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Post-transplant complications

Increased incidence of EBV-associated lymphoproliferative disorders after allogeneic stem cell transplantation from matched unrelated donors due to a change of T cell depletion technique

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Summary:

Here, the influence of T *vs* T and B cell depletion on the incidence of EBV-associated lymphoproliferative disorder (EBV-LPD) after bone marrow transplantation (BMT) from a matched unrelated donor (MUD) is analyzed. From 1982 to 1997 the soy bean agglutinin/sheep red blood cell (SBA/SRBC) method was used for T cell depletion. This technique is well established, but the use of SRBC has a risk of transmitting prions or viruses. Therefore, a new T cell depletion method was introduced, using CD2 and CD3 monoclonal antibodies (CD2/3 method) instead of SRBC. Unfortunately, this led to an unexpected high number of EBV-LPDs in patients receiving transplants from MUDs. SBA depletion was reintroduced and combined with the CD2/3 method (SBA/CD2/3) in this patient population, later replaced by B cell-specific (CD19 and CD22) antibodies (CD3/19/22 method). The number of T ($\times 10^5$/kg) and B ($\times 10^5$/kg) cells in the graft was 1.5 $\pm$ 0.8 and 2 $\pm$ 1 (T/B ratio 0.75), 2.2 $\pm$ 2.0 and 41 $\pm$ 21 (ratio 0.055), 5.0 $\pm$ 0.0 and 2 $\pm$ 1 (ratio 2.5), 2.5 $\pm$ 1.2 and 10 $\pm$ 6 (ratio 0.25) using the SBA/SRBC, CD2/3, SBA/CD2/3 and CD3/19/22 techniques, respectively. When B cell depletion was performed (SBA/SRBC, SBA/CD2/3, CD3/19/22) four out of 31 patients (13%) receiving a BMT from a MUD developed an EBV-LPD. Without B cell depletion (CD2/3) this occurred in five out of seven patients (71%) ($P < 0.05$). A T/B cell ratio in the graft of $\geqslant$0.25 seems sufficient to significantly reduce the incidence of EBV-LPD after BMT from MUDs.

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After bone marrow transplantation (BMT) from a matched unrelated donor (MUD), transplant-related morbidity and mortality are increased compared to BMT from a matched related donor (MRD). This is largely due to an increased incidence of graft-versus-host disease (GVHD) and infectious complications.[1,2] T cell depletion reduces the incidence and severity of GVHD[3] but also has important side-effects. Curtis *et al*[4] showed that the risk of Epstein–Barr virus-associated lymphoproliferative disorders (EBV-LPD) was strongly associated with T cell depletion of donor marrow. Use of antithymocyte globulin (ATG) and of grafts from unrelated or HLA-mismatched related donors were other adverse risk factors for the development of EBV-LPD. The risk for EBV-LPD varied according to the techniques used for T cell depletion, being lowest when the Campath-1 method was used, which, in contrast to T cell-specific monoclonal antibodies (Mabs), removed both T and B cells.[4]

In this report the influence of the T *vs* T and B cell depletion method on the incidence of EBV-LPD among MUD transplant recipients is described, as it was observed at our institute. From 1982 to 1997 the soy bean agglutinin/sheep red blood cell (SBA/SRBC) method was used for T cell depletion. This technique has been well established, but the use of SRBC has a risk of transmitting prions or viruses. Therefore, a new T cell depletion method was introduced, the immunorosette (IR) technique, using tetrameric complexes with T cell-specific CD2 and CD3 Mabs instead of sheep red blood cells. Unfortunately, this led to an unexpectedly high number of EBV-LPDs in patients receiving transplants from MUDs. Since it was suspected that this was caused by the relatively high number of B cells in the graft, SBA depletion, which results in B cell depletion of 1 to 1.5 log, was reintroduced and combined with CD2/3 depletion from May 1998 (SBA/CD2/3 method). In March 1999, SBA was replaced by B cell-specific (CD19 and CD22) antibodies (CD3/19/22 method).

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Table 1 Number of BMTs according to depletion technique

Depletion technique	*MUD patients*	*NE*	*MRD patients*	*NE*
SBA/SRBC	19	5	202	54
CD2/3	11	4	62	9
SBA/CD2/3	6	0		
CD3/19/22	13	2		

SBA/SRBC = soy bean agglutinin/sheep red blood cell; SBA/CD2/3 = SBA agglutination followed by depletion with anti-CD2 and CD3 Mabs; CD2/3 = depletion with anti-CD2 and CD3 Mabs; CD3/19/22 = depletion with anti-CD3, CD19 and CD22 Mabs; NE = not evaluable because of early death.

Methods

Patients

From November 1985 to April 2000 a total of 313 patients received allogeneic BMT, 49 from a MUD and 264 from a matched related donor (MRD). Eleven MUD and 63 MRD transplant recipients died within 6 months after BMT from relapse or transplant-related toxicity, apart from EBV-LPD, and were therefore not evaluable for this study. The interval of 6 months was chosen because most EBV-LPDs develop during the first 6 months post transplant.[4] The distribution of patients according to T cell depletion method used is shown in Table 1. Characteristics of recipients of MUD transplants are described in Table 2. All patients were treated on clinical protocols approved by the local investigation review board and gave informed consent.

Table 2 Characteristics of recipients of MUD transplants

	SBA/SRBC	*CD2/3*	*SBA/CD2/3*	*CD3/19/22*
No.	14	7	6	11
Age	29 (17–47)	28 (18–37)	31 (22–47)	32 (18–48)
Diagnosis				
ALL	5	1	1	2
AML	0	3	0	4
CML	6	1	2	4
MDS	1	0	1	1
SAA	2	2	2	0
Sex				
F/F	3	0	0	3
M/M	5	3	3	4
M/F	5	1	2	3
F/M	1	3	1	1
CMV				
–/–	2	2	3	3
+/+	1	2	1	2
–/+	5	1	1	4
+/–	6	2	1	2
GVHD				
Acute				
I/II	10	4	2	5
III/IV	1	1	0	2
None	3	2	4	4
Chronic				
L	3	1	0	0
E	6	0	1	0
NE	0	3	0	1
None	5	3	5	10

NE = not evaluable; L = limited; E = extensive.

Transplantation procedure

Conditioning regimens consisted of cyclophosphamide (60 mg/kg/day) on each of 2 successive days, followed by total body irradiation (600 cG/day) on each of 2 successive days. The graft was infused after the second TBI fraction (day 0). ATG (Imtix Sangstat, Amstelveen, The Netherlands) was given to MUD patients before cyclophosphamide was started, at a total dose of 20 mg/kg intravenously. It was lowered to a total dose of 8 mg/kg from March 1999. All patients received cyclosporine from day –2 at a dose of 3 mg/kg/day by continuous infusion for 4 weeks; thereafter it was given orally for 4–6 weeks at a dose that gave comparable levels, and then tapered. Cyclosporin was discontinued within 3 months of transplantation if no active GVHD was observed. Infection prevention for all patients consisted of ciprofloxacin, fluconazole and amphotericin B given orally until granulocyte counts exceeded 500 cells/mm^3. Cephalothin was given intravenously for 10 days from day +3. Co-trimoxazole and valacyclovir were given orally from day +1 until 12 months post BMT. GVHD was diagnosed according to the Seattle criteria.[5] Acute GVHD grade I was treated with topical corticosteroids; grade II or higher was treated with systemic corticosteroids as described.[6] Extensive chronic GVHD was treated with systemic corticosteroids, sometimes combined with cyclosporin. For the first 4 months post transplant, CMV-seropositive patients who demonstrated reactivation of CMV infection or those who were treated with high-dose corticosteroids received pre-emptive or prophylactic therapy, respectively, with ganciclovir at a dose of 2.5 mg/kg intravenously twice a day for 14 days.[7]

HLA matching

In all MRD patient–donor pairs, class I antigens (A, B and Cw) were analyzed by serological typing, and in the event of doubt, low resolution molecular typing was performed. Class II antigens (DRB1, DRB3, DRB4, DRB5 and DQB1) were analyzed by serological typing until 1993, and after 1993 by low resolution molecular typing with sequence specific primers. In MUD patient–donor pairs HLA analysis was performed as in MRD recipients until 1993. Thereafter, class I antigens (A, B) were analyzed by serological typing, and in the event of doubt low resolution molecular typing was performed. Class I Cw and class II antigens (DRB1, DRB3, DRB4, DRB5 and DQB1) were analyzed by low resolution molecular typing with sequence-specific primers and DRB1, B3, B4 and B5 antigens were also defined by high resolution typing.

T and B cell depletion

SBA/SRBC T cell depletion was performed as described.[8] The immunorosette depletion technique (CD2/3) was performed as described by Slaper-Cortenbach *et al*.[9] In short, tetrameric complexes (CLB, Amsterdam, The Netherlands) were formed by addition of crosslinking RaMIgG1 Mabs

to a mixture of murine IgG1 Mabs, one directed against glycophorin A in the membrane of human erythrocytes and another against T cell-specific antigens (CD2 or CD3). These complexes were then bound to donor erythrocytes (in the case of a MUD transplant obtained from a healthy O rhesus-negative donor from the blood bank) and the coated erythrocytes were washed. After addition of the coated erythrocytes to the bone marrow buffy coat cells prepared using the COBE 2991, immunorosettes formed. These immunorosettes were removed using Ficoll density separation (d = 1.077 g/cm^3). In the SBA/CD2/3 method Ficoll density separation was used to prepare mononuclear cells, and subsequently SBA was used with the CD2 and CD3 tetrameric complexes instead of SRBC. The CD3/19/22 method used tetrameric complexes with CD3, CD19 and CD22 Mabs for depletion of T and B cells. All monoclonal antibodies were tested for viral and bacterial contamination and were, therefore, biosafe. In all T/B cell depletion procedures the residual number of T cells was counted, and nonmanipulated T cells (from a small BM fraction that was set apart before the stem cell manipulation started) were added to obtain a low fixed number of T cells.[6] This fixed number of T cells in the graft differed per depletion technique (SBA/SRBC: 1×10^5 T cells/kg; CD2/3: 1×10^5 T cells/kg; SBA/CD2/3: 5×10^5 T cells/kg; CD3/19/22: 2×10^5 T cells/kg). In some grafts the residual number of T cells after depletion was above the fixed number and no T cell add-back was performed in these grafts. T/B cell depletion was evaluated by Facs analysis (FACScan; Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA, USA) on unmanipulated bone marrow and depleted marrow using monoclonal antibodies (CD2-FITC, CD3-PE, CD19-PE, CD20-FITC, CD45-PERCP (Becton Dickinson)).

EBV-LPD

EBV-LPD was diagnosed by standard histologic criteria.[10] CD20 antibodies were used to assess the B cell origin of the LPD. The presence of EBV infection was determined immunohistochemically by detection of EBNA-2 and LMP-1 proteins and with *in situ* hybridization to detect EBV-encoded RNA (EBER).

Statistics

Data are expressed as mean $\pm$ s.d. Mean differences between groups were assessed by the Mann–Whitney *U* test or chi squared analysis. Calculations were performed using SPSS/PC+ 8.0 (SPSS, Chicago, IL, USA).

Results

Incidence of EBV-LPD

The incidence of EBV-LPD is summarized in Table 3. When B cell depletion was performed (SBA/SRBC, SBA/CD2/3, CD3/19/22), four out of 31 patients (13%) receiving BMT from a MUD developed an EBV-LPD, this being the cause of death in three patients. Without B cell depletion (CD2/3), five out of seven patients (71%) receiving BMT from a MUD developed an EBV-LPD and all died of this disease. This resulted in a significant difference ($P < 0.05$) between B cell-depleting techniques and a non-B cell-depleting technique concerning the incidence of EBV-LPDs. In contrast to MUD recipients, among patients receiving BMT from an HLA-identical sibling donor the incidence of EBV-LPD was similar when a T and B cell depletion method was used (SBA/SRBC) compared to a T without B cell depletion method (CD2/3). The incidences were 5 and 4%, respectively.

T and B cell numbers in the graft

T and B cell counts in the MUD grafts according to depletion technique are shown in Table 4. The SBA/CD2/3 group received significantly more T cells ($P < 0.05$), compared to all other groups. This was not due to depletion failure as can be seen from the log depletion reached. In fact, the SBA/CD2/3 was the most efficient T cell depletion method. As has been described earlier, after T cell depletion the residual number of T cells was counted, and nonmanipulated T cells were added to obtain a low fixed number of T cells (1–5 $\times$ 10^5 T cells/kg recipient weight). In the SBA/CD2/3 group this number was set at 5×10^5/kg.

In the SBA/SRBC-depleted grafts, B cell numbers were not measured. We can assume, however, that the SBA/SRBC method will yield a B cell depletion comparable to the SBA/CD2/3 method (confirmed by experiments in the laboratory). B cell numbers were also not measured in CD2/3-depleted grafts of unrelated donors. When the incidence of EBV-LPD increased dramatically in MUD recipients and this was suspected as being due to the relatively high number of B cells in the graft, SBA agglutination was performed prior to CD2/3 depletion. After that B cells were measured in all grafts (CD2/3-depleted grafts from MRDs, SBA/CD2/3 and CD3/19/22-depleted grafts from MUDs). The CD2/3 group (data derived from MRD transplants) received significantly more B cells compared to all other groups: $41 \pm 21 \times 10^5$/kg *vs* 2 ± 1 (SBA/CD2/3) and 10 ± 6 (CD3/19/22); $P \leqslant 0.001$.

The T/B cell ratio in the graft according to the depletion method was 0.75 for the SBA/SRBC group (ratio was calculated using the B cell count from the SBA/CD2/3 method), 0.055 for the CD2/3 method, 2.5 for the SBA/CD2/3 group and 0.25 for the CD3/19/22 technique. It should be noted that in this last group ATG dose was reduced. Therefore, *in vivo* T cell depletion due to ATG in this group is expected to be less than in the other three groups.[11]

Discussion

The most important observation of this report is the significantly increased incidence of EBV-LPD in patients receiving BMT from an unrelated donor, which was T but not B cell-depleted, with an immunorosette technique, using CD2 and CD3 Mabs. Among MRD patients the incidence of EBV-LPD was not influenced by B cell depletion. The SBA/SRBC depletion method was abandoned in 1997 because the use of SRBC has a risk of transmitting prions

Table 3 EBV-LPD according to depletion technique

Depletion technique	*MUD patients (n)*	*EBV-LPD*	*DOD*	*MRD patients (n)*	*EBV-LPD*	*DOD*
SBA/SRBC	14	3 (21%)	2	148	7 (5%)	5
CD2/3	7	5 (71%)	5	53	2 (4%)	0
SBA/CD2/3	6	0 (0%)	0			
CD3/19/22	11	1 (9%)	1			

EBV-LPD = EBV-associated lymphoproliferative disease; DOD = died of EBV-LPD.

Table 4 Depletion of T and B cells from marrow grafts of matched unrelated donors

Techique	*T cell count in graft ($\times 10^5$/kg)*	*Log depletion T cells*	*B cell count in graft ($\times 10^5$/kg)*	*Log depletion B cells*	*ATG dose*	*Ratio T/B*
SBA/SRBC[a]	1.5 ± 0.8	2.3 ± 0.6	ND		HD	0.75
CD2/3[b]	2.2 ± 2.0	2.3 ± 0.4	41 ± 21	0.2 ± 0.1	HD	0.055
SBA/CD2/3	5.0 ± 0.0	3.2 ± 0.5	2 ± 1	2.0 ± 0.2	HD	2.5
CD3/19/22	2.5 ± 1.2	3.0 ± 0.7	10 ± 6	1.1 ± 0.2	LD	0.25

[a]To calculate the T/B cell ratio, B cell count from the SBA/CD2/3 group was taken.
[b]B cell count was measured in a group of 24 patients receiving a transplant from a MRD.
ATG dose: HD = high dose = 20 mg/kg; LD = low dose = 8 mg/kg.

or viruses. The Mabs used in the immunorosette technique were biosafe (screened for viral and bacterial contamination). The CD2/3 depletion method had been proven to give similar T cell depletion (Table 4). Engraftment and hematopoietic recovery were comparable for the two techniques (data not shown). Up to 1998 there were no reports showing the importance of B cell depletion for prevention of EBV-LPD. Resting memory B cells are thought to be the natural reservoir of EBV within the body.[12] B cell load of patient origin has been largely destroyed due to the pretransplant myeloablative conditioning regimen. Gratama *et al*[13,14] showed that latently EBV-infected host cells can also be eliminated after BMT. Therefore, theoretically, B cell depletion of the graft along with T cell depletion might improve immunological control of EBV infection post-transplant. Indeed, Cavazzana *et al*[15] showed that none of 19 patients receiving transplants from a partially matched related donor (PMRD) developed EBV-LPD when *ex vivo* T and B cell depletion was performed, whereas seven out of 19 historical controls developed EBV-LPD when only T cell depletion was carried out. Two other studies showed that B cell depletion might be of benefit for decreasing the incidence of EBV-LPD.[16,17] Our report emphasizes the importance of B cell depletion in patients receiving T cell-depleted grafts from matched unrelated donors. In MRD recipients T cell depletion without B cell depletion did not result in an increased incidence of EBV-LPD. Therefore, aside from *ex vivo* T cell depletion, there have to be other factors which impair immune surveillance of the Epstein–Barr virus in MUD recipients. It was recently shown that TCD together with use of ATG and a high $CD34^+$ cell count in the graft were the only factors influencing EBV reactivation post transplant.[18] The effect of $CD34^+$ cell count of the graft on the incidence of EBV reactivation might be explained by the infusion of a higher number of B cells together with larger stem cell grafts when $CD34^+$ stem cell selection is performed. All our MUD patients received ATG, giving additional *in vivo* T cell depletion.[11] In MUD recipients, the ratio of T and B cells in the graft seems to be very important for EBV surveillance. When a high dose of ATG was used pretransplant, a T/B cell ratio of 2.5 was sufficient to prevent EBV-LPD in six patients (SBA/CD2/3 group). When low-dose ATG was used, a ratio of 0.25 did not prevent EBV-LPD totally: one out of 11 patients developed EBV-LPD (CD3/19/22 group). The actual T/B cell ratio *in vivo* might have been higher in this group due to the less severe *in vivo* T cell depletion. The optimal T/B cell ratio is currently not known and will be dependent on several factors such as use and dosage of ATG. The number of patients in our study is limited, so further studies are necessary to establish the degree of B cell depletion needed for efficient prevention of EBV-LPD in patients receiving T cell-depleted grafts from donors other than HLA-matched siblings. In the study of Cavazzana *et al*[15] B and T cell counts in the grafts were $5 \pm 8.5 \times 10^5$/kg and $1.5 \pm 1.7 \times 10^5$/kg, respectively. This gave a ratio of 0.3 and no EBV-LPD was observed post transplant. These and our data suggest that a ratio $\geqslant 0.25$ can markedly reduce EBV-LPD in such patients.

In conclusion, our data show that the incidence of EBV-associated lymphoproliferative disorders in recipients of allogeneic bone marrow transplants from matched unrelated donors is increased when T cell depletion of the graft is performed, without B cell depletion. We replaced the SBA/SRBC technique with a new technique, based on the use of Mabs instead of SRBC. This resulted in a disproportionally higher number of residual B cells in the graft and, consequently, in a dramatic increase in EBV-LPD in recipients of T cell-depleted stem cell transplant from matched unrelated donors.

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