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Minor Histocompatibility Antigen-specific Cytotoxic T Cell Lines, Capable of Lysing Human Hematopoietic Progenitor Cells, Can Be Generated In Vitro by Stimulation with HLA-identical Bone Marrow Cells

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Summary

Recipient-antidonor alloreactivity before HLA genotypically identical bone marrow transplantation (BMT) between donor-recipient pairs that are negative in the mixed lymphocyte reaction (MLR), the cell-mediated lympholysis (CML) assay, and the lymphocyte crossmatch was not detectable in the majority of cases, using recipient peripheral blood lymphocytes (PBL) collected before BMT as responder cells and donor PBL as stimulator cells. However, when donor bone marrow mononuclear cells (BMMNC) instead of PBL were used as stimulator cells, we could detect donor-specific alloreactivity in 7 of 10 HLA genotypically identical donor-recipient pairs. To demonstrate that this alloreactivity was minor histocompatibility (mH) antigen specific and not directed against HLA class I splits or variants, two cytotoxic T lymphocyte (CTL) lines were tested in further detail against phytohemagglutinin (PHA) blasts from pairs of HLA genotypically identical siblings positive for the HLA class I restriction molecule. Both CTL lines recognized mH antigens, as illustrated by the differential recognition of PHA blasts of one of the two siblings from several pairs. The potential role of these mH antigen–specific CTLs in bone marrow graft rejection was demonstrated by the mH antigen–specific growth inhibition of hematopoietic progenitor cells from the original bone marrow donor and from HLA class I restriction molecule–positive individuals who expressed the mH antigens on their PBL and BMMNC. Our assay can be used in HLA genotypically identical BMT to detect a recipient-antidonor response, directed against cellularly defined mH antigens expressed on donor HPC, BMMNC, and PBL, before transplantation.

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Acute graft-vs.-host disease (aGVHD)[1] represents one of the major complications after allogeneic bone marrow transplantation (BMT) with unmodified grafts. Removal of T lymphocytes from the marrow graft has been effectively used to prevent aGVHD (1, 2). However, T cell depletion is associated with an increased risk of graft failure (3–7), probably due to reduced immune suppression as a result of removing immunocompetent donor cells from the graft. This may lead to immune-mediated graft failure by residual alloreactive recipient cells directed against antigenic determinants expressed on donor cells from the graft, especially in recipients who are sensitized by previous blood transfusions (8–15). Graft failure may also be influenced by several other mechanisms, such as recipient stromal cell defects (16), viral infections (16–18), and loss of accessory cell function due to the T cell depletion procedure (19).

Immune-mediated graft rejection after transplantation of a marrow graft from an HLA-identical sibling donor appears to be caused by CTLs of recipient origin (20–22). Minor histocompatibility (mH) antigens, which are recognized in the context of HLA class I antigens (23, 24), are the target structures for these CTLs. Residual immunocompetent T cells that have survived the conditioning regimen (25–28) may recognize and lyse donor cells that express mH antigens. Previously, we have shown that the mH antigens HY and HA-3 are expressed on human hematopoietic progenitor cells (HPC) and that these cells are subject to lysis by mH antigen–specific CTLs (29). The clinical relevance of these findings was illus-

[1] *Abbreviations used in this paper:* aGVHD, acute graft-vs.-host disease; BMMNC, bone marrow mononuclear cells; BMT, bone marrow transplantation; CML, cell-mediated lympholysis; GM, granulocyte/macrophage; HPC, hematopoietic progenitor cells; mH, minor histocompatibility.

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trated in a sensitized recipient who rejected her HLA-identical marrow graft (25).

The occurrence of graft rejection after HLA genotypically identical BMT cannot be accurately predicted since no assays are available to detect recipient-derived immunocompetent T lymphocytes reactive with donor cells, before BMT. Reactivity or nonreactivity of the pretransplant MLR does not correlate with the occurrence of graft rejection after HLA genotypically identical BMT (30); only in those recipients who are strongly sensitized to allo-antigens by multiple blood transfusions can antidonor reactivity be detected (8, 13, 25, 31).

Here, we report a method that can be used to detect alloimmune reactivity of recipient cells against mH antigenic determinants expressed on cells of donors who are negative in the MLR, the cell-mediated lympholysis (CML) assay, and the lymphocyte crossmatch before HLA genotypically identical BMT. Using HLA genotypically identical donor bone marrow mononuclear cells (BMMNC) as stimulator cells and recipient PBL collected before BMT as responder cells, we were able to generate mH antigen–specific CTL lines. These CTL lines did not only lyse donor PBL but also inhibited donor HPC growth in an antigen-specific manner. Therefore, this assay provides a method to study mechanisms responsible for bone marrow graft rejection after HLA-identical BMT.

Materials and Methods

Patients. All patients suffered from acute myeloid or lymphocytic leukemia and were transplanted in first or second complete remission with bone marrow from their HLA genotypically identical siblings, who are negative in the MLR, the CML assay, and lymphocyte crossmatch. All patients had received blood products that were leukocyte depleted to prevent allo-immunization (32).

Collection of PBL. PBL were isolated from heparinized blood by Ficoll-Isopaque (1.077 g/cm^3) density centrifugation (1,000 *g*, 20 min, 20°C). The interphase cells were harvested and washed twice in RPMI 1640 plus 5% FCS (Gibco Laboratories, Grand Island, NY). For cryopreservation, the cells were resuspended at a concentration of 10^7 cells/ml in cryopreservation medium consisting of 70% RPMI, 20% FCS, and 10% DMSO, frozen, and preserved in liquid nitrogen. Immediately before use, the cells were thawed for 1 min in a 37°C waterbath, diluted in RPMI plus 20% FCS, and washed twice in the same medium. The cells were then resuspended in RPMI plus 15% pooled human serum (RPMI plus 15% serum).

Collection of Bone Marrow. Normal human bone marrow was obtained, after informed consent, from donors for BMT by aspiration from the posterior iliac crests. The marrow suspension was diluted in RPMI plus 5% FCS and centrifuged over Ficoll-Isopaque. The interphase BMMNC were harvested, washed twice in RPMI plus 5% FCS, diluted at a concentration of 10^7 cells/ml in cryopreservation medium, and frozen using a computer-controlled freezer (Cryoson, Middenbeemster, The Netherlands), as described (33). Immediately before use, the cells were thawed, washed, and resuspended in RPMI plus 15% human serum.

PHA Blasts. 10^7 PBL were cultured in RPMI plus 15% serum and 0.2% PHA M (Difco Laboratories, Detroit, MI) for 3 d. The cells were then washed and cultured further in the absence of PHA in medium consisting of RPMI plus 15% serum and 40 U rIL-2/ml. After 2–3 d of culture in this medium, cells were used as targets in a ^{51}Cr release assay.

Generation of Recipient-derived T Lymphocyte Lines Using Donor PBL as Stimulator Cells. Recipient PBL collected before BMT were used as responder cells at a concentration of 10^6 cells/ml. They were stimulated with 2 × 10^6/ml irradiated (30 Gy) HLA genotypically identical donor PBL and cultured in 24-well tissue culture plates in medium consisting of RPMI plus 15% serum. After this initial step, three different culture protocols were used.

First, after 7 d of culture, medium was replaced by RPMI plus 15% serum and 40 U rIL-2/ml. On day 10, the responder cells were restimulated with irradiated (50 Gy) EBV-transformed donor lymphocytes, and this was repeated once weekly, while twice weekly, culture medium was replaced by RPMI plus 15% serum and 40 U rIL-2/ml.

Second, effector cells were cloned after 9 d of culture by limiting dilution at seven concentrations ranging from 300 to 0.3 cells/well. The cells in each well were cultured in the presence of 2 × 10^4 irradiated allogeneic PBL and 10^4 irradiated EBV-transformed donor lymphocytes in 0.2 ml of RPMI plus 15% serum, 1% leukoagglutinin (Leuko-A; Pharmacia Fine Chemicals, Espoo, Finland), and 40 U rIL-2/ml. After 8–10 d, growing wells were split and assayed for cytotoxicity against donor PHA blasts. Alternatively, growing wells were split and analyzed for the expression of the CD8 antigen with a fluorescence-activated cell sorter (FACScan; Becton Dickinson Immunocytometry Systems, Mountain View, CA) and then assayed for cytotoxicity against donor PHA blasts. Cells that were selected based on their cytotoxic activity or CD8 antigen expression were further expanded using irradiated allogeneic PBL and EBV-transformed donor lymphocytes. Cell sublines were derived from wells plated at concentrations ranging from 300 to 10 cells/well.

Third, responder cells were restimulated with irradiated donor PBL on day 4 and depleted of CD4$^+$ cells on day 8 by complement-mediated cytotoxicity, as described (34). Briefly, 1–2 × 10^7 cells were resuspended in 1 ml RPMI plus 15% serum, and incubated with an equal volume of a 100-fold dilution of RIV-6 (anti-CD4 specificity) mAb in RPMI for 30 min at 4°C. Baby rabbit complement (Pel-Freez Biologicals, Rogers, AR) of prescreened batches was then added to a final concentration of 50%. The suspension was incubated again for 1 h at 37°C. Finally, cells were washed three times in RPMI plus 10% FCS, resuspended in RPMI plus 15% serum at the appropriate cell concentration, and cloned by limiting dilution. When a sufficient number of effector cells could be harvested, a ^{51}Cr release assay was performed using PHA blasts and EBV-transformed cells from donor and recipient as target cells.

Generation of Recipient-derived T Lymphocytes Lines Using Donor BMMNC as Stimulator Cells. Recipient PBL collected before BMT were used as responder cells at a concentration of 10^6 cells/ml. They were stimulated with 2 × 10^6/ml irradiated (30 Gy) HLA genotypically identical donor BMMNC and cultured in 50-ml tissue culture flasks in RPMI plus 15% serum. On day 7, medium was replaced by RPMI plus 15% serum and 20% T cell growth factor (TCGF; Biotest, Offenbach, FRG). On day 14, responder cells were restimulated with irradiated donor BMMNC in a responder/stimulator ratio of 1:10, together with RPMI plus 15% serum and 20% TCGF. On day 21, responder cells were restimulated with irradiated EBV-transformed donor lymphocytes. When enough cells could be harvested, a ^{51}Cr release assay was performed using PHA blasts and EBV-transformed cells from donor and recipient as target cells. In some cases, recipient-derived PBL were stimulated with donor-derived PBL, using this culture protocol.

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^{51}Cr Release Assay. Standard ^{51}Cr release assays were performed as described (35). Briefly, target cells consisting of PHA blasts, EBV-transformed cells, or BMMNC were labeled with 0.1 ml $Na_2{}^{51}CRO_4$ (100 μCi) for 1 h in a 37°C waterbath, washed three times with HBSS, and then resuspended in RPMI plus 15% serum at a concentration of 5×10^4 viable cells/ml. 0.1 ml of the effector cell suspension and 0.1 ml of the target cell suspension were added to each well of a round-bottomed microtiter plate at E/T ratios ranging from 40:1 to 10:1. To measure spontaneous release of ^{51}Cr, 0.1 ml of the target cell suspension was added to 0.1 ml RPMI plus 15% serum without effector cells, while maximum release was determined by adding 0.1 ml of the target cell suspension to 0.1 ml of a Zaponine solution. The percentage of lysis was determined as follows: 100× (experimental release cpm − spontaneous release cpm)/(maximum release cpm − spontaneous release cpm).

Cell-mediated Lysis of HPC. 1.25×10^5 BMMNC in 0.2 ml medium consisting of 33% FCS and 66% α-modified Eagle's MEM (α-MEM; Flow laboratories, Irvine, Scotland) was mixed with CTLs at E/T cell ratios varying from 4:1 to 0.5:1.

The cell mixture was centrifuged (1,000 *g*, 15 s) to establish cell-cell contact between CTLs and BMMNC, and then incubated for 6–18 h in a fully humidified atmosphere of 5% CO_2 at 37°C. After incubation, the cells were resuspended, and 1.05 ml culture medium was added to a final concentration of 20% FCS, 40% α-MEM, 40% of a 2.25% methylcellulose solution in α-MEM, and 100 U/ml glycosilated recombinant human granulocyte/macrophage (GM) CSF (Sandoz-Schering Plough, Basel, Switzerland). Subsequently, 1 ml semisolid medium containing 10^5 BMMNC was incubated in a fully humidified atmosphere of 5% CO_2 and 37°C, and cultured for 10 d. The number of CFU-GM colonies, defined as granulocytic, monocytic of eosinophilic aggregates of >20 cells, were scored on day 10 under an inverted microscope.

As a control, to establish the necessity of cell-cell contact between CTLs and BMMNC, and to exclude the possibility of nonspecific inhibition of HPC growth due to the presence of cytotoxic cells in the semi-solid culture medium, CTLs were added to BMMNC immediately before plating. CTLs were irradiated (20 Gy) before use to prevent colony formation by these cells.

Calculations and Normal Values. The level of 100% growth was defined as the number of colonies cultured from 10^5 untreated BMMNC. The number of colonies in these cultures was always within the normal range for our laboratory (CFU-GM: 160 ± 101 [mean ± SD]/10^5 BMMNC plated). In cellular cytotoxicity assays, the percentages of surviving HPC were calculated by dividing the total number of colonies by the number of colonies in the untreated control cultures.

Phenotype of CTL Lines. The phenotypes of the CTL lines and clones were analyzed on a FACscan using mAbs against the CD3, CD4, and CD8 antigens (Becton Dickinson Immunocytometry Systems).

Results

As shown in Table 1, no donor-specific lysis could be detected using donor PBL as stimulator cells and recipient PBL as responder cells irrespective of the three culture protocols used; in some cases, cytotoxicity directed against donor EBV cells or the allogeneic feeder cells was observed. The cell lines and the cell sublines that were obtained after these culture protocols consisted of a mixture of $CD4^+$ and $CD8^+$ cells. Most of the cell clones were $CD4^+$ when not enriched for $CD8^+$ cells before cloning (data not shown). Cloning after depletion of $CD4^+$ cells resulted in generation of predominantly $CD8^+$ clones. The proliferative capacity of the cell

Table 1. *Phenotype and Lytic Activity of Recipient-derived Cell Lines, Sublines, and Clones, Generated by Stimulation with Donor PBL*

				Percent lysis in ^{51}Cr release assay*			
	Phenotype			PHA blasts			EBV cells
Effector cells	CD3	CD4	CD8	Donor	Recipient	Allo PBL‡	Donor
Cell line:							
JI	95§	93	10	0	0	ND	ND
JI'‖	95	5	80	7.5	10	ND	47
SND	ND	ND	ND	2	0	ND	ND
ST	96	75	10	3	6	ND	ND
Cell subline:							
JI 11	98	5	50	0	0	20	39
DC 9	95	6	60	0	1	32	24
Cell clone:							
SND 27	99	3	80	2	0	0	0
ST 156	98	0	94	1	1	0	14

* E/T ratio = 40:1.
‡ Allogeneic PBL (feeder cells).
§ Percentage of fluorescence.
‖ After enrichment for $CD8^+$ cells.

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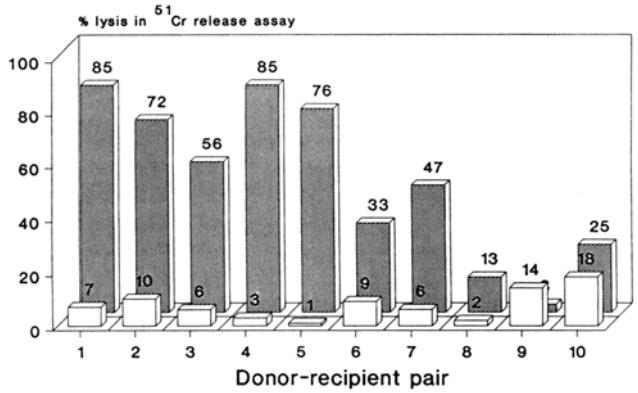

Figure 1. Cytotoxicity against donor (▨) and recipient (□) PHA blasts of recipient-derived CTL lines generated by stimulation with HLA genotypically identical donor bone marrow cells, as measured in a ^{51}Cr release assay. E/T ratio = 40:1. CTL lines derived from pairs 1 and 2 were studied in further detail.

lines was strong in all cases tested, resulting in $>10^8$ cells after 3 wk of culture.

In contrast to the results obtained with PBL as stimulator cells, the use of HLA genotypically identical donor BMMNC as stimulator cells resulted in a strong specific cytotoxic response, i.e., only donor PHA blasts and not recipient PHA blasts were lysed in 7 of 10 donor-recipient combinations tested, demonstrating donor-specific allo-reactivity (Fig. 1). In four of these donor-recipient pairs (pairs 1, 6, 7, and 10), both donor-derived BMMNC and PBL were used separately as stimulator cells, and cultures were performed under identical conditions, i.e., addition of TCGF on day 7 and restimulation with original stimulator cells on day 14. In two of four pairs, and antidonor response was only detected after stimulation with donor BMMNC (pairs 1 and 6), while in one combination (pair 7), antidonor reactivity was also present after stimulation with donor-derived PBL. In one combination (pair 10), no donor-specific cytotoxicity could be generated. Two other cell lines, derived from recipients 8 and 9, showed no donor-specific cytotoxicity and had a similar proliferative capacity as the other cell lines, resulting in $>3 \times 10^7$ cells after 3 wk of culture. All cell lines tested consisted of a mixture of $CD4^+$ and $CD8^+$ cells.

The CTL line from recipient 1 (CTL line 1) recognized a mH antigen, restricted by HLA-A2 or HLA-B44. The mH antigen specificity was demonstrated by the differential recognition of one of six $HLA\text{-}A2^+$ and $HLA\text{-}B44^+$ HLA genotypically identical sibling pairs and three of eight $HLA\text{-}B44^+$ HLA genotypically identical sibling pairs (Table 2). Both siblings of two of the $HLA\text{-}A2^+$ and $HLA\text{-}B44^+$ pairs were recognized by CTL line 1, whereas siblings of the remaining three pairs were both negative for expression of the mH antigen. Siblings of one of the five remaining $HLA\text{-}B44^+$ pairs were both positive for mH antigen expression, whereas the other four sibling pairs were negative. As shown in Table 2, PHA blasts of both siblings of 29 $HLA\text{-}A2^+$ HLA genotypically identical sibling pairs were recognized by the CTL line from recipient 2 (CTL line 2), whereas the

Table 2. *mH Antigen Specificity of Recipient-derived CTL Lines Generated by Stimulation with Donor Bone Marrow Cells*

CTL line	Target cells obtained from pairs of HLA genotypically identical siblings	HLA restriction element	Percent lysis in ^{51}Cr release assay*	
			Sibling 1	Sibling 2
	No. of pairs			
1‡	2	A2 + B44	56	82
	1	A2 + B44	80	8
	3	A2 + B44	1 ± 1§	1 ± 1
	1	B44	77	100
	3	B44	79 ± 36	3 ± 2
	4	B44	2 ± 3	9 ± 3
2‖	29	A2	66 ± 15	66 ± 18
	2	A2	66	4
	1	B37	30	28
	2	B37	24	5
	1	B37	1	0

* E/T = 40:1
‡ HLA type: *A2 B44* B27 C1 DR5,7,12 DQ2,3,7.
§ Mean ± SD.
‖ HLA type: *A2* A3 B7 *B37* C6 C7 DR4,6,10,14 DQ1.

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Table 3. *Cytotoxicity of mH Antigen-specific CTL Lines Against ^{51}CR-labeled PHA Blasts and BMMNC*

CTL line	Target	HLA restriction element	Target cells: PHA blasts 40:1*	PHA blasts 20:1	PHA blasts 10:1	BMMNC 40:1	BMMNC 20:1	BMMNC 10:1
1	Donor	ALL	28‡	16	16	30	22	11
	Patient	ALL	6	0	0	0	0	0
	A	B44	3	0	0	8	6	1
	B	B44	43	47	36	8	9	1
	C	B44	28	21	16	25	11	8
	D	Absent	6	6	6	13	9	0
2	Donor	ALL	61	58	40	48	42	40
	Patient	ALL	0	0	0	ND	ND	ND
	X	B37	39	34	34	50	41	40
	Y	B37	19	19	17	54	56	46
	Z	Absent	0	0	0	7	10	5

* E/T ratio.
‡ Percentage lysis in ^{51}Cr release assay.

PHA blasts of two HLA-A2$^+$ HLA genotypically identical sibling pairs were differentially recognized, indicating that this cell line recognized an HLA-A2-restricted mH antigen. The PHA blasts of two of four HLA-B37$^+$, A2$^-$ HLA genotypically identical sibling pairs were found to differentially express the mH antigen, whereas in one pair, PHA blasts from both siblings were recognized, and in the last pair, no mH expression was found. These results show that CTL line 2 also recognized an HLA-B37-restricted mH antigen.

The frequency of mH antigen expression as determined by the number of positive reactions, and expressed as a percentage of the total number of tested individuals, was 42%

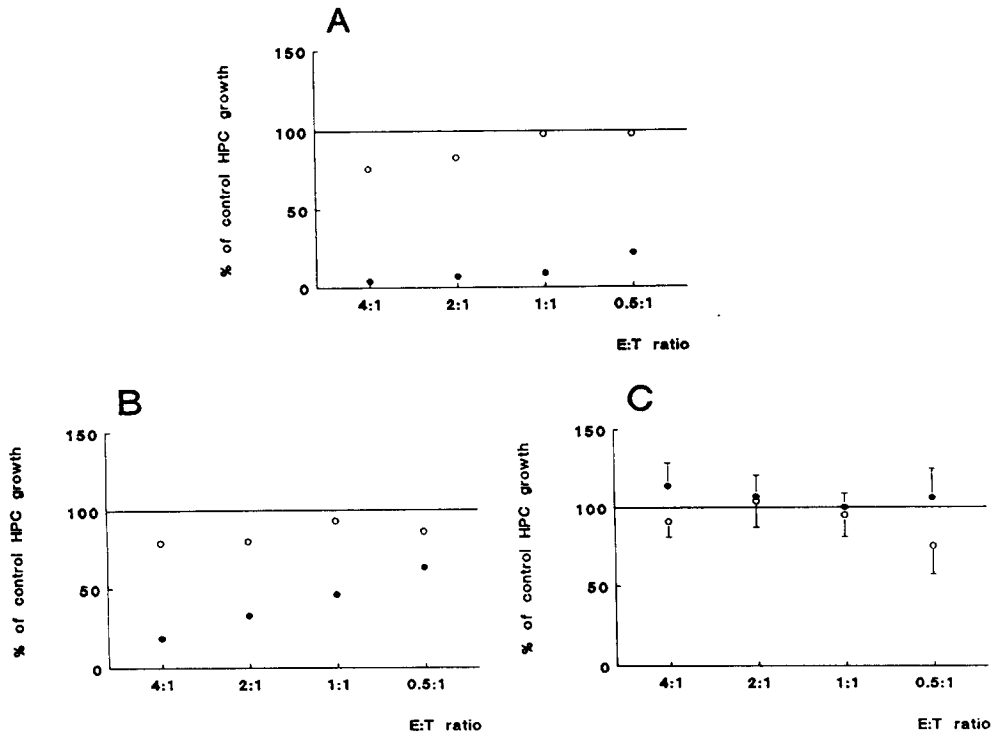

Figure 2. Growth of HPC from the bone marrow donor (*A*) and mean growth of HPC from two unrelated HLA-B44$^+$ targets (*B*), expressed as a percentage of control HPC growth after culture with CTL line 1 with (●) or without (○) a 6-h preincubation period with BMMNC. (*C*) Mean HPC growth of three HLA-A1-negative targets and the HLA-B44$^+$ mH antigen–negative target A, after culture with an HLA-A1-restricted anti-HA-3 CTL clone and CTL line I, respectively, with (●) or without (○) a 6-h preincubation period with BMMNC (vertical bars indicate SD).

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for the HLA-A2- and/or HLA-B44-restricted mH antigen (n = 12) and 31% for the HLA-B44-restricted mH antigen (n = 16), recognized by CTL line 1. For the HLA-A2-restricted mH antigen (n = 62) and the HLA-B37-restricted mH antigen (n = 8) recognized by CTL line 2, the frequencies of expression were 97 and 50%, respectively.

To study their potential role in bone marrow graft rejection, the two mH antigen–specific CTL lines were assayed for cytotoxicity against HPC and BMMNC collected by aspiration from the posterior iliac crests from several individuals, either positive or negative for mH antigen expression on PHA blasts or negative for expression of the relevant HLA class I restriction molecules (Table 3). Results obtained in ^{51}Cr release assays using PHA blasts and BMMNC as targets correlated well with results obtained in the assays for HPC growth inhibition in most cases (CTL line 1: Table 3 and Fig. 2, *A* and *B*; CTL line 2: Tables 3 and 4). Individuals expressing the mH antigen on their PHA blasts and BMMNC also showed HPC growth inhibition, whereas individuals negative for mH antigen expression on PHA blasts and BMMNC or individuals lacking the relevant HLA class I restriction element showed no HPC growth inhibition. An exception to these observations was the absence of lysis of BMMNC from individual B by CTL line 1 (Table 3), whereas PHA blasts and HPC were lysed.

CTL line 1 showed a dose-dependent inhibition of HPC growth of the bone marrow donor (Fig. 2 *A*) and of two unrelated HLA-B44$^+$ individuals expressing the mH antigen on their PHA blasts (Fig. 2 *B*). CTL line 2 inhibited HPC growth of the bone marrow donor and of two unrelated HLA-B37$^+$ individuals almost completely even, at the lowest E/T cells ratios (Table 4). Fig. 2 *C* shows that an HLA-A1-restricted anti-mH CTL clone (the anti-HA-3-specific CTL clone [23, 29]) and CTL line 1 did not inhibit HPC growth when target cells did not express the relevant HLA class I restriction molecule or the mH antigen, respectively, demonstrating that the presence of CTLs per se did not induce nonspecific growth inhibition of HPC.

Table 4. *mH Antigen–specific Recognition of HPC by CTLs*

Effector	Target	HLA restriction element	Percent of control HPC growth*	
			+‡	−§
CTL line 2	Donor‖	B37	0	70
	X¶	B37	2	174
	Y	B37	0	122
	Z	Absent	105	120
Anti-HA-3 clone	Donor‖	Absent	66	96
	X¶	A1	11	71
	Y	A1	2	97
	Z	Absent	93	119

* Percentage of HPC growth at E/T ratio of 0.5:1.
‡ Preincubation period of 6 h.
§ No preincubation period.
‖ BMMNC from bone marrow donor.
¶ BMMNC from an unrelated individual.

Discussion

Before HLA genotypically identical BMT, the detection of allo-reactivity between donor and recipient has not been possible because no assay was sufficiently sensitive (30, 36, 37). Only in patients who were strongly sensitized to alloantigens by multiple blood transfusions (8, 13, 15, 23, 31) and in patients who were transplanted with HLA-matched unrelated donors (38) has it been possible to detect allo-reactivity between donor and recipient before BMT. The patients in our study group had received leukocyte-depleted blood products to prevent allo-immunization (32). Only a small percentage (9%) of patients transfused with such blood products have been reported to develop allo-antibodies (39). In the patients studied, allo-antibodies were only present in patients 4 and 8, as demonstrated by decreased 1-h increments after platelet transfusions from six random donors. No antidonor immune response could be detected in the patients studied, using the standard MLR, the CML assay, and the lymphocyte crossmatch before BMT.

In this report, we demonstrate that, using genotypically identical BMMNC as stimulator cells, it is possible to generated mH antigen–specific CTL lines from recipient PBL collected before BMT. Only once was an antidonor-specific cytotoxic response observed after stimulation with donor-derived PBL, while all other attempts to generate donor-specific cytotoxicity using PBL as stimulator cells followed by expansion with donor EBV cells or allogeneic PBL resulted in cytotoxic reactivity against EBV or allogenic feeder cells only. Modification of this protocol, e.g., cloning at an early stage of the immune response and depletion of CD4$^+$ proliferative T cells to prevent overgrowth of EBV-specific T lymphocytes, was unsuccessful in generating specific CTLs. Because, until now, all described mH antigen–specific CTLs are CD8$^+$ and HLA class I restricted (23, 40), depletion of CD4$^+$ T cells by anti-CD4 mAb and complement could have resulted in a growth advantage of CD8$^+$ mH antigen–specific CTLs. However, no such effect was observed. These results are in accordance with reports by others and our own group showing that it was not possible to isolate mH antigen–specific CTLs before BMT or kidney transplantation from recipient-derived peripheral blood, using donor PBL as stimulator cells. Such responses could only be obtained after in vivo sensitization (23, 41, 42). This is probably due to better or different antigen presentation in vivo, resulting in an increased frequency of mH antigen–specific CTLs.

Because the target cells in bone marrow graft rejection are HPC, we next used BMMNC of the HLA genotypically identical bone marrow donor as stimulator cells and recipient PBL, collected before BMT as responder cells. Using this protocol,

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we were able to establish CTL lines in 7 of 10 donor-recipient combinations. In two of four donor-recipient combinations where either donor BMMNC or PBL were used as stimulator cells under identical culture conditions, donor-specific cytotoxicity was observed only after stimulation with BMMNC and not after stimulation with PBL. The absence of cytotoxicity in the three remaining T cell lines was not due to an insufficient number of cells because proliferation was similar to the other seven CTL lines. The cytotoxicity of the two CTL lines derived from recipients 1 and 2, which were tested in further detail, proved to be directed against mH antigens that were not only expressed on cells of bone marrow donor, but also on cells of many unrelated individuals positive for the HLA class I restriction molecule. This was shown by the differential recognition of PHA blasts obtained from pairs of HLA genotypically identical siblings by both CTL lines, demonstrating that the observed cytotoxicity is mH antigen specific and not directed against splits or variants of HLA class I molecules. The expression of the same mH antigen on cells of a large number of unrelated individuals implicates that these antigens are widely distributed in the population and are not unique for one individual. As a consequence, it may be possible to type for a limited number of frequently occurring mH antigens, which may give sufficient information about the potential allo-reactivity between donor and recipient, either in the host-vs.-graft or the graft-vs.-host direction.

The absence of lysis of ^{51}Cr-labeled BMMNC from individual B, whose PHA blasts are lysed and whose HPC are specifically growth inhibited by CTL line 1, may be caused by a lower expression of the HLA class I restriction molecule or of the mH antigen itself on the surface of relatively mature myeloid precursor cells. Similarly, we previously observed a decreased reactivity of an HLA-A1-restricted HY-specific CTL line with day 4 cluster-forming cells and mature myeloid cells from HLA-A1$^+$ males as compared with PBL and day 10 CFU-GM (43). The reactivity of the CTL lines with PBL and HPC demonstrates the potential role of mH antigen–specific CTLs in bone marrow graft rejection.

No graft rejection was observed in the 10 recipients. The actual occurrence of graft rejection will depend on the balance between immunocompetent donor and recipient lymphocytes after BMT. Apparently, in these 10 recipients, the conditioning regimen was effective in abrogating recipient antidonor alloreactivity. Our observations indicate that the occurrence of graft rejection after BMT is not predominantly dependent on qualitative antigenic disparity between donor and recipient but appears to be the result of quantitative residual antidonor reactivity of the recipient lymphocytes that survived the conditioning regimen. Therefore, particularly in BMT with T cell–depleted marrow, intensifying pretransplant conditioning regimens or post-transplant immunosuppression may decrease the incidence of graft rejection. Comparison of mH antigen–specific cytotoxic activity present before and after BMT in patients with full engraftment or graft rejection may provide information about the relative importance of different mH antigen–specific CTLs and the necessity of matching for these mH antigens. These questions are currently under investigation in our laboratory.

In conclusion, we have demonstrated that mH antigen–specific CTL lines can be generated in vitro in a high percentage of HLA genotypically identical donor-recipient pairs using donor BMMNC as stimulator cells and recipient PBL, collected before BMT, as responder cells. These CTLs are capable of lysing HPC and may therefore play a role in BM graft rejection after HLA genotypically identical BMT.

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