Cytotoxic Effects of Anti-CD5 Radioimmunotoxins on Human Tumors in Vitro and in a Nude Mouse Model

Jill Marie Manske, Donald J. Buchsbaum, David E. Hanna, and Daniel A. Vallera

Department of Therapeutic Radiology, Section on Experimental Cancer Immunology [D. J. B., D. A. V.] and Department of Laboratory Medicine/Pathology [J. M. M.], University of Minnesota, Minneapolis, MN 55455; and the Department of Radiation Oncology, University of Michigan, Ann Arbor, MI 48109-0010 [D. J. B., D. E. H.]

ABSTRACT

An immunoconjugate, consisting of both toxin and radionuclide on the same antibody molecule, was synthesized by cross-linking the phytotoxin ricin to the T101 monoclonal antibody recognizing the CD5 cluster of differentiation antigens on the surface of normal and malignant T-cells. The hybrid molecule was then labeled with iodine-125 by an iodine monochloride procedure. This radioimmunotoxin (RIT), which selectively bound to the CD5-positive CEM human leukemia cell line, was selectively inhibitory to antigen-positive cells in protein synthesis inhibition assays. RIT was only 3.0-7.8-fold less toxic and was 1.1-1.6-fold slower than unlabeled immunotoxin in inhibiting protein synthesis. Because of the radioclonal moiety, the RIT also provided information related to biodistribution and pharmacokinetics. Four days following intratumoral injection, more than 125-fold greater activity was found in CEM tumors implanted in nude mice as compared to normal tissues. The mean blood half-life for RIT was 25.7 h and for radiolaographed antibody, 91.3 h. Intratumoral injections of RIT selectively induced regression of established CEM tumors. To our knowledge, these studies are the first to demonstrate that a single immunoconjugate can combine the advantages of both a catalytic toxin and radionuclide for cancer therapy.

INTRODUCTION

Radiolabeled antibodies (1-9) and antibody-toxin conjugates (10-28) are among the reagents that have shown potential in cancer research. Since these immunoconjugates differ in their biochemical applications and limitations, we evaluated the potential advantage of dual-tagging the same MoAb with the toxin ricin and the radionuclide 125I. T101 is a MoAb that recognizes the CD5 cluster on the surface of normal and malignant human T-cells (29). This antibody was chosen because (a) we have previously studied the selectivity, potency, and internalization of T101 as an intact ricin IT (15, 24, 30, 31); (b) the University of Minnesota BMT team has used T101-ricin ex vivo in allogeneic BMT for graft-versus-host-disease prophylaxis and in autologous BMT for purging residual leukemia cells from autografts (12, 19); (c) T101 antibody has been used clinically for in vivo treatment of ALL (21 and C11) and CLL (21, 34); and (d) T101 antibody has been radio-

Received 4/11/88; revised 8/31/88; accepted 9/21/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to identify this fact.

1 This work is part of a doctoral thesis by J. M. Manske to fulfill the requirements of the Graduate School of the University of Minnesota and is Center for Experimental Transplantation and Cancer Research publication no. 33. This work was supported in part by National Cancer Institute grants R01-CA-31618, R01-CA-43368, R01-CA-36725, and R01-CA-21737, American Cancer Society Grant IM-502, and the Minnesota Medical Foundation.

2 To whom requests for reprints should be addressed, at Department of Therapeutic Radiology, Box 367 UHMC, Harvard Street at East River Road, University of Minnesota, Minneapolis, MN 55455.

3 Scholar of the Leukemia Society of America.

4 The abbreviations used are: MoAb, monoclonal antibody; IT, immunotoxin; BMT, bone marrow transplantation; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; RIT, radioimmunotoxin; MBS, maleimidobenzoyl-N-hydroxysuccinimide ester; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ICI, iodine monochloride; TCA, trichloroacetic acid; FCS, fetal calf serum; PBS, phosphate-buffered saline.

5 Clusters of differentiation antigens were defined at the International Workshops on Human Leukocyte Differentiation Antigens.

6 The costs of publishing this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to identify this fact.
were mixed and incubated for 30 min at room temperature. Precipitated protein was pelleted. The pellet and supernatant were counted in a gamma counter. The percent TCA precipitable counts was determined using the following equation:

\[
\% \text{ TCA precipitable counts} = \frac{\text{cpm pellet}}{\text{cpm pellet + cpm supernatant}} \times 100
\]

To determine which components of IT had incorporated \( ^{125}\text{I} \), samples of radiolabeled IT were treated in solution with TCA precipitable counts and resolved by gradient SDS-PAGE according to the method described by Laemmli (40). \( ^{125}\text{I} \) labeled proteins were visualized by autoradiography of the dried gels using Kodak X-OMAT AR film.

Cell Lines. CEM (41) is a T-cell line derived from a patient with T-cell ALL. The cells express CD5. Daudi, derived from a Burkitt’s lymphoma (42), and the Raji Burkitt’s lymphoma cell line do not express CD5. Cell lines were maintained at 37°C in 5% CO2. Cells were passaged every 2–3 days in RPMI 1640 supplemented with 10% FCS and antibiotics.

Immunofluorescence Analysis. The binding of unconjugated MoAb or IT to CEM cells was determined by standard immunofluorescence procedures. In brief, 104 cells/0.1 ml PBS were incubated with various concentrations of primary MoAb or IT at 30 min at 4°C in the presence of 0.015% sodium azide. After incubation, cells were washed and incubated with secondary fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Meloy Laboratories Inc., Springfield, VA). Cells were washed, diluted, and analysed using a FACS IV (Becton Dickinson, Mountain View, CA) flow cytometer.

Immunoreactivity. The immunoreactivity of radiolabeled T101 and T101-IT preparations was measured using an in vitro direct cell binding assay. All binding assays were performed under conditions of antigen excess as measured by flow cytometry. CEM, Daudi, or Raji leukemia cells were suspended in RPMI 1640 medium containing 5% FCS at a concentration of 2 \( \times 10^6 \) cells/ml. Three-tenths ml of the cell suspension (6 \( \times 10^6 \) cells) was placed into glass test tubes, and a volume of \( ^{125}\text{I} \)-T101 or \( ^{125}\text{I} \)-T101-IT containing 0.04 \( \mu \)Ci of activity (at a final concentration of 0.13 \( \mu \)Ci/ml) was added to duplicate tubes. Tubes were incubated at 37°C for 1 h with shaking, and then counted in a well-type gamma counter to determine the total radioactivity added. Antigenic shedding was not a problem since our previous studies showed minimal loss of CD5 from CEM cells within 1 h at 37°C (30) and unpublished studies showed little difference in comparative binding results at 4°C and 37°C. After three washes with 1 ml RPMI 1640 medium containing 10% FCS, the tubes were re counted to determine the percentage of bound radiolabeled antibody. Percent binding was calculated according to the formula:

\[
\% \text{ Binding} = \frac{\text{cpm in sample after washing} - \text{background cpm}}{\text{cpm in sample before washing} - \text{background cpm}} \times 100
\]

The same procedure was used to determine the binding of \( ^{125}\text{I} \)-IgG2a.

Protein Synthesis Inhibition. Cells were treated using a variation of our previously described protocol (43). Washed cells (2 \( \times 10^6 \)/200 \( \mu \)l) were incubated with or without IT or RIT in leucine-free RPMI 1640 containing 10% heat-inactivated dialyzed FCS. Samples were treated as duplicates; one set of samples was incubated in 150 mM lactose and the other was not. All samples were incubated at 37°C in a humidified atmosphere of 5% CO2/95% air. At designated intervals aliquots of cells were harvested, and 10-\( \mu \)l aliquots (104 cells) were transferred to triplicate wells of 96-well flat-bottom tissue culture plates (CoStar, Cambridge, MA). Each well was pulsed for 2 h at 37°C with 1 \( \mu \)Ci \( ^{3}\text{H} \)-leucine (128 Ci/mmole; Amersham, Arlington Heights, IL). Cells were then harvested with an automatic cell harvester, washed, and dried. \( ^{3}\text{H} \)-Leucine incorporation was determined using standard scintillation counting techniques. Results were expressed as percent lactose control using the following formula:

\[
\% \text{ Control} = \frac{\text{cpm treated sample}}{\text{cpm untreated sample}} \times 100
\]

in vitro Cytotoxicity Test. Varying concentrations of T101-IT or T101-RIT were added to 2 \( \times 10^6 \) cells for a final volume of 200 \( \mu \)l. Duplicate samples were incubated with or without lactose (150 mM). Cells were preincubated with RIT or IT for 2 h at 37°C, washed twice, and plated at 104 cells/well in 100 \( \mu \)l RPMI 1640/10% FCS in 96-well tissue culture plates. Samples were incubated at 37°C in a humidified atmosphere of 5% CO2/95% air. On designated days, the number of viable cells was determined by trypan blue dye exclusion. At these times, remaining cells were fed 50 \( \mu \)l of RPMI 1640/10% FCS.

Mice. BALB/c nude (nu/nu) male mice, 4–5 weeks old, were obtained from Charles River, Inc., Wilmington, MA. Mice were kept under sterile conditions in cages with filter bonnets. They were maintained on Purina Lab Chow mouse diet and were given water supplemented with hypochlorite ad libitum.

Establishing CEM or Daudi Tumors in Nude Mice. CEM cells, 107/0.5 ml RPMI, or Daudi cells, 5 \( \times 10^6 \)/0.5 ml RPMI 1640, were injected s.c. into the flanks of nude mice. Mice were examined every 2–3 days until palpable tumors were detected.

Tumor Treatment with RIT. CEM and Daudi tumors were selected on the basis of size. Palpable tumors of 0.25–0.99 cm3 were treated by intratumoral injection using a previously established procedure (24). Intratumoral injection of 5 \( \mu \)g RIT was preceded by 0.7 ml i.v. injection of 500 mM lactose in PBS. Previous studies demonstrated the need for lactose to obtain specificity in this model (24). The injected RIT was also diluted into 500 mM lactose prior to injection. Treatments were repeated 3 days later using 2.5 \( \mu \)g RIT. Controls received no injections. All tumor-bearing animals were observed for 50 days or until death.

Tumor size was measured as two perpendicular diameters, and the approximate surface area was calculated as a product of the two measurements.

Biodistribution. For \( \text{in vivo} \) distribution studies following intratumoral administration of 5 \( \mu \)Ci \( ^{125}\text{I} \)-T101-rrc containing 5 \( \mu \)g of IT, animals bearing CEM or Daudi tumors were injected i.v. with 0.7 ml of 500 mM lactose prior to injection of RIT. One ml of 500 mM lactose was given i.p. two times per day, in the morning and evening, in an attempt to reduce the nonspecific binding of IT to normal tissues by the galactose binding site of ricin. Animals were dissected at 4 days after injection. Samples of blood, tumor, spleen, liver, heart, lung, kidney, muscle, skin, small intestine, stomach, and femur were counted in a well-type scintillation detector. Blood clearance measurements were made by serial eye bleeding beginning at 4-h and then at 24-h intervals in groups of five BALB/c mice injected i.v. with 2 \( \mu \)Ci of \( (2 \mu \)g) RIT or \( ^{125}\text{I} \)-T101 over a 6-day period. Samples were counted in a well-type scintillation detector. These animals received i.v. lactose prior to RIT administration, and i.v. injections of lactose twice daily as described. All animals received potassium iodide in their drinking water starting 2 days prior to administration of radiolabeled materials.

RESULTS

Radiolabeling of T101-IT or T101 and \textit{in Vitro} Binding. When 500-\( \mu \)g aliquots of T101-IT were labeled with 1.3 mCi of \( ^{125}\text{I} \) using five equivalents of ICI, the labeling efficiency was about 36% and the RIT had a specific activity of 1.0 \( \mu \)Ci/\( \mu \)g with an average iodine to antibody ratio of 1.8. T101 and normal IgG2a labeled in the same manner had similar labeling efficiencies and similar specific activities. TCA precipitation of MoAb or IT after radiolabeling indicated that at least 80% of the radio-nucleotide incorporated into protein was precipitated. The stability of our reagents in serum has not been determined. Autoradiographs of reduced SDS-PAGE of radiolabeled reagents showed that all constituents of the IT, i.e., MoAb, A chain, and...
cells were treated with ricin over a dose range of 8 log.s. The average amount of T101-RIT bound to 6 x 10^6 CEM cells in vitro was 36.6%. The average amount of ^125I-labeled T101 bound to an equal number of CEM cells was 31.5%. The average quantities of T101-RIT and ^125I-labeled T101 bound to equal numbers of Daudi cells were 2.2 and 2.6%, respectively. The binding of ^131I-labeled normal IgG2a to CEM and Daudi cells was 1.4 and 3.5%, respectively.

Reactivity of T101 and T101-ricin was also examined by indirect immunofluorescence analysis in four experiments (not shown). The CEM cell line was 88–95% positive when reacted with 10 μg/ml T101. A similar concentration of T101-ricin yielded 92–96% positive CEM cells. Saturation of CEM cells by T101 was reached when 10^6 cells were treated with 25–50 μg/ml T101. Daudi cells were only minimally reactive (0–15% positive) with T101. Control mouse myeloma protein IgG2a was also minimally reactive (0–6% positive) with CEM cells.

Protein Synthesis Inhibition. Protein synthesis inhibition assays were performed since the primary action of ricin is at the level of the 60S ribosome. T101-RIT and T101-IT were tested against CEM and Daudi cell lines for their comparative ability to inhibit protein synthesis. The results of a representative experiment are shown in Fig. 1. After a 4-h incubation (Fig. 1A), T101-RIT inhibited CEM protein synthesis 0, 29, and 60% at 0.01, 0.1, and 1 μg/ml, respectively. IT was more toxic inhibiting protein synthesis 0, 65, and 64%, respectively, at the same concentrations. Both T101-RIT and T101-IT had minimal inhibitory effect on Daudi cells. A more pronounced selectivity of the reagents was measured against the cell lines following a longer preincubation period of 8 h (Fig. 1B). RIT inhibited CEM protein synthesis 48, 73, and 93% at 0.01, 0.1, and 1 μg/ml, respectively. IT inhibited CEM protein synthesis 61, 93, and 93%, respectively.

After a 4-h incubation in the absence of lactose (not shown), RIT and IT were equally inhibitory to CEM and Daudi. Since the immunoconjugate binds by the galactose binding site in the absence of lactose, these findings suggest that labeling did not affect the toxin portion of the hybrid molecule.

To determine the differential toxicity of CEM and Daudi cells, cells were treated with ricin over a dose range of 8 logs. Cells were exposed to ricin over the course of the entire assay. The IC50 for CEM cells was 0.2 ng/ml. The IC50 for Daudi cells was 2 ng/ml. Thus, Daudi cells were 10-fold more resistant to ricin than CEM cells in our experiments.

Kinetcs of Inhibition. T101-RIT plus lactose was tested against CEM cells in kinetic studies. The rate of protein synthesis decreased rapidly after a lag period of 2–3 h (Fig. 2A). When percentage of control protein synthesis and time were plotted in a log/linear fashion, lines plotted by linear regression analysis denoted first order, single hit kinetics. The time required to reduce protein synthesis 1 log, denoted as T10, at a concentration of 1, 0.1, and 0.01 μg/ml of RIT, was 7.6, 12, and 24 h, respectively. Faster rates of inhibition were noted for T101-IT (Fig. 2B). After a lag period of 0–4 h, 1, 0.1, and 0.01 μg/ml showed T10 values of 7.6, and 15.2 h, respectively. Thus, IT inhibited protein synthesis only 1.1 to 1.6-fold faster than RIT. We noted similar rates of protein synthesis inhibition in cells treated with 0.1 and 1 μg/ml of IT, suggesting saturation at these higher concentrations. Saturation was not obtained with 0.1 and 1 μg/ml RIT treatments. When 1 μg/ml RIT was used to treat cells in the absence of lactose (Fig. 2A), there was no lag period, and the T10 was 5 h. Treatment with 1 μg/ml IT without lactose also resulted in no lag period and a T10 of 4 h (Fig. 2B).

Inhibition of Tumor Cell Viability as Measured by an in Vitro Cytotoxicity Assay. To determine the selective toxicity of RIT in long-term assays, cells were preincubated with RIT for 2 h, washed, and measured for viability by trypan blue dye exclusion. T101-RIT in the presence of lactose was toxic to CEM cells over a concentration range of 0.01–1.0 μg/ml (Fig. 3A). By Day 2, cells treated with 1 and 0.1 μg/ml RIT were effectively inhibited and did not recover by Day 6. Although 0.01 μg/ml T101-RIT was toxic, higher concentrations of RIT demonstrated superior tumor cell inhibition. T101-IT at the same concentrations was toxic to CEM cells (Fig. 3B). Growth of cells pretreated with either 0.1 or 1 μg/ml IT was inhibited by Day 2 and did not recover within the remaining days of the

Table 1 In vitro binding of radioimmunotoxin and radiolabeled immunoglobulin to human leukemia cell lines

<table>
<thead>
<tr>
<th>Binding (%)</th>
<th>CEM</th>
<th>Daudi</th>
<th>Raji</th>
</tr>
</thead>
<tbody>
<tr>
<td>T101-ricin</td>
<td>36.6 (N = 8)</td>
<td>2.2 (N = 5)</td>
<td>1.0 (N = 2)</td>
</tr>
<tr>
<td>T101</td>
<td>31.5 (N = 10)</td>
<td>2.6 (N = 8)</td>
<td>0.8 (N = 2)</td>
</tr>
<tr>
<td>IgG2a</td>
<td>1.4 (N = 2)</td>
<td>3.5 (N = 2)</td>
<td>1.5 (N = 1)</td>
</tr>
</tbody>
</table>
CEM and Daudi cell lines. Cells were pretreated with RIT or IT at concentrations with two intratumoral injections of RIT plus i.v. lactose (Fig. 3, G and //).

Since 0.01 ng/ml IT was inhibitory at Day 2 but RIT was not.

Assay. These data show that T101-IT is more toxic than RIT, since 0.01 µg/ml IT was inhibitory at Day 2 but RIT was not. T101-RIT and T101-IT did not exhibit any differences in toxicity at higher concentrations. Our viability studies show that T101-ricin IT are no more than 10-fold more toxic than RIT and are in agreement with protein synthesis studies. Similar kinetics were observed when T101-RIT and T101-IT were tested without lactose at all concentrations (Fig. 3, C and D). In the presence of lactose (Fig. 3, E and F), RIT and IT were minimally toxic to Daudi cells at Day 2 or 3 posttreatment. After an initial decrease in cell number on Day 2, cell numbers in all groups increased on Days 3–6. Neither IT nor RIT-treated cells recovered to the levels of untreated controls. Without lactose, both T101-RIT and T101-IT were toxic to Daudi cells (Fig. 3, G and H).

In Vivo Antitumor Effect of T101-RIT. The in vivo efficacy of T101-RIT was evaluated against established CEM tumor xenografts. Nude mice bearing CEM tumors of an average size± SD of 0.57 ± 0.26 cm² (range of 0.25–0.99 cm²) were treated intratumorally with as little as 0.3-0.6 ng ricin killed all mice. Lower doses of ricin did not kill mice, but did not cause tumors to regress. In the same study, free T101 also did not cause tumors to regress.

When the in vivo RIT data was plotted as a percentage of the pretreatment tumor size, a marked decrease in CEM tumor size was still evident. However, the untreated Daudi tumors showed a slower increase in percent initial tumor size than the RIT treated group because of their large initial size.

Biodistribution and Pharmacokinetics of T101-RIT and 125I-T101. Table 2 shows the biodistribution of T101-RIT in CEM tumor-bearing mice given i.v. and i.p. lactose. 4 days after intratumoral injection, the highest concentration of T101-RIT was in tumor (about 32% of the injected dose/g), with relatively small amounts in various normal tissues. 4 days following intratumoral injection, more than 125-fold greater activity was found in tumor than in normal tissues. Similar results were obtained in two CEM tumor-bearing mice at 1 day after intratumoral injection (data not shown). More than 10-fold lower levels of tumor uptake were seen in two animals bearing Daudi tumors 1 and 4 days following intratumoral injection. 1 day
Fig. 3. The effects of T101-RIT and T101-IT on the viability of antigen-positive (CEM) and antigen-negative (Daudi) cells. Cells were treated with RIT or IT at concentrations of 0.01, 0.1, 1.0, or 3.0 μg/ml for 2 h. Cells were then washed and plated. Viability was assessed by trypan blue dye exclusion assays on Days 2, 3, 4, and 6. Control cultures were incubated in lactose without RIT or without IT. A, CEM cells treated with T101-RIT in lactose; B, CEM cells treated with T101-IT in lactose; C, CEM cells treated with T101-RIT without lactose; D, CEM cells treated with T101-IT without lactose; E, Daudi cells treated with T101-RIT in lactose; F, Daudi cells treated with T101-IT in lactose; G, Daudi cells treated with T101-RIT without lactose; H, Daudi cells treated with T101-IT without lactose. The number of viable cells is plotted against time (days).

Fig. 4. Growth of CEM and Daudi tumors following treatment with T101-RIT or T101-IT. Five to 10 mice with palpable tumors (0.25–0.99 cm²) were given intratumoral injections of 5 μg RIT in 500 μl lactose. Treatments were repeated 3 days later with 2.5 μg RIT. Controls received no injections. All mice treated with IT received an initial i.v. injection of 0.7 ml lactose (500 μl). Tumor size (cm²) is plotted against day of tumor measurement. SD ranged from 0.24 to 4.8 cm².

Table 2 Distribution of T101-RIT in nude mice bearing CEM tumors

The concentration of T101-RIT in tissues of nude mice bearing CEM tumors that weighed 0.4 g was determined. Samples of tissue were counted 4 days after intratumoral injection of 5 μg (5 μCi) T101-RIT. All animals received lactose prior to RIT administration.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% injected dose/g</th>
<th>Mouse 1</th>
<th>Mouse 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.26</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>32.06</td>
<td>31.53</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.05</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.05</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0.07</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0.09</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.09</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>0.04</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>0.13</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Femur</td>
<td>0.03</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. Pharmacokinetics of 125I-labeled T101-ricin and 125I-labeled T101 in the blood of BALB/c mice. Individual blood concentrations for each group of five mice are plotted. Blood clearance measurements were made by serial eyeblooding from mice injected i.v. with 2 μCi (2 μg) of each reagent. The mice received i.v. lactose prior to RIT administration, and twice daily thereafter.

Blood clearance curves for T101-RIT and [125I]T101 from groups of five BALB/c mice treated with lactose are shown in Fig. 5. Straight lines fit by regression analysis (not shown) and analyzed by two sample t-tests were significantly different from each other (P < 0.001). The standard deviation of the slopes was only 5% of the average for the animals injected with RIT, and 15% of the average for the animals injected with [125I]T101. The deviation from linearity was minimal. The mean blood half-life ± SD of T101-RIT and [125I]T101 were 25.7 ± 1.5 h and 91.3 ± 13.3 h, respectively.

DISCUSSION

A primary goal of these studies was to establish a radiolabeling procedure that would result in minimal inactivation of
binding and/or toxicity of IT. Because of our background in the use of MoAb cross-linked to the lysines of ricin via a thioether linkage (12, 15, 18, 19, 24, 25, 30, 31), we employed MBS as a linking agent. An iodine monochloride labeling procedure (39) based on the iodination of tyrosine was used to prohibit the labeling of the same amino acid. Also, the ICI method is relatively gentle, avoiding the use of oxidizing agents that may damage protein (38, 39). By this method, at least 80% of the radionuclide was incorporated and attached to the protein in the RIT.

In viability assays using trypan blue dye exclusion, no difference in toxicity between IT and RIT was observed on antigen-positive cell lines at the higher concentrations tested. However, at the lowest concentration (0.01 μg/ml), RIT was not as toxic as IT. Although viability assays are a good measure of long-term activity, the primary action of ricin is inhibition of protein synthesis at the level of the 60S ribosomal subunit. Thus, protein synthesis inhibition assays were performed.

When IC50s are compared, RIT are only 3.0–7.8-fold less toxic than unlabeled IT. This result supports the findings of our in vitro cytotoxicity studies. The slight decrease in toxicity could be due to damage during the radiolabeling procedure, resulting in loss of either antibody or toxin activity. However, T101-IT and RIT exhibited similar toxicity against CEM cells in the absence of lactose, suggesting that differences were attributable to an effect on antibody rather than on toxin. Moreover, similar toxicity patterns were observed when IT and RIT were tested with and without lactose on the antigen-negative Daudi cell line. Although the radiolabeling procedure slightly altered the ability of IT to inhibit protein synthesis and cell viability at lower concentrations, differences in activity were minimal at higher concentrations (Fig. 1, A and B, and Fig. 3, A and B).

Kinetic analysis of RIT and IT, in which data were plotted as log response against time, indicated a first order single-hit process. Comparison of the time taken to inhibit 1 log of protein synthesis (T10) showed that IT was only 1.1–1.6-fold faster than RIT in inhibiting protein synthesis. Both RIT and IT exhibited the characteristic lag periods observed in earlier studies with IT (37, 43). Such lag periods were not observed with toxins alone. The T10 for IT at 0.1 and 1 μg/ml were similar (7.6 and 7.0 h). This implies that maximal inactivation was reached and may reflect saturation. Such was not the case for RIT (12 and 7.6 h) at the same concentrations, which may indicate some inactivation of antibody by iodination.

Our data provide evidence that RIT retained specificity: (a) RIT selectively bound CEM cells and not Daudi cells. An irrelevant antibody control labeled with 125I did not bind cells. (b) When IC50s are compared, RIT are about 2,000-fold more toxic to CEM cells than to Daudi cells in protein synthesis inhibition assays (Fig. 1). Notably, Daudi were 10-fold more resistant to ricin than CEM. Thus, we estimate RIT were about 200-fold more selective in their inhibition of CEM than Daudi. (c) RIT localized selectively in vivo (Table 2).

No enhancement of activity using RIT instead of IT was apparent in the in vitro studies. Although this does not suggest a therapeutic advantage for conjugating 125I to IT, our failure to observe additional killing with the radiolabeled reagent is likely attributable to our choice of radionuclide, low specific activity, and in vitro assays. Other investigators have shown toxicity with 125I-labeled MoAb at high specific activities after cryogenic incubation for several weeks (35, 44), resulting in a greater radionuclide decay (half-life for 125I is 60 days) and energy deposition. Studies showed that 125I-labeled T101 MoAb at a cell bound concentration of 4–52 dpm/cell produced 92–99% cytotoxicity of human malignant T-cell lines in vitro after 10 days exposure (35). In comparison, the contribution of 125I to in vitro cytotoxicity in our experiments was probably low, since our cell bound concentration was at least 2 logs lower and the time of exposure was shorter.

Several factors may influence the cytotoxic potential of RIT. Perhaps radionuclides with greater energies and different types of emission may be necessary to sufficiently increase the toxicity of an IT for treatment of macroscopic solid tumors. Radionuclides under consideration include 131I, which has been used therapeutically against a variety of tumors (1–3, 6, 7), copper-67, rhenium-186, and yttrium-90 (45, 46). Energy deposition from these radionuclides would occur at a distance from the binding site of RIT. Thus, such radionuclides might enhance IT toxicity by destroying proximal cancer cells that do not bind antibody or by killing resistant cancer cells that survive toxin exposure due to (a) failure to internalize toxin, or (b) low expression of the appropriate surface antigen. At this point, we have no evidence that indicates a higher risk of nontarget reactivity with RIT, but this issue requires further detailed investigation. Another factor influencing cytotoxic potential is dose rate. If the radionuclide emits ionizing irradiation at a low dose rate, most cells would be able to repair damage. Rapid decay and multiple hits using radionuclides such as α particle emitters may lessen or obviate repair.

Xenogeneic nude mouse tumor models have been used to test the antitumor efficacy of IT (24, 26, 47, 48). In the current studies, we found that 75% of CEM tumor-bearing mice showed tumor regression by Day 15 after treatment with RIT. By Day 30, four of eight original mice were alive. Although none of these mice were tumor-free, the tumors were relatively small (2.43 ± 2.1 cm²), as compared to untreated controls (9.95 ± 3.5 cm²), indicating that RIT treatment did have an effect. Although less dramatic, a significant reduction in Daudi tumor size was also apparent. These findings are in agreement with our previously published data showing that selectivity of intact ricin IT is not absolute in vivo in this model (24). The differences in tumor growth rate between untreated and treated Daudi tumors were probably due to a small amount of RIT escaping the lactose blockade, 125I, or the fact that the untreated Daudi tumors were generally larger at the outset. Mortality observed in mice bearing CEM tumors and treated with RIT is not likely attributed to the chronic toxicity of RIT since pathological examination of these treated mice showed limited liver and splenic damage. Mortality is more likely attributed to infections of the nude mice.

In contrast to our in vitro studies, our calculations show that the 125I in our RIT may have contributed to CEM tumor growth inhibition in our in vivo studies. The concentration of T101-RIT measured in our CEM tumor study was about 2 dpm/viable cell at 4 days after injection (Table 2). Studies show that this dose is sufficient to kill T-leukemia cells (35).

RIT showed similar specificity when compared to T101-IT in our previously published studies using the same in vivo nude mouse model (24, 26). These results confirm the reactivity and selectivity of our RIT conjugate. However, findings in the nude mouse model must be interpreted with caution, since xenogeneic tumor models represent artificial systems, and the CEM tumor has historically proven sensitive to toxins and chemotherapeutic reagents.

We also determined the biodistribution of RIT within the tumor-bearing host. In order for RIT to be useful, they must specifically localize and remain in the tumor rather than in...
normal tissue where they could cause damage. Our RIT remained in the tumor at least 4 days after intratumoral injection, with relatively small amounts distributed among normal tissues. After 4 days, more than 125-fold greater activity was found in tumor as compared to other tissues. Localization of RIT to tumor might be attributable to the higher affinity of the RIT molecule based on the presence of ricin B chain, which has a high affinity for galactose receptors on the cell surface. However, much lower levels of binding occurred in Daudi tumors. The data on CEM tumor growth inhibition and retention of RIT in CEM tumors supports the concept that there was integrity of the dual-labeled immunoconjugate in vivo.

The more rapid blood clearance observed for our T101-RIT as compared to T101 correlates with reports from other investigators who found that antibody-toxin conjugates cleared more rapidly than native antibodies (49–54). Autoradiographic studies of RIT analyzed by SDS-PAGE show label present on both antibody and toxin. Thus, it should be pointed out that we may be following the clearance of MoAb, ricin and/or dehalogenated label with RIT. The speed of removal may differ. Thus, the dual-labeled immunoconjugate is less than perfect as suggested by the differential blood clearance. Others have reported differences in the clearance of T101 when iodin-111, 125I, or 131I were used as tracers for antibody (8).

Our results suggest that radiolabeled intact ricin IT may be safe for treating localized cancers, such as ovarian cancer, and useful for treatment of inoperable solid tumors. It would be interesting to investigate the selectivity of our reagent in an i.p. tumor model using intact ricin IT (55). Unpublished animal studies show that intact ricin IT or RIT may have limited clinical applicability because of undesirable toxicity due to the galactose binding site of ricin. It may be possible to chemically block or modify the B chain of ricin at the genomic level to block or remove the galactose binding region and render these agents suitable for clinical use.

In conclusion, our studies demonstrate that a single immunoconjugate can combine the advantages of both toxin and radionuclide. The catalytic nature of our reagent is attributed to toxin, since radiolabeled antibodies do not inhibit protein synthesis (56). Our studies show that radionuclide and toxin can be attached to the same molecule in order to determine biodistribution and pharmacokinetics during cancer therapy. IT can be radiolabeled with 125I using the iodine monochloride procedure without significantly reducing toxicity or specificity. RIT can selectively induce localized tumor regression in nude mice with minimal nonspecific ricin or radiation toxicity.

In our opinion, the presence of two agents (toxin/radiouclide) on the same molecule may provide more useful and potent reagents for tumor destruction. Although 125I may not be an appropriate choice for therapy, we anticipate that [125I]-T101-ricin will serve as a prototype for the synthesis of more selective and potent RIT. We have radiolabeled T101-ricin with yttrium-90 to produce a RIT that is selectively toxic to tumor cells in vitro and in vivo (56). The observation that dual-labeled immunoconjugates retain activity after being synthesized encourages further studies of RIT for potential radioimmunotherapy of malignant diseases.

ACKNOWLEDGMENTS

The authors wish to acknowledge the exceptional editorial skills of M. J. Hildreth. We thank Hybritech Inc. for generously providing T101 monoclonal antibody. *Manuscript submitted for publication.

REFERENCES


ANTI-CD5 RADIOIMMUNOTOXINS


Cytotoxic Effects of Anti-CD5 Radioimmunotoxins on Human Tumors *in Vitro* and in a Nude Mouse Model

Jill Marie Manske, Donald J. Buchsbaum, David E. Hanna, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/24_Part_1/7107

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/48/24_Part_1/7107.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.