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It has been reported that DCs activated $CD4^+$ T cells via MHC class II-restricted presentation [3–5]. We showed that the effector function of Ag-specific $CD4^+$ T cells was impaired in a second coculture when these T cells were stimulated with Ag-pulsed IL-10-induced semi-mDCs in a first coculture. These results suggest that IL-10-induced semi-mDCs may present internalized soluble Ag–Ag-specific $CD4^+$ T cells via the interaction between MHC class II-antigenic peptide and TCR with low interaction of costimulatory molecules, and this event may induce an anergic state in Ag-specific $CD4^+$ T cells.

We showed that the coculture with allogeneic cellular fragment-pulsed normal mDCs enhanced the cytolytic activity of allogeneic fibroblast specific-Tc cells in an Ag-dependent manner. Therefore, normal mDCs may activate these Tc cells via cross-presentation of the phagocytosed necrotic cellular fragments in the booster phase, and this mechanism may involve the normal mDC-mediated delivery of Ag-specific signal to TCR ligands plus signal to costimulatory molecules in Tc cells. On the other hand, these Tc cells cocultured with allogeneic cellular fragment-pulsed IL-10-induced semi-mDCs had reduced their activity in Ag-dependent and -independent manners. Our results suggest that this Ag-dependent suppression may involve IL-10-induced semi-mDC-mediated delivery of Ag-specific signal to TCR ligands via the cross-presentation pathway plus a poor signal to costimulatory molecules in Tc cells in the booster phase resulting in the induction of an anergic state in Ag-specific $CD8^+$ T cells.

We showed the Ag-non-specific suppression of the effector function of allogeneic fibroblast-specific Tc cells following the stimulation with unpulsed IL-10-induced semi-mDCs as well as IL-10-induced semi-mDCs that had been pulsed with allogeneic cellular fragments prepared from unrelated donor-derived necrotic fibroblasts in the second coculture. The precise mechanism responsible for the Ag-independent suppressive effect of IL-10-induced semi-mDCs remains unknown, the interaction between unknown surface-bound molecule(s) on IL-10-induced semi-mDCs and receptor(s) on $CD8^+$ T cells may account for this phenomenon.

Vaccination with tumor-associated Ag-pulsed normal iDCs has been shown to be useful for tumor immunotherapy in clinical trials [21]. In contrast, repetitive stimulation of CB naïve $CD4^+$ T cells with allogeneic iDCs resulted in the induction of their anergic state whereas repetitive stimulation with allogeneic mDCs induced a strong Th response [15]. Furthermore, a single injection of antigenic peptide-pulsed iDCs led to the specific inhibition of the effector function of Ag-specific $CD8^+$ T cells whereas injection of antigenic peptide-pulsed mDCs enhanced the responses of Ag-specific $CD4^+$ and $CD8^+$ T cells in humans [22]. However, clinical application of self or allogeneic peptide-pulsed iDCs may not be suitable for the treatment of autoimmune diseases or organ transplantation, because the injected iDCs are not likely to remain immature in vivo after recirculation and homing to the damage tissues where chronic inflammation is present [23]. On the other hand, we showed that IL-10-induced semi-mDCs retained the capacity to induce an Ag-specific anergy in effector T cells even in their mature state. Thus, further modification of human DCs may facilitate their use for treatment or prevention of immunopathological diseases. To test our hypothesis, the modification of DCs and preclinical studies in animal immunopathogenic models are being conducted in our laboratories.

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the targeted allogeneic fibroblasts were matched with allogeneic fibroblasts used in the priming condition. The coculture with allogeneic cellular fragment-pulsed normal mDCs enhanced their cytolytic activity as compared with Ag-specific Tc cells alone. Furthermore, similar CTL response was observed among Ag-specific Tc cells alone and Ag-specific Tc cells cocultured with unpulsed normal mDCs and normal mDCs pulsed with allogeneic cellular fragments prepared from unrelated donor-derived necrotic fibroblasts. These results indicate that normal mDCs can increase cytolytic activity in Ag-specific Tc cells in the booster phase. In contrast, the coculture with allogeneic cellular fragment-pulsed IL-10-induced semi-mDCs had reduced their activity. We also observed a lower reduction in their lytic activity when cocultured with unpulsed IL-10-induced semi-mDCs and IL-10-induced semi-mDCs that had been pulsed with allogeneic cellular fragments prepared from unrelated donor-derived necrotic fibroblasts. These results indicate that IL-10-induced semi-mDCs that had been pulsed with cellular fragments derived from necrotic cells to induce a hyporesponsiveness in Ag-specific Tc cells.

4. Discussion

The findings reported here suggest that IL-10-induced semi-mDCs may present the internalized soluble antigenic protein and cross-present the phagocytosed allogeneic cellular fragments prepared from necrotic fibroblasts to induce an Ag-specific anergy in Th1 and Tc cells.

We showed that the inflammatory stimulations failed to elicit a fully immunostimulatory maturational change in IL-10-treated iDCs. On the other hand, the maturation-associated changes of the phenotype (CD83 expression) and the endocytosis were observed at various degrees. Therefore, IL-10 selectively impaired the maturational process in DCs.

We showed that allogeneic T cells primed with IL-10-induced semi-mDCs as well as IL-10-treated iDCs failed to respond to further stimulation with normal mDCs. We observed that IL-10-treated iDCs and IL-10-induced semi-mDCs failed to directly induce cell death in responding T cells, and these types of DCs did not express the cell surface molecules inducing apoptosis, such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (data not shown). Therefore, these results exclude the possibility that these types of DCs regulate T-cell response via cell death of responding T cells. Our findings suggest that these types of DCs may impair the function of allogeneic T cells via an induction of their anergic state. Thus, an anergy-inducing ability of IL-10-induced semi-mDCs as well as IL-10-treated iDCs may involve the allogeneic Ag-specific delivery of signal to TCR ligands with a poor signal to costimulatory molecules because these types of DCs displayed moderately high expressions of MHC molecules and low expressions of costimulatory molecules as compared with their counterparts.

In contrast to a previous report [17], we did not detect anergic T cells when allogeneic T cells were primed with normal iDCs and then restimulated with normal mDCs. Indeed, the expression levels of HLA and costimulatory molecules were higher in our normal iDCs than their counterpart. Thus, the discrepancy might be due to the cell preparation and experimental design. Collectively, T-cell anergy may be induced by APCs when the degree of T cell activation in the priming condition is much lower than that on restimulation.

Unlike allogeneic normal mDCs which increased $CD4^+CD25^+CD154^+$ T cells as well as IFN-γ-and IL-2-producing $CD4^+$ T-cell subsets, allogeneic IL-10-induced semi-mDCs induced not only $CD4^+CD25^+$ $CD152^+$ subpopulations but also IL-10-producing $CD4^+$ T-cell subsets. $CD4^+CD25^+CD152^+$ T cells reportedly act as regulatory T (Tr) cells to suppress the responses of alloreactive or self-reactive Th1 cells, and this subpopulation of T cells are supposed to maintain immunologic self-tolerance or control autoimmunity [14,15]. On the other hand, stimulation of $CD4^+$ T cells with tolerant DCs, such as iDCs and 1α,25-dihydroxyvitamin D_3-treated iDCs, could induce a high proportion of $CD4^+CD25^+CD152^+$ Tr-like cells [16,17]. Although the mechanism underlying the generation of $CD4^+$ Tr-like cells by tolerant DCs remains unknown, it is possible that tolerant DCs may convert naïve $CD4^+$ T cells into $CD4^+$ Tr-like cells. On the other hand, $CD4^+$ Tr cells reportedly can induce naïve $CD4^+$ T cells to themselves differentiate into $CD4^+$ Tr cells (infectious tolerance) [17]. These phenomena imply that tolerant DCs may provide the environment promoting naturally existed $CD4^+$ Tr cell-mediated "infectious tolerance." Further study will be needed to test these possibilities.

Albert et al. [18] reported that DCs acquired Ag from apoptotic cells and activated MHC class I-restricted Tc cells. On the other hand, it has been suggested that DCs that have captured apoptic cells induce tolerance whereas DCs efficiently present the phagocytosed cellular fragments derived from necrotic cells to T cells [19]. In addition, Ferlazzo et al. [20] reported that DCs efficiently cross-primed MHC class I-restricted Tc cells when pulsed with both apoptotic and necrotic cells but not soluble cell-derived lysates. Although the reason for the discrepancy in the ability of DCs that have phagocytosed apoptotic cells to activate T cells via cross-presentation remains unclear, this phenomenon might be influenced by the status of DCs (e.g., maturity) or the character of apoptotic cells, such as their immunogenicity or ability to induce DC maturation.

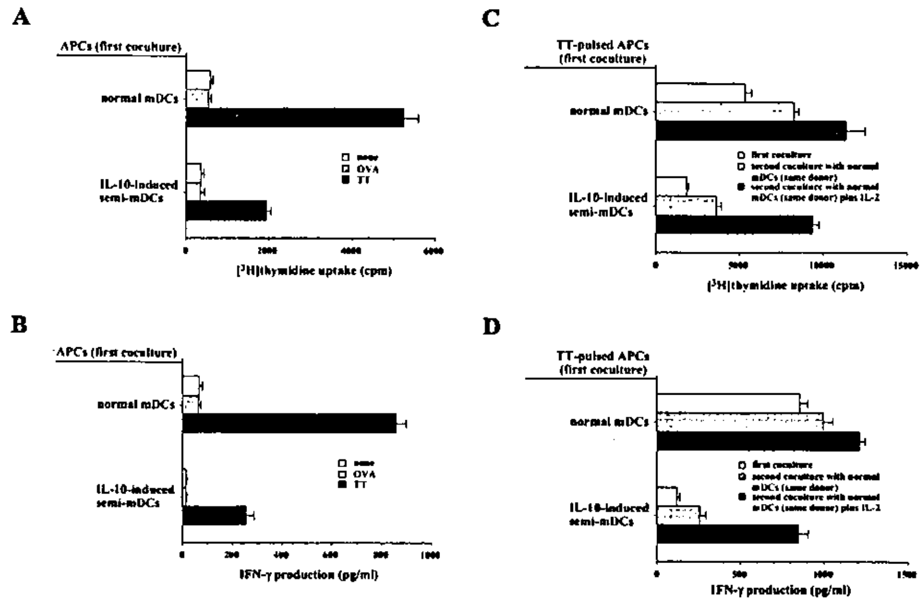


Fig. 4. Ag-pulsed IL-10-induced semi-mDCs induce an Ag-specific anergy in Th1 cells. (A, B) TT-primed Th1 cells were cocultured with unpulsed, TT-pulsed, or OVA-pulsed normal mDCs or IL-10-induced semi-mDCs (TNF-α-stimulated). (C, D) TT-primed Th1 cells were cocultured with TT-pulsed normal mDCs or IL-10-induced semi-mDCs (TNF-α-stimulated) in a first coculture. After 3 days, Th1 cells were rescued, cultured for 5 days in medium containing IL-2 (10 U/ml), and subsequently restimulated with TT-pulsed normal mDCs (TNF-α-stimulated) in the presence or absence of IL-2 (100 U/ml). Proliferation (A, C) and IFN-γ production (B, D) on day 5 were assessed. The results are representative of five experiments with similar results.

coculture (Figs. 4C and D). In contrast, TT-specific Th1 cells cocultured with TT-pulsed IL-10-induced semi-mDCs markedly reduced their activity in response to TT-pulsed mDCs in a second coculture (Figs. 4C and D). These results indicate that IL-10-induced semi-mDCs that had been pulsed with soluble proteins induce a state of tolerance in Ag-specific Th1 cells.

It has been reported that DCs internalized the cellular fragments, and processed them for cross-presentation to T cells [3–5]. We therefore tested whether IL-10-induced semi-mDCs that had been pulsed with allogeneic cellular fragments prepared from necrotic fibroblasts induced a state of tolerance in allogeneic fibroblast-specific $CD8^+$ T cells (Fig. 5). The established allogeneic fibroblast-specific $CD8^+$ T cells only showed a CTL activity when

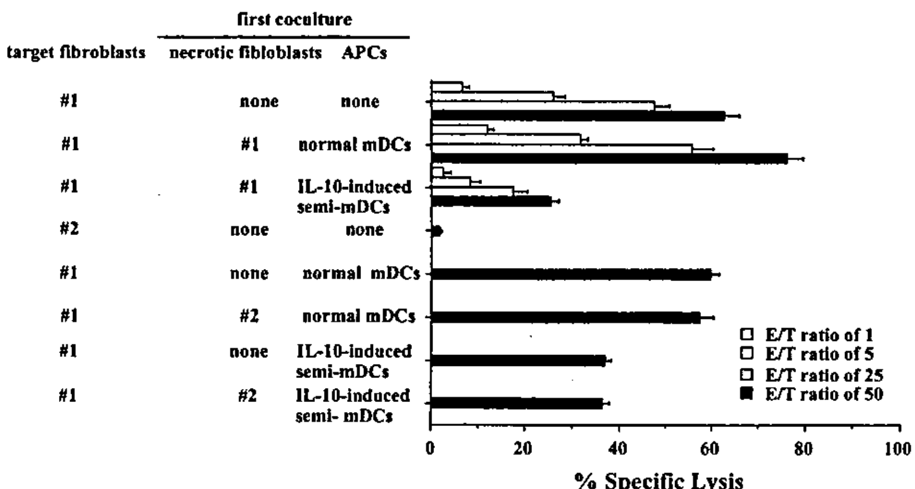
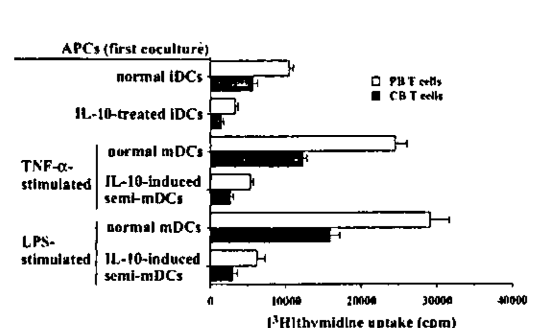


Fig. 5. Ag-pulsed IL-10-induced semi-mDCs induce a tolerance in allogeneic fibroblast-specific Tc cells. Allogeneic fibroblast-specific Tc cells were cocultured with or without normal mDCs or IL-10-induced semi-mDCs (TNF-α-stimulated) that had been pulsed with or without the necrotic fibroblasts used in the priming culture (#1) or unrelated donor-derived necrotic fibroblasts (#2). After coculture, Tc cells were rescued, cultured for 5 days in medium containing IL-2 (10 U/ml), and subsequently subjected to cytotoxicity assay against the allogeneic fibroblasts used in the priming culture (#1) or unrelated donor-derived necrotic fibroblasts (#2). The results are representative of five experiments with similar results.

A



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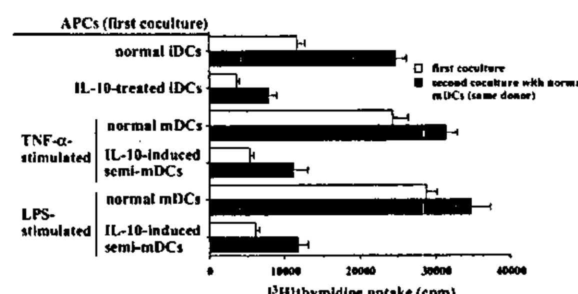

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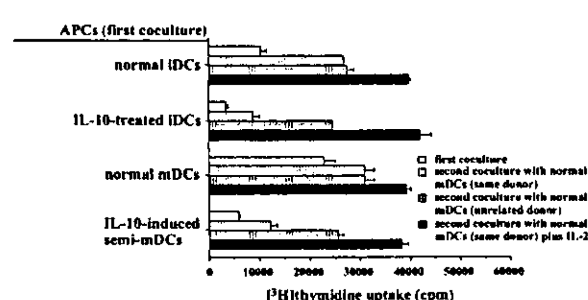

Fig. 2. Induction of an allogeneic Ag-specific anergy in T cells by IL-10-induced semi-mDCs. (A) PB T cells or CB T cells were cultured with or without the indicated types of DCs. (B) PB T cells were primed with or without the indicated types of DCs in a first coculture. After 3 days, T cells were rescued, cultured for 5 days in medium containing IL-2 (10 U/ml), and subsequently restimulated with normal mDCs (TNF-α-or LPS-stimulated) generated from the same donor. (C) PB T cells were primed with or without the indicated types of DCs in a first coculture. After 3 days, T cells were rescued, cultured for 5 days in medium containing IL-2 (10 U/ml), and subsequently restimulated with normal mDCs (TNF-α-stimulated) generated from the same donor or an unrelated donor in the presence or absence of IL-2 (100 U/ml) in a second coculture. [^{3}H]thymidine incorporation was determined after 5 days. The results are representative often experiments with similar results.

3.4. *Induction of tolerance in Ag-specific Th1 and Tc cells by Ag-pulsed IL-10-induced semi-mDCs*

Normal mDCs are effective for the presentation of the processed Ag to Ag-specific T cells leading to their activation as compared with normal iDCs [5]. In a first coculture, TT-pulsed normal mDCs, but not unpulsed

Table 2
The cell surface phenotype and the ability to produce cytokines in CD4$^+$ T cells following stimulation with allogeneic IL-10-induced semi-mDCs

Type of CD4$^+$ T-cell subsets ($n = 5$)	% Positivity		
	Cocultured with		
	Unprimed	Normal mDCs	IL-10-induced semi-mDCs
CD4$^+$CD25$^+$CD154$^+$	23 ± 1	31 ± 4	6 ± 2
CD4$^+$CD25$^+$CD152$^+$	3 ± 1	5 ± 3	23 ± 3
CD4$^+$IFN-γ$^+$	30 ± 4	63 ± 8	10 ± 4
CD4$^+$IL-2$^+$	25 ± 3	48 ± 6	5 ± 2
CD4$^+$IL-4$^+$	20 ± 4	12 ± 4	4 ± 2
CD4$^+$IL-10$^+$	8 ± 3	4 ± 2	23 ± 4

Unprimed or primed CD4$^+$ T cells recovered from the coculture with allogeneic normal mDCs or IL-10-induced semi-mDCs were assayed for the phenotype and the cytokine production by flow cytometry as described in Section 2.

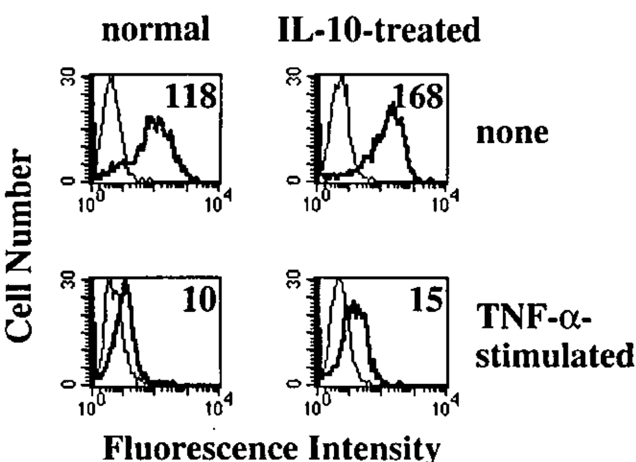

Fig. 3. Endocytosis of antigenic material by IL-10-induced semi-mDCs. The indicated types of DCs were cultured with medium alone (thin lines) or FITC-OVA (1 μg/ml) (thick lines). After a 60-min pulse at 37 °C, cells were washed and the internalization of FITC-OVA by cells was analyzed by flow cytometry. The values shown in the flow cytometric profiles are MFI, and the value of the background FITC-staining was less than 6. The results are representative of 10 experiments with similar results.

and OVA-pulsed normal mDCs, induced Ag-specific proliferation and IFN-γ production in TT-specific CD4$^+$ T cells, indicating that this activated CD4$^+$ T cells was type-1 Th (Th1) cells (Figs. 4A and B). On the other hand, TT-pulsed IL-10-pretreated mDCs elicited weak responses (Figs. 4A and B).

It has been reported that antigenic peptide-pulsed IL-10-treated DCs induced an Ag-specific anergy in T cells [8,9]. We therefore examined whether IL-10-induced semi-mDCs that had been pulsed with soluble antigenic protein induced an anergy in Ag-specific Th1 cells. TT-specific Th1 cells restimulated with TT-pulsed normal mDCs exhibited potent responses when TT-pulsed normal mDCs were used as APCs in a first

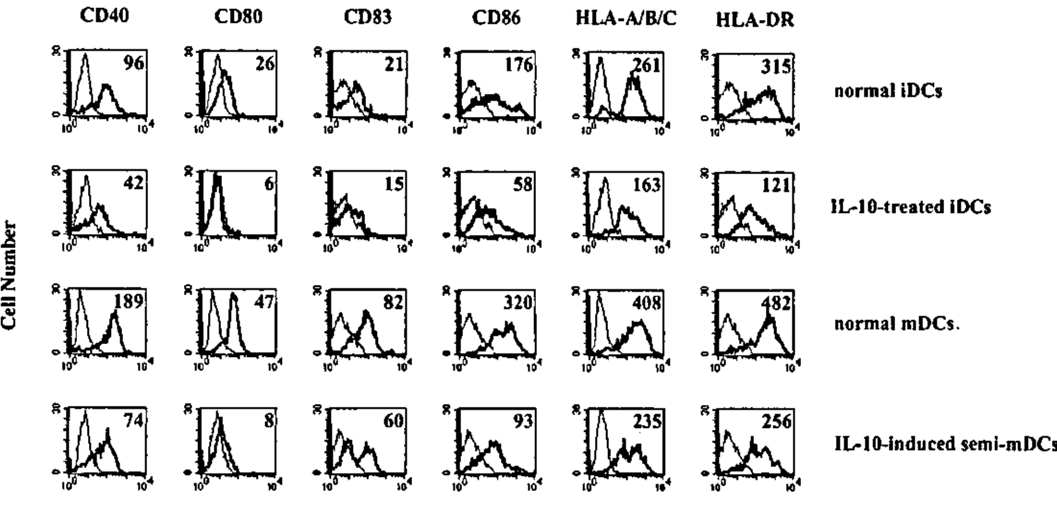

Fig. 1. Phenotypic profile of IL-10-induced semi-mDCs. Cells were stained with stated mAbs (thick lines) or isotype-matched mAb (thin lines). Cell surface expression was analyzed by FACS. The values shown in the flow cytometric profiles are MFI, and the value of the background FITC- or PE-staining was less than 6. The results are representative often experiments with similar results.

Table 1
Expression levels of MHC and costimulatory molecules in IL-10-induced semi-mDCs

Type of DCs (n = 10)	MFI					
	CD40	CD80	CD83	CD86	HLA-A/B/C	HLA-DR
Normal iDCs	92 ± 13	27 ± 4	22 ± 5	172 ± 14	275 ± 21	320 ± 18
IL-10-treated iDCs	40 ± 8	6 ± 1	14 ± 3	50 ± 12	158 ± 23	115 ± 18
TNF-α-stimulated normal mDCs	182 ± 15	56 ± 14	84 ± 10	338 ± 36	417 ± 18	512 ± 38
TNF-α-stimulated IL-10-induced semi-mDCs	65 ± 23	8 ± 2	62 ± 5	85 ± 21	245 ± 26	266 ± 27
LPS-stimulated normal mDCs	206 ± 32	75 ± 12	92 ± 13	368 ± 42	433 ± 28	554 ± 42
LPS-stimulated IL-10-induced semi -mDCs	68 ± 24	10 ± 4	72 ± 13	190 ± 19	267 ± 48	287 ± 36

The indicated types of DCs were stained with the stated mAbs or isotype-matched mAbs, and cell surface expression was analyzed by FACS. The value of the background FITC- or PE-staining was less than 6.

iDCs, allogeneic T cells primed with of IL-10-induced semi-mDCs were hyporesponsive to further stimulation with same donor-derived normal mDCs, but not with unrelated donor-derived normal mDCs. Furthermore, the addition of IL-2 to a second culture overcame the hyporesponsiveness. These results indicate that, like IL-10-treated iDCs, IL-10-induced semi-mDCs retain the ability to induce an anergy in T cells.

We also examined the cell surface phenotype in $CD4^+$ T cells recovered from the first coculture of $CD4^+$ T cells with allogeneic normal mDCs or IL-10-induced semi-mDCs (Table 2). Flow cytometric analysis showed that $CD4^+CD25^+CD154^+$ or $CD4^+CD25^+CD152^+$ subpopulations were naturally existed in $CD4^+$ T cells at approximately 3%, respectively. Stimulation with allogeneic normal mDCs increased $CD4^+CD25^+CD154^+$ subpopulations in the recovered $CD4^+$ T cells by approximately 10-fold. On the other hand, $CD4^+$ $CD25^+CD152^+$ subpopulations were detected in the recovered $CD4^+$ T cells at approximately 20% following the coculture with allogeneic IL-10-induced semi-mDCs.

We further examined the ability of the recovered $CD4^+$ T cells to produce cytokines (Table 2). The coculture with allogeneic normal mDCs increased IFN-γ- and IL-2-producing $CD4^+$ T-cell subsets whereas IL-4- and IL-10-producing $CD4^+$ T-cell subsets were decreased. On the other hand, IL-10-producing $CD4^+$ T-cell subsets were increased whereas IFN-γ -, IL-2-, and IL-4-producing $CD4^+$ T-cell subsets were decreased following the coculture with or IL-10-induced semi-mDCs.

3.3. The ability of IL-10-treated DCs to internalize soluble Ag

Normal iDCs capture Ags through their endocytic capacity via receptor-mediated endocytosis, clathrin-coated pits, and fluid phase endocytosis to present them to T cells [3–5]. Fig. 3 shows that IL-10-treated iDCs exhibited a higher ability to endocytose FITC-OVA than normal iDCs, and this ability was significantly reduced in both cell types.

cells or > 95% $CD3^+CD8^+$ cells) were washed twice with PBS, before being cultured in medium containing IL-2 (10 U/ml) for 5 days, and used for subsequent experiments.

2.7. Mixed leukocyte reaction (MLR) and Ag presentation assay

T cells and their subsets (10^5) were cultured in 96-well plates (Coster, Cambridge, MA) with irradiated (15 Gy from a ^{137}Cs source) unpulsed or Ag-pulsed autologous/allogeneic APC (10^4). Thymidine incorporation was measured on day 5 by an 18-h pulse with [^{3}H]thymidine (1 μCi/well, specific activity, 5 Ci/mmol; Amersham Life Science, Buckinghamshire, UK). In another experiment, the culture supernatants were collected, and assayed for interferon (IFN)-γ production by ELISA (Endogen, Woburn, MA). The sensitivity of the assay was >2 pg/ml.

2.8. Cytotoxicity assay

Allogeneic fibroblast-specific $CD8^+$ T cells ($10^4 - 5 \times 10^5$) were cultured with $Na_2{}^{51}CrO_4$ (100 μCi/10^6 cells, NEN Life Science Products, Boston, MA)-labeled allogeneic fibroblasts used in the priming condition or allogeneic fibroblasts derived from unrelated donor (10^4) for 4 h at various effector cell to target cell ratios (E/T ratios) from 1 to 50. The supernatants were harvested, the radioactivity was measured, and the percentage of specific lysis was calculated [12]. The value of spontaneous release cpm was less than 10% of the total release cpm.

2.9. Assay for T-cell anergy

To detect a T-cell anergy by DCs, the response of allogeneic T cells, which were recovered from a first coculture (an induction phase) with various types of DCs, were determined in a second coculture with normal mDCs (an effector phase) according to the previous reports [8,9] with some modifications. Alternatively, Ag-specific $CD4^+$ and $CD8^+$ T cells obtained from a priming culture of PBMCs with the respective Ag were stimulated with Ag-pulsed normal mDCs or IL-10-induced semi-mDCs in a first coculture (a booster phase). Subsequently, the responses of these T-cell subsets were examined in a second coculture with Ag-pulsed normal mDCs (for Ag-specific $CD4^+$ T cells) or the targeted fibroblasts (for Ag-specific $CD8^+$ T cells) in a second coculture (an effector phase). In these experiments, we collected similar numbers of T cells and their subsets from the first coculture, and we adjusted for the number of recovered T cells with similar viability for the second coculture. In brief, allogeneic PB T cells (5×10^6) were primed with irradiated immature or mature types of normal or IL-10-treated DCs (5×10^5) for 3 days. After incubation, these T cells were negatively selected with anti-CDllc mAb (BD PharMingen) in combination with anti-mouse IgG mAb conjugated immunomagnetic beads (Dynal, Oslo, Norway) and rested in medium containing IL-2 (10 U/ml) for 5 days. PB T cells (10^5) were restimulated with TNF-α- or LPS-induced normal mDCs (10^4) generated from the same donor used in the first culture or from another unrelated donor in the presence or absence of IL-2 (100 U/ml). Alternatively, $CD4^+$ T cells recovered from the first coculture of $CD4^+$ T cells with allogeneic normal mDCs or IL-10-induced semi-mDCs were assayed for the phenotype and the cytokine production by flow cytometry as described above. In another experiments TT-primed $CD4^+$ T cells or allogeneic fibroblast-specific $CD8^+$ T cells (5×10^6) were cocultured for 3 days with irradiated Ag-pulsed autologous normal mDCs or IL-10-induced semi-mDCs (5×10^5) in a first coculture (a booster phase). After incubation, these T cells prepared as described above were assayed for Ag-specific proliferation and lysis against allogeneic fibroblasts in a second coculture (an effector phase).

3. Results

3.1. Phenotypic profile and allogeneic T-cell stimulatory ability of IL-10-induced semi-mDCs

IL-10 converted iDCs into immunostimulatory inactive iDCs, which exhibited the reduced expressions of MHC and costimulatory molecules (Fig. 1 and Table 1) and stimulation of allogeneic T cells (Fig. 2A) compared to normal iDCs. Furthermore, this type of DCs induced an allogeneic Ag-specific anergy in T cells (Figs. 2B and C) [8,9]. We first examined the effect of inflammatory stimuli on the maturation-associated changes of IL-10-treated iDCs. Although TNF-α-or LPS-stimulated IL-10-treated iDCs expressed CD83 as a maturation marker for DCs [13], this expression level was lower than that in normal mDCs (Fig. 1 and Table 1). Unlike in normal mDCs, the stimulation with TNF-α and LPS failed to fully upregulate the expression of MHC and costimulatory molecules in IL-10-treated iDCs (Fig. 1 and Table 1), and these expressions paralleled the stimulatory ability of allogeneic T cells (Fig. 2A). Therefore, we referred to TNF-α-or LPS-stimulated IL-10-treated iDCs as IL-10-induced semi-mDCs in subsequent experiments.

3.2. Induction of allogeneic Ag-specific anergy in T cells by IL-10-induced semi-mDCs

We examined the ability of IL-10-induced semi-mDCs to induce an anergy in allogeneic T cells (Figs. 2B and C). Allogeneic T cells primed with normal iDCs and mDCs responded vigorously to restimulation with same donor-derived normal mDCs. Similar to IL-10-treated

erythrin (PE). Antihuman CD3, CD4, CD8, CD45RA, CD45RO, CD56, and HLA-DR mouse immunoglobulin G1 (IgG1) MoAbs (Becton Dickinson, Mountain View, CA, USA) were used. The cells were also stained with FITC- or PE-labeled isotype-matched IgG1 controls (Becton Dickinson) and thereafter analyzed with FACSCalibur and CellQuest software (Becton Dickinson).

For determination of the cytokine production profile of $CD4^{bright}$ helper T-cells, PBMNCs were separated after red blood cell lysis with FACS lysing solution (Becton Dickinson). These cells were incubated for 4 hours with 25 ng/mL phorbol myristate acetate (PMA), 1 μg/mL ionomycin (IoM), and 10 μg/mL brefeldin A; stained with phycoerythrin cychrome 5–labeled antihuman-CD4 MoAbs; and permeablized with FACS permeablizing solution (Becton Dickinson). FITC-labeled antihuman interferon γ (IFN-γ), PE-labeled antihuman interleukin 4 (IL-4) mouse MoAbs, and isotype-matched control antibodies (FastImmune Cytokine System, Becton Dickinson) were used for cytoplasmic staining. After washing, $CD4^{bright}$-gated events were analyzed.

2.3. Cytokine Induction from Stimulated PBMNCs

Cytokine production by patient-derived PBMNCs was assessed with a semiquantitative reverse transcription (RT)-PCR method. Amplification of 1 μg complementary DNA from PMA- and IoM-stimulated patient- and healthy volunteer–derived PBMNCs was performed with a SuperTaq Premix kit (Sawady Technology, Tokyo, Japan) with specific primers for β-actin, granulocyte-macrophage CSF (GM-CSF), IL-4, IL-6, IL-12p40, IL-12p35, IFN-γ, and tumor necrosis factor α (TNF-α) (Continental Laboratory Products, San Diego, CA, USA).

2.4. In Vitro Responsiveness to Mitogens

For the detection of mitogenic responses of patient-derived lymphocytes, the patient's PBMNCs were isolated by Ficoll density gradient, and 5×10^5 cells were incubated in each well of a 96-well plate, stimulated with 20 μg/mL phytohemagglutinin (PHA), or 7 μg/mL concanavalin-A (ConA) for 64 hours and then pulsed with 12.5 μCi ^3H-labeled thymidine for 8 hours. Pulsed cells were harvested and the β irradiation was measured with a scintillation counter. Normal values for mitogenic responses among volunteer donor PBMNCs were 26,000 to 53,000 counts per minute for PHA-L stimulation, and 20,000-48,000 counts per minute for ConA stimulation.

2.5. Allogeneic Mixed Leukocyte Reaction

Allogeneic mixed leukocyte reaction (allo-MLR) with the patient's PBMNCs 136 days after CBT was performed as described below. Patient-derived PBMNCs (5×10^4 cells) or those derived from 3 healthy HLA-DRB1–disparate donors were irradiated with 150 Gy from an electric x-ray source (MBR-1505 R2, Hitachi, Tokyo, Japan) as stimulator cells and cocultured with 5×10^4 donor- or patient-derived PBMNCs as responder cells for 5 days in 96-well plates and then pulsed with 1 μCi ^3H-labeled thymidine for 8 hours. Pulsed cells were harvested, and the β irradiation was measured.

2.6. Immunoglobulin Production

The patient's serum Ig concentrations were monitored. Intravenous Ig was administered as a supplement to maintain a serum IgG level higher than 1000 mg/dL. Endogenous Ig production was monitored by measurement of the serum concentration of IgA and IgM.

3. Results

3.1. Flow Cytometry

Results of phenotypic analyses of the patient's PB T-cells are presented in Figure 1. Marked proliferation of $CD3^+CD56^+$ natural killer (NK) T-cells and $CD3^-CD56^+$ NK cells were recognized on day 48 after CBT concurrently with graft rejection (Figure 1A, B). This finding suggested that cellular immune responses were activated. These types of cells may have a potential effect on the cell-mediated graft rejection and elimination process of DNA-impaired cells in the course of ARS, because they have cytotoxic effects involving the killer inhibitory molecules and major histocompatibility complexes (MHCs). Relatively higher percentages of $CD45RA^+$-naive T-cells were documented throughout the course (Figure 1A, C). Mature memory T-cells may be abrogated after fatal neutron irradiation, and newly generated and differentiated T-cells from hematopoietic progenitors were dominant, reflecting recipient immune immaturities.

Intracellular staining of $CD4^{bright}$ helper T-cells showed dominant IFN-γ production and $IFN\text{-}\gamma^+$ $IL\text{-}4^-$ populations throughout the course (Figure 2). Interestingly, IFN-γ production was transiently activated concomitant with graft rejection. These results indicate the existence of the helper T-cell subtype 1 (Th1)-dominant Th1/Th2 axis and suggest cell-mediated immune function overcomes humoral immunity. IFN-γ also may play an important role in graft rejection and DNA-impaired cell elimination.

3.2. Cytokine Induction from Stimulated PBMNCs

We detected transcriptional expressions of GM-CSF, IL-4, IL-6, IL-12p40, IL-12p35, IFN-γ, and TNF-α messenger RNA (mRNA) after stimulation with PMA and IoM by the RT-PCR method. However, the expression levels did not differ from those of the healthy volunteer donor (data not shown). Thus there were no obvious differences in cytokine mRNA expression of PBMNCs between the patient and healthy donors after stimulation.

3.3. In Vitro Responsiveness to Mitogens

To clarify the functional activation and maturation of the patient-derived T-cells, we examined the mitogenic responses of these T-cells in vitro (Figure 3). Patient-derived T-cells did not respond to mitogens such as PHA or ConA. This finding

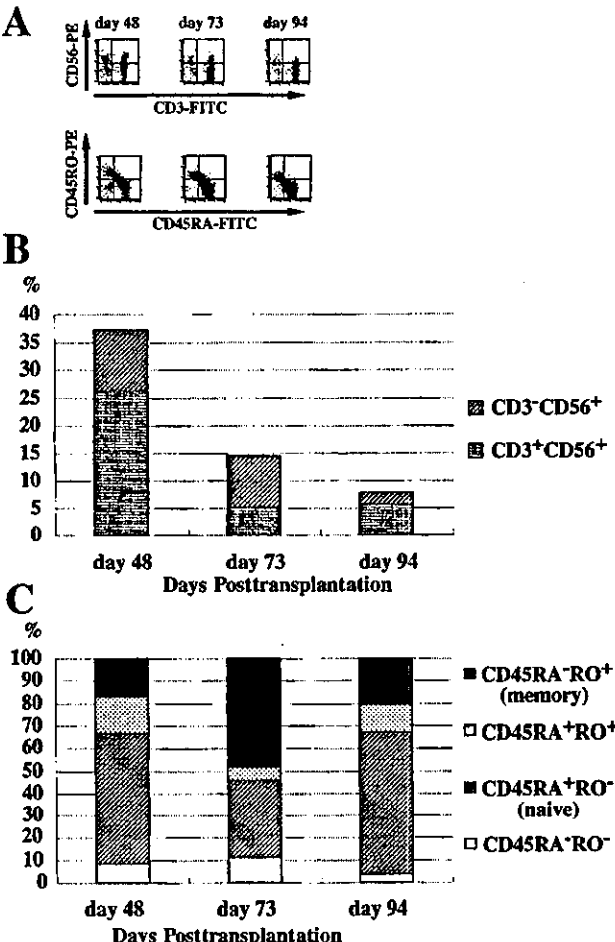

Figure 1. A, Flow cytometric analysis of patient-derived peripheral blood mononuclear cells (PBMNCs). Patient PBMNCs were separated with Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden), stained with monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE), and thereafter analyzed with FACSCalibur and CellQuest software (Becton Dickinson, Mountain View, CA, USA). B, Lymphocyte subsets expressing $CD3^-CD56^+$ (natural killer cell subset) and $CD3^+CD56^+$ (natural killer T-cell subset) cells. C, Lymphocyte subsets expressing $CD45RA^+CD45RO^-$ (naive) and $CD45RA^-CD45RO^+$ (memory) cells.

suggested that the patient's immune responses were severely impaired.

3.4. Allogeneic Mixed Leukocyte Reaction

Even more surprisingly, an allo-MLR with HLA-DRB1–disparate unrelated healthy volunteer donors showed the patient-derived PBMNCs to be unresponsive to stimulation with allogeneic antigen-presenting cells and to have impaired antigen-presenting capacities against allogeneic responder cells (Figure 4). These results indicate that the allogeneic antigen presenting and responding capacities of autologous recovered PBMNCs were completely abrogated and that tolerable acceptance of transplanted cadaver donor–derived skin grafts ensued.

3.5. Immunoglobulin Production

Endogenous Ig-producing capacity measured as the serum IgA and IgM concentrations were suppressed until 120 days after the accident (Figure 5). Intrinsic IgA and IgM production transiently increased in concordance with autologous hematopoietic recovery. Thereafter intrinsic production decreased and stabilized at a lower level until the patient began to suffer from fatal pneumonia. These results suggest that the patient's immune system was fatally impaired despite the number of autologous hematopoietic cells recovered.

4. Discussion

We present the case of a male adult, lethally irradiated by mixed γ-rays and fast neutrons in the criticality accident in Tokaimura [18-23]. Determination of the irradiation dose is a complex problem with mixed γ-ray and neutron radiation, because neutrons cause nonuniform irradiation owing to rapid attenuation through the human trunk. Neutrons, heavy particles, are absorbed by water and release an enormous amount of atomic energy in the penetrated human body. The biological effects of neutrons may be more severe on the immune system than are those of γ-rays. Koike and Ando, using a murine model, described the relative biological effects (RBE) of fast neutrons [24]. They determined the RBE of neutrons were 1.7-fold (mean 1.70; 95% CI, 1.66-1.74) those of γ-rays, according to the median lethal dose within 30 days ($LD_{50/30}$) of murine gut toxicity. However, bone marrow toxicity was 1.16-fold (mean 1.16; 95% CI, 1.14-1.18) of γ-rays in their experiments. Our patient absorbed 3.0 Gy of neutrons and 4.7 Gy of γ-rays according to the dose estimation of ^{24}Na in the PB sample [19], and it was calculated as 9.8 GyEq by RBE 1.7 on the basis of the results of the experiment with gut toxicity. However, the absorbed dose was estimated as 8.2 GyEq with the use of RBE 1.16 of bone marrow $LD_{50/30}$. The unexpected autologous recovery may be ascribed to nonuniform irradiation by neutrons and overestimation of absorbed dose with gut toxicity–oriented dosimetry. Although the estimated bone marrow toxicity was lower than gut toxicity, autologous recovered hematopoiesis possessed severely impaired immune function in vitro. These findings suggest that the immune function of radiation victims is difficult to restore by autologously recovered hematopoiesis.

Irradiation with 12 Gy or more results in death from non–bone marrow toxicities. The survival advantage of HSCT is limited to a narrow dose window from 8 to 12 Gy. Findings after the accident at the Chernobyl nuclear power plant suggested that hematological intervention with HSCT for ARS had only a limited role in the treatment of radiation victims [1,2]. These limitations stemmed from a lack of HLA-matched donors, a requirement for additional immunosuppression, and the risk of GVHD, which worsen the ARS. To overcome these limitations, optimal reduction of the risk of acute GVHD is

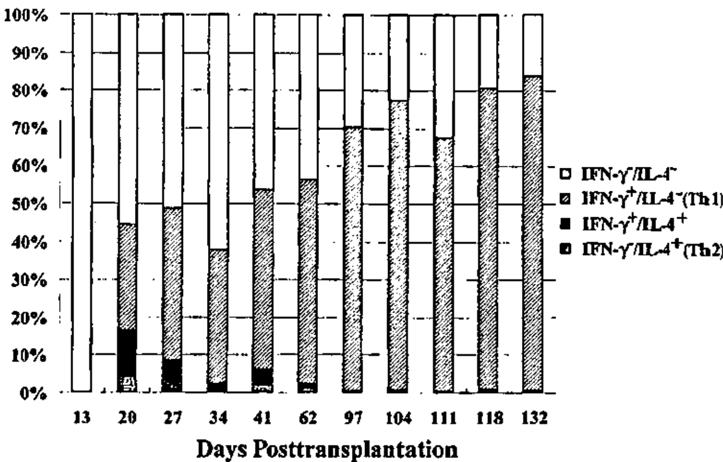

Figure 2. Cytoplasmic staining of interferon γ (IFN-γ) and interleukin 4 (IL-4) in the CD4^{bright} gate. The patient's peripheral blood mononuclear cells were stimulated with phorbol myristate acetate and ionomycin under the treatment of brefeldin A and stained with fluorescein isothiocyanate–labeled antihuman IFN-γ mouse monoclonal antibody (MoAb), phycoerythrin (PE)-labeled antihuman IL-4 mouse MoAb, and PE cychrome 5–labeled antihuman-CD4 MoAb. CD4^{bright} gate was analyzed with FACSCalibur and CellQuest Software (Becton Dickinson, Mountain View, CA, USA). Th1 and Th2 indicate helper T-cells subtypes 1 and 2.

preferred. We selected CB as the source of HSCT to avoid donor acquisition delay and fatal GVHD. In many clinical studies, a lower incidence and lesser extent of acute GVHD have been reported even in HLA 1-locus to 3-loci mismatched unrelated donor/recipient parity in CBT [5-9,11,12,14,15]. The feasibility of CBT for adult recipients has been established in cases with a sufficient number of stem cells infused [13-17]. We previously described the reduced cytotoxic activities and Fas-ligand (FasL) cell surface expression on CB T-cells after CD3 and CD28 costimulation compared with those on adult PB T-cells in vitro [25]. Less stringent donor/recipient HLA compatibility allows the mismatched CBT and smaller CB donor pool as the store for emergency.

Donor/recipient mixed chimerism induces a significant advantage in the treatment of ARS and avoids the risk of GVHD [26]. Table 1 shows the comparative theoretical hypothesis of each chimeric state. Autologous recovery needs no conditioning or extended immunosuppression, and it carries no risk of GVHD, although immune function is severely impaired, as in our case. Complete chimerism carries the risk of worsening of regimen-related toxicity and GVHD, although immune function may be normal. Stable

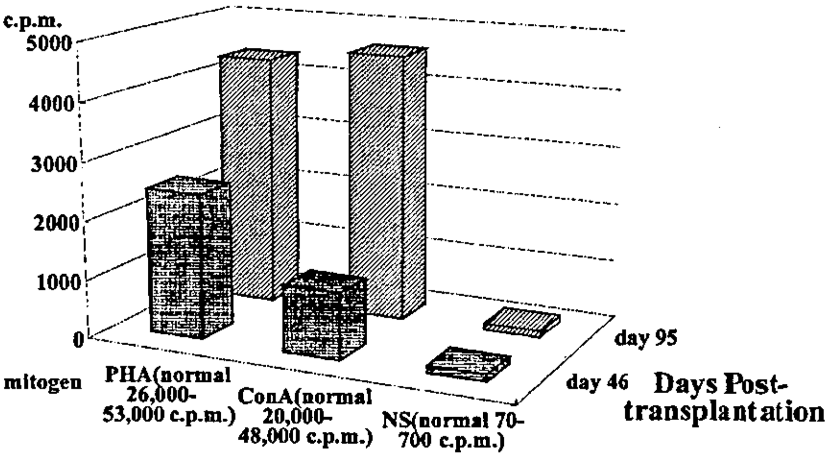

Figure 3. In vitro responsiveness to mitogens. The patient's peripheral blood mononuclear cells were isolated by Ficoll-Hypaque technique (Pharmacia Biotech, Uppsala, Sweden), and 5×10^5 cells were incubated in each well of a 96-well plate, stimulated with 20 μg/mL phytohemagglutinin (PHA) or 7 μg/mL concanavalin-A (ConA) for 64 hours, and then pulsed with 12.5 μCi ^{3}H-labeled thymidine for 8 hours. Incorporation of ^{3}H was measured with a β-scintillation counter. NS indicates normal saline solution.

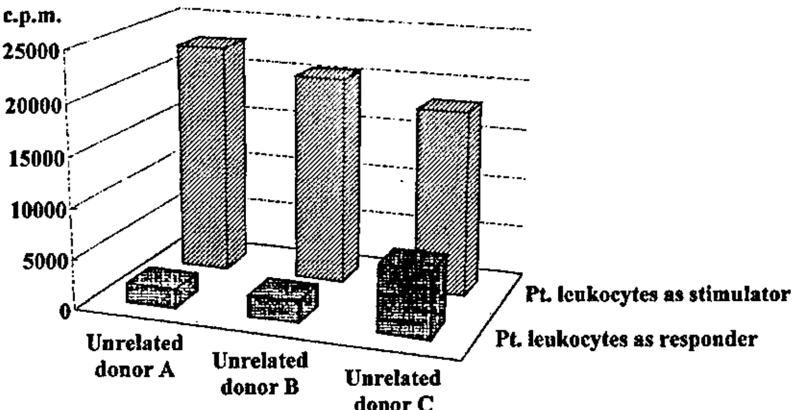

Figure 4. Allogeneic mixed leukocyte reaction. On day 136 after cord blood transplantation, patient- or HLA-DRB1–disparate healthy volunteer donor-derived peripheral blood mononuclear cells (PBMNCs) (5×10^4 cells) were irradiated with 150 Gy (electric x-ray source; MBR-1505 R2, Hitachi, Tokyo, Japan) as stimulator cells and cocultured with 5×10^4 donor- or patient-derived PBMNCs as responder cells for 5 days in the 96-well plate then pulsed with 1 µCi/well ^{3}H-labeled thymidine for 8 hours. Incorporation of ^{3}H was measured with a β-scintillation counter (c.p.m.). Pt. indicates patient.

mixed chimerism needs a lower intensity of conditioning, carries no risk of GVHD, and may promote moderate immune function despite the necessity for further extended immunosuppression. Radiation burns progressed after the recovery of autologous granulocytes in our experience. Appropriate immunosuppression may be mandatory to hamper the worsening of ARS.

Probable disadvantages of the use of CB sources are insufficient stem cell doses for adult recipients, resulting in delayed hematopoietic recovery, and the high incidence of graft rejection. Greater volume of donor doses for CB banks, appropriate conditioning with posttransplantation immunosuppression, use of multiple units of CB, and augmentation of stem cell expanding/homing capacities are mandatory for the successful engraftment of CB. Another victim in this accident, who was irradiated at a higher dose (16-24.5 GyEq), underwent transplantation with PB stem cells mobilized from an HLA-matched sibling with G-CSF. He obtained rapid hematological recovery and donor-derived complete chimerism. The granulocyte count rose to more than 500/mm^3 within 16 days after the accident (25 days in our case). However, the patient died within 82 days after the accident of GI bleeding and burns. Transplanted skin grafts failed to engraft in that case [27]. ARS worsened immediately after engraftment of donor-derived hematopoiesis; this finding suggested that granulocytes had a potential role in the worsening of ARS. FasL, which is expressed on the T-cell surface, also may be involved in the cell-mediated cytotoxicity

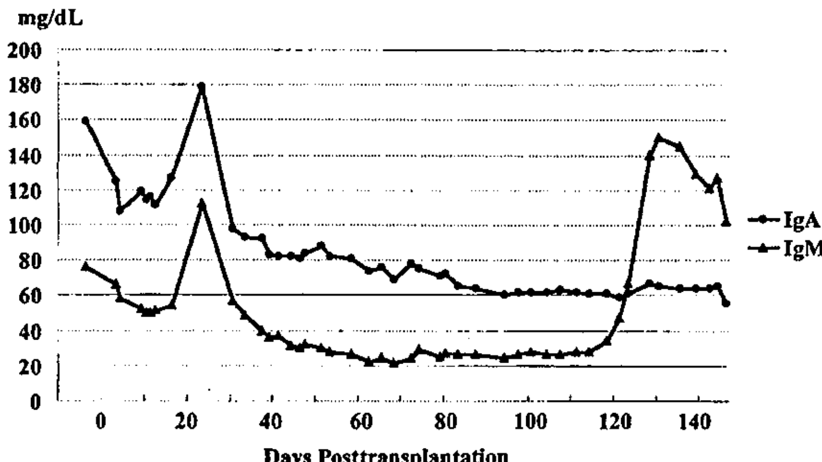

Figure 5. Endogenous immunoglobulin (Ig) production. Serum Ig concentrations of the patient were monitored. Intravenous Ig was supplemented to maintain the serum IgG level higher than 1000 mg/dL. Endogenous Ig production was monitored by measurement of the serum concentrations of IgA and IgM (mg/dL).

Table 1.
Comparison of Autologous Hematopoietic Recovery and Donor-Derived Chimerism*

Aim of Transplantation/ Target Point	Intensity of Conditioning	Risk of GVHD/ Worsening of ARS	Need Extended Immunosuppression	Immune Function
Autologous recovery [21-23]	No or less†	None†	No†	Dysfunctional‡
Stable mixed chimerism	Mild	None†	Yes‡	Moderate
Complete chimerism [27]	Intensive‡	Potent‡	No†	Normal†

*GVHD indicates graft-versus-host disease; ARS, acute radiation syndrome.
†Advantage for the treatment of ARS.
‡Disadvantage involving the treatment of ARS.

of ARS. Delayed recovery of granulocytes and reduced expression of FasL on the CB T-cell surface [25] may ameliorate ARS after CBT.

The results of immunological examination of this patient are summarized below. A transient increase in NK/NKT-cells immediately after CBT and a relatively high proportion of naive T-cells were documented (Figure 1). Th1 were dominant throughout the course, compared with Th2 (Figure 2). NK cells, Th1, and their functional product IFN-γ play an important role in organ damage in ARS. These types of cells may also contribute to the eradication of irradiated DNA-damaged cells and transplanted organ grafts, because they were activated at the time of CB graft rejection and worsening of ARS. More surprisingly, heavily irradiated autologous marrow-derived PB showed a defective mitogenic response (Figure 3) and severely impaired allo-MLR (Figure 4). These dysfunctional immune responses against mitogens and alloantigens may contribute to the durable engraftment of transplanted cadaver donor–derived skin grafts. Endogenous Ig production was activated at the initiation of autologous hematopoietic recovery but gradually decreased and was markedly suppressed until 120 days after CBT (Figure 5). These findings suggest that appropriate immunosuppression is mandatory for the hematological intervention of ARS to avoid worsening of ARS and to encourage maintenance of stable mixed chimerism. Immune function was relatively well preserved during the mixed chimeric state immediately after CBT, but severely impaired immune function was documented after autologous hematopoietic recovery.

In summary, fatal mixed neutron and γ-ray irradiation cause severe impairment of immune function examined in vitro after autologous hematopoietic recovery, although autologous hematopoietic recovery has been recommended on the basis of experience in Chernobyl. Complete chimera carries a potential risk of GVHD and worsening of ARS [27]. Stable mixed chimerism obtained with CBT may become an ideal platform for minimizing the risk of GVHD and worsening of ARS. Recently, a nonmyeloablative conditioning regimen with multiple units of CB had promising results in phase I clinical trials, and that strategy is optimal for the treatment of radiation victims (J.E. Wagner, University of Minnesota, personal communication). Further investigation is needed into hematological intervention in ARS and for rescue from the non–bone marrow toxicities affecting the lungs and GI tract that influence clinical outcome in cases of higher-dose irradiation. The urgent progress of regeneration biology is warranted.

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Cord blood stem cells

Transient hematopoietic stem cell rescue using umbilical cord blood for a lethally irradiated nuclear accident victim

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Summary:

We performed stem cell rescue and allogeneic skin transplantation on a lethally neutron-irradiated nuclear accident victim. HLA-DRB1 mismatched unrelated umbilical cord blood cells (2.08 × 10^7/kg recipient body weight) were transplanted to an 8–10 Gy equivalent neutron-irradiated patient because of a lack of a suitable bone marrow or peripheral blood donor. Pre-transplant conditioning consisted of anti-thymocyte γ-globulin alone, and GVHD prophylaxis was a combination of cyclosporine (CYA) and methylprednisolone (mPSL). Granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), and thrombopoietin (TPO) were concurrently administered after transplantation. The absolute neutrophil count reached 0.5 × 10^9/l on day 15, the reticulocyte count rose above 1% on day 23, and the platelet count was over 50 × 10^9/l on day 27, respectively. Cytogenetic studies of blood and marrow showed donor/recipient mixed chimerism. Rapid autologous hematopoietic recovery was recognized after withdrawal of CYA and mPSL. Repeated pathological examinations of the skin revealed no evidence of acute GVHD. Eighty-two days after the irradiation, skin transplantation was performed to treat radiation burns. Almost 90% of the transplanted skin engrafted. Immunological examination after autologous hematopoietic recovery revealed an almost normal T cell count. However, immune functions were severely impaired. The patient died from infectious complication 210 days after the accident.

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Keywords: umbilical cord blood; hematopoietic stem cell; skin transplantation; neutron irradiation; acute radiation syndrome

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The feasibility and efficacy of allogeneic hematopoietic stem cell transplantation for accidentally irradiated individuals remain unclear because of the high incidence of transplant-related mortality including acute graft-versus-host disease (GVHD) and ensuing complications, that are exacerbated by coincident radiation injury.[1] Target organs of acute GVHD are mainly the skin, gastro-intestinal tract, and liver, which are also susceptible to acute radiation syndrome (ARS).[2] Main causes of death after allogeneic bone marrow transplantation for irradiated individuals are burns, acute GVHD and interstitial pneumonitis with or without adult respiratory distress syndrome (ARDS).[1,2]

Umbilical cord blood is a useful stem cell source because the incidence and extent of acute GVHD associated with its use are low as compared to conventional bone marrow,[3–10] allowing for three HLA loci donor/recipient mismatching.[4,7,8] In addition, umbilical cord blood can be used for urgent triage for numerous victims without donor acquisition delay, because it has already been screened and cryopreserved. Thus, umbilical cord blood is an ideal stem cell source for accidentally irradiated individuals.

Neutrons cause severe cutaneous blistering and desquamative changes on irradiated skin fields several weeks after irradiation.[2] Systemic neutron irradiation induces fatal systemic destruction of the skin barrier that plays an important role in host defense against exogenous pathogens.

In the present study, we examined the feasibility and efficacy of unrelated HLA-disparate umbilical cord blood and cadaver skin transplantation for the rescue of a lethally irradiated recipient. We also examined donor/recipient chimerism and immunological reconstitution in the early phase of umbilical cord blood transplantation (CBT) for ARS.

Case report

A 39-year-old male, who had been lethally irradiated with neutrons at 8–10 Gy equivalent (Eq) in a nuclear accident,[11] was referred to our hospital on 4 October 1999 for hematopoietic stem cell transplantation. He had been irradiated by a critical fission reaction at the uranium-processing plant by JCO, Co., Ltd, in the village of Tokai in the Ibaraki Prefecture, Japan, on 30 September 1999. Symptoms

Table 1 Dosimetry of the patient based on (A) lymphocyte count nomogram, (B) time of ^{24}Na decay from peripheral blood samples, and (C) ringed chromosome analysis. The relative biological effect of neutrons was calculated as 1.7-fold that of γ-rays. (R.B.E., 1.7)

(A) Estimated irradiated dose using decreased curve of lymphocyte count	6–10 GyEq
(B) Estimated irradiated dose calculated by the half-life of ^{24}Na contained in the peripheral blood	10.4 GyEq
(C) Estimated irradiated dose using ringed chromosome analysis	7.8 GyEq

immediately after the accident were vomiting within 30 min, redness and edema of the skin, and marked lymphocytopenia with granulocytosis on examination of the blood. His consciousness level was normal. Bone marrow examination suggested that there was little hope of autologous hematopoietic reconstitution, and the same results were suggested by dosimetry (Table 1).

We intended to treat him with stem cell transplantation to avoid infection during the granulocytopenic period of ARS.[2] However, there was no suitable related bone marrow or peripheral blood stem cell donor, and several months would have been required to co-ordinate an unrelated donor from the Japan Marrow Donor Registry. We searched for an HLA-matched stem cell source at the Japan Cord Blood Bank Network, with permission from the Ministry of Health and Welfare, Japan. We finally found an HLA-DRB1 one locus-mismatched unrelated umbilical cord blood in the Tokai Cord Blood Bank, a member of the Japan Cord Blood Bank Network. Characteristics of this umbilical cord blood are shown in Table 2. It was serologically negative for HBV, HCV, HIV1/2, ATLL, and syphilis. Both the maternal and cord blood were negative for CMV-IgG and CMV-IgM (examined by ELISA; enzyme-linked immuno-solvent assay). Physical examination and screening tests for inherited disorders of the donor infant were normal 6 months after delivery. The institutional review board consented to this transplant. After signed informed consent had been obtained, the umbilical cord blood was transplanted. Before the transplant, both sternal and iliac bone marrow became markedly hypocellular. The bone marrow nucleated cell counts decreased to 0.5×10^9/l and 0.7×10^9/l (normal 7–20 $\times 10^9$/l in our institute), respectively.

Conditioning consisted of anti-thymocyte equine γ-globulin (ATG) (2.5 mg/kg/day) for two successive days (total 5 mg/kg). No cytotoxic drugs were used to avoid regimen-related toxicities. The dose of ATG was modified to a half of the conventional dose to avoid delay in lymphocyte recovery during the post-transplant period. After conditioning, a total of 2.08×10^7 umbilical cord blood-derived mononuclear cells was infused. GVHD prophylaxis consisted of cyclosporine (CYA; 3 mg/kg once daily i.v. infusion over 10 h from the day before CBT; day −1) and methylprednisolone sodium succinate (mPSL; 4 mg/kg i.v. on day −3, 2 mg/kg from day −2 to day 0). These drug doses were gradually tapered (see Figure 1a). Granulocyte colony-stimulating factor (G-CSF) (5 μg/kg/day from day −4, and 10 μg/kg/day from day −1 to day 16), erythropoietin (EPO) (100 IU/kg/day from day −1 to day 20), and thrombopoietin (TPO) (5 μg/kg/day from day 3 to day 16) were concurrently administered intravenously to assist in a rapid recovery of tri-lineage hematopoiesis. On day 15, the neutrophil count reached 0.5×10^9/l. We therefore considered that the umbilical cord blood had successfully engrafted. The reticulocyte count rose above 1% of total red blood cells on day 23, and the platelet count reached 50×10^9/l on day 27, respectively (Figure 1a–c).

On day 9, the fluorescence *in situ* hybridization technique using X- and Y-chromosome-specific probes (Y-probe FISH) showed that almost half of the cells analyzed had donor-derived XX signals and the others recipient-derived XY signals, indicating that mixed chimerism was present (Figure 2a–c). Throughout the post-transplant course, no evidence of acute GVHD was seen, but blistering of the right hand developed, rapidly extending to both the forearms and plantars. Repeated examination of cutaneous (right hand and leg on day 24, right forearm, right shank, right buttock, and abdominal skin on day 40, and both hips on day 94), and gastro-duodenal mucosae (day 40) revealed no evidence of acute GVHD. As we had obtained mixed chimerism that could induce immunological tolerance between donor and recipient, we reduced the doses of CYA

Table 2 HLA typing of recipient, umbilical cord blood donor, and skin-graft donor. Condensed type shows mismatched HLA antigen, and italic type shows high-resolution DNA typing using the PCR-SBT method

	Recipient		*Umbilical cord blood donor*		*Skin-graft donor*	
HLA-A	A11	A24	A11	A24		
	1101	*2402*			*2402101*	
HLA-B	B52	B62	B52	B62		
	5201	*15011*			*15011*	
HLA-C	Cw9		Cw3	Cw12		
	w0303	*w1202*				
HLA-DRB1	**DR14**	DR15	**DR13**	DR15		
	1406	*1502*	***1302***	*1502*	**04051**	**15011**

Characteristics of infused cord blood.
Collected volume 114 ml at collection (recipient BW 66 kg).
Total nucleated cells 2.08×10^7/kg.
CFU-GM 4.61×10^4/kg.
$CD34^+$ cells (at thawing) 7.11×10^4/kg.

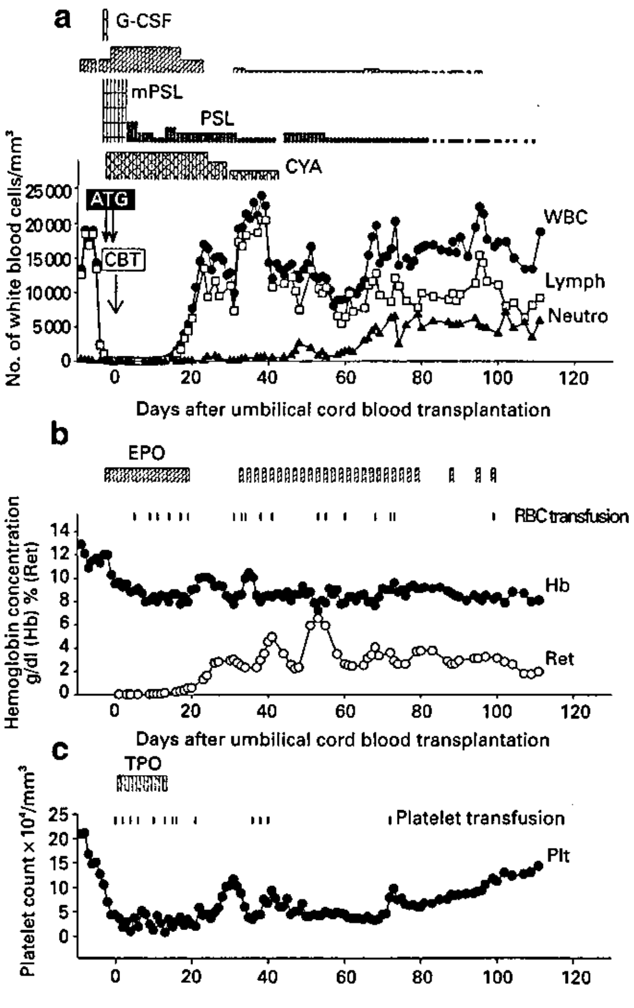

Figure 1 Treatment course and hematopoietic recovery from acute radiation syndrome. (a) Medication and recovery of white blood cells: •, white blood cell count; ▲, neutrophil count; □, the lymphocyte count; G-CSF, granulocyte colony-stimulating factor; mPSL, methylprednisolone; PSL, prednisolone; CYA, cyclosporine; ATG, anti-thymocyte equine gamma-globulin; CBT, umbilical cord blood transplantation. (b) Hemoglobin concentration and reticulocyte count: ●, hemoglobin concentration; O, reticulocyte count; EPO, erythropoietin; |, transfusion of red blood cells. (c) Platelet count: ●, platelet count; TPO, thrombopoietin; |, transfusion of platelets.

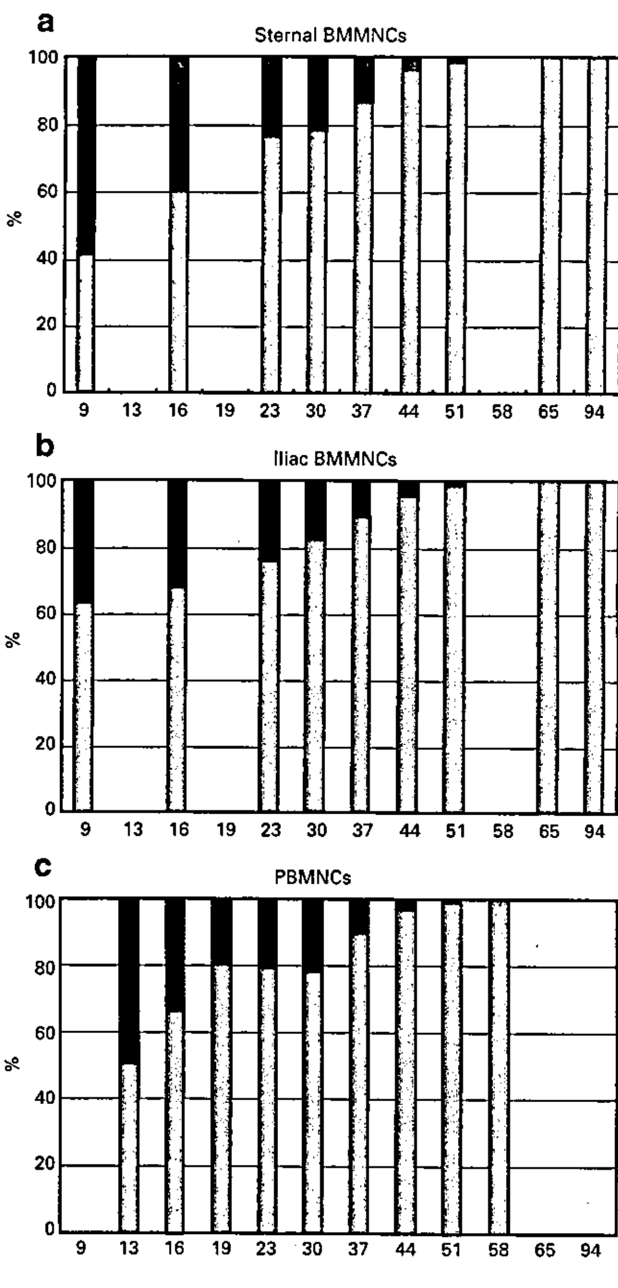

Figure 2 The chimeric state analysis of the patient-derived BM and PBMNCs using fluorescence-labeled *in situ* hybridization of the X and Y chromosome-specific probes: solid columns, percentages of donor-derived XX signals; shaded columns, percentages of recipient-derived XY signals. (a) Analysis of sternal bone marrow (sternal), (b) iliac bone marrow (iliac), and (c) peripheral blood mononuclear cells (PBMNCs).

and mPSL to ameliorate the post-transplant immunosuppressive state. Thereafter, unexpected rapid autologous hematopoietic recovery occurred, and almost all the PBMNCs analyzed developed recipient-derived XY signals by Y-probe FISH up to 50 days after CBT.

In spite of there being no evidence of acute GVHD, neutron-burn induced blistering and desquamation occurred. Deep dermal neutron burns progressed to cover 67% of the total body surface area (BSA) including the face, and both upper and lower extremities from day 60 to day 80 after CBT. Palliative care including skin-dressing and hygiene treatment was not effective and methicillin-resistant *Staphylococcus aureus* (MRSA) was isolated from skin exudates. In order to relieve the pain, the exudates, and the infections of the desquamated skin lesions, we transplanted skin from a cadaver donor to both the victim's hands and arms (15% of BSA) on day 72 after CBT. Allogeneic cultured skin grafts were also transplanted to both legs (20% of BSA) on day 80 after CBT, and to the face (10% of BSA) on day 114. Almost 90% of this skin engrafted, relieving the pain, massive exudates, and infection.

On day 131 after CBT, his stool became positive for occult blood, and thereafter he developed progressive anemia. Finally, he became red blood cell transfusion depen-

dent. Gastric fiberoptic examination revealed acute gastric mucosal lesions in the antrum of the stomach causing diffuse bleeding. He suffered from obstructive sleep apnea due to ARS-related stomatitis, and clot formation in his buccal cavity. He aspirated the purulent exudates from the nasal cavity and developed MRSA pneumonia on day 153 after the accident. Acute renal failure developed as a result of the long-term administration of nephrotoxic antibiotics, including vancomycin and arbekacin. He was diagnosed as having ARDS based on the criteria outlined at a consensus conference of ARDS. We failed to resuscitate him with a tracheotomy, the open lung approach based on the protective ventilation strategy, and the use of mPSL 2 mg/kg/day. In spite of all our efforts, he died from respiratory and multi-organ failure on day 201 after CBT (day 210 after the accident).

Patient and methods

Patient materials

All materials examined were collected from this patient after signed informed consent, and used with his permission.

Dosimetry

Dosimetry was based on the lymphocyte count, blood ^{24}Na concentration, and chromosome analysis of peripheral blood mononuclear cells, as described previously.[2,11]

Analysis of chimerism

A fluorescence-labeled *in situ* hybridization (FISH) technique using X and Y sex chromosome-specific probes was employed to examine bone marrow (BM) and peripheral blood (PB) mononuclear cells (MNCs). Chromosomal analyses of BM and PBMNCs were also performed. On day 20 after transplantation, we separated the peripheral blood mononuclear cells by density gradation using Lymphoprep LY (Nycomed, Oslo, Norway), Percoll LY (Amersham Pharmacia Biotech, Uppsala, Sweden), and Percoll GR (Amersham Pharmacia Biotech). After the separation, we analyzed the karyotypes of the separated mononuclear cells and granulocytes.

Monitoring of immunological reconstitution

An absolute lymphocyte count, including CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$, was carried out using a Biometric IMAGN 4T8 assay kit (Becton Dickinson, Mountain View, CA, USA). Cytokine production by patient-derived PBMNCs was assessed using a semi-quantitative RT-PCR method. Amplification of 1 μg cDNA from PMA- and IoM-stimulated patient- and normal healthy volunteer-derived PBMNCs was performed with a SuperTaq Premix kit (Sawady Technology, Tokyo, Japan) using specific primers for β-actin, GM-CSF, IL-4, IL-6, IL-12p40, IL-12p35, IFN-γ, and TNF-α (Continental Laboratory Products, San Diego, CA, USA).

For the detection of mitogenic responses of patient-derived lymphocytes, the patient's PBMNCs were isolated by Ficoll density gradation, and 5×10^5 cells were incubated in each well of a 96-well plate, stimulated with 20 μg/ml phytohemagglutinin (PHA), or 7 μg/ml concanavalin-A (ConA) for 64 h, and then pulsed with 12.5 μCi ^{3}H-labeled thymidine for 8 h. Pulsed cells were harvested and the β-irradiation was measured with a scintillation counter. Normal values for mitogenic responses among volunteer donor PBMNCs were 26000–53000 counts per minute for PHA-L stimulation, and 20000–48000 counts per minute for Con-A stimulation.

Allogeneic mixed leukocyte reaction (allo-MLR) was performed as below. Patient- or HLA-DRB1 disparate three donor-derived PBMNCs (5×10^4 cells) were irradiated to 150 Gy as stimulator cells and co-cultured with 5×10^4 donor- or patient-derived PBMNCs as responder cells, for 5 days in 96-well plates, and then pulsed with 1 μCi ^{3}H-labeled thymidine for 8 h. Pulsed cells were harvested and the β-irradiation was measured.

Immunoglobulin concentrations from the patient were also monitored.

Results

Dosimetry

Dosimetry of the patient is described in Table 1. As is usual with neutron irradiation, distribution of the estimated dose was heterogeneous. It is very difficult to estimate accurately the dose of irradiation received by victims during the ideal transplantation time immediately after the accident.

Analysis of chimerism

FISH, using X- and Y-sex chromosome-specific probes, showed that almost 60% of BMMNCs were donor CB-derived XX cells in the sternal bone marrow, thought to be irradiated at a relatively high dose. Almost 40% of BMMNCs showed donor-derived XX signals in the iliac bone marrow, which had had a lower estimated irradiation dose due to attenuation of neutrons through the trunk. Almost half of all PBMNCs exhibited donor-derived XX signals, the rest XY signals. Donor-derived XX signals gradually decreased in intensity in both BMMNCs and PBMNCs with a reduction in CYA (Figure 2a–c).

Chromosomal analysis of sternal BM, iliac BM, and PB MNCs, showed complex chromosomal abnormalities, which were mainly non-clonal complex single cell abnormalities (SCA) (Figure 3a–c). We examined the fractional chromosome analysis to compare chimerisms of the mononuclear cell and granulocyte fractions. The granulocyte-enriched fraction indicated that 91.8% of granulocytes had recipient origin 46, XY chromosomes, and only 7.8% of granulocytes had the donor-derived 46, XX, chromosome. However, the lymphocyte and monocyte-enriched fraction possessed 24.3% and 26.8% of donor-derived 46, XX chromosome, respectively. These data indicated that only a small fraction of granulocytes was differentiated from donor-derived myeloid progenitor cells, but a quarter of

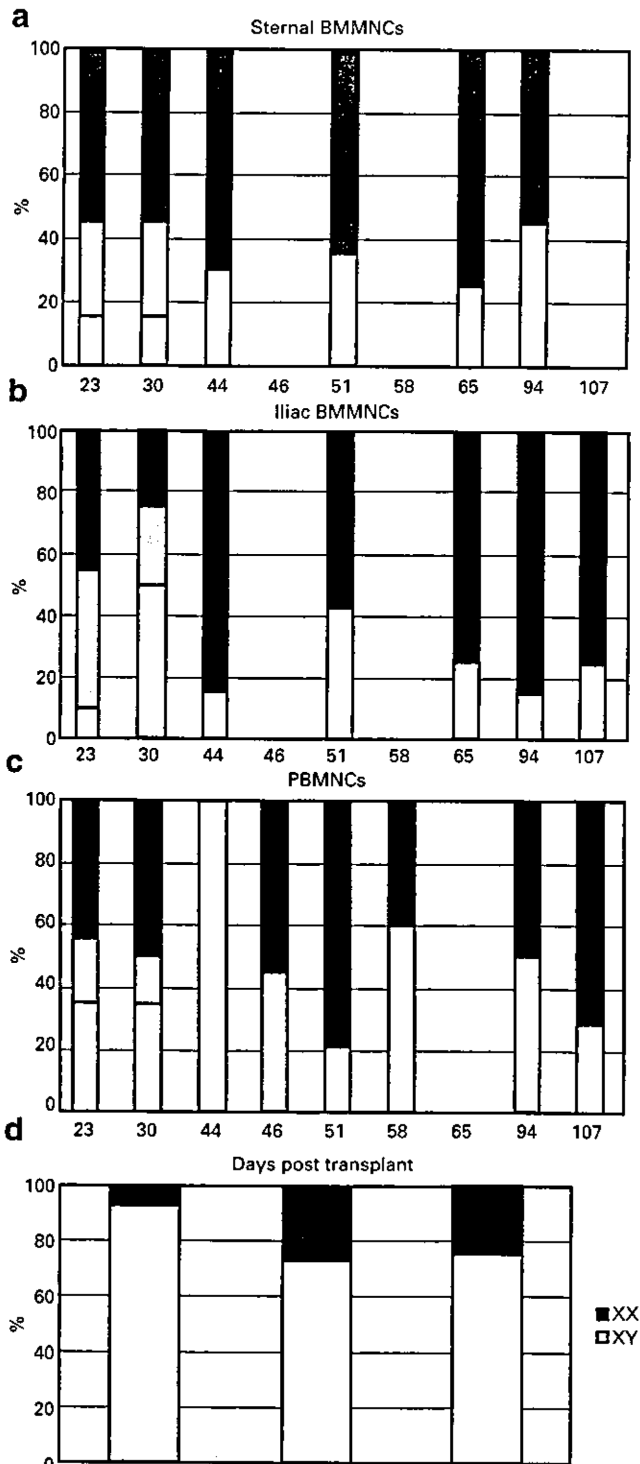

Figure 3 Chromosomal analysis of patient-derived BM or PBMNCs: open columns, percentages of normal karyotype cells; striped columns, percentages of cells with 46,XY, inv (9), thought to be a normal variant type chromosomal translocation; solid columns, percentages of cells possessing the non-clonal complex chromosomal abnormalities, which are so called single cell abnormalities (SCA). (**a**) Analysis of sternal bone marrow (sternal), (**b**) iliac bone marrow (iliac), (**c**) peripheral blood mononuclear cells (PBMNCs), and (**d**) analysis of day 20 PBMNCs and granulocytes.

lymphocytes and monocytes were of donor origin (Figure 3d).

Clonal mutations including chromosomal translocation t(4;11)(q23;q24) were detected only in the PB- and iliac BM-derived PHA blasts on day 107 after CBT (Table 3).

Monitoring of immunological reconstitution

The number of T cells after transplantation in this patient is shown in Figure 4. The number of $CD3^+CD4^+$ cells rose to above $1.0 \times 10^9/l$ about 80 days after transplantation. In contrast, the number of $CD3^+CD8^+$ cells began to progressively increase in spite of the administration of CYA. After termination of CYA treatment, the number of both $CD3^+CD4^+$ and $CD3^+CD8^+$ cells further increased and that of $CD3^+CD8^+$ cells reached as high as $3.0 \times 10^9/l$. During treatment, CMV reactivation was detected by PCR and antigen detection methods using C9 and C10 monoclonal antibodies. However, the CMV rapidly disappeared after administration of ganciclovir. Concurrently, the number of $CD3^+CD8^+$ T cells abruptly increased.

We detected transcriptional expressions of GM-CSF, IL-4, IL-6, IL-12p40, IL-12p35, IFN-γ, and TNF-α mRNA after stimulation with PMA and IoM using the RT-PCR method. However, the expression levels did not differ from those of the healthy volunteer donor (data not shown). Thus, there were no obvious differences in cytokine mRNA expression of PBMNCs between the patient and healthy donor.

To clarify the functional activation and maturation of the patient-derived T cells, we examined the mitogenic responses of these T cells *in vitro* (data not shown). Patient-derived T cells did not respond to mitogens including PHA or ConA, suggesting that the patient's immune responses were severely impaired. Even more surprisingly, an allogeneic mixed leukocyte reaction with third-party HLA-DRB1 disparate unrelated donors showed the patient-derived PBMNCs to be unresponsive to allogeneic antigen-presenting cells, and to have impaired antigen-presenting capacities against allogeneic responder cells (data not shown). Endogenous immunoglobulin producing capacities were also suppressed until 120 days after the accident (data not shown). These results suggest that the patient's immune system was fatally impaired in spite of the number of autologous hematopoietic cells which had recovered.

Discussion

Here, we present the case of an adult male, who was lethally irradiated with neutrons from a critical fission reaction.[11] The accidents at the Chernobyl and Soreq nuclear power plants[1,2] suggest that bone marrow transplantation has only a limited role in the treatment of victims of radiation. The limitation stems from a lack of HLA-matched donors, a requirement for additional immunosuppression and the risk of GVHD. Given these experiences, transplants should probably be considered only for victims who have received doses in the range of 8–12 Gy without serious skin injuries, severe internal contamination or conventional injuries.[2] We selected umbilical cord blood as the hemato-

Table 3 Chromosomal analysis of BM and PBMNCs after PHA blastoid formation

Iliac BMMNCs					
Days post-transplant					107
SCA					10
46,XY,t(4;11)(q23;q24) or 46,XY,der(4)t(4;11)(q23;q24),der(11)(t(4;11)add(4)(q31)					5
46,XY,add(2)(p11),–5,–7,add(12)(q24),–17,+mar4,+mar5,+mar6					3
46,XY,t(3;3)(p14;p23)					2
Total					20
PBMNCs					
Days post-transplant	46	51	58	95	7
SCA		1	4		11
46,XY	3	2	9		3
46,XY,t(4;11)(q23;q24) or 46,XY,der(4)t(4;11)(q23;q24),der(11)(t(4;11)add(4)(q31)					2
46,Y,t(X;13)(p22;q12),t(7;14)(p13;q32)					2
46,XY,t(1;3)(q11;p11),del(6)(q23q25),add(7)(q11),–8,add(10)(p11),–14,–17,–19,+mar1,+mar2,+mar3,+mar4					2
Total	3	3	13	0	20

SCA = single cell abnormalities.

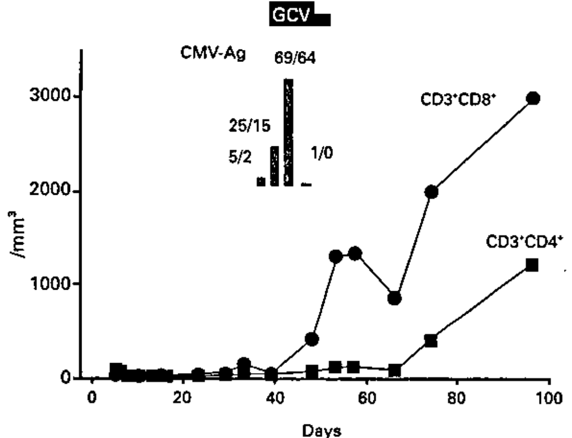

Figure 4 Absolute T cell number before and after CBT: ●, absolute number of $CD3^+CD8^+$ cells; ■, absolute number of $CD3^+CD4^+$ cells; CMV-Ag, CMV antigenemia; GCV, ganciclovir. Bars and numbers in the inset show the results of CMV-Ag.

poietic stem cell source to avoid donor acquisition delay and fatal GVHD. During the optimum time for stem cell transplantation, we closely observed our patient as there was no hope of autologous hematopoietic recovery according to the results of dosimetry and the clinical manifestations.

Umbilical cord blood T cells contain a relatively large number of naive T cells and produce fewer inflammatory cytokines, resulting in an amelioration of acute GVHD.[6] In clinical studies, a low incidence of and less acute GVHD with CBT have been reported.[4,5,7–9] Rocha *et al*[9] described a statistically significant reduction in the incidence of aGVHD and cGVHD in CBT recipients from HLA-matched siblings, compared to that seen with bone marrow. We previously described that the lower incidence of acute GVHD was related to the reduced post-membrane phosphorylation activities and Fas ligand (FasL) cell surface expression on cord blood T cells compared to that on adult PB T cells.[12] The absence of acute GVHD in our patient may be related not only to these characteristics of umbilical cord blood T cells but also to the donor/recipient mixed chimerism.[13,14] Storb *et al* in Seattle established the ideal stable mixed chimeric state between donor and recipient using a canine model.[15–19] Storb *et al* also described the use of stable mixed chimerism in the treatment of non-malignant hematological disorders, including hemoglobinopathies (β-thalassemia), inherited T cell deficiencies,[18] and autoimmune disorders.[19] In the non-malignant settings as with bone marrow failures and ARS, donor-derived hematopoiesis sufficiently compensates the defective host-derived hematopoiesis with mixed chimerism, without a risk of GVHD.

Dysfunctional regulation of FasL[12] may also induce a less potent graft-protecting effect, destroying recipient residual bone marrow cells and allowing autologous reconstitution. Rubinstein *et al*[8] reported 13 recipients with autologous reconstitution among 562 unrelated umbilical cord blood recipients (2.3%) after myeloablative conditioning. Decreased cytotoxic properties of CB T cells may contribute to autologous reconstitution after CBT.

In our case, ARS recrudesced after IS taper and granulocyte recovery. Further continuation of IS would be needed for the treatment of ARS and for the maintenance of stable mixed chimerism. We discussed the indication for granulocyte transfusions until engraftment after CBT in this case, but we did not undertake this, because of the danger of worsening the ARS. Only intensive antibiotic therapy, using vancomycin and arbekacin was given. The worsening of the cutaneous ARS after neutrophil recovery supports the integrity of this decision.

Many studies show a correlation between transfused cell numbers and the time to engraftment of the CBT.[4,7–9] The time required to reach an absolute neutrophil count greater than 0.5×10^9/l ranged from 25 to 42 days among recipients who were transplanted with 2×10^7 nucleated cells/kg of umbilical cord blood in the New York experience.[8] In the present case, recovery was faster than we had predicted. This recovery may be attributed to hematopoietic growth factors (G-CSF/EPO/TPO) although the effectiveness of this combination of growth factors has not yet been established.

Although several studies have examined T cell recovery after CBT,[4,10,20–22] little is known about immune reconstitution after myeloablative doses of irradiation. We documented the early immune reconstitution after a two loci HLA-mismatched CBT in an adult recipient compared to that seen with bone marrow, avoiding the use of ATG and mPSL in the conditioning and GVHD prophylaxis. A lower incidence of, and reduced GVHD may permit early termination of immunosuppressive therapy, resulting in early immune reconstitution after CBT.[10] Thomson *et al*[20] examined immune recovery after unrelated umbilical cord blood transplantation in childhood. They reported similar immune recovery after CBT, without delayed $CD8^+$ T cell recovery, compared to that reported for other stem cell sources. However, there are some reports of delayed immune recovery after CBT, in which transplant procedures involved ATG and mPSL for conditioning and GVHD prophylaxis.[21,22] In the present case, hematopoesis was supported by autologous hematopoietic recovery, and not comparable to previous studies that attained donor-derived complete chimerism. However, dysfunctional immunological reconstitution was observed soon after CBT, concurrently with irradiated autologous hematopoietic recovery. The profile of immunological reconstitution is summarized as follows: relatively normal numbers of $CD3^+CD4^+$, $CD3^+CD8^+$, normal cytokine mRNA expression in PBMNCs, defective mitogenic responses and impaired reactivity against third-party allogeneic PBMNCs of patient-derived PBMNCs. Immunoglobulin-producing capacities were suppressed until 4 months after irradiation. These results indicated severe impairment of immune function although autologous hematopoiesis recovered after ARS.

Skin grafts were obtained from a single cadaver donor not tested for HLA alleles. However, these grafts survived until the patient's death. The engraftment rate of cultured allografts is usually more than 40% in our experience, so we were able to obtain better results than was our prediction. The successful skin engraftment may be ascribed to post-radiation immunosuppression including a lack of mitogenic or allo-MLR responses of T cells.

In summary, CB is a useful stem cell source because of the potential for rapid acquisition, and the low incidence of GVHD associated with its use.[3–10,23] The relatively weak cord blood T cell-derived effect via down-regulating FasL[12] may induce donor/recipient mixed chimeric tolerance, which allows autologous hematopoietic reconstitution and ameliorates the risk of GVHD. Avoidance of transplant-related mortality including GVHD, and protection from opportunistic infections are the purposes of clinical intervention for ARS. We discontinued further IS therapy as we were afraid of infection, but the subsequent graft rejection was complicated by a severely immunosuppressed state, because the recovered autologous hematopoietic cells had aberrant chromosomes, and severely impaired immune functions, as the result of the fatal neutron irradiation. Donor-specific tolerance without non-specific immunosuppression established by stable mixed chimerism is the ideal platform for intervention against ARS. Antigen-specific immunosuppression is desirable for the suppression of HVG reaction.[24]

We were able to temporarily rescue the hematopoietic and dermal stem cells by allogeneic transplantation. However, the targeted organs of ARS are not limited to only bone marrow and skin, but the lungs and GI tract are also susceptible to ARS. To overcome the limitations of transplantation procedures, an improvement in 'regenerative medicine' that may compensate for the defective stem cells is desirable. Further evaluation is needed to improve stem cell rescue for nuclear accident victims.

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