Antitumor Activity of L6-Ricin Immunotoxin against the H2981-T3 Lung
Adenocarcinoma Cell Line in Vitro and in Vivo

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ABSTRACT

The monoclonal antibody L6 recognizes a determinant that is expressed on lung, breast, colon, and ovarian carcinomas and is present only at trace levels in normal tissues. L6 was covalently linked to intact ricin by a thioether bond to produce an immunotoxin (IT). Gel analysis revealed that this IT was heterogeneous, but mostly one monoclonal antibody molecule linked to one ricin molecule. The L6-ricin IT selectively bound and was selectively toxic to L6-positive H2981-T3 adenocarcinoma cells in protein synthesis inhibition assays in which lactose was added to block the native ricin binding site. Clonogenic studies showed that 1 μg/ml L6-ricin could inhibit about 99.99% of H2981-T3 growth in a limiting dilution assay, even in the presence of a 20-fold excess of human bone marrow cells. Treatment of bone marrow cells with the same dose of L6-ricin resulted in the growth of ample numbers of bone marrow progenitor cells (colony-forming units-mixed, colony-forming units granulocyte/macrophage, and blast-forming units erythroid) after 14 days.

We also evaluated the antitumor effect of L6-ricin administered intratumorally with lactose against established H2981-T3 tumors in a nude mouse model. Thirty % of the tumor-bearing animals responded completely to single-dose treatment, while 60% gave partial responses. The in vivo effects were not absolutely specific, since irrelevant anti-CD5 IT also induced tumor regression in this model (10% responded completely, while 30% gave partial responses). However, irrelevant IT gave higher systemetic toxicity (50% mortality) than L6-ricin (23% mortality). The nonspecific activity of IT was possibly due to Fc binding, which was demonstrated in vitro, or due to ricin B-chain binding. Ricin alone was too toxic for sustained tumor protection. Unconjugated L6 had no antitumor effect. The data suggest that L6-ricin may be useful for in vitro purging of autologous bone marrow from patients with solid tumors and marrow involvement and for in vivo regional therapy of L6-positive carcinomas.

INTRODUCTION

IT are antibodies covalently linked to bacterial or plant toxins (reviewed in Ref. 1). The antibody moiety specifically binds the IT to cells expressing the appropriate antigen, while the toxin catalytically inhibits protein synthesis and causes cell death. Intact ricin is particularly effective and has been conjugated to a variety of MoAb for preclinical studies (2–5). Clinical trials have evaluated intact ricin IT for donor T-cell depletion prior to allogeneic bone marrow transplantation (6) and for the purging of leukemia cells from autologous bone marrow grafts (7). We (8) and others (9) reported the therapeutic efficacy of ricin IT on human lymphoma xenografts in nude mice. The efficacy of this treatment can be increased by combining it with chemotherapy (10) or using radioimmunotherapy (11, 12).

The ricin molecule consists of two M, 32,000 subunits joined by a disulfide bond. The A-chain subunit is an enzyme which inactivates the 28S component of the 60S subunit of ribosomes (13). The B-chain subunit contains a binding site that binds to terminal nonreducing galactose residues on the surface of all eukaryotic cells. Studies have shown that B-chain facilitates A-chain activity (14). B-chain also slows IT trafficking to the lysosomes, likely protecting the A-chain from lysosomal degradation (15). Native ricin binding can be prevented by co-incubating IT with lactose, which blocks the galactose binding site of the B-chain, rendering the IT more immunologically specific (16).

The MoAb L6 recognizes a carbohydrate antigen that is strongly expressed in lung, breast, colon, and ovarian carcinomas (17, 18), while this antigen is minimally expressed on normal human cells. In this study, we have investigated the therapeutic potential of L6-ricin IT in vitro against a human adenocarcinoma, H2981-T3. This is in view of the fact that major centers are currently evaluating the efficacy of strategies designed to eliminate residual tumor cells from autologous bone marrow grafts in patients with solid tumors (19–22). Reagents destroying tumor cells and not bone marrow progenitor cells could be used to purge marrow in vitro, permitting the clinical use of more aggressive therapy by radiation and/or anticancer drugs.

Intact ricin IT may also be useful for the regional treatment of cancers (3, 23), such as ovarian carcinomas which often occur in areas amenable to intracavitary treatment (24). Therefore, we investigated the activity of L6-ricin against H2981-T3 carcinomas in vivo, using a nude mouse model.

MATERIALS AND METHODS

Antibodies. L6 is an IgG2a mouse MoAb recognizing a carbohydrate determinant expressed on most human carcinomas. Normal adult tissues express no more than trace amounts of this antigen (17). The hybridoma resulted from a fusion of BALB/c splenocytes (immunized against human non-small cell carcinoma) and NS-1 mouse myeloma cells. T101 is an IgG2a mouse MoAb that recognizes CD5, a M, 67,000 glycoprotein on normal and malignant T-lymphocytes (25).UCHT1 is an IgG1 MoAb which recognizes the CD3 (p20) component of the T-cell receptor (26). Human γ-globulin was obtained from Sigma Chemicals (St. Louis, MO).

Immunotoxin Preparation. L6 anti-CD5 were linked to intact ricin via a covalent thioether linkage, which has been previously described (2). Briefly, ricin was reacted with m-maleimidobenzoyl-N-hydroxysuccinimide ester at a 3:1 molar ratio to attach maleimide linkage residues to the lysines of ricin. Monoclonal antibodies were prepared for cross-linking by reduction of disulfide bonds with 91 mM dithiothreitol for 30 min. Derivatized ricin was then mixed with the sulhydral-containing MoAb, resulting in the formation of a nonreducible thioether linkage...
between the two species. In a typical conjugation, 5.0 mg of purified MoAb were reduced and reacted with 12.4 mg of m-maleimido benzoyl-N-hydroxysuccinimide ester-derivatized ricin to give a molar ratio of ricin to MoAb of 6:1. IT was purified by high performance liquid chromatography on a 21.5-× 600-mm SW size exclusion column (Torry Soda, Japan and Beckman Instruments) to remove unreacted ricin. Unreacted MoAb was removed by passage over a Sepharose 4B affinity column, which binds the galactose binding site of ricin B-chain. Purified IT was eluted from the column with 50 mM lactose. The yield of purified IT was 20–40% of the concentration of MoAb used.

**Gel Analysis.** Analysis was performed on a 10% PAGE plate (Daiichi Pure Chemicals, Tokyo, Japan), using a Mini Protein II slab cell (Bio-Rad, Richmond, CA). Five μg of IT were suspended in sample buffer (40 mM Tris buffer, pH 6.8, 1.5% SDS, 7.5% glycerol, 0.005% bromophenol blue), boiled for 5 min, and added to the plate. Electrophoresis was performed using a constant current (26 mA), for a running time of 100 min, in electrode buffer (0.025 M Tris buffer, 0.192 M glycine, 0.1% SDS). The plates were stained with Coomassie blue, dried, and scanned using a Du-62 spectrophotometer with gel-scanning accessory (Beckman Instruments, Fullerton, CA). The photometry was performed at 560-nm wavelength. Quantitation of the bands was carried out by densitometric analysis using the Gel Scan Soft Pack Module software package (Beckman Instruments, Fullerton, CA).

**Target Cell Line and Tumor.** The cell line H2981 was established at Oncogen Corporation (Seattle, WA) from a primary human adenocarcinoma of the lung (17) and was further propagated at the University of Minnesota under the designation H2981-T3. The cells grew as adherent monolayers in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1% glutamine, and 1% penicillin/streptomycin, at 37°C in a 5% CO₂/95% air atmosphere. Once a week, cells were removed from monolayers with a PBS solution containing 0.05% trypsin and 0.02% EDTA and were passed. Before resuspended cells were used for in vitro assays, they were incubated at 37°C for 1 h to allow reexpression of receptors that may have been damaged by trypsinization (FACS studies of the kinetics of reexpression of the L6 antigen showed that reexpression was complete by this time). Prior to Scatchard analysis, adherent cells were scraped from the flasks rather than trypsinized. The human T-cell leukemia line CEM was maintained at 37°C in 5% CO₂/95% air and passed every 3 days in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS, 1% glutamine, and 1% penicillin/streptomycin.

**Immunofluorescence of Cell Lines.** The binding of unconjugated MoAb and IT to H2981-T3 and CEM cells was determined by indirect immunofluorescence. In brief, 10⁴ cells in 0.1 ml were incubated with various concentrations of MoAb or IT for 30 min at 4°C, in the presence of 0.1% NaN₃. After incubation, cells were washed and incubated with 50 μl of a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse γ-globulin (G Meloy Laboratories, Springfield, VA). Cells were washed 3 times, fixed in 400 μl 1% paraformaldehyde, and analyzed using a FACS IV (Becton Dickinson, Mountain View, CA).

To test for the presence of the L6-defined antigen in tumors not cured by treatment with L6-ricin, relapsing tumor that had initially responded to L6-ricin treatment (95% tumor size reduction) was rechallenged by treatment with 1.6 ricin, relapsing tumor that had initially responded to L6-ricin treatment (95% tumor size reduction) was rechallenged by treatment with 1.6 ricin, relapsing tumor that had initially responded to L6-ricin treatment (95% tumor size reduction) was rechallenged by treatment with 1.6 ricin, relapsing tumor that had initially responded to L6-ricin treatment (95% tumor size reduction) was rechallenged by treatment with 1.6 ricin. As a negative control, one sample was incubated with 100 ng/ml L6 or anti-CD5 MoAb, respectively, and allowed to adhere to plastic. The medium was then replaced by 100 μl of the same incubation medium containing the appropriate amount of IT and 200 mM lactose. After a 2-h incubation, quadruplicate wells were washed twice and incubated for 24 h in leucine-free RPMI 1640 containing 20% dialyzed FCS and 1% penicillin/streptomycin. The cells were then pulsed for 4 h at 37°C with 1 μCi [³H]leucine (American, Arlington Heights, IL) per well. The monolayers were washed twice in serum-free medium, resuspended with trypsin-EDTA (5 min, 37°C), and harvested onto glass filter paper using an automatic harvester (Brandel, Gaithersburg, MD). Filter paper discs were dried, and [³H]leucine incorporated into the cells was determined by using standard scintillation counting techniques. The results were expressed as percentages of the lactose control.

**Radiolabeling.** L6 MoAb and ricin were labeled with iodine-125 using an iodine-monochloride micro-method which has been previously described (28). Briefly, 200-μg aliquots of MoAb or ricin were labeled with 3 mCi of radionuclide and 12 equivalents of ICI. Free iodide ions were removed by passage over a Dowex 1-X4 resin column. The contents of the column were collected into vials containing 5% human serum albumin. When ricin was radiolabeled, the column was washed with 1 ml of 100 mM lactose to block ricin binding. A 20% loss of protein content during the labeling procedure was assumed, based on previous studies (11), and all concentrations were adjusted using this value.

To determine the amount of label incorporated into protein, 10% TCA was added to the radiolabeled reagents, after which the tubes were mixed and incubated for 30 min at room temperature. Precipitated protein was pelleted. The pellet and supernatant were counted in a well-type gamma counter (Gamma 5500; Beckman Instruments, Fullerton, CA). The percentage of TCA-precipitable counts was determined using the following equation:

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

**Scatchard Analysis.** Binding of radiolabeled MoAb and ricin was assayed using a modification of previously described techniques (15, 29–32). Target cells (10⁴/tube) were washed and resuspended in 100 μl RPMI, 5% FCS, containing increasing amounts of radiolabeled MoAb or ricin. Binding of the labeled reagents was lowered 100-fold by adding unlabeled protein. Thus, it was possible to incubate with saturating protein concentrations without surpassing the counting limits of the gamma counter used. The protein concentrations used for binding studies ranged from 3.9 ng/ml to 390 μg/ml for L6 MoAb and from 2.25 to 180 μg/ml for ricin. The binding at each protein concentration was determined in duplicate samples. Nonspecific binding of the radiolabeled ligand was determined for each protein concentration by adding excessive amounts (20 times the saturating concentration) of unlabeled ligand to the suspension prior to incubation with radioligand. (The percentage of bound activity under blocked conditions was subtracted from bound activity under unblocked conditions. The resulting activity was taken as the corrected, specific, bound activity. The cell suspensions were vortexed and incubated for 1 h at 4°C. Preliminary experiments indicated that binding of both radiolabeled L6 and ricin...
was complete by 1 h. After incubation, 90% of the cell suspension were in a gamma counter. Pellets and supernatants were counted through the oil at 10,000 x g. Scatchard analysis was performed using H2981-T3 cells, to determine the association constant of the equilibrium binding of labeled L6, the number of L6 binding sites per cell, and the number of ricin binding sites per cell. The data were plotted as the number of molecules bound to each cell at a given dilution versus the ratio of bound MoAb to free MoAb divided by the concentration of cells in cells/liter. The amount of free MoAb was corrected for the immunoreactive fraction, and the corrected binding data for specific binding were considered for the Scatchard representation. K∞ values were derived from the slopes of the linear regression lines and expressed as liters/mol (M⁻¹). The binding sites were expressed as n/cell.

Clonogenic Assay. Neoplastic target cells were assayed for clonogenic growth by limiting dilution, as previously described (33).

Measurement of Normal Bone Marrow Progenitor Cells in Mixed Colony Assays. Human bone marrow, obtained from normal donors following informed consent, was cultured for progenitor cells using a modification of a previously described technique (34). Briefly, cells suspended in culture medium at concentrations ranging from 0.5 x 10⁵ to 2 x 10⁶ were plated in triplicate in 15-mm wells (Nunc, Roskilde Denmark). Culture medium consisted of Iscove's modified Dulbecco's medium with 30% FCS, 5 x 10⁻² x 2-mercaptoethanol 0.01 g/ml methycellulose, 100 μg recombinant granulocyte/macrophage colony-stimulating factor, 5 ng/ml recombinant interleukin 3, and 1 unit/ml recombinant erythropoietin. The recombinant cytokines were kindly provided by Genetics Institute (Cambridge, MA). After 14 days in culture at 37°C in 4% CO₂/96% air, colonies were visually scored using a stereo dissecting microscope. Mixed colonies containing erythroid and nonerythroid cells were scored as CFU-mix. Pure erythroid colonies were scored as BFU-E, and nonerythroid colonies were scored as CFU-GM.

Animals. BALB/c (nu/nu) male mice, 4 weeks old, were obtained from Charles River (Wilmington, MA) or Simonsen Laboratories (Gilroy, CA). The animals were kept under sterile conditions in cages with filter bonnets. They were maintained on Purina Lab Chow mouse diet and given water, supplemented with hypochlorite, ad libitum.

Establishment of H2981-T3 Tumors in Nude Mice. H2981-T3 cells (5 x 10⁶ in 0.1 ml RPMI) were injected s.c. into the flanks of nude mice. Palpable tumors were detected after 2–3 weeks. Tumor establishment in the animals was 80–90%. Tumor size was evaluated by measuring three perpendicular diameters of each tumor. Multiplication of these diameters yielded an index for tumor size, given in cm³. Statistical comparisons of tumor sizes between various treatment groups were performed by Student's t test at different time points after treatment. Tumors were read blindly.

Tumor Treatment with Immunotoxins. Tumors with a size index of between 0.3 and 0.6 cm³ were treated by intratumoral injection of 5 or 10 μg of L6-ricin, control anti-CD3-ricin, or control anti-CD5-ricin. The intratumoral injections were preceded by 0.5-ml i.v. injection of 750 mM lactose solution per mouse. The intratumoral injections contained 300 mM lactose in PBS and 5 or 10 μg/0.1 ml IT. Control animals which only received lactose were included and usually had tumors of 3–6 cm³ by day 30. Mice treated with unconjugated ricin did not receive a lactose blockade prior to treatment. Responses were considered complete if there was no detectable tumor on day 30. Responses were defined as partial if tumors were <50% of the size of the untreated control tumor, a definition established by the World Health Organization.

RESULTS

Gel and HPLC Analysis of Immunotoxins. Two different batches of L6-ricin and two different batches of anti-CD5-ricin were tested. High performance liquid chromatography profiles were similar if not identical for all (not shown). Data from SDS-PAGE under nonreducing conditions and gel analysis by scanning are shown in Fig. 1. Gel profiles of L6-ricin (Fig. 1, lanes 1 and 2) and anti-CD5-ricin (Fig. 1, lanes 3 and 4) revealed four major IT bands exceeding M₀, 150,000. The protein bands corresponded to IT species of molecular weights 170,000, 190,000, 220,000, and 260,000. Scanning showed that the largest band (on a percentage basis) for all IT was at M₀, 220,000 (43.3–51.6%). These bands contained over twice the amount of any other IT band and likely represent one ricin molecule linked to one antibody molecule. Only a small amount of M₀, 260,000 species was present for all IT (0.1–6.0%). This band likely represents two ricin molecules linked to one antibody molecule. There were no major differences in IT bands (bands of M₀, >150,000) obtained with either L6-ricin or anti-CD5-ricin. The M₀, 170,000 and 190,000 species likely represent antibody fragments linked to ricin and represent <9.4% and <16.3% of the total protein, respectively. We did not attempt to remove these species. Gel scanning revealed 0.3–7.8% contaminating free MoAb and 2.3–8.9% free ricin. L6-ricin showed a band at M₀, 125,000 that was not present for anti-CD5-ricin. The band could represent one light chain reannealed to two reannealed heavy chains, while the band at M₀, 100,000 for all IT could represent reannealed heavy chain. The band of anti-CD3-ricin that was used for in vivo studies showed less than 5% free ricin (not shown).

Affinity and Binding Sites of ¹²⁵I-Ricin on H2981-T3 Cells. Since the native galactose binding site of ricin is present on the surface of eukaryotic cells, a difference in the number of galactose binding sites on H2981-T3 and the control CEM line might influence our results. Thus, we studied the binding of ricin to H2981-T3 and CEM cells. Table 1 shows the Scatchard calculations of the binding data. Ricin bound to H2981-T3 cells with an association constant, K∞, of 1.8 x 10⁶ M⁻¹ (average of two experiments) and each tumor cell had an average of 16 x 10⁶ receptors. CEM cells had a similar affinity of 3.7 x 10⁶ M⁻¹ and 9 x 10⁶ ricin binding sites/cell (average of two experiments). The data indicate that the binding of ricin to H2981-T3 and CEM cells is comparable. Affinity of L6 MoAb and L6...
Table 1  
Affinity of ricin and number of ricin receptors on H2981-T3 and CEM cells as measured by Scatchard analysis

One million cells were incubated with increasing concentrations of 125I-labeled ricin for 60 min at 4°C. Cell-associated radioactivity was separated from unbound activity by centrifugation through an oil layer, as described in "Materials and Methods." Bound and free radioactivity (cpm) were analyzed by the Scatchard method. The analysis was performed without lactose.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line</th>
<th>Kd (mM)</th>
<th>Ricin receptor sites/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H2981-T3</td>
<td>1.5 x 10^6</td>
<td>21 x 10^6</td>
</tr>
<tr>
<td>2</td>
<td>H2981-T3</td>
<td>2.0 x 10^4</td>
<td>11 x 10^4</td>
</tr>
<tr>
<td>3</td>
<td>CEM</td>
<td>5.1 x 10^5</td>
<td>10 x 10^5</td>
</tr>
<tr>
<td>4</td>
<td>CEM</td>
<td>2.2 x 10^4</td>
<td>8 x 10^4</td>
</tr>
</tbody>
</table>

Table 2  
Binding of MoAb L6 and L6-ricin IT to H2981-T3 cells as measured by flow cytometry

Indirect immunofluorescence was performed with one million L6-positive H2981-T3 cells or CDS-negative CEM cells. Cells were washed, treated with MoAb or IT at 4°C for 30 min, washed again, and then stained with fluorescein isothiocyanate-goat anti-mouse γ-globulin G. Binding was measured by flow cytometry using a FACS IV and expressed as percentage of positive cells. For each cell line, background binding was determined by incubating cells with mouse myeloma protein followed by fluorescein isothiocyanate-goat anti-mouse γ-globulin G.

<table>
<thead>
<tr>
<th>MoAb or IT</th>
<th>Concentration (μg/ml)</th>
<th>Positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6</td>
<td>1</td>
<td>53.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>70.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>95.1</td>
</tr>
<tr>
<td>L6-ricin</td>
<td>1</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>81.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>81.3</td>
</tr>
<tr>
<td>Anti-CD5</td>
<td>1</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.6</td>
</tr>
<tr>
<td>Anti-CD5-ricin</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>34.4</td>
</tr>
</tbody>
</table>

ND, not determined.

L6 receptor number on H2981-T3 cells were also determined. Scatchard analysis revealed a Kd of 1.6 x 10^8 M^-1 for the L6 antigen-MoAb interaction and 170,000 L6 receptors/cell. All Scatchard plots were curvilinear. The r^2 value for the regression analysis was 0.97.

Binding of MoAb or Immunotoxin to Target Cells. The binding of unconjugated MoAb or IT to H2981-T3 (L6 positive, CDS negative) and to CEM (L6 negative, CDS positive) cells was determined by indirect immunofluorescence at various protein concentrations. A representative experiment is shown in Table 2. The cell suspensions contained 200 mM lactose to block the binding of ricin B-chain. At 1, 10, and 50 μg/ml, L6 MoAb bound to 53.5, 70.7, and 95.1% of the H2981-T3 cells and L6-ricin bound to 5.0, 81.2, and 81.3% at the same concentrations. This indicates that conjugation to ricin had minimal effect on MoAb binding. Binding to the control cell line CEM was minimal for both L6 MoAb and L6 IT. Conversely, anti-CD5 and anti-CD5-ricin IT bound to CEM cells, while binding to H2981-T3 cells only occurred at high IT concentrations which might overcome the lactose blockade.

Antigenic Modulation of L6 on H2981-T3 Cells. To determine the degree of L6-induced modulation of the receptor for MoAb L6 on H2981-T3 cells, these cells were incubated with 0.1, 1.0, or 10.0 μg/ml MoAb L6 for 2 h at 37°C. We measured 7.3, 6.5, and 5.1% modulation, respectively (Table 3). As a positive control, we measured the modulation of CD5 on PBMC after incubation with the anti-CD5 antibody. Anti-CD5 was chosen as a control because a detailed study of the modulation of CD5 was previously published (27, 35). As a further control, we studied CD5 modulation of a CD5-positive neoplastic cell line, CEM. Modulation of CD5 on PBMC was 6–15-fold higher than the modulation of the defined L6 antigen on H2981-T3 cells (49.8 and 55.2% at 1.0 and 10.0 μg/ml antibody). Modulation of CD5 on CEM cells was 2-fold higher (14.2 and 16.7% at 1.0 and 10.0 μg/ml antibody). A prolonged incubation time with MoAb L6 (up to 16 h) did not increase the modulation of the L6-defined antigen, whereas the modulation of CD5 on PBMC increased more than 30% (data not shown). We conclude that the L6-defined antigen does not modulate well.

In Vitro Cytotoxic Activity of L6-Ricin Immunotoxins. To evaluate the cytotoxic potential of L6-ricin IT, we incubated adherent H2981-T3 cells with various concentrations of the IT for 2 h at 37°C in a lactose-containing medium (Table 4). At concentrations of 0.01, 0.1, 0.3, 1.0, and 5.0 μg/ml, L6-ricin IT reduced protein synthesis to 66.2, 50.5, 42.1, 23.5, and 7.9%, respectively. The IC50 value (i.e., the concentration of L6-ricin IT inhibiting protein synthesis to 50% of control) was 0.1 μg/ml. When L6-ricin was incubated with a 200-fold excess of unconjugated MoAb L6, inhibition of protein synthesis was blocked 35-fold (IC50 = 3.5 μg/ml). Blocking with IgG decreased the inhibition of protein synthesis 7-fold (IC50 = 0.7 μg/ml). Blocking was inefficient at 5 μg/ml, which was probably due to the inefficiency of the lactose blockade (36).

When treated with 0.01, 0.1, and 1.0 μg/ml levels of a control IT, anti-CDS-ricin, the protein synthesis of H2981-T3 cells as compared to controls, was 70.7, 73.0, and 52.7%, respectively. Thus, the IC50 value for anti-CDS-ricin was 10-fold higher (1 μg/ml) than the IC50 value for L6-ricin. The effect of anti-CDS-ricin could be blocked almost completely by addition of 1 mg/ml human IgG, suggesting that ricin IT can enter and kill cells in more ways than binding of specific antibody or B-chain.

Effect of L6-Ricin on the Clonogenic Growth of H2981-T3 Cells. To determine the potency of L6-ricin, 10^5 adherent H2981-T3 cells were treated with 1 μg/ml L6-ricin for 24 h at 37°C and then plated in a limiting dilution assay following resuspension of trypsinized cells. Comparison of the colony counts for L6-ricin-treated cells versus nontreated controls gave an estimate of 4.1-log killing of clonogenic cells on day 7. In a second experiment, we observed 3.5-log kill. In an experiment in which H2981-T3 cells were admixed with a 20-fold higher number of human bone marrow mononuclear cells, we measured about 4-log kill of H2981-T3.

Effect of L6-Ricin on in Vitro Cultured Normal Bone Marrow Progenitor Cells. To determine the toxicity of L6-ricin to human stem cells, bone marrow was preincubated with L6-ricin, studied CD5 modulation of a CD5-positive neoplastic cell line, CEM. Modulation of CD5 on PBMC was 6–15-fold higher than the modulation of the defined L6 antigen on H2981-T3 cells (49.8 and 55.2% at 1.0 and 10.0 μg/ml antibody). Modulation of CD5 on CEM cells was 2-fold higher (14.2 and 16.7% at 1.0 and 10.0 μg/ml antibody). A prolonged incubation time with MoAb L6 (up to 16 h) did not increase the modulation of the L6-defined antigen, whereas the modulation of CD5 on PBMC increased more than 30% (data not shown). We conclude that the L6-defined antigen does not modulate well.
washed, and then cultured for the growth of CFU-mix, BFU-E, and CFU-GM after 14 days. In Table 5, experiment 1, L6-ricin at 1 µg/ml did not inhibit CFU-mix or BFU-E; CFU-GM were inhibited by 64%. In a second experiment with cells from a different donor, CFU-mix was inhibited by 40%, BFU-E was inhibited by 24%, and CFU-GM was inhibited by 31%.  

In Vivo Treatment of Established H2981-T3 Tumors with Intratumoral Ricin. Before evaluating the antitumor efficacy of ricin IT in vivo, the effect of free ricin on tumor growth was examined. Nude mice were s.c. inoculated with 5 x 10^6 H2981-T3 cells. Three weeks later, they had tumors with a size index of 0.3–0.6 cm^3, which were treated with 0.2, 0.4, 0.6, or 1.0 µg ricin in 100 µl PBS. Control animals received 100 µl lactose. Intratumoral injection of ricin had a dose-dependent inhibitory effect on tumor growth in vivo. As shown in Fig. 2, tumors injected with 0.2, 0.4, or 0.6 µg of ricin grew more slowly than the controls. The difference in tumor size indices between animals treated with 0.4 µg ricin and controls was significant, as shown by Student’s t test on day 30 after treatment (P < 0.021). However, at a dose of 0.6 µg, two of the four treated animals died within 6 days due to ricin toxicity. These animals showed a characteristic wasting syndrome with a purpuric rash distributed over the whole body, which appeared 1–2 days after treatment. At a dose of 1 µg ricin, all of the six treated animals died within 5 days. Animals which survived treatment with ricin for longer than 6 days usually recovered from the toxic effects within 1 or 2 weeks.  

Antitumor Activity of in Vivo Treatment of Established H2981-T3 Tumors with Intratumoral L6-Ricin and Anti-CD5-Ricin plus Lactose. To determine antitumor efficacy, nude mice bearing H2981-T3 tumors with a size index of 0.3–0.6 cm^3 were randomly allocated to different treatment groups, after which 5 or 10 µg of L6-ricin IT in 100 µl of 300 mM lactose were injected into each tumor. Five min prior to the application of IT, the animals received 500 µl of 750 mM lactose i.v. The same dose of lactose alone was given to the control mice. Fig. 3A shows a representative experiment. Both of the two groups treated with 5 or 10 µg of L6-ricin showed a marked inhibition of tumor growth, in comparison to the lactose controls. This effect was dose dependent, since 10 µg of L6-ricin caused more tumor inhibition than 5 µg. The difference in tumor size between either IT group and lactose controls was significantly different in a Student’s t test on day 30 (10 µg L6-ricin versus lactose control, P < 0.001; 5 µg L6-ricin versus controls, P < 0.001). Of the six animals treated with 5 µg L6-ricin, reexamination on day 45 showed stabilization in one case, relapse in three cases, and two cures. Of the four survivors treated with 10 µg L6-ricin, two cures and two relapses were apparent upon reexamination on day 45. The administration of 50 µg unmodified MoAb L6 had no effect on established tumors.  

To evaluate the specificity of L6-ricin treatment, we determined the effect of anti-CD5-ricin IT on H2981-T3 tumors, since anti-CD5-ricin did not bind to H2981-T3 cells according to FACS analysis. Fig. 3B shows the growth curves of tumors treated with 5 or 10 µg of anti-CD5-ricin, in comparison to lactose controls. Anti-CD5-ricin had a dose-dependent inhibi-
L6-RICIN ANTITUMOR ACTIVITY

Fig. 3. Growth of H2981-T3 tumors following intratumoral administration of L6-ricin (A) and anti-CD5-ricin (B) in nude mice. Groups of 4–7 animals bearing H2981-T3 tumors between 0.3 and 0.5 cm³ were given injections of 0 (□), 5 (■), or 10 μg (△) IT in 300 mg lactose. Animals received 500 mIU of 750 mIU lactose i.v. 5 min prior to the application of IT. Tumor size is plotted against the day of measurement; the data points represent the means and SE in each treatment group. The data in the parentheses are the number of survivors after 30 days over the total number of treated animals in each group.

Table 6 Survival and antitumor response after treatment of established H2981-T3 tumors with ricin IT

<table>
<thead>
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<th>Dose (μg)</th>
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<th>Number of animals</th>
<th>Antitumor responses</th>
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<td></td>
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<td>Survivors</td>
</tr>
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<td>10</td>
</tr>
<tr>
<td>5</td>
<td>L6-ricin</td>
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<tr>
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<td>Anti-CD5-ricin</td>
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DISCUSSION

The antigen defined by L6 MoAb is expressed on the surface of neoplastic lung, breast, colon, and ovarian tissue. L6-ricin IT may be used for in vitro purging of residual tumor cells from the bone marrow of carcinoma patients with marrow involvement, prior to autologous bone marrow transplantation. This technique is used to allow more aggressive radiochemotherapy for high risk patients, followed by a rescue dose of their own marrow. L6-ricin may prove useful for this purpose, since our studies showed that, at a concentration (1 μg/ml) that was...
sufficient to inhibit about 99.99% of clonogenic growth, there was still ample growth of normal bone marrow progenitors. It should be noted that the 37–64% reduction of CFU-GM could be due to expression of the L6 antigen on accessory cells that promote CFU-GM growth in vitro or due to Fc-mediated L6 ricin toxicity.

Gel analysis showed that both L6-ricin and anti-CD5-ricin displayed four major bands in excess of $M$, 150,000. Scanning showed that the most abundant band was at $M$, 220,000, likely representing one ricin molecule linked to an antibody molecule. However, a small amount (0.1–6.0%) of $M$, 260,000 species, likely representing two ricins linked to one antibody, was also present. Contaminating free antibody and ricin were minimal. L6 bound with an affinity of $1.6 \times 10^{8}$ M$^{-1}$ to the adenocarcinoma cell line H2981-T3. An average of $1.7 \times 10^{5}$ receptors/cell were expressed. Similar values have been reported for L6 MoAb on Ch-27 cells (17). Ricin binding studies showed that ricin bound similarly to H2981-T3 and CEM cells. Thus, differences obtained with the two cell lines could not be attributed to ricin binding differences.

Binding of L6-ricin to H2981-T3 cells incubated with lactose was specific, as shown by immunofluorescence. In contrast, inhibition of protein synthesis by L6-ricin in the presence of lactose was not entirely selective, and blocking studies with unconjugated L6 and human IgG showed that both a specific and a nonspecific component of L6-ricin were active. Approximately 5 times greater specific than nonspecific activity was apparent. An irrelevant anti-CD5 IT showed a 10-fold higher IC$_{50}$ value for inhibition of protein synthesis of H2981-T3 than did L6-ricin, even in the presence of lactose. The inhibition of the irrelevant IT could be entirely blocked by human IgG. Taken together, these findings indicate that some part of the antibody moiety other than the variable region, probably the Fc region, can also mediate IT activity. Others have reported nonspecific binding of abrin IT on human melanoma cells that did not involve the galactose binding site of the B-chain (37). However, in contrast to our findings, preincubation with human F(ab')$_2$ showed no effect in their studies, and binding was dependent on the cell line used.

We observed low modulation of L6 antigen on H2981-T3 cells, as compared to the modulation of CD5 on CEM and PBMC. Since the translocation of ricin A-chain into the cytosol is a prerequisite for killing, perhaps the antitumor efficacy of L6-ricin is attributed to the translocation-enhancing capabilities of ricin B-chain (15). Notably, L6 IT made with A-chain devoid of B-chain were not cytotoxic (data not shown).

There are several reasons for evaluating IT in a lung adenocarcinoma growing in nude mice. (a) Intratumoral injection obviates two major problems associated with IT therapy: limited tumor localization (38) and rapid elimination of IT from the bloodstream by the liver and the spleen (11, 39, 40). (b) IT constructed with intact ricin can be used, which has higher cytotoxicity but lower specificity than A-chain IT (8, 15, 41). (c) Intratumoral injections may be used as an adjunct therapy for treating carcinomas localized to particular areas, e.g., ovarian carcinomas in the peritoneal cavity.

A tumoricidal effect was observed with intratumoral injections of L6-ricin IT, corresponding to our in vitro findings. Regressions were noted after a single injection of 5 or 10 µg of IT. The antitumor effect in this system was more impressive than in our previously published work using anti-CD5-ricin IT with CEM lymphomas (8), since there was a higher incidence of cures with tumors of similar size. However, the L6 model showed a higher incidence of tumor regressions induced by treatment with nonspecific anti-CD5-ricin IT. Perhaps Fc binding caused indiscriminate tumor kill. Alternatively, H2981-T3 carcinomas are not as well vascularized as the soft CEM lymphomas, the former being whitish and the latter being reddish tumors. The carcinomas may not be as easily saturated after i.v. injection of lactose.

Although selectivity in tumor regression was not absolute, the systemic administration of lactose is important in our model. In the absence of lactose prior to IT treatment, all animals treated with 10 µg of anti-CD5-ricin died. In contrast, only 50% of the mice died who had received lactose prior to anti-CD5-ricin. This indicates that lactose reduces the toxicity of the irrelevant IT. The toxicity of L6-ricin was not affected by lactose, suggesting that the L6 determinant kept the IT localized to the tumor.

We also studied the effects of ricin alone on tumor growth. Efficacious treatment of neoplasms in humans (42) and in animal models with unconjugated ricin has been reported (43). We were able to identify a dose of ricin (0.4 µg) which could induce a significant antitumor effect without significant toxicity. However, the effect was partial, since relapse occurred. Higher concentrations were toxic, while lower concentrations were not effective at all. In the CEM system, lower concentrations (0.3 µg) of ricin were lethal without a detectable tumoricidal effect (8). The different effects in the two systems cannot be explained by a difference in numbers of galactose receptors, since Scatchard analysis of ricin binding showed only slight differences. There may be differences in tumor vascularization and, therefore, different clearances of ricin in CEM and H2981-T3.

A comparison of the toxicity caused by the relevant (L6-ricin) or irrelevant (anti-CD5-ricin or anti-CD3-ricin) IT at a dose of 10 µg reveals a higher systemic toxicity for the irrelevant IT (8% survivors for anti-CD3-ricin and 50% survivors for anti-CD5-ricin) than for L6-ricin (77% survivors). This may be explained by the ability of L6-ricin to bind to the tumor, while the control IT displayed nonspecific binding. The higher toxicity seen with 10 µg anti-CD3-ricin in comparison to anti-CD5-ricin might be due to a difference in subclasses of the two antibodies and differences in Fc binding properties. The treatment of tumors with L6-ricin did not lead to a selection of antigen-negative cells in relapsing tumors, as could be shown by staining a relapsed tumor with L6 MoAb. Thus, it may be possible to increase the therapeutic efficacy of L6-ricin by repeated applications of the IT.

In conclusion, we observed that L6-ricin is a relatively selective IT with a high cytotoxic potency. Thus, L6-ricin may be useful for the in vitro removal of residual tumor cells from autologous bone marrow for patients with solid tumors. Comparisons of the therapeutic and toxic effects of intratumoral treatment using L6-ricin, the irrelevant IT, and free ricin show that the toxicity of L6-ricin in this system can be mediated by at least three different sites on the molecule: the specific antibody binding site, a nonspecific antibody binding site (probably Fc), and the ricin B-chain. The specific and nonspecific binding of ricin IT can contribute to the overall therapeutic effectiveness of intratumoral treatment, a situation that might be advantageous from interstitial tumor treatment of carcinomas but undesirable for systemic treatment. Clearly, the lack of absolute specificity in vivo remains a concern. For the systemic use of L6 IT, it may be necessary to synthesize more selective agents, such as an IT containing ricin with a manipulated B-chain (44–46) or Fab fragments coupled to toxin.

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Antitumor Activity of L6-Ricin Immunotoxin against the H2981-T3 Lung Adenocarcinoma Cell Line *in Vitro* and *in Vivo*

Heinz Schmidberger, Laurel King, Larry C. Lasky, et al.