Elimination of Clonogenic T-Leukemic Cells from Human Bone Marrow Using Anti-M, 65,000 Protein Immunotoxins

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ABSTRACT

Two anti-M, 65,000 protein (p65) murine monoclonal antibodies, T101 and VIII-1, were conjugated to intact ricin. Toxicity of the resulting immunotoxins (IT) was measured against leukemic cell lines treated alone and in the presence of excess bone marrow using a highly sensitive colony inhibition assay. Cells were pretreated with IT in the presence of lactose to block the native binding of ricin.

The IT proved to be potent cytotoxins for the p65-positive cell lines, CEM and MOLT-4. Treatment with T101-ricin (1000 ng/ml) inhibited clonogenic activity of these lines by more than 5.1 logs. Less than 1 log of the inhibition at this dose was due to nonspecific killing by IT. Notably, the presence of excess bone marrow did not reduce IT toxicity against the leukemic populations. Comparison of IT concentrations which inhibited 50% of clonogenic activity showed that T101-ricin was 140- to 540-fold and VIII-1-ricin was 12- to 192-fold more toxic to p65-positive than to p65-negative cell lines. Neither unconjugated anti-p65 nor IT prepared with an irrelevant antibody inhibited clonogenic activity. Blocking of IT toxicity by unconjugated antibody further demonstrated that the antibody moiety of the IT directed the selective toxicity. We found that T101-ricin was more toxic for CEM cells than was VIII-1-ricin, even though blocking studies indicated that the two antibodies bind to proximal or identical epitopes. This report is unique in that an IT was shown to specifically eliminate greater than 99.99% of leukemic cells from human bone marrow. These findings indicate the utility of T101-ricin as an in vitro reagent for autologous bone marrow transplantation in treatment of T-cell leukemia.

INTRODUCTION

Therapy for human leukemia is currently limited by the toxicity of chemoradiotherapy to bone marrow stem cells. This limitation can be overcome if high-dose aggressive therapy is followed by BMT to replace the damaged marrow. Two factors limit the broader application of BMT: the requirement for HLA-matched donor BM and the development of graft-versus-host disease, which sometimes occurs even with matched sibling transplants. Both problems can be circumvented by using autologous marrow. However, in order for disease-free engraftment to occur, the autologous marrow must be purged of residual leukemia cells without destroying hematopoietic stem cells.

ABSTRACT

Antibodies which have been conjugated to the potent toxin ricin offer a promising approach to clean-up of autologous marrow. Ex vivo treatment with such IT has been shown to eliminate most T- or B-cells present in rodent marrow without damaging the ability of the marrow to reconstitute lethally irradiated recipients (9, 23, 30, 31). Similar IT with specific toxicity for human leukemia/lymphoma cells have been reported (18, 19, 24). The efficacy of IT to kill "the last" leukemic cell still remains an issue.

The toxiricin, isolated from the seeds of Ricinus communis, consists of 2 M, 30,000 subunits linked by a disulfide bond (17). The toxic effect is mediated by the A chain, which enzymatically inactivates eukaryotic ribosomes completely inhibiting protein synthesis. The B chain binds to galactose residues on the cell surface, thereby enabling the entry of ricin into the cell. B chain also facilitates transport of A chain to ribosomes by an unknown mechanism (35). Treatment with intact ricin IT requires the presence of lactose to competitively block the galactose-binding site of the B chain (34). Under these conditions, the specificity of the IT resides in the antigen binding site of the antibody.

In the present study, we used a colony assay to evaluate and compare the specific cytotoxic effects of 2 anti-T-cell IT on T-leukemic cell lines. Both IT were directed to the p65 antigen expressed on mature thymocytes and peripheral blood T-cells (13). We report for the first time that an IT can specifically eliminate more than 99.99% of clonogenic T-cells, even in the presence of excess human BM. The prevalence of p65 on human T-cell leukemias (8) and the limited toxicity of the anti-p65 ricin IT for human pluripotent stem cells in in vitro assays (18, 25) indicate that these reagents may be extremely useful for the ex vivo elimination of leukemic cells in autologous BMT.

MATERIALS AND METHODS

Human Cell Lines. Leukemic cell lines used in this study included CEM and MOLT-4, derived from patients with T-cell ALL; HPB-Null and NALM-6, both pre-B-ALL lines; and Reh, a non-T-, non-B-ALL cell line. The general characteristics of these cell lines have been summarized (15). All lines were originally obtained from Dr. Jun Minowada (Hines Veterans Administration Hospital, Hines, IL). The cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% ambient air. The tissue culture medium consisted of RPMI 1640 containing 10% heat-inactivated FCS, streptomycin (100 μg/ml), and penicillin (100 IU/ml). All reagents were purchased from Grand Island Biological Co. (Grand Island, NY).

Human BM. Heparinized BM was obtained from normal volunteers who gave informed consent according to guidelines of the University of Minnesota Committee on the Use of Human Subjects in Research. Mononuclear cells were isolated from Ficoll-Hypaque gradients (2), washed 3 times, and irradiated (4000 rads) using a 137Cs irradiator.

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Antibodies. T101, an IgG2a murine MOAB, binds to p65 expressed on normal and malignant cells of T-cell lineage (21). (T101 was generously provided by Hybritech, Inc., La Jolla, CA.) VII-1, an IgG1 murine MOAB produced by immunizing BALB/c mice with the MOLT-3 cell line, precipitates a M, 65,000 molecule on MOLT-3 cells. (VIII-1 was generously provided by Kallestad Laboratories, Inc., Austin, TX) VIII-1 and Leu-1 (32) (Becton Dickinson, Mountain View, CA) demonstrate identical patterns of reactivity by immunofluorescence with peripheral blood mononuclear cells and human leukemic cell lines. Moreover, these 2 MOAB cross-block in immunofluorescence studies using peripheral blood cells, suggesting that they react with the same p65 antigen. 5

TA-1, an IgG2a MOAB, binds to greater than 90% of peripheral blood T-cells, approximately 75% of thymocytes, and monocytes (11). TA-1 recognizes gp170/95 (10). Anti-Thy-1.2, an lgG2b MOAB, reacts with gp25 expressed on murine T-cells (12).

IT. IT were prepared using m-maleimidobenzoyl-N-hydroxysuccinimide ester to cross-link MOAB to intact ricin via a covalent thioether bond. The details of the procedure have been published previously (34). 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 2 ricin moieties per antibody.

Immunofluorescence. Reactivity of unconjugated MOAB with cell lines was determined by direct or indirect immunofluorescence under noncapping conditions as described previously (1). Primary antibodies were used at saturating concentrations, and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Tago, Inc., Burlingame, CA) diluted 1:20 was used as a second antibody. Thy-1.2 expression was determined by direct immunofluorescence using fluorescein isothiocyanate conjugated anti-Thy-1.2 (New England Nuclear, Boston, MA). Background staining using control ascitic fluid or IgG2a myeloma protein (UPC 10; Litton-Bionetics, Charleston, SC) as primary antibody was less than 1% and usually 0%. Fluorescent staining of cells was determined by a Zeiss fluorescent microscope equipped with Phloem epillumination and by FACS IV analysis.

Treatment of Cells with Immunotoxin. Washed leukemic cells were treated under standard conditions (25) with IT or intact ricin. Ten million cells/ml were incubated with IT in RPMI 1640 containing 2% HSA (Cutter Laboratories, Inc., Berkeley, CA) and 100 mM lactose (Difco Laboratories, Detroit, MI) for 2 hr at 37° in a humidified atmosphere of 5% CO2 in air. Control cultures were incubated with medium containing 100 mM lactose plus ricin. For experiments in which leukemic cells were mixed with excess BM, the combined cell concentration for IT treatment was always 107/ml. Treated cells were washed once (400 g, 10 min) in medium containing 100 mM lactose plus 1% HSA and again in medium containing 15 mM lactose plus 1% HSA. Cells were then counted using a hemacytometer and resuspended in α-modified minimal essential medium (KC Biologicals, Lenexa, KY) containing 10% FCS and antibiotics.

Blocking experiments were performed by pretreating 2 x 10⁴ MOLT-4 leukemic cells with unconjugated purified antibody (100 μg/ml) (T101, VII-1, TA-1) at 4° for 30 min. IT diluted in lactose was added to the unwashed cells, and treatment continued as described above.

Colony Assay. Leukemic cell lines were cloned in vitro using a slight modification of the methylcellulose system described by Izazaurie et al. (5). Cell suspensions were added to α-minimal essential medium containing 10% FCS, antibiotics, and 0.3% methylcellulose (4000 cps) to give a final cell concentration of 2.5 to 5.0 x 10⁴/ml, unless otherwise indicated. For certain cell lines, increased cloning efficiencies could be obtained by adding growth stimulators to the culture system (conditioned medium, irradiated T-feeder cells) as described by Izazaurie et al. (5). Methylcellulose mixtures were vortexed well, and 0.1-ml aliquots were dispensed into wells of 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). At least 4 replicate wells were plated for each control population. In a colony inhibition assay, the maximum level of cytotoxicity that can be measured is determined by the number of clonogenic cells plated.

For example, if one plated 1,000 treated cells from a population with a cloning efficiency of 40%, the maximum measurable log kill in treated cultures if no colonies formed would be 2.6. Similarly, if one plated 10,000 treated cells (4,000 clonogenic cells), the maximum measurable log kill would be 3.6. In our experiments, 6 to 10 replicate wells (1,500 to 2,500 total cells) were plated from control cultures and cultures treated with low doses of IT; this permitted a maximum measurable log kill of 2.78 to 3.0, assuming a cloning efficiency of 40% for untreated cultures.

For large scale experiments, 300 to 700 replicate wells were plated, permitting a maximum measurable log kill of 5.2.

 Cultures were examined using an inverted phase-contrast microscope, and groups of 20 or more cells were scored as "colonies." The number of colonies within each well was counted on Days 7 to 14, depending on the cell line. For control cultures, the mean± S.D. was determined from replicate well counts, and the cloning efficiency was calculated. For treated cultures, the total number of colonies was determined. The results were then expressed by the following calculations.

% of control response
\[
\frac{\text{No. of colonies in IT-treated cultures}}{\text{Population cloning efficiency} \times \text{no. of cells plated}} \times 100
\]

RESULTS

Colony Assay for Human Leukemic Cell Lines. We used a colony inhibition assay to test the effectiveness of intact ricin IT in eliminating leukemic T-cells. In our assay, a group of 20 or more cells was scored as a "colony." The kinetics of colony growth was examined using untreated and low-dose IT-treated cell lines. The T-cell lines grew more rapidly than did the non-T-cell lines. Therefore, MOLT-4 and CEM colonies were scored on Days 7 to 9; Reh, NALM-6, and HPB-Null colonies were scored on Days 11 to 14. After these time intervals, the size of the colonies increased, but the number of colonies did not. At the time of scoring, the vast majority of colonies contained more than 30 cells (Fig. 1). The cloning efficiencies of all cell lines varied from experiment to experiment, exhibiting a range of 10 to 89%. Nevertheless, within an experiment, there was a linear relationship between the number of cells plated and the number of colonies which developed (Chart 1). We found that IT exhibited greater toxicity in experiments with cloning efficiencies of less than 10%. Therefore, these anomalous experiments were excluded.

Effect of Ricin on Clonogenic Leukemic Cells. The ricin

8 D. Murray and D. A. Vallera, unpublished data.  

Anti-p65 Ricin IT
sensitivity of each cell line was determined according to our standard IT protocol by pretreating cells with ricin (3, 10, 30, 100, 300, and 500 ng/ml) in the presence of 100 mM lactose. Lactose alone did not inhibit colony formation because, in 7 independent experiments, the mean colony formation by cells pretreated with lactose was as great or greater than that by cells treated with medium alone. Therefore, in these and subsequent experiments, lactose-treated cultures were used as controls to calculate the percentage of control colony formation. For each cell line, a ricin dose-response curve was generated, and the experiments, lactose-treated cultures were used as controls to determine the IC50. The IC50 for CEM and MOLT-4 was 60 to 65 ng/ml, for Reh and NALM-6, 110 ng/ml, and for HPB-Null, 170 ng/ml. The non-T-cell lines, therefore, were approximately 2 to 3 times more resistant than were the T-cell lines.

**Immunofluorescence.** The binding of unconjugated MOAB to human leukemic cell lines was determined by immunofluorescence using a fluorescence-activated cell sorter (Table 1). MOAB T101 and VIII-1 each bound strongly to greater than 95% of CEM and MOLT-4 cells but did not bind to Reh, NALM-6, or HPB-Null. The antigen gp170/95 detected by TA-1 was weakly expressed by a minority of MOLT-4 cells. Monoclonal anti-Thy-1.2 did not bind to the human cell lines but reacted strongly with C57BL/6 murine thymus cells.

**Effect of IT on Leukemic Cell Line Colony Formation.** T101-R was extremely effective at inhibiting colony growth by CEM and MOLT-4 cells (Chart 2). Treatment with 100 ng/ml inhibited CEM clones 2.8 logs. The same dose completely inhibited colony formation by MOLT-4 cells. Since no colonies formed from the 1200 to 1300 treated clonogenic cells plated in these experiments, we can conclude that T101-R effected at least 3.1-log inhibition of MOLT-4 cells.

Treatment with a control IT made with anti-Thy-1.2, which does not bind to the leukemic cells (Table 1), did not inhibit colony formation by MOLT-4 and CEM. Rather, at concentrations of 300 and 1000 ng/ml, a 25 to 35% increase in cloning efficiency was observed (data not shown).

T101-R did not strongly inhibit colony formation by the p65-negative cell lines (Chart 2), even at a concentration of 1000 ng/ml. Concentrations greater than 1500 ng/ml were required to reduce colony formation by 50%. Therefore, comparison of the IC50 with adjustment for ricin sensitivity indicated that T101-R was 140 to 540 times more toxic to positive targets than negative targets.

Another IT directed to the p65 antigen was prepared using the MOAB VIII-1. VIII-1-R completely inhibited colony formation by MOLT-4 cells at a concentration of 300 ng/ml, corresponding to at least 3.0-log inhibition. The same concentration inhibited 94% (1.2 logs) of CEM colonies. Higher concentrations were increasingly toxic to CEM clonogenic cells, but 3-log inhibition was not observed even with concentrations as high as 1500 ng/ml. Therefore, VIII-1-R was not as potent as T101-R in its inhibition of CEM. However, like T101-R, VIII-1-R was specific for targets which express p65; neither HPB-Null nor Reh was significantly inhibited by VIII-1-R at these concentrations (Chart 2).

**Blocking of T101-R-specific Toxicity by Free Antibody.** To determine conclusively that the antibody moiety of our IT mediated specificity, we attempted to block IT binding by pretreating cells with unconjugated antibody. Table 2 depicts the results of these experiments.

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>T101</th>
<th>VIII-1</th>
<th>TA-1</th>
<th>Anti-Thy-1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>97.7 (101.0)</td>
<td>95.8 (51.6)</td>
<td>18.3 (1.7)</td>
<td>0.2 (0)</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>97.6 (92.5)</td>
<td>97.0 (57.1)</td>
<td>44.0 (7.6)</td>
<td>0.2 (0)</td>
</tr>
<tr>
<td>HPB-Null</td>
<td>4.0 (1.0)</td>
<td>4.0 (0)</td>
<td>4.3 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>Reh</td>
<td>2.1 (0.2)</td>
<td>1.0 (0)</td>
<td>2.5 (0.3)</td>
<td>ND</td>
</tr>
<tr>
<td>NALM-6</td>
<td>2.3 (0)</td>
<td>0.3 (0)</td>
<td>0.4 (0)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, mean fluorescence of test sample with background fluorescence subtracted.

### Table 2

<table>
<thead>
<tr>
<th>Blocking antibody</th>
<th>T101-R treatment (ng/ml)</th>
<th>No. of colonies</th>
<th>% of control response</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>91.0 ± 10.0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>10.0 ± 2.6</td>
<td>2.0 ± 1.6</td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T101</td>
<td>0</td>
<td>95.5 ± 9.1</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>92.0 ± 6.6</td>
<td>111.0 ± 15.0</td>
<td>96</td>
</tr>
<tr>
<td>100</td>
<td>94.0 ± 3.3</td>
<td>79.8 ± 11.0</td>
<td>89</td>
</tr>
<tr>
<td>VIII-1</td>
<td>0</td>
<td>98.8 ± 7.1</td>
<td>120 ± 13.0</td>
</tr>
<tr>
<td>10</td>
<td>103.0 ± 7.5</td>
<td>96.8 ± 8.8</td>
<td>101</td>
</tr>
<tr>
<td>100</td>
<td>62.7 ± 9.8</td>
<td>85.5 ± 7.1</td>
<td>74</td>
</tr>
<tr>
<td>TA-1</td>
<td>0</td>
<td>101.0 ± 9.0</td>
<td>140.3 ± 9.1</td>
</tr>
<tr>
<td>10</td>
<td>8.8 ± 1.5</td>
<td>1.8 ± 1.0</td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Colonies scored on Day 7. Each well received 250 plated cells.

*a* Mean percentage of lactose control calculated for Experiments 1 and 2.

*b* Mean ± S.D. of quadruplicate wells from each treatment.

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Human BM aspirates are often contaminated with peripheral blood T-cells. To determine if the presence of normal p65-positive cells would reduce the toxicity of T101-R against leukemic targets, a mixture containing normal BM and leukemic cells was treated with T101-R. BM mononuclear cells were irradiated prior to mixing and treatment to prevent colony formation by normal hematopoietic stem cells, which would interfere in scoring of leukemic colonies. In each experiment, control wells plated with irradiated BM alone did not yield colonies. Preliminary experiments demonstrated that colony formation remained linear over a wide range of cell concentrations, despite the presence of 10-fold excess irradiated BM (Chart 1). Moreover, excess BM did not affect the cloning efficiency of MOLT-4 and CEM cells when the plating cell mixtures contained 10-, 100-, and 1000-fold excess marrow (data not shown). The cloning efficiency of HPB-Null was augmented by low (10-fold) concentrations of excess marrow, unchanged by higher concentrations (100-fold), and strongly inhibited by the highest concentration (1000-fold) tested.

Leukemic cells were mixed with 20- to 40-fold excess BM and subsequently treated with various concentrations of T101-R. The presence of excess BM did not change the dose-response curves of T101-R with MOLT-4 or CEM (Chart 2) or HPB-Null (data not shown). The results of treatment with T101-R (1000 ng/ml) are summarized in Table 3. No colonies developed in treated cultures of CEM and marrow cells or MOLT-4 and marrow cells, indicating at least 5.1-log inhibition of CEM clonogenic cells and at least 3.2-log inhibition of MOLT-4 clonogenic cells. These results are thus no different than those from comparable experiments lacking the BM.

**DISCUSSION**

The present studies extend our previous findings that MOAB ricin IT can be used ex vivo in human BMT to eliminate specific disease-causing cells from the marrow grafts. We showed previously that IT directed against different T-cell determinants...
(including p65) can be used for graft-versus-host-disease prophylaxis in allogeneic BMT. Each IT selectively inhibited T-cell proliferation and function but did not inhibit normal hematopoietic progenitors unless used at much higher concentrations (18, 25, 27). In the present studies, we determined the efficacy of 2 anti-proliferation and function but did not inhibit normal hematopoietic prophylaxis in allogeneic BMT. Each IT selectively inhibited T-cell (8). The toxic effects of p65 IT were tested by pretreating leukemic cell lines with IT in the presence of lactose and assaying for the inhibition of colony formation in a methylcellulose culture system.

Colony assays have been used to determine the susceptibility to chemotherapeutic agents of normal and malignant cells from a variety of mouse and human tumors (22, 33). The assay must fulfill certain criteria before it can be useful in this capacity (22). (a) Cloning efficiency must be sufficiently high to assess activity of the cytotoxic agent. In our experiments, cloning efficiencies ranged from 10 to 89%. (b) There must be a linear relationship between the number of colonies obtained and the number of cells plated. A linear relationship, especially at low plating concentrations, indicates that each colony is formed from a single cell and that culture conditions are sufficient to support colony formation by rare cells which may have escaped cytotoxic effects of the therapeutic treatment. We demonstrated a linear relationship in our colony culture system. (c) Control experiments must demonstrate that cells which die from the therapeutic treatment do not inhibit the clonogenic activity of those which survive the treatment. This was demonstrated for the selected plating concentrations used in our experiments.

The exquisite sensitivity of a colony assay is particularly important for assessing the efficacy of IT to purge autologous BM of residual leukemic cells. Studies in rodents have shown that at least 99.9% (3 logs) of leukemic cells must be eliminated from autologous grafts to prevent leukemia in the transplant recipient (9, 23). Using our colony assay, we found that treatment with T101-R (300 ng/ml) killed more than 99.9% of the leukemic cell targets, and 1000 ng/ml killed 99.9992% and at least 99.9993% of CEM and MOLT-4 cells, respectively. To simulate the clinical situation, in some experiments, leukemic cells were mixed with excess normal BM cells prior to IT treatment. Importantly, the presence of excess normal BM cells, some of which express p65, did not reduce the toxicity of T101-R for leukemic targets.

We have shown previously that T101-R causes minimal toxicity to normal hematopoietic stem cells at the concentrations used in our experiments (18, 25). Treatment of normal BM with 300 ng T101-R/ml, a dose which inhibited 99.9% (3 logs) of leukemic cells, induced no inhibition of stem cell activity as detected in the granulocyte, erythroid, macrophage, megakaryocyte colony-forming unit and erythroid blast-forming unit-erythrocye in vitro assays (18, 25). Treatment with 1000 ng T101-R/ml, which effected 5-log inhibition of leukemic cells, inhibited stem cell activity only 20 to 49%. Patients at the University of Minnesota have received BM treated with IT (300 to 600 ng/ml) and have all engrafted without delay (4). Doses of 300 to 600 ng T101-R/ml, therefore, would appear to be feasible for clinical treatment. Moreover, the low level of stem cell inhibition does not necessarily preclude use of 1000 ng/ml or even higher doses. There is no published evidence that stem cell activity detected in vitro correlates with successful engraftment. However, should high-dose treatment impede engraftment, a higher concentration of lactose can be used to block the nonspecific killing due to ricin-mediated binding.

Target specificity of IT in the presence of lactose was mediated by the antigen-binding region of the MOAB because: (a) IT made with an irrelevant MOAB, anti-Thy-1.2, did not inhibit leukemic lines. (b) A comparison of the doses at which 50% inhibition was achieved with adjustment for differential ricin sensitivity indicated that T101-R was 140- to 540-fold and VIII-1-R was approximately 12- to 192-fold more toxic to p65-positive than to p65-negative targets. (c) The toxicity of T101-R was specifically blocked by parent MOAB (T101) as well as by a different MOAB (VIII-1) directed to the same determinant. In contrast, a MOAB (TA-1) of the same IgG subclass but directed to a different determinant did not block toxicity.

High concentrations of T101-R induced some nonspecific killing of antigen-negative targets due to ricin-mediated binding. Because the non-T-cell lines were approximately 2 to 3 times more resistant to ricin than were the T-cell lines and because clonogenicity of these lines was inhibited 15 to 30% following treatment with 1000 ng T101-R/ml, we estimate that 30 to 90% inhibition of the T-cell lines may have been caused by IT which bound via ricin rather than antibody. Therefore, since T101-R at 1000 ng/ml effected 5.1- and greater than 5.2-logs inhibition of CEM and MOLT-4, respectively, and 0.2 to 1.0 log was due to nonspecific toxicity, we conclude that 4.1 to 5.0 logs of killing were attributable to the specificity of our reagent. The added nonspecific killing may prove advantageous for preventing relapse to autologous BMT by eliminating a maximum number of residual leukemic cells. On the other hand, it may prolong the period to engraftment, since some normal stem cells may be damaged.

In vitro incubation with pharmacological agents (7) is one method of autologous marrow treatment which has been tried. This approach is complicated by the nonselective toxicity of such agents for stem cells as well as leukemic cells. Antibody and complement have been used to purge autologous marrow in rodent models (9, 28, 29) and in humans (20), but with variable success. In part, this is due to the variable biological potency and nonspecific stem cell cytotoxicity of exogenous complement. Another drawback to this approach is that many potentially valuable antibodies cannot be used because of their failure to activate complement. An IT made with ricin A chain and directed to human immunoglobulin light chains inhibited 99% (or 2 logs) of Daudi cells in a clonogenic assay without reducing stem cell activity (16). In the human system, IT containing A chain are far less toxic than those containing intact ricin (27), although specific toxicity can be augmented with activating agents such as ammonium chloride (6).

The 4.1 to 5.0 log specific inhibition observed with T101-R is greater than that which has been reported for other immunotoxins (3, 6, 9, 16, 23, 25, 34). We found that IT synthesized with 35.1 (14) and TA-1 (11) were much less potent against CEM and MOLT-4 cell lines than was T101-R. It is probable that the lower toxicity of these IT is due to the lower affinities of the 2 MOAB as compared to T101 and to the low level of expression of p50 and gp170/95 on the target cell lines. The differential ability of the MOAB to induce modulation probably also plays a role in the
IT toxicity. Whereas T101 readily induces modulation, 35.1 induces only partial modulation (14), and TA-1 induces no detectable modulation.7 These considerations indicate that only certain MOAB may yield IT that are clinically useful. Our findings with T101-R and VIII-1-R emphasize this point.

Precipitation of a M, 65,000 molecule and similar patterns of binding to normal and malignant leukocytes by immunofluorescence suggest that the MOAB T101 and VIII-1 detect the same antigen. The ability of VIII-1 to block T101-R toxicity against clonogenic leukemic cells is strong additional evidence that they bind to proximal or identical epitopes. However, IT prepared with the 2 MOAB were not equally toxic in our experiments, since T101-R was more inhibitory to GEM than was VIII-1-R. This effect was not as pronounced when the 2 IT were tested against MOLT-4. Possible explanations for the observed differences in IT potency include differences in antibody subclass, affinity, or perhaps subtle differences in the way the 2 MOAB are linked to ricin. Differences in the manner in which the 2 cell lines internalize IT might explain their differential sensitivity to the 2 p65 IT. Investigation of the immunochemical characteristics (e.g., avidity, epitope binding) and biological effects (e.g., modulation) of the 2 MOAB may lead to the synthesis of more effective IT. Moreover, our system may prove useful for defining the important parameters which control IT toxicity.

Our studies indicate that T101-R has clinical possibilities as a reagent for autologous BMT. The expression of the p65 target antigen in the vast majority of cases of T-ALL contributes to the potential utility of T101-R. Nevertheless, it is well known that leukemic cells freshly derived from patients are markedly more heterogeneous in antigen expression than cultured leukemic cell lines. Thus, treatment with a cocktail of potent IT directed to a variety of T-lineage markers may increase the likelihood of eliminating leukemic cells from autologous marrow, especially the clonogenic cells in the disease, the phenotype of which is currently unknown. Our demonstration of 4- to 5-log killing indicates the tremendous utility of IT in BMT. Moreover, our model may serve as a prototype for selecting IT for use in autologous BM to treat a wide range of neoplasms.

REFERENCES


Fig. 1. Morphology and cellular composition of MOLT-4 colonies. MOLT-4 cells were pretreated in 100 mM lactose 2 hr at 37°C, washed twice, and plated at 250 cells/well in methylcellulose culture as described. A, representative area of 7-day culture (x 30); B, higher magnification showing loosely compact colonies of 40 to 100+ cells (x 164).
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