# Growth Inhibition of Clonogenic Leukemic Precursor Cells by Minor Histocompatibility Antigen-specific Cytotoxic T Lymphocytes

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## Summary

Minor histocompatibility (mH) antigens appear to play a major role in bone marrow transplantation (BMT) using HLA-identical donors. Previously, we reported the isolation of major histocompatibility complex (MHC)-restricted mH antigen-specific cytotoxic T lymphocytes (CTL) from patients with graft-vs.-host disease or rejection after HLA-identical BMT. We have demonstrated that mH antigens can be recognized on hematopoietic progenitor cells, and residual recipient CTL specific for mH antigens expressed on donor hematopoietic progenitor cells may be responsible for graft rejection in spite of intensive conditioning regimens in HLA-identical BMT. Here, we investigated whether mH antigen-specific CTL directed against the mH antigens HA-1 to HA-5 and the male-specific antigen H-Y were capable of antigen-specific inhibition of in vitro growth of clonogenic leukemic precursor cells. We demonstrate that mH antigen-specific CTL against all mH antigens tested can lyse freshly obtained myeloid leukemic cells, that these mH antigen-specific CTL can inhibit their clonogenic leukemic growth in vitro, and that this recognition is MHC restricted. We illustrate that leukemic (precursor) cells can escape elimination by mH antigen-specific CTL by impaired expression of the relevant MHC restriction molecule. We suggest that mH antigen-specific MHC-restricted CTL may be involved in vivo in the graft-vs.-leukemia reactivity after BMT.

After allogeneic bone marrow transplantation (BMT)[1] for malignant hematologic diseases, relapse of the disease is one of the major complications. Since T lymphocytes have been removed from the bone marrow graft to prevent the occurrence of acute graft-vs.-host disease (aGVHD), an increased incidence of relapse of the leukemia has been found (1). Furthermore, in HLA-identical BMT, an inverse correlation has been found between the occurrence of GVHD mediated by donor-derived T lymphocytes and a relapse of leukemia (1). These observations have led to the hypothesis that donor-derived T lymphocytes may be responsible for the graft-vs.-leukemia (GVL) reactivity in allogeneic BMT. Since this GVL reactivity has not been observed in transplants between homozygous twins, it has been suggested that alloantigen-specific CTL may be responsible for the GVL reactivity after transplantation. In BMT between HLA-identical sibling donors, donor-derived alloreactive lymphocytes directed against recipient antigens can not be directed against MHC antigens, and are therefore by definition directed against minor histocompatibility (mH) antigens (2, 3).

mH antigens appear to play a major role in BMT using HLA-identical donors (2, 3). Previously, we reported the isolation of MHC-restricted mH antigen-specific CTL from patients with GVHD or rejection after HLA-identical BMT (2, 4–6). We have demonstrated that mH antigens can be recognized on hematopoietic progenitor cells (HPC) (7–9), and that residual recipient CTL specific for mH antigen expressed on donor HPC may be responsible for graft rejection in spite of intensive conditioning regiments in HLA-identical BMT (6). Furthermore, mH antigen disparity between donor and recipient has been associated with the occurrence of

[1] *Abbreviations used in this paper:* aGVHD, acute graft-vs.-host disease; AML, acute myeloid leukemia; BMT, bone marrow transplantation; CML, chronic myeloid leukemia; GVL, graft-vs.-leukemia; HPC, hematopoietic progenitor cells; mH, minor histocompatibility.


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GVHD after BMT (2). Particularly in chronic GVHD, there appears to be a strong anti-mH antigen reactivity against the recipient mH antigens, probably associated with a low incidence of relapse after transplantation (1–3, 10, 11).

To study whether the GVL reactivity may be caused by donor-derived CTL specific for mH antigen expressed on recipient tissues, we investigated whether mH antigen–specific CTL were capable of antigen-specific inhibition of in vitro growth of clonogenic leukemic precursor cells. We demonstrate that mH antigen–specific CTL can lyse freshly obtained myeloid leukemic cells, and can inhibit the in vitro growth of clonogenic leukemic precursor cells. We therefore suggest that mH antigen–specific MHC-restricted CTL may be involved in the in vivo GVL reactivity after BMT.

## Materials and Methods

*Leukemic Samples.* Samples of leukemic cells from patients suffering from acute myeloid leukemia (AML) or chronic myeloid leukemia (CML) were obtained, after informed consent, from the peripheral blood or bone marrow of the patients before treatment. Patients who had >95% morphologically recognizable leukemic cells in their peripheral blood or bone marrow samples were selected. The cells were centrifuged over Ficoll Isopaque (density 1.077/$cm^3$, 1,000 *g*, 20 min) and the interphase cells were harvested. The cells were cryopreserved in liquid nitrogen as described previously (12). Leukemic samples were selected that gave rise to >100 leukemic colonies per $10^4$ cells plated in semisolid medium cultures. Before use, the cells were thawed, washed twice, and resuspended in IMDM containing 15% prescreened human AB serum.

*Lymphocyte Populations.* The lymphocyte populations were generated by stimulating peripheral blood cells from the patient with PHA (0.8 μg/ml for 3 d), followed by culturing at least 3 d in the absence of PHA in the presence of human rIL-2 (50 U/ml) (9). Using FACS® (Becton Dickinson & Co., Mountain View, CA), the lymphocyte suspensions where shown to contain >90% $CD3^+$ cells.

*Generation of mH Antigen–specific CTL Clones.* mH antigen–specific CTL lines were generated by stimulating donor-originated peripheral blood cells obtained from recipients after HLA genotypically identical BMT with irradiated peripheral blood cells obtained from the recipients before transplantation, as described previously (2–5). The mH antigen–specific CTL lines were subsequently cloned by limiting dilution in the presence of recipient-derived EBV-transformed lymphoblastoid cell lines (EBV lines) and third party peripheral blood feeder cells in the presence of IL-2. CTL clones were obtained with specific cytotoxic activity for each of the mH antigen specificities described previously (2), i.e., HA-1, HA-2, HA-3, HA-4, or HA-5 (Van Els, C.A.C.M., J. D'Amaro, E. Blokland, A. Bakker, P.J. Van den Elsen, J.J. van Rood, and E. Goulmy, manuscript submitted for publication). The MHC class I–restricted mH antigen specificity of these $CD8^+$ CTL clones was demonstrated by differential recognition of lymphocytes from various HLA genotypically identical sibling pairs, and population studies (2). In a random population, the frequency of the mH antigens on the lymphocytes from individuals expressing the relevant class I restricting molecule was 68% for HA-1, 96% for HA-2, 87% for HA-3, 16% for HA-4, and 8% for HA-5 (data not shown). In some experiments, an HLA-A2-restricted HY-specific CTL clone was used, generated from a female patient rejecting the HLA-identical bone marrow graft from a male donor (4). HA-1-, HA-2-, HA-4-, and HA-5-specific recognition was shown to be restricted by HLA-A2. HA-3 recognition was restricted by HLA-A1 (2).

*$^{51}Cr$ Release Assay.* Standard $^{51}Cr$ release assays were performed as described (13). Briefly, target cells consisting of lymphocyte populations or leukemic cells were labeled with 0.1 ml $Na_2\,^{51}CrO_4$ (100 μCi) for 1 h at 37°C, washed three times, and resuspended in RPMI plus 15% serum at a concentration of $5 \times 10^4$ viable cells/ml. 0.1 ml of the effector cell population 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 10:1 to 0.1:1. 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. Maximum release was determined by adding 0.1 ml of the target cell suspension to 0.1 ml of Zaponine solution. The percentage of specific lysis was determined as follows: 100× (experimental release cpm − spontaneous release cpm)/(maximum release cpm − spontaneous release cpm).

*Cell-mediated Inhibition of Clonogenic Leukemic Precursor Cell Growth.* $10^4$ leukemic cells in 0.1 ml medium consisting of IMDM plus 15% human AB serum was mixed with CTL at E/T ratios varying from 10:1 to 0.1:1. The cell mixture was centrifuged (1,000 *g*, 15 s) to establish cell-cell contact between CTL and leukemic cells, and then incubated for 4 h at 37°C. After incubation, the cells were resuspended and cultured as a single cell suspension at a concentration of $10^4$ leukemic cells/ml in 30-mm culture dishes in 1 ml IMDM containing 20% prescreened human AB serum, 20% PHA-stimulated leukocyte-conditioned medium (PHA-LCM), 100 U/ml recombinant human granulocyte/macrophage CSF, human transferrin, $5 \times 10^{-5}$ M mercaptoethanol, and methylcellulose at a final concentration of 1.1%. The cells were incubated in the fully humidified atmosphere of 5% $CO_2$ and 37°C, and leukemic colonies, consisting of at least 20 cells, were counted after 10–14 d of culture. Morphological and/or cytogenetic analyses of several representative leukemic colonies isolated from the cultures were performed. Inhibition of leukemic growth was expressed as the percentage decrease of the number of colonies as compared with control cultures, grown in the absence of effector cells. To control for antigen-nonspecific inhibition of colony growth due to the secretion of humoral inhibitory factors into the culture medium, effector cells were also plated together with the leukemic cells at the same E/T ratios directly in the semisolid medium, without preincubation, to prevent cell-cell contact.

## Results

The PHA-stimulated lymphocytes from patients with leukemia of various subtypes according to the FAB classification were HLA typed. Since the mH antigen–specific CTL clones were shown to be HLA-A1 or HLA-A2 restricted, only patients expressing HLA-A1 and/or -A-2 on their lymphocytes were selected for the study. Table 1 shows the characteristics of the patients and the leukemic samples used. Representative leukemic colonies were isolated from the cultures, and morphological and/or cytogenetic analysis of these colonies revealed that the cells recovered had characteristics identical to the original leukemic population. In one of the two cases with CML, the progeny harvested from the colonies showed additional cytogenetic abnormalities (data not shown). Furthermore, the high percentage of the leukemic cells in the samples used, in combination with the high plating efficiency, excluded the possibility of a significant contribu-

**Table 1.** *Characteristics of the Leukemic Cells*

| Patient | HLA type | FAB classification | Karyotype | No. of colonies per $10^4$ cells |
|---|---|---|---|---|
| 1 | A2, B27,44 | AML M1 | 46XX | 243 |
| 2 | A1,9 B8,w41 | AML M2 | 46XX | 189 |
| 3 | A1,2 B49,w56 | AML M4 | 46XX,-7, + t(1;7)(q11;p11) | 577 |
| 4 | A1,3 Bw57,35 | AML M5 | 46XX | 310 |
| 5 | A2,3 B7,27 | AML M2 | 47XY, + 13 | 249 |
| 6 | A2,26 B7,44 | AML M1/2 | 46XX | 624 |
| 7 | A2, B8,40 | AML M5 | 46XX | 259 |
| 8 | A2, B7,27 | CML CP* | 46XX, t(9;22) | 326 |
| 9 | A1,2 B8,13 | CML CP* | 46XY, t(9;22) | 182 |

* Chronic phase.

tion of colonies derived from residual normal progenitor cells present in the cell suspension (Table 1).

A good correlation was found between the antigen-specific lysis of the lymphocytes and the leukemic cells in a $^{51}Cr$ release assay, and the inhibition of the clonogenic leukemic precursor cell growth by the mH antigen–specific CTL clones. Fig. 1 shows a representative experiment with the cells of a patient with AML. CTL clones directed against HA-3 or HLA-A1 that gave dose-dependent lysis of the lymphocytes of the patient showed similar cytotoxicity against the AML blasts (Fig. 1, *A* and *B*). When cell-cell contact between the CTL clones and the leukemic cell population was established for 4 h before plating the cells as a single cell suspension in the semisolid culture medium, strong dose-dependent, antigen-specific inhibition of the clonogenic leukemic growth was observed (Fig. 1 *C*). When the effector/target cells were plated directly together as a single cell suspension in the semisolid medium, thereby prohibiting direct cell-cell contact between effector cells and target cells, no growth inhibition was observed. This illustrated that cell-cell contact was necessary for the observed growth inhibition (Fig. 1 *D*) (7, 8).

Results of experiments with cells of three representative patients with AML of different subtypes, and of one of the two patients with CML, are shown in Fig. 2. The left panels show the result of lysis of lymphocytes or leukemic cells by the mH antigen–specific CTL clones or anti-HLA class I CTL clones in a 4-h $^{51}Cr$ release assay. The data are expressed as means ± SD of the highest E/T ratios used (10:1 or 5:1) in three to nine experiments performed in duplicate. The right panels illustrate the inhibition of the clonogenic leukemic

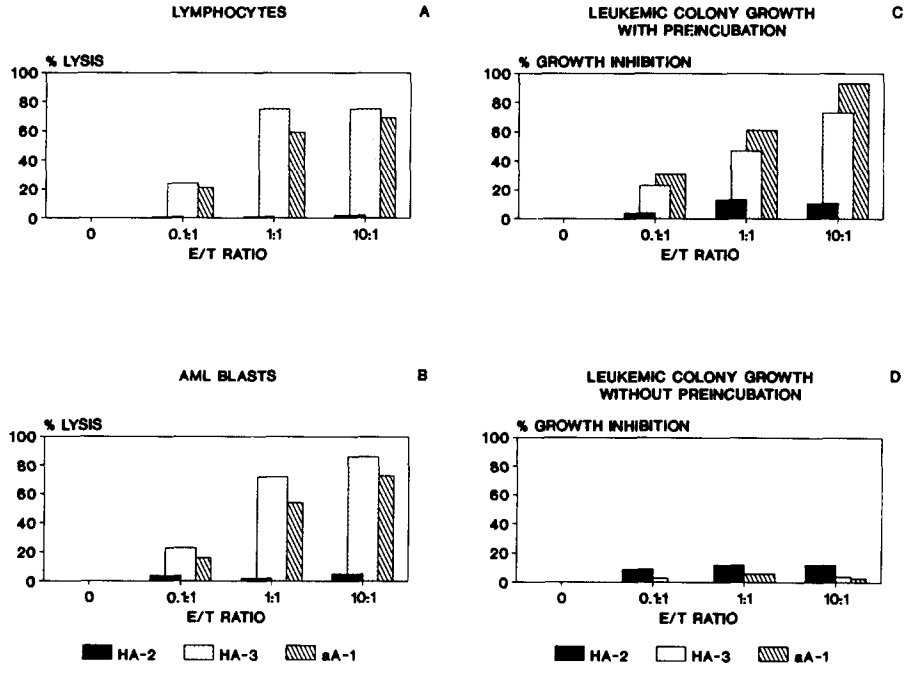


**Figure 1.** Cell-mediated lysis of lymphocytes or leukemic cells, and cell-mediated inhibition of leukemic colony growth by CTL clones. Lysis of lymphocytes or leukemic blast cells by the CTL clones was measured using a standard $^{51}Cr$ release assay in duplicate at E/T ratio's varying from 10:1 to 0.1:1 (*left panels*). Growth inhibition by the CTL clones was measured by preincubating the leukemic cells before plating with irradiated effector cells at E/T ratio's varying from 10:1 to 0.1:1 (*C*). As a control, effector cells and leukemic cells at the same E/T ratios were plated directly in the semisolid medium, without preincubation, to prevent cell-cell contact (*D*).

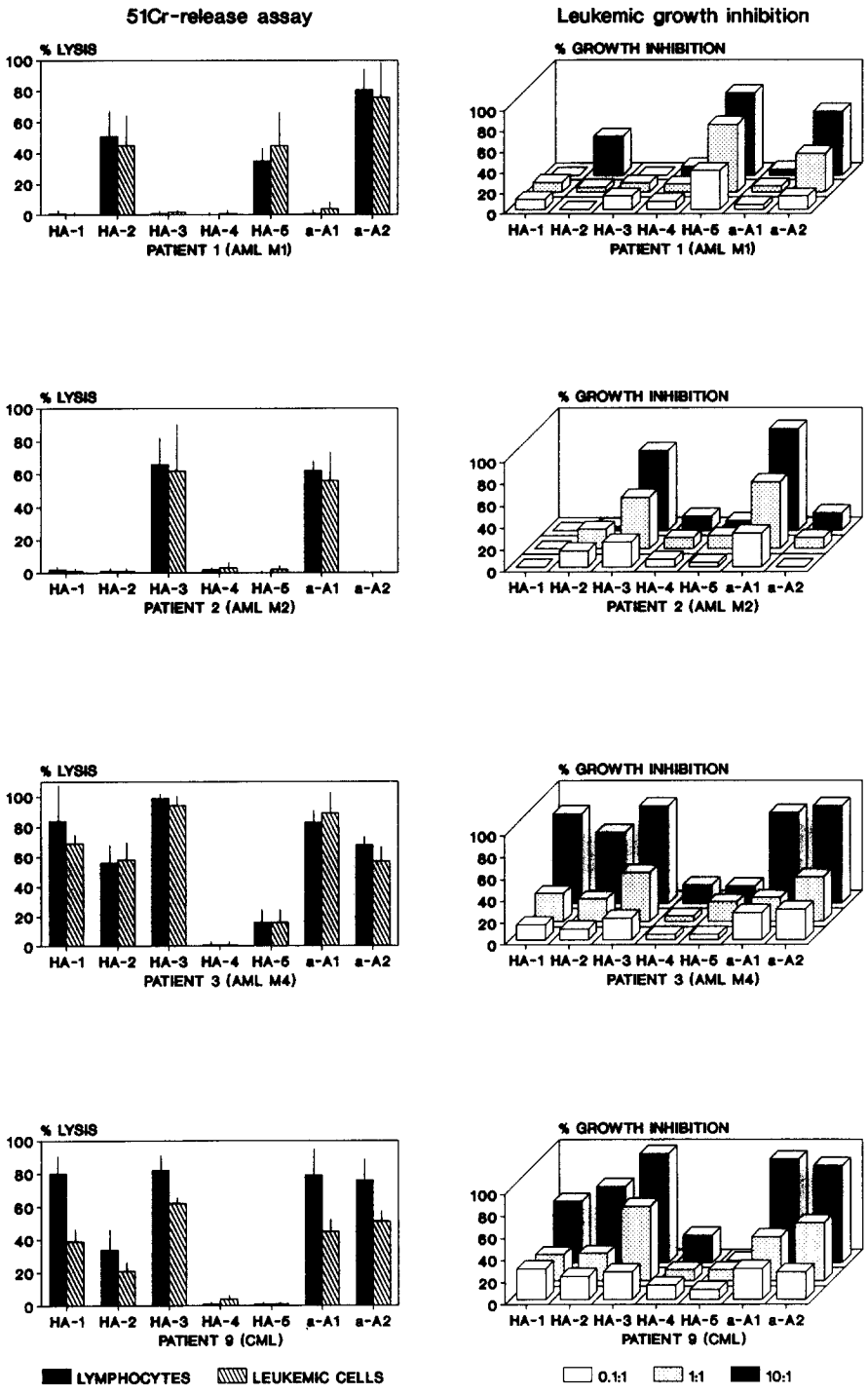


**Figure 2.** mH antigen-specific lysis of lymphocytes or leukemic cells, and inhibition of leukemic colony formation by mH antigen-specific CTL clones. The left panels show the results of lysis of lymphocytes or leukemic cells by the mH antigen-specific CTL clones and/or by HLA-A1 or -A2 CTL clones. The data are expressed as mean ± SD of the highest E/T ratios used (10:1 or 5:1) in three to nine experiments performed in duplicate. The right panels illustrate the inhibition of clonogenic leukemic colony growth after 4 h of preincubation of the leukemic cells with CTL clones at E/T ratios varying from 10:1 to 0.1:1.

colony growth after a 4-h preincubation of the leukemic cells with the CTL clones. $10^4$ leukemic cells were incubated with $10^5$, $10^4$, or $10^3$ irradiated (20 Gy) CTL in IMDM with 15% serum. Then, the cells were resuspended and plated as single cell suspensions in semisolid medium cultures as described in Materials and Methods. Percentage growth inhibition was expressed as percentage decrease in the number of colonies counted after 14 d as compared with control leukemic colony growth in the absence of effector cells. As a control for nonspecific inhibition of leukemic growth by factors secreted by the CTL clones, in all experiments leukemic cells were also cultured in the presence of the same number of effector cells in the semisolid culture medium without the 4-h preincubation in liquid medium. These cultures did not show significant growth inhibition as compared with the control culture in the absence of effector cells (data not shown).

**Table 2.** *Reactivity of mH-specific Cytotoxic T Cell Clones with Normal Lymphoblasts, Leukemic Cells, and Clonogenic Leukemic Precursor Cells*

| | Patients | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CTL clone | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| HA-1: | | | | | | | | | |
| Lympholysis* | − | − | + + | − | − | − | − | + + | + + |
| Leukemic lysis* | − | − | + + | − | − | − | − | + | + |
| Growth inhibition‡ | − | − | + + | − | − | − | − | + + | + + |
| HA-2: | | | | | | | | | |
| Lympholysis* | + + | − | + + | − | − | + | + + | (+) | + |
| Leukemic lysis* | + | − | + + | − | − | − | + | (+) | (+) |
| Growth inhibition‡ | + | − | + + | − | − | − | (+) | − | + + |
| HA-3: | | | | | | | | | |
| Lympholysis* | − | + + | + + | + + | − | − | − | − | + + |
| Leukemic lysis* | − | + + | + + | + + | − | − | − | − | + + |
| Growth inhibition‡ | − | + + | + + | + + | − | − | − | − | + + |
| HA-4: | | | | | | | | | |
| Lympholysis* | − | − | − | − | − | − | − | − | − |
| Leukemic lysis* | − | − | − | − | − | − | − | − | − |
| Growth inhibition‡ | − | − | − | (+) | − | − | − | − | (+) |
| HA-5: | | | | | | | | | |
| Lympholysis* | + | − | − | − | (+) | − | − | − | − |
| Leukemic lysis* | + | − | − | − | (+) | − | − | − | − |
| Growth inhibition‡ | + + | − | − | − | + | − | − | − | − |
| Anti-A1: | | | | | | | | | |
| Lympholysis* | − | + + | + + | + + | − | − | − | − | + + |
| Leukemic lysis* | − | + + | + + | + + | − | − | − | − | + |
| Growth inhibition‡ | − | + + | + + | + + | − | − | − | − | + + |
| Anti-A2: | | | | | | | | | |
| Lympholysis* | + + | − | + + | − | + + | + + | + + | + + | + + |
| Leukemic lysis* | + + | − | + + | − | + + | − | + | + + | + + |
| Growth inhibition‡ | + + | − | + + | − | + + | − | + + | + + | + + |
| Anti-H-Y: | | | | | | | | | |
| Lympholysis* | | | | | + + | | | − | + + |
| Leukemic lysis* | | | | | + | | | − | + |
| Growth inhibition‡ | | | | | + + | | | − | + + |

Results show specific lysis of target cells or inhibition of clonogenic leukemia growth.
Mean lysis or inhibition of three to nine experiments is presented.
−: 0–20% lysis or inhibition; (+): 20–30% lysis or inhibition; +: 30–50% lysis or inhibition; + +: >50% lysis or inhibition.
* Percentage specific lysis of lymphocytes or leukemic cells in $^{51}Cr$ release assay.
‡ Percentage inhibition in clonogenic leukemic precursor cell assay.

Effector cells plated in the absence of leukemic cells did not form colonies (data not shown). It was demonstrated that similar to the lysis in the $^{51}Cr$ release assay, antigen-specific dose-dependent inhibition of the clonogenic leukemic precursor cell growth was observed, even at very low E/T ratios (Fig. 2, *right panels*).

The results of the experiments with all nine patients are summarized in Table 2. A strong correlation between the antigen-specific lysis of the lymphocytes, the lysis of the leukemic cells, and the inhibition of the clonogenic leukemic precursor cell growth was observed for all mH antigen–specific CTL clones tested. In addition, the HLA-A2-restricted anti-

H-Y CTL clone showed strong reactivity with both lymphocytes and leukemic cells from HLA-A2-positive male patients. As expected, no lymphocytes or leukemic cells from patients negative for HLA-A1 and -A2 showed reactivity with the CTL clones (data not shown). Only lymphocytes or leukemic cells from HLA-A1-positive patients showed reactivity with the anti-HA-3 CTL clone, and only lymphocytes or leukemic cells from HLA-A2-positive patients showed reactivity with the anti-HA-1, -HA-2, -HA-4, -HA-5, and -HLA-A2-restricted H-Y CTL clones, illustrating that similar to the lymphocytes, the recognition of the leukemic cells is HLA class I restricted (Table 2).

In one case, patient 6, the AML cells showed no reactivity with the anti-HA-2 CTL clone, although the lymphocytes were clearly lysed (Table 2). Furthermore, the control anti-HLA-A2 clone did not react with the leukemic (precursor) cells. Serological typing of the leukemic cells revealed that the leukemic cell population did not express the class I HLA-A2/B44 antigens, whereas the other haplotype (HLA-A26/B7) was normally expressed, as well as both class II haplotypes. Therefore, impaired recognition of this leukemic cell population by the anti-HA-2 CTL clone appeared to be due to the loss of expression of the relevant class I restriction molecule.

## Discussion

Our results show that leukemic (precursor) cells express mH antigens. CTL clones directed against these mH antigens are not only capable of lysis of freshly obtained leukemic cells, but are capable of antigen-specific growth inhibition of clonogenic leukemic precursor cells, illustrating that the leukemic precursor cells are susceptible targets for alloreactive MHC-restricted mH antigen–specific T lymphocytes.

Recently, we showed that anti-host mH antigen–specific reactivity can be found after HLA-identical BMT not only in patients with GVHD, but also in patients without clinical signs of GVHD, or after resolution of acute GVHD (14). Consequently, the existence of anti–recipient mH CTL in vivo does not always imply destruction of the GVHD target organs. This is compatible with the differential expression of mH antigens on keratinocytes (15; De Bueger, M.M., A. Bakker, J.J. van Rood, and E. Goulmy, manuscript submitted for publication). Previously, we illustrated that mH antigens may be differentially recognized on normal hematopoietic progenitor cells (8). We have shown that the mH antigens HY and HA-3 are expressed on peripheral blood monocytes and lymphocytes, as well as hematopoietic progenitor cells (7–8). In contrast, CTL lines specific for the mH antigens HA-1, HA-2, HA-4, and HA-5 could lyse lymphocytes, but did not recognize hematopoietic progenitor cells from the same individuals, indicating differential expression of mH antigens on hematopoietic cells (8). These results indicate that the expression of several mH antigens appeared to be tissue specific, and some of these antigens may be differentiation antigens (3, 7, 8).

We have demonstrated that residual recipient CTL specific for mH antigens expressed on donor hematopoietic progenitor cells may be responsible for graft rejection in spite of intensive conditioning regiments after HLA-identical BMT (6). These results illustrated that also in vivo, mH antigen–specific CTL can be responsible for the elimination of hematopoietic progenitor cells. Similarly, donor-derived mH antigen–specific CTL may be capable of lysis of leukemic precursor cells in vivo. Particularly in chronic GVHD, when strong donor anti–recipient mH antigen reactivity is present in the patient, a low incidence of relapse of the leukemia appears to occur (1–3, 10, 11). These results suggest that also in vivo, mH antigen–specific CTL may be responsible for a GVL effect. Since mH antigens may be differentially expressed on normal recipient tissue, and since the occurrence of anti–recipient mH CTL in vivo (14) is not always correlated with GVHD, it may be hypothesized that certain mH antigen reactivities after transplantation may cause a graft-vs.-leukemia reactivity without concurrent GVHD.

In conclusion, in this report we demonstrate that mH antigens can be recognized on fresh leukemic cells by MHC-restricted CTL. These mH antigen–specific CTL are capable of growth inhibition of clonogenic leukemic precursor cells in vitro, and may therefore be responsible for the GVL reactivity that has been hypothesized in HLA-identical BMT. Furthermore, it may be conceivable that donor-derived CTL clones that recognize the neoplastic target cells from the recipient but not the normal host cells of the tissues that are involved in GVHD may be used as an adjuvant immunotherapy in a treatment of leukemia.

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