole blood donation		
Age[b]		
16–17 year	11.86	(4.62–30.45)***
18–19 year	16.33	(6.40–41.69)***
20–29 year	11.16	(4.52–27.58)***
30–39 year	4.97	(1.95–12.68)***
Predonation diastolic pressure <70 mmHg	0.46	(0.36–0.59)***
Sleep duration[c]		
<6 h	5.84	(4.14–8.25)***
6–6.9 h	1.66	(1.15–2.38)***
7–7.9 h	2.00	(1.39–2.87)***
First-time donors	3.86	(3.03–4.90)***
Circulating blood volume <4.30 l	2.47	(1.45–4.20)***
400 ml Whole blood donation		
Age[b]		
18–19 year	12.13	(9.64–15.26)***
20–29 year	6.31	(5.13–7.76)***
30–39 year	3.23	(2.60–4.00)***
40–49 year	1.84	(1.45–2.34)***
Female	1.28	(1.17–1.40)***
BMI <25	1.67	(1.48–1.89)***
Predonation pulse ⩾90 beats/min	1.25	(1.11–1.40)***
Predonation systolic pressure <100 mmHg	0.71	(0.59–0.85)***
Predonation diastolic pressure <70 mmHg	0.77	(0.71–0.85)***
Sleep duration[b]		
<6 h	4.05	(3.56–4.60)***
6–6.9 h	1.92	(1.68–2.19)***
7–7.9 h	1.58	(1.37–1.82)***
Time after eating ⩾4 h	1.10	(1.01–1.19)*
First-time donors	4.21	(3.85–4.60)***
Circulating blood volume <4.30 l	1.69	(1.53–1.88)***
Apheresis donation		
Age[b]		
18–19 y	1.58	(1.18–2.11)**
Female	4.48	(3.95–5.07)***
BMI < 25	1.30	(1.08–1.57)**
Predonation pulse ⩾90 beats/min	1.90	(1.66–2.18)***
Predonation diastolic pressure <70 mmHg	0.86	(0.76–0.98)*
Sleep duration[c]		
<6 h	3.96	(3.33–4.69)***
6–6.9 h	1.54	(1.32–1.80)***
7–7.9 h	1.60	(1.37–1.87)***
Time after eating ⩾4 h	1.92	(1.73–2.14)***
First-time donors	5.10	(4.11–6.34)***
Circulating blood volume <4.30 l	2.06	(1.71–2.47)***

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
[a] Odds ratio (OR) adjusted for age group, BMI group, pulse group and systolic blood pressure group.
[b] The risk was compared to age group of 50 years and older.
[c] The risk was compared to sleep duration of 8 h and more.

effects in recipients of transfusions. According to the nationwide donor records, adverse reactions have been reported for the fiscal years of 2007, 2008 and 2009 at the rate of 1.11%, 1.16% and 1.15%, respectively (JRC internal report). For the same years, in Tokyo the rates were 1.15%, 1.25% and 1.28%, respectively, with the majority of the incidences, 67.4%, 65.6% and 65.0%, respectively, consisting of VVR. This was in spite of the introduction of predonation hydration, following reports on decreasing such incidences by drinking water [8,9]. The analyses on risk factors of blood donation show that even a notably low risk population, males of higher weight, is not free from VVR.

Eder et al. [5] reported that regional practices could cause differences in incident rates. We also recognized an effect from the environment, as the VVR group more often consisted of donors at the mobile bus sites, which have a less spacious and less relaxed environment. The smaller ratio of the mobile setup locations for the VVR group (5.0%) compared to the control group (9.3%) may also reflect that the donors at mobile setup locations are often acquainted with each other, as these sites are usually at companies and community centres. As well, there was a tendency for a concentration of VVR cases in the peak afternoon time that often makes for a crowded and busy

environment. With age, it was reported that the reaction rates increase with decreasing age for teenaged donors [5,10]. We also observed an increase in risk with younger age in 400WB subgroup analysis, but it was not clear for the 200WB subgroup, presumably because the number is relatively small.

The weight of donors has been reported as a factor for VVR [2,3,8], but for this analysis we used BMI and circulating BV, which is calculated from the height, weight and sex of the donor. In our multivariate analyses we chose a BMI dichotomized at 25, for BMI $\geqslant$25 is regarded as overweight, and we had a low OR for this group in the preliminary analysis. According to the national health and nutrition survey of Japan [11], 19% of women and 30% of men between 20 and 69 years old had a BMI $\geqslant$25, so the ratio of overweight individuals was lower in our donor group, at 18%. The effect of BMI on the VVR risk was not significant for female donors, while it was significant for male donors, in sex subgroup analysis. BMI was also not significant for WB200 donors in donation subgroup analysis, probably because of the small size of the group with BMI $\geqslant$25 in this subgroup, due to our practice of encouraging donors to switch from WB200 to WB400 when the donor's weight is more than 50 kg.

The circulating BV was dichotomized at the median, 4.30 l, and was a significant factor in every analysis. This is in agreement with Wiltbank et al. [6], whose report showed that a smaller BV is associated with a higher OR, especially for those with a BV <3.5 l. It was reported that greater blood loss was associated with presyncopal reactions [12]. We did not include volume loss as one of our factors, because the volume loss for the VVR group is rather small compared with donors without adverse reaction, as we stop the collection in cases when the donor has a symptom of VVR during the collection, which is when the majority of VVR occurs.

A predonation pulse $\geqslant$ 90/min was a significant risk in analyses of the WB400, apheresis and sex subgroups. It is possible that stress or anxiety mounts before the donation, as a minority of donors had shown VVR even before the haemoglobin test. Our result is in accordance with the report by Wiltbank et al. [6], which had shown that compared to the group with a pulse of 65–90/min, >90 is a significantly high risk and <65 is a significantly low risk. We dichotomized the group at a pulse of 90/min, as the OR for a pulse of 65–90 and <65 were similar in our preliminary analysis.

The predonation systolic BP of <100 mmHg was shown to be a significant low risk factor in analyses of the WB400 subgroup. We dichotomized the group at 100 mmHg as the preliminary analysis had shown a similar OR for the groups with a BP of 100–140 and of >140 mmHg. The adjustment for the final analyses included age, pulse and systolic BP, as the younger the age, the lower the BP. There are reports that a higher systolic BP had shown a lower risk compared to normal BP, and lower BP was not significantly different [2,6]. It is possible that the demographics of the subjects caused the different results, as more than 9% of our analysed population had a BP <100 mmHg, while the report by Wiltbank et al. [6] showed it was 4%. A predonation diastolic BP <70 mmHg compared with $\geqslant$70 was a low risk factor in analyses for the sex subgroups, and every subgroup of donation type. There is a report showing a lower diastolic BP associated with a higher VVR rate, explaining that younger donors have lower BP [13]. This discrepancy with our results could be explained by our multivariate analyses being adjusted for factors including age. Also, there is a possibility that the donors who have a relatively low BP are used to having it, or that the BP change in VVR is relatively small.

In our practice, donors who had not eaten for a while, skipped their last meal or felt hungry were advised to come back after they had something to eat, or were sent for a snack and drink first. So the donors who stated at reception that they had gone $\geqslant$4 h since their last meal had eaten at the time of venipuncture. There is a possibility that the donors who did not eat for $\geqslant$4 h had been relatively busy and were under stress when they dropped into the donation site.

A short sleep duration was shown to be a significant risk factor for VVR, especially when it was less than 6 h. Tomasulo et al. [14] had shown that it was not necessary to consider the sleep history, since the sleep duration was not significantly different between the control donors and those with a reaction. In their study, the mean of the sleep duration for first time female donors was 7.8 h for the control and 7.7 h for those with a reaction, while for first time male donors the mean was 7.4 h for both. As our data for sleep hours is categorical and not numerical data, we cannot compare the sleep hours between their study and ours. Still, in our study the difference between the VVR group and the control group was significant. We have regular donors who work the night shift, so the sleep duration is not to be applied generally, but sufficient sleep is an important health factor, as Breslow and Enstrom [15] reported. The relatively high OR for groups with <6 h sleep should be considered when the donor belongs to a subgroup that is at higher risk, such as young, first-time donors, females or having a circulating BV <4.3 l.

The risk analyses of syncope reflected that for VVR, as the majority had been VVR cases. Young, female and first-time donors had been reported to be more likely in the syncopal group than general donors [16]. We have also shown that donors with a small BV and a short sleep duration should be added to the high risk groups.

Though our analyses of VVR was prompted by a serious accident related to syncope, it is difficult to apply all of the results directly to actual practice: It is essential to recruit young and first-time donors for the future of the blood supply, and also female donors, who made up 32.5% of the donors in Japan in the fiscal year of 2009. Well informed consent for blood donation is important [17]. To prevent serious consequences, the donors need to be informed about the posture to take when symptoms occur. Also, we should try to recruit donors from low risk groups.

The study showed the VVR risk factors and particularly the importance of the duration of sleep. From our analysis, the amount of sleep obtained the night prior to a blood donation should be considered at the reception of donors.

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Impact of graft-versus-host disease on outcomes after allogeneic hematopoietic cell transplantation for adult T-cell leukemia: a retrospective cohort study

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Allogeneic hematopoietic cell transplantation (HCT) is an effective treatment for adult T-cell leukemia (ATL), raising the question about the role of graft-versus-leukemia effect against ATL. In this study, we retrospectively analyzed the effects of acute and chronic graft-versus-host disease (GVHD) on overall survival, disease-associated mortality, and treatment-related mortality among 294 ATL patients who received allogeneic HCT and survived at least 30 days posttransplant with sustained engraftment. Multivariate analyses treating the occurrence of GVHD as a time-varying covariate demonstrated that the development of grade 1-2 acute GVHD was significantly associated with higher overall survival (hazard ratio [HR] for death, 0.65; $P = .018$) compared with the absence of acute GVHD. Occurrence of either grade 1-2 or grade 3-4 acute GVHD was associated with lower disease-associated mortality compared with the absence of acute GVHD, whereas grade 3-4 acute GVHD was associated with a higher risk for treatment-related mortality (HR, 3.50; $P < .001$). The development of extensive chronic GVHD was associated with higher treatment-related mortality (HR, 2.75; $P = .006$) compared with the absence of chronic GVHD. Collectively, these results indicate that the development of mild-to-moderate acute GVHD confers a lower risk of disease progression and a beneficial influence on survival of allografted patients with ATL. (*Blood.* 2012;119(9):2141-2148)

Introduction

Adult T-cell leukemia (ATL) is a mature T-cell neoplasm that is causally associated with a retrovirus designated human T-cell leukemia virus type I (HTLV-I).[1-4] HTLV-I is endemic in southwestern Japan, sub-Saharan Africa, the Caribbean Basin, and South America.[3,4] In Japan, more than 1 million people were estimated to be infected with HTLV-I. Although the majority of HTLV-I–infected individuals remain asymptomatic throughout their lives, ~ 5% develop ATL at a median age of 40 to 60 years.[4,5]

ATL is categorized into 4 clinical variants according to its clinical features: smoldering, chronic, acute, and lymphoma types.[6] The acute and lymphoma variants of ATL have an extremely poor prognosis, mainly because of resistance to a variety of cytotoxic agents and susceptibility to opportunistic infections; the median survival time is ~ 13 months with conventional chemotherapy,[7,8] although encouraging results have been recently reported with the use of novel agents such as mogamulizumab.[9-11]

Over the past decade, allogeneic hematopoietic cell transplantation (HCT) has been increasingly performed with the aim of improving dismal prognosis of patients who developed ATL.[12-18] Notably, some patients with ATL who relapsed after allogeneic HCT were shown to achieve remission only with the cessation of immunosuppressive agents, raising the question of whether the graft-versus-leukemia effect against ATL can be induced as part of graft-versus-host reaction.[19,20] In 1 study, among 10 patients who experienced relapse of ATL after transplantation and were withdrawn from immunosuppressive therapy, 8 developed graft-versus-host disease (GVHD), and 6 of them subsequently achieved

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complete remission of ATL.[19] Similar observations have been rarely reported in other aggressive mature lymphoid neoplasms,[21] suggesting the unique susceptibility of ATL to graft-versus-host reactions. Recently, a combined analysis of 2 prospective studies including 29 ATL patients in total undergoing allogeneic HCT suggested that development of mild acute GVHD favorably affected overall survival and progression-free survival.[22] However, the impact of GVHD on the outcome of allogeneic HCT in ATL needs to be verified in a much larger cohort. We previously conducted a nationwide retrospective study to evaluate the current results of allogeneic HCT for ATL, and we confirmed that a substantial proportion of patients with ATL can enjoy long-term, disease-free survival after transplantation: the overall survival rate at 3 years among patients who received transplants in complete remission and not in complete remission was 51% and 26%, respectively.[23] Using the same cohort, we further evaluated the effects of acute and chronic GVHD on long-term outcomes of allografted patients with ATL.

Methods

Collection of data

Data on 417 patients with acute or lymphoma type ATL who had undergone allogeneic bone marrow, peripheral blood, or cord blood transplantation between January 1, 1996, and December 31, 2005, were collected through the Japan Society for Hematopoietic Cell Transplantation (JSHCT), the Japan Marrow Donor Program (JMDP), and the Japan Cord Blood Bank Network (JCBBN), the 3 largest HCT registries in our country; their roles were detailed previously.[23] The patients were included from 102 transplant centers; the data were updated as of December 2008. The study was approved by the data management committees of JSHCT, JMDP, and JCBBN, as well as by the institutional review boards of Kyoto University Graduate School of Medicine, where this study was organized.

Inclusion and exclusion criteria

Patients were included in the analysis if the following data were available: age at transplantation, sex of the recipient, donor type, stem cell source, agents used in the conditioning regimen and GVHD prophylaxis, the maximum grade and day of occurrence of acute GVHD, and the day of neutrophil recovery. Acute GVHD was reported according to the traditional criteria,[24] except that 1 patient was considered to have late-onset acute GVHD at day 133; neutrophil recovery was considered to have occurred when an absolute neutrophil count exceeded 0.5×10^9/L for 3 consecutive days after transplantation. Patients who missed any of these data (n = 37), who had a history of prior autologous or allogeneic HCT (n = 8), who had received an ex vivo T cell–depleted graft (n = 1), who experienced primary or secondary graft failure (n = 24) were excluded from the analysis. Because the association between the occurrence of acute GVHD and disease-associated mortality was difficult to evaluate in the event of early toxic death, patients who died within 30 days of transplantation (n = 53) also were excluded from the study. Among these 53 patients, 22 were evaluable for acute GVHD: grade 0 in 17 patients, grade 1-2 in 3 patients, and grade 3-4 in 2 patients. Two physicians (J.K. and T.I.) independently reviewed the quality of collected data, and 294 patients in total (158 males and 136 females), with a median age of 51 years (range, 18-79 years), were found to meet these criteria and included in the study: 163 patients from JSHCT, 82 patients from JMDP, and 49 patients from JCBBN. No overlapping cases were identified. Of these 294 patients, the effects of chronic GVHD, reported and graded according to using traditional criteria,[25] were considered evaluable for the 183 patients who survived at least 100 days after transplantation with complete information on the type and the day of occurrence of chronic GVHD.

End points

The primary end point of the study was the effect of acute GVHD on overall survival, defined as the period from the date of transplantation until the date of death from any cause or the last follow-up. The secondary end points of the study included the impact of acute GVHD on disease-associated and treatment-related mortality, and the impact of chronic GVHD on overall survival, disease-associated mortality, and treatment-related mortality. Reported causes of death were reviewed and categorized into disease-associated or treatment-associated deaths. Disease-associated deaths were defined as deaths from relapse or progression of ATL, whereas treatment-related deaths were defined as any death other than disease-associated deaths.

Statistical analysis

The probability of overall survival was estimated by the Kaplan-Meier method. Treatment-related and disease-associated mortality were estimated with the use of cumulative incidence curves to accommodate the following competing events[26]: disease-associated death for treatment-related mortality and treatment-related deaths for disease-associated mortality. Data on patients who were alive at the time of last follow-up were censored. Semi-landmark plots were used to illustrate the effects of GVHD on overall survival and cumulative incidence of disease-associated and treatment-related deaths. For patients with acute or chronic GVHD, the probability of overall survival and the cumulative incidences of disease-associated and treatment-related deaths were plotted as a function of time from the onset of acute or chronic GVHD. Day 24.5, the median day of onset for acute GVHD, was termed as the landmark day in patients without acute GVHD. In the case of patients without chronic GVHD, day 116, the median day of onset for chronic GVHD, was termed as the landmark day.

Univariate and multivariate Cox proportional hazards regression models were used to evaluate variables potentially affecting overall survival, whereas the Fine and Gray proportional subdistribution hazards models were used to evaluate variables potentially affecting disease-associated and treatment-related mortality.[27] In these regression models, the occurrence of acute and chronic GVHD was treated as a time-varying covariate.[28] In the analysis of acute GVHD, patients were assigned to the "no acute GVHD group" at the time of transplantation and then transferred to the "grade 1-2 acute GVHD group" or to the "grade 3-4 acute GVHD group" at the onset of the maximum grade of acute GVHD. In the analysis of chronic GVHD, patients were assigned to the "no chronic GVHD group" at the time of transplantation and then transferred to the "limited chronic GVHD group" or to the "extensive chronic GVHD group" at the onset of the maximum grade of chronic GVHD. The variables considered were the age group of the recipient ($\leq$ 50 years or $>$ 50 years at transplantation), sex of the recipient (female or male), disease status before transplantation (complete remission, disease status other than complete remission, or unknown), intensity of conditioning regimen (myeloablative, reduced intensity, or unclassifiable), type of GVHD prophylaxis (cyclosporine-based, tacrolimus-based, or other), type of donor (HLA-matched related donor, HLA-mismatched related donor, unrelated donor for bone marrow, or unrelated cord blood), time from diagnosis to transplantation (within 6 months, $>$ 6 months, or unknown), and year of transplantation (1995-2002 or 2003-2005). We classified the intensity of conditioning regimen as myeloablative or reduced intensity based on the working definition by Center for International Blood and Marrow Transplant Research if data on dosage of agents and total-body irradiation (TBI) used in the conditioning regimen were available.[29] For 110 patients for whom such information was not fully available, we used the information on conditioning intensity (myeloablative or reduced intensity) reported by treating clinicians. The cutoff points for year of transplantation were chosen such that we could make optimal use of the data with a proviso that the smaller group contained at least 30% of patients. In the analysis of the effect of chronic GVHD, the prior history of grade 2-4 acute GVHD also was added to the multivariate models. We also assessed the interaction between acute GVHD and the intensity of conditioning regimen in the multivariate models. Only factors with a P value of less than .10 in univariate analysis were included in the multivariate models. In addition, the heterogeneities of the effects of grade 1-2 or grade 3-4 acute GVHD on overall survival according to background transplant characteristics were evaluated by the forest plots stratified by variables included in the regression analyses. Furthermore, landmark analysis treating the development of acute GVHD as a time-fixed covariate was performed to confirm