Correlation of Minor Histocompatibility Antigen-Specific Cytotoxic T Lymphocytes With Graft-Versus-Host Disease Status and Analyses of Tissue Distribution of Their Target Antigens

By Dietger Niederwieser, Alfred Grassegger, Josef Auböck, Manfred Herold, David Nachbaur, Agathe Rosenmayr, Anneliese Gächter, Walter Nussbaumer, Sabine Gaggl, Michael Ritter, and Christoph Huber

Peripheral blood mononuclear cells (PBMC) from 17 patients receiving HLA-identical sibling bone marrow grafts were stimulated with host pretransplant PBMC. Cytotoxic T-cell lines (TCL) with specificity for host pretransplant patients receiving HLA-identical sibling bone marrow transplantation, and (2) their target antigens are simultaneously expressed on several host cell lines, including lymphoblastoid cell lines, PHA blasts, leukemic cells, and K. We also extend previous findings by showing that, besides the expression of the nominal MiHA, the density of the restricting class I MHC elements also crucially determines the extent of TCL lysis. Because of its capacity to enhance class I MHC antigen expression, IFN-γ represents a key cytokine for determining the susceptibility of MiHA targets for lysis by TCL and clones, and in all patients an MiHA-specific clone recognized host leukemic cells and also inhibited host leukemic cell growth in a colony inhibition assay.

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Submitted June 30, 1992; accepted December 4, 1992.

Supported by the Austrian Research Fund “Zur Förderung der wissenschaftlichen Forschung” Project No. 8603 and the Research Fund of the Max Planck Gesellschaft.

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BONE MARROW transplantation (BMT) represents the therapy of choice for treatment of various malignant and nonmalignant diseases.1-7 Despite the use of HLA-identical sibling donors, graft-versus-host disease (GVHD) remains a frequent complication after BMT, affecting approximately half of all recipients.8-11 GVHD is mediated by donor lymphocytes recognizing so-called minor histocompatibility antigens (MiHA) on HLA-identical host cells.12,14 In contrast to the functionally and biochemically well-characterized major histocompatibility complex (MHC) molecules,15,16 knowledge about the structure, distribution, and function of MiHA antigens is still rather limited.13,19 The only well-defined MiHA in mice is the maternally transmitted MiHA MTF and the nuclear myxovirus-resistance protein Mx.20,21

Molecules associated with the male-specific H-Y antigen were described as MiHA in humans.22,23 Recent experiments indicate that MiHA are peptides from endogeneous cell proteins that bind to MHC and are recognized by the antigen-binding T-cell receptors.13,22,24-27

This study was aimed at isolating host-specific T-cell lines (TCL) from the blood stream of patients who underwent MHC-identical allogeneic BMT, and to correlate their occurrence with clinical expression of GVHD. Also investigated were the tissue distribution of MiHA, the patterns of MHC restriction of host-reactive TCL lysis, and their interaction with GVHD targets of epithelial or leukemic origin.

PATIENTS AND METHODS

Patients. Seventeen (11 male and 6 female) patients undergoing BMT from their HLA-identical and MLC-negative sibling donors for treatment of hematologic malignancies (acute myelogenous leukemia [n = 4]; acute lymphoblastic leukemia [n = 2]; lymphoma [n = 4]; myelodysplastic syndrome [n = 1]); and chronic myelogenous leukemia [n = 4]) and severe aplastic anemia (n = 2) were investigated. Clinical characteristics of these patients are given in Table 1. Patients with hematologic malignancies were conditioned with cyclophosphamide (2 × 60 mg/kg/d) and total body irradiation (either as single dose 10 Gy or fractionated 2 × 2 Gy/d on 3 consecutive days), whereas patients with severe aplastic anemia (n = 2) were pretreated with cyclophosphamide 50 mg/kg/d for 4 days. One patient with lymphoma (unique patient number [UPN] 57) received carmustin (BCNU), etoposide (VP-16), and cyclophosphamide without irradiation before BMT. Cyclosporin was used as GVHD prophylaxis alone (n = 14)28 or in combination with methotrexate (n = 3).29 Nine of the 17 patients developed GVHD grades I through IV and were subsequently treated with high-dose methylprednisolone (Table 1).

Isolation of peripheral blood mononuclear cells (PBMC). Heparinized PB was taken from donors before BM harvest, from patients
before transplantation and during GVHD, or, at the latest, 2 months after BMT. PBMC were separated on Ficoll-Isoopaque (Lymphoprep, Nyegard, Norway), washed three times, frozen, and stored in liquid nitrogen.

Epstein-Barr virus (EBV) transformation. Retransformation recipient PBMC was resuspended in tissue culture medium at concentrations of $2 \times 10^6$ mell and mixed with the same amount of a mycoplasma-free EBV producer line B95-8 supernatant and 200 ng/ml cyclosporin (Sandimmun; Sandoz, Vienna, Austria). On day 6, and every third day thereafter, 50% of the culture medium was replaced with fresh medium. Cyclosporin was added to the culture until the third week.

Phytohemagglutinin (PHA) blasts. PBMC ($1 \times 10^6$/mL) from the host or donor were incubated with 1% PHA (Difco, Detroit, MI) in a humidified 5% CO$_2$/95% air atmosphere at 37°C for 72 hours. For further growth, cells were cultured in 20 U/mL recombinant IL-2 (Hoffmann-LaRoche, Basel, Switzerland).

Establishment of cytotoxic TCL. TCL were established from $2 \times 10^6$ posttransplant recipient PBMC harvested during GVHD or, in the absence of GVHD, at the latest 2 months after BMT, stimulated with $2 \times 10^6$ irradiated (30 Gy) pretransplant recipient PBMC, and cultured in RPMI 1640 (Biochrom, Berlin, Germany) medium containing 100 U/mL penicillin (Biochemie, Vienna, Austria) and 20 mg/L gentamicin (Biochemie) supplemented with 1% human AB pool serum. On day 6, cells were placed in fresh culture medium ($2 \times 10^5$ cells/mL), restimulated with $4 \times 10^5$/mL irradiated (50 Gy) EBV-transformed, pretransplant recipient PBMC, supplemented with 15% pool serum and 2% Lymphoblast-Highly Purified (HP; Biotech, Frankfurt, Germany) as an interleukin-2 (IL-2) source, and plated in a 24-well Costar plate (2 mL/well). Every third day, 50% of the medium was replaced with fresh medium and 20% of Lymphoblast-T (Biotech) final concentration added to the culture. On day 14, cells were counted, adjusted to $2 \times 10^5$/mL responder cells, and restimulated with $2 \times 10^5$ irradiated (50 Gy) EBV-transformed pretransplant recipient PBMC and $2 \times 10^5$ donor cells/mL as feeder cells supplemented with 20% final concentration Lymphoblast-T (Biotech). The cells were fed every other day and restimulated weekly with the same cell mixture as on day 14. TCL obtained after 3 weeks were used as polyclonal effector cells in cytotoxic assays.

Establishment of clones. On day 14, TCL were counted and diluted to 0.5 cells/well in a U-shaped 96-well plate (Falcon; Becton Dickinson, Oxnard, CA). The cells were restimulated with 20,000 cells/well from an irradiated (30 Gy) stimulator pool of at least four healthy blood donors matching the TCL in one HLA-antigen and 10,000 irradiated (50 Gy) EBV-transformed recipient pretransplant cells/well. Finally, 20 U/mL recombinant IL-2 (Hoffmann-LaRoche) and 1% PHA-M (Difco) were added to the culture. After 6 days of culture, host-specific cytotoxic clones were selected and further cultured as described above. All clones and TCL were tested routinely for mycoplasma contamination (Gen-Probe; Gen-Probe Inc, San Diego, CA) and were negative.

Isolation and culture of keratinocytes. Keratinocytes from the host or donor were isolated by trypsin-EDTA (Biochrom) at 2°C. Epidermal cells (EC) were frozen and stored in liquid nitrogen or expanded as previously described. Briefly, $2 \times 10^6$ fresh EC were plated in 75-cm$^2$ flasks (Falcon; Becton Dickinson) containing 1.8$ \times 10^5$ irradiated (60 Gy) 3T3 feeder cells (a generous gift of Dr. H. Green, Harvard University, Boston, MA). Cultures were kept in a 3:1 mixture of Dulbecco Vogt modification of Eagle’s medium in Ham’s F12 medium (Flow Laboratories, Irvine, UK) supplemented with 10% fetal calf serum (FCS; Flow), 5 mg/mL insulin (Sigma, Munich, Germany), $2 \times 10^{-9}$ mol/L triiodo-L-thyronine (Serva, Heidelberg, Germany), $10^{-10}$ mol/L choleratoxin (Calbiochem-Behring, La Jolla, CA), 5 mg/mL transferrin (Calbiochem-Behring), 0.4 mg/mL hydrocortisone (Calbiochem-Behring), and $8 \times 10^{-4}$ mol/L adenine (Boehringer Mannheim, Vienna, Austria). The cultures were incubated at 37°C in a 10% CO$_2$/90% air atmosphere and, after 2 days of culture, human recombinant epidermal growth factor (a generous gift of Dr. D. Green, Harvard University, Boston, MA) was added to a final concentration of 10 ng/mL. Medium was changed three times weekly until K were confluent. For further expansion, K were subcultivated as described above. K of these secondary cultures were used as targets after incubation with human recombinant interferon-γ (rIFN-γ; Genentech, South San Francisco, CA; specific activity, $2 \times 10^7$ IU/mg protein) or human rIFN-α (Boehringer Ingelheim, Ingelheim, Germany; specific activity, $3.2 \times 10^8$ IU/mg protein) for 72 hours with concentrations ranging from 1 to 1,000 IU/mL.
55 cpm/cell. For PHA blasts, 1.1 cpm/cell; for PHA blasts, 1.1 ± 0.9 cpm/cell; for leukemia cells, 0.45 ± 0.1 cpm/cell; for K, 5.3 ± 2.8 cpm/cell; for K pretreated with IFN-γ, 6.65 ± 4.4 cpm/cell; for K pretreated with IFN-α, 4.2 ± 2.3 cpm/cell. For cytotoxic assays, 5 × 10^5 labeled target cells were mixed with the effector cells (TCL or clones) in U-shaped microtiter plates at various ratios ranging from 25:1 to 3:1. After 4 hours of incubation at 37°C in a humidified 5% CO2/95% air atmosphere, 0.1 mL of the supernatant was removed with a semiautomatic harvesting device and counted with a gamma-sciillation counter (LKB, Stockholm, Sweden). Results are expressed as percentage of specific lysis according to the formula:

\[
\text{Experimental Release} - \text{Spontaneous Release} \times 100
\]

### Results

#### Host-specific cytotoxic TCL are exclusively established from patients with GVHD

PBMC isolated from 17 allogeneic BMT recipients after grafting were stimulated with irradiated pretransplant PBMC. Primary mixed leukocyte cultures (MLC) were then supplemented with natural IL-2 and restimulated with MiHA⁺ lymphoblastoid cells derived from the respective recipient’s pretransplant PBMC. Under these conditions and from a total of 59 TCL grown, cytotoxic TCL with specificity for host cells were exclusively obtained from those 9 patients presenting with grades II through IV GVHD. No such cytotoxic TCL were established in the 8 patients with GVHD grades 0 through 1.

The possibility that cytotoxicity was directed against MiHA⁺ lymphoblastoid cells derived from the respective recipient’s pretransplant PBMC. Under these conditions and from a total of 59 TCL grown, cytotoxic TCL with specificity for host cells were exclusively obtained from those 9 patients presenting with grades II through IV GVHD. No such cytotoxic TCL were established in the 8 patients with GVHD grades 0 through 1. These differences were statistically significant (Table 2, P < .0001). TCL were tested for their capacity to lyse donor or host pretransplant PHA blasts. Lysis of all 9 TCL was restricted to host targets, and was therefore designated MiHA-specific (Table 2, P < .01). The possibility that cytotoxicity was directed against MiHA⁻ lymphoblastoid cells derived from the respective recipient’s pretransplant PBMC. Under these conditions and from a total of 59 TCL grown, cytotoxic TCL with specificity for host cells were exclusively obtained from those 9 patients presenting with grades II through IV GVHD. No such cytotoxic TCL were established in the 8 patients with GVHD grades 0 through 1. These differences were statistically significant (Table 2, P < .0001). TCL were tested for their capacity to lyse donor or host pretransplant PHA blasts. Lysis of all 9 TCL was restricted to host targets, and was therefore designated MiHA-specific (Table 2, P < .01). The possibility that cytotoxicity was directed against MiHA⁺ lymphoblastoid cells derived from the respective recipient’s pretransplant PBMC. Under these conditions and from a total of 59 TCL grown, cytotoxic TCL with specificity for host cells were exclusively obtained from those 9 patients presenting with grades II through IV GVHD. No such cytotoxic TCL were established in the 8 patients with GVHD grades 0 through 1. These differences were statistically significant (Table 2, P < .0001). TCL were tested for their capacity to lyse donor or host pretransplant PHA blasts. Lysis of all 9 TCL was restricted to host targets, and was therefore designated MiHA-specific (Table 2, P < .01). The possibility that cytotoxicity was directed against MiHA⁺ lymphoblastoid cells derived from the respective recipient’s pretransplant PBMC. Under these conditions and from a total of 59 TCL grown, cytotoxic TCL with specificity for host cells were exclusively obtained from those 9 patients presenting with grades II through IV GVHD. No such cytotoxic TCL were established in the 8 patients with GVHD grades 0 through 1. These differences were statistically significant (Table 2, P < .0001). TCL were tested for their capacity to lyse donor or host pretransplant PHA blasts. Lysis of all 9 TCL was restricted to host targets, and was therefore designated MiHA-specific (Table 2, P < .01). The possibility that cytotoxicity was directed against MiHA⁺ lymphoblastoid cells derived from the respective recipient’s pretransplant PBMC. Under these conditions and from a total of 59 TCL grown, cytotoxic TCL with specificity for host cells were exclusively obtained from those 9 patients presenting with grades II through IV GVHD. No such cytotoxic TCL were established in the 8 patients with GVHD grades 0 through 1. These differences were statistically significant (Table 2, P < .0001). TCL were tested for their capacity to lyse donor or host pretransplant PHA blasts. Lysis of all 9 TCL was restricted to host targets, and was therefore designated MiHA-specific (Table 2, P < .01).
Table 2. Functional Characteristics of TCL Grown From the PB of Patients With GVHD Grades 0-1 and Grades II-IV

<table>
<thead>
<tr>
<th></th>
<th>GVHD Grades 0-1</th>
<th>GVHD Grades II-IV</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of TCL</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>No. of TCL with specificity for host cells</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>No. of TCL without specificity for host cells</td>
<td>8</td>
<td>0</td>
<td>P &lt; .0001*</td>
</tr>
</tbody>
</table>

Specific lysis of host PHA blasts (%)

- E:T ratio 25:1:
  - No. of TCL: 1 (0-5), 53 (36-93) (P < .01†)
- E:T ratio 12:1:
  - No. of TCL: 0 (0-2), 50 (24-80) (P < .01†)
- E:T ratio 6:1:
  - No. of TCL: 0 (0-2), 41 (16-71) (P < .01†)
- E:T ratio 3:1:
  - No. of TCL: 0 (0-3), 25 (0-54) (P < .01†)

Spontaneous release (cpm):

- E:T ratio 25:1:
  - 219 (77-777), 288 (191-288)
- E:T ratio 12:1:
  - 2,334 (525-3,906), 2,734 (477-8,948)
- E:T ratio 6:1:
  - 100 (0-0), 100 (0-0)
- E:T ratio 3:1:
  - 693 (336-2,023), 832 (363-2,510)

Specific lysis of donor PHA blasts

- E:T ratio 25:1:
  - 0 (0-7), 2 (0-10)
- E:T ratio 12:1:
  - 0 (0-5), 2 (0-5)
- E:T ratio 6:1:
  - 0 (0-3), 2 (0-7)
- E:T ratio 3:1:
  - 0 (0-4), 1 (0-3)

Spontaneous release (cpm):

- E:T ratio 25:1:
  - 271 (208-1,323), 249 (135-806)
- E:T ratio 12:1:
  - 2,417 (621-4,241), 2,734 (1,002-8,045)
- E:T ratio 6:1:
  - 100 (0-0), 100 (0-0)
- E:T ratio 3:1:
  - 606 (273-1,685), 1,005 (641-1,355)

Values are the median, with minimum and maximum in parentheses.

* X² test.
† Lysis of TCL from PBMC of patients with GVHD grades II-IV versus lysis of TCL from patients with GVHD grades 0-1 (Student’s t-test) and MiHA-specific TCL lysis of host PHA targets versus donor PHA targets (Student’s t-test).

FACS analysis of cytotoxic TCL with specificity for host cells showed a predominant CD8 positivity (79.9%) and only a low percentage (16.9%) of CD4+ cells (Table 3, UPN 53). In contrast, TCL without specificity for host cells were CD4+ (91.8%) and CD8- (Table 3, UPN 58). All TCL expressed the CD3 marker and consistently lacked the CD16 (NK-cell associated) marker (Table 3).

The two TCL UPN 37 and 53 were further cloned, and clones displaying host specificity were expanded for further testing. Host-specific cytotoxic clones also showed a CD3+, CD8+, CD16-, CD4- phenotype (Table 3, UPN 53 clone 7) and the noncytotoxic clones a CD3+, CD8-, CD16+, CD4+ phenotype (Table 3, UPN 53 clone 2).

Expression of MiHA on various hematopoietic and non-hematopoietic host targets. Cytotoxic TCL specific for MiHA were used to screen various hematopoietic and non-hematopoietic cells from the respective hosts for expression of MiHA. Lymphoblastoid cell lines, PHA blasts, leukemic cells, and K of host and of donor origin were used as targets. MiHA, as defined by specific lysis, were detected on both hematopoietic and nonhematopoietic cells, but the amount of target cell killing was variable (Fig 1). Host lymphoblastoid cell lines and PHA blasts were more susceptible to lysis than K and leukemic cells. Expression of MiHA on these targets...
Fig 2. Lysis of various hematopoietic and nonhematopoietic host targets by a clone isolated from PBMC from a patient with GVHD (UPN 37). (●) Lymphoblastoid cell lines, host; (○) PHA blasts, host; (□) leukemia cells, host; (Δ) K, host; (▲) PHA blasts, donor; (■) K + IFN-γ (1,000 U/mL), host.

Class I MHC represents the restricting element of MiHA-specific TCL responses and its expression crucially controls target cell lysis. Published evidence suggests that recognition of MiHA+ targets by TCL is restricted by class I MHC antigens.24,25,38-42 Our MoAb blocking experiments also confirmed these findings (Fig 3). Treatment with MoAb against common determinants of MHC class I but not class II antigens inhibited cytotoxicity. Blocking by MoAb against framework determinants of MHC class I antigens was more efficient than MoAb against polymorphic determinants of HLA-A or HLA-B antigens. Results were further supported in experiments using clones (Fig 4). As shown, cytotoxicity of the clones (from UPN 37) was blocked by anti-MHC class I antigens and anti–HLA-A2 MoAb. In contrast, MoAb against MHC class II were not inhibitory.

Because MiHA-specific TCL responses had been shown to be restricted by class I MHC, the possibility was considered that relative resistance of certain target cells to lysis might be caused by inappropriate expression of the restricting determinants. To test this, quantitative radioimmunometric measurements of class I or class II MHC antigen densities were performed on a panel of hematopoietic and nonhematopoietic targets. Results are shown in Table 4. Quantitative expression of MHC antigens clearly differed among the various cells analyzed, showing high MHC antigen expression on T blasts and lymphoblastoid cell lines and low or absent on leukemic cells and resting K (Table 4). Considering the enormous differences in size of K and PHA blasts or lymphoblastoid cell lines, these differences in MHC class I antigen expression were even more pronounced. After incubation of K with rIFN-γ using doses up to 1,000 U/mL MHC class I antigen, expression values increased in a dose-dependent manner. As discussed in the previous paragraph, there was a close correlation between the amount of MHC class I antigen expression on different targets and their susceptibility to lysis (Figs 1 and 2).

Further evidence for class I MHC antigens as restricting elements of MiHA recognition by TCL was obtained in studies using PHA blasts from a panel of 57 unrelated donors. Results are demonstrated in Fig 5; no specific lysis was seen with donors who failed to share at least one HLA-A or -B antigen with the TCL effector line. On the other hand, PHA blasts sharing HLA-A24 (15%), HLA-A31 (67%), HLA-B44 (55%), or HLA-B5 (38%) were all susceptible in a portion...
of the test population. No evidence was obtained for restriction of the MiHA-specific TCL response in this panel for a single HLA specificity. This was different in patient UPN 37, in whom the haplotypes HLA-A3, B5 and HLA-A2, B7 were involved. Testing of PHA blasts of 19 unrelated donors with clones derived from this patient strongly suggest that this MiHA-specific TCL response is restricted by HLA-A2 (Fig. 6).

By its capacity to enhance class I MHC antigen expression IFN-γ renders K targets susceptible to lysis by MiHA-specific TCL. As discussed in the previous paragraph, K representing the natural targets for GVHD exhibited a low constitutive expression of class I MHC antigens and also display relative resistance to lysis by MiHA-specific cytotoxic TCL. We tested whether preexposure of K targets to IFN-γ would render them susceptible to lysis by TCL. Results shown in Fig 7 indicate that IFN-γ, but not IFN-αα, renders these cells susceptible to lysis by MiHA-specific TCL. Lysis of IFN-γ preincubated cells was again inhibited by anti-class I MHC MoAb (data not shown), demonstrating the crucial role of increased MHC class I antigen expression. To exclude that lysis of K exposed to IFN-γ might be due to a toxic effect on K, natural killing experiments were performed. No difference in susceptibility to lysis by NK cells between IFN-γ-treated or untreated K was found (Table 5).

MiHA are expressed on host leukemic cells. In 3 patients suffering from CML, cytotoxic TCL specific for MiHA were used to test the presence of these antigens on host leukemic cells. As shown in Fig 1, leukemic cells were quite resistant to the lytic attack of TCL. This effect was studied in more detail in a chromium release assay and leukemia colony inhibition test, using clones from patient UPN 37. In the Cr release assay, leukemic target cells were recognized by clones (specific lysis, 40%; E:T, 25:1; see Fig 2). The presence of MiHA on host leukemic cells was also confirmed by the leukemia colony inhibition assay. Growth of the patient’s tumor cell colonies was drastically inhibited by the clone isolated during GVHD. This effect was host leukemia-specific, because growth of third-party, HLA-mismatched leukemia cells was not affected by the clone (Fig 8).

**DISCUSSION**

We demonstrate here that (1) host-specific cytotoxic T lymphocytes are detected exclusively in the PB of patients with acute GVHD after allogeneic BMT between HLA-identical siblings and (2) their target antigens are simultaneously expressed on several host cell lines, including lymphoblastoid cell lines, PHA blasts, leukemic cells, and K.

Our data, which indicate that successful establishment of MiHA-specific TCL is restricted to patients with extensive

### Table 4. Radioimmunometric Measurement of MHC Class I and Class II Antigen Densities on Various Hematopoietic and Nonhematopoietic Targets (n = 4; cpm means ± SE)

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Pretreatment</th>
<th>MHC Class I</th>
<th>MHC Class II</th>
<th>Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoblastoid cell lines</td>
<td>—</td>
<td>6,784 ± 664</td>
<td>7,918 ± 901</td>
<td>337 ± 36</td>
</tr>
<tr>
<td>PHA blasts</td>
<td>—</td>
<td>10,396 ± 3,538</td>
<td>5,166 ± 1,288</td>
<td>263 ± 65</td>
</tr>
<tr>
<td>Leukemic cells</td>
<td>—</td>
<td>2,910 ± 826</td>
<td>1,232 ± 118</td>
<td>276 ± 25</td>
</tr>
<tr>
<td>K</td>
<td>—</td>
<td>4,391 ± 930</td>
<td>395 ± 131</td>
<td>259 ± 84</td>
</tr>
<tr>
<td>K IFN-γ (10 U/mL)</td>
<td>—</td>
<td>5,293 ± 1,299</td>
<td>403 ± 122</td>
<td>198 ± 22</td>
</tr>
<tr>
<td>K IFN-γ (100 U/mL)</td>
<td>—</td>
<td>7,062 ± 1,872</td>
<td>1,656 ± 742</td>
<td>208 ± 41</td>
</tr>
<tr>
<td>Leukemic cells</td>
<td>—</td>
<td>2,910 ± 826</td>
<td>1,232 ± 118</td>
<td>276 ± 25</td>
</tr>
<tr>
<td>K IFN-γ (1,000 U/mL)</td>
<td>—</td>
<td>9,007 ± 2,104</td>
<td>5,723 ± 2,405</td>
<td>332 ± 136</td>
</tr>
</tbody>
</table>

Fig 5. Lysis of PHA blasts from 57 unrelated donors differing in degree of HLA-matching by an MiHA-specific TCL from patient UPN 14.

Fig 6. Lysis of PHA blasts from 19 unrelated donors differing in degree of HLA-matching by a clone (UPN 37).
GVHD, contradict previous reports by Van Els et al., but are supported by our previous experiments in which the frequency of MiHA-specific cytotoxic T cells (CTL-p) was assessed by limiting dilution analyses (LDA) subsequent to allogeneic BMT.44 We observed that MiHA-specific CTL-p frequencies were undetectable in recipients of HLA-identical BMT lacking clinical signs of extensive GVHD. However, high frequencies were detected in all 3 patients suffering from grades II through IV GVHD.44 LDA of MiHA-specific CTL-p were also performed in 8 of the cases studied here. Four of them neither developed extensive GVHD nor gave rise to MiHA-specific cytolytic TCL and also displayed MiHA-specific CTL-p frequencies of less than 1/300,000. Of the other 4 patients, two developed grade II GVHD, gave rise to establishment of MiHA-specific weakly cytolytic TCL, but also exhibited MiHA-specific CTL-p frequencies below the detection limit. The last 2 patients presented with grade III GVHD, gave rise to highly cytolytic MiHA-specific TCL and clones, and displayed very high frequencies of MiHA-specific CTL-p of 1/13,833 and 1/7,933, respectively. In both these cases, CTL-p frequencies were below the detection limit before onset of GVHD. Taken together, our data support the view that extensive GVHD is accompanied by massive enlargement of the MiHA-specific CTL-p pool and that this favors the establishment of specific TCL or clones. The contradiction between the present results and those presented by Van Els et al.43 might be explained by different detection methods and immunosuppression used in the prophylaxis of GVHD. Furthermore, timing might play an important role in isolating TCL. In patient UPN 49, TCL were isolated exclusively during GVHD, and not 10 days after GVHD had been successfully treated with high-dose methylprednisolone.

We further investigated the presence of MiHA on different host tissues and the role of the density of the restricting class I MHC elements. The presence of MiHA on hematopoietic cells and K has already been reported by Goulmy and others.19,22,23,38 We found that MiHA recognized by donor antihost TCL are simultaneously expressed on host lymphoblasts, on host K, and in one case on host leukemia cells and therefore not restricted to a single cell lineage. The presence of MiHA on cells of different tissues, however, did not result in an equal susceptibility of these cells to TCL. The amount of cell killing varied among target cells and paralleled the constitutive expression of the restricting class I MHC antigens.

We now expand previous information in mice45 by demonstrating that, at least in part, differences in the surface density of the restricting class I MHC determinants between hematopoietic and epithelial targets account for this unexpected and so-far unexplained relative resistance of epithelial cells. Data presented here with MiHA-specific class I MHC-restricted TCL thus confirm our previous findings of relative resistance of MHC-disparate K to lysis by MHC-specific TCL.46 In this case, too, pretreatment of K with IFN-γ enhanced their susceptibility to MHC-specific TCL, and their response was also inhibited by anti-class I MHC antibodies.46

The view of the crucial role of class I MHC expression on GVHD target tissues and of IFN-γ regulating this key parameter is also supported by recent in vitro and in vivo find-

### Table 5. IFN-γ Pretreated and Untreated K Are Equally Susceptible to NK Lysis (n = 4; means ± SE)

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Pretreatment</th>
<th>Specific Lysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>—</td>
<td>12.75 ± 3.28</td>
</tr>
<tr>
<td>K</td>
<td>IFN-γ (1,000 U/mL)</td>
<td>14.00 ± 4.92</td>
</tr>
<tr>
<td>K 562</td>
<td>—</td>
<td>50.00 ± 7.44</td>
</tr>
</tbody>
</table>

*Fig 7. Lysis of native or cytokine-treated K by an MiHA-specific cytotoxic TCL isolated from four patients with GVHD (mean ± SE). Lysis of donor K was negative (data not shown). (○) Untreated K, host; (●) K + IFN-γ (1,000 U/mL), host; (□) K + IFN-α (1,000 U/mL), host; (*) versus K IFN-γ P < .04.

*Fig 8. Inhibition of leukemic colony formation from the host (■) but not from HLA-mismatched third-party leukemia (□) by a clone.*
ings. IFN-γ and tumor necrosis factor-α (TNF-α) have been shown to be directly involved in GVH reactions using an in vitro human skin explant model. In vivo, endogenous IFN-γ and TNF-α levels are elevated before and during clinical manifestation of GVHD. IFN-γ is known to enhance endogenous expression of IFN-γ. Neutrophil antibodies to TNF-α or IFN-γ were shown to prevent or modify GVHD. Increased serum levels of both the nonpolymorphic and the polymorphic chain of IL-2 receptor-B, indicating enhanced biosynthesis, were demonstrated to proceed and accompany GVHD.5,23

Understanding the role of IFN-γ in the pathomechanisms of GVHD has several implications. First of all, it provides an explanation for the frequent association between infections and GVHD.5,11,14,23 Infection triggers the release of cytokines, which in turn can enhance alloreactivity by increasing MHC class I antigen expression on target cells.54 In addition, the role of IFN-γ might explain tolerance mechanisms at target level and tissue specificity of GVHD. Local production of IFN-γ might induce local GVHD and certain tissues are less inducible to express restricting MHC determinants. This analysis also provides a rationale for using cytokine modulation to prevent GVHD.

Leukemic cells have previously been shown to be susceptible to lysis mediated by MiHA-specific cytotoxic T lymphocytes.6 However, in our analysis we found that a clone isolated from a patient after BMT recognized leukemic cells, K, and lymphoblastoid cells from the host. Although our search for graft-versus-leukemia effects mediated by an MiHA-specific clone is so far limited to one case, the data presented clearly demonstrate that clones that lyse K only after pretreatment with IFN-γ effectively kill and inhibit growth of native host leukemic cells. This raises the hope that GVHD and antileukemic resistance can be differentiated on the basis of their different cytokine requirements. Obviously, much more data are required to assess the relevance of this observation. Such studies are now in progress at our institution.

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