Recombinant Toxins Containing the Variable Domains of the Anti-Tac Monoclonal Antibody to the Interleukin-2 Receptor Kill Malignant Cells From Patients With Chronic Lymphocytic Leukemia

By Robert J. Kreitman, Vijay K. Chaudhary, Robert W. Kozak, David J.P. Fitzgerald, Thomas A. Waldmann, and Ira Pastan

We have previously shown that the variable domains of the monoclonal antibody anti-Tac (anti-Tac(Fv)) can be fused to derivatives of Pseudomonas exotoxin (PE) or diphtheria toxin (DT) to produce recombinant immunotoxins that kill interleukin-2 (IL-2) receptor-bearing cells. We now report that two of these single-chain recombinant immunotoxins, anti-Tac(Fv)-PE40KDEL and DT388-anti-Tac(Fv), are cytotoxic toward peripheral blood mononuclear cells (PBMCs) from patients with chronic lymphocytic leukemia (CLL). In anti-Tac(Fv)-PE40KDEL, anti-Tac(Fv) is genetically fused to the amino terminus of PE40KDEL, a recombinant form of PE which contains amino acids 253-608 of PE and the -KDEL mutation at the carboxyl terminus. In DT388-anti-Tac(Fv), anti-Tac(Fv) is fused to the carboxyl terminus of the first 388 amino acids of DT. PBMCs from 14 patients were incubated with the recombinant toxins for 60 hours, and [3H]-leucine incorporation was measured. Anti-Tac(Fv)-PE40KDEL was cytotoxic to 7 of the 14 patient samples, with half-maximal inhibition of protein synthesis (IC50) achieved at 1.2 to 9 ng/mL (1.8 to 13 × 10^-11 mol/L). DT388-anti-Tac(Fv) was cytotoxic to 11 of the 14 samples, with IC50 ranging from less than 1 to 250 ng/mL. DT388-IL-2, in which the first 388 amino acids of DT are attached to IL-2, was marginally cytotoxic toward only 4 of 13 CLL samples tested with IC50 ranging from 100 to 550 ng/mL. Trypan blue staining of cells from several patients indicated that inhibition of protein synthesis correlated with cell death. Binding assays using [3H]-anti-Tac indicated that the CLL cells from nine of the patients contained between 400 and 2,500 sites per cell. Cells from another patient, which were resistant to both anti-Tac(Fv)-PE40KDEL and DT388-anti-Tac(Fv), had less than 100 sites per cell. We conclude that anti-Tac(Fv)-PE40KDEL and DT388-anti-Tac(Fv) can kill CLL cells which have low numbers of IL-2 receptors, and should be investigated further for therapy of this disease.

This is a US government work. There are no restrictions on its use.

Pseudomonas exotoxin (PE) is a 66-Kd protein which, like diphtheria toxin (DT), kills cells by binding to a receptor, internalizing via a coated pit, translocating its active fragment into the cytosol, and enzymatically adenosine diphosphate (ADP)-ribosylating elongation factor-2 (EF2). The x-ray crystallographic structure of PE indicates three major domains, and mutational analysis has elucidated which domains are responsible for the several steps necessary to kill cells. Domain Ia, composed of amino acids 1 through 252, functions to bind the toxin to the PE receptor. Domain III (amino acids 400 through 613) contains the enzymatic activity that ADP ribosylates EF2. Domain II (amino acids 253 through 364) undergoes proteolytic processing and is responsible for translocating to the cytosol the 37-Kd carboxyl terminus of PE that contains the ADP ribosylating activity. DT also undergoes proteolytic processing, but its amino terminus contains the ADP ribosylating activity and is translocated to the cytosol. Accordingly, in chimeric DT-containing toxins such as DAB486IL-2,6 DAB486MSH,12 and DAB486EGF,13 the ligand replaces the toxin's binding domain at the carboxyl terminus. Conversely, in PE-containing chimeric toxins, such as IL-2-PE4014 and CD4-PE40,15 the ligand replaces the toxin's binding domain at the amino terminus.

We have recently reported the activities of recombinant toxins made from a derivative of the monoclonal antibody (MoAb) anti-Tac, which binds to the p55 subunit of the IL-2 receptor. Using recombinant DNA techniques we fused the variable heavy chain (VH) and light chain (VL) regions of anti-Tac to each other through a peptide linker to make a single chain antigen binding protein. This was in turn fused to domains II and III of PE, and the resulting chimeric toxin was termed anti-Tac(Fv)-PE40.16 The recombinant immunotoxin anti-Tac(Fv)-PE40 was cytotoxic toward cells bearing p55 such as T-cell leukemia cell lines,16 activated human lymphocytes,17 and fresh cells from patients with adult T-cell leukemia (ATL),18 but not toward...
normal lymphocytes. To improve the activity of the toxin, we mutated the carboxyl terminus from -REDLK to -KDEL, and found that anti-Tac(Fv)-PE40KDEL was several-fold more cytotoxic than anti-Tac(Fv)-PE40 toward target cells. Another recombinant immunotoxin was made using diphtheria toxin by fusing anti-Tac(Fv) to the carboxyl terminus of the first 388 amino acids of DT. A similar truncated form of DT had been previously shown to make an active toxin when fused with IL-2. DT388-anti-Tac(Fv), like anti-Tac(Fv)-PE40, was also cytotoxic toward p55 bearing cells. We also made DT388-IL-2, which contains IL-2 instead of anti-Tac(Fv) at the carboxyl terminus of the first 388 amino acids of DT.

The goal of the present study was to determine the cytotoxic activities of anti-Tac(Fv)-PE40, anti-Tac(Fv)-PE40KDEL, DT388-anti-Tac(Fv), and DT388-IL-2 toward malignant cells isolated from patients with CLL.

MATERIALS AND METHODS

Patients. Fourteen CLL patients were examined. One of the 14 patients had prolymphocytic leukemia (patient 1); the remainder had the usual type of B-cell CLL. Table 1 gives the clinical characteristics of the patients.

Recombinant toxins and control molecules. As shown in Fig 1, anti-Tac(Fv)-PE40, anti-Tac(Fv)-PE40KDEL, DT388-anti-Tac(Fv), and DT388-IL-2 are encoded by the plasmids pVC70108, pSS7010841, pVCDT1-anti-Tac(Fv), and pVCDT1-IL-2, respectively. The plasmids were expressed in Escherichia coli and the recombinant proteins purified as described. All toxins used were greater than 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). Protein concentrations of toxins were determined by the Warburg assay or by standard UV spectrophotometric methods. Anti-Tac was purified by Hazleton Laboratories (Vienna, VA) and Hoffmann La Roche (Nutley, NJ). UPC-10 was purchased from Sigma (St Louis, MO). The LL2 MoAb was provided by Immunomedics (Newark, NJ). Fluorescent activated cell sorting (FACS) analysis was performed by Fast Systems (Gaithersburg, MD).

Cells and cell culture. HUT-102 cells were maintained in RPMI containing 10% heat-inactivated fetal bovine serum (FBS). Anticoagulated venous blood was obtained from patients and used for experiments within 24 hours. The blood was diluted with phosphate-buffered saline (PBS), centrifuged over Ficoll (Organon Teknika, Durham, NC), and the mononuclear cells used for cytotoxicity assays.

Cytotoxicity assays. Cells were incubated with toxins at 1 × 10⁶ cells/mL in leucine-poor media (88% leucine-free RPMI, 2% RPMI containing leucine 50 μg/mL, and 10% FBS) in 96-well plates containing 0.1 mL per well. After 60 hours, each well was pulsed with 2 μCi of [³H]-leucine diluted to 0.05 mL with leucine-free RPMI. After 6 to 8 hours of labeling, the cells were harvested onto protein-binding glass fiber filters and leucine incorporation determined with a Betaplate (Pharmacia-LKB, Gaithersburg, MD) scintillation counter. The medians of triplicate experiments, which usually varied from their means by less than 5%, were used for calculations. Before calculating IC₅₀, all values obtained with and without toxin addition were reduced by the level of [³H]-leucine incorporation in cells treated with cycloheximide 10 μg/mL. This was done to correct for [³H]-leucine accumulating in the absence of protein synthesis. HUT-102 cells were assayed like the patient cells, except that the cell density was 4 × 10⁶/mL, the volume per well was 0.2 mL, the incubation time with toxins was 16 hours, and the cells were pulsed with only 1 μCi of [³H]-leucine per well for 3 to 4 hours before harvesting.

Trypan blue staining. CLL peripheral blood mononuclear cells (PBMCs) were incubated with toxins as described above for 1 week instead of 60 hours. Cells were diluted 1:1 with 0.2% trypan blue, and the number of viable cells determined by light microscopy. The medians of triplicate experiments, which usually varied from their means by less than 10%, were used for data analysis.

Binding assay. Cells from patients were washed twice with binding buffer (Dulbecco's modified Eagle's medium [DMEM] containing 50 mmol/L BES pH 6.8 and bovine serum albumin [BSA] 1 mg/mL), resuspended in binding buffer, and added in 0.15 mL vol to 96-well U-bottom plates. For a given patient, each well contained the same number of cells, and between the patients the number of cells per well was 1.2 to 20 × 10⁶. To the cells was added 0.05 mL vol of binding buffer containing varying amounts of [³H]-anti-Tac, with or without a 1,000-fold excess of unlabeled humanized anti-Tac. The choice of humanized anti-Tac was made based on availability from the company. After about 2 hours of incubation at 4°C with intermittent resuspension of the cells, the 96-well plate was centrifuged and the cells washed twice with 0.2 mL binding buffer and once with 0.2 mL of PBS. The cells in each well were resuspended in 0.1 mL of PBS and 0.01 mL of 10 N NaOH was added. The cells were kept cold during the washing steps. The dissolved cells were then counted in scintillation vials after neutralizing with 0.5 mL of 0.4 mol/L AcOH. The means of duplicate experiments, each of which usually varied from their means by less than 5%, were used to construct Scatchard plots.
PLASMID  PROTEIN  STRUCTURE  CYTOTOXICITY ON HUT-102 (ng/ml)

<table>
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<th>STRUCTURE</th>
<th>CYTOTOXICITY</th>
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<td>Anti-Tac(Fv) - PE40</td>
<td>GLU</td>
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</tr>
<tr>
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<td>Anti-Tac(Fv) - PE40KDEL</td>
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<tr>
<td>pRK7010841M</td>
<td>Anti-Tac(Fv) - PE40KDEL-Asp553</td>
<td>ASP</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>pVC70108</td>
<td>Anti-Tac(Fv) - PE40</td>
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<td>GLU</td>
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<td>Anti-Tac(Fv) - PE40KDEL-Asp553</td>
<td>ASP</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Fig 1. Chimeric toxins and control molecules, and the plasmids encoding them. V, and V, represent the heavy and light variable domains of anti-Tac, respectively, and are connected by a peptide linker. Box II represents domains II and IB of PE (amino acids 253 through 399). Box III represents domain III of PE up to amino acid 608, and is followed by the carboxyl terminal amino acids indicated. The indicated aspartate or glutamate is at position 563 of PE. Box DT represents amino acids 1 through 388 of DT. IL-2 includes amino acids 2 through 131 of human IL-2. The extra amino acids IPEGEA following IL-2 and HMAD linking DT with IL-2 are not shown. Cytotoxic activities of the toxins on HUT-102 cells are shown as IC50s, the concentrations necessary for 50% inhibition of protein synthesis.

RESULTS

Our goal is to develop toxin-based therapies for diseases that are not curable by conventional chemotherapy. For this to be successful the cells must contain unique growth factor receptors or other antigens on their surface. Because cells from patients with CLL contain IL-2 receptors, we chose to examine the malignant cells from 14 patients with CLL for their sensitivity to several chimeric toxins. These toxins contain anti-Tac(Fv), the combining site of an antibody directed at the p55 subunit of the IL-2 receptor.

Patient characteristics. Table 1 shows the clinical characteristics of the patients. Patient 1 had the prolymphocytic variant of CLL. All patients had B-cell disease, based on positive FACS staining for CD19 and CD20 (data not shown). Patients ranged in age from 52 to 77. White blood cell (WBC) counts ranged from 10,500 to 306,000/mm³. In all patients, the vast majority of the cells were lymphocytes, by light microscopical examination of Wright-stained cytopsins of the cells (data not shown). The patients were evenly divided with respect to stage of disease, with 5 patients having A, 5 patients having B, and 4 patients having C disease, according to the Binet staging system. Over half of the patients were untreated at the time blood was obtained, while the others were treated with chemotherapy. Cells from patients 1 and 2 were examined twice for toxin sensitivity, with 17 and 15 months separating the two assays, respectively. During the second assay, patient 1 was untreated and patient 2 was treated with fludarabine. Cells from patients 5 and 7 through 9 were examined twice over 1 to 5 months, and during this time the treatment did not vary significantly.

Activity of anti-Tac(Fv)-PE40KDEL. The cytotoxic effect of anti-Tac(Fv)-PE40 and anti-Tac(Fv)-PE40KDEL was evaluated by incubating cells from patients with CLL with the recombinant toxins for 60 hours and pulse labeling the cells for 6 to 8 hours with [3H]-leucine. Table 2 shows that anti-Tac(Fv)-PE40KDEL was very cytotoxic toward cells from half (7 of 14) of the patients. The IC50s varied from 1.2 to 9 ng/mL for patients with sensitive cells. Cells from patient 1 showed an IC50 of 9 ng/mL during the first assay, and 1.2 ng/mL 17 months later. Cells from patient 2 were also assayed twice, 15 months apart, and the IC50s were 1.5 and 3 ng/mL. For patients 1 and 2, cytotoxicity curves are depicted in Figs 2 A and B, respectively, for the first assays. In cells from patient 1, anti-Tac(Fv)-PE40KDEL at 100 ng/mL resulted in just over 50% inhibition of protein synthesis for the first assay (Fig 2A), but greater than 85% inhibition for the second assay (data not shown). Cells from patients 7 through 12 were resistant to anti-Tac(Fv)-PE40KDEL, showing less than 50% inhibition of protein synthesis even at 1,000 ng/mL (Table 2, Fig 2, G through L). With anti-Tac(Fv)-PE40, the IC50s for cells of patients 2 and 6 were 500 and 40 ng/mL, respectively, and greater than 1,000 ng/mL for the other 12 patients (data not shown). Thus, anti-Tac(Fv)-PE40KDEL was very cytotoxic toward cells from half of the 14 CLL patients, and was much more effective than anti-Tac(Fv)-PE40.
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Activity of DT388-anti-Tac(Fv). In an identical manner, cells from the 14 patients with CLL were evaluated for their response to DT388-anti-Tac(Fv). Table 2 shows the IC₅₀ of protein synthesis indicated that greater than 50% of the cells were killed, we used trypan blue which stains cells with nonfunctional cell membranes. We examined the cells 1 week after the addition of toxins by light microscopy, and counted the cells that were still able to extrude the dye. We tested cells from patients 1, 2, and 13, and found that those cells sensitive to anti-Tac(Fv)-PE40KDEL or DT388-anti-Tac(Fv) by protein synthesis were also sensitive by the viability assay. Moreover, at a given toxin concentration (1, 10, or 100 ng/mL), the percent of cell death was greater than the percent of inhibition in protein synthesis. Conversely, cells from patient 1 were resistant to DT388-anti-Tac(Fv) by both assays. Thus, inhibition of protein synthesis in the CLL cells by recombinant immunotoxins correlated with cell death.

Activity of *diphtheria toxin* targeted by IL-2. To determine the activity of a DT derivative directed by IL-2, we tested DT388-IL-2 toward cells from 13 of the 14 CLL patients. Table 2 shows that cells from 9 of the 13 patients were not affected by DT388-IL-2. There was a small cytotoxic effect on the cells from four patients, with IC₅₀ ranging from 100 to 550 ng/mL. The remaining nine patients showed no significant response, even at 1,000 ng/mL. Thus, DT388-IL-2 was not an effective cytotoxic agent against these cells.

**Cytotoxic specificity.** We performed several control experiments to prove that the cytotoxicity of anti-Tac(Fv)-PE40KDEL and DT388-anti-Tac(Fv) was specific and required both binding and ADP-ribosylation activity. To determine if only binding of anti-Tac(Fv) to the p55 subunit was responsible for the cytotoxicity of the recombinant toxins, we incubated cells from each patient with anti-Tac at 2,500 ng/mL, and found no significant cytotoxicity (data not shown). This indicates that the binding of the antibody variable region is insufficient for cytotoxicity. To determine if the activity of anti-Tac(Fv)-PE40KDEL required ADP ribosylation activity, we tested the cells with anti-Tac(Fv)-PE40KDEL, and found no significant cytotoxicity (data not shown). Moreover, it probably internalizes like the active toxin, because PEDS553 was shown to internalize and translocate the same fragments to the cytosol as wild type PE. Anti-Tac(Fv)-PE40KDEL was tested on cells from all 14 patients and in each case showed no cytotoxicity (data not shown), indicating that cytotoxicity of anti-Tac(Fv)-PE40KDEL required ADP ribosylation activity. To ascertain that cytotoxicity also required binding to the p55 subunit of the IL-2 receptor, we performed competition experiments using the anti-Tac antibody at 2,500 ng/mL to block binding of either anti-Tac(Fv)-PE40KDEL or DT388-anti-Tac(Fv). Figure 3 shows the results of competition experiments performed on cells from patients 2 and 11. In each case, 2,500 ng/mL of the irrelevant IgG 2a control antibody LL2 effectively blocked the cytotoxic activity of DT388-anti-Tac(Fv). Similarly, in cells from patients 1 and 2, anti-Tac at 2,500 ng/mL blocked the

Table 2. Cytotoxicity of Recombinant Toxins Toward CLL Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>Anti-Tac(Fv)-PE40KDEL</th>
<th>DT388-anti-Tac(Fv)</th>
<th>Tac Receptors Sites/Cell</th>
<th>Tac + by FACs (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>9-1.2</td>
<td>&gt;1,000</td>
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</tr>
<tr>
<td>2</td>
<td>1.5-3</td>
<td>&lt;1-60</td>
<td>550</td>
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</tr>
<tr>
<td>3</td>
<td>1.6</td>
<td>1.5</td>
<td>200</td>
<td>—</td>
</tr>
<tr>
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<tr>
<td>6</td>
<td>1.8</td>
<td>16</td>
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<tr>
<td>7</td>
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*IC₅₀ were the calculated concentrations necessary for 50% inhibition of protein synthesis. [H]-Murine anti-Tac was used to quantify receptor number in cells of patient 11 and the first assays in cells of patients 7 and 8. [H]-Humanized anti-Tac was used in the remaining binding assays. For the CLL cells, kds for murine and humanized [H]-anti-Tac were 1.1 to 2.3 × 10⁻¹⁰ mol/L and 1.0 to 3.3 × 10⁻¹⁰ mol/L, respectively. Using the same binding assay with HUT-102 cells, 2.7 × 10⁻¹⁰ sites/cell (kd = 1.4 × 10⁻¹⁰ mol/L) was obtained with [H]-murine anti-Tac, and 5 × 10⁻⁸ sites/cell (kd = 3.5 × 10⁻¹⁰ mol/L) with [H]-humanized anti-Tac.

Cells from patients 1, 2, 5, and 7-9 were examined twice; a hyphen separates results from each of the two assays, with data from the first assay before the hyphen.
Fig 2. Sensitivity of CLL cells to recombinant toxins. Cytotoxicity of anti-Tac(Fv)-PE40KDEL (○) and DT388-anti-Tac(Fv) (△) toward cells from CLL patients 1 through 14 (panels A through N), and normal PMBCs (panel O). The dashed line indicates the level of [3H]-leucine incorporation halfway between the level in the absence of toxin (Y-intercept), and the level in the presence of cycloheximide 10 μg/ml. The IC₅₀ for a particular toxin is the concentration at which the cytotoxicity curve crosses this midline.

Determination of receptor numbers. To quantitate the numbers of p55 subunits per cell from CLL patients, we performed binding studies using [3H]-anti-Tac. Aliquots of cells (0.2 mL/well) were incubated for approximately 2 hours at 4°C with increasing amounts of [3H]-anti-Tac in the presence and absence of a 1,000-fold excess of unlabelled humanized anti-Tac.⁵ The choice of the humanized form of anti-Tac was made based on availability from the company. After washing and dissolving the cells, the bound antibody was quantitated on a scintillation counter. Figure 4A shows the amount of [3H]-anti-Tac specifically bound to the cells of patient 1 at increasing nanogram amounts of [3H]-anti-Tac added. The Scatchard plot of this data is shown in Fig 4B. Table 2 lists receptor numbers for the 10 patients tested; patient 1 has about 1,000 receptors per cell. Figure 4, C and D, show similar data for patient 5, who also had 1,000 receptors/cell. For patients 2, 7, and 8, receptor numbers were determined twice, in assays performed several weeks apart. Table 2 shows that the two measurements of receptor number for these patients are less than twofold different. Thus, CLL cells from 8 of the 10 patients had 400 to 1,500 p55 subunits per cell. The dissociation constants (kds) were all between 1.0 and 3.3 × 10⁻¹⁰ mol/L.

Affinity of anti-Tac(Fv)-PE40KDEL and DT388-anti-Tac(Fv) for p55. To examine the possibility that differences in the sensitivity of the CLL cells to anti-Tac(Fv)-PE40, anti-Tac(Fv)-PE40KDEL, and DT388-anti-Tac(Fv) were partly caused by differences in binding of the recombinant toxins to the cells, we performed [3H]-anti-Tac displacement assays. Aliquots of cells from several CLL patients...
Single Chain Immunotoxins Against CLL

Fig 3. Competition of the cytotoxicity of DT388-anti-Tac(Fv). Cytotoxicity of DT388-anti-Tac(Fv) with either an excess of anti-Tac (Δ), an excess of the irrelevant antibody LL2 (□), or DT388-anti-Tac(Fv) alone (▲); (A) Cells from patient 2. (B) Cells from patient 11.

(0.2 mL) were incubated with a constant amount of humanized [3H]-anti-Tac and varying amounts of either anti-Tac(Fv)-PE40, anti-Tac(Fv)-PE40KDEL, or DT388-anti-Tac(Fv). Table 3 shows the concentration of the immunotoxins needed to displace [3H]-anti-Tac by 50% (EC50). EC50s for anti-Tac(Fv)-PE40 and anti-Tac(Fv)-PE40KDEL varied by less than 50%. Anti-Tac(Fv)-PE40KDEL bound to HUT-102 cells with about threefold higher affinity than DT388-anti-Tac(Fv) (EC50 = 2.5 ng/mL and 6.5 ng/mL, respectively). Anti-Tac(Fv)-PE40KDEL bound to cells from patients 1, 2, 8, 13, and 14 about 2.5- to 4.5-fold better than DT388-anti-Tac(Fv). UPC-10, the murine IgG 2a control antibody, showed no displacement of [3H]-anti-Tac from the CLL cells (data not shown). Thus, the affinity of anti-Tac(Fv)-PE40KDEL for p55 on patient cells and HUT-102 cells was the same as anti-Tac(Fv)-PE40 and a few-fold higher than DT388-anti-Tac(Fv). Moreover, binding did not correlate with cytotoxicity.

Time course study of cytotoxicity. To determine whether 60 hours was the optimum time to incubate the cells with recombinant toxins, we incubated cells with anti-Tac(Fv)-PE40KDEL (patients 1, 2, 13, and 14) or DT388-anti-Tac(Fv) (patients 2, 8, and 13) for either 20, 40, or 60 hours before labeling with [3H]-leucine. We found that in the prolymphocytic leukemia cells from patient 1, the IC50s with anti-Tac(Fv)-PE40KDEL were 5.5, 4.5, and 1.2 ng/mL at 20, 40, and 60 hours, respectively. In contrast, in the other CLL cells the cytotoxic effect of anti-Tac(Fv)-PE40KDEL or DT388-anti-Tac(Fv) was not evident unless 40 or 60 hours of incubation was used. Between 40 and 60 hours, the IC50s with anti-Tac(Fv)-PE40KDEL improved from 3- to more than 10-fold, while those with DT388-anti-Tac(Fv)
improved less than 2.5-fold. The cells did not lose sensitivity with time. Thus, to examine the cytotoxic effect of either recombinant toxin against cells from CLL patients, the 60-hour incubation was most appropriate.

**DISCUSSION**

We are interested in the use of recombinant toxins composed of anti-Tac(Fv) for the treatment of malignancies or autoimmune diseases where the target cells express the p55 subunit of the IL-2 receptor. In the current study, we found that cells from 7 of 14 patients with CLL were very sensitive to anti-Tac(Fv)-PE40KDEL (IC₅₀ = 16 ng/mL). Cells from 9 of the 14 patients were very sensitive to DT388-anti-Tac(Fv), and cells from an additional two patients showed a weak response to this recombinant toxin. In contrast, DT388-IL-2 or anti-Tac(Fv)-PE40 were much less effective at killing cells from CLL patients.

Sensitivity of CLL cells to anti-Tac(Fv)-PE40KDEL or DT388-anti-Tac(Fv). We found that cells from 11 of 14 patients were sensitive to DT388-anti-Tac(Fv), while cells from 7 of 14 patients were sensitive to anti-Tac(Fv)-PE40KDEL. Cells from six of the patients were very sensitive to both toxins. Cells from five patients were over 10-fold more sensitive to DT388-anti-Tac(Fv) than to anti-Tac(Fv)-PE40KDEL. While two patients were over 10-fold more sensitive to anti-Tac(Fv)-PE40KDEL. In contrast, HUT-102 cells and samples of fresh cells from ATL patients were more sensitive to anti-Tac(Fv)-PE40KDEL than DT388-anti-Tac(Fv) (unpublished data, 1990-1992). The binding data (Table 3) shows that differences in affinity for p55 cannot explain the cytotoxicity difference between these two molecules on CLL cells. Therefore, the difference in activity between anti-Tac(Fv)-PE40KDEL and DT388-Tac(Fv) must be caused by differences in the efficiency of which each of the two recombinant toxins are metabolized by the target cell. For a recombinant toxin to kill a cell it must enter the cell by endocytosis, and be processed to an active fragment that is translocated to the cytosol. It is possible that either the protease necessary to process PE or the protease used to process DT is absent in cells from some CLL patients. Alternatively, the carboxyl terminal sequence of anti-Tac(Fv)-PE40KDEL or the amino terminal sequence of DT388-anti-Tac(Fv), both of which are important for cytotoxicity but not ADP ribosylation activity, may not be functional in cells from some patients. Regardless of the basis for differential toxicity, anti-Tac(Fv)-PE40KDEL and DT388-anti-Tac(Fv) might broaden the spectrum of activity of recombinant toxins toward CLL.

**Targeting CLL with IL-2.** We found that DT388-IL-2 had little if any cytotoxic activity toward cells from CLL patients (Table 2). We also tested IL-2-PE⁴⁰ on four patient samples and found no sensitivity in cells from patients 1 and 12 through 14 (data not shown). IL-2-PE⁴⁰ contains full-length PE carrying four mutations in domain Ia to abolish binding to the PE receptor. It is very cytotoxic to HUT-102 cells (IC₅₀ = 2 ng/mL), but is much less cytotoxic than anti-Tac(Fv)-PE40 toward fresh cells from ATL patients. We have recently found that fresh cells from ATL patients are often much less sensitive to DT388-IL-2 than to DT388-anti-Tac(Fv) (unpublished data, 1990-1992). It is possible that malignant PBMCs from CLL patients have very low numbers of high affinity IL-2 receptors, and IL-2 toxins must bind to the p55 subunits. If so, the difference in the IC₅₀ of anti-Tac(Fv) (~10⁻¹⁰ mol/L) and IL-2 (~10⁻⁸ mol/L) for binding to p55 may lead to the difference in cytotoxicity of DT388-anti-Tac(Fv) and DT388-IL-2 toward those cells.

The DT388-IL-2 used in our study is very similar to DAB₃₈⁹IL-2, which has been described previously, and is likely to have the same cytotoxic activity. DAB₃₈⁹IL-2 was reported to be fourfold more cytotoxic than DAB₃₈⁹IL-2; the IC₅₀ of DAB₃₈⁹IL-2 on HUT-102 cells was reported to be about 1.5 ng/mL, which is a few-fold lower than our IC₅₀ for DT388-IL2 in a similar assay. Between Thr-386 of DT and Pro-2 of IL-2 (where the glycine that follows methionine is considered the first amino acid of DT), DT388-IL-2 contains the amino acids RPHMAD, while DAB₃₈⁹IL-2 contains HA. Also, DT388-IL-2 contains the extra amino acids IPEGEA following amino acid 131 of IL-2. It is interesting that DAB₃₈⁹IL-2 in a clinical trial has resulted in a partial remission in a CLL patient. Perhaps the malignant cells in that patient had high numbers of high affinity IL-2 receptors. It is also possible that the clonogenic cells responsible for malignant cell proliferation in CLL have higher numbers of IL-2 receptors than do cells found in the peripheral blood of such patients.

**Importance of the carboxyl terminus of PE.** We have previously shown that anti-Tac(Fv)-PE40KDEL, which contains KDEL following amino acid 608 of PE, is several-fold more cytotoxic toward cell lines than anti-Tac(Fv)-PE40. Moreover, anti-Tac(Fv)-PE40KDEL was several-fold more cytotoxic than anti-Tac(Fv)-PE40 toward fresh ATL cells (unpublished data, 1990-1992). It is unclear why the IC₅₀ for anti-Tac(Fv)-PE40 was greater than 20-fold more than that of anti-Tac(Fv)-PE40KDEL on cells from CLL patients 2 and 6, and is greater than 1,000 ng/mL on cells from the remaining 12 CLL patients (data not shown). The binding data in Table 3 indicate that both recombinant toxins bind with equal affinity to p55. Moreover, previous data indicate that changes in amino acids past position 602 of PE do not affect ADP ribosylation activity. We believe the carboxyl terminus delivers PE to a cellular compartment where translocation of the enzymatic portion of the toxin into the cytoplasm occurs, and that this process is more efficient.
with -KDEL than the native -REDLK sequence at the carboxyl terminus. It is possible that the -REDLK carboxyl terminus may be missing in cells from most CLL patients. Alternatively, both the -REDLK and -KDEL pathways may be present in CLL cells, but a recombinant toxin using the less efficient pathway for translocation may not kill the cell even if it saturates the relatively few binding sites. Cells not sensitive to toxins. Cells from 2 of the 14 patients tested were not sensitive to any of the toxins used (Table 2, Fig 2, G and J). One of these had less than 100 p55 subunits per cell. The other had about 700 receptors. Low receptor numbers probably contributed to resistance in at least the former case. Normal PBMCs were also resistant to all active toxins tested. In addition to the data shown in Table 2, we tested several other normal samples of PBMCs and Ficoll-purified BM cells, and found no sensitivity to the toxins in concentrations up to 10,000 ng/mL (data not shown).

CONCLUSION

We have shown that anti-Tac(Fv)-PE40KDEL and DT388-anti-Tac(Fv) are effective in killing malignant PBMCs from a high percentage of CLL patients, despite the presence of low numbers of receptors on those cells. These recombinant immunotoxins appear much more effective than either DT388-IL-2 or anti-Tac(Fv)-PE40. The clinical utility of these molecules will depend on how the malignant progenitor cells in CLL patients differ from malignant PBMCs from patients, and how toxic the immunotoxins are in humans. Based on our data, we believe anti-Tac(Fv)-PE40KDEL and DT388-anti-Tac(Fv) should be studied further as potential agents for the treatment of CLL.

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Recombinant toxins containing the variable domains of the anti-Tac monoclonal antibody to the interleukin-2 receptor kill malignant cells from patients with chronic lymphocytic leukemia

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