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Rejection of bone-marrow graft by recipient-derived cytotoxic T lymphocytes against minor histocompatibility antigens

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A female patient showed rejection of a T-lymphocyte-depleted bone-marrow graft from her phenotypically HLA-identical father. Before bone-marrow transplantation, there was strong recipient anti-donor cellular cytotoxic reactivity directed against several minor histocompatibility (mH) antigens, including the male-specific H-Y antigen. After conditioning treatment, no recipient anti-donor cytotoxic activity could be detected, and good graft function was shown a month after transplantation. Thereafter, however, graft function deteriorated rapidly, while recipient-derived anti-donor cellular cytotoxic reactivity, against similar mH antigens, reappeared. The recipient-derived cytotoxic T lymphocytes could completely inhibit growth of donor haemopoietic progenitor cells both before and after bone-marrow transplantation. Thus, cytotoxic T lymphocytes can survive very intensive conditioning regimens, and residual recipient cytotoxic T lymphocytes directed against mH antigens expressed on donor haemopoietic progenitor cells may cause graft rejection after HLA-identical T-lymphocyte-depleted bone-marrow transplantation.

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Introduction

T-lymphocyte depletion of bone-marrow grafts has been successfully used to reduce the incidence of graft-versus-host-disease after allogeneic HLA-identical bone-marrow transplantation [1] However, this procedure has resulted in a higher rate of graft failure [2-5] The mechanisms of graft failure have not been elucidated, though failure may be due to immune-mediated rejection of the graft caused by residual immunocompetent recipient cells that have survived the pretransplant conditioning regimen

We report here the detailed analysis of graft rejection in a female bone-marrow transplant recipient of a T-lymphocyte-depleted graft from her phenotypically HLA-identical father.

Case-report

4 years before bone-marrow transplantation, this 32-year-old woman was treated for Hodgkin lymphoma (mixed cellularity, clinical stage IIIB) with radiation therapy and courses of chemotherapy A complete remission was obtained 3 years later, persisting pancytopenia developed and she required red-cell and platelet transfusions A sample of bone marrow showed a myelodysplastic syndrome (refractory anaemia with excess of blasts, FAB type III) [6] Cytogenetic analysis of bone marrow and blood showed a normal female karyotype During the year before transplantation there were repeated life-threatening infections with *Pseudomonas aeruginosa*, and continuous intravenous antibiotic treatment was required We decided to carry out allogeneic bone-marrow transplantation with a graft donated by her phenotypically HLA-identical father The bone-marrow graft was depleted of T lymphocytes by means of murine monoclonal antibodies (anti-CD5 and anti-CD8) and baby rabbit complement After depletion, the graft contained 20×10^6 nucleated cells and $0{\cdot}9 \times 10^6$ CD3-positive cells per kg body weight The conditioning regimen for transplantation consisted of total body irradiation (single dose 9 Gy, dose rate 22 cGy/min, with lung shielding to a cumulative dose of 6 Gy, additional shielding of the left kidney to a cumulative dose of 3 Gy) on day −8 before bone-marrow transplantation, and cyclophosphamide (60 mg/kg daily) on days −5 and −4 Since her risk of graft rejection was thought to be high because of pretransplant sensitisation, the patient was treated with horse antithymocyte globulin (ATG, Upjohn, Kalamazoo) 20 mg/kg daily, intravenously on days −3, −2, −1, and on the day of transplantation (day 0) ATG was continued at a dose of 5 mg/kg daily every 2 days after transplantation until day +12 Methylprednisolone (10 mg/kg daily) was given from day −5 to day +12 Cyclosporin (3 mg/kg daily by continuous infusion) was started on day −1 and continued to day +14 From that time onwards, cyclosporin was given orally at a dose of 3 mg/kg, three times a day Engraftment was noted on day +20, and good graft function was documented on day +36 (neutrophils 9×10^8/l, reticulocytes 50 per 1000) After day +43, graft function deteriorated, resulting in complete pancytopenia on day +64 On day +85 leukaemic cells were observed in the peripheral blood and bone marrow The patient's condition thereafter deteriorated rapidly and she died 105 days after bone-marrow transplantation

Methods

Cultures of cytotoxic T lymphocytes were established by incubating $5–10 \times 10^6$ recipient mononuclear cells as responder cells with $5–10 \times 10^6$ irradiated (20 Gy) donor mononuclear cells as stimulator cells in RPMI medium plus 15% pooled, prescreened,

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TABLE I—REACTIVITY OF RECIPIENT ANTI DONOR CYTOTOXIC T LYMPHOCYTE LINE ESTABLISHED BEFORE TRANSPLANTATION

Target cells	Sex	HLA antigen sharing	% specific lysis*
Donor	M	A1, A2, B8, Bw61, Cw2, Cw7, DR3, DR4	95
Recipient	F	A1, A2, B8, Bw61, Cw2, Cw7, DR3, DR4	0
1	M	A1, DR3	83
2	M	A1, B8, DR3	98
3	M	A1, A2, B8, DR3, DR4	90
4	M	A1, B8, DR3	94
5	M	A1, DR4	81
6	M	A1	46
7	M	A1	68
8	M	A2	2
9	M	DR3	1
10	M	None	0
11	M	None	3
12	F	A2	3
13	F	A2	1
14	F	A2	0
15	F	A2	30
16	F	A1, A2, B8, Cw7, DR3	12
17	F	A1, A2, Cw2	16
18	F	A1, A2, B8, DR3	43
19	F	A1, B8, DR3	34
20	F	A1, B8, DR3	35
21	F	A1, B8, DR3	18
22a†	M	A1, Cw2	87
22b	F	A1, Cw2	6
23a†	F	A1, DR4	55
23b	F	A1, DR4	4

*% specific lysis of phytohaemagglutinin stimulated blasts in ^{51}Cr release assay
†Target cells 22 a and b 23 a and b were obtained from pairs of genotypically HLA identical siblings

heat-inactivated human AB serum After 6 days of culture, responder cells were harvested and used in a cell-mediated lympholysis assay [7] Alternatively, responder cells were expanded by restimulation with 10^6 irradiated (20 Gy) mononuclear cells or irradiated (50 Gy) Epstein-Barr-virus (EBV) transformed B cells from the donor (5×10^5 cells per 10^5 responder cells) in RPMI with 20% T-cell growth factor (Biotest, Offenbach, West Germany) plus 15% serum Fresh medium was added every 3 or 4 days and new stimulator cells were added once a week In this way, proliferating cell lines were established that could be kept in culture for many weeks Cytotoxic reactivity was tested in a chromium-51-release assay [7] Surface marker analysis with monoclonal antibodies against CD3, CD4, CD8, or CD16 antigens was carried out as described previously [8]

Cell-mediated cytotoxicity against haemopoietic progenitor cells was investigated as described previously [9] Briefly, 5×10^5 effector cells were incubated with 5×10^4 bone-marrow mononuclear cells in 0 5 ml medium (RPMI 1640 plus 15% serum) for 4–18 h Thereafter, the cell mixture was washed once in RPMI plus 15% serum and cultured for the myeloid (CFU-GM), erythroid (CFU-E/BFU-E), and the multipotent (CFU-GEMM) haemopoietic progenitor cells The percentage growth in these cultures was calculated as the ratio of colonies grown in the presence of effector cells to colonies observed in untreated control cultures

Results

The patient and her father had identical HLA phenotypes, as defined by serological tissue typing techniques Typing of other family members showed that the patient had inherited the A1, B8, Bw6, Cw7, DR3, DQw2 haplotype from her mother and shared the A2, Bw61, Bw6, Cw2, DR4, DQw3 haplotype with her father There was no reactivity in crossmatches between leucocytes, platelets, and erythrocytes from the recipient and serum from the donor or the opposite combinations However, there was strong recipient anti-donor reactivity in both the mixed lymphocyte reaction (relative response index 78%) and the cell-mediated lympholysis assay, no donor anti-recipient reactivity was observed

TABLE II—CHROMOSOME ANALYSIS OF RECIPIENT CELLS AND ESTABLISHMENT OF RECIPIENT DERIVED CYTOTOXIC T LYMPHOCYTE (CTL) LINES

Day of sampling	Chromosome analysis	% specific lysis* of CTL line with	
		Donor cells	Recipient cells
−10	100% XX	81	0
−6	ND	79	1
+28	ND	11	2†
+35	100% XY	4	0†
+64	100% XY	39	5
+88	39% XY, 11% XX, 50% XO‡	26	3
+96	45% XX, 55% XO‡	48	0
+105	100% XO‡, −7, −21, 6q	36	4

*In ^{51}Cr release assay with phytohaemagglutinin stimulated blasts as target cells effector/target ratio 40/1
†Phenotype CD3+ CD4+ CD8 CD16 Phenotype of all other cytotoxic T lymphocyte lines CD3+ CD4 CD8+ CD16
‡Recipient origin shown by polymorphism on chromosome 3
ND—not done

By means of a panel of target cells, we showed that the anti-donor cytotoxic T-lymphocyte line established from the recipient before transplantation had cytotoxic reactivity against several minor histocompatibility (mH) antigens, including the male-specific antigen H-Y (table I), there was substantial lysis of all HLA-A1-positive male target cells The male specificity of the cytotoxic T-lymphocyte line was confirmed by means of a subclone (data not shown) However, substantial lysis of several female target cells was also observed, indicating additional cytotoxic reactivity against other mH antigens This reactivity seemed to be restricted by the HLA A1/B8 or HLA A2 antigens Differential lysis of target cells from pairs of genotypically HLA-identical siblings confirmed that these reactivities were directed against mH antigens

TABLE III—REACTIVITY OF ANTI DONOR CYTOTOXIC T LYMPHOCYTE LINES ESTABLISHED BEFORE AND AFTER TRANSPLANTATION (BMT)

Target	Sex	HLA antigen sharing	% specific lysis	
			Before BMT	After BMT
24	M	A1, B8, DR3	>40	>40
25	M	A1, B8, DR3	>40	>40
26	M	A2, DR4	15–40	15–40
27	M	A2	15–40	15–40
28	M	A2, DR4	15–40	<15
29	M	A2	>40	15–40
30	M	A2	15–40	15–40
31	M	A2, DR3	15–40	<15
32	M	A2, Cw7	<15	<15
33	M	Cw7, DR4	<15	<15
34	F	A1, B8, Cw7, DR3	>40	>40
35	F	A1, B8, DR3	>40	>40
36	F	A1, B8, DR3	>40	>40
37	F	A1, Cw7	<15	<15
38	F	A2, B8, DR3	>40	>40
39	F	A2, Cw2	15–40	<15
40	F	A2	<15	15–40
41	F	A2, DR4	<15	<15
42	F	DR3	<15	<15
43	F	None	<15	<15

TABLE IV—GROWTH OF HAEMOPOIETIC PROGENITOR CELLS (HPC) AFTER INCUBATION WITH ANTI-DONOR CYTOTOXIC T-LYMPHOCYTE (CTL) LINES*

—	% colony growth of untreated bone-marrow cells	
	Donor HPC	Control† HPC
Pretransplant CTL line		
CFU-GM	6	102
CFU-E	40	97
BFU-E	12	114
CFU-GEMM	0	100
Post-transplant CTL line		
CFU-GM	2	96
CFU-E	20	86
BFU-E	11	88
CFU-GEMM	0	90

*Effector/target ratio 10/1
†Bone marrow from a female donor whose phytohaemagglutinin stimulated blasts were not lysed in the ^{51}Cr-release assay

Chromosome analysis[10] of recipient bone marrow and peripheral blood cells after bone-marrow transplantation is presented in table II. During the first 60 days only donor-type metaphases could be detected, whereas from day 88 onwards there was progressive loss of cells with donor-type metaphases. They were replaced by recipient-type cells with normal metaphases and leukaemic cells with an XO karyotype. The latter cells were also of recipient origin as shown by chromosome polymorphism.

After total body irradiation 6 days before transplantation anti-mH reactivity was still found (table II) on stimulation of recipient mononuclear cells with irradiated donor mononuclear cells. No anti-donor lymphocyte lines could be generated from the recipient on days −4, −1, +8, +12, +15, or +21. However, from day +28 to day +105, T-lymphocyte lines could be established from the recipient after specific stimulation with donor cells (table II). Specific anti-donor cytotoxicity was observed on days +64, +88, +96, and +105. All these lines were of recipient origin, as shown by their normal 46XX karyotype on chromosome analysis.

A comparison of the cytotoxic reactivity of T-lymphocyte lines established before and after transplantation is shown in table III. HLA-restricted anti-mH reactivity could be shown both before and after transplantation. The cytotoxic reactivity patterns obtained before and after transplantation were similar, although there were some differences in HLA-A2-restricted anti-mH reactivity (table III).

There was strong inhibition of donor CFU-GM, CFU-E, BFU-E, and CFU-GEMM colony growth on incubation with anti-donor cytotoxic T-lymphocyte lines established before and after transplantation, whereas no such inhibition was observed with haemopoietic progenitor cells from an unrelated individual. Thus, the cytotoxic reactivity of the recipient cells against donor haemopoietic progenitor cells was antigen specific (table IV).

Discussion

We have investigated the mechanism of graft failure in a patient who underwent T-lymphocyte-depleted bone-marrow transplantation. During pretransplant analysis this patient was found to be immunised against several mH antigens. Anti-mH activity was still detectable after total body irradiation, but it disappeared after completion of the immunosuppressive conditioning regimen. Good graft function was demonstrated about a month after transplantation, and routine cytogenetic analysis at this time showed only donor-type metaphases. However, even during this period of good engraftment of donor cells, T-lymphocyte lines of recipient origin could be established after specific in-vitro stimulation with donor cells. At first this anti-donor response was merely proliferative. However, at the time of clinical graft failure, host-derived cytotoxic T lymphocytes were able to lyse donor T cells and haemopoietic progenitor cells. Finally, only autologous (host-derived) normal and leukaemic cells were detectable and the patient died.

Cytotoxic T-lymphocyte lines established before and after bone-marrow transplantation showed several anti-mH cytotoxic specificities, including the male-specific H-Y antigen. The cytotoxic reactivity of the effector cells was similar before and after transplantation, although there were some differences in HLA-A2-restricted specificities afterwards. In a previous female patient treated by our group[11] an HLA-restricted H-Y-specific T-cell response from the peripheral blood could be elicited after rapid rejection of an unmodified bone-marrow graft from her genotypically HLA-identical brother. These observations and others[12,13] show that immunocompetent cells can survive intensive conditioning regimens. In allogeneic bone-marrow transplantation with undepleted grafts, residual immunocompetent cells in the recipient, capable of rejecting the graft, may be suppressed or eliminated by donor T lymphocytes present in the graft. Indeed, the engraftment-promoting effect of T lymphocytes has been shown in several animal models.[14,15] When the bone-marrow graft is depleted of T lymphocytes to prevent graft-versus-host disease, this immunosuppressive effect of donor T lymphocytes is also lost, resulting in a greater risk of graft rejection.[2-5] This report shows that prior sensitisation of the recipient against donor antigens may even further raise the risk of graft rejection.[16]

We have identified the expression of several mH antigens on haemopoietic progenitor cells and shown that they can be recognised by cytotoxic T lymphocytes as possible target structures. Since mH antigens seem to be differentially expressed on haemopoietic progenitor cells,[17] not all mH antigens may be equally important in bone-marrow graft rejection. The recipient cytotoxic T lymphocytes directed against mH antigens in our patient before and after transplantation could completely inhibit growth of donor haemopoietic progenitor cells; this anti-mH reactivity was therefore the cause of the graft rejection.

In conclusion, this study has shown that it may not be possible to eradicate all immunocompetent cells in recipients of bone-marrow transplantation, even with highly immunosuppressive conditioning regimens. Furthermore, complete chimerism, as determined by cytogenetic analysis of bone marrow and peripheral blood cells after transplantation, does not exclude the presence of residual immunocompetent recipient T cells capable of graft rejection. These recipient T cells can be detected in vitro by specific stimulation with donor cells. Finally, cytotoxic T cells against mH antigens that are present before bone-marrow transplantation can be directly involved in the rejection of HLA-identical bone-marrow grafts. Pretransplant immunisation, for example by blood transfusions, may be an important factor in this process.

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REFERENCES

1 Neudorf S, Filipovich A, Ramsay N, Kersey J Prevention and treatment of acute graft versus host disease *Sem Hematol* 1984, **21:** 91–100

2 Martin PJ, Hansen JA, Buckner CD, et al Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts *Blood* 1985, **66:** 664–72

3 Hows J, Apperley J, Yin J, et al T-cell depletion with Campath-1 to prevent GVHD *Exp Hematol* 1985, **13** (suppl 17) 114

4 Barrett AJ, Poynton CH, Flanagan P Shaw PJ, McCarthy D Factors associated with rejection in bone marrow depleted of T-lymphocytes by Campath-1 monoclonal antibody *Bone Marrow Transpl* 1986, **1** (suppl 1) 95 (abstr)

5 Patterson J, Prentice HG, Gilmore M, et al Analysis of rejection in HLA-matched T-depleted bone marrow transplants *Exp Hematol* 1985, **13** (suppl 17) 117

6 Bennett JM, Catovsky D, Daniel MT, et al Proposals for the classification of the myelodysplastic syndromes *Br J Haematol* 1982, **51:** 189–99

7 Goulmy E HLA-A, −B restriction of cytotoxic T cells In Ferrone S, Solheim BG, eds HLA-typing methodology and clinical aspects, vol 2 New York CRC Press, 1982, 105–22

8 Voogt PJ, Goulmy E, Fibbe WE, Veenhof WFJ, Brand A, Falkenburg JHF Minor histocompatibility antigen H-Y is expressed on human hematopoietic progenitor cells *J Clin Invest* 1988, **82:** 906–12

9 Voogt PJ, Fibbe WE, Veenhof WFJ, et al Cell-mediated lysis of human hematopoietic progenitor cells *Leukemia* 1987, **1:** 427–31

10 Paris Conference (1971) Standardization in human genetics *Cytogenetics* 1972, **11:** 313–62

11 Goulmy E, Termijtelen A, Bradley BA, van Rood JJ Y-antigen killing by T cells of women is restricted by HLA *Nature* 1977, **266:** 544–45

12 Reisner Y, Ben-Bassat I, Doner D, Kaploon A, Schwartz E, Ramot B A primate preclinical model for bone marrow transplantation definite proof for host radioresistant clonable T cells *Exp Hematol* 1985, **13:** 321 (abstr)

13 Butturini A, Seeger RC, Gale RP Recipient immune competent T lymphocytes can survive intensive conditioning for bone marrow transplantation *Blood* 1986, **68:** 954–56

14 Walma EP, Vriesendorp HM, Zurcker C, van Bekkum DW Engraftment of stem cell enriched bone marrow fractions in MHC-identical dogs after fractionated total body irradiation *Transplantation* 1987, **43:** 818–23

15 Schwartz E, Lapidot T, Gozes D, Singer TS, Reisner Y Abrogation of marrow allograft resistance in mice by total body irradiation correlates with eradication of host clonable T cells and alloreactive cytotoxic precursors *J Immunol* 1987, **138:** 460–65

16 Dennert G, Anderson CG, Warner J T killer cells play a role in allogeneic bone marrow graft rejection, but not in hybrid resistance *J Immunol* 1985, **135:** 3729–37

17 Voogt PJ, Goulmy E, Veenhof WFJ, et al Cellularly defined minor histocompatibility antigens are differentially expressed on human hematopoietic progenitor cells *J Exp Med* 1988, **168:** 2337–47