Preclinical Studies on the Use of Selective Antibody-Ricin Conjugates in Autologous Bone Marrow Transplantation

By John E. Leonard, Raymond Taetle, Dong To, and Kasper Rhyner

Whole-ricin immunoconjugates were synthesized with the pan-T cell antibodies T101 and 3A1 and assayed in the presence of 0.1 mol/L lactose. Their toxicity for cell lines, peripheral blood T lymphocytes, and normal bone marrow progenitors was compared with that of whole ricin. In the presence of 0.1 mol/L lactose, normal cells and cell lines exhibited the following sensitivities to ricin: 8392 (human malignant B cell line) < E rosette-positive lymphocytes < bone marrow progenitors < E402 (human T ALL) < CEM (human T ALL). Ricin sensitivities correlated with ricin binding as determined by immunofluorescence. In the presence of lactose, peripheral blood T cells were resistant to 0.1 mol/L ricin, but a similar concentration of T101-ricin inhibited normal and malignant T colony formation by >98%. 3A1-ricin was slightly less effective. At a conjugate concentration of 0.1 mol/L, bone marrow progenitor colony formation was inhibited by 30% or less; T101-positive cells were at least tenfold more sensitive than normal progenitors. When mixtures of 10% CEM cells and marrow cells were incubated with T101-ricin, 95% of CEM colonies were killed, and 96% of marrow granulocyte/macrophage progenitors survived. Some free ricin was released from immunotoxin-treated cells, producing minimal inhibition of protein synthesis or cell growth. We conclude that (a) normal blood cells and malignant cell lines exhibit varying degrees of ricin sensitivity in the presence of lactose; (b) T101-ricin is at least tenfold more toxic to T lymphocytes than to bone marrow progenitor cells and is effective in mixtures of normal and malignant cells; and (c) treatment of infiltrated marrow with anti-T cell immunotoxins should safely remove target T cells without excessively damaging normal progenitors or producing excessive free ricin. Anti-T cell, whole-ricin immunotoxins merit trials for autologous transplantation. © 1985 by Grune & Stratton, Inc.

A LLOGENEIC bone marrow transplantation is effective therapy for acute nonlymphocytic leukemia (ANLL) and has a role in therapy of acute lymphocytic leukemia and non-Hodgkin's lymphoma. The number of patients treated with allogeneic transplants is small and limited by availability of compatible donors and patient age. Graft-versus-host disease (GVHD) is also common in older patients and is a major cause of mortality in ANLL patients receiving allogeneic transplants.

Recently, autologous transplantation has been proposed as an alternative therapy for leukemia and lymphoma patients who are without available donors. In the procedure, marrow is removed from patients and incubated in vitro with drugs or specific antibodies to remove residual leukemia cells. Supralethal (and, it is hoped, curative) therapy is then given, and the patient is "rescued" by infusion of treated, autologous marrow. The feasibility of this approach has been demonstrated in experimental animal systems and clinical trials.

Because of their specificity, monoclonal antibodies are appropriate reagents for "purging" marrow of malignant cells, and preliminary trials using monoclonal antibodies and complement for autologous transplantation have recently been reported. However, many monoclonals do not fix complement, or require high antibody concentrations for complement-mediated cell killing. To broaden the available spectrum of antibodies and increase their efficacy, murine monoclonal antibodies have recently been complexed to highly toxic plant lectins, such as ricin. These chimeric molecules are effective in vitro and have been used successfully in animal transplantation models.

Ricin consists of two dissimilar polypeptide chains held together by a single disulfide bond. The B (binding) chain binds the toxin to the cell surface and facilitates toxin internalization. The enzymatically active A chain blocks cellular protein synthesis activity. Because it acts catalytically, perhaps as few as one to two molecules of cytosolic A chain can kill a cell. However, immunotoxins prepared with ricin A chain are variably cytotoxic to antigen-positive cell targets. Thus, we and others have prepared selective immunotoxins using whole ricin. In contrast to ricin A chain conjugates, these immunotoxins are consistently effective in vitro but must be used in the presence of lactose. In the present studies, we have conducted preclinical studies demonstrating that T cell immunotoxins are suitable for use in autologous marrow transplantation.

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MATERIALS AND METHODS

Materials

Electrophoretically pure ricin was obtained from Calbiochem-Behring (San Diego). The pan-T cell antibodies T101 and 3A1 were purified from mouse ascites fluid by ammonium sulfate precipitation and chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described.19 Polycrylamide gel electrophoresis showed the purified antibodies to be greater than 95% homogeneous. T101 is an IgG2 antibody that recognizes a 65,000-dalton antigen on all T cells and on chronic lymphocytic leukemia (CLL) lymphocytes.20 3A1 is an IgG1 that recognizes a 40,000-dalton antigen on all T cells and some early myeloid cells.21 Affinity-purified goat anti-racine antibody was obtained from Vector Labs (Burlingame, Calif). Nuclease-treated rabbit reticuloocyte lysate, Brome mosaic virus, and other necessary components were obtained from Promega Biotech (Madison, Wis).

Conjugate Synthesis and In Vitro Conjugate Exposure of Cells

Whole-racine immunotoxins were constructed and purified as previously described.24 Purified antibodies (5 mg/ml) were reacted with a seven-to tenfold molar excess of the cleavable cross-linking reagent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate) for 30 minutes at 23 °C and the reaction stopped by dialysis v phosphate-buffered saline (PBS), pH 7.4. The 2-pyridyl disulfide content of the derivatized antibodies was determined by measuring the absorbance at 343 nm after reduction of a diluted protein sample with 50 mmol/L dithiothreitol.21 Approximately 3 mol of 3-(2-pyridyl dithio) propionyl groups per mole of antibody was introduced. As determined by indirect immunofluorescence, the binding of derivatized T cell antibodies was identical to that of the native immunoglobulins.

Native ricin, suspended in PBS, pH 7.4, containing 50 mmol/L lactose, was reacted with a sevenfold molar excess of SPDP as described. After overnight dialysis v the same buffer, the ricin derivative was reduced at pH 5.5 by the addition of 1 mol/L dithiothreitol to a final concentration of 25 mmol/L. The reduction was allowed to proceed for 30 minutes at 23 °C, and the reduced racine derivative was dialyzed at 4 °C against PBS, pH 6.0, containing 1 mmol/L ethylenediaminetetraacetic acid (four changes) and sucrose. The racine derivative was then derivatized with the antibody at a fourfold molar excess. After overnight incubation at 23 °C, the free racine was removed by protein A-Sepharose chromatography; unconjugated antibody was removed by chromatography on Sepharose 4B.22 The purified conjugates were judged to be free of contaminating antibody or ricin by electrophoresis in polycrylamide gels containing sodium dodecyl sulfate. Approximately 2 mol of ricin per mole of antibody was coupled. Conjugates were filter sterilized, diluted as necessary with sterile PBS, and stored at 4 °C. Under these conditions the conjugates were stable for at least four months.

Cells were incubated with various concentrations of purified ricin or immunotoxin in the presence of 0.1 mol/L lactose for one hour in air at 37 °C, then they were washed three times and plated in relevant growth or colony assays. Unless otherwise indicated, all incubations were performed in the presence of lactose to block ricin B-chain binding to galactose cell surface receptors.23

Cell Free Protein Synthesis Assays

Protein synthesis assays consisted of 35 µL nuclease-treated lysate, 1 µL 1 mmol/L amino acid mixture (minus methionine), 2 µL Brome mosaic virus at 0.5 µg/µL, 7 µL sterile water, and 5 µL of 35S-methionine (800 Ci/µmol) at 10 µCi/µL; the total reaction volume was 50 µL. Reaction mixtures were incubated at 30 °C and triplicate 2-µL aliquots were removed every ten minutes for one hour and precipitated with trichloroacetic acid as described in the user information supplied by Promega. Precipitates were collected on Whatman (Clifton, NJ) GF/C filters and analyzed by standard scintillation counting techniques. All protein synthesis values were corrected for nonspecific incorporation by subtracting background (minus RNA) values. Ricin was added to a final concentration of 1 nmol/L; the final T101-racine concentration was 0.5 nmol/L and thus equivalent to ricin on an equimolar concentration basis. In all assays less than 30% of the available methionine was consumed.

Protein Assays

Protein concentrations were determined by the method of Lowry et al24 using bovine gammaglobulin as the standard.

Established Cell Lines

The cell lines used have been described previously.25-28 CEM and 8402 are T cell, acute lymphocytic leukemia (ALL) lines positive for T101 and 3A1 antibodies. HEL is an erythroleukemia line; HL60 was derived from a patient with promyelocytic leukemia. 8392 is a malignant B cell line derived from a patient with histiocytic lymphoma. Other cell lines used were the multipotential myeloid cell line K562, the T ALL cell lines MOLT-4 and HPB-ALL, the non-T-ALL cell lines NALM-6 and HPB-NULL, the B cell line WIL-2, and the Burkitt lymphoma cell line NAMALWA.

Cell lines were maintained in RPMI 1640 media with 10% fetal bovine serum. Log phase cells with viabilities exceeding 90% were used for all studies. Viable cell counts were determined using trypan blue exclusion.

Normal T Cell Colony-Forming Assays

Peripheral blood was obtained from normal volunteers and T cell colonies grown as previously described.29 E rosette-positive T cells were cultured at a density of 4 x 10⁶ per milliliter in 1% methylcellulose containing 15% fetal bovine serum, RPMI 1640, 20% T cell-conditioned medium,30 and irradiated, normal, mononuclear cells (3,000 rad) at 3 x 10⁶ per milliliter. The mixture was vortexed thoroughly and, after clearing, plated in triplicate, 1-µL aliquots in 35-mm tissue culture dishes (Lux Plastics, Newbury Park, Pa). The plates were incubated for seven days at 37 °C in 7.5% CO₂, and aggregates of 40 cells were scored as colonies on days 5 through 7.

Assays for Normal Bone Marrow Progenitors

Bone marrow cells from normal volunteers were collected in heparin, diluted with medium, and separated by centrifugation through Ficoll-Hypaque (Pharmacia).31 The interface cells were collected, washed, treated with immunotoxin or ricin, and plated as described later.

Granulocyte/macrophage progenitors (CFU-GM) were grown as previously described.32 The final culture mixture contained 2 x 10⁵ per milliliter normal bone marrow mononuclear cells, Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY), 15% fetal bovine serum (JMI International, Kansas City, Kan), and 10% placenta-conditioned medium,33 as a source of colony-stimulating factor (CSF), in 0.3% agar. Triplicate 1-µL cultures were incubated in 35-mm tissue culture dishes with 2-mm grid squares (Lux Plastics) in 7.5% CO₂, for 12 days at 37 °C. After seven and 14 days, aggregates of ten to 40 cells were scored as clusters; those greater than 40 cells were scored as colonies, using an inverted microscope.

Bone marrow erythroid progenitors (BFU-E) were cultured in triplicate 1-µL aliquots in 1% methylcellulose as previously described.34 The final culture mixture contained 15% FCS, 1MEM, 1% deionized bovine serum albumin, 0.1 mmol/L α-thioglycerol, 5% medium conditioned by phytohemagglutinin (PHA)-stimulated...
mononuclear cells, 3 U/mL erythropoietin (Hyclone, Logan, Utah), and 2 × 10^5 per milliliter marrow mononuclear cells. Morphologically, typical bursts were scored after 14 days of incubation in 5% CO₂ at 37 °C. About 20% of BFU-E grown in this manner contained other myeloid elements, including eosinophils, granulocytes, and macrophages; megakaryocytes were not identified. In vitro sensitivities for these mixed colonies and BFU-E were identical.

**Incubation of Mixtures of Malignant and Normal Marrow Cells With Immunotoxin**

Mixtures of 10% CEM and 90% normal marrow cells in medium containing lactose were incubated with 0.1 nmol/L T101-rinic at 37 °C in air for one hour, washed, and plated in 1% methylcellulose with or without a source of CSF. Cells were plated at final concentrations of 10^4 per milliliter CEM and 2 × 10^5 per milliliter normal marrow. In cultures without CSF, CEM colonies grew as round aggregates of large monomorphic cells that tended to float in methylcellulose; normal CFU-GM did not proliferate. In cultures with CSF, seven- to ten-day CFU-GM containing polymorphic cells of varying sizes grew as dispersed planar aggregates along the floor of the culture dish. These colonies were clearly distinguished from CEM colonies by morphology, and colony counts of each cell type plated along agreed closely with counts obtained in cell mixtures (see Results).

**Ricin Release Experiments**

Mid-log phase cultures of CEM and 8402 cells (5 × 10⁵ per milliliter) were treated for 30 minutes at 37 °C with either T101-PDP (the pyridyldithiopropionate derivative of T101) or T101-rinic (in the presence of lactose) or ricin (no lactose) at a final concentration of 1 nmol/L. The cultures were washed three times with fresh growth medium, resuspended to 5 × 10⁵ cells per milliliter, and cultured for either 24 hours (CEM) or 48 hours (8402) at 37 °C. After the culture period, an aliquot of cells was resuspended in Dulbecco’s Modified Eagle’s Medium without leucine and assayed for protein synthesis activity (primary cultures) using a 1.5-hour pulse of ³H-leucine (0.5 μCi). The remainder of the cells from primary cultures were removed and the supernatants used directly after dilution with medium or dilution with medium containing lactose (0.1 mol/L final concentration). Fresh cells were resuspended in these media at a final concentration of 5 × 10⁵ per milliliter (secondary cultures). Secondary cultures were incubated 24 hours at 37 °C and subsequently assayed for protein synthesis.

**RESULTS**

**Differential Effects of Ricin/Lactose on Cell Lines**

The effect of ricin on HL-60, HEL, CEM, 8392, and 8402 cells is shown in Fig 1. The data shown are the means of three independent studies. The cells were incubated with 0.1 mol/L lactose and various concentrations of ricin for 60 minutes at 37 °C, washed, and cultured for six days. When treated in the presence of lactose (ricin/lactose), the B cell line 8392 was resistant to concentrations of ricin up to 100 nmol/L. HL-60 cells were also relatively resistant to ricin/lactose; 20 nmol/L ricin was needed to inhibit HL-60 cell growth by 50% (ID₅₀) on day 3. ID₅₀ values for other cell lines varied by as much as 3 logs. Thus, although modest variations in ricin sensitivities were observed at low ricin concentrations, these increased markedly at concentrations above 1 nmol/L ricin. Similar results were obtained with several other cell lines, including K562, HPB-NULL, and NAMALWA. Results with colony-forming assays for CEM and 8392 (Fig 3) were similar to those shown in Fig 1. Treatment of cell lines with 0.1 nmol/L ricin in the absence of lactose resulted in complete inhibition of growth and colony formation (data not shown). At lower concentrations of ricin, modest variations in toxin sensitivity were observed, and these variations decreased with the ricin concentration. The ID₅₀ values for CEM, 8392, and 8402 cells treated with ricin alone were between 2 and 4 pmol/L, with 8392 being the most resistant. These data indicate that cell lines vary in their sensitivity to ricin whether assayed in the presence or absence of lactose. Because nonspecific killing by immunotoxins is due to ricin B-chain binding that is not inhibited by lactose, cells such as 8392 are not as likely to reflect nonspecific toxicity as are more sensitive cells, such as CEM.

We considered that variation in ricin sensitivity in the presence of lactose might be due to differences in ricin binding. Ricin binding by a number of cell lines is shown in Table 1; the cell lines are listed in order of decreasing binding as determined by immunofluorescence intensity and the percentage of labeled cells. The T cell lines CEM, NALM-6, and 8402 bound the greatest amounts of ricin, whereas K562 and 8392 bound the least amount of toxin. These binding data correlate with ricin sensitivities determined at 1 nmol/L toxin as measured in liquid culture and clonogenic assays (Figs 1 and 3), and suggest that variable
sensitivities to ricin/lactose are due in part to differences in ricin binding.

When normal bone marrow CFU-GM were treated with various concentrations of ricin, plated, and assayed for colony formation seven days later, (Fig 2), the ID$_{50}$ was approximately 0.1 nmol/L; surprisingly, progenitors treated with ricin plus lactose exhibited a similar value. The data presented are the means of triplicate determinations in three independent experiments. These results are in contrast to ricin treatment of cell lines and peripheral blood T cells, which exhibited considerably higher ricin ID$_{50}$ values in the presence of 0.1 mol/L lactose. Fourteen-day cultures produced similar results. These results indicate that lactose may not protect all cells from ricin toxicity, and it is fortuitous that CFU-GM appear relatively resistant to this toxin.

**Inhibition of T Colony Formation by TiOl-Ricin, 3A1-Ricin, and Ricin**

Figure 3 shows the effects of immunotoxin or ricin treatment (in the presence of lactose) on colony formation by the T cell line CEM, peripheral blood T lymphocytes, and the B cell line 8392. The data shown are the means of three independent studies. At ricin concentrations of < 1 nmol/L (with lactose), growth of normal T colonies was not affected, but at a concentration of 1 nmol/L, colony formation was inhibited by 46%. As predicted by the ricin-binding data (Table 1) and the liquid culture studies, the T cell line CEM was sensitive to 1 nmol/L ricin even in the presence of 0.1 mol/L lactose. Although CEM colony formation was completely blocked after treatment with 1 nmol/L ricin plus lactose, only minimal inhibition was observed at lower concentrations.

In contrast to the effects of ricin/lactose on peripheral blood T cells, treatment of these cells with 0.1 nmol/L TiOl-ricin in the presence of lactose inhibited colony formation by 98%, and complete inhibition of colony formation was observed at higher immunotoxin concentrations. No inhibition of colony formation was observed at conjugate concentrations below 0.1 nmol/L (data not shown). Treatment of normal T cells with 3A1-ricin produced dose-dependent killing over a concentration range of 0.1 to 3.0 nmol/L. Treatment of CEM cells with either 0.1 nmol/L TiOl-ricin or 3A1-ricin (data not shown) completely blocked colony formation. Similar results were obtained with 8402.

Results comparable to those shown in Fig 3 were obtained with liquid cultures; concentrations of TiOl-ricin or 3A1-ricin greater than 0.1 nmol/L completely abrogated CEM or normal T cell growth (data not shown). Treatment of cells from the B lymphoma line 8392 with 3 nmol/L TiOl-ricin or 3A1-ricin (data not shown) in the presence of lactose was without effect. Similar results were obtained after conjugate treatment of HL-60 (data not shown).
immunotoxin treatment with TlOl-ricin or 3A1-ricin in the presence of lactose (Figs 2 and 4) are compared, it appears that the conjugate is approximately 20-fold less toxic to the progenitors than ricin on an equimolar ricin basis. To determine whether this reduced nonspecific toxicity of the immunotoxin was due to damage of the ricin moiety during conjugation, protein synthesis inhibition by ricin and T101-ricin was determined in a cell-free lysate system. Assays were conducted in the absence of lactose and at equimolar ricin concentrations. In the absence of ricin or immunotoxin, protein synthesis was linear for approximately 30 minutes and reached a plateau value after 60 minutes of approximately 2.1 pmol total protein synthesized per microgram RNA. In the presence of 1 nmol/L ricin or 0.5 nmol/L T101-ricin, protein synthesis was inhibited, respectively, to 3% and 6% of the control value. The entire experiment has been repeated with similar results.

**Effects of T101-Ricin on Mixtures of CEM and Bone Marrow Cells**

Because T101-ricin was by far the most effective of the two immunotoxins, further studies to rule out toxic effects on normal cells were performed. Mixing of CEM and normal marrow cells did not appear to inhibit CEM colony growth (Table 2). Addition of

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Percent Positive</th>
<th>Mean Intensity of Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM (PBS control)</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>CEM</td>
<td>100</td>
<td>148</td>
</tr>
<tr>
<td>NALM-6</td>
<td>99</td>
<td>130</td>
</tr>
<tr>
<td>8402</td>
<td>100</td>
<td>112</td>
</tr>
<tr>
<td>HL-60</td>
<td>95</td>
<td>105</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>99</td>
<td>109</td>
</tr>
<tr>
<td>WIL-2</td>
<td>98</td>
<td>92</td>
</tr>
<tr>
<td>HPB-ALL</td>
<td>98</td>
<td>85</td>
</tr>
<tr>
<td>NAMALWA</td>
<td>99</td>
<td>75</td>
</tr>
<tr>
<td>HEL</td>
<td>87</td>
<td>72</td>
</tr>
<tr>
<td>HPB-NULL</td>
<td>90</td>
<td>53</td>
</tr>
<tr>
<td>K562</td>
<td>94</td>
<td>48</td>
</tr>
<tr>
<td>8392</td>
<td>79</td>
<td>37</td>
</tr>
</tbody>
</table>

These data show that whole-ricin immunotoxins, when assayed in the presence of 0.1 mol/L lactose, are selectively effective against T cell targets, are more effective than ricin plus lactose, and show similar levels of nonspecific cytotoxicity.

**Bone Marrow Progenitor Colony Formation After Immunotoxin Treatment**

Figure 4 shows growth of normal BFU-E and CFU-GM after treatment with T101-ricin or 3A1-ricin in the presence of 0.1 mol/L lactose. The data presented are the means of three independent experiments, and standard errors for data points were 10% or less. Treatment with either conjugate at a final concentration of 1 nmol/L produced 50% inhibition of granulocyte-macrophage colony formation; approximately 25% inhibition was observed for erythroid progenitors. At a T101-ricin concentration of 0.1 nmol/L, a concentration at which colony formation by normal T cells and T cell lines was inhibited by approximately 2 logs, bone marrow progenitor colony formation (CFU-GM and BFU-E) was inhibited by less than 30%. Regardless of the conjugate used, BFU-E was less sensitive to nonspecific cytotoxic effects of the immunotoxins than CFU-GM.

When the ID_{50} values for growth inhibition of CFU-GM treated with ricin or T101-ricin in the presence of
Table 2. Effect of T101-Ricin on Mixtures of CEM and Normal Marrow Colonies

<table>
<thead>
<tr>
<th>Cells Plated</th>
<th>Control</th>
<th>CFU-GM</th>
<th>CEM Colonies</th>
<th>0.1 nmol/L T101-Ricin</th>
<th>CFU-GM</th>
<th>CEM Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>−</td>
<td>NA</td>
<td>1,316 ± 395*</td>
<td>NA</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>NA</td>
<td>1,173 ± 221</td>
<td>NA</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Marrow</td>
<td>−</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NT</td>
</tr>
<tr>
<td>+</td>
<td>154 ± 46†</td>
<td>NA</td>
<td>1,173 ± 591</td>
<td>0</td>
<td>71 ± 73</td>
<td>73 ± 73</td>
</tr>
<tr>
<td>CEM + marrow</td>
<td>−</td>
<td>0</td>
<td>1,194 ± 582</td>
<td>149 ± 48</td>
<td>73 ± 73</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>155 ± 39</td>
<td></td>
<td></td>
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</tbody>
</table>

CEM, bone marrow cells, or a combination were incubated alone or with 0.1 nmol/L T101-ricin in the presence of 0.1 mol/L lactose, washed, and plated. Cells were grown alone or with placenta conditioned medium as a source of CSF and CFU-GM or CEM colonies distinguished by morphology. NA, not applicable; NT, not tested.

Colonies/10⁴ CEM cells; x ± SE, three experiments.
†CFU-GM/2 x 10⁴ marrow cells; x ± SE, three experiments.

CSF inhibited CEM colony growth by 12% and had the expected stimulatory effect on normal CFU-GM colony growth. When a mixture of 10% CEM and 90% normal marrow cells was incubated with 0.1 nmol/L T101-ricin in the presence of lactose, washed, and plated, 96% of CFU-GM survived, whereas 95% of CEM colonies were destroyed. These data indicate that the immunotoxin is effective in mixtures of normal and malignant cells, and that toxic, cell-bound ricin was not released into cultures after plating (see later).

Release of Ricin by Immunotoxin-Treated Cells

To further ensure that ricin (as immunotoxin) bound to cells and infused into patients would not be released later in vivo, we directly assessed release of toxic materials from immunotoxin-treated cells. Table 3 shows that after incubation with ricin, CEM and 8402 cells released toxic material into supernatant fluids. Toxicity was blocked by 0.1 mol/L lactose, indicating that it was due to ricin. When CEM cells were incubated with immunotoxin plus lactose, no toxic material was present, but lactose-inhibitable toxic material was present in 8402 supernatants. However, this toxicity was diluted out rapidly, indicating the presence of only small amounts of ricin. HL-60 cells plated simultaneously in agar with T101-ricin–treated T cells resulted in no inhibition of HL-60 cluster or colony formation (data not shown), confirming that significant amounts of ricin were not released by the T cells. These data indicate that the release of free ricin by immunotoxins is minimal, and that 0.1 mol/L lactose protects cells from released toxin.

DISCUSSION

In these studies we characterized in vitro effects of anti-T cell immunotoxins prepared with whole ricin. This approach was used because previous studies showed A chain immunotoxins were not consistently effective in vitro. Anti-T cell antibodies were

Table 3. Effect of Released Ricin on Secondary Cultures

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Dilution</th>
<th>Percent Control</th>
<th>Protein Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T101-PDP</td>
<td></td>
<td>100 ± 3</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>T101-Ricin</td>
<td></td>
<td>1 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Ricin</td>
<td></td>
<td>1 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Secondary cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T101-PDP</td>
<td>Neat</td>
<td>100 ± 13</td>
<td>100 ± 1</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>97 ± 7</td>
<td>111 ± 6</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>89 ± 1</td>
<td>94 ± 6</td>
</tr>
<tr>
<td></td>
<td>Neat + 0.1 mol/L Lactose</td>
<td>108 ± 41</td>
<td>73 ± 2</td>
</tr>
<tr>
<td>T101-Ricin</td>
<td>Neat</td>
<td>95 ± 33</td>
<td>66 ± 6</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>87 ± 16</td>
<td>85 ± 6</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>87 ± 3</td>
<td>98 ± 1</td>
</tr>
<tr>
<td></td>
<td>Neat + 0.1 mol/L Lactose</td>
<td>85 ± 7</td>
<td>85 ± 6</td>
</tr>
<tr>
<td>Ricin</td>
<td>Neat</td>
<td>35 ± 1</td>
<td>49 ± 3</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>62 ± 8</td>
<td>78 ± 8</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>79 ± 5</td>
<td>88 ± 7</td>
</tr>
<tr>
<td></td>
<td>Neat + 0.1 mol/L Lactose</td>
<td>93 ± 15</td>
<td>101 ± 6</td>
</tr>
</tbody>
</table>

Effect of released ricin on unexposed cells. The primary and secondary cultures were treated as described in Materials and Methods. The final reagent concentrations were 1 nmol/L during treatment of the primary cultures. The data represent the means ± SE of triplicate determinations from two separate experiments. Column A: CEM cells; column B: 8402 cells.
chosen as initial vectors because binding and toxicity for bone marrow cells had been studied previously. Relevant normal and cell line targets were also available, and colony assays for these cells were well described. T cell leukemias and lymphomas are relatively rare tumors, but they have assumed increased importance in areas where human T leukemia virus is endemic. T cell immunotoxins may also be useful for treating allogeneic marrow to prevent GVHD.

Certain aspects of our studies suggest that caution must be used in the in vitro evaluation of whole-ricin immunotoxins. Although Vallera et al reported that normal T cells and bone marrow progenitor cells exhibited equal sensitivities to ricin whether assayed in the presence or absence of lactose, we found that normal T cells, cell lines, and normal marrow cells exhibit varying ricin sensitivities. Most of this variance occurs at concentration ranges unlikely to be used for autologous transplantation, but one should not assume that diverse cells will show equivalent nonspecific toxicity with whole-ricin immunotoxins. Binding studies suggest that ricin toxicity is a function of cell surface-bound toxin, and our immunofluorescence data support this contention. Therefore, in evaluation of whole-ricin immunotoxins, controls for nonspecific and specific effects must be chosen carefully and based on proposed in vitro immunotoxin concentration and intrinsic sensitivity of cell targets.

Anti-T cell, whole-ricin immunotoxins were effective against antigen-positive targets. Although peripheral blood T lymphocytes were resistant to 0.1 nmol/L ricin in the presence of 0.1 mol/L lactose, a similar concentration of T101-ricin plus lactose inhibited colony formation by 98%. Less inhibition of T colony formation was observed with 0.1 nmol/L 3A1-ricin when assayed in the presence of lactose, suggesting that this immunoconjugate may be less well internalized than T101-ricin, and therefore less suitable for use in autologous transplantation. Similar results were recently reported by Strong et al; in a comparison of T101-ricin and VIII-1-ricin, the former was much more effective despite equivalent target cell binding. Thus, specific cytotoxic effects are observed with these conjugates, and their effects are not due to intrinsic ricin sensitivities.

In the presence of lactose, bone marrow progenitors showed much greater sensitivity to ricin than to either whole-ricin conjugate (compare Figs 2 and 4). Because approximately 2 mol of ricin was coupled to each mole of anti-T cell antibody, a conjugate concentration of 1 nmol/L is roughly equivalent to 2 nmol/L of ricin. When assayed in the absence of lactose, T101-ricin alone inhibited bone marrow CFU-C by 99%. Further, when ricin and T101-ricin were assayed for their ability to inhibit protein synthesis in a cell-free reticulocyte lysate system, both reagents produced similar levels of inhibition, suggesting that the ricin moiety of the immunotoxin was not damaged during conjugation. These data suggest that in the presence of lactose, whole-ricin conjugates are less toxic to bone marrow progenitors than are equivalent amounts of ricin used under similar conditions. Further, if one assumes that the ID<sub>50</sub> for T101-ricin treatment of T cells in the presence of lactose is <0.1 nmol/L (Fig 3), then T101-ricin is at least tenfold more toxic to antigen-positive target cells than to bone marrow progenitors (ID<sub>50</sub> ≈ 1 nmol/L; Fig 4). Thus, safe immunotoxin concentration ranges for removing malignant cells from human bone marrow would be between 0.1 and 0.3 nmol/L. T101-ricin plus lactose was effective in mixtures of marrow and malignant cells, indicating that normal marrow cells do not impede its action. The margin of protection for normal CFU-GM appeared even greater in mixing studies than when marrow cells alone were incubated with immunotoxin. This may indicate that antigen-positive targets bind immunotoxin and protect normal cells from nonspecific ricin toxicity. The 10% CEM mixture used here is higher than the percentage of malignant cells in remission marrows, and the number of proliferating malignant cells is much greater. Thus, the immunotoxin should be effective when used to treat remission T leukemia marrow.

A potential caveat in the use of whole-ricin conjugates for bone marrow treatment is the release of free ricin or conjugate from target cells after infusion into the patient. The marrow-mixing experiments suggest that this will not be a major problem. However, the data in Table 3 show that toxic material can be released on target cell death, and effects of lactose indicate that this material is free ricin. We estimated that approximately 0.1 μg ricin per kilogram of body weight might remain after treatment of bone marrow cells with 1 nmol/L immunoconjugate in the presence of 0.1 mol/L lactose. Fodstad has recently reported that patients tolerated whole ricin in concentrations up to approximately 23 μg/m<sup>2</sup> or approximately 0.6 μg/kg. Thus, the amount of free ricin complexed to the immunotoxin and infused into the patient should not produce excessive toxicity even if marrow is not washed after in vitro exposure.

This work suggests three conclusions relevant to the use of whole-ricin immunoconjugates for removal of malignant T cells from bone marrow: Primary cells and cell lines exhibit varying degrees of sensitivity to ricin, with and without lactose, and antibody–ricin conjugates with lactose. In the presence of lactose,
normal peripheral blood T lymphocytes and other T65-positive T cells are at least tenfold more sensitive to 0.1 nmol/L T101-ricin in vitro than are normal bone marrow progenitor cells, and the conjugates are effective on mixtures of normal and malignant cells.1 Treatment of infiltrated bone marrow with cell-specific immunotoxins in the presence of lactose should safely remove target cells without unduly damaging the marrow progenitor cells or leaving toxic amounts of ricin behind. Thus, T101 whole-ricin immunotoxins merit clinical trials for removing malignant T cells from human bone marrow.

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Preclinical studies on the use of selective antibody-ricin conjugates in autologous bone marrow transplantation

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