Allogeneic T-cell clones able to selectively destroy Philadelphia chromosome-bearing (Ph1+) human leukemia lines can also recognize Ph1- cells from the same patient

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Allogeneic T-Cell Clones Able to Selectively Destroy Philadelphia Chromosome-Bearing (Ph\(^+\)) Human Leukemia Lines Can Also Recognize Ph\(^-\) Cells From the Same Patient

By Kurt R. Oettel, Osvaldo H. Wesly, Mark R. Albertini, Jacquelyn A. Hank, Othon Iliopolis, Jeffrey A. Sosman, Karl Voelkerding, Shi-Qi Wu, Steven S. Clark, and Paul M. Sondel

Immune competent cells in bone marrow allografts have been associated with a graft-versus-leukemia (GVL) effect. To further characterize effector mechanisms that may be involved in this GVL phenomenon, we have previously established an in vitro model to identify allogeneic T-cell clones that selectively mediate cytotoxicity against a patient's leukemic cells, but not against nonleukemic lymphocytes from the same patient. We have modified this in vitro model to test whether the Ph\(^+\) chromosome and the P210 fusion protein it controls have a detectable role in leukemia-specific recognition by allogeneic T-cell clones. In this report, T-cell lines reactive with allogeneic Ph\(^+\) chromosome-bearing (Ph\(^+\)) chronic myeloid leukemia (CML) cell lines were derived and selected to be minimally reactive with Ph\(^-\) negative (Ph\(^-\)) lymphoid lines from the same patient. However, after prolonged culture, these same T-cell lines also mediated significant destruction of the Ph\(^+\) target cells from the same patients. These T-cell lines specifically recognized cells from the allogeneic CML patient to which they were sensitized, and were not contaminated by an outgrowth of natural killer cells. Furthermore, subclones could be derived from these T-cell lines, and some of these subclones again showed selective killing of the allogeneic Ph\(^+\) leukemia cell lines, and not of the Ph\(^-\) cell line from the same patient. Analyses of T-cell receptor (TCR) genes showed the alloreactive T-cell lines and the Ph\(^+\) selective subclones derived from them to be of the same clonal origin. This suggests that the same T cells reacting with antigens expressed on the nonleukemic Ph\(^-\) targets can at times selectively and preferentially kill the allogeneic Ph\(^+\) cells. As the same TCR that recognizes Ph\(^+\) cells also can recognize the Ph\(^-\) targets, it appears that the Ph\(^+\) chromosome does not play a detectable role in recognition by these allogeneic T-cell clones. This in vitro observation may provide a model for evaluating the relationship between GVL and graft-versus-host disease effects.

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C L I N I C A L AND experimental evidence support the presence of an antileukemic effect mediated by immune competent cells in the donated marrow after an allogeneic bone marrow transplant (BMT) for the treatment of leukemia.\(^{1,2}\) Despite active research on this subject and accumulating clinical data supporting the existence of this phenomenon,\(^{2}\) no single effector population or mediator has been identified as the cell or factor responsible for the graft-versus-leukemia (GVL) effect. In addition, the finding that this GVL effect is observed most frequently in patients who also develop graft-versus-host disease (GVHD)\(^{3}\) has led to speculation that perhaps the observed antileukemic effect may be a part of this larger and more vigorous antihost immune effect. A number of investigators have attempted to separate the antileukemic GVL effect from the GVHD in experimental animal models and in clinical BMT studies.\(^{5}\) Some investigators have suggested that cytokines,\(^{5,6}\) non-major histocompatibility complex (MHC)-restricted effector cells such as natural killer (NK) cells\(^{11,14}\) or other mediators\(^{15}\) may play a major role in the GVL phenomenon. Others have proposed T lymphocytes as the primary mediators of the antileukemic effect.\(^{6,16}\)

Several observations suggest that non-MHC-restricted NK cells can be involved in the GVL effect. First, NK cells are among the initial immune competent mononuclear cells found to repopulate the bone marrow after BMT.\(^{19}\) Second, cytotoxic effects against both autologous and allogeneic leukemic and lymphoma cells can be mediated by these NK cells after stimulation with IL-2.\(^{20}\) Furthermore, leukemic murine recipients of allogeneic T-cell-depleted BM are protected from leukemic relapse by in vivo stimulation with interleukin-2 (IL-2) treatment, suggesting an in vivo antileukemic effect mediated by IL-2–responsive NK cells.\(^{21}\)

Clinically, the concept that T cells mediate GVL is supported by the finding that recipients of BMs that have been depleted of T cells in an effort to decrease GVHD complications, have a much higher rate of leukemic relapse after BMT than patients receiving intact marrows.\(^{22}\) Thus, T cells are somehow important in preventing leukemic relapse after BMT. Unfortunately, those who undergo BMT with non–T-cell–depleted BMs also have a much higher incidence of GVHD. Furthermore, recent preliminary data from Champelin et al\(^{23}\) have suggested that selective removal of CD8\(^+\) T cells from the donated marrow preserves the CD4\(^+\) helper T-cell population and decreases the incidence of GVHD, while maintaining an incidence of relapse comparable to non–T-cell–depleted marrow recipients. In contrast to this, a recent report by Sykes et al\(^{24}\) using a murine model have shown that IL-2 given early after BMT can reduce GVHD and preserve GVL effects by selectively inhibiting CD4\(^+\) T-cell activity.

If T cells are the predominant cell population mediating the GVL phenomenon,\(^{24}\) it is uncertain what structures on the leukemic cells are being presented to the T-cell receptor (TCR) by the MHC antigens of the leukemic patient. It remains uncertain whether leukemic target cells express MHC-
restricted peptides that are absent from nonleukemic cells.\textsuperscript{25,26} In previous reports,\textsuperscript{16,17} we have shown that highly selected T-cell lines and clones from normal donors can preferentially recognize allogeneic leukemic cells and mediate reproducible cytotoxicity against these leukemic cells.

Studies currently underway in our laboratory seek to identify whether leukemia-specific antigens are recognized by T cells to mediate the observed in vitro antileukemic effect. One such “leukemia-specific” peptide recognized by human T cells in vitro is derived from the retinoic acid receptor \( a \) chain-fusion protein expressed selectively by acute promyelocytic leukemia cells.\textsuperscript{27}

In this present study, we have attempted to extend our previous findings by using an allogeneic system in which chronic myeloid leukemia (CML) cells are known to express a leukemia specific molecule: the P210\textsuperscript{BCR-ABL} fusion protein, controlled by the Ph\( ^1 \) chromosome consisting of the 9:22 translocation.\textsuperscript{28-30} We hoped to generate allogeneic T-cell clones selectively reactive to Ph\( ^1 \) leukemic cells, perhaps by recognition of this P210\textsuperscript{BCR-ABL} fusion protein or recognition of other antigenic molecules induced or influenced by this leukemia-specific protein. We have identified allogeneic T-cell clones that mediate selective cytotoxicity of these Ph\( ^1 \) leukemic cells. However, with prolonged culturing these same clones began to mediate destruction of Ph\( ^1 \) cell lines derived from B cells of the same leukemic patient. Thus, these T-cell clones that selectively react with the Ph\( ^1 \) cells are not exclusively recognizing an antigen specifically expressed only by the Ph\( ^1 \) cells. These results suggest that the same population of T cells may display leukemia-selective cytotoxicity (potentially analogous to GVL), and subsequently develop the ability to also lyse nonleukemic tissues.

**MATERIALS AND METHODS**

**Lymphocytes From Healthy Donors**

As previously described\textsuperscript{16,17} peripheral blood mononuclear cells (PBMCs) from healthy volunteer donors were used to be responding and effector cells in allosimulation and establishment of long-term cell lines were collected using sterile heparinized syringes, separated on Ficoll-Hypaque gradient and washed twice with RPMI 1640 supplemented with 2 mmol/L-glutamine, 100 U/mL penicillin, 100\( \mu \)g/mL streptomycin (Flow Laboratories, McLean, VA), 25 mmol/L HEPES buffer, and 10% heat-inactivated AB human serum (Pel-Freeze, Brown Deer, WI); this medium is designated RPMI-HS. PBMCs from six donors used in these experiments were HLA typed, and these donors are KO (HLA-A3,2 B27,44,Bw4 C2,4 DR2,7 DRw53 DQw1), JH (HLA-A1,3 B8,14,Bw6 DR3,6 DRw53 DQ3), PS (HLA-A5,6 B18,38,Bw6 DR5,6), D4 (HLA-A3,36 B14,18,Bw6 DR4,6 DRw52 DQ1), SV (HLA-A2,3 B7,49 DR and DQ typing not performed), and OI (HLA-A1,25 B35,51, C4 DR11,14). Some PBMCs were cryopreserved in 10% dimethyl sulfoxide by controlled-rate freezing and stored at \(-135^\circ\text{C}\) for use in later experiments.

**Generation of Leukemic and Nonleukemic B-Lymphoblastoid Cell Lines Using Epstein-Barr Virus (EBV) Viral Transformation**

Paired B-lymphoblastoid cell lines from leukemic and nonleukemic B cells used as target cells and stimulatory cells in these studies were a generous gift from Dr Bayard Clarkson at Memorial Sloan-Kettering, New York, NY. These cell lines were isolated as previously described.\textsuperscript{39,30} Briefly, PBMCs from patients with Ph\( ^1 \) leukemia were collected and transformed in vitro with EBV. Cell lines that grew spontaneously were tested for Ph\( ^1 \) expression by karyotype. For some patients, both Ph\( ^1 \) and Ph\( ^1 \) EBV-transformed B-cell lines were identified.\textsuperscript{27,28} Paired Ph\( ^1 \) and Ph\( ^1 \) EBV-transformed B-lymphocyte cell lines derived from two of these patients, (patients 8 and 35) were used in these present studies. As reported previously,\textsuperscript{29,30} the Ph\( ^1 \) translocation-bearing B-cell lines from these two patients are designated SK-CML 8Bt and SK-CML 35Bt, whereas the paired nontranslocated (Ph\( ^1 \)) B-cell lines from these same two patients are designated SK-CML 8BN and SK-CML 35BN, and are referred to here as 8Bt, 35Bt, 8BN, and 35BN. These four cell lines were examined for P210\textsuperscript{BCR-ABL} synthesis by immunoprecipitation of cell extracts with anti-ABL sera. P210\textsuperscript{BCR-ABL} was only detected in the Ph\( ^1 \) cell lines.\textsuperscript{27,28} HLA typing was performed on each of these four cell lines. Serological typing for HLA phenotype was kindly performed by the HLA laboratory at Memorial Sloan Kettering and showed that lines 8Bt and 8BN were identical and expressed HLA-A1,2,9,32, Bw52,Bw51,B8,12,18,39 DR3,6 DRw52 DQ1,2; and that both 35Bt and 35 BN were identical and expressed HLA-A1,2,3,9,30 Bw52,B16,35,51, Bw4, Bw6, DR1, DR5, MB1, MB3, and MT2. As reported previously on transformed cell lines, serological typing with alloantisera from multiple donors showed extra alleles for the HLA-A and B loci on these transformed cells. likely reflecting increased cross-reactivity and sensitivity to complement in these in vitro assays.\textsuperscript{31} Class II typing of 8Bt and 8BN was performed by the University of Wisconsin HLA lab (Madison, WI), and showed both were identical and expressed DR3,6, DRw52, and DQw1.2.

**Generation of T-Cell Lines After Sensitization With Allogeneic Leukemia In Vitro**

In vitro allosensitization was performed by using a modification of previously described methods.\textsuperscript{32} Briefly, 1 \times 10^6 PBMCs from each of six healthy donors were cultured in 10 mL of RPMI-HS with 10 \times 10^6 irradiated (4,000 cGy) Ph\( ^1 \) cells (from either line 8BT or 35BT) for 7 days. Sixteen separate allosensitizations were performed in 25-cm\(^2\) flasks (Costar, Cambridge, MA). Cells from four of the six donors were primed with 8BT, whereas 5 of these 6 donors were primed with 35BT. Some donors were primed to Ph\( ^1 \) cells more than once. At the end of 7 days in culture, viable cells were transferred from the flask and cloned by limiting dilution at 1 or 10 cells per well in 96-microtiter U-bottom wells and cultured with irradiated Ph\( ^1 \) cells (the same line used in the initial priming) at 1 \times 10^4/well, autologous PBMCs at 5 \times 10^4/well, and IL-2 at 100 U/mL (Hoffmann-LaRoche, Nutley, NJ) final concentration in a total volume of 200 \( \mu \)L/well. These limiting dilution cultures were then incubated for at least 2 weeks and periodically checked visually for macroscopic growth. Cultures that showed macroscopic growth were then expanded by restimulating the cells harvested from these 200-\( \mu \)L cultures in a total volume of 1.2 mL in six microwells with the same concentration of IL-2 and stimulators as described previously. These were incubated for 1 week and then harvested for testing in \(^51\text{Cr}\) release assays.

**Long-term Maintenance of Isolated Cell Lines and Subcloning**

Cell lines that were isolated by limiting dilution analyses were cultured in vitro for long-term periods (greater than 6 months). These cells were expanded and restimulated in 96-microwell U-bottom plates each week with irradiated Ph\( ^1 \) cells (the same cell line used in the initial priming) 1 \times 10^4/well, autologous or allogeneic PBMCs at 5 \times 10^4/well and IL-2 at 100 U/mL final concentration in a total volume of 200 \( \mu \)L. Three cell lines that were cultured for long time periods were subcloned. Subcloning was performed by limiting...
dilution of cell lines at a concentration of 1 cell/well and restimulating with irradiated Ph"* cells (the same used in the initial priming) 1 x 10^6/well, autologous or allogeneic PBMCs at 5 x 10^6/well and IL-2 at 100 U/mL final concentration in a total of 200 μL. These limiting dilution cultures were then incubated for at least 2 weeks and periodically viewed using light microscopy for macroscopic growth. Cultures that showed macroscopic growth were expanded and tested for cytotoxicity in the same manner as previously isolated cell lines.

**Chromium Release Assay**

Target cells were labeled with 250 μCi ⁵¹Cr and incubated at 37°C for 1.5 to 2 hours. Targets were then washed with serum-free RPMI media, counted, and diluted. Effector cells were diluted to the appropriate effector/target (E/T) ratios and placed in 0.1 mL of media per well of a 96 round-bottom microwell plate. Target cells (5 x 10⁶ cells/mL) in 0.1 mL were added to effectors and the plates were incubated at 37°C and 5% CO₂ for 4 hours before harvesting with a Skatron harvesting system (Sterling, VA). Radioactive ⁵¹Cr release was subsequently counted on a gamma counter. The percent cytotoxicity was calculated by 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 cetrizine detergent (Sigma Chemical Co, St Louis, MO), respectively. In many of the analyses, the percent cytotoxicity values from the E/T ratios were converted to lytic unit values. A lytic unit value is defined as the number of effectors resulting in 20% lysis of 5 x 10^⁶ target cells. Lytic units are expressed as LU/10⁶ cells.

**Monoclonal Antibodies (MoAbs) Used for Phenotyping and Antibody Blocking Tests**

MoAbs reactive against CD3, CD4, CD8, HLA-DR, HLA-DP, HLA-DQ, and the TCRβ/δ (WT31) were purchased from Becton Dickinson (Mountain View, CA). Anti-class I (W632) was obtained from a hybridoma cell line (American Type Culture Collection, Rockville, MD) and used as a culture supernatant. For cytotoxic blocking tests, MoAbs were added to effector cells (anti-CD3, CD4, CD8, or TCR) or to target cells (anti-HLA-DR, HLA-DP, HLA-DQ, anti class I) 45 minutes before mixing target and effector cells, and incubated at 4°C. The antibody was not washed out before mixing of cells. All antibodies including isotype controls (IgG1 and IgG2a) were used at saturating conditions as follows: anti-CD3, CD4, and CD8 at 1:200 final concentration; anti-class I (HLA-A,B,C) at 1:4 final concentration of culture supernatant; and anti-HLA-DR, HLA-DP, and HLA-DQ at 1:20 final concentration. For phenotyping, fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated MoAbs were used. Ten thousand events were analyzed for each marker using a Becton Dickinson FACScan flow cytometer, selecting positive fluorescence gating using appropriate FITC- or PE-labeled isotype control MoAbs.

**Molecular Analyses**

Molecular analyses consisted of initial Southern blot analyses for the TCRβ and TCRγ gene rearrangements after HindIII, BamHI and EcoRI restriction endonuclease digestion, essentially as described in a prior report, followed by Southern blot analysis for TCRβ and TCRγ gene rearrangements. For the Southern blots, approximately 15.0 μg/lane of digested genomic DNA was fractionated on a 0.7% agarose gel and transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH). Hybridization was performed overnight at 42°C with 1.0 to 1.5 x 10⁶ cpm/mL ³²P oligonucleotide-labeled probe.

**Table 1. Cytogenetic Analysis of Ph'⁺ and Ph'⁻ B-Lymphoblastoid Cell Lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No. of Cells Examined</th>
<th>Karyotype (% of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8BN</td>
<td>200</td>
<td>46,XY (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46,XY, -6, +der(8)t(8;13)q(9:22)</td>
</tr>
<tr>
<td>8BT</td>
<td>200</td>
<td>46,XY (100%)</td>
</tr>
<tr>
<td>35BN</td>
<td>200</td>
<td>46,XY (100%)</td>
</tr>
<tr>
<td>35BT</td>
<td>200</td>
<td>92,XXYY,2xt(6;22)(q43;q11) (100%)</td>
</tr>
</tbody>
</table>

Detailed chromosome analysis was performed on two paired Ph'⁺ and Ph'⁻ cell lines from patients no. 8 and 35. Chromosome preparations were made by treating cultured cells with colcemid (GIBCO) at a final concentration of 0.1 μg/mL for 3 hours. The cells were then exposed to a hypotonic treatment of 0.075 mol/L KCl at 37°C for 15 minutes followed by fixation in 3:1 methanol-glacial acetic acid for three times. Slides were made by dropping the fixed cells onto cold wet slides that were allowed to air dry. A modified trypsin G-banding technique was used to band the chromosomes. The cells were then examined using an Olympus photomicroscope.

The TCRβ probe used was a cDNA clone derived from Jurkat 2 that was obtained from Dr T.W. Mak. It contains nucleotides 100 through 870 cloned into the Pst I site of pBR322. The TCRγ probe used was obtained from Dr T.H. Rabbits and is the 700-bp HindIII-EcoRI insert of pH60, a genomic clone containing Jy1. Specific bands were visualized by autoradiography at ~80°C for 3 to 5 days.

**RESULTS**

**Characterization of Paired Ph'⁺ and Ph'⁻ Cell Lines 8BT, 8BN, 35BT, and 35BN**

Cytogenetic and molecular cytogenetic analyses (fluorescence in situ hybridization using a BCR/ABL probe) were performed on metaphase and interphase preparations from these four cell lines to clarify their homogeneity (Table 1). Both 8BN and 35BN lines showed normal 46, XY karyotypes with no translocations or karyotypic abnormalities noted in any of the cells examined. The Ph'⁺ line from patient 8, 8BT, showed homogeneous presence of the anticipated 9;22 translocation, which is characteristic of the Ph⁺ chromosome, as well as homogeneous presence of a translocation involving chromosomes 8 and 13. The karyotype for line 35BT showed homogeneous presence of the 9;22 Ph⁺ translocation, and showed a stable duplication of all chromosomes, yielding homogeneous tetraploidy.

Molecular analysis for the presence of the Ph⁺ chromosome was performed by Southern blot hybridization for each of these cell lines using a probe complementary to the breakpoint cluster region on chromosome 22. Both 8BN and 35BN lines showed normal germline patterns with no evidence of the Philadelphia t(9;22) translocation. In contrast, both 8BT and 35BT lines showed patterns indicative of the presence of the Philadelphia t(9;22) translocation.

Southern blot analyses of Ig gene rearrangement patterns were also analyzed for each of these four cell lines. Each showed a clonal pattern that differed for each cell line (data not shown). Thus, line 8BT is a clonal B-cell line that has a different rearrangement pattern from clonal line 8BN, indicating that lines 8BT and 8BN arose independently from patient 8. Similarly, line 35BT is a clonal line that has a different rearrangement pattern from clonal line 35BN, indi-
Table 2. Number of T-Cell Lines Evaluated for Cytotoxic Reactivity With PhT- Targets

<table>
<thead>
<tr>
<th>Cells/well as plated</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cell lines tested</td>
<td>1,871</td>
<td>576</td>
</tr>
<tr>
<td>T-cell lines showing &gt;10% lysis of PhT- targets on initial testing</td>
<td>654</td>
<td>403</td>
</tr>
<tr>
<td>T-cell lines showing selective lysis* on the PhT- targets on initial testing</td>
<td>63</td>
<td>0</td>
</tr>
<tr>
<td>T-cell lines showing PhT- selective lysis* after 1 wk</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>T-cell lines showing PhT- selective lysis* after 3 wks</td>
<td>7†</td>
<td>0</td>
</tr>
<tr>
<td>T-cell lines showing PhT- selective lysis* after 3 mos</td>
<td>3‡</td>
<td>0</td>
</tr>
</tbody>
</table>

Repetitive testing of cell lines isolated from 16 different primings of six different healthy donors against two different PhT- cell lines (some healthy donors were primed more than once with the same PhT- cell line). T-cell lines were derived by limiting dilution analysis (LDA) at either 1 cell/well or 10 cells/well after priming in bulk culture (25 cm² flasks) for 7 days. No cell lines grown out from 10 cells/well in LDA showed selective cytotoxicity on the PhT- target. Cell lines were initially tested on PhT+ and PhT- targets alone. Cell lines that showed selective cytotoxicity were expanded in 96-well microtiter plates, maintained continuously in vitro and subsequently tested weekly for cytotoxicity on the PhT+ and PhT- targets.

* Selective lysis is defined here as cytotoxicity on the PhT- target greater than 10% with at least twofold greater lytic unit values on the PhT+ target than on the paired PhT- target.
† These seven lines include lines designated KO14, OI31, OI11, KO95, KO181, JH109, and KO134.
‡ These three lines include lines designated OI11, KO181, and OI31.

...
cell lines after 6 months of prolonged culturing. In fact, as shown in Table 2, most of the cell lines that initially showed selective cytotoxicity on the Ph1+ target either lost cytotoxicity against the Ph1+ cells, or became reactive with the Ph1+ target cells within 3 months. Figure 2 shows four T-cell lines that selectively killed the Ph1+ target in the initial screening and several subsequent testings. Yet after 2 to 6 months in vitro, each of these four cell lines showed similar destruction of Ph1+ and Ph1- targets.

**Acquired destruction of Ph1+ targets reflects a change in**

**function of previously Ph1+-selective T-cell lines.** The T-cell lines that selectively recognize the Ph1+ targets described above were maintained continuously in culture as were the Ph1+ and Ph1- target cells for the studies described above, and shown in Table 1 and Figs 1 and 2. The acquisition of reactivity to the Ph1+ targets shown in Fig 2 could have resulted from a change in cytotoxic selectivity by the T cells themselves, or by a change in susceptibility of the Ph1+ target cells to recognition or lysis by the T cells. This issue was addressed by assaying effector T-cell populations and target
populations that had been previously cryopreserved and, therefore, not continually maintained in culture. A portion of T-cell lines from cell lines OI11 and OI31 were cryopreserved early after their derivation and at a time when these T-cell lines exhibited selective cytotoxicity on the Ph⁺ targets (designated OC for original culture). These cryopreserved T-cell lines were thawed and tested for cytotoxicity on the Ph⁺ and Ph¹⁺ targets in parallel with samples of these same effector cell lines that had been maintained continuously in culture and had gained the ability to lyse the Ph¹⁺ targets (Fig 3). The cryopreserved OC T-cell lines retained their ability to selectively kill the Ph⁺⁺ cells upon thawing, whereas cells from the same T-cell lines that were cultured continuously and tested in parallel lysed both the Ph⁺⁺ and Ph¹⁺ targets. Note that the continuously cultured lines showed a somewhat greater level of cytotoxicity on the Ph⁺⁺
little killing of the Ph\(^{1+}\) targets (data not shown). These experiments prove that the change in cytotoxicity pattern by T-cell lines that originally showed selective killing on the Ph\(^{1+}\) targets (such as IO11 and OI31) after prolonged culture is caused by changes in the functional selectivity of these T-cell lines and not caused by a change in the susceptibility of the Ph\(^{1+}\) target cell line to recognition or lysis by these T-cell lines.

Continuously cultured Ph\(^{1+}\)-reactive T-cell lines retain allospecificity. The increased capacity of these Ph\(^{1+}\)-reactive T-cell lines to lyse the Ph\(^{1+}\) target cells after prolonged culture (ie, time point +2 in Fig 2, A to D) raised the possibility that a small contaminating population of NK cells, or T cells with lymphokine-activated killer (LAK) activity had overgrown these cultures. However, these cultures (both original and continuously cultured populations) showed virtually no cytotoxicity against any of 4 separate LAK-susceptible targets, including Daudi, Raji, Molt, or K562 cell lines (Table 3). Furthermore, antibody-blocking studies showed that killing by these continuously cultured T-cell lines of the Ph\(^{1+}\) and the Ph\(^{1+}\) targets could be inhibited by anti-CD3 and anti-CD4 MoAbs, but not MoAbs to CD8 (data not shown). The acquisition of the ability to lyse the Ph\(^{1+}\) cell line did not diminish the level of cytotoxicity on the Ph\(^{1+}\) cell line; in fact the level of cytotoxicity on the Ph\(^{1+}\) cell line generally increased in parallel with the acquisition of

![Graph showing cytotoxicity patterns](image)

**Fig 3.** The change in cytotoxicity patterns is caused by a change in function of the T-cell lines and not to a change in susceptibility by the Ph\(^{1+}\) or Ph\(^{1+}\) targets. Cryopreserved samples of T-cell lines that showed selective lysis of Ph\(^{1+}\) targets when first isolated after limiting dilution analysis (for the purposes of this assay are called OC) were thawed and tested for cytotoxicity in parallel with the same T-cell line that had been grown continuously for at least 3 months (designated lines OI31 and OI11). All cytotoxic tests shown here were performed on the same day using the same Ph\(^{1+}\) and Ph\(^{1+}\) target cells. Data shown are for percent cytotoxicity at E:T ratios of 10:1. Lytic unit values are expressed on the top of each bar graph.

cells than did the freshly thawed OC lines. This assay documents the selective recognition of the Ph\(^{1+}\) targets by the OC, but not by the continuously cultured T-cell lines when tested simultaneously and in parallel on the same Ph\(^{1+}\) and Ph\(^{1+}\) target cells. Separate experiments compared the continuously cultured Ph\(^{1+}\) target cells with samples of the same targets cryopreserved previously (when the selective recognition of the Ph\(^{1+}\) targets by these T-cell lines had been observed). These target cells were thawed and recultured 1 week before the assay. Alloreactive T-cell lines mediated comparable killing on the cultured and recently thawed targets, whereas the OC T-cell lines showed significant destruction of the fresh and cryopreserved Ph\(^{1+}\) targets, but very

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Line KO181 Original Culture</th>
<th>Line KO181 Continuous Culture</th>
<th>Line OI11 Original Culture</th>
<th>Line OI11 Continuous Culture</th>
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<tbody>
<tr>
<td>8BN</td>
<td>1.0</td>
<td>11.4</td>
<td>3.7</td>
<td>50.6</td>
</tr>
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<td>46.5</td>
<td>60.3</td>
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</tr>
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<td>54.0</td>
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<td>0.8</td>
</tr>
<tr>
<td>35BT</td>
<td>56.3</td>
<td>73.2</td>
<td>-1.5</td>
<td>0.1</td>
</tr>
<tr>
<td>K562</td>
<td>0.5</td>
<td>-0.7</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Molt-4</td>
<td>0.3</td>
<td>0.0</td>
<td>2.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Daudi</td>
<td>2.3</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Raji</td>
<td>0.3</td>
<td>0.7</td>
<td>0.2</td>
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<td>NT</td>
<td>NT</td>
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<td>LCL no. 55</td>
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<td>0.5</td>
<td>NT</td>
<td>NT</td>
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<td>LCL no. 115</td>
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<td>47.1</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>TK8</td>
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</tbody>
</table>

The T-cell lines KO181 and OI11, which were isolated to show selective cytotoxicity on the Ph\(^{1+}\) target and subsequently acquired reactivity on the Ph\(^{1+}\) targets, do not show a complete loss of specificity as these T-cell lines still maintain allospecificity. Two samples of two different T-cell lines (KO181 and OI11) are shown in this assay. One sample of each cell line is shown as tested shortly after initial isolation and is called original culture, and one sample of each cell line is shown after a period of in vitro culture (at least 2 months) and is called continuous culture. Target cells are the 8BN, 8BT, 35BN, and 35BT paired cell lines from CML patients; the K562, Molt-4, Daudi, and Raji tumor lines susceptible to LAK cytotoxicity; three allogeneic EBV-transformed LCL lines from donors sharing HLA-DR antigens with patient 35 (nos. 1, 55, and 115) or allogeneic EBV-LCL line TK8 that was not HLA typed. In all experiments shown in this table, the T-cell lines were tested at E:T ratios between 20 and 30 in a standard 4-hour \(^{51}Cr\) release assay, and data are presented as % cytotoxicity.
Table 4. Phenotyping Data of Original and Continuously Cultured T-Cell Lines

<table>
<thead>
<tr>
<th>Line K0181</th>
<th>Original</th>
<th>Line K0181</th>
<th>Continuous Culture</th>
<th>Line O111</th>
<th>Original</th>
<th>Line O111</th>
<th>Continuous Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>92.3</td>
<td>97.7</td>
<td>98.1</td>
<td>98.9</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>97.3</td>
<td>99.8</td>
<td>98.9</td>
<td>94.2</td>
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<td></td>
</tr>
<tr>
<td>CD4</td>
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<td>98.6</td>
<td>99.8</td>
<td>99.4</td>
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<td>CD5</td>
<td>67.4</td>
<td>53.7</td>
<td>60.9</td>
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</tr>
<tr>
<td>CD8</td>
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<td>0.1</td>
<td>20.2</td>
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</tr>
<tr>
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<td>1.3</td>
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<td></td>
</tr>
<tr>
<td>CD25</td>
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<td>89.2</td>
<td>99.0</td>
<td>98.9</td>
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<td></td>
</tr>
<tr>
<td>TCRαβ̂</td>
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<td>99.8</td>
<td>97.2</td>
<td>98.1</td>
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</tr>
</tbody>
</table>

The phenotypic pattern of the indicated T-cell lines (see legend for Table 3) remains stable despite the change in cytotoxicity on Ph⁺ targets. Values shown represent the percent of cells showing positive antibody staining by flow cytometry. All phenotyping data for original lines was done in parallel with cytotoxicity tests where lysis on the Ph⁺ target did not exceed 5% and lysis on the Ph⁻ target was at least 30% at a 20:1 to 30:1 E:T ratio. Phenotyping data for continuous-culture cell lines was done in parallel with assays showing cytotoxicity on the Ph⁻ and Ph⁺ cell lines greater than 30% at a 20:1 to 30:1 E:T ratio.

The phenotypic pattern of Allogeneic Antileukemic T-cell Clones 3397.

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<tr>
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<tr>
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Detectable cytotoxicity on the Ph⁻ cell line. Note that the continuously cultured O111 line showed reactivity to the Ph⁺ and Ph⁻ targets from patient 8, but not to targets from patient 35 and allogeneic LCLs from line TK6. The continuously cultured line K0181 showed strong destruction of Ph⁺ and Ph⁻ lines from patient 35. In addition, line K0181 mediated weaker killing of Ph⁺ cells and minimal killing of Ph⁻ cells from patient 8 (Table 3). This same continuously cultured K0181 line was tested on three separate EBV-transformed LCL lines that had partial matching for HLA-D alleles with patient 35; only one of these three lines (LCL no. 115) was significantly destroyed. These data show that these continuously cultured T-cell lines had gained the ability to destroy the Ph⁻ targets from the initial donor, but did not destroy LAK-susceptible targets or most allogeneic LCL lines. This suggests that these T-cell lines had gained the ability to recognize, in an alloreactive manner, the Ph⁺ targets from the leukemia donor to which they were initially sensitized.

Cell surface characteristics of effector cells in culture. Effector cell lines obtained by limiting dilution cultures, described above, were evaluated by flow cytometry for cell surface phenotype. These lymphocyte cultures, both before and after acquisition of reactivity to Ph⁺ targets, showed nearly identical staining patterns for virtually all cell surface markers assayed (Table 4). This includes stability for CD3 and CD4 expression, and no change in the minimal expression of CD16 and weak expression of CD56, documenting that the continuing cultures retain cloned T-cell patterns, and are not cultures that have been overgrown with IL-2 activated NK cells. One exception to this phenotype stability was the acquisition of CD8 for approximately 20% of the cells in cell line O111. As 99% of this cell line was CD4⁺ these 20% CD8⁺ cells were CD8⁺/CD4⁺ double positive. Once these CD8⁺/CD4⁺ cells were noted, weekly phenotyping of this O111 continuously cultured cell line was performed over the next 6 weeks and showed expression of CD8 ranging from 5% to 25% of the cells (data not shown). It was possible that this gain in double-positive CD4/CD8 T cells may somehow be associated with the change in cytotoxicity pattern observed. To test whether CD8 expression was associated with acquisition of lysis by line O111 of the Ph⁺ target, CD8 depletions were performed. After CD8 depletion by magnetic bead separation, the remaining cells were tested for phenotype expression and lytic ability. These CD8-depleted populations of cell line O111 still retained the ability to lyse both Ph⁺ and Ph⁻ targets (data not shown). Thus the variable increase in CD8 expression of continuously cultured line O111 does not account for the acquisition of lysis seen repeatedly on the nonleukemic target.

Functional heterogeneity in T-cell line O111 and its subclones. The continuously cultured cell line O111 was able to lyse both the Ph⁺ and Ph⁻ targets. To evaluate the potential clonal homogeneity of cell line O111, subclones were generated and tested for lysis of both the Ph⁺ and Ph⁻ targets. Eighty-nine subclones were evaluated (data not shown): 42 showed an alloreactive pattern with cytotoxicity against both Ph⁺ and Ph⁻ targets, whereas 26 showed selective destruction of the Ph⁺ target. The remaining 21 subclones showed an indeterminant pattern. This functional difference could imply that cell line O111 is not clonal (despite derivation from a 1 cell per well initial culture), but actually contains at least two populations of cells with different specificities caused by different TCR structures; one population is alloreactive, and the other shows selective reactivity to the 8BT targets. Alternatively, cell line O111 may actually be a single clone with homogenous TCR usage. However, if this is the case, then the changes in target selectivity with culturing may reflect physiologic changes that influence effector-target interactions that are unrelated to TCR specificity. If, in fact, line O111 was a homogeneous clone, then the recovery of subclones from continuously cultured line O111, which retain leukemia reactivity with Ph⁺ targets yet do not react well with the Ph⁻ targets, would indicate that the subcloning process may have induced some physiologic change in some of the cloned O111 cells that allow T-cell populations derived from them to again distinguish Ph⁺ from Ph⁻ cells. Therefore, the clonal identity of line O111 and of a Ph⁺-reactive subclone (O111-15) was evaluated.

Genomic DNA was prepared from continuously cultured cell line O111 and from the subclone O111-15, which demonstrated the original capability to selectively lyse Ph⁻ targets. This DNA was digested with restriction enzyme HindIII, and analyzed for the TCR gene rearrangement pattern by Southern blot analysis after hybridization with TCRβ and TCRγ gene probes. The initial Southern blot hybridization of HindIII-digested DNA from line O111 and subclone O111-15 with the TCRβ probe showed identical rearrangement patterns. This TCRβ probe was then removed and the blot was rehybridized with a TCRγ probe that again showed

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identical rearranged bands in OI11 and subclone OI11-15 (Fig 4, A and B). Confirmatory blot analyses were then performed with BamHI- and EcoRI-digested DNA from line OI11 and subclone OI11-15. The Southern blot hybridization of BamHI-digested DNA with the TCRβ probe showed similar band patterns for OI11 and OI11-15 that were also similar to the germline pattern; however, hybridization of EcoRI-digested DNA showed identical TCRγ rearrangement patterns in both clones. Likewise hybridization of EcoRI blots with the TCRβ probe showed identical rearranged bands, but did have a decreased band intensity by one of the approximately 6-kb rearranged fragments (Fig 4, C and D). These composite results are strongly suggestive of similar clonal origins for cell lines OI11 and OI11-15. The TCR
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Vβ, Jβ, and CDR3 sequence of 0111 and 0111-15 was then examined to determine whether their functional heterogeneity would correlate with sequence divergence in the antigen binding region. Both 0111 and 0111-15 are Vβ 6.9, Jβ 1.1, and have identical CDR3 amino acid sequences (data not shown). Thus, these two clones are identical as evaluated by restriction fragment length polymorphism analysis and have an identical TCR sequence for Vβ, Jβ, and CDR3.

More limited analyses were possible for two subclones (0111-58 and 0111-33) that showed selective recognition of Ph+ targets. These subclones had identical rearranged fragments as cell line 0111 and subclone 0111-15 after HindIII digestion and hybridization with the TCRβ gene probe and a similar germline configuration as cell line 0111 and 0111-15 after BamHI digestion and hybridization with the TCRβ gene probe. Additional molecular analyses of subclones 0111-58 and 0111-33 were not possible because of an insufficient amount of DNA.

Further confirmation of the physiologic change in clonal function was obtained by repeated growth and analyses of the 26 subclones of line 0111 that appeared to show selective lysis of Ph+ cells. After 1 month of culture, 8 subclones maintained continued selective recognition of Ph+ cells and 18 had an alloreactive recognition pattern, with comparable destruction of the 8BT and 8BN targets. After 2.5 months of culture, only subclone 0111-15 retained preferential lysis of Ph+ cells; after 3.5 months of culture, subclone 0111-15 also showed an alloreactive pattern with strong destruction of both 8BT and 8BN targets.

DISCUSSION

Many investigators have attempted to identify the underlying mechanisms of T-cell recognition and activation of antileukemic responses in both autologous and allogeneic systems.8,48 Human T cells that appear to specifically recognize autologous human leukemia cells without recognizing autologous remission lymphocytes have occasionally been identified.41,42 In murine models, T-cell reactivity to syngeneic virally induced leukemias have been used to identify MHC-restricted virally encoded peptides.43 Furthermore, murine T cells can show responses to synthetic peptides corresponding to the TCR of the P210BCR-ABL protein found in human CML.44 Results such as these suggest that human leukemia-specific peptides, in addition to other MHC-presented peptides, might be specifically recognized by leukemia cells by autologous or allogeneic T cells.45 A variety of approaches could potentially enable the application of these clones for clinical purposes.

We have evaluated human lymphocytes for antileukemic reactivity using an in vitro allogeneic system. For most leukemia patients, we and others have had difficulty using these same culture systems to detect remission T cells from leukemia patients with the capability of specifically recognizing autologous leukemia. This suggests that T cells from most leukemia patients might not be effective at mediating detectable autologous leukemia-specific responses in vitro using these techniques. One problem may be the need for leukemia-specific peptides and TCRs that recognize them; these essential components of a leukemia-specific response might be lacking for some patients. Furthermore, T cells from tumor-bearing individuals may show a triggering dysfunction associated with T-cell functional deficiency.52

Thus, we chose to evaluate an allogeneic system because of the clinical data supporting an antileukemic allogeneic GVL effect. In murine models, GVL reactivity has been reported in donor host combinations differing only for minor histocompatibility antigens48 as well as combinations differing for major histocompatibility antigens.49,50 To compare alloreactive T-cell lines with leukemia-reactive lines, we used an allogeneic in vitro system that crosses an HLA barrier because reproducible human T-cell reactivity to minor locus alloantigens is not readily detectible in vitro.53 We have previously reported the isolation and characterization of allogeneic T cells with selective reactivity to human leukemia, but we have not identified a leukemia-specific antigen responsible for this type of reactivity. Similarly, the concept that allogeneic T cells can mediate an antileukemic effect has been highlighted by recent clinical reports.54-58 These reports document the occasional reinduction of chronic phase or induce an apparent complete remission of CML by donor leukocyt transusions used as the sole treatment for relapsed CML after BMT.

In this report we tested whether allogeneic T cells primed with irradiated Ph+ B-cell lines from patients with Ph+ CML might generate clones that selectively react to the leukemia cells. We focused our attention on CML-derived target cells where specific genetic translocations produce leukemia-specific molecules, namely the P210BCR-ABL fusion protein.59,60 Using EBV-immortalized B-cell lines from two separate CML patients expressing the P210BCR-ABL chimeric molecule and paired P210BCR-ABL-negative EBV-immortalized cell lines from these same patients, we were able to screen T-cell clones for preferential destruction of the Ph+ cells. We isolated T-cell lines from several donors that mediated selective reactivity to the P210BCR-ABL-expressing target cell line and not to the paired Ph+ target cell line. With continued culture, these T-cell lines that were preferentially reactive toward Ph+ cells gradually acquired the ability to destroy the Ph+ target cell line from the same patient. Cytotoxicity assays and phenotypic analyses proved that this acquired pattern of reactivity was not caused by an outgrowth of activated NK cells from the effector T cell population. No LAK activity was mediated by these lines, which showed a phenotypic pattern consistent with CD3+, CD4+ clonal origin.

Another possibility for this acquired cytotoxicity on the Ph+ target could be outgrowth of separate alloreactive T-cell clones from our T-cell lines. The homogeneous pattern of surface phenotype markers on our T-cell lines argues against this. Furthermore, we analyzed T-cell lines which had acquired the ability to lyse the Ph+ target and generated subclones from them. Some subclones reacted with both Ph+ and Ph+ targets, whereas others selectively lysed the Ph+ target. Southern blot analyses and probing for TCR γ and β rearrangement patterns documented the similar clonal origin and homogeneity for cell line 0111 and one of its subclones that exhibited a different in vitro pattern of target recognition. Identical DNA sequence analysis was seen for the TCR Vβ, Jβ, and CDR3 regions from line 0111, which were obtained when this line recognized both Ph+ and Ph+.
targets, and for its derivative subclone O111-15, which showed a Ph' reactive pattern. Similar results were suggested by DNA blot analysis for two additional subclones: from O111. Thus, clonally derived T cells that express the same TCRβ variable sequence mediate recognition of the allogeneic Ph' target cells, but show differential ability to destroy the Ph' target. These results suggest that these initial T-cell clones mediated selective cytotoxicity on the Ph' targets, but with further culturing these same clones acquired the ability to kill the Ph' targets.

One initial hypothesis accounting for clones able to selectively kill Ph' cells is recognition of leukemia-specific peptides presented by MHC molecules. The experiments presented here were designed to potentially test this hypothesis using leukemic cells that express the leukemia-specific P210BCR-ABL fusion protein. If the P210BCR-ABL fusion protein is immunogenic, then identification of T cells that selectively recognize Ph' cells may be a means to detect anti-P210 immunity. However, in these experiments the T-cell lines that selectively lysed the Ph' targets gradually gained the ability to specifically recognize the allogeneic Ph' target that does not express P210. Therefore, these T cells that react with Ph' target cells do not have TCRs that specifically recognize the P210BCR-ABL molecules. Similarly, as these same T-cell clones can recognize the Ph' targets, they must not be recognizing a peptide that requires the presence in any way, of the Ph' chromosome.

In all experiments presented here, to maintain reproducibility between experiments, we have used EBV-transformed B cells from normal donors or from CML patients as target cells. The ability of certain T-cell clones (such as O111) to recognize only the Ph' and Ph' target cells from the allogeneic donor to which it was sensitized (patient 8), and not allogeneic LAK-susceptible targets or other third party allogeneic LCL targets (35BT, 35BN, or TK6), argues that these T cells are specifically recognizing alloantigens. Thus, we have referred to them as "allospecific" T-cell lines. As killing can be blocked by anti-CD3 and anti-CD4 MoAbs, it seems likely that killing is specifically directed to allogeneic class II MHC antigens (although this was not proven). Although this was not directly tested because EBV-lymphocytes or leukemic cells from patients 8 or 35 were not available to test as target cells, it remains possible that this killing is directed against EBV determinants presented by these allogeneic MHC molecules.

How might these allospecific T-cell clones transiently distinguish between the Ph' and the Ph' targets? We must account for alloreactive clones that selectively recognize both Ph' and Ph' cells indicating TCR-mediated recognition of an alloantigenic determinant expressed by the paired Ph' and Ph' cell lines from the same patient. However, at times these same alloreactive clones selectively and preferentially kill the Ph' targets. This means that these alloreactive clones can recognize the Ph' targets. However, when these lines selectively recognize the Ph' targets using the same TCR, only the Ph' target and not the Ph' target induces an effective lytic interaction. Thus, there must be a difference at some level between the Ph' and Ph' targets to allow effective lytic function by the alloreactive T cells when they are selectively destroying the Ph' cells. For example, there might exist adhesion structures on the Ph' cell that quantitatively or qualitatively allow better interactions with the T cells that selectively destroy them than the analogous adhesion molecules on the Ph' cells. When these same T-cell clones are able to kill both Ph' and Ph' targets, this implies that the T cells have modified their function to interact comparably with the adhesive structures on both the Ph' and Ph' cells. Alternatively, these T-cell clones may modify, quantitatively or qualitatively, the molecular mechanisms they use to induce target cell lysis (ie, perforin, esterases, tumor necrosis factor, etc). If so, there may be differences between the Ph' and Ph' targets in their susceptibility to these lytic mechanisms. The Ph' cells could be more sensitive to the lytic machinery used by these T-cell clones when they are selectively lysing the Ph' targets. When the T-cell clones are alloreactive, they might use lytic pathways (ie, perforin) to which both the Ph' and Ph' cells are susceptible.

The identification of T cells that destroy Ph' cells to a greater degree than Ph' cells, even if transiently, further provides in vitro leads for characterizing and potentially controlling the GVL effect. Nevertheless, these experiments indicate that the Ph' chromosome and the P210BCR-ABL leukemia specific molecule it generates are not sufficiently immunogenic to allow specific anti-P210 reactive allogeneic T-cell clones to be readily detected by this in vitro sensitization and screening approach.

ACKNOWLEDGMENT

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