Use of Multiple T Cell-Directed Intact Ricin Immunotoxins for Autologous Bone Marrow Transplantation

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The monoclonal antibodies (MoAb) T101, G3.7, 35.1, and TA-1 were conjugated to intact ricin using a thioether linkage. These MoAb detect, respectively, the CD5[gp67], CD7[p41], CD2[p50], and [gp95,170] determinants that are found in the vast majority of cases of T cell acute lymphocytic leukemia (T-ALL). The resulting immunotoxins (ITs) and an equimolar mixture of these ITs were evaluated as potential pustuliferous reagents for autologous transplantation in T-ALL. Leukemic cell lines were used to compare the kinetics of protein synthesis inactivation mediated by each IT. The cells were treated with IT in the presence of lactose in order to block the native binding of ricin. The observed rates of protein synthesis inactivation correlated with target antigen expression detected by fluorescence-activated cell sorter analysis. Of the four ITs, T101-ricin (T101-R) exhibited the fastest rate of inactivation, followed in order by G3.7-ricin, TA-1-ricin, and 35.1-ricin. At concentrations >300 ng/mL, a cocktail containing an equimolar amount of all four ITs (referred to as the four-IT cocktail) exhibited kinetics that were as fast or faster than those of T101-R. The long-term cytotoxic effects of individual ITs and the four-IT cocktail were evaluated using a sensitive clonogenic assay. Each IT was specifically cytotoxic and inhibited 1 to 4 logs of clonogenic leukemic cells at doses (300 to 600 ng/mL) that can be used clinically. The four-IT cocktail was highly cytotoxic; a concentration of 300 ng/mL inhibited >4 logs of leukemic cells while sparing the majority of committed (CFU-GM, CFU-E) and pluripotent (CFU-GEEM) hematopoietic stem cells. The determination of both short-term kinetics of protein synthesis inactivation and longer-term inhibition of clonogenic growth allowed new insight into cell killing by IT. Our results suggest that ITs continue to act on clonogenic target cells for a period of three to five days. Interestingly, the four-IT cocktail was not as potent against clonogenic leukemic cells as T101-R alone, although it exhibited kinetics of protein synthesis inhibition that were as fast as those of T101-R alone. This finding suggests that internalized ITs may differ in the length of time they remain active within the cell. Our results also demonstrate the importance of using several different assays to evaluate IT reagents.

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A VARIETY OF immunologic and pharmacologic strategies are currently being evaluated for the ex vivo purging of neoplastic cells from patient bone marrow grafts. These autologous grafts are reintroduced into remission leukemic patients after aggressive suprarectal chemotherapy and irradiation. The objective of all strategies is to deplete neoplastic cells while leaving unharmed the pluripotent hematopoietic cells which repopulate the patient's marrow after reinfusion. The authors1,2 and others3,4 have demonstrated that immunotoxins (ITs)—antibodies chemically linked to ricin or other potent toxins—offer a high degree of selective and potent cytotoxicity. Our approach is based on extensive clinical experience using ricin-IT to eliminate graft-v-host disease—causing cells from allogeneic bone marrow grafts.4,4 Our experience has demonstrated that with our conditions of ex vivo treatment, intact ricin-ITs selectively eliminate antigen-positive targets without endangering engraftment and without causing ricin intoxication.

Ricin consists of two 30-kilodalton subunits linked by a disulfide bond.5 The A chain enzymatically inactivates eu-karyotic ribosomes, resulting in complete inhibition of protein synthesis, thereby causing cell death. The B chain facilitates transport of A chain into the cytoplasm.6 The B chain also binds to galactosyl residues on the cell surface, and treatment with intact ricin-IT requires the presence of lactose to competitively block this galactosyl-binding site.7 In the presence of lactose, ITs bind via their antibody-binding sites to antigen-positive target cells and exhibit minimal toxicity to stem cells.

Effective ITs require target selective monoclonal antibodies (MoAb). The selection of MoAb for elimination of T cell acute lymphocytic leukemia (T-ALL) cells from autologous marrow is made difficult by the phenotypic heterogeneity encountered in this disease.8 Over the past year, a study at our institution has identified four determinants (CD2, CD5, CD7, and [gp95,170])2,3 which are expressed frequently in T-ALL. Because these determinants are not expressed on normal hematopoietic precursor cells,4 the MoAb recognizing them are attractive candidates as ITs for purging of leukemic cells.

In the present study, we evaluated ITs directed to these determinants as potential reagents for autologous marrow transplantation. We compared the specific cytotoxic effect of each IT on leukemic cell lines using a sensitive clonogenic assay and kinetic analysis of protein synthesis inactivation. Each IT was found to be specifically cytotoxic to cells bearing target antigens. Because combined use of ITs may be required to eliminate the heterogeneous leukemic cells present in remission marrow, we evaluated cocktails of these ITs. A cocktail containing all four ITs eliminated >4 logs of clonogenic leukemic cells at doses that spared >70% of the pluripotent hematopoietic progenitor cells (CFU-GEEM).

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in normal human marrow. Thus, we believe that this cocktail may effectively eliminate occult neoplastic cells in the auto-
logous marrow grafts from patients with T-ALL/lymphoma
without compromising engraftment.

The assay systems we used to evaluate ITs covered
different time frames. The results obtained with these com-
bined assays provide new insight into cell killing mediated by
ITs and demonstrate the advantage of our approach for the
evaluation of ITs.

MATERIALS AND METHODS

Antibodies. Four murine MoAb which bind to different
determinants on human T lymphocytes were selected. T101, an IgG2a,
recognizes the CD5[T,667] determinant; 35.1, an IgG2a, rec-
ognizes the CD2[T,p50] molecule associated with the E rosette recep-
tor; G3.7, an IgG1, recognizes the CD7[T,p41] determinant; and
TA-1, an IgG2a, recognizes the 95/170-kilodalton glycoprotein on
peripheral blood T cells, monocytes, and some bone marrow myelo-
monocytic precursors. 7 Serologic cluster designations were assigned
at the First (Paris, 1982) and Second (Boston, 1984) International
Workshops on Human Leukocyte Differentiation Antigens.8,9
Monoclonal GK1.5 9 binds the p52 determinants on murine T
lymphocytes. All MoAb were purified over protein A-Sepharose
(Pharmacia, Uppsala, Sweden). 10

Immunotoxin synthesis. Details of the procedure to covalently
link intact ricin to MoAb by using the cross-linking agent M-
maleimidobenzyl-N-hydroxysuccinimide ester (MBS) have been
previously reported. 11 Briefly, ricin was treated with MBS to intro-
duce maleimide residues, and purified MoAb was partially reduced
with dithiothreitol to generate free sulfhydryl groups. The two
species were mixed, forming a non-reducible thiether bond. The
MoAb-ricin conjugates were purified by high-pressure liquid chro-
matography and affinity chromatography. Analysis of IT by sodium
dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated
little or no unconjugated antibody or ricin and revealed conjugate
species with one or two ricin moieties per antibody.

Cell lines. MOLT7 12 and MOLT4 13 were derived from patients
with T-ALL and express membrane markers characteristic of T
cells. KM 3 and NALM-6 2 were derived from non-T-ALL and
exhibit characteristics of B cell precursors. Cells were maintained as
previously described. 1

Protein synthesis inhibition. For kinetic analysis, leukemic cells
were treated with ITs using a modification of our standard protocol.4
Washed cells (10 5 per milliliter) were incubated with or without IT
in leucine-free RPMI 1640 containing 10% heat-inactivated dia-
lyzed fetal calf serum (FCS) and 100 mmol/L lactose at 37 °C in a
humidified atmosphere of 5% CO2/95% air. At designated intervals,
aliquots of cells were diluted tenfold and 100-μL volumes (10 5 cells)
were transferred to triplicate wells of 96-well flat-bottom tissue
culture plates (Costar, Cambridge, Mass). Each well was pulsed for
two hours at 37 °C with 1 μCi of [3H]-leucine (140 Ci/mmol;
Amersham, Arlington Heights, Ill), harvested with an automated
cell harvester (Brandel, Gaithersburg, Md), washed with water, and
dried. 5[3H]-Leucine incorporation was determined using standard
scintillation counting techniques. In order to compare experiments
easily, results were expressed as a percentage of the lactose control.
Using this technique, we found that [3H]-leucine incorporation was
directly related to the number of cells per well over a range of 10 5
to 10 7 cells.

Colonies assay. For IT comparisons based on inhibition of clono-
genic growth, our standard IT treatment protocol was used without
modification. 4 Ten million cells per milliliter were incubated with IT
in RPMI 1640 containing 2% human serum albumin ([HSA] Cutter
Laboratories, Berkeley, Calif), and 100 mmol/L of lactose for two
hours at 37 °C in a humidified atmosphere of 5% CO2 in air. After
treatment, cells were washed twice in lactose medium.

The colony assay for human leukemic cell lines has been
previously described in detail.1 Following treatment with IT, washed
leukemic cells were suspended in o-minimum essential medium
(MEM) (KC Biologicals, Lenexa, Ky) containing 10% FCS, anti-
biotics, and 0.8% methylcellulose at a cell concentration of 2.5 × 10 3
per milliliter. Cells were dispensed into 96-well flat-bottom micro-
titer plates; eight to 1,000 replicate wells were plated depending
on the IT treatment concentration. Cells were incubated at 37 °C in a
humidified atmosphere of 5% O2/5% CO2/90% N2. After seven to
14 days, cultures were evaluated and groups of 20 or more cells
were scored as colonies. For control cultures, the means ± SD were
determined from eight replicate wells, and the cloning efficiency was
calculated. The total number of colonies in treated cultures was
counted. The results were expressed using the formula:

\[ \text{% control response} = \frac{\text{cloning efficiency} \times \text{No. of cells plated}}{100} \]

Stem cell assays. The effect of IT on normal committed (CFU-
GM, CFU-E) and pluripotent (CFU-GEMM) bone marrow precu-
sor cells was evaluated using a modification 2 of the technique
described by Fauser and Messner. 1 Mononuclear cells, obtained
from normal bone marrow by Ficoll-Hypaque density centrifuga-
tion, were cultured (1.5 × 10 6 per milliliter) in 1 mL of Iscove's
modified Dulbecco's medium containing 0.9% methylocellulose, 30%
fresh human plasma, 5% medium conditioned by lymphocytes
stimulated with phytohemagglutinin, 6.5 × 10 -3 mol/L 2-mercap-
noethanol, and 1 unit of erythropoietin (Connaught Labs, Willow-
dale, Ontario, Canada). Cultures were incubated at 37 °C in a
humidified atmosphere of 5% CO2/95% air and evaluated after 14
days. The results are derived from three replicate dishes.

Immunofluorescence analysis of cell lines. The binding of
unconjugated MoAb to leukemic cell lines was determined by
standard indirect immunofluorescence. 4 One million washed
leukemic cells were incubated with 0.6 μg/mL (similar to concentra-
tion of MoAb in clinical doses of IT) or 10 μg/mL (near-saturating
concentration) of MoAb or mixtures of MoAb. Mixtures contained
equimolar concentrations of the MoAb (totaling 0.6 or 10 μg/mL).
Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG
(Tago, Burlingame, Calif) served as secondary antibody. Cells were
analyzed using a FACS IV (Becton Dickinson, Mountain View,
Calif). Fluorescence profile data were plotted with the aid of an
Apple II computer (Apple Computer, Inc, Cupertino, Calif) as
histograms of relative intensity over 256 channels (x axis) v
the number of cells in each channel (y axis). The background fluo-
cescence has been subtracted from these histograms. Background
staining was determined using an IgG2a murine myeloma protein
(UPC 10; Litton-Bionetics, Charleston, SC) as primary antibody.
All MoAb were assayed in the same experiment.

Reactivity of MoAb with patient bone marrow. Thirty-four
patients with T-ALL from the University of Minnesota and affili-
ated institutions were included in this study. Newly diagnosed and
relapse patients, both children and adults, were tested. Bone marrow
samples were obtained with informed consent under the guidelines of
the Committee for Use of Human Subjects in Research at the
University of Minnesota. Mononuclear cells, obtained by Ficoll-
Hypaque density centrifugation, were tested by indirect immuno-
fluorescence with 35.1, TA-1, T101, either G3.7 or 3A1 (both detect
the CD7 determinant), 12 and an equimolar mixture of the four
MoAb. In some cases, cell numbers were not sufficient to test all
MoAb. Fluorescence was determined using a Spectrum III cyto-
fluorograph (Ortho Diagnostics, Raritan, NJ). Samples were
defined as positive if >20% of the cells were reactive.
RESULTS

MoAb reactivity in T-All. Bone marrow from 34 patients with T-ALL were assayed for reactivity with MoAb to identify determinants that might be potential targets for IT-mediated purging of bone marrow autografts. 35.1, which detects the CD2 (p50/E rosette receptor), reacted with 29 of 33 patients tested (88%). TA-1, an anti-gp95,170, reacted in 16 of the 17 patients tested (94%). T101, which detects the CD5 determinant, was positive in 17 of 18 patients (94%). The CD7 determinant detected by 3A1 or G3.7 was expressed in all patients tested (seven patients, 100%). Notably, staining with a mixture of all four MoAb yielded a percentage of positive cells that was as high or higher than that obtained with any single MoAb (24 cases tested).

Immunofluorescence analysis of leukemic cell lines. Representative fluorescence-activated cell sorter (FACS) histograms of the T cell lines MOLT3 and MOLT4 following treatment with 0.6 μg/mL of MoAb or MoAb mixtures are shown in Fig 1. This concentration was tested because it approximates the optimal concentration used for patient IT treatment in our institution. Greater than 90% of MOLT3 cells bound T101, G3.7, and TA-1. Essentially identical histograms were obtained when cells were treated with 10 μg/mL (data not shown). Reactivity of 35.1 with MOLT3 was concentration dependent: 27% of cells were positive with 10 μg/mL, whereas only 19.8% were positive with 0.6 μg/mL. This finding indicates that P50 sites were not saturated at the lower antibody concentration. In contrast to MOLT 3, MOLT4 reacted strongly only with T101 (88.5%). The percentage of TA-1-positive cells ranged from 27% to 45.7%, depending on MoAb concentration used for treatment. MOLT4 reacted only slightly with G3.7 and 35.1; 9.2% and 9.8% of cells, respectively, were positive after treatment with 10 μg/mL of these MoAb. The lower reactivity of MOLT4 with TA-1, 35.1, and G3.7 at 0.6 μg/mL, as compared with 10 μg/mL, indicates that these MoAb were not saturating at the lower concentration.

An equimolar mixture of T101 and TA-1 stained MOLT 3 to yield a higher fluorescence intensity and slightly higher percentage of positive cells than single MoAb at the same concentration. An equimolar mixture of all four MoAb gave a more pronounced increase than the two-MoAb mixture. In contrast, MOLT4 cells showed only a slight (but nevertheless reproducible) increase in fluorescence intensity and the percentage of positive cells when stained with MoAb mixtures. The non-T cell lines NALM-6 and KM3 showed no reactivity with the four MoAb individually or with two- or four-MoAb mixtures. All fluorescence studies were performed with MoAb since we have demonstrated that ITs give FACS profiles that are very similar to those obtained with unconjugated MoAb (data not shown).

Effect of IT on kinetics of protein synthesis inhibition. Ricin conjugates of the four T cell MoAb were tested for their ability to inhibit the rate of protein synthesis in MOLT 3 cells. Cells were incubated with IT in the presence of lactose, and at defined time intervals over a 24-hour period, the incorporation of [3H]-leucine was measured. The results, expressed as a percentage of the control level, are plotted in Fig 2 as a function of time. After a lag period of one to four hours, the rate of protein synthesis decreased linearly for each IT. These kinetics indicate a first-order single-hit process. The rate of inactivation was concentration dependent, with higher concentrations giving faster rates. However, in the case of T101-ricin (R) (Fig 2A) concentrations >300 ng/mL had little additional effect in reducing protein synthesis. G3.7-R approached maximal inactivation at 600 ng/mL (Fig 2B). TA-1-R and 35.1-R showed increasing rates even at 1,000 ng/mL (Fig 2C and D). Concentrations >1,000 ng/mL were not tested because such treatment has previously been shown to overcome the lactose blockade and to result in some IT binding via ricin B chain.

The ITs differed in their effectiveness against MOLT3. This is most clearly demonstrated by comparing the time required for each IT (1,000 ng/mL) to reduce protein synthesis by 1 log (90%). This value, denoted T10, was 24 hours for T101-R, 35 hours for G3.7-R, 47 hours for TA-1-R, and 57.5 hours for 35.1-R. Thus, nearly a 2½-fold difference in inactivation rates was observed in this system between the fastest (T101-R) and the slowest IT (35.1-R).

To evaluate the effectiveness of multiple ITs directed to different membrane determinants, MOLT 3 cells were treated with a cocktail containing two or four ITs. The two-IT cocktail contained equal concentrations of TA-1-R and T101-R. Equimolar amounts of T101-R, TA-1-R, G3.7-R, and 35.1-R comprised the four-IT cocktail. The four-IT cocktail gave faster inactivation rates than the two-IT cocktail at all concentrations tested (Fig 3), and both cocktails gave faster rates than G3.7-R, TA-1-R, and 35.1-R individually. At 100 ng/mL total IT, T101-R showed faster inactivation than either cocktail (Fig 3A). At 300 and 1,000 ng/mL, however, inactivation by the four-IT cocktail was as fast as or faster than by T101-R (Fig 3B and C); the four-IT cocktail (1,000 ng/mL) showed a T10 value of 20.5 hours.
Fig 2. Kinetics of inhibition of protein synthesis in the MOLT3 cell line by anti-T cell intact ricin conjugates. Time plotted is the incubation time plus one half of the two-hour [3H]-leucine pulse. Six time points (1, 3, 5, 7, 9, and 23 hours) were assayed; for clarity, not all points are shown. Each point represents the mean of two to four experiments, each assayed in triplicate. Numbers represent the concentrations of IT added to the cells at time zero. Control cultures were incubated with lactose without IT. Lines were determined from all data points by least-squares linear regression analysis.

Inactivation by the two-IT cocktail was not as rapid as by T101-R at any concentration tested; the T_{50} value for the two-IT cocktail (1,000 ng/mL) was 27 hours.

The question arises as to whether the effectiveness of the four-IT cocktail was due solely to T101-R. To evaluate the contribution of T101-R, the percentage of residual protein synthesis observed after 24-hour treatment with the cocktails (Fig 3A through C) is replotted in Fig 3D as a function of the concentration of T101-R in the cocktails. For example, the 6.4% residual activity observed after treatment with 1,000 ng/mL of four-IT cocktail is plotted at 250 ng/mL because T101-R comprises one quarter of the total four-IT concentration. The modified dose–response curve of the two-IT cocktail closely follows that of T101-R alone; the entire inhibitory effect, therefore, may possibly have been due to T101-R. In contrast, inhibition by the four-IT cocktail at concentrations above 300 ng/mL (75 ng/mL of T101-R) was greater than expected if mediated by T101-R alone; this indicates that the other ITs in the cocktail also contributed to the observed level of inhibition.

Three types of experiments were performed to evaluate the specificity of the four-IT cocktail. First, the antigen-negative

Fig 3. Comparison of kinetics of inhibition of protein synthesis in MOLT3 cells by individual IT and cocktails containing two or four ITs. The kinetic curves for individual ITs are those presented in Fig 2. 2IT, an equimolar mixture of T101-R and TA-1–R; 4IT, an equimolar mixture of T101-R, TA-1–R, G3.7-R, and 35.1-R. Cells were treated with a total concentration of 100 ng/mL (A), 300 ng/mL (B), and 1,000 ng/mL (C). In D, the percentage of protein synthesis remaining after 24 hours of incubation with IT is plotted as a function of the T101-R concentration present during treatment. Data are derived from four separate experiments in which the cocktails were compared side-by-side to either T101-R or G3.7-R. Points represent the means of these separate determinations. Lines were fit by least-squares linear regression analysis.
cell line NALM-6 was treated with up to 1,000 ng/mL of each of the four ITs comprising the cocktail; <10% inhibition of protein synthesis was observed in each case. Treatment with up to 1,500 ng/mL of the four-IT cocktail inhibited protein synthesis <6%. The NALM-6 line is approximately twofold more resistant to ricin in the presence of 100 mmol/L of lactose than MOLT3. Second, MOLT3 cells were treated with an IT synthesized using GK1.5, a MoAb specific for murine T cell subpopulations but unreactive with MOLT3 by immunofluorescence. Treatment with 1,000 ng/mL of this control IT induced no inhibition of protein synthesis. Third, MOLT3 cells were treated with an equimolar mixture of the unconjugated MoAb comprising the four-IT cocktail; 1,000 ng/mL of this mixture augmented protein synthesis to 155% of the control value. This finding demonstrates that the observed toxicity of the four-IT cocktail was mediated by the ricin moiety of the IT.

To determine if cocktails might be used effectively against patients’ cells that express low levels of one or more of the target antigens, MOLT4 cells were treated with four-IT cocktail and the inhibitory effect was compared to that of individual ITs. Two of the ITs, G3.7-R and 35.1-R, were minimally toxic to MOLT4; 24 hours after treatment with 1,000 ng/mL, protein synthesis was reduced only 16% and 28%, respectively. Such low inhibition is consistent with the low to negligible levels of CD2 and CD7 antigens detected on these cells by FACS analysis (Fig. 1). In contrast, identical treatment of these cells with T101-R and TA-1-R reduced protein synthesis by 92% and 76%, respectively. The kinetics of inactivation by the cocktail and T101-R are shown in Fig 4. The cocktail mediated slower inactivation than T101-R at 100 and 600 ng/mL; however, at 1,000 ng/mL the inactivation rate equaled that observed with T101-R. The data are replotted in Fig 4D as a modified dose–response curve based on T101-R concentration within the cocktail. Although the curves diverge slightly at concentrations >75 ng/mL (ie, 300 ng/mL cocktail), the similarity between them suggests that the three other ITs contribute little additional inhibition to that mediated by T101-R.

**Effect of IT on leukemic cell clonogenic growth.** Clonogenic assays provide a precise determination of the proportion of cells ultimately killed by IT. For clonogenic assays, MOLT3 cells were treated for two hours with various concentrations of individual ITs and the four-IT cocktail, washed, and plated. Resulting colonies, expressed as a percentage of lactose-treated cultures, are shown in Fig 5. In these experiments the cloning frequencies of untreated MOLT3 cells ranged from 13% to 51%. The cocktail and individual ITs inhibited colony growth in a dose-dependent fashion. Each of the four ITs were cytotoxic to MOLT3. 35.1-R inhibited 0.8 logs at 1,000 ng/mL; TA-1-R mediated 1.5 logs and G3.7-R mediated 3.85 logs of inhibition at this dose. T101-R was the most effective inhibitor of MOLT3; a dose of only 30 ng/mL mediated 2.1 logs of inhibition, and 100 ng/mL mediated 4.4 logs of inhibition. Using doses which effected 1 log (90%) of inhibition for comparison, we can conclude that T101-R was 12-fold more potent to these cells than G3.7-R, more than 25-fold more potent than TA-1-R, and more than 150-fold more potent was 35.1-R.

The four-IT cocktail was also highly effective against clonogenic MOLT3 cells (Fig 5). Treatment with 100 ng/mL inhibited 2.3 logs and 300 ng/mL inhibited 4.1 logs of colony growth. The cocktail was clearly more effective than G3.7-R, TA-1-R, and 35.1-R. Surprisingly, the cocktail was less effective than T101-R at the concentrations tested and at extrapolated higher doses. Repploting the cocktail results as a function of T101-R concentration suggested that, against this cell target, the other IT contributed little to the killing mediated by the extremely potent T101-R. Nevertheless, from a clinical perspective, it is significant that the cocktail eliminated more than 4 logs of clonogenic leukemic cells at a concentration of 300 ng/mL. In contrast to the effect on
MOLT3, the four-IT cocktail, at doses up to 1,000 ng/mL, had no effect on colony formation by the antigen-negative target KM3 (data not shown).

Effect of pretreatment period. The length of IT treatment differed for the protein synthesis assay (up to 24 hours) and the clonogenic assay (two hours). This difference may have accounted for the disparity in results between the two assays for the four-IT cocktail relative to T101-R. To test this hypothesis, MOLT3 cells were treated with IT for two hours, washed, and then assayed 24 hours later for [3H]-leucine uptake. In parallel experiments, cells were incubated with IT for 24 hours and assayed directly for [3H]-leucine uptake. In both cases, the cocktail was as inhibitory as or more inhibitory than T101-R alone (at total IT concentrations >300 ng/mL). These results indicate that the decreased effectiveness of the cocktail in the clonogenic assay was not due to a shorter treatment with IT.

Toxicity to normal stem cells. In two experiments, bone marrow mononuclear cells were incubated for two hours with various concentrations of cocktail in the presence of lactose, washed, and assayed by standard in vitro culture techniques. As shown in Table 1, about 20% of CFU-GEMM were inhibited by treatment (300 ng/mL) which effected 4.1 logs of inhibition of leukemic T cells. CFU-GM and CFU-E were slightly more sensitive to four-IT treatment, although the highest concentration tested inhibited progenitor cells by only 30% to 40%. Notably, the morphology and cellular composition of CFU-GEMM colonies in control and treated (up to 1,000 ng/mL IT) cultures were identical. These results suggest that purging with four-IT cocktail would not significantly inhibit the repopulation capacity of autologous marrow grafts.

DISCUSSION

Methodologies to remove residual leukemic cells from autologous bone marrow are being studied intensively in a number of laboratories. Many groups are employing IT based on the selective and potent cytotoxicity of these agents1-4 and their clinical advantages for in vitro depletion of select cell populations.23 We have proposed that purging with mixtures of ITs directed to different determinants might improve therapy, but little study has been directed to this approach.26,27

In the present study, we evaluated a cocktail of four intact ricin ITs—T101-R, G3.7-R, TA-1-R, and 35.1-R. Our four-IT cocktail proved to be highly effective, eliminating >4 logs of clonogenic leukemic cells while sparing most pluripotent hematopoietic precursor cells (CFU-GEMM). Individually, the component ITs were specifically cytotoxic to leukemic T cells, inhibiting 1 to 4 logs of clonogenic cells at doses that can be used clinically.7

Individual ITs exhibited different rates of protein synthesis inactivation. T101-R gave the fastest kinetics, followed in order by G3.7-R, TA-1-R, and 35.1-R. The relative potency

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<th>CFU-E</th>
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<tr>
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<td>Mean ± SD</td>
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*Expressed as a percentage of the average number of colonies in cultures treated with lactose without IT.
of individual ITs based on inhibition of clonogenic growth correlated well, with a ranking based on the rate of protein synthesis inactivation. The actual percentage of inhibition detected in the two assays differed because the protein synthesis assay measures a short-term effect (24 hours) whereas the clonogenic assay measures a long-term effect (seven to nine days). We would expect to see increased inhibition beyond 24 hours using the protein synthesis assay, were it not that the sensitivity of this assay is limited to 1 to 2 logs. Based on the observed time required to inhibit 1 log of protein synthesis in kinetic studies, we estimate that ITs require three to five days to effect the 1 to 4+ logs of inhibition observed in the clonogenic assay. Because the doubling time of MOLT3 cells in culture is one to two days, we conclude that ITs inhibit cell proliferation before they inhibit total protein synthesis in target cells.

The relative efficacy of ITs directed to different determinants correlated to the expression of those determinants on target cells as determined by FACS analysis. However, other factors must also have influenced the relative effectiveness of our ITs. For example, T101-R and G3.7-R exhibited very similar fluorescence on MOLT3 but differed 1½-fold in the maximal rate of protein synthesis inhibition (based on $T_{1/2}$ values) and differed 12-fold in the concentration required to inhibit one log of leukemic cell clonogenic growth. It is possible that our FACS results, which were obtained using indirect immunofluorescence, may not accurately reflect antigen number. Alternatively, receptor number alone may not predict IT-mediated toxicity. This is known to be true in certain cases for diphtheria toxin and ricin. Characteristics of the receptors themselves may also account for the observed differences among ITs. Receptor-mediated endocytosis is the likely mechanism by which ITs enter the cell, and various proteins differ in the rate at which they undergo endocytosis. Perhaps antibody affinity plays a major role in the observed differences. Previous studies have demonstrated the importance of high antibody affinity for effective ITs. Subtle differences in the way the MoAb are linked to ricin may also play a role.

Our cocktail of four ITs exhibited faster kinetics than G3.7-R, TA-1-R, and 35.1-R alone at all concentrations; however, the kinetics approached those of T101-R only at concentrations $>300$ ng/mL. This suggests that at low concentrations the effect of T101-R was diluted by the less potent ITs within the cocktail. Above 300 ng/mL, however, the cocktail exhibited rates of inactivation that could not be attributed to T101-R. In contrast to the four-IT cocktail, the two-IT cocktail exhibited kinetics that did not approach those of T101-R at any concentration. Since the two-IT cocktail contained twice the T101-R concentration of the four-IT cocktail at each dose, these findings provide further evidence that within the four-IT cocktail, ITs other than T101-R contributed to the observed inhibition.

Our kinetic findings suggested that the four-IT cocktail at concentrations $>300$ ng/mL would be as effective as or more effective than T101-R treatment alone. We were surprised to find, therefore, that in the clonogenic assay, even high concentrations of the cocktail would not mediate killing as effectively as T101-R. The disparity between the two assays was not due to the IT incubation period, which can influence the binding of low-affinity ITs to target cells.

One explanation for the disparity between the two assay systems may be that the clonogenic subpopulation evaluated in the clonogenic system differed in phenotype from the bulk population evaluated in the protein synthesis assay. A more intriguing possibility is that T101-R, due to some feature of the MoAb or the p67 receptor, continued to act on target cells for a longer period than the other ITs, and thereby was able to effect higher levels of clonogenic inhibition than the cocktail. Agents that increase intralysosomal pH potentiate the toxicity of A chain ITs, possibly by protecting the A chain from degradation. Perhaps, the p67 receptor or the T101 MoAb are also able by some unknown mechanism to protect the toxic A chain from intralysosomal degradation.

We believe that our four-IT cocktail may mediate better elimination of residual leukemic cells from remission bone marrow than T101-R alone. Our experimental findings may not appropriately reflect the true therapeutic advantage of the four-IT cocktail because T-ALL cell lines strongly express CDS[p67] on virtually all cells, and thus may be much more sensitive to T101-R than fresh cells. In contrast, normal T cells represent a heterogeneous population and we have previously demonstrated that against this mixed population, an IT cocktail was more effective than single ITs. Fresh leukemic cells are also much more heterogeneous than cultured cell lines. For example, in our study, the proportion of CDS[p67]-positive cells in leukemia marrow ranged from 0% to 84%. Notably, in our survey, we found that the percentage of reactivity with a cocktail of the four MoAb was as high or slightly higher than with any individual MoAb. We have isolated leukemic cell clones which resisted treatment with high concentrations of T101-R and have shown that resistance was likely related to lower levels of CDS[p67] expression. This is an additional argument that treatment with a single IT may not provide adequate purging of autologous marrow.

The question arises as to whether or not IT cocktails for autograft purging should be tailored to each T-ALL patient. The results of our kinetic studies with MOLT4 indicated that toxicity of the four-IT cocktail was mediated primarily by the T101-R component. These results imply that there is no advantage to including ITs in a cocktail if the target determinants are absent or expressed in low levels on the target leukemic cells. However, there are major problems involved in tailoring IT cocktails to individual patients. T cell malignancies have undergone phenotypic changes following chemotherapy; thus, the phenotype at diagnosis or relapse may not be a reliable basis for selecting ITs to treat remission bone marrow. In addition, certain determinants vary in expression during the cell cycle. Clearly, the lack of information regarding the phenotype of the clonogenic cell in T-ALL, and whether or not this varies from patient to patient, remains a major problem.

Because our four-IT cocktail is directed to determinants expressed in the vast majority of cases of T-ALL, we believe that its use will increase the likelihood of eliminating residual leukemic cells, especially the crucial clonogenic cells, from remission bone marrow. Our cocktail is specific and highly effective.
effective; a concentration of only 300 ng/mL eliminated 4.1 logs of clonogenic MOLT3 cells but inhibited only 30% (0.2 logs) of pluripotent stem cell activity. This concentration represents half the dose which has been used safely ex vivo to purge allogeneic marrow of GVHD-causing cells. Higher concentrations of the cocktail up to 1,000 ng/mL might be considered for clinical application, since these doses were not more toxic to stem cells and yet would mediate increased inhibition of leukemic cell targets.

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