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Specific Recognition of Human Leukemic Cells by Allogeneic T Cells: II. Evidence for HLA-D Restricted Determinants on Leukemic Cells That Are Crossreactive With Determinants Present on Unrelated Nonleukemic Cells

By Jeffrey A. Sosman, Kurt R. Oettel, Stephen D. Smith, Jacquelyn A. Hank, Paul Fisch, and Paul M. Sondel

Transplantation of immunocompetent cells present within allogeneic bone marrow has been associated with the elimination of residual host leukemia, both in animal tumor models and in patients receiving marrow transplants for leukemia. This observation has been called the "graft-versus-leukemia effect." We have attempted to study this phenomenon in vitro by characterizing the cytolytic response of T cells from normal donors after in vitro activation with allogeneic leukemic cells. As expected, most T cells that react against an allogeneic patient's leukemic cells recognize their foreign HLA antigens and lyse the patient's nonleukemic remission lymphoid cells. In addition, we have shown that a small fraction of the T cells recognize and lyse foreign leukemic targets without lysis of nonmalignant remission targets from the same leukemic patient. These T cells have been isolated and characterized as CD3+, CD4+ cells expressing the α/β T cell receptor (TCR). Their lysis appears to reflect specific antigen recognition mediated via the CD3-TCR complex and interactions involving the CD4 receptor. Some of these "leukemic specific" T cell lines, which are restricted by HLA class II molecules, can also lyse occasional nonleukemic cells from certain unrelated donors. This recognition appears to involve crossreactive determinants shared by the leukemic cells and the unrelated allogeneic nonleukemic cells. These specific interactions may represent an in vitro model of the graft-versus-leukemia effect.

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A LLGENEIC BONE MARROW grafting with immunocompetent donor cells can suppress the growth of leukemic cells, a phenomenon known as the "graft-versus-leukemia (GVL) effect." This antileukemic effect has been documented in both experimental tumor models and retrospective analyses of human clinical bone marrow transplants. The mechanism underlying this phenomenon and its relationship to graft-versus-host disease (GVHD) is poorly understood. The immune mechanisms responsible for the GVL effect may involve any one of the following: (1) alloreactive donor T cells recognizing foreign major histocompatibility complex (MHC) antigens on host normal and neoplastic tissue; (2) induction of non-MHC-restricted lymphokine-activated killer (LAK) cells; (3) release of cytokines with antitumor effects; or (4) allogeneic donor T cells recognizing antigens uniquely or selectively presented on host's leukemic blasts.

Several decades of research have focused on the identification of "tumor-specific antigens" speculated to be present on human tumor cells and that could be recognized by a patient's own T cells. Numerous experimental tumor models in laboratory animals apparently express such tumor-specific antigens that are recognized by antigen-specific syngeneic T cells. Some of these tumor antigens have been associated with oncornoviral encoded peptides. Nevertheless, at the present time there are only a few examples documenting T cells mediating antigen-specific (MHC-restricted) responses to autologous human tumor. For example, some patients bearing malignant melanoma show MHC-restricted T cell responses to their autologous tumor. Comparable analyses of other types of tumors have not documented analogous T-cell specificity.

The relative inability to identify autologous tumor-specific T cell responses for most human tumors has multiple explanations. One explanation is that human tumors may express potentially immunogenic tumor-associated antigens, but the cancer patient may have an acquired or inherited immune response defect preventing the recognition of these antigens. If this latter possibility is the case, then the demonstration of such tumor-associated antigens might require T cells from healthy allogeneic donors in order to detect their presence.

We have explored the capacity of allogeneic T cells to recognize differences between normal lymphoid cells (or Epstein-Barr virus [EBV]-transformed lines) and leukemic lymphoid cells. Matzinger and Bevan have previously speculated that the relative high frequency of cells capable of mediating allospecific responses reflected a summation of many different clonal responses to allogeneic MHC antigens presenting a variety of self peptides. Recently, others have published data supporting this concept. We hypothesize that certain T cells present in a donor repertoire may recognize alloanitgigen plus peptides that are preferentially expressed on the leukemic population compared with the autologous non-malignant lymphoid cells. Furthermore, in animal models, malignancies have been identified that appear to express altered or "alien" histocompatibility antigens. If T cells with this specificity exist, then they may play a role in mediating the specific GVL effect.

Our previous studies have documented the isolation of several allogeneic T cell lines that specifically lyse leukemic cells but do not lyse the same patient's remission lymphocytes. Inadequate numbers of leukemic cells prevented the further characterization of the leukemic reactive
T cells. The experiments presented here confirm and extend these preliminary findings using cryopreserved leukemic cells, non-transformed lymphoid leukemic cell lines, and EBV-transformed remission B cells from the same patient as stimulator and target cells. In this report, we have isolated cloned T cell lines with specificity for allogeneic leukemic targets. We have shown that their recognition of leukemic targets involves the T cell receptor-CD3 complex and CD4 accessory molecules on the effector cells and MHC class II antigens on the leukemic cell. Some of these cloned T cell lines can also lyse occasional non-leukemic cells from unrelated donors, apparently via the recognition of crossreactive determinants shared by both target cell populations and restricted by MHC class II antigens. The pattern of crossreactivity was different for the various cytotoxic T lymphocyte (CTL) lines, suggesting that a variety of epitopes may be recognized on the leukemic cells, or different MHC determinants are presenting the same epitope. While the further characterization of the molecules on the leukemic cells recognized by these effector cells may be important, we believe that documenting the existence of normal allogeneic T cells, which can differentiate leukemic from normal lymphoid cells using their T cell receptor, has important implications for tumor immunity and immunotherapy.7,16

METHODS

Lymphocytes and bone marrow cells. Peripheral blood mononuclear cells (PBMC) from healthy volunteer donors were obtained by Ficoll-Hypaque sedimentation of anticoagulated whole blood as previously described.17 Heparinized bone marrow aspirates were obtained from leukemic patients and purified on a Ficoll-Hypaque gradient. Some PBMC and leukemic samples were cryopreserved in 10% dimethyl sulfoxide by controlled rate freezing and stored in liquid nitrogen for later use.19

Generation of leukemic and lymphoblastoid cell lines. Leukemic cell lines were generated by procedures previously reported.39,40 Briefly, patient samples were collected in preservative-free heparin, separated on a Ficoll-Hypaque gradient, and washed with McCoy’s 5A media. Cells were initially plated in agar as a single cell suspension and grown at 37°C in a Heraeus incubator gassed with 5% O2, 6% CO2, and 89% N2. Cells were fed fresh media and transferred to new plates after 2 to 4 weeks and transferred into a flask after 3 to 6 weeks in culture. After steady-state growth was maintained in suspension cultures, the cells were gradually weaned to a growth environment without added mitogens, thiols, conditioned media, or interleukins. The cell line SUP-B26 (AT-leuk) was established from the spinal fluid of a patient with acute lymphoblastic leukemia at first relapse. The SUP-B28 cell (AT-2) was established from the same patient (AT) when he subsequently relapsed in the bone marrow. Cell lines SUP-B2 (EL), SUP-B16 (ER-1), SUP-B19 (ER-2), SUP-B24 (MM), SUP-B27 (KD), and SUP-B7 (ST) were all derived from children with acute lymphoblastic leukemia (common type and pre-B cell type). The SUP-B12 cell line was established from a patient with a Burkitt’s lymphoma. There is general agreement between established cell lines and the patient’s tumor cells based on morphology, cytochemical staining pattern, immunophenotype, immunogenotype, and karyotype.41 The SUP-B30 (AT-LCL) cell line is a lymphoblastoid cell line (EBV positive) obtained from the patient AT from which cell line SUP-B26 and SUP-B28 were established using previously described methods.41

Other lymphoblastoid cell lines (LCL) were generated by incubating PBMC with an EBV-containing supernatant as previously described.43 The natural killer (NK) sensitive leukemic cell line, K562,44 the Daudi cell line, a relatively NK-resistant Burkitt lymphoma,45 and the Raji cell line were obtained through the American Type Culture Collection (ATCC; Rockville, MD). All cell lines were maintained in vitro by standard methods.

Monoclonal antibodies for phenotyping and blocking experiments. Monoclonal antibodies (MoAbs) reactive against CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2b), HLA-DR (monomorphic), HLA-DQ (monomorphic), T cell receptor (TCR) α/β (WT31), and Leu-19 (NKH-1) were purchased from Becton-Dickinson (Mountain View, CA). MoAb reactive against TCR-delta (TCR-d) was obtained from T-Cell Science (Cambridge, MA).46 Anti–HLA-class I (monomorphic) (W6/32) was obtained from a hybridoma (obtained from ATCC, Rockville, MD) and used as a culture supernatant. Anti–HLA-DP (B7.21) was obtained from purified hybridoma ascites as a gift from Nancy Robinson (Minneapolis, MN).47 For phenotyping, indirect immunofluorescence was done with the MoAb and developed with fluorescein-labeled goat anti-mouse immunoglobulin (Couleur, Hialeah, FL). Ten thousand cells were analyzed for each marker on an Ortho Cytofluorograph-Systems Model 50 flow cytometer (Ortho, Raritan, NJ). For cytolytic blocking experiments, MoAbs were added to effectors (anti-CD3, anti-CD4, anti-CD8, IgG1, control) or to 111Cr-labeled targets (anti-class I, anti-DP, anti-DQ, anti-DR, IgG1, control, and IgG,A control) 30 minutes before assay at 4°C. All antibodies were used at saturating conditions (unless indicated otherwise in the text or legend) as follows: anti-CD3 (IgG1) at 1:200 final dilution; anti-CD8 (IgG1) at 1:200; anti-CD4 (IgG1) at 1:200; anti-class I (HLA-A,B,C) (IgG,A) at 1:4; anti-DR (IgG1) at 1:100; anti-DQ (IgG1) at 1:20, anti-DR (IgG,A) at 1:20; mIgG1, control at 1:100; and mIgG,A control at 1:100. All antibodies remained in culture throughout the cytotoxicity assay.

In vitro allosensitization and derivation of allogeneic CTL lines and clones. In vitro activation was performed using a modification of previously described methods.27 PBMC were diluted in tissue culture medium: RPMI 1640 supplemented with 24 mmol/L HEPES buffer, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Flow Laboratories, McLean, VA), and 10% heat-inactivated AB human serum (Pel-Freeze, Brown Deer, WI); this medium is designated HS-RPMI. All allosensitization was carried out for 6 days in 25 cm2 flasks (Costar, Cambridge, MA) containing responder cells at 1 × 106/mL and irradiated (6,000 cGy) allogeneic leukemic cell lines cells at 1 × 104/mL or irradiated (10,000 cGy) allogeneic LCL at 1 × 104/mL in a total volume of 10 to 15 mL.

Viable cells were then harvested and plated at 1 or 10 cells per well in 96-microwell round bottom plates (Costar, Cambridge, MA) with irradiated AT-Leuk (5 × 104 per well), irradiated autologous PBMC (5 × 104 per well) and interleukin-2 (HL-2; 100 U/mL). Microwell plates were incubated for 2 to 6 weeks at 37°C and observed for macroscopic cell growth in individual wells. Wells positive for growth were expanded and screened for lytic activity by a standard 3Cr release assay. Subsequently, cells lines of interest were subcloned by the same technique at 0.3 cells per well.

Maintenance of cytolytic cell lines. Cell lines were cultured in 96-round bottom well tissue culture plates (Costar) in HS-RPMI. Cell lines were split and expanded weekly with 5 × 104 irradiated leukemic cells per well, 5 × 104 irradiated autologous PBMC, and IL-2 (50 U/mL) (Hoffman-LaRoche, Nutley, NJ). These cell lines have remained in culture for up to 6 months.

Proliferative assay. Responding cells were placed in tissue culture media in 96-round bottom microwell plates in a volume of 0.2 mL. T cell lines were cultured at 1.0 to 5.0 × 104 cells per well for 48 to 72 hours with the respective stimulators. All cultures were set up in triplicate. Stimulating cells were irradiated before culture (6,000
cGy for AT-Leuk; 10,000 cGy for LCL lines). The cultures were pulsed by adding 1 μCi of "H-labeled methyl thymidine ("H-TdR) (New England Nuclear, Boston, MA) 12 to 24 hours before the termination of the culture. Radiolabeled cells were harvested onto filter discs with a MASH device (Otto Hiller, Madison, WI), and "H-TdR incorporation was quantitated by liquid scintillation counting. Data are expressed as mean counts per minute (cpm) ± SD.

Cell-mediated lysis. Target cells (including cell lines and freshly cryopreserved leukemic blasts) were labeled with 250 μCi 51Cr for 1.5 to 2 hours at 37°C. Targets were then washed and resuspended in media. Effector cells were diluted to three appropriate effector-target (E:T) ratios and placed in 0.1 mL of media in 96-microwell round bottom plates in triplicate. Target cells (5 x 10^4 cells in 0.1 mL media) were added to the wells. Plates were centrifuged at 200g for 5 minutes and incubated at 37°C in 5% CO₂ for 4 hours. After incubation, plates were again centrifuged at 500g for 10 minutes and harvested with a Skatron harvesting system (Sterling, VA), and 51Cr was counted in a gamma counter. The percentage cytotoxicity was calculated by using the formula:

\[
\text{% Cytotoxicity} = \frac{\text{Test cpm} - \text{Spontaneous cpm}}{\text{Maximal cpm} - \text{Spontaneous cpm}} \times 100
\]

Spontaneous and maximum release values were determined by incubating target cells in media or cetrimide detergent (Sigma Chemical, St Louis, MO), respectively.

In some analyses, percent cytotoxicity values from some three E:T ratios were converted to E:U ratios. Only one lytic unit (LU) was defined for these studies as the number of effectors resulting in 20% lysis of 5 x 10^4 target cells. Lytic units are expressed as LU/10^3 cells. In some experiments, effectors of the presence of MoAbs on cytotoxicity are assayed; effector or target cells were incubated with the MoAbs at 4°C for 30 minutes before assay. MoAbs were present in microwells for the duration of the assay.

Competitive inhibition studies. For cold target competitive studies, unlabeled target cells were serially diluted to achieve various ratios of unlabeled (cold):labeled target cells as previously described. Effector cells followed by unlabeled target cells, followed by 51Cr-labeled target cells (5,000 cells) were serially added to each well for the 4-hour incubation.

Limiting dilution assay of precursor frequency. The limiting dilution assay (LDA) method used to assess frequency of precursor cytotoxic lymphocytes was essentially as described by MacDonald et al., and described previously in detail. Responding cells were stimulated with allogeneic leukemic or LCL cell lines in 96-microwell plates in the presence of IL-2 and autologous feeder cells for 8 days and then tested for cytotoxicity against the Daudi cell line, the leukemic, and the LCL cell lines.

RESULTS

In vitro stimulation with an allogeneic leukemic cell line and subsequent cloning by limiting dilution to isolate cytolytic cells with specificity for the allogeneic leukemia. A leukemic cell line designated AT-leuk was originally derived from a central nervous system recurrence of an 8-year-old male with acute lymphoblastic leukemia (ALL). These ALL cells displayed L-1 morphology and expressed both the common ALL antigen (CALLA) and HLA-DR antigens, as did the AT-leuk line. The cell line was derived under hypoxic conditions as previously described. Experiments presented in this report were initiated approximately 6 months after the establishment of the leukemic cell line. Remission peripheral blood from this same patient was previously obtained and an EBV-transformed cell line was generated from the remission B cells. This line was designated AT-LCL. In addition, a small number of cryopreserved leukemic blasts were available for select experiments. These cells came from a bone marrow aspirate obtained at relapse that contained greater than 80% blasts by morphology. Serologic typing for HLA phenotype showed both AT-leuk and AT-LCL lines were identical and were HLA-A3,24, B8,25 (60), Bw6, DR5,6,DRw52,DQw2,3. HLA-DP typing was not performed. Initially, bulk cultures were established in which fresh responding PBMC from a normal unrelated volunteer donor KO (HLA-A3,23 B27,44,B4, C2,4 DR2,7,DRw53,DQw1) were stimulated with irradiated AT-leuk, AT-LCL, other leukemic cell lines (see Methods), or IL-2 (200 U/mL). After 6 days in culture, cells were harvested and tested in a cell mediated lysis (CML) assay against a variety of targets. As shown in Fig 1, cultures stimulated with either AT-leuk or AT-LCL lysed both allogeneic targets (AT-leuk or AT-LCL). KO PBMC stimulated with unrelated leukemic lines or IL-2 mediated only minimal lysis against AT-LCL or AT-Leuk compared with those cultures stimulated with the AT lines (Fig 1 and data not shown). These data suggest that the majority of effector cells present after in vitro priming did not preferentially lyse AT-leuk compared with AT-LCL. This was also supported by LDA experiments; most precursors that generated lytic activity against AT-leuk were also able to lyse AT-LCL (data not shown). This was previously demonstrated for allogeneic T cell responses to fresh leukemic cells.

The limiting dilution technique was used to isolate cells that were cytolytic for the AT-leuk cell line. In this approach, responding cells from donor KO were first primed in culture to the allogeneic leukemia (AT-leuk) before cloning. All microwells showing evidence of macroscopic cell growth after 2 to 6 weeks in vitro were expanded further and then screened in triplicate for lytic activity against a limited panel of targets in a standard CML assay (Table 1). Lytic activity was considered present (+) in this initial screening if specific lysis of the target was greater than 10%. Of the 135 cultures analyzed, 91 (55 + 19 + 9 at 10 cells per well and 4 + 1 + 0 at 1 cell per well) lysed the AT-leuk target and 12 (9 plated initially at 10 cells per well and 3 plated initially at 1 cell per well) demonstrated lytic activity for the leukemia, but not Daudi or the AT-LCL line on the initial screening (Table 1). Cultures that demonstrated specificity in their lysis for the leukemia were expanded for further characterization and subcloned at 0.3 cell per well whenever possible. Other T cell lines reactive with both AT-leuk and AT-LCL were also expanded as alloreactive controls.

Initial characterization of T cell lines and clones with specificity for allogeneic leukemia. Several of the cell lines shown in Table 1 that demonstrated lysis of the leukemic target (AT-leuk) were further expanded, subcloned, and repeatedly rescreened for lysis of AT-leuk and AT-LCL. In Table 2 a representative experiment showing the results obtained with some of these cell lines and their subclones is presented. Some T cell lines lysed the leukemic target to a much greater degree than the remission LCL cells; these are designated "allo-leuk reactive" lines. In contrast, "allo-
reactive" cell lines lysed both leukemia and LCL targets to a similar degree. Furthermore, the freshly isolated cryopreserved leukemic cells were generally lysed by most of the alloreactive and allo-leuk reactive cell lines shown in Table 2; only lines 10-120 and 1-12 were not able to significantly lyse the fresh leukemic cells. The maximum lysis of the fresh leukemic cells by the allo-leuk reactive T cells was usually less than that of the leukemic line (AT-leuk), but was far above the minimal lysis of AT-LCL mediated by the allo-leuk reactive lines.

The phenotypic characterization of these cytolytic lines is also shown in Table 2. As expected, they marked as CD3+, but unexpectedly, all of the allo-leuk reactive lines were also CD4+. Several lines (10-17 and 10-31) were examined for expression of WT31 (TCR α/β chains), TCR-δ chains, and Leu-19 (a marker on NK and activated T cells). Both lines 10-17 and 10-31 demonstrated 100% of cells positive for WT31, no cells positive for TCR-δ, and variable degrees of staining for the Leu-19 marker (20% to 40%). To better characterize the specificity of the lysis mediated by these allo-leuk reactive CTL lines, we next determined their pattern of lysis against a panel of allogeneic leukemia and EBV-transformed B cell lines.

Specificity of lytic activity mediated by allo-leuk reactive CTL. Several targets were selected to be tested in a standard CML assay using the allogeneic CTL lines and clones as effectors. In addition to AT-leuk, AT-LCL, and cryopreserved fresh leukemic blasts, target cells included: (1) AT-2, a separately derived leukemic cell line from the same patient (AT); (2) allogeneic CALL+ and CALL− leukemic cell lines derived similarly to AT-leuk, but obtained from seven separate unrelated patients; (3) hematopoietic cell lines sensitive to NK or LAK lysis (Daudi, K562, and Raji); and (4) a large number of HLA-typed LCL cells from unrelated donors. Results from these experiments are presented in Table 3. All five allo-leuk reactive CTL lines tested lysed the AT-2 leukemic line from the same patient, further demonstrating that the lysis by the allo-leuk reactive CTL was not restricted to a unique determinant found only on the one AT-leuk line that may have been induced by in vitro culture. No significant lysis was observed against the other allogeneic lymphoblastic leukemia target cell lines (CALL+).

**Table 1. Cloning by Limiting Dilution**

<table>
<thead>
<tr>
<th>Cells/Well as Initially Plated</th>
<th>Wells Showing Growth</th>
<th>Wells Screened for Cytotoxicity</th>
<th>No. Wells Showing Lysis of AT-LCL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nonlytic</td>
</tr>
<tr>
<td>10</td>
<td>123/180</td>
<td>116</td>
<td>31</td>
</tr>
<tr>
<td>1</td>
<td>21/180</td>
<td>19</td>
<td>11</td>
</tr>
</tbody>
</table>

Primed cells derived from a bulk culture of KO + AT-leuk were harvested and cloned at 10 and 1 cell per well in the presence of IL-2, AT-leuk, and KO PBMC. At each responding cell concentration, 180 wells were tested.

*Expanded wells were tested in a standard CML assay in triplicate wells at two different E:T ratios to 2 to 6 weeks. Initially, E:T ratios were not determined because of low cell yields. All wells were screened against AT-LCL, AT-leuk, and Daudi cell lines. Numbers designate the number of wells showing significant lysis against the targets listed in each column. Lysis was considered significant if there was greater than 10% specific lysis. Some of the wells that showed specific lysis of only AT-leuk did not repeat this pattern on further analysis. Ultimately, 4 of the initial 12 cultures that appeared specific for AT-leuk were expanded and retained the same pattern of lysis. No wells lysed only AT-LCL on the initial analysis.
Table 2. Characterization of Allo-Activated Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Phenotype*</th>
<th>AT-Leuk Lysis (LU)†</th>
<th>AT-LCL Lysis (LU)</th>
<th>Fresh Leuk§ Lysis (LU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allo-leuk reactive lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-20</td>
<td>CD3, CD4</td>
<td>590</td>
<td>33</td>
<td>175</td>
</tr>
<tr>
<td>10-17</td>
<td>CD3, CD4, WT31</td>
<td>393</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>10-17.21</td>
<td>CD3, CD4</td>
<td>234</td>
<td>18</td>
<td>120</td>
</tr>
<tr>
<td>10-31</td>
<td>CD3, CD4, WT31</td>
<td>179</td>
<td>38</td>
<td>140</td>
</tr>
<tr>
<td>10-31.2</td>
<td>CD3, CD4</td>
<td>227</td>
<td>38</td>
<td>170</td>
</tr>
<tr>
<td>10-120</td>
<td>CD3, CD4</td>
<td>173</td>
<td>31</td>
<td>19</td>
</tr>
<tr>
<td>Alloreactive lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-12</td>
<td>CD3, CD4, 10% CD8</td>
<td>521</td>
<td>746</td>
<td>33</td>
</tr>
<tr>
<td>1-17</td>
<td>CD3, 80% CD8</td>
<td>610</td>
<td>390</td>
<td>174</td>
</tr>
<tr>
<td>10-51</td>
<td>CD3, CD4</td>
<td>311</td>
<td>144</td>
<td>124</td>
</tr>
<tr>
<td>Primary cultures§</td>
<td>KO + AT-LCL</td>
<td>—</td>
<td>197</td>
<td>542</td>
</tr>
<tr>
<td>KO + AT-leuk</td>
<td>—</td>
<td>283</td>
<td>263</td>
<td></td>
</tr>
</tbody>
</table>

Cell lines were all derived by limiting dilution. Data expressed are from a representative experiment of the 3 to 15 separate assays performed on the different targets with these effectors.

*Surface phenotype was characterized by direct and indirect immunofluorescence and flow cytometric analysis. Unless otherwise indicated, the phenotype designations shown indicate that greater than 90% of the cells tested were positive.

†Lysis is defined as lytic units (LU) per 10⁶ effector cells calculated from three E:T ratios.

§Fresh leuk (AT) represents cryopreserved leukemic blasts obtained from a bone marrow aspirate at relapse that contained greater than 80% blasts by morphology.

Table 3. Specificity of Lytic Activity of Allo-Leuk Reactive Lines

<table>
<thead>
<tr>
<th>Targets</th>
<th>Cytotoxicity by T Cell Lines*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-20</td>
</tr>
<tr>
<td>AT-leuk line</td>
<td>++++</td>
</tr>
<tr>
<td>AT-leuk fresh</td>
<td>++++</td>
</tr>
<tr>
<td>AT-LCL</td>
<td>+</td>
</tr>
<tr>
<td>AT-2 line</td>
<td>++++</td>
</tr>
<tr>
<td>Leukemic lines</td>
<td></td>
</tr>
<tr>
<td>(Pre-B) leukemic lines (2)</td>
<td>NT</td>
</tr>
<tr>
<td>CALLA leukemic lines (4)</td>
<td>—</td>
</tr>
<tr>
<td>Burkitt cell line</td>
<td>—</td>
</tr>
<tr>
<td>Hematopoietic cell lines</td>
<td></td>
</tr>
<tr>
<td>Daudi</td>
<td>—</td>
</tr>
<tr>
<td>KB62</td>
<td>—</td>
</tr>
<tr>
<td>Raji</td>
<td>NT</td>
</tr>
<tr>
<td>HL-A-D Typd LCL †</td>
<td>4-LCL DR5</td>
</tr>
<tr>
<td>5-LCL DR2</td>
<td>++</td>
</tr>
<tr>
<td>6-LCL DR7, w9</td>
<td>—</td>
</tr>
<tr>
<td>56-LCL DR2</td>
<td>—</td>
</tr>
<tr>
<td>75-LCL DR1</td>
<td>—</td>
</tr>
<tr>
<td>76-LCL DR6</td>
<td>++++</td>
</tr>
<tr>
<td>102-LCL DR8</td>
<td>—</td>
</tr>
<tr>
<td>105-LCL DR7</td>
<td>—</td>
</tr>
<tr>
<td>157-LCL DR7</td>
<td>—</td>
</tr>
<tr>
<td>PS-LCL DR5,6</td>
<td>—</td>
</tr>
<tr>
<td>106-LCL DR7</td>
<td>NT</td>
</tr>
<tr>
<td>125-LCL DR2</td>
<td>NT</td>
</tr>
<tr>
<td>131-LCL DR8</td>
<td>NT</td>
</tr>
<tr>
<td>BM-LCL DR2,4</td>
<td>—</td>
</tr>
</tbody>
</table>

In all experiments shown in this table the T cell lines were tested in a standard 4 hour CML assay with three E:T ratios with the highest ratio being between 20 and 30:1.

Abbreviation: NT, not tested.

*Cytotoxicity by the T cell lines represents the maximal specific lysis at the highest E:T ratio (20 to 30:1) tested with the following symbols: —, less than 10%; ++, 10% to 20%; +++, 20% to 30%; ++++, 30% to 40%; +++++, greater than 40%.

†All LCL lines were specifically lysed by control allospecific cultures or lines at 21% to 65% cytotoxicity (data not shown).
or pre-B cell), one of which expressed the same DR typing as AT-leuk (DR5,6). This finding suggests that the specificity is directed at some epitope other than CALLA. The NK/LAK sensitive hematopoietic cell lines, Daudi, K562, and Raji, were not lysed, further confirming the specificity of these CTL lines. Screening 15 unrelated allogeneic LCL cells as targets revealed specific lysis of isolated individual LCL cells by certain T cell lines. For example, CTL line 10-31 and its subclone 10-31.2 and CTL line 10-120 all lysed the 105-LCL (DR7,DQw1,2,DRw53); the CTL line 1-20 lysed 76-LCL (DR5,6,DQw1,2,DRw52) and, to a lesser degree, 5-LCL (DR2,DQw1,2,DRw52). Other LCL cells with expression of similar HLA-D typing were not lysed. These results were further addressed in later experiments described below.

The role of T-cell associated and MHC molecules in the lysis mediated by the allo-leuk reactive CTL on the leukemic targets. We next evaluated the role of certain cell surface molecules on the effector and target cells in the recognition and lysis of the leukemic cells. To address this question, cytotoxic assays were carried out in the presence of various MoAbs. Antibodies were added at saturating conditions either to the effector cells (anti-CD3, anti-CD4, anti-CD8) or to the labeled target cells (anti-MHC class I, anti-HLA-DP, anti-HLA-DR) for 30 minutes at 4°C before the assay. Antibodies remained in culture throughout the assay period. As shown in Fig 2, the T cell lines (10-17 and 10-31) were effectively inhibited by anti-CD3 and anti-CD4. For line 10-31 and subclone 10-31.2 (data not shown), anti-DP also inhibited leukemic lysis (Fig 2B). Furthermore, anti-CALLA antibodies were ineffective at blocking lysis of leukemia by any of the CTL tested (data not shown). These results support a role for the CD3-TCR complex in the lysis of the leukemia.32 The CD4 receptor for MHC class II molecules is also likely to be involved in the interaction of effector and leukemic target.33 Furthermore, for cell line 10-31 and its subclone (10-31.2), DP molecule on the target cell appears to be playing a critical role; it may be recognized directly or could be presenting an undefined antigen to the effector (10-31).

Other T cell lines, including 10-17 (Fig 2A), 10-120, and 1-20, were all blocked in the presence of anti-CD3 and anti-CD4 but were not inhibited by any of the antibodies against framework structures on HLA-A,B,C,DQ, and DP determinants that were rigorously assayed at various dilutions. Furthermore, line 1-20 but not 10-17 or 10-120 was inhibited by the anti-DR antibody (data not shown). Flow cytometric staining revealed that the amounts of all three MHC class II molecules (DR, DQ, DP) on the surface of the AT-leuk line were not increased compared with that expressed on the AT-LCL line (median fluorescence channel of 645; DP: 585; DQ: and 741; DR for AT-LCL and 530; DP: 351; DQ: and 650; DR for AT-leuk). These data indicate that the DP and DR molecules are involved in the presentation of determinants to cell lines 10-31, 10-31.2, and 1-20, respectively. However, with regard to the other T cell lines (such as 10-17 and 10-120), the data suggest that other molecules on the leukemic cell, not recognized by these antibodies, may play a role in antigen presentation or that epitopes on MHC molecules recognized by these antibodies are distinct from the epitopes that may be involved in the recognition of antigen on the leukemia by these CTL. Alternative molecules on the leukemic cells presenting these
antigens could include other MHC molecules not recognized by these antibodies or MHC-like molecules, such as CD1 that can evidently present antigens.

Cold target blocking studies support the presence of determinants on the leukemic cells absent from the remission LCL cells. To further investigate the mechanism of the specificity of these allogeneic CTL for the leukemic targets, we performed a series of experiments examining the ability of cold (unlabeled) target cells to inhibit the lysis of \(^{51}\)Cr-labeled leukemic targets. Initially, a set of T cell lines that exhibited allo-leuk reactivity (10-17 and 10-31) or alloreactivity (10-51) were assayed for AT-leuk lysis in the presence of varying numbers of cold targets, including AT-leuk, AT-LCL, and an unrelated B-cell leukemia line (BM-leuk). Results shown in Fig 3 demonstrate that, while the lysis of AT-leuk mediated by the alloreactive CTL line 10-51 is effectively blocked in the presence of cold AT-leuk or cold AT-LCL, the lysis mediated by allo-leuk reactive CTL lines 10-17 and 10-31 is specifically blocked only by the unlabeled AT-leuk. Control experiments showed that unlabeled AT-leuk did not block alloreactive CTL directed at an unrelated target, and BM-leuk could effectively block allospecific CTL against labeled BM-leuk (data not shown). These results further support the existence of determinants on AT-leuk absent from AT-LCL that are recognized by the T cell lines 10-17 and 10-31. Furthermore, this differential blocking pattern (Fig 3A versus 3B and 3C) indicates that the alloreactive T cell line, 10-51, appears to interact with different recognition units on the AT-leuk than do the allo-leuk reactive CTL lines.

Experiments presented in Table 3 revealed that the CTL line 10-31 not only lysed AT-leuk but also one of the allogeneic LCL (105-LCL). This lysis was specific since other allogeneic CTL, including lines 10-17 and 1-20, mediated no significant lysis against the 105-LCL target. Therefore, we asked whether the determinants recognized on 105-LCL by cell line 10-31 were in fact crossreactive with those recognized on AT-leuk by CTL line 10-31, using cold target blocking analysis. As shown in Table 4, cold AT-leuk markedly inhibited the lysis of the AT-leuk target mediated by lines 10-17 and 10-31, while the cold AT-LCL (which were not lysed by these effector CTL) showed relatively little inhibition of lysis. Unlabeled 105-LCL did not inhibit the lysis of the AT-leuk target by the cell line 10-17. In contrast, cold 105-LCL markedly inhibited the lysis of AT-leuk by CTL line 10-31. These data support the concept that determinants recognized on 105-LCL are similar or crossreactive with those recognized on AT-leuk by the 10-31 cell line. Therefore, at least with respect to CTL line 10-31, specificity against allogeneic leukemia is not directed against determinants that are recognized uniquely on the leukemic cells, but may involve recognition of shared or crossreactive determinants expressed on at least some nonleukemic third party cells.

The HLA-DP molecule is involved in both the recognition of allogeneic leukemia and the unrelated LCL cell line (105-LCL) by the CTL line 10-31. We next evaluated whether the shared or crossreactive determinants recognized by CTL line 10-31 on the unrelated allogeneic LCL also required recognition of HLA-DP molecules. As shown in Table 5, lysis of AT-leuk and 105-LCL mediated by line 10-31, or the lysis of AT-leuk by line 10-17, was inhibited by anti-CD3. In addition, anti--HLA-DP antibody inhibited the lysis of both AT-leuk and 105-LCL mediated by line 10-31.
Table 4. 105-LCL Specifically Blocks Lysis of AT-Leuk Mediated by CTL Line 10-31

<table>
<thead>
<tr>
<th>T Cell Line</th>
<th>AT-Leuk</th>
<th>AT-LCL</th>
<th>105-LCL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25:1</td>
<td>100:1</td>
<td>25:1</td>
</tr>
<tr>
<td>10-17</td>
<td>13.6</td>
<td>1.1</td>
<td>71.5</td>
</tr>
<tr>
<td>10-31</td>
<td>37.1</td>
<td>1.1</td>
<td>68.1</td>
</tr>
</tbody>
</table>

Lysis of AT-leuk by T cell lines was assayed at an E:T ratio of 10:1 in the presence of cold unlabeled targets in a standard 4-hour CML assay. Control lysis in the absence of cold targets was 46.3% for line 10-17 and 28.5% for line 10-31. Percent cytotoxicity was converted to percent control lysis for each cold target:unlabeled target ratio. Data are shown for percent control lysis using 25 and 100 cold targets for each labeled target.

In contrast, the anti-DP antibody did not inhibit line 10-17 lysis of AT-leuk, and anti-DQ antibody did not significantly inhibit lysis of either target by line 10-31 or line 10-17. These results suggest that the line 10-31 requires recognition of the DP molecules on both AT-leuk and 105-LCL. Whether the determinants recognized are identical and how they relate to the DP molecules expressed on normal lymphoid cells will require further analyses.

Specific proliferative responses to allogeneic leukemic cells by the allo-leuk reactive T cells. Other T cell functions have been proposed to be equally or even more important than cytotoxicity in mediating antitumor responses.35,36 Proliferative responses involve T cell recognition and signal transduction as do cytolytic responses, but additionally involve the release of cytokines that may function in an autocrine and paracrine manner.14 Furthermore, proliferation is another way to assess the specificity of T cell responsiveness to a target or stimulator population that is not potentially complicated by differences in target susceptibility to lysis. For these reasons, we have studied the proliferation of these CTL lines in a 72 hour 3H-TdR incorporation assay. T cell lines at 2 × 10^6 cells per well were cultured in the presence of media, irradiated AT-leuk, irradiated AT-LCL, or IL-2 at 100 U/mL. Various concentrations (from 10^3 to greater than 10^3 cells per well) of irradiated AT-leuk and AT-LCL were tested as stimulator cells; the concentrations shown in Fig 4 induced the optimal stimulation (cpm) versus background (media or irradiated stimulators alone) (data for other stimulator concentrations not shown). As shown in Fig 4, alloreactive T cells (10-51 and 1-17) proliferated better to AT-LCL than to AT-leuk. In contrast, the allo reactive CTL (10-17, 10-31, and 1-120) showed much better proliferation in response to the AT-leuk line than to the AT-LCL line. In fact, line 10-17 demonstrated no significant proliferation to AT-LCL when compared with the AT-leuk stimulus. IL-2 induced variable responses among the different T cell lines studied. Additional studies showed that AT-leuk supernatants did not induce proliferation of any of the T cell lines (data not shown). Therefore, with regard to both proliferation and cytolytic, these allo-leuk reactive CTL lines demonstrate specificity for the allogeneic leukemia.

DISCUSSION

The graft versus leukemia phenomenon is one of a few documented examples in which a presumably immunologic manipulation has been associated with antitumor effects in man.23,24 While the mechanism remains uncertain, this antileukemic effect suggests a role (either direct or indirect) for allogeneic T cells in the recognition and elimination of residual leukemia. Furthermore, murine leukemia models and clinical bone marrow transplant experience suggest the antileukemic role of allogeneic T cells may be independent of their ability to induce detectable GVHD.9,10 In contrast, the experiments presented here have focused on the response of normal donor lymphocytes against allogeneic leukemia as an in vitro model of the GVL response. The mechanism by which donor lymphocytes may eliminate residual foreign leukemia in vivo is poorly understood. We have directed our efforts at studying cellular cytotoxicity as one possible mechanism, although we are aware that in vivo destruction in some animal models can be mediated by cells without in vitro cytolytic activity.35,36

Studies we conducted earlier suggested the presence of T cells with TCR α/β that do not express NK/LAK activity but appear to specifically lyse an allogeneic leukemia target.36 The previous studies described the isolation and preliminary

Table 5. The HLA-DP Molecule is Involved in the Recognition of 105-LCL by Line 10-31

<table>
<thead>
<tr>
<th>T Cell Line</th>
<th>Media</th>
<th>Anti-CD3</th>
<th>IgG1 Control</th>
<th>Anti-DP</th>
<th>Anti-DQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LU</td>
<td>1,477</td>
<td>17</td>
<td>2,023</td>
<td>218</td>
<td>2,429</td>
</tr>
<tr>
<td>% inhibition*</td>
<td>—</td>
<td>98.9</td>
<td>(-36.9)</td>
<td>78.5</td>
<td>(-64.5)</td>
</tr>
<tr>
<td>10-17</td>
<td>1,726</td>
<td>17</td>
<td>1,796</td>
<td>1,816</td>
<td>1,119</td>
</tr>
<tr>
<td>% inhibition*</td>
<td>—</td>
<td>99.1</td>
<td>(-4.1)</td>
<td>(-6.2)</td>
<td>35.2</td>
</tr>
</tbody>
</table>

Line 10-17 and line 10-31 were assayed for their lysis of AT-leuk or 105-LCL in the presence of saturating conditions of various antibodies. Antibodies were added to effector or to target cells before the assay as described in Methods. Results are expressed as LU (20% lysis/10^6 effectors). Effector cells were assayed at three E:T ratios (10:1, 3:1, and 1:1). Target cells were chromium labeled as previously described.

*Percent inhibition is computed from the baseline lytic units in the presence of media alone. Negative values for inhibition are above the baseline values in media.
characterization of CTL with specificity for allogeneic leukemia. In this report, we have confirmed and extended those initial findings using leukemic cells and cell lines from a separate patient with a CALL+ leukemia. We have isolated a series of T cell lines and clones derived from a normal donor that specifically stimulate an allogeneic lymphoid leukemic line and not its matched lymphoid LCL line. These CTL also lysed fresh cryopreserved leukemic blasts, indicating that these T cells did not recognize neo-antigens induced by long-term culture of the AT-leuk line. A separate group of similarly derived T cell lines from the same allogeneic donor lysed both leukemic and LCL lines with similar efficacy. Our characterization of the “allo-leuk reactive” CTL revealed their surface phenotype as CD3+ , CD4+ and TCR α/β+ . Their lysis was blocked by the anti-CD3 and anti-CD4 antibodies, and in some cases (CTL lines 10-31, 10-31.2, and 1-20) by an anti-DP or anti-DR antibody, respectively. Cytotoxic cell-target cell conjugation studies failed to reveal enhanced nonspecific adhesion between the allo-leuk reactive CTL and the leukemic target as an explanation for the specificity of its lysis of the leukemic target (data not shown). Cold target blocking studies demonstrate that only the appropriate allogeneic leukemic target, not the matched LCL line, could inhibit the lysis of the leukemic target by the allo-leuk reactive CTL. Several of the allo-leuk reactive CTL also lysed other unrelated allogeneic LCL cell in addition to the leukemic line. Furthermore, with respect to one CTL line (10-31), the unrelated LCL line could specifically inhibit the leukemic lysis mediated by these effector cells documenting that the unrelated LCL and the AT-leuk expressed a determinant that was shared or crossreactive and was not detected on the patient’s own LCL (AT-LCL). The different “allo-leuk reactive” CTL lines showed different patterns of specificity on a panel of unrelated LCL targets. This may indicate a variety of “leukemic specific” epitopes on the AT-leuk or a variety of different MHC determinants (“histotopes”) presenting the same epitope on the leukemic cell. Each CTL line with a distinct pattern of specificity on allogeneic LCL targets may recognize a different antigen complex of epitope plus MHC determinant. Lysis of this unrelated LCL line by the CTL line 10-31 was also inhibited by anti–HLA-DR antibody, indicating that the crossreactive determinant on the two cell lines AT-leuk and 105-LCL involves or is presented by HLA-DR. Finally, several of the allo-leuk reactive CTL lines also demonstrated preferential proliferation in response to the allogeneic leukemic stimulus.

Taken together, these data support the presence of T cells in a normal donor’s repertoire that are able to recognize differences between allogeneic normal lymphoid cells and leukemic lymphoid cells. These T cells appear to conform to the rules of T cell recognition with use of the CD3-TCR complex in their antigen recognition.57 Furthermore, these CTL lines appear to be restricted or responsive to signals induced by target MHC class II molecules. Lastly, it appears that whatever is recognized on the leukemic cell line may be identical or crossreactive with class II restricted determinants on unrelated third party nonleukemic lymphoid cell lines.

These data indicate the likely presence of antigens on leukemic cells that are absent on at least some normal lymphocytes and recognized by some allogeneic CTL. Target cell lines (AT-leuk, AT-LCL, 105-LCL, and 76-LCL) were screened and found to be negative for mycoplasma infection, making this type of contamination an unlikely explanation for the HLA-D restricted pattern of specificity. The nature of this antigen has not been identified. One possible explanation
may be the presence of a B cell lineage differentiation marker that is expressed preferentially on the leukemic cell line. To address this point, we attempted antibody blocking of leukemic lysis with an anti-CALLA antibody. This antibody failed to inhibit the lysis of the leukemia by these specific CTL lines. Additionally, other CALL-1 leukemic cell lines were not lysed by these CTL. It is still possible that one of many other B cell differentiation markers may be involved. It is likely that whatever determinants are recognized on the leukemic cell are presented in the context of class II MHC antigens based on the phenotype of the effector cells and antibody blocking studies with anti-CD4 and anti-DR or anti-DR antibodies.53

We favor two hypotheses regarding the nature of these leukemic cell associated antigens. The first relates to the hypothesis that alloimmune responses are made up of an array of different responses to both alloantigens plus various self peptides.31-34 Recently, Marrack and Kappler proposed the presence of self peptides associated uniquely with B cells that are recognized by an alloreactive T cell hybridoma.35 It is possible that the allo-leuk reactive T cells we have described are recognizing MHC class II alloantigens plus self peptides that are uniquely or preferentially expressed on the leukemic cell but not on the normal lymphoid cell line. These peptides could be unique to the leukemia, or more likely, preferentially expressed by the leukemic cell. The fact that other unrelated LCL lines are also lysed by some of these same CTL suggests that these peptides could also be presented by some, but not all (ie, not AT-LCL), normal lymphoid B cells as well.

An alternative hypothesis is based on earlier studies in animal tumor models and some human tumor studies that suggest the presence of "alien" or altered MHC expression on these tumors.34,35 The results from a number of laboratories indicate the presence of altered or "alien" MHC class I or class I-like molecules on the surface of some tumor cells. It is possible that "alien" MHC antigens are present on the leukemic line we are studying, and that these antigens crossreact with MHC antigens on the unrelated third party LCL cells, which are also lysed by the allo-leuk reactive CTL lines. If this hypothesis is true, then these antigens would likely relate to MHC class II molecules since the effector cells are CD4+ and antibodies to CD4 and class II molecules may inhibit the leukemic lysis.53

At this point we can only hypothesize about the nature of the leukemic cell associated antigens recognized by these CTL. Further studies are needed to biochemically characterize these antigens on the leukemic cells. Recently, techniques have been described where sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of membrane proteins has allowed the dissection of antigens involved in T cell recognition and proliferation.60,61 By applying these techniques, or by analyzing the HLA-DR and -DP proteins on the leukemic cells, we hope to be able to demonstrate more definitively the presence of unique antigens on the leukemic cells. These studies may be especially difficult in lieu of our recent understanding of the nature of T-cell antigen recognition (small peptides presented in the cleft of the MHC molecule). For example, it is possible that the leukemic cell may be merely presenting self peptides differently (minimally altered or increased amounts) than the normal lymphoid cell line does, thereby enabling specific recognition by the allogeneic responding T cell.31-34 Earlier, clonal analysis of limiting dilution cultures from eight different normal allogeneic donors and five leukemic targets supported the existence of allogeneic cytotoxic T cells specific for leukemic targets and not remission cells. The ability to expand T cell lines and clones with this specificity in both studies66 supports these earlier findings and suggests that they are applicable to other allogeneic responders and leukemic targets. To confirm the significance of these findings and determine their in vivo significance, experimental animal systems will be required. These in vivo systems will assess both antileukemic and antithost effects of alloleukemic reactive T cells.

Future studies are needed to enable more specific approaches at directing immune responses against human malignancy.62 Adoptive immunotherapy with allogeneic lymphocytes63 might potentially involve transfer of purified tumor reactive allogeneic cells such as those studied here, if they can be propagated in sufficient numbers. Immune interventions in the context of the GVL effect after allogeneic bone marrow transplantation or the treatment of syngeneic malignancy with biologic response modifiers (such as IL-2, interferons, tumor necrosis factor, etc) should be enhanced by a better understanding of immune recognition of tumor cells, which in vitro studies such as this may potentially allow.54

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