A Ricin A Chain-Containing Immunotoxin That Kills Human T Lymphocytes In Vitro

By Paul J. Martin, John A. Hansen, and Ellen S. Vitetta

An immunotoxin specific for human T lymphocytes was prepared by coupling an IgG\(_\alpha\)-anti-CD3 murine monoclonal antibody (64.1) to purified ricin A chain (64.1-A). Treatment of blood mononuclear cells with this immunotoxin at a concentration of \(1.7 \times 10^{-8}\) mol/L for two hours at 37 °C in the presence of 20 mmol/L NH\(_4\)Cl decreased phytohemagglutinin-stimulated protein synthesis by 95%. In addition, a sensitive culture assay showed that fewer than 0.03% T cells remained after treatment of human bone marrow mononuclear cells with 64.1-A at a concentration of \(1.7 \times 10^{-8}\) mol/L. The inhibition of protein synthesis could be prevented by preincubating cells with unconjugated 64.1 antibody but not by preincubating cells with a control IgG\(_\alpha\) antibody that binds to a different T cell antigen (CD5). At concentrations up to \(1 \times 10^{-8}\) mol/L, 64.1-A had little effect on blood mononuclear cells from baboons or human myeloid precursors (CFU-GM), which do not express the CD3 antigen recognized by 64.1. Taken together, these results indicate that the toxicity of 64.1-A was specific and that 64.1-A may be a useful reagent for depleting T cells from donor marrow as a means of preventing acute graft-v-host disease after allogeneic bone marrow transplantation.

WHEN TECHNIQUES were introduced for the generation of monoclonal antibodies of defined specificity,\(^1\) it was immediately recognized that these new reagents might have valuable therapeutic applications. Monoclonal antibodies have been infused in vivo in attempts to treat neoplastic diseases\(^2\) and graft-v-host disease (GVHD).\(^3\) They have also been used in vitro to remove malignant cells from autologous marrow grafts\(^4\) or T cells from allogeneic grafts.\(^5\) With few exceptions, however, the available monoclonal antibodies are ineffective at fixing human complement or triggering cytolytic effector cells.\(^6,9\) For this reason, investigators have synthesized hybrid molecules composed of antibodies covalently linked to such toxins as ricin, abrin, gelonin, pokeweed antiviral protein (PAP), and diphtheria toxin (DT).\(^10-16\)

Many immunotoxins have used ricin or its A chain as the toxic moiety.\(^17\) Immunotoxins prepared with intact ricin\(^18\) can be used for in vitro applications when treatment must be carried out in the presence of galactose or lactose in order to prevent nonspecific binding of the holotoxin B chain to cell surface galactosyl residues. In this report, we described the synthesis and characterization of an immunotoxin comprising an anti-CD3 monoclonal antibody and purified ricin A chain. We demonstrate that this immunotoxin induces specific in vitro killing of human T lymphocytes. Thus, the data suggest that this new reagent could potentially be used for depleting T cells from donor marrow as a means of preventing acute GVHD after allogeneic bone marrow transplantation.

**MATERIALS AND METHODS**

**Preparation of cells.** Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood by centrifugation over Ficoll-Hypaque (SG 1.077) and monocytes were isolated by adherence to plastic. Bone marrow was aspirated from the posterior iliac crest of healthy volunteers into heparinized syringes and the marrow mononuclear cells were isolated by centrifugation over Ficoll-Hypaque. Informed consent was obtained from blood and marrow donors.

**Monoclonal antibodies.** Monoclonal antibody 64.1 is a murine IgG\(_\alpha\) that recognizes the CD3 antigen of human T lymphocytes.\(^19\) This antigen is also recognized by antibodies Leu-4 and OKT3. Monoclonal antibody 10.2 is a murine IgG\(_\alpha\) that recognizes the CD5 antigen of human T lymphocytes.\(^20\) This antigen is also recognized by antibodies Leu-1, OKT1, and TIOI. Purified RPC-5 (Litton Bionetics, Charleston, SC), a murine myeloma protein of the IgG\(_\alpha\) subclass, was used as an irrelevant control antibody. The amount of antibody in ascites fluids was quantitated by competitive radioimmunoassays.

**Preparation of antibody-toxin conjugates.** Antibody 64.1 was purified from ascites fluid by protein A-Sepharose affinity chromatography.\(^21\) Highly purified ricin A chain was purchased from XOMA Corporation (San Francisco) and was verified to be free of contaminating B chains by a whole cell toxicity assay and by determining that the LD\(_{50}\) in mice was 1 mg.\(^22\) Antibody and purified ricin A chain were conjugated with the heterobifunctional cross-linker N-succinimidyl-3(2-pyridyldithio)propionate (SPDP, Pharmacia, Piscataway, NJ), followed by Sephacryl S-200 gel filtration to remove free ricin A chains.\(^23\) Conjugation of antibody and ricin A chain was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by radioimmunoassay with the use of rabbit anti-mouse Ig-coated microtiter plates and \(^{125}\)I-labeled rabbit anti-ricin A chain antiserum.\(^24\) Each molecule of antibody contained one to two molecules of ricin A chain. Conjugates were stabilized with carrier protein (reduced and alkylated human IgG) and stored at \(-70^\circ\) C.

**Treatment of cells with immunotoxin.** Peripheral blood mononuclear cells (5 \(\times\) \(10^5/50\) μL) were incubated for two hours at 37 °C with immunotoxin diluted in RPMI 1640 medium containing 12% fetal calf serum (FCS) and 20 mmol/L NH\(_4\)Cl. Cells were washed three times and resuspended in 100 μL of leucine-free RPMI containing 15% pooled human serum. Sixteen hours later, 25 μL of phytohemagglutinin (PHA) (12.5 μg/mL) were added, and the cultures were incubated for 48 hours at 37 °C. The cultures were pulsed with 1 μCi of \(^3\)H-leucine four hours before harvesting. Cells were harvested using a MASH apparatus, and leucine incorporation was quantitated by scintillation counting.

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Bone marrow mononuclear (BM) cells (5 x 10^6/500 μL) were treated similarly, and aliquots (1 x 10^6) were cultured for seven days in 1 mL RPMI 1640 medium containing 15% pooled human serum and 1 μg PHA. Viable cells were stained by indirect immunofluorescence using a mixture of anti-T cell antibodies as described previously.\(^24\) Brightly stained T cell blasts were enumerated by flow microfluorimetry (FACS IV, Becton Dickinson, Mountain View, Calif). This assay can reliably detect as few as 0.03% viable T cells.\(^24\) Aliquots of treated BM cells were also cultured in 0.3% agar containing 20% FCS and 50 UmL placental conditioned medium as a source of colony-stimulating activity. Myeloid colonies (CFU-GM) were enumerated as described elsewhere.\(^25\)

RESULTS

Specific inhibition of protein synthesis in T cells in 64.1-A. As shown in a representative experiment (Table 1), inhibition of PHA-stimulated protein synthesis in T cells could be achieved by incubating PBMCs for two hours at 37 °C with 64.1-A either in the presence or absence of 20 mmol/L NH₄Cl. The concentration of 64.1-A required to achieve 50% inhibition of protein synthesis (IC₅₀) in the absence of NH₄Cl was approximately 1 x 10⁻¹⁰ mol/L. In the presence of NH₄Cl, the same effect was achieved with approximately 1 x 10⁻¹⁰ mol/L 64.1-A. Thus, treatment with 64.1-A was 1,000-fold more efficient when incubations were carried out together with NH₄Cl. The dose–response curve for 64.1-A did show some variation, which was greatest at low immunotoxin concentrations. Results were more uniform at high immunotoxin concentrations. In the presence of NH₄Cl the IC₅₀ of 64.1-A was 2 x 10⁻¹² mol/L (Fig 1). In contrast, the control RPC-5-A was 100,000-fold less toxic than 64.1-A when used under identical conditions (IC₅₀ = 1 x 10⁻⁷ mol/L).

Adding untreated monocytes to 64.1-A–treated PBMCs did not reverse the inhibition of PHA-stimulated protein synthesis (data not shown), indicating that damage to accessory cells\(^26\) could not account for the effect of 64.1-A. Preincubation of PBMCs with unconjugated 64.1 antibody prevented the toxic effect of 1.7 x 10⁻¹⁰ mol/L of the 64.1-A (Fig 2). In contrast, preincubation of PBMCs with the anti-T cell antibody 10.2 (anti-CD5) had no effect on the cytotoxicity of 64.1-A. These results indicated that the anti-T cell effect of 64.1-A was caused by specific binding of the immunotoxin to CD3-positive cells. It was also evident that under these conditions, the unconjugated 64.1 antibody was not capable of inhibiting PHA-stimulated protein synthesis.

The CD3 antigen recognized by antibody 64.1 is not expressed on T cells from lower primates.\(^2\) Thus, it was not possible to determine whether the inhibition of PHA-stimulated protein synthesis might be in any way mediated by nonspecific binding of 64.1-A. Treatment of baboon PBMCs with 64.1-A had only a modest effect on P1-IA (Fig 3),

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<th>Table 1. Effect of NH₄Cl on Lysis of T Cells by 64.1-A*</th>
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<td>64.1-A Concentration (mol/L)</td>
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PBMCs were treated with serial tenfold dilutions of 64.1-A immunotoxin in the presence or absence of 20 mmol/L NH₄Cl. Numbers in the table represent cpm (X ± SEM) ³H-leucine incorporation in sextuplicate microcultures assayed 48 hours after PHA stimulation.

*Numbers in parentheses represent the percentage of cpm in control microcultures containing neither 64.1-A nor NH₄Cl. Calculations were corrected for a background of 265 cpm in wells containing no cells.
which was somewhat greater than the slight nonspecific effect of RPC-5-A on human T cells.

Specific inhibition of T cell growth by 64.1-A. Immunotoxin-treated marrow cells were cultured for seven days in medium containing PHA and then stained with monoclonal anti-T cell antibodies. It was found that T cell blasts were not detected after treatment of marrow mononuclear cells with 64.1-A at concentrations ≥1.7 × 10^{-9} mol/L (Fig 4). Human committed myeloid precursors assayed as CFU-GM do not express CD3. Treatment of marrow mononuclear cells with 64.1-A at concentrations ≤1.7 × 10^{-9} mol/L had little or no effect on the in vitro growth of CFU-GM (Fig 5). Taken together, these results demonstrated the feasibility of using 64.1-A to achieve effective specific in vitro killing of T cells without evident nonspecific toxicity to other cells.

**DISCUSSION**

In this report, we describe the use of ricin A chain conjugated to a murine monoclonal anti-CD3 antibody to achieve specific lysis of human T lymphocytes. The assay systems used for testing this immunotoxin were selected on the basis of its intended use for in vitro depletion of mature T cells from donor marrow as a means of preventing acute GVHD after allogeneic bone marrow transplantation. For this purpose, peripheral blood and bone marrow cells from normal donors were used to establish the experimental preclinical model. One of the ways used to assess the activity of the immunotoxin was to determine its effect on protein synthesis. This required the use of mitogen to induce T cell activation. PHA was selected because it can activate a high proportion of human T cells. In our assay system, the addition of PHA to the cultures was delayed in order to avoid any possible alteration of immunotoxin binding or internalization. This precaution may have been unnecessary in view of the results of Kernan et al., which showed that the effect of an anti-CD5-A immunotoxin was not influenced by the time interval between treatment of the cells and PHA stimulation. We chose to assay \(^3\)H-leucine incorporation rather than \(^3\)H-thymidine incorporation because ricin A chain primarily interferes with protein synthesis by catalytic inactivation of the 60S subunit of ribosomes. In separate experiments, we have found a close correlation between \(^3\)H-leucine incorporation assayed two days after PHA stimulation and \(^3\)H-thymidine incorporation assayed one day later.

Protein synthesis in human T lymphocytes was readily inhibited by the 64.1-A immunotoxin, and the inhibition was greatly enhanced by \(\text{NH}_4\text{Cl}\), similar to findings reported for other ricin A chain immunotoxins. It is possible that the increased lysosomal pH caused by incubation of cells in \(\text{NH}_4\text{Cl}\) may decrease intracellular proteolytic inactivation of A chains. When treatment of PBMCs was carried out for two hours at 37°C in the presence of 20 mmol/L \(\text{NH}_4\text{Cl}\), 1.7 × 10^{-10} mol/L 64.1-A inhibited PHA-stimulated protein synthesis by 90%. A 1,000-fold excess of unconjugated 64.1 antibody was required in order to prevent the toxicity of 64.1-A at this concentration. In separate experiments, we have found that the amount of surface binding occurring after incubation of T cells with 1.7 × 10^{-10} mol/L 64.1-A is
below the threshold of sensitivity for detection by indirect immunofluorescence and flow microfluorimetry (unpublished observations, Oct 1984). Taken together, these findings indicate that binding of less than 5,000 molecules of 64.1-A is sufficient for effective transport of ricin A chain to the cytosol.

The ability of unconjugated 64.1 antibody to inhibit the effect of the 64.1-A immunotoxin indicated that specific binding represented one pathway by which the immunotoxin could gain entry into T cells. The possibility that such reagents might gain entry by other pathways was evaluated by testing the effect of a control immunotoxin of irrelevant specificity (RPC-5-A) and by testing the effect of the 64-A immunotoxin on cells that do not express the CD3 antigen recognized by 64.1 antibody. At sufficiently high concentrations, RPC-5-A and 64.1-A were found to have to detectable nonspecific toxicity. The effect of RPC-5-A on human PBMCs was similar to the effect of 64.1-A on human CFU-GM. However, 64.1-A was more toxic for baboon PBMCs than for human CFU-GM. In separate experiments, we have found that preincubating baboon PBMCs with either 10.2 antibody for 64.1 antibody had little, if any, effect on the toxicity of 64.1-A. Thus, it appears that the nonspecific effects of immunotoxins may vary on different cells.

The results of this study suggest that treatment of donor marrow with 64.1-A may provide a degree of T cell depletion sufficient to prevent acute GVHD after HLA-identical allogeneic bone marrow transplantation. In previous studies we have shown that culturing T cell-depleted marrow for seven days in medium containing PHA can reliably detect as few as 0.03% residual viable T cells. With the use of monoclonal antibodies and rabbit complement, we have further demonstrated that depletion of T cells in donor marrow to <0.03% as detected by this assay can be sufficient to decrease the incidence of acute GVHD in recipients of HLA-identical allogeneic marrow grafts but was associated with an increased risk of graft failure. The data presented here demonstrate that a similar depletion of T cells from bone marrow can be achieved by in vitro treatment with 1.7 x 10^{-9} mol/L 64.1-A for two hours at 37 °C in the presence of 20 mmol/L NH_{4}Cl. In the concentration range between 1.7 x 10^{-9} mol/L and 1.7 x 10^{-8} mol/L, 64.1-A had a negligible effect on marrow CFU-GM.

Treatment of bone marrow with 64.1-A will not remove the CD3-negative subpopulation of T rosette cells known to contain large granular lymphocytes that have natural killer activity. It is not yet known whether such cells can initiate GVHD in humans. However, evidence from murine models has suggested that natural killer cells do not cause GVHD. In preliminary experiments we have found that CD3-negative E rosette-forming cells can proliferate in response to PHA and IL 2. Thus, limiting dilution assays that use these reagents cannot be used to determine the number of CD3-positive cells remaining after treatment of marrow with 64.1-A.

Our results and those of others have shown that effective killing of normal or malignant human T cells can be achieved with the use of ricin A chain conjugated to antibodies that recognize CD3, CD5, and CD7. However, not all T cell surface antigens may represent targets through which effective killing can be achieved by ricin A chain-conjugated monoclonal antibodies. Further studies are needed to elucidate the factors that govern the efficacy of ricin A chain-conjugated monoclonal antibodies.

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A ricin A chain-containing immunotoxin that kills human T lymphocytes in vitro

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