CELLULARLY DEFINED MINOR HISTOCOMPATIBILITY ANTIGENS ARE DIFFERENTIALLY EXPRESSED ON HUMAN HEMATOPOIETIC PROGENITOR CELLS

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Allogeneic bone marrow transplantation (BMT)[1] has become a major therapeutic modality in the treatment of various malignant and nonmalignant hematologic disorders (1-6). However, graft-vs.-host disease (GvHD) causes much morbidity and mortality after transplantation (7, 8). Since GvHD is mediated by immunocompetent T cells in the graft (9, 10), depletion of T cells from the bone marrow graft has been successfully applied to reduce the incidence of this complication (11, 12). On the other hand, T cell depletion from the graft has led to an increased incidence of graft failure (13-16), which could in part be prevented by increasing the intensity of the pretransplant conditioning regimen (16-20).

As a result of the genetic disparity between donor and recipient, graft failure may be due to an immune-mediated rejection by immunocompetent cells in the recipient, provided that these cells are not eliminated by the pretransplant conditioning and/or by the donor T cells present in the graft. In HLA-nonidentical transplants, the high incidence of graft rejection (21, 22) can be explained by the general tissue distribution of these histocompatibility antigens, and, in particular, by the expression of HLA class I and class II antigens on hematopoietic progenitor cells (HPC) (23-26). However, after the introduction of T cell depletion of the graft to prevent GvHD, ~15% graft rejections have also been observed in HLA-identical transplants (13-16). In these cases, antigenic determinants outside the HLA system, called minor histocompatibility (mH) antigens (27), are likely to be involved in the pathogenesis of graft rejection. If mH antigens are expressed on HPC, an immune response in the recipient, directed against these target structures on donor cells, may lead to elimination of the hematopoietic stem cells from the graft. Therefore, mapping of mH antigens on HPC is of major importance for understanding the mechanism of rejection of the hematopoietic graft in bone marrow transplantation.

[1] *Abbreviations used in this paper:* a-MEM, α-modified Eagle's MEM; BMT, bone marrow transplantation; GvHD, graft-vs.-host disease; HPC, hematopoietic progenitor cells; mH, minor histocompatibility; TCGF, T cell growth factor.

Until now, no antibodies against human mH antigens have been available. To study the expression of mH determinants on cell populations, specific anti-mH CTL lines have to be used. Goulmy et al. (28–30) have been able to generate several anti-mH CTL lines from patients who were sensitized in vivo for these antigens by multiple blood transfusions, or from patients sensitized in vivo for these antigens by allogeneic BMT. Recognition of these mH antigens appeared to be HLA class I restricted. Apart from the H-Y antigen, expressed on cells of male individuals, five other mH antigens have been characterized by Goulmy (30) in family and population studies.

Previously, we demonstrated the expression of the H-Y antigen on human hematopoietic progenitor cells (31), indicating that an immune response by the recipient directed against this determinant may lead to rejection of the graft in allogeneic BMT. Here, we investigated the expression of five mH antigens, designated HA-1–5, on hematopoietic progenitor cells, using a cell-mediated cytotoxicity assay (32). We report the expression of the mH HA-3 determinant on human HPC, whereas expression of the HA-1, -2, -4, and -5 antigens on these cells is either absent or extremely low, as measured by antigen-specific growth inhibition of colony forming cells by cytotoxic T cell lines. Thus, mH antigens appear to be differentially expressed on human HPC.

Materials and Methods

Generation of Cytotoxic T Cell Lines against mH Antigens. The anti-mH CTL lines were established as described previously (29, 30). Briefly, 10^7 PBMC were isolated from patients after bone marrow transplantation. These cells were used as responder cells, and stimulated by PBMC that were isolated from the bone marrow transplant recipient before BMT. After 6 d of culture, the effector cells were harvested, and further expanded by weekly stimulation of 10^5 cells with 10^6 stimulator mononuclear cells from the recipient before BMT, in the presence of 20% T cell growth factor (TCGF; Biotest, Offenbach, Federal Republic of Germany). In this way CTL lines were established, and cryopreserved in liquid nitrogen. Before use, the CTL lines were thawed for 1 min in a 37°C waterbath, washed, and further expanded for another 3–5 d in 20% TCGF in RPMI 1640 + 15% prescreened human AB serum.

Phenotype of the CTL Lines. The phenotypes of the CTL lines were analyzed using mAbs against CD2, CD3, CD4, CD8, CD16, and CD19 antigens and FACS (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Cytotoxic Activity of CTL Lines. Cytotoxic activity of the CTL lines was measured in a 4-h ^{51}Cr-release assay as described earlier (33), using PHA-stimulated PBL as target cells. Family studies and large panels of randomly selected HLA-typed donors were used to define the specificity of the CTL lines and to identify the restricting HLA antigens, as described previously (30).

Preparation of Bone Marrow Cells. Normal human bone marrow was obtained, after informed consent, by aspiration from the posterior iliac crests of donors for bone marrow transplantation. The cells were collected in HBSS with 100 U/ml preservative-free heparin, and were centrifuged (1,000 g, 30 min, 20°C) over Ficoll-Isopaque (1.077 g/cm^3). In some experiments partially purified bone marrow cells were used, i.e., depleted of monocytes and T lymphocytes by incubation with carbonyl-iron particles (45 min, 37°C), and subsequent centrifugation over Ficoll-Isopaque (34); the interphase cells were then collected, incubated with 2-aminoethylisothiouroniumbromide–pretreated SRBC cells, and centrifuged over Ficoll-Isopaque to deplete T lymphocytes (35). In most cases bone marrow mononuclear cells, or partially purified cells were cryopreserved in liquid nitrogen, as described previously (36). Immediately before use, the cells were thawed for 1 min in a 37°C waterbath, diluted in hepes-buffered RPMI plus 20% FCS (Gibco Laboratories, Grand Island, NY) at 0°C, washed once in the

same medium, and then washed in RPMI plus 15% AB serum. The cells were resuspended in RPMI plus 15% AB serum at concentrations of 1-5 × 10⁵ viable cells/ml.

Cell-mediated Cytotoxicity Assay (32). A quantity of 1.25 × 10⁵ mononuclear bone marrow cells, or 0.25 × 10⁵ enriched bone marrow cells in 0.25 ml RPMI + 15% AB serum was mixed with an equal volume of this medium containing CTL. The E/T ratios varied from 1:2 to 20:1. The cell mixture was centrifuged (1,000 g, 15 s) to establish cell-cell contact between CTL and the bone marrow cells, and then was incubated for 4-18 h at 37°C in fully humidified air with 5% CO₂. After incubation, the cell mixture was washed once in RPMI plus 15% AB serum, resuspended in α-modified Eagle's MEM (a-MEM; Flow Laboratories, Inc., Irvine, UK), and subsequently cultured for CFU-GM, BFU-E/CFU-E, and CFU-GEMM. All CTL lines were irradiated (20 Gy) before use to prevent colony formation by these cells.

CFU-GM. A quantity of 0.2-1 × 10⁵ bone marrow cells was cultured in 1 ml medium containing 20% FCS (Rehatuin, Kankakee, IL) 20% leukocyte-conditioned medium (37), 20% a-MEM, and 40% methylcellulose 2.25% in a fully humidified atmosphere of 5% CO₂ and 37°C in 35-mm plastic petri dishes. CFU-GM colonies, defined as granulocytic, monocytic, or eosinophilic aggregates of >20 cells were scored under an inverted microscope on day 10.

CFU-E/BFU-E. A quantity of 0.2-1 × 10⁵ bone marrow cells was cultured in 1 ml medium containing 20% FCS (Rehatuin), 20% leukocyte-conditioned medium, 5% 10⁻³ M 2-ME, 5% Iscove's modified Dulbecco's medium with 1 U/ml erythropoietin (step III, Connaught Laboratories Ltd., Willowdale, Canada), 5% deionized serum albumin (Sigma Chemical Co., St. Louis, MO), 5% human transferrin, and 40% methylcellulose 2.25% in 35-mm plastic petri dishes in a fully humidified atmosphere of 5% CO₂ at 37°C. CFU-E, defined as clusters of 8-64 hemoglobinized cells were scored on day 7. The number of BFU-E was scored on day 14.

CFU-GEMM. A quantity of 0.2-1 × 10⁵ bone marrow cells was cultured in 1 ml medium containing 30% ABO-compatible human heparin plasma, 7.5% PHA leukocyte-conditioned medium (38), 5% 10⁻³ M 2-ME, 5% deionized serum albumin, 5% human transferrin, 7.5% Iscove's modified Dulbecco's medium with 1 U/ml erythropoietin, and 40% methylcellulose 2.8% in 35-mm plastic petri dishes in a fully humidified atmosphere of 5% CO₂ at 37°C. CFU-GEMM, defined as colonies containing at least both erythroid and myeloid cells (39), were scored on day 18.

Normal Values and Calculations of HPC Growth. Control 100% growth was defined as the number of colonies cultured from 10⁵ untreated bone marrow mononuclear cells. Normal values of HPC growth from mononuclear bone marrow cells in our laboratory are 182 ± 15 for CFU-GM day 10; 121 ± 12 for BFU-E; 149 ± 6 for CFU-E; and 16 ± 1 for CFU-GEMM (mean ± SE). In cellular cytotoxicity assays, the number of surviving HPC was expressed as percentage of the total number of colonies in the untreated control cultures.

Results

All cytotoxic T cell lines showed the characteristic phenotype of cytotoxic T cells (CD2, CD3, CD8⁺, and CD4, CD16, CD19⁻). The CTL lines against the mH antigens HA-1, -2, -4, and -5 all expressed the same restricting HLA-A2 antigen, whereas HLA-A1 was the restriction molecule for the anti-HA-3 CTL line. All anti-mH CTL lines only showed specificity for the antigen they were directed against when the target cells expressed the restricting HLA antigen identical to the effector cells (Table I). All bone marrow donors used were typed for the mH antigens using their PHA-stimulated PBL as target cells in a standard ⁵¹Cr-release assay.

Previous studies had shown that inhibition of HPC by incubating the bone marrow cells for 4 h with antigen-specific CTL lines could be clearly demonstrated using E/T ratios from 1:2 to 4:1, leading to nearly complete inhibition of colony formation

TABLE I

Characteristics of the Anti-mH Cytotoxic T Lymphocyte Lines

mH specificity	HLA restriction molecule	Percent lysis of PHA blasts in ^{51}Cr-release assay*		Membrane phenotype[‡] (percent positive cells)					
		Positive donors	Negative donors	CD2	CD3	CD4	CD8	CD16	CD19
HA-1	A2	44 ± 2	1 ± 2	ND	ND	ND	ND	ND	ND
HA-2	A2	51 ± 7	9 ± 2	97	90	16	76	1	1
HA-3	A1	85 ± 3	4 ± 2	98	94	3	95	3	3
HA-4	A2	52 ± 4	7 ± 1	99	98	2	98	7	2
HA-5	A2	79 ± 8	14 ± 3	97	94	4	95	5	4

* E/T ratio, 20:1, mean ± SE of five experiments.
[‡] Using an indirect immunofluorescence technique, as described in Materials and Methods.

by the progenitor cells in the highest E/T ratios (32). When the anti-HA-3 CTL line was incubated for 4 h with bone marrow cells from HA-3^{+} donors, a strong inhibition of CFU-GM, BFU-E, and CFU-GEMM was observed at all E/T ratios used (Fig. 1), showing that the mH HA-3 antigen is strongly expressed on human HPC. Although CFU-E was inhibited to a lesser degree, dose-dependent inhibition was observed until 80% at the highest E/T ratio (4:1). HPC of HA-3^{-} HLA-A1^{+} donors were not inhibited by the anti-HA-3 CTL lines, demonstrating that the growth inhibition was mH antigen specific.

In contrast to the mH antigen HA-3, no growth inhibition of CFU-GM, BFU-E, CFU-E, and CFU-GEMM from donors expressing the HA-1, HA-2, HA-4, or HA-5 antigen could be obtained after a 4-h incubation of the bone marrow mononuclear cells with the anti-HA-1, -2, -4, or -5 CTL lines at these E/T ratios (Figs. 2–5). The PHA-stimulated target cells from these donors could easily be lysed by these anti-mH CTL lines.

The possibility was considered that the expression of the HA-1, -2, -4, and -5 antigens on HPC was not absent, but much lower than that of the HA-3 antigen. To further increase the sensitivity of the assay, we therefore enriched bone marrow cell suspensions for HPC by monocyte and T cell depletion (34, 35). These suspensions
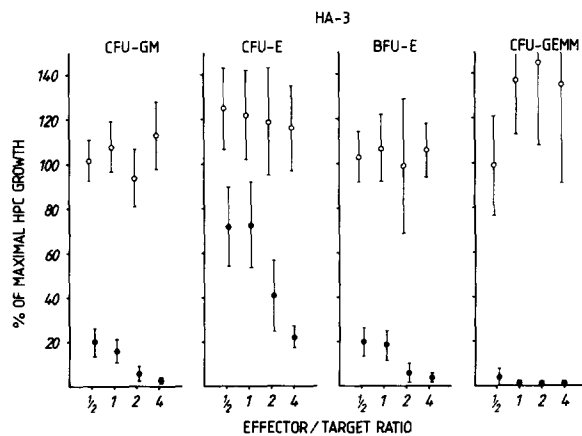

FIGURE 1. Growth of hematopoietic progenitor cells (mean ± SE) after incubation of bone marrow cells from HA-3^{+} donors (closed circles, n = 8) or HA-3^{-} donors (open circles, n = 8) with anti-HA-3 CTL, as compared with untreated controls.
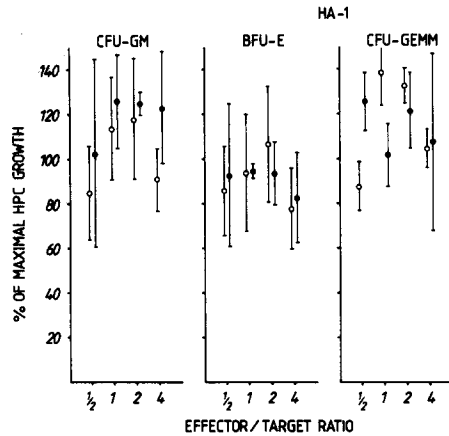
HA-1

CFU-GM   BFU-E   CFU-GEMM

FIGURE 2. Growth of hematopoietic progenitor cells (mean ± SE) after incubation of bone marrow cells from HA-1$^+$ donors (closed circles, n = 3) or HA-1$^-$ donors (open circles, n = 4) with anti-HA-1 CTL, as compared with untreated controls.

were incubated for 18 h with the anti-mH lines at E/T ratios of 20:1 (Table II). Although this procedure increased the sensitivity of the assay, it resulted, as demonstrated previously (32), in nonspecific inhibition of HPC growth (Table II). Despite this nonspecific inhibition, there appeared to be some differences in growth inhibition of HPC from mH$^+$ and mH$^-$ bone marrow donors, using the anti-mH HA-2, and possibly HA-5 CTL lines as effector cells. Thus, there may be a low expression of these antigens on HPC. Due to technical difficulties, the anti-HA-1 CTL line could not be tested in these experiments. Finally, to determine the killing efficacy of unstimulated cells by the anti-mH CTL lines, the lysis of unstimulated peripheral blood T lymphocytes was compared with that of PHA-stimulated lymphocytes (Table III). Although lysis of unstimulated T lymphocytes was somewhat less efficient than that of PHA blasts, all mH$^+$ donors showed lysis of their periferal blood T lymphocytes, and there was no difference in this respect between HA-2, HA-3, and HA-5. These results show that whereas the HA-3 antigen was demonstrated to be strongly expressed on HPC, the HA-1, -2, -4, and -5 antigens seem to be absent or expressed to a much lower extent.
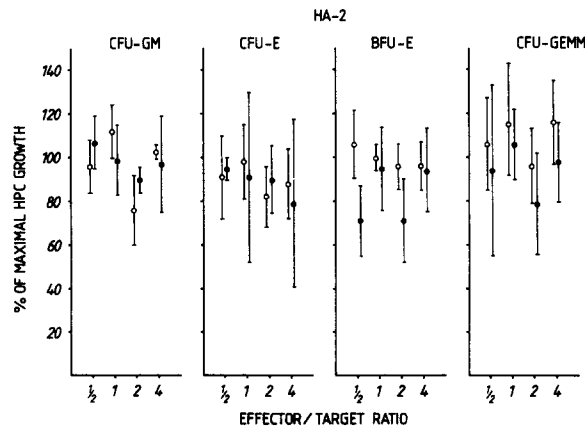
HA-2

CFU-GM   CFU-E   BFU-E   CFU-GEMM

FIGURE 3. Growth of hematopoietic progenitor cells (mean ± SE) after incubation of bone marrow cells from HA-2$^+$ donors (closed circles, n = 7) or HA-2$^-$ donors (open circles, n = 7) with anti-HA-2 CTL, as compared with untreated controls.
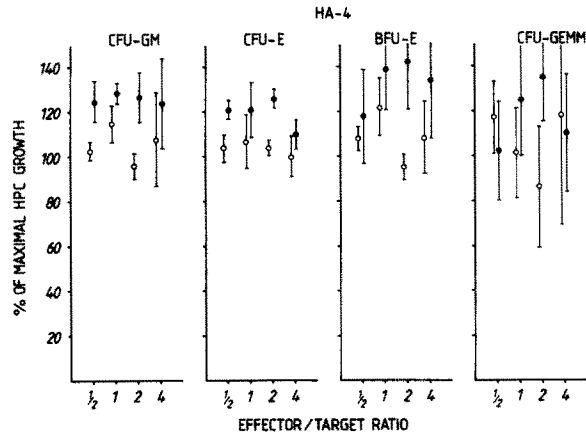
HA-4

CFU-GM   CFU-E   BFU-E   CFU-GEMM

FIGURE 4.   Growth of hematopoietic progenitor cells (mean ± SE) after incubation of bone marrow cells from HA-4⁺ donors (*closed circles, n* = 5) or HA-4⁻ donors (*open circles, n* = 5) with anti-HA-4 CTL, as compared with untreated controls.

Discussion

In this study, we investigated the expression of five mH antigens on human HPC using antigen-specific, HLA-restricted CTL lines. A CTL line specific for one of these antigens, HA-3, showed strong HLA-restricted inhibition of HPC growth in vitro from HA-3⁺, but not from HA-3⁻ donors, indicating that this HA-3 antigen is highly expressed on HPC. Similar to HA-3, we previously demonstrated strong HLA-restricted inhibition of male, but not of female, HPC by CTL lines against the mH antigen H-Y (31), showing that the H-Y antigen is also highly expressed on HPC of (male) donors. In contrast, CTL lines against the mH antigens HA-1, -2, -4, and -5 showed no or only minor antigen-specific inhibition of HPC growth even at high E/T ratios. Although recognition of the HA-3 antigen is restricted by HLA-A1, while recognition of all the other mH antigens is restricted by HLA-A2, several arguments make it unlikely that the observed differences in growth-inhibition of HPC by the anti-mH-CTLs are due to differences in the recognition of the restricting HLA antigens.

Firstly, we have demonstrated previously that anti-HLA-cytotoxic T lymphocytes

HA-5

CFU-GM   CFU-E   BFU-E   CFU-GEMM
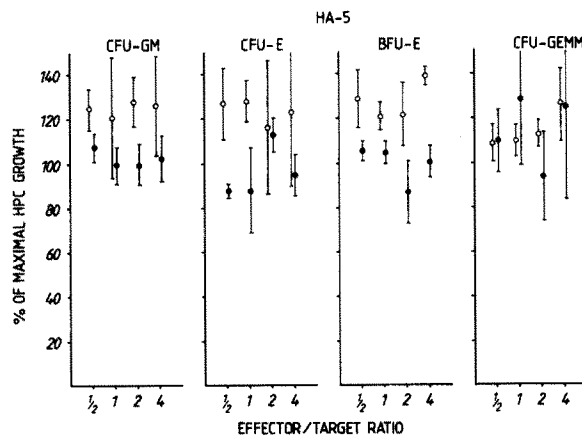

FIGURE 5.   Growth of hematopoietic progenitor cells (mean ± SE) after incubation of bone marrow cells from HA-5⁺ donors (*closed circles, n* = 4) or HA-5⁻ donors (*open circles, n* = 4) with anti-HA-5 CTL, as compared with untreated controls.

Table II

Colony Growth of Hematopoietic Progenitor Cells after Incubation of Bone Marrow Target Cells with Effector Cells in a High Effector/Target Cell Ratio and a Prolonged Incubation Time

	Percentage growth of HPC*					
	CFU-GM		BFU-E		CFU-GEMM	
Anti-mH-CTL	mH positive donors	mH negative donors	mH positive donors	mH negative donors	mH positive donors	mH negative donors
HA-2	37 ± 4	77 ± 7	41 ± 6	65 ± 6	36 ± 14	63 ± 11
HA-3	2 ± 3	110 ± 10	0 ± 0	81 ± 5	0 ± 0	117 ± 7
HA-4	69 ± 6	53 ± 4	83 ± 8	75 ± 8	58 ± 4	53 ± 6
HA-5	37 ± 5	42 ± 4	50 ± 6	70 ± 7	40 ± 6	68 ± 11

E/T ratio of 20:1; incubation time, 18 h.
* Percentage growth (mean ± SE) of untreated controls (n = 6).

can efficiently cause growth inhibition of HPC (32, Voogt, P. J., J. H. F. Falkenburg, W. E. Fibbe, W. F. J. Veenhof, M. Hamilton, B. A. van Krimpen, and R. L. H. Bolhuis, submitted for publication). This has been shown for several HLA specificities, such as HLA-A2, HLA-B7 (32), and HLA-Cw3, (Voogt, P. J., et al., submitted for publication).

Secondly, we have shown that recognition of another mH antigen, HY, can occur using different HLA-restricting antigens, such as HLA-A1, HLA-A2, or HLA-B7 (31). These studies demonstrated that HPCs of all male donors tested were inhibited in an antigen-specific way by the anti-HY-CTLs, provided the donors were positive for the restricting HLA specificity (31).

Finally, our results using nonstimulated T lymphocytes of mH⁺ donors show that lysis of these target cells occurred in all cases tested, albeit if somewhat less efficiently when compared with PHA blasts (Table III). However, there was no difference in this respect between the various anti-mH CTL lines tested.

Thus, the cellularly defined mH antigens appear to be differentially expressed on human HPC. A similar phenomenon has been described for the major histocompatibility class II antigens, HLA-DR but not HLA-DQ, being expressed on HPC (23, 24).

A cytotoxic alloimmune response of the recipient directed against polymorphic membrane determinants present on donor cells may cause bone marrow graft rejection, provided that these polymorphic antigens are expressed on the donor HPC. Therefore, the mH antigens HA-3 and H-Y, which are strongly expressed on HPC,

Table III

Lysis of PHA-stimulated or -nonstimulated T Cells by Anti-mH Cytotoxic T Lymphocyte

	PHA blasts*		T lymphocytes*	
mH specificity	Positive donors	Negative donors	Positive donors	Negative donors
HA-2	46 ± 3‡	4 ± 2	26 ± 1	3 ± 2
HA-3	75 ± 4	2 ± 1	42 ± 2	3 ± 2
HA-5	72 ± 5	6 ± 2	36 ± 3	1 ± 1

* Percent lysis in ⁵¹Cr-release assay at E/T ratio of 20:1.
‡ Mean ± SE of three to four experiments.

may function as target structures for T lymphocyte–mediated rejection of the HPC from the graft. In contrast, a cytotoxic alloreaction of the recipient against the mH antigens HA-1, HA-2, HA-4, and HA-5, which are weakly or not present at all on HPC, may not necessarily cause rejection of HPC if the donor is positive for these antigens.

Both GvHD and graft rejection are serious complications after allogeneic BMT, even between HLA genotypically identical siblings. Whereas severe GvHD occurs in many patients transplanted with an unmodified graft, graft rejection is a frequent complication in patients transplanted with a graft, depleted of T lymphocytes to prevent GvHD. We previously showed that disparity for the HA-1, -2, -4, or -5 antigens between donor and recipient is correlated with an increased risk of GvHD (30). However, HA-3 incompatibility appeared not to be associated with GvHD (30). Conversely, this study suggests that HA-3, but not HA-1, -2, -4, or -5 incompatibility may be a risk factor for graft rejection. These findings implicate that typing for mH antigens could be of importance in the decision whether or not T lymphocyte deple-tion of the graft should be performed in an individual transplantation between HLA-genotypically identical siblings. Furthermore, in HLA-identical unrelated BMT, if more than one phenotypically identical donor would be available, typing for mH antigens may be of help to select the most suitable donor.

In conclusion, our results show that cellularly defined mH antigens are differen-tially expressed on human HPC. Further mapping of mH antigens on HPC is of major interest for gaining insight into the mechanisms of graft rejection. Matching for these antigens between donor and recipient may decrease the risk of graft rejec-tion in allogeneic BMT.

Summary

Previously, five CTL lines directed against minor histocompatibility (mH) an-tigens designated HA-1–5 have been established from peripheral blood of patients after allogeneic bone marrow transplantation (BMT), and have been characterized using population and family studies. All cell lines showed specific HLA class I–re-stricted lysis of PHA-stimulated peripheral blood target cells from donors positive for the particular mH antigens. After 4 h of incubation of the mH antigen HA-3-specific CTL line with bone marrow cells from HA-3$^+$ donors, complete class I–restricted inhibition of colony growth of the hematopoietic progenitor cells was observed even at low E/T ratios, indicating that the HA-3 antigen is strongly ex-pressed on hematopoietic stem cells. Therefore, this antigen may be a target struc-ture in the immune-mediated rejection of the hematopoietic graft in case of incom-patibility for this determinant between donor and recipient in allogeneic BMT. In contrast, incubation of bone marrow cells with the antigen-specific anti-HA-1, -2, -4, and -5 CTL lines did not result in growth inhibition of the hematopoietic pro-genitor cells tested. After a prolonged incubation time and using a very high E/T ratio, progenitor cells from HA-2$^+$ or HA-5$^+$ donors were killed to some extent by the anti-mH-specific CTL lines, although the growth inhibition observed was minor and variable. Our results show that mH antigens are differentially expressed on human hematopoietic progenitor cells. Therefore, only some of these antigens may be targets in immune-mediated rejection of the bone marrow graft.

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