Table 4. Differentiation potential of paired secondary clones

Mouse	No. of clones	Clone type, no. (%)				
		MTB-MTB	MTB-MB	MTB-TB	MB-TB	T-B
101	22	12 (54.5)	5 (22.7)	1 (4.5)	3 (13.6)	1 (4.4)
109	11	9 (81.8)	2 (18.2)	0 (0)	0 (0)	0 (0)
113	10	6 (60)	2 (20)	2 (20)	0 (0)	0 (0)
Total	43	27 (62.8)	9 (20.9)	3 (7)	3 (7)	1 (2.3)

Differentiation potential of paired secondary clones was determined by PCR tracking strategy based on integration site analysis of DP cells developed in secondary recipients.

Individual thymus-repopulating SRC clones have different self-renewal capacities

Consistent with our earlier observations in the primary recipient mice, the proportion of MTB clones present in the $CD34^+$ cell population decreased as the differentiation capacity of the clones became limited (Figure 3C). In addition, note that 30.3% of MTB clones in the secondary recipient mice were no longer found in the $CD34^+$ cell population, contrasting with the initial finding that all MTB clones in the primary mice were found in the $CD34^+$ cell population (Figure 2E). Therefore, phenotypic and possibly functional differences exist between MTB clones in the primary and secondary recipient mice, although both were capable of repopulating and giving rise to multilineage progenitors in the host. To assess how the MTB clone cell division affected the status of daughter MTB clones, we examined how many of in vivo–expanded MTB-MTB clone pairs were also found in the $CD34^+$ cell population and classified into 3 groups (Figure 3D). First, in 44.4% of the MTB-MTB clone pairs, both daughter clones were found in the $CD34^+$ cell population. Second, in another 44.4% of MTB-MTB clone pairs, only one of the daughter clones was found in the $CD34^+$ cell population. These results indicated that the former type clones have relatively higher SRC activity and that, as a result of unequal distribution of SRC activity in 2 daughter clones after cell division, 1 of the daughter clones exited from the $CD34^+$ stem cell pool. Finally, in 11.1% of MTB-MTB clone pairs, neither daughter clone was detected in the $CD34^+$ stem cell pool, which may reflect the extensive replication required to repopulate both primary and secondary recipients that eventually leads to exhaustion of the stem cells. These results demonstrate the heterogeneity among clones that repopulate both the primary and secondary recipient mice.

To further confirm our findings of heterogeneity of SRC clones, we quantitatively examined what was the relative clone size ratio of each MTB-MTB clone pair in the $CD34^+$ stem cell pool by RQ-PCR. In the majority of MTB-MTB clone pairs, the proportion of individual clone in each clone pair varied widely (Figure 3E). Interestingly, only a small proportion of MTB-MTB clone pairs (no. 29-3, no. 29-18, and no. 29-45) were found to be equally distributed to each paired recipient. Those clone pairs that were able to repopulate equally in a paired secondary recipient may have more extensive self-renewal capacity. The results indicated that individual thymus-repopulating SRC clones have different self-renewal capacities, which is a basis for a hierarchically organized stem cell pool.

We also performed tertiary transplantation and analyzed SRC clones found in the recipients. The level of engraftment in tertiary recipients was less than 1% in BM and 0.1% in the thymus (n = 4). We performed integration site analysis on unseparated BM MNCs of tertiary recipients and obtained 17 clones from 4 mice. The status of individual tertiary SRC clones in the secondary recipients was examined by clone tracking analysis using the LAM-PCR products from tertiary graft as a starting point of clonal analysis. We found that all SRC clones in the tertiary mice were detected in the $CD34^+$ stem cell pool of secondary recipient (Table S3 summarizes the results of the integration site analysis of SRCs). The results of tertiary transplantation experiment confirmed that only HSC clones that continuously replicate themselves in the $CD34^+$ stem cell pool could produce descendents to maintain long-term hematopoiesis.

Discussion

This study provides the first direct evidence for the multilineage differentiation and self-renewal of human HSCs at the single-cell level in vivo using PCR tracing analysis of individual thymus-repopulating clones. We demonstrated that polyclonal thymus-repopulating clones with multilineage differentiation and self-renewal abilities were able to maintain long-term human hematopoiesis. This study revealed several features of human HSCs that are important biologically and clinically. First, self-renewal division of individual thymus-repopulating clones resulted in clonal expansion of cells with multilineage differentiation in vivo. Second, a single thymus-repopulating clone produced progeny that were heterogeneous in SRC activity. Third, as a result of continuous division and/or advancement of lineage commitment, some of these multipotent thymus-repopulating clones were destined to limit their capability for multilineage differentiation and self-renewal. Our study indicated that, although the majority of thymus-repopulating clones loose their self-renewal potential, a relatively small proportion of thymus-repopulating clones retain extensive self-renewal potential. Therefore, the self-renewal capacity distinctly different in individual thymus-repopulating clones may cause a hierarchically organized stem cell pool.

Controlling the copy number of the virus vector in each transduced cell is important for our clonality analysis. We recently reported the direct evidence for single virus integration per cell in a transplantation study.[24] When EGFP-transduced $CD34^+$ cells expanded in vitro were divided and transplanted into multiple recipient mice, the unique integration site representing individual clones were detected in multiple mice; in other words, multiple mice were engrafted with the same clone. If a cell contains more than one integration site and assessed as "different" clones, these different clones should be detected in the same recipients. However, none of the clones demonstrated the identical engraftment pattern. In addition, clonal tracking analyses in this study clearly demonstrated that the individual SRC clones were both qualitatively and quantitatively heterogenous. Everything being considered, our infection condition achieves one copy per cell. Even if there is a slight possibility that the number of copies per cell is more than one, the clonality is not denied. Because a virus gene integrates into the host genome at a random site, progenies having a common integration site are developed from a single clone. It could affect the numeric calculation of the number of clones, but it would not influence our interpretation of the result.

Although the concept of HSCs was proposed decades ago and is well accepted, little experimental data regarding multipotency of human HSCs is available. Using a genetic marking strategy in both experimental[6-12] and clinical studies,[28-33] it has been suggested that hematopoietic reconstitution after transplantation is attributed to oligoclonal or polyclonal HSC activity and that the repopulation capacity of individual HSCs is substantially heterogeneous. However, the results of these studies, such as the presence of transgene expression in B lymphocytes and myeloid cells and the detection of similar genomic bands in multiple hematopoietic recipient organs, were not sufficient to unequivocally determine the multipotency of human HSCs at the single-cell level. Recently, Schmidt et al[34] reported clonal evidence for multilineage

human hematopoietic differentiation from IL-2Rcγ gene-transduced CD34+ cells transplanted into patients with X-linked SCID. Considering that the IL-2Rcγ has been known to play a critical role in lymphoid development,[35] the observations based on the IL-2Rcγ gene–expressing clone may not reflect authentic hematopoietic development because of possible lineage commitment redirection mediated by cytokines that act on IL-2Rcγ. Although the HSC's potential was elaborately assessed clonally in vitro,[36,37] there exists some concerns; the lineage commitment could be easily fluctuated by culture conditions; homing of HSCs to thymus was neglected. The field has thus far lacked experimental evidence showing that purified human HSCs possess the potential for multilineage differentiation at the single-cell level. In this study, we succeeded in demonstrating that a single HSC gave rise to myeloid, T-, and B-lymphoid lineage cells by using LAM-PCR–based clonal tracing analysis in highly purified lymphomyeloid cell populations. Our results now provide data supporting the multipotency of a single human HSC.

Transplantation of prospectively isolated donor cells in mice has demonstrated that only HSCs can sustain thymopoiesis for a long period.[38,39] A lack of reliable in vivo experimental models for human thymocyte reconstitution and clonal stem cell assays has precluded determining whether the same applies to human HSCs. In this study, we used the thymus-repopulating potential of individual SRC clones as a means to analyze human HSCs at a single-cell level in vivo. Previously, we have demonstrated that human T lymphocytes derived from CD34+ cells and developed in NOG recipient mice bore polyclonal Vβ TCR and responded not only to mitogenic stimuli but also to allogeneic human cells, which reflects normal human T-lymphoid cell development.[16-19] By analyzing individual thymus-repopulating clones in the NOG recipient, we successfully demonstrated that human thymus-repopulating cells were derived from a single multipotent-type HSC clone and were capable of maintaining long-term thymopoiesis in vivo. This is the first clonal evidence that multipotent HSCs contribute to long-term human thymopoiesis.

Self-renewal is essential for HSCs to maintain homeostasis in the blood system by the sequential generation of mature blood cells throughout a lifetime. To maintain the total pool of HSCs, this ability must be passed on to at least one of the daughter cells in each division. If both daughter cells undergo terminal differentiation, HSCs will eventually be lost. On the other hand, if both daughter cells retain stem cell properties, the total number of HSCs will increase, resulting in expansion of HSCs.[40] In fact, an increase in the number of murine[41,42] (and human[43,44]) repopulating cells was reported in serial transplantation studies. Limiting dilution analysis showed that secondary recipients contained larger numbers of HSCs than was originally injected. These results suggest that HSCs can replicate vigorously under certain conditions. However, it has been shown that HSCs intrinsically limit their potential for self-renew.[45-47] Recently, Ema et al[48] observed the self-renewal ability of HSCs by single murine HSC serial transplantation experiments and statistically determined that the number of HSCs increased in the BM of recipients, although the mean activity of individual HSCs was reduced. Taken together, this finding suggests that, although HSCs replicate themselves, which results in expansion of HSCs, they sequentially loose their potential as a HSC. Our clonal analysis of thymus-repopulating cells in paired secondary recipients provides direct evidence to address this innate property of HSCs in vivo.

Our finding that the same multipotent HSC clone was detected in paired secondary recipients (MTB-MTB type) indicates that a single SRC clone can self-replicate to produce 2 daughter cells with multilineage differentiation and self-renewal potential, leading to the in vivo expansion of SRCs. It has been considered that SRCs that can engraft and give rise to multilineage cells in secondary recipients are self-renewed HSCs. Thus, these MTB-MTB clones in this study could be defined as HSCs. However, when the MTB-MTB clone pairs were further examined whether they remained in the stem cell pool, one of the daughter clones in the pair was no longer found in the CD34+ cell population in approximately half of MTB-MTB clone pairs. Furthermore, the stem cell phenotype was not retained in 11.1% of MTB-MTB clones. Considering that 100% of MTB clones in primary recipients possess the stem cell phenotype, these results indicate that SRCs with the stem cell phenotype progressively decrease during serial transplantation, leading to exhaustion of SRCs. This is consistent with our finding that the proportion of clones with the stem cell phenotype decreased as the clone committed to specific lineages. By assessing the phenotype of self-replicated multilineage clone pairs, determined by the presence of common integration site in CD34+ cell population, we were able to reveal the status of HSCs during aging. The loss of stem cell phenotype may be caused by extensive replication required for hematopoietic reconstitution in recipient. Although the total SRC population appears to expand, our data indicate that the ability of individual SRCs, in more than half of the clones, may become restricted during long-term hematopoiesis in vivo.

Our clonal tracking analysis clearly demonstrated that heterogeneity in the self-renewal capacity of individual multipotent SRC clones underlies the differences of clonal longevity in the stem cell pool (Figure 4). Although most of the SRC clones loose their self-renewal potential, a relatively small proportion of SRC clones

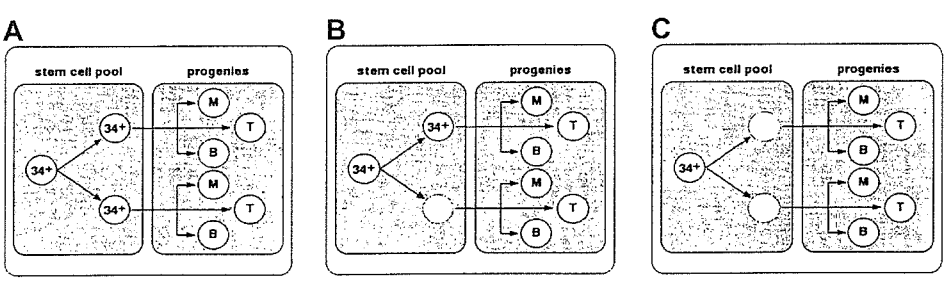

Figure 4. Schema of in vivo expansion. (A) A HSC replicates and produces 2 daughter cells, both of which retain the HSC phenotype. The paired daughter HSCs in the stem cell pool contribute to hematopoiesis; even so, the self-renewal activity of the parent HSC may be equally distributed to both daughters or may be skewed to either daughter cell. (B) As a result of heterogeneous HSC replication, one of the daughter HSCs loses the stem cell potential and therefore exits from the stem cell pool, but still remains in the progenitor pool. (C) Both paired daughter cells have lost their HSC potential, leading to exhaustion from the stem cell pool. 34 indicates CD34+ stem/progenitor cells; M, CD33+ myeloid lineage cells; B, CD19+ B-lymphoid lineage cells; T, CD3+ (spleen) or CD4/CD8 double-positive (thymus) T-lymphoid lineage cells.

(3 of 43 total secondary descending clones) are able to continuously self-renew. Our strategy which combined lineage-cell sorting and LAM-PCR enabled identification of the MTB clone that continuously self-renews in the stem cell pool and represents the long-term HSC. Our study provides a method that can accurately evaluate in vivo properties of human HSCs, and further studies may lead to elucidation of the mechanisms of self-renewal of the long-term human HSC at the single-cell level. It has been demonstrated that long-term leukemic stem cells have extensive self-renewal potential, and their hierarchic organization of stem cell pool was notably similar to the normal HSC compartment.[49,50] These findings propose the idea that some forms of leukemia imitate a system of the normal long-term HSC and retain or acquire the extensive self-renewal capacity. The clonal analysis for properties of long-term HSCs in vivo will be a powerful tool to understand the mechanisms for tumor initiation, progression, and relapses and will lead to efficient use of HSCs in clinical transplantation medicine.

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bjh short report

Allogeneic haematopoietic cell transplantation from alternative donors with a conditioning regimen of low-dose irradiation, fludarabine and cyclophosphamide in Fanconi anaemia

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Summary

A pilot study was undertaken using a fludarabine-based conditioning regimen to improve haematopoietic cell transplantation (HCT) from alternative donors in 27 Fanconi anaemia (FA) patients. Patients were conditioned with 150–180 mg/m^2 of fludarabine, 40 mg/kg of cyclophosphamide, 5–10 mg/kg of antithymocyte globulin, and 300–450 cGy of thoracoabdominal/total body irradiation. One patient who received unrelated cord blood transplantation failed to engraft, another patient died of sepsis. The 1-year overall survival was 96·3% (95% CI, 89–100). This conditioning regimen exerted an immunosuppressive effect that enabled durable engraftment in alternative donor HCT without severe toxicity.

Keywords: Fanconi anaemia, haematopoietic cell transplantation, alternative donors, fludarabine, tacrolimus.

Allogeneic haematopoietic cell transplantation (HCT) is the only treatment option with curative potential for Fanconi anaemia (FA). HCT from a human leucocyte antigen (HLA)-identical sibling is an established therapy, but the results of HCT from alternative donors have been disappointing, with a survival rate of only 29–38% due to the high rate of graft rejection or severe graft *versus* host disease (GVHD) (Gluckman *et al*, 1995; Guardiola *et al*, 2000; MacMillan *et al*, 2000). We describe 27 FA patients who received HCT from an alternative donor following a fludarabine monophosphate (Flu)-based conditioning regimen.

Patient characteristics and donor information are given in Table I. The median age at diagnosis of FA (range), the median age at transplantation (range), the median interval between diagnosis and transplantation (range) were 4·8 years (0·8–8·8), 8·1 years (2·6–28·6), and 4·0 years (0·9–21·1) respectively. The complementation group was analysed in 16 patients; nine were group A, seven were group G (Niedernhofer

Table I. Patient characteristics, disease status and donors.

n	Age at diagnosis (years)	Age at HCT (years)	Sex	Complementation group	Status at HCT	Cytogenetics	Prior therapy	Transfusions prior to HCT (*n*)	Donor	HLA match
1	0·8	2·6	M	Unknown	SAA	46, XY	None	25	BM-URD	6/6
2	3·2	5·1	F	G	SAA	46, XX	Prednisolone	6	BM-URD	6/6
3	6·4	12·4	F	A	MDS (RA)	46, XX, t(18;20)(p11;q11)	Androgen, prednisolone	3	BM-URD	5/6 (DRB1)
4	8·8	11·6	M	A	SAA	46, XY	Androgen	3	BM-URD	5/6 (DRB1)
5	2·3	5·6	M	G	SAA	46, XY	Androgen	3	BM-URD	6/6
6	4·0	12·9	F	Unknown	SAA	46, XX	Androgen	55	BM-URD	6/6
7	7·5	28·6	F	Unknown	MDS (RAEB), with 6% blasts	46, XX, dup(1)	Androgen	23	BM-URD	5/6 (DRB1)
8	5·0	16·6	M	A	MDS (RA)	46, XY, der(11)	Androgen	>100	BM-RD	3/6 (A, B, DR)
9	6·8	7·9	F	A	SAA	46, XX	Androgen	<10	BM-URD	6/6
10	2·5	7·2	M	Unknown	SAA	46, XY	Androgen	>100	BM-URD	6/6
11	5·0	9·4	M	G	SAA	46, XY	None	3	BM-URD	6/6
12	5·3	7·4	M	Unknown	SAA	46, XY	None	34	BM-URD	6/6
13	5·3	8·4	M	A	SAA	46, XY	Androgen, prednisolone	7	BM-URD	6/6
14	4·0	12·9	F	G	SAA (CA)	46, XY, der(7), t(1;7)(q21;q22)	Prednisolone	37	BM-URD	6/6
15–1	1·8	6·0	M	Unknown	SAA	46, XY	Alkaloid	>20	UCB-URD	5/6 (B)
15–2	1·8	6·1	M	Unknown	SAA	46, XY	UCBT	>20	BM-RD	4/6 (B, DR)
16	5·5	6·4	F	G	SAA (CA)	45, XX, add(3)(q21), −17	Androgen, prednisolone	3	BM-URD	6/6
17	5·8	11·1	M	G	MDS (RAEB), with 8% blasts	46, XY, der(13)t(1;13)(q11;q14), add(6)(p25)	Androgen	33	BM-URD	5/6 (B)
18	6·0	13·5	F	Unknown	SAA	46, XX	None	40	BM-URD	6/6
19	3·0	6·4	F	Unknown	SAA	46, XX	None	10	BM-RD	5/6 (DRB1)
20	4·6	6·8	F	Unknown	MDS (RAEB), with 8% blasts	46, XX, add(3)(q26), der(7), add(7)(p22)	None	17	BM-URD	5/6 (DR)
21	8·8	15·2	F	A	SAA	46, XX	Androgen	15	BM-URD	5/6 (DRB1)
22	5·8	12·7	F	A	SAA	46, XX	Androgen, prednisolone	25	BM-URD	5/6 (DR)
23	6·8	8·4	F	A	MDS (RA)	46, XX	None	5	BM-URD	5/6 (B)
24	1·8	3·1	F	Unknown	SAA	46, XX	None	10	BM-URD	6/6
25	3·3	7·0	M	Unknown	SAA	46, XY	None	3	BM-URD	6/6
26	2·0	4·3	M	A	MDS (RA)	46, XY	Androgen	7	BM-URD	6/6
27	4·0	9·0	M	G	SAA	46, XY	None	5	BM-URD	6/6

HCT, haematopoietic cell transplantation; M, male; SAA, severe aplastic anaemia; BM, bone marrow; URD, unrelated donor; F, female; MDS, myelodysplastic syndrome; RA, refractory anaemia; RAEB, refractory anaemia with excess blasts; RD, related donor; CA, chromosomal abnormality; UCB, unrelated cord blood; UCBT, unrelated cord blood transplantation.

et al, 2005). Twenty-one patients were diagnosed with limited malformation syndrome and six patients with extensive malformation syndrome, based on the number of anatomical sites involved (Guardiola *et al*, 2000). Eleven of 27 patients had received more than 20 transfusions and 14 of 27 patients received androgen therapy.

The protocol of alternative donor HCT for life-threatening FA was approved by the institutional review board, and written informed consent was obtained from patients and/or their parents. The conditioning regimen consisted of the following: either thoraco-abdominal irradiation (TAI) (150 × 3 cGy on days −9 to −8) in patients with severe aplastic anaemia (SAA)/myelodysplastic syndrome (MDS)-refractory anaemia (RA), or total body irradiation (TBI) (150 × 3 cGy on days −9 to −8) in patients with MDS-RA with excess blasts, Flu 30 × 6 mg/m^2 (days −7 to −2), cyclophosphamide (CY) 10 × 4 mg/kg (days −5 to −2) and antithymocyte globulin (ATG, Thymoglobuline; IMTIX Sangstat, Lyon, France) 2·5 × 4 mg/kg (days −5 to −2). The first patient received 150 × 2 cGy of TAI because of marked short stature. After 10 patients had been treated, the dose of irradiation in patients with SAA or MDS-RA was decreased to 300 × 1 cGy (day −8), the dose of Flu was decreased to 25 × 6 mg/m^2 (days −7 to −2), and the dose of ATG was decreased to 1·25 × 4 mg/kg (days −5 to −2). GVHD prophylaxis was carried out with short-term methotrexate (MTX) (15 mg/m^2, day 1; 10 mg/m^2, days 3, 6 and 11) and continuous intravenous infusion of tacrolimus 0·03 mg/kg (days −1 to 28); moreover, mycophenolate mofetil (MMF) 15 mg/kg/d (days 14–42) was added in three patients according to each institutional protocol. When patients were able to take oral medication, at 4 weeks post-transplant, intravenous tacrolimus was switched to oral administration. All patients received granulocyte colony-stimulating factor from day 5 until neutrophil counts exceeded 1·5 × 10^9/l. HCT was performed between 15 November 2000 and 5 November 2004.

Table II. Transplant characteristics.

n	Conditioning	TNC/kg (×10^8)	Engraftment	Maximum organ toxicity (Bearman) grade II	Maximum GVHD		Outcome
					Acute	Chronic	
1	TAI (3)/Flu (180)/CY (40)/ATG (10)*	4·0	Yes	None	0	0	Alive, 56·3 months
2	TAI (4·5)/Flu (180)/CY (40)/ATG (10)	4·2	Yes	None	1	0	Alive, 53·7 months
3	TAI (4·5)/Flu (180)/CY (40)/ALG (40)	1·5	Yes	Stomatitis	2	0	Alive, 53·5 months
4	TAI (4·5)/Flu (180)/CY (40)/ALG (60)	2·5	NE	None	NE	NE	Dead, day 4 (sepsis)
5	TAI (4·5)/Flu (180)/CY (40)/ATG (10)	3·9	Yes	None	0	0	Alive, 52·3 months
6	TAI (4·5)/Flu (180)/CY (40)/ATG (10)	5·0	Yes	None	0	0	Alive, 49·9 months
7	TBI (4·5)/Flu (180)/CY (40)/ATG (10)	3·8	Yes	None	0	0	Alive, 49·3 months
8	TBI (4·5)/Flu (180)/CY (40)/ATG (10)	1·4	Yes	Bladder	0	Limited	Alive, 48·4 months
9	TAI (4·5)/Flu (180)/CY (40)/ATG (10)	6·1	Yes	Stomatitis	0	Limited	Alive, 45·8 months
10	TAI (4·0)/Flu (180)/CY (40)/ATG (10)	3·0	Yes	None	0	0	Alive, 43·9 months
11	TAI (3)/Flu (150)/CY (40)/ATG (10)	4·8	Yes	Liver	0	0	Alive, 43·7 months
12	TAI (3)/Flu (150)/CY (40)/ATG (5)	4·7	Yes	None	0	Extensive	Alive, 40·7 months
13	TAI (3)/Flu (150)/CY (40)/ATG (5)	4·4	Yes	None	0	0	Alive, 40·4 months
14	TAI (3)/Flu (150)/CY (40)/ATG (5)	3·0	Yes	Stomatitis	1	Limited	Alive, 40·3 months
15–1	TAI (3)/Flu (150)/CY (40)/ATG (5)	0·64†	NE	None	NE	NE	Re-BMT, 1·3 months
15–2	TAI (3)/Flu (150)/CY (40)/ATG (5)	5·2	Yes	None	0	0	Alive, 36·7 months
16	TAI (3)/Flu (150)/CY (40)/ATG (5)	4·5	Yes	None	1	Limited	Alive, 37·4 months
17	TBI (4·5)/Flu (150)/CY (40)/ATG (5)	1·9	Yes	Kidney	3	Extensive	Alive, 36·4 months
18	TAI (3)/Flu (150)/CY (40)/ATG (5)	2·8	Yes	None	0	0	Alive, 33·2 months
19	TAI (3)/Flu (150)/CY (40)/ATG (5)	2·6	Yes	None	3	0	Alive, 25·7 months
20	TBI (4·5)/Flu (150)/CY (40)/ATG (10)	4·5	Yes	None	0	0	Alive, 25.5 months
21	TAI (3)/Flu (150)/CY (40)/ATG (5)	4·2	Yes	Stomatitis	0	Extensive	Alive, 22·8 months
22	TAI (3)/Flu (150)/CY (40)/ATG (5)	1·6	Yes	Liver	0	0	Alive, 20·8 months
23	TAI (3)/Flu (150)/CY (40)/ATG (5)	5·5	Yes	Stomatitis, liver	0	Limited	Alive, 16·3 months
24	TAI (3)/Flu (150)/CY (40)/ATG (5)	4·8	Yes	None	1	0	Alive, 14·5 months
25	TAI (3)/Flu (150)/CY (40)/ATG (5)	4·6	Yes	None	0	0	Alive, 9·9 months
26	TAI (3)/Flu (150)/CY (40)/ATG (5)	4·7	Yes	Liver	0	0	Alive, 8·9 months
27	TAI (3)/Flu (150)/CY (40)/ATG (5)	5·6	Yes	Stomatitis, liver	0	0	Alive, 7·9 months

Two patients received ALG instead of ATG.

ALG, antilymphocyte globulin; TAI, thoraco-abdominal irradiation; TBI, total body irradiation; Flu, fludarabine; CY, cyclophosphamide; ATG, antithymocyte globulin; TNC, total nucleated cells; GVHD, graft *versus* host disease; BMT, bone marrow transplantation; NE, not evaluable.

*The number in parentheses indicates the dose and units are Gy with TAI and TBI, mg/m^2 with Flu, mg/kg with CY and ATG.

†Post-thawing cell dose.

The transplant characteristics are given in Table 2. Twenty-five of 27 patients exhibited prompt haematopoietic recovery. The median time to neutrophil recovery to more than $0{\cdot}5 \times 10^9$/l was 17 d (range 11–25), and to an unsupported platelet count of more than 20×10^9/l, 22 d (range 14–43). Two patients were not evaluable for haematological reconstitution as one underwent graft rejection and the other died on day 4. One patient who showed graft failure after the first unrelated cord blood transplantation received a second successful bone marrow transplantation from an HLA-two loci-mismatched mother with the same conditioning. All chimaeric studies performed after 8 weeks post-transplant revealed full donor chimaerism. No patient displayed a greater than grade III regimen-related toxicity using the criteria of Bearman *et al* (1989) at any evaluation point. The incidence of organ toxicity did not differ in relationship with the dose of irradiation or Flu.

Acute GVHD (grades I–III) according to the Seattle criteria (Glucksberg *et al*, 1974) developed in seven of 26 evaluable patients, grade I in four patients, grade II in one and grade III in two patients. Chronic GVHD was observed in eight of the 26 evaluable patients, with a limited form in five patients and an extensive form in three patients. No patients died of GVHD. One patient died of methicillin-resistant *Staphylococcus aureus* sepsis on day 4. The overall survival rate estimated by the product-limit method (Kaplan & Meier, 1958) was 96·3% (95% CI, 89–100) and the median follow up was 37·1 months (range 7·9–56·3 months). Karnofsky scale was 100% in all patients. Transplant-related mortality at 12 months was 3·6%, and no late tumours occurred during the same follow-up period.

Both graft rejection and GVHD were major causes of failure in alternative donor HCT for FA in previous studies that used conditioning regimens that included 400–600 cGy of limited field irradiation or TBI, as well as 15–120 mg/kg of CY with or without ATG (Gluckman *et al*, 1995; Guardiola *et al*, 2000; MacMillan *et al*, 2000). Recently, Flu has been used as part of a conditioning regimen that facilitates engraftment without increased toxicity in FA patients (Boulad *et al*, 2000; Boyer *et al*, 2003). One of the aims of this study was to improve the engraftment rate after alternative donor HCT in FA by adding Flu to low-dose TAI/TBI, low-dose CY and low-dose ATG (Seidel *et al*, 2005). Because 25 of 26 evaluable patients achieved engraftment without severe toxicity, Flu may be considered as a keydrug for the conditioning of alternative HCT in FA.

The other aim of this study was to prevent moderate to severe acute GVHD after alternative donor transplants. The combination of tacrolimus plus short-term MTX considerably decreased the incidence of severe acute GVHD in HLA-matched unrelated bone marrow transplantation despite ethnic differences (Nash *et al*, 1996; Morishima *et al*, 2002). In this study, low-dose ATG was administered prior to transplant and ATG also might exert a preventive effect on GVHD in conjunction with both tacrolimus and MTX.

Many variables including older patient age, lower platelet counts, extensive malformations, and the use of androgen before transplant have been reported to be associated with a worse outcome (Gluckman *et al*, 1995; Guardiola *et al*, 2000). These variables and the number of transfusions pretransplantation were not significantly different between this study and previous reports. Complementation groups included in this study were Groups A and G, and this might have contributed to the good result.

This study provides evidence that low-dose irradiation and Flu-based preconditioning regimen can be used satisfactorily in alternative HCT for FA; however, long-term observation of secondary cancers will be required to determine the therapeutic utility of this approach.

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EXPERIMENTAL HEMATOLOGY

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Development of human–human hybridoma from anti-Her-2 peptide–producing B cells in immunized NOG mouse

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Objective. **Numerous monoclonal antibodies have been developed for the purpose of medical treatments, including cancer treatment. For clinical application, the most useful are human-derived antibodies. In this study, we tried to prepare designed antigen-specific antibodies of completely human origin using immunodeficient mouse.**

Methods. **Nonobese diabetic/severe combined immunodeficient/IL-2 receptor γ null mouse (NOG) mouse was used to reconstitute the human immune system with umbilical cord blood hematopoietic stem cells (CB-NOG mouse) and to prepare human-derived Her-2-epitope–specific antibodies. Hybridoma lines were prepared by fusing the human myeloma cell line Karpas707H.**

Results. **Serum of immunized NOG mouse contained human-derived immunoglobulin M (IgM) antibodies specific for a short peptide sequence of 20 amino acids, including the epitope peptide of apoptotic Her-2 antibody CH401. Hybridoma lines were successfully prepared with spleen B cells obtained from the immunized CB-NOG mouse. One of these cell lines produced human IgM against the epitope peptide that can recognize surface Her-2 molecule.**

Conclusion. **We could produce human-derived IgM antibody against Her-2 epitope peptide in CB-NOG mouse, succeeding in generation of human hybridoma-secreting IgM against a given peptide.**

Passive monoclonal antibody (mAb) therapy has been accepted as a treatment for cancers and autoimmune diseases for the past decade [1,2]. For patients affected by cancer, passive mAb therapy is of benefit mainly because the immune reaction of most patients is suppressive. To date, several antibodies such as Trastuzumab (Herceptin) and Rituximab have been already under practical use, and some are under clinical investigation. Therapeutic effectiveness of these antibodies, to some extent, promotes development of new monoclonal antibodies related to the disease [3]. In addition to cancer patients, anti-tumor necrosis factor-α mAb of human–mouse chimera mAb (Infliximab), or of completely humanized mAb (Adalimumab) are used for suppression of T-cell function in rheumatoid arthritis and Crohn's disease, and are proved to be relatively successful [4,5].

In consideration of clinical application, therapeutic antibody is ideal to be humanized. Today, almost all antibodies for clinical use are derived from mouse, at least in part, although they were humanized by techniques of molecular biology [6]. Recently, the mouse line carrying human chromosome fragments containing immunoglobulin (Ig) gene cluster was developed, which made it possible to prepare completely human-type antibodies produced by mouse B cells [7]. However, these mice produce IgG with sugar chains of mouse origin. Although sugar chains are

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thought to have low immunoreactivity induced by species-specificity, the risk to induce human anti-mouse antibody production remains [8]. Therefore, the most ideal system is that of antibodies of human origin produced by human B cells.

We have developed a human immune system reconstituted in the mouse environment by transplanting human hematopoietic stem cells in immunodeficient mouse [9,10]. In comparison with nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice, NOD-SCID–IL-2Rγ knockout mouse (NOG) showed higher engraftment of human hematopoietic cells and higher proportion of T-cell development in the xenogenetic environment [11]. In a previous study, we reconstituted the human immune system in NOG mouse with umbilical cord blood hematopoietic stem cells (CB-NOG) and found that immunization of 2,4-dinitrophenol-conjugated keyhole limpet hemocyanin (DNP-KLH) induces antigen-specific human IgM production [12]. These findings indicate that the reconstituted human immune system has developed potential for producing antibodies against the immunized antigens, although the net number of human T and B cells per head in CB-NOG mice is less than one tenth that in normal mouse.

To obtain human-derived antibody to effectively suppress tumor cells or mass, we can immunize CB-NOG mice with a particular epitope, which is recognized by an apoptotic antibody. Among the reported monoclonal antibodies against Her-2 established by mouse and the epitopes reported [13–16], some possessed suppressive effect for tumor growth. Ishida and collaborators [17] established a humanized monoclonal antibody termed CH401, which has an epitope different from Herceptin and induces apoptosis to Her-2–expressing cells efficiently. In this article, we focus on the capacity of human B cells developed in mouse environment to produce antigen-specific antibodies, to analyze if CH401-recognizing epitope can induce human B cells to produce epitope-specific antibody in CB-NOG mouse. As a result, we determined that the CB-NOG mouse produced specific antibodies against a short peptide of 20 amino acids carrying the CH401 epitope. Consequently, the antibody-producing B cells were fused with a human myeloma cell line, Karpas707H [18], to establish a hybridoma line.

Materials and methods

Mice

NOD/Shi-scid, common γc-null (NOD/SCID/γc-null; NOG) mice were provided from the Central Institute for Experimental Animals (Kawasaki, Japan). BALB/c mice were purchased from Crea Japan Inc. (Kawasaki, Japan). All mice were kept under specific pathogen-free conditions in the animal faculty located at the Tokai University School of Medicine (Isehara, Japan).

Human hematopoietic stem cells

Human umbilical cord blood was obtained from full-term healthy newborns immediately after vaginal delivery. Informed consents were obtained according to Institute guidelines, and this work was approved by Tokai University Human Research Committee. Mononuclear cells (MNCs) were separated by Ficoll-Paque gradient centrifugation. $CD34^+$ cells were purified from MNCs using the two-step MACS system (Miltenyi Biotec, Gladbach, Germany). The purity was >98%.

Transplantation

Nine-week-old NOG mice were irradiated sublethally with 2.5 Gy prior to transplantation, and $CD34^+$ cells were transplanted intravenously. Eight weeks after transplantation, peripheral blood was collected via orbit under inhalation anesthesia. MNCs were prepared and reconstitution efficiencies were calculated by hCD45 expression analyzed by fluorescein-activated cell sorting (FACS) analysis.

Peptides

Her-2 peptide includes the sequence identified as the epitope of an apoptotic anti-Her-2 antibody, CH401, which was determined using multiple antigen peptides (MAP) peptides with a partial amino acid sequence of Her-2 (Miyako et al., manuscript in preparation). The sequence of the peptide is N:163-182 ((YQDTILWK-DIFHKNNQLALT-BBB)8-K4K2KB). This peptide was synthesized using Rink amide resin (0.4–0.7 mmol/g) and peptide synthesizer ACT357 (Advanced Chemtech, Louisville, KY, USA). On the 96-well plate, each of these MAP peptides was coated and the cross-reactivity with CH401 was examined by enzyme-linked immunosorbent assay (ELISA).

Monoclonal antibodies, flow cytometry, and cell sorting

All cells were analyzed using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). For each analysis or sorting, living gate white blood cells or lymphocytes were further gated on human $CD45^+$ cells. Mouse anti-human mAbs included peridinine chlorophyll protein-conjugated CD45, T-cell receptor (TCR), CD1a, fluorescein isothiocyanate (FITC)-conjugated CD4, IgM, phycoerythrin-conjugated CD4, CD8, IgD, and allophycocyanin (APC)-conjugated CD19.

Immunization, EBV transformation, and hybridoma preparation

Eight to 10 weeks after transplantation, mice were immunized intraperitoneally with 2,4-dinitrophenylated keyhole limpet haemocyanin (DNP-KLH; 100 μg/alum/head), toxic shock syndrome toxin-1 (TSST-1; 25 μg/alum/head), Her-2 extracellular domain (25 μg/alum/head) or Her-2 epitope peptide (100 μg/FCA/head). Mice were immunized every 2 weeks. Four days after the last booster, mice were sacrificed and spleen cells were collected. Epstein-Barr virus (EBV) transformation was performed by conventional method. Four weeks after EBV treatment, culture supernatants were collected and specific-antibody–producing clones were selected by ELISA, as described previously [9]. The cells secreting specific antibodies were expanded and fused with a human myeloma cell line, Karpas707H, using polyethylene glycol. Hybridomas were selected by Ouabine and HAT. Positive clones were selected by ELISA.

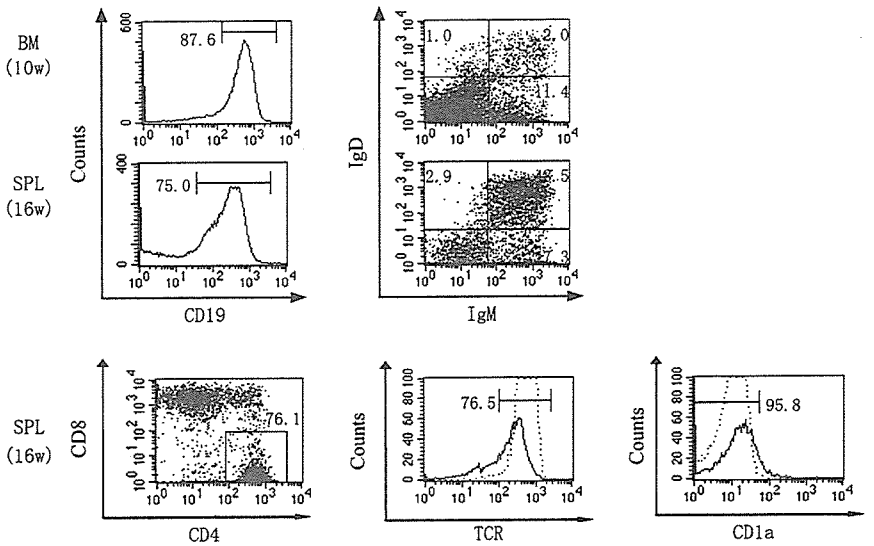

Figure 1. Reconstitution of human immune system in nonobese diabetic/severe combined immunodeficient/gammac(null) mouse with umbilical cord blood $CD34^+$ cells. Fluorescein-activated cell sorting analysis of lymphoid tissue cells. Bone marrow (BM) cells were prepared 10 weeks after transplantation. Spleen (SPL) cells were prepared 16 weeks after transplantation. CD4SP cells were enclosed in a square and the percentage was shown above the square. T-cell receptor (TCR) and CD1a expression was analyzed for the CD4SP-gated fractions. B cells were gated for $CD19^+$ cells, and immunoglobulin (Ig) IgM and IgD double-staining patterns are shown.

Results

CB-NOG can produce specific antibodies against ordinary protein antigens

We reported previously that IgM+IgD+mature type human B cells are detectable in the spleen and bone marrow of NOG mouse reconstituted with CB-derived $CD34^+$ cells (CB-NOG mouse), although the majority of IgM^+ cells in bone marrow are IgD-negative immature B cells [12]. In addition, as shown in Figure 1 of this study, the spleen of these CB-NOG mice were shown to contain CD4 and CD8 single-positive human T cells, with high expression of TCR. The pattern of mature T and B cells as shown in Figure 1 was observed in the mice with relatively high reconstitution ratio of human-derived cells (>20%, data not shown). Thus, for examination of human antibody production specific to a given antigen, we used CB-NOG mice, the engraftment of which was >20% in the 8th to 10th week after transplantation of human CB cells. These CB-NOG mice were immunized with Her-2 extracellular domain (exHer-2), DNP-KLH as a hapten-carrier system, or whole protein TSST-1 intraperitoneally with alum biweekly (Fig. 2A). Two experiments were performed using CB-NOG mouse groups, each of which was transplanted with one-donor–derived $CD34^+$ cells (Table 1).

Antigen-specific IgM was detected in the sera of mice immunized with three kinds of antigens in both experiments, but specific IgG was not detected even after five boosters in all CB-NOG mice (data not shown). Reconstitution efficiency, which was determined by the proportion of human $CD45^+$ cells in the peripheral blood and specific IgM producibility were not statistically correlated in the immunized mice (Fig. 2B). Moreover, the immunized CB-NOG mouse showed very little positive correlation between total serum IgM and specific IgM producibility (Fig. 2C). Among the immunized mice with each protein antigen, the ratio of mice producing specific antibody against exHer-2 was lower (3/6) in comparison with that of mice immunized with DNP-KLH (4/4) and TSST-1 (2/2 in experiment 1 and 7/11 in experiment 2) (Table 1).

Detection of human IgM antibody specific for Her-2-peptide antigen

We have previously reported on the preparation of a humanized antibody termed CH401, which recognizes extracellular domain of Her-2 different from the recognition site of Herceptin [19]. Moreover, we recently identified an epitope peptide recognized by CH401 (Miyako et al., manuscript in preparation). In consideration of the clinical application of oligo-clonal antibodies carrying various epitopes, such epitope-specific antibodies would be useful. Then, we tried to raise the specific IgM antibody against an epitope peptide of CH401 in the immunized CB-NOG mice based on the above result that CB-NOG mice can produce human-derived IgM antibodies specific for the immunized exHer-2 protein antigen. However, because Her-2 is originally a membrane protein, the question remained whether immunization of a Her-2–derived peptide could promote production of antibodies against the CH401 epitope peptide (20 amino residues), which recognize Her-2 whole chain expressed on the cell surface. For that, we examined whether immunization of the peptide alone can induce efficiently specific antibody

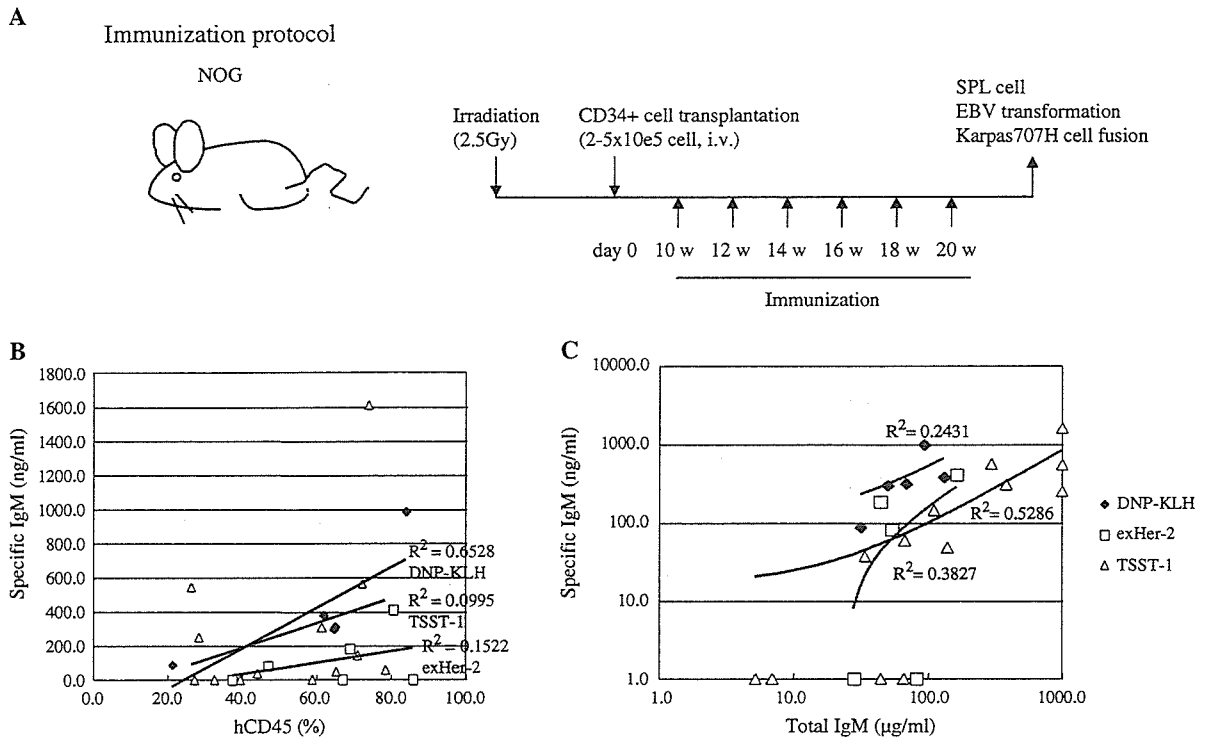

Figure 2. Correlation of specific antibody with reconstitution efficiency. (**A**) Nine-week-old nonobese diabetic/severe combined immunodeficient/ gammac(null) (NOG) mice were irradiated and transplanted with $CD34^+$ cells purified from cord blood. Ten weeks after the transplantation, immunization was started biweekly. Four days after the last booster, splenocytes were prepared and fused with Karpas707H. (**B**) Specific immunoglobulin M (IgM) of the mice vs hCD45 (%) of the mice for experiment 1 data. R^2 = 0.6528 for 2,4-dinitrophenol-conjugated keyhole-limpet-hemocyanin (DNP-KLH), R^2 = 0.1522 for exHer-2, R^2 = 0.0995 for toxic shock syndrome toxin-1 (TSST-1). Totally not so high correlation between the concentration of specific antibodies and efficiency of engraftment was observed. (**C**) Specific IgM/total IgM were analyzed for experiment 1 data shown in Table 1. Samples with specific antibodies were used for estimating the coefficients of correlation. R^2 = 0.2431 for DNP-KLH, R^2 = 0.3827 for Her-2, R^2 = 0.5286 for TSST-1.

production or whether co-immunization with cells expressing Her-2 on their surface provides synergistic or additional effect on anti-peptide antibody production. An immunization protocol we performed was shown in Figure 2A and Table 2. For peptide immunization, we used MAP because MAP peptides were reported to have similar immunogenicities to hapten-carrier antigens [20,21].

Ten and 20 weeks after $CD34^+$ cell transplantation (pre-immunization and a week after the fifth immunization), sera were collected and ELISA was performed for measuring serum level of anti-peptide antibodies (Table 3). In experiment 2-1, 16 NOG mice transplanted with human CB cells obtained from one donor were divided into three groups and immunized with epitope peptide alone (P), SV22, a NIH3T3-derived cell line expressing Her-2 (S), or epitope peptide plus SV22 (P/S), respectively. Peptide-specific IgM antibody was substantially detectable in 2/3 CB-NOG mice receiving epitope peptide alone. In comparison, immunization of SV22 or of SV22 plus epitope peptide led the ratio of anti-peptide–producing mice to 100%. The similar tendency was found in experiment 2-2, in which CB-NOG mice received another donor-derived $CD34^+$ CB cells. These results showed that a peptide immunization is capable of producing the peptide-specific antibody in CB-NOG mice. Although the ratio of antibody-producing mice was higher in mice immunized with SV22 cells plus peptide than in mice immunized with peptide alone, the same high efficiency was also found with immunization with SV22 cells, suggesting that the Her-2 epitope has sufficient antigenicity in the whole Her-2 molecule on the cell surface without additional immunization with the peptide.

Human hybridoma preparation using human myeloma cell line Karpas707H

Next, we tried to establish human–human hybridoma by fusing B cells prepared from the spleen of immunized CB-NOG mouse with a human myeloma cell line. As for human myeloma cell line, Karpas707H was used. Specificity of antibodies secreted in the culture supernatant was analyzed by ELISA, results of which are shown in Table 4. Only four Her-2–specific hybridoma lines were obtained from the mice immunized with exHer-2, while 32 hybridoma lines were obtained for DNP-KLH specific B cells. As CB-NOG mice produce mainly IgM isotype for antigen-specific antibody, spleen cells of the mice immunized with Her-2 peptide and SV22 were transformed with EBV before the fusion with

Table 1. Summary of specific antibody production in CB-NOG

Ag	Total IgM (μg/mL)	hCD45 (%)	Specific IgM (ng/mL)
	Experiment 1-1		
DNP-KLH	131.9	62.0	375.3
DNP-KLH	93.4	84.1	981.8
DNP-KLH	50.2	64.8	294.7
DNP-KLH	31.9	21.4	86.0
exHer-2	162.8	80.7	409.5
exHer-2	110.7	37.4	0.0
exHer-2	54.1	47.0	81.8
exHer-2	44.7	69.0	182.5
exHer-2	28.5	67.1	0.0
exHer-2	82.2	86.0	0.0
TSST-1	139.2	65.3	47.9
TSST-1	34.2	44.1	36.8
	Experiment 1-2		
TSST-1	>1000	26.6	543.1
TSST-1	>1000	28.5	250.3
TSST-1	>1000	74.1	1610.4
TSST-1	110.1	71.1	143.4
TSST-1	295.8	72.4	561.0
TSST-1	381.3	61.5	305.3
TSST-1	65.9	59.0	0.0
TSST-1	45.2	32.7	0.0
TSST-1	67.1	78.7	58.5
TSST-1	5.3	27.4	0.0
TSST-1	7.0	39.5	0.0

Total immunoglobulin M (IgM), specific IgM, and percentage of human $CD45^+$ cells in bone marrow were compared. Percentage of $CD45^+$ cells was determined by fluorescein-activated cell sorting. Total and specific IgM levels were determined by enzyme-linked immunosorbent assay.
CB-NOG = NOG mouse reconstituted with CB-derived $CD34^+$ cells; DNP-KLH = 2,4-dinitrophenol-conjugated keyhole-limpet-hemocyanin; exHer-2 = Her-2 extracellular domain; TSST-1= toxic shock syndrome toxin-1.

Karpas707H. As a result, we obtained specific antibody-producing hybridoma lines in both experiments, with or without EBV treatment. Consequently, totally 20 hybridoma lines producing peptide-specific antibody were obtained from the mice of experiment 2-1 (P/S-1, S and P/S-2). Moreover, 28 peptide-specific IgM-producing hybridoma lines were obtained in P/S-3 mice (experiment 2-2). One of these clones was expanded and anti-peptide IgM was collected from the supernatants. FACS analysis showed that the antibody specifically recognized surface Her-2 expressed on A20, though the intensity was not so high as that of Herceptin (Fig. 3). However, we could not detect any apoptotic effect of this antibody on Her-2–expressing cancer cells under the presence of human complement (data not shown).

These results suggest that NOG mice can produce antigen-specific IgM against very short epitope peptides. Antibody-producing B cells were successfully fused with a human myeloma cell line, and antibody-producing hybridoma was obtained. Using this system, we prepared hybridoma lines of completely human type secreting anti-Her-2 IgM.

Discussion

There is increasing evidence showing that clinical trials of certain humanized monoclonal antibodies are prospective [1,2], although their therapeutic effect is not always constitutive [22]. On the other hand, there is an argument that such antibodies may cause production of new anti-mouse antibodies during the continuous treatments because most of the therapeutic antibodies are generated from the human-mouse chimera antibody [8]. In this study, we examined the capacity of human B cells developed in mouse environment to produce antigen-specific antibodies, particularly against certain peptides, in vivo. For this, the human immune

Table 2. Immunization protocol for Her-2–related antigens

	Experiment 2-1				
	Immunization no.				
Group	1	2	3	4	5
1 (P)	Peptide	Peptide	Peptide	Peptide	Peptide
2 (P/S)	Peptide	Peptide	Peptide	SV22	SV22
3 (S)				SV22	SV22
	Experiment 2-2				
1 (P)	Peptide	Peptide	Peptide	Peptide	Peptide
2 (P/S)	Peptide	Peptide	Peptide	SV22	SV22
3 (H)	Her-2	Her-2	Her-2	Her-2	Her-2
4 (H/S)	Her-2	Her-2	Her-2	SV22	SV22
5 (S)				SV22	SV22

CB-NOG mice were immunized with Her-2–related antigens. In the experiment 2-1, epitope peptide and SV22 were used for the priming and booster. In the experiment 2-2, recombinant Her-2 extracellular domain protein (exHer-2) was also used.
CB-NOG = NOG mouse reconstituted with CB-derived $CD34^+$ cells; H = exHer-2; H/S = exHer-2 and SV22; P = peptide; P/S = peptide and SV22; S = SV22.

Table 3. Summary of anti-Her-2 antibody production in CB-NOG mice

	Experiment 2-1					
	10 Weeks after transplantation		20 Week after transplantation			
Ag	Total IgM (μg/mL)	hCD45 (%)	Total IgM (μg/mL)	Total IgG (ng/mL)	Her-2 IgM (ng/mL)	Anti-Peptide IgM (ng/mL)
P	90.7	81.4	287.9	13,548.9	0	334.4
P	58.2	30.8	418.6	2533.3	0	0
P	76.6	62.6	308.9	22,379.7	0	34.2
P/S	28	85.0	98.8	1237	18	138.4
P/S	153.7	82.1	373.4	55	0	65
P/S	104.9	88.3	185.6	26,855.3	0	59.2
P/S	94.1	83.7	250.2	0.361	0	53.6
P/S	98.2	63.8	228.2	10,788.9	0	60.7
P/S	123	63.0	208.4	0	0	52
P/S	7.6	51.8	96	0	3.6	50.4
P/S	55.7	45.5	368.3	0	0	37.6
P/S	325.1	64.5	424.8	0	0	111.2
P/S	133.6	71.1	166.9	0	0	106.9
S	47.5	72.8	194.8	801.2	0	153.7
S	108.6	61.8	324.1	4866.8	0	48.7
S	108.6	29.8	231	411.3	0	143.6
	Experiment 2-2					
P		88.3			0.0	0.0
P		80.5			0.0	0.0
P/S		83.0			0.0	5.0
P/S		57.1			0.0	12.5
P/S		90.1			0.0	0.0
P/S		39.5			725.9	613.8
H	ND	64.9	ND	ND	0.0	129.8
H		52.1			0.0	0.0
H		93.8			0.0	0.0
H		74.1			0.0	29.0
H/S		70.4			0.0	0.0
H/S		94.7			0.0	193.3
S		87.4			0.0	234.5
S		77.4			0.0	30.6

CB-NOG mice were immunized by the protocols as shown in Table 2. Total immunoglobulin M (IgM) and hCD45 (%) were determined 10 weeks after transplantation. Specific IgM, total IgM, and total IgG were determined at week 20.
CB-NOG = NOG mouse reconstituted with CB-derived $CD34^+$ cells; H = exHer-2; Her-2 = exHer-2 used for enzyme-linked immunosorbent assay (ELISA); H/S = exHer-2 and SV22; ND = not determined; P = peptide; Peptide = epitope peptide used for ELISA; P/S = peptide and SV22; S = SV22.

system in immunodeficient mouse was reconstituted by transplantation of human cord blood $CD34^+$ cells (CB-NOG), and they were thereafter immunized with given antigens. Then, antibody-producing human B cells obtained from the spleen of immunized CB-NOG mice were fused with a human myeloma cell line, which was recently established by Karpas et al. [18] to generate human–human hybridoma.

Among immunodeficient mice used for transplantation of human tissues, NOG mice were reported to be the most acceptable for human leukocytes [11]. Moreover, several groups including us showed that the human immune system could be efficiently reconstituted in NOG mice after transplantation of $CD34^+$ human cord blood cells [10,12,23,24]. Moreover, we demonstrated that the CB-NOG mice produced the antigen-specific antibody when they were immunized with DNP-KLH [12]. In this study, we examined whether peptide-specific antibodies are produced in the reconstituted CB-NOG mice when they were immunized with a MAP peptide with a small region of the Her-2 epitope peptide in Freund's complete adjuvant. In these mice, IgM production against a given short peptide antigen was detected with a relatively high frequency, which was not different from that of mice immunized with the ordinary protein antigens, such as a Her-2 extracellular domain. Compared with the peptide immunization, tumor cells expressing Her-2 on their surface induced anti-Her-2 peptide antibody production in a markedly high frequency. Peptide immunization did not have additive effect on the specific antibody induction when the Her-2–expressing cells were used for the antigen, as the frequency of antibody-producing mice and the antibody concentration in the sera were

Table 4. Summary of hybridoma production by CB-NOG B cells and Karpas707H

Ag	Mouse[a]	EBV	Cell[b] (x10e5)	Colony (n)	Total antibody (colony, n)		Specific antibody (colony, n)	
					IgM	IgG	IgM	IgG
H	4	–	921	142	21	15	4	ND
P/S-1	1	+	366	31	ND	ND	8	ND
P/S-2	1	+	255	14	ND	ND	2	ND
P/S-3	1	+	641	54	ND	ND	28	ND
S	1	+	358	48	ND	ND	10	ND
DNP-KLH	4	–	351	58	32	0	32	0

CB-NOG B cells were fused with Karpas707H and the yield was shown.
CB-NOG = NOG mouse reconstituted with CB-derived $CD34^+$ cells; DNP-KLH = 2,4-dinitrophenol-conjugated keyhole-limpet-hemocyanin; EBV = Epstein-Barr virus; H = exHer-2; Ig = immunoglobulin; ND = not determined; P/S = peptide and SV22; S = SV22.
Antibody production was determined by enzyme-linked immunosorbent assay for culture supernatants. Four clones were exHer-2–specific. Forty-eight clones were epitope peptide-specific. One of the clones stably produced antibodies for more than 7 months.
[a]Mouse pooled for one fusion experiment.
[b]Total cell number used for one fusion experiment.

not so different between the peptide-immunized and nonimmunized groups. These results indicate that immunization of a certain peptide derived from the membrane protein can induce specific antibodies in CB-NOG mice, but that its antigenicity is not as strong as the whole molecule including the epitope sequence expressed on the cell surface.

Only IgM was detectable as antigen-specific antibodies in the sera of CB-NOG mice when mice were immunized with either peptide or ordinary protein antigens. In consideration of the characters of CB-NOG mice, this result might not be unexpected: the majority of spleen B cells of CB-NOG are $CD5^+B1$ cells [12], which are recognized as being the major IgM producer. However, human B cells developed in CB-NOG mice have a potential to produce IgG, because nonspecific IgG was detected in the CB-NOG mice immunized with Her-2 peptide. Very recently, Ishiawa et al. [25] reported development of specific IgG against ovalbumin in newborn NOG mice receiving transplantation of human stem cells, although the antibody level was not high enough. Therefore, it is possible that the cognate interaction mediated by a certain antigen on major histocompatibility class (MHC) II between B cells and T cells may occur in a low frequency, presumably because most human T cells developed in the mouse thymus of CB-NOG mice are restricted with mouse MHC. Consequently, only a low proportion of B cells can recognize helper T cells by human MHC restriction to secrete antigen-specific IgG (in preparation).

To date, most human monoclonal antibodies were prepared from mouse–human hybridoma lines prepared by human B cells and mouse myeloma cell lines, mainly because the adequate human myeloma cell line was not available. Recently, Karpas and his collaborators established a myeloma cell line, Karpas707H, which expresses a low level of human light chain without its secretion. Using this myeloma cell line, they succeeded in establishing genuine

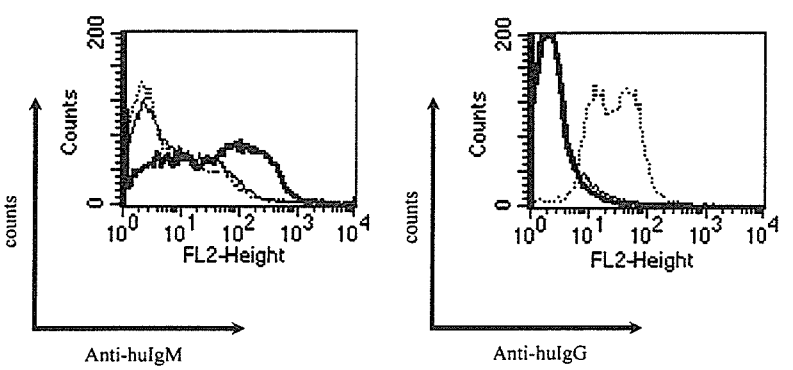

Figure 3. Recognition of surface Her-2 by human anti-Her-2 immunoglobulin M (IgM). Anti-Her-2 IgM was prepared from human hybridoma and purified. Mouse B-cell lymphoma (A20) expressing Her-2 was stained with the anti-peptide IgM antibody prepared from one of the hybridoma lines or Herceptin (IgG). Phycoerythrin-conjugated anti-human IgM (Anti-huIgM) or anti-human IgG (Anti-huIgG) was used for the secondary antibody. Cells were washed and analyzed by fluorescein-activated cell sorting analysis. Bold lines show the staining pattern with anti-peptide IgM; dotted lines show that of Herceptin. Solid thin lines show that of only secondary antibody.

human hybridomas by fusing with EBV transformed human B cell line producing anti-HIV-1 IgG and with fresh cells obtained from tonsils or peripheral blood cells. According to their process, we fused Karpas707H with spleen cells obtained from CB-NOG mice immunized with given antigens. Corresponding to the spectrum of the immunoglobulin type in the serum of the immunized CB-NOG mice, all the hybridomas we established secreted IgM antibody against Her-2 peptide (Table 4). Among these, one of the hybridoma lines maintained production of anti-Her-2 peptide antibody in the culture for more than 7 months without losing IgM secretion ability, and the partially purified antibody recognized Her-2 transfectants. Corresponding to the serum Igs, some of the hybridoma lines were found to secrete nonspecific IgG antibodies, indicating that at least EBV transformation of B cells in vitro does not disturb the fusion of Karpas707H with spleen B cells producing either IgG or IgM in CB-NOG.

IgG-type monoclonal antibody is considered more useful for clinical usage mainly because of its low molecular weight, high specificity, and multifunctions. However, IgM may not always be invalid for clinical application. For example, IgM antibodies against HIV induce more efficient cytolysis for infected cells in a complement-mediated manner presumably because the complement binding ability of IgM is higher than IgG [26]. Although our monoclonal IgM antibody showed very low cytotoxic activity against Her-2–positive tumor in vitro under the presence of mouse serum (data not shown), several groups reported that IgM antibodies possess anti-tumor function in the presence of complements [27–31]. Thus, the method described in this article will be useful in obtaining genuine human monoclonal antibody to the surface molecule on tumor cells, even if the antibody is IgM.

In summary, we demonstrated that CB-NOG mice, which were prepared from immunodeficient NOG mice reconstituted with human immune system, have potential for producing peptide-antigen–specific IgM antibodies, although most B cells developed in the mice are $CD5^+$ cells. We could produce human-derived IgM antibody against Her-2 epitope peptide in the CB-NOG mice. By fusing splenic B cells from the immunized NOG mice with a human myeloma cell line, we succeeded to generate genuine human hybridoma secreting antigen-specific IgM against a given peptide.

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bjh research paper

Outcome in patients with Wiskott–Aldrich syndrome following stem cell transplantation: an analysis of 57 patients in Japan

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Summary

A total of 57 patients with Wiskott–Aldrich syndrome (WAS) were studied after undergoing stem cell transplantation (SCT) in Japan between January 1985 and December 2004. Eleven patients received transplants from human leucocyte antigen (HLA)-matched related donors, 10 from HLA-mismatched related donors, 21 from unrelated bone marrow donors, and 15 from unrelated cord blood donors. Nine of the 57 patients rejected the initial graft. The overall 5-year survival rate was 73·7% and the 5-year failure-free survival rate was 65·7% (failure was defined as rejection or death). The overall 5-year survival rates for patients receiving bone marrow and cord blood from unrelated donors were both 80·0%. Based on univariate analysis, the factors associated with poor survival were: transplantation from an HLA-mismatched related donor, patient age of more than 5 years at the time of transplantation, and a conditioning regimen other than busulfan and cyclophosphamide (BU-CY) or busulfan, cyclophosphamide and antithymocyte globulin (BU-CY-ATG). In a multivariate analysis, a conditioning regimen other than BU-CY and BU-CY-ATG was the only independent factor associated with transplantation failure. Given the improved outcome for WAS patients following transplantation from an unrelated donor, we conclude that patients with WAS should receive SCT as soon as possible after diagnosis.

Keywords: Wiskott–Aldrich syndrome, stem cell transplantation, immunodeficiency, unrelated donor.

Wiskott–Aldrich syndrome (WAS) is an X-linked disorder of haematopoietic cells characterised by thrombocytopenia with small platelets, eczema and progressive immunodeficiency (Wiskott, 1937; Aldrich *et al*, 1954; Perry *et al*, 1980). The condition is caused by mutations in the *WASP* gene at Xp11.22 (Derry *et al*, 1994), and has a worldwide distribution with an estimated incidence of 4 per one million live male births (Somerville & Forsyth, 1993). Diagnostic measures for WAS patients have progressed markedly since identification of the responsible gene (*WASP*) and its product. We have established a method of flow cytometric analysis of intracellular WASP (Yamada *et al*, 2000), which can be used for WAS screening and is potentially useful for the clinical evaluation of WAS patients after stem cell transplantation (SCT) (Yamaguchi *et al*, 2002). A suspected diagnosis of WAS diagnosis following screening can be confirmed by subsequent mutation analysis;

thus, early diagnosis of WAS is now possible, whereas it was previously difficult for younger children.

Untreated patients with typical WAS have a poor prognosis, with the major causes of death being infection, bleeding and lymphoproliferative disorders. Although splenectomy generally increases platelet count and reduces the risk of major haemorrhage (Lum *et al*, 1980; Mullen *et al*, 1993; Litzman *et al*, 1996), the risk of death from sepsis is increased. Allogeneic SCT has been recognised as an effective method for curing WAS patients who have HLA-matched siblings (Rimm and Rappeport, 1990; Bortin *et al*, 1994), but the number of WAS patients with such a sibling is only small. Although patients who do not have an HLA-matched sibling can undergo SCT from an HLA-mismatched related donor or an unrelated donor, the results to date have been extremely poor (Brochstein *et al*, 1991; Fischer *et al*, 1991). In particular, low survival rates are likely for patients receiving transplants from related donors other than HLA-identical siblings, or from unrelated donors for boys older than 5 years, according to the National Marrow Donor Program (Filipovich *et al*, 2001). The use of cord blood stem cell transplantation (CBSCT) has become common for many diseases, but only a few examples of this procedure for patients with WAS have been reported (Knutsen & Wall, 2000; Kaneko *et al*, 2003; Knutsen *et al*, 2003; Tsuji *et al*, 2006). Herein, we report the outcomes and prognostic factors of 57 WAS patients who underwent SCT in Japan since 1985.

Patients and methods

Between January 1985 and December 2004, 57 WAS patients who were registered with the Committee for Stem Cell Transplantation of the Japanese Society of Pediatric Haematology underwent a total of 64 SCTs. The age of the patients at transplantation ranged from 3 months to 19 years, and the median age was 1·6 years. The origin of the stem cells was bone marrow (BM), peripheral blood (PB) stem cells, and cord blood (CB) stem cells for 40, 2 and 15 patients respectively. The donors were HLA-matched siblings (in eight cases), siblings with a single locus mismatch (5/6 matched, two cases), HLA-matched parents (three cases), a parent with a single locus mismatch (5/6 matched, one case), parents with two loci mismatches (4/6 matched, four cases), parents with three loci mismatches (3/6 matched, two cases), an uncle with a single locus mismatch (5/6 matched, one case), HLA-matched unrelated donors (25 cases), unrelated donors with one HLA locus mismatch (5/6 matched, 10 cases), and an unrelated donor with two loci mismatches (4/6 matched, one case). One patient received transplantation using CD34-positive selection, and transplantation using T-cell depletion was not performed in any patients. A conditioning regimen of busulfan, cyclophosphamide and anti-thymocyte globulin (BU-CY-ATG) was used in 20 patients, and a BU-CY regimen was employed in 13 patients. Radiation-containing regimens (total body irradiation, thoraco-abdominal irradiation and total lymphoid irradiation) were used for 14 patients. Prophylaxis for graft-*versus*-host disease (GVHD) was performed using ciclosporin A and short term methotrexate in 28 patients, ciclosporin A alone in seven patients, ciclosporin A and methyl prednisolone in five patients, tacrolimus and short term methotrexate in eight patients, methotrexate alone in four patients, tacrolimus and methyl prednisolone in three patients, and tacrolimus alone in one patient.

Statistical analysis

The data were analysed as of December 1, 2005. Failure-free survival (FFS) was defined as survival with treatment response, whereas death, rejection and secondary malignancy were considered to be treatment failures. Analyses of overall survival (OS) and FFS were performed using the Kaplan–Meier method, with differences compared by log-rank test. Multivariate stepwise regression analysis was performed to explore the independent effects of variables that showed a significant influence on outcome in univariate analysis. Statistical analyses were performed using Dr SPSS II for Windows (release 11.0.1J, SPSS JAPAN Inc., Tokyo, Japan).

Results

Nine of the 57 patients who underwent SCT rejected their graft; six of these patients received a second transplantation. An additional patient received a second transplantation because of mixed chimaerism. Fourteen patients died, and the causes of death were infection (nine patients), GVHD (three patients), bleeding (one patient) and thrombotic microangiopathy (one patient). Four patients died following rejection, but no patients suffered a secondary malignancy. Acute GVHD ≥ grade II developed in 36·8% of all patients, and in 27·3% of HLA-matched related SCT recipients, 40·0% of HLA-mismatched related SCT recipients, 33·3% of unrelated bone marrow transplantation (BMT) recipients and 46·7% of unrelated CBSCT recipients. Chronic GVHD occurred in 40·4% of all patients, and in 36·4% of HLA-matched related SCT recipients, 60·0% of HLA-mismatched related SCT recipients, 47·6% of unrelated BMT recipients and 33·3% of unrelated CBSCT recipients (Table I). Seven patients received a second transplantation: six for failure to engraft or graft failure, and one for mixed chimaerism, and the interval between the two transplantations ranged from 1 to 36 months. Two of the seven patients had the same donor for both transplantations, three patients received a transplantation from an HLA-mismatched related donor following graft failure after an initial transplantation from an unrelated donor, and one patient who had mixed chimaerism had different unrelated donors for each transplantation; all six patients are still alive without graft failure. The seventh patient underwent a second transplantation from an HLA-matched sibling following graft rejection after the first transplantation from an HLA-mismatched related donor; however, this patient died from sepsis.

The OS rate 5 years after transplantation was 73·7 ± 6·1% and the 5-year FFS rate was 65·7 ± 6·6% (Figs 1 and 2).

