Correlation between \textit{in Vivo} Toxicity and Preclinical \textit{in Vitro} Parameters for the Immunotoxin Anti-B4-blocked Ricin\textsuperscript{1}

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ABSTRACT

Anti-B4-blocked ricin (Anti-B4-bR) is an immunotoxin comprised of the anti-B4 monoclonal antibody and the protein toxin, “blocked ricin.” In blocked ricin, the galactose-binding sites of the ricin B-chain which mediate nonspecific binding to cells are blocked by covalently linked affinity ligands prepared from N-linked oligosaccharides of fetuin. Blocked ricin consists of two species, one with two covalently attached ligands and one with three covalently attached ligands. In a Phase I dose escalation clinical trial, Anti-B4-bR was administered to patients with relapsed and refractory B-cell neoplasms by 7-day continuous infusion. Although several different lots of Anti-B4-bR had similar IC\textsubscript{50} values as determined by \textit{in vitro} cytotoxicity testing on cultured human cell lines, these lots differed in their \textit{in vivo} toxicity when administered to patients. Thus, IC\textsubscript{50} values alone were not sufficient to predict \textit{in vivo} toxicity. We report that the degree of cell kill at concentrations of drug that saturate the B4 antigen and murine 50% lethal dose values provide additional parameters that may be predictive of \textit{in vivo} cytotoxicity. Furthermore, we performed detailed cytotoxicity studies of the ricin species containing two and three covalently attached ligands, respectively. \textit{In vitro} cytotoxicity testing using these samples revealed that Anti-B4-bR made with blocked ricin containing two covalently attached ligands is capable of depleting five logs of target cells in an \textit{in vitro} cytotoxicity assay, while Anti-B4-bR comprised of blocked ricin with three ligands can deplete only one log of cells. Log cell kill at antigen saturating concentrations, murine 50% lethal dose and biochemical analysis of the composition of blocked ricin are therefore important considerations for establishing the potential efficacy and safety of Anti-B4-bR.

INTRODUCTION

Monoclonal antibody-toxin conjugates, known as immunotoxins, bind to antigens on the surface of malignant cells and may both specifically direct cytotoxicity to those cells and circumvent mechanisms of tumor cell resistance by using distinct cytotoxic mechanisms (1, 2). Anti-B4-bR\textsuperscript{2} is an immunotoxin comprised of the anti-B4 (CD19) monoclonal antibody and the protein toxin, “blocked ricin” (3). The CD19 antigen is expressed on nearly all normal and neoplastic B-cells, and is a potential target for immunotoxin therapy. The characterization of blocked ricin containing two covalently attached ligands is capable of depleting five logs of target cells in an \textit{in vitro} cytotoxicity assay, while Anti-B4-bR comprised of blocked ricin with three ligands can deplete only one log of cells. Log cell kill at antigen saturating concentrations, murine 50% lethal dose and biochemical analysis of the composition of blocked ricin are therefore important considerations for establishing the potential efficacy and safety of Anti-B4-bR. Anti-B4-bR for clinical use is required to demonstrate appropriate potency, selectivity, and specificity as determined by \textit{in vitro} cytotoxicity assays by using Namalwa, a cell line expressing B4, and Molt-4, a cell line that lacks surface B4. An initial Phase I dose escalation trial used four lots of Anti-B4-bR to treat 25 patients with relapsed and refractory B-cell neoplasms (10). We demonstrated that the maximal tolerated dose was 50 \textmu g/kg/day when Anti-B4-bR was administered by daily 1-h infusions for 5 consecutive days. The dose-limiting toxicity was defined by transient, dose-related, reversible Grade 3 elevations in serum transaminases. By using daily bolus infusions, serum levels capable of depleting up to 5 logs of malignant cells in \textit{in vitro} were obtained for less than 6 h. Preclinical animal studies indicated that higher doses of Anti-B4-bR could be administered with reduced nonspecific toxicity if the immunotoxin was administered by prolonged continuous infusion. Thus, a second Phase I dose escalation trial was undertaken in patients with refractory B-cell neoplasms in which Anti-B4-bR was administered by 7-day continuous infusion.

In the present report, we discuss the clinical observation that some lots of Anti-B4-bR that were used to treat some patients on the continuous infusion trial did not lead to increasing clinical toxicity when administered at increasing doses. Further evaluation of these lots showed that despite similar IC\textsubscript{50} values to those of lots used in the bolus infusion trial, their log kill on Namalwa cells were different. We concluded, therefore, that the IC\textsubscript{50} values for specific and nonspecific \textit{in vitro} cytotoxicity alone were not adequate for preclinical cytotoxicity assessment of Anti-B4-bR. In this report, we describe the clinical, biological, and biochemical differences among these lots of Anti-B4-bR. This has led to enhanced understanding of the mechanism of Anti-B4-bR cytotoxicity and to the development of new tests for preclinical screening.

MATERIALS AND METHODS

Materials. Ricin D [nomenclature of Wei and Koh (11)] was purchased from Inland Laboratories (Austin, TX). The monoclonal antibody anti-B4 was purchased from Brunswick BioTechnetics (San Diego, CA), and was purified as described previously (4, 12). Rabbit anti-ricin immunoglobulin, fluorescein-labeled goat anti-rabbit immunoglobulin, and rabbit globin mRNA were from Sigma Chemical Co. (St. Louis, MO). A rabbit reticulocyte lysate system for cell-free protein synthesis, which included tryptophan, isoleucine (specific radioactivity, 146.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate was purchased from Pierce Chemical Co. (Rockford, IL). The reactive affinity ligand for blocking the galactose-binding sites of ricin was prepared by chemical and enzymatic modification of glycoproteins containing triantennary N-linked oligosaccharides derived from fetuin (3, 9).

Cell Lines. The CD19 antigen-positive human B-lymphoblastoid cell line Namalwa (ATCC CRL 1432) derived from a Burkitt’s lymphoma, and the CD19 antigen-negative human cell line Molt-4 (ATCC CRL 1582) derived from an acute T-lymphoblastic leukemia were from the American Type Culture Collection (Bethesda, MD). Cells were grown in RPMI 1640 medium supplemented with 10% heat-treated (56°C for 30 min) fetal calf serum and 2 mm \textgamma-glutamine at 37°C in a
humidified atmosphere containing 5% CO₂, and were maintained in exponential growth as asynchronous cultures. For binding assays, cells were transferred into a medium (AB-medium) comprising minimal essential medium (Cellgro, Herndon, VA) containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.0, and 2.5% (v/v) pooled human AB serum (Pel Freeze Biologicals, Rogers, AR).

Preparation of Anti-B4-blocked Ricin. Blocked ricin was prepared from ricin by chemically blocking the galactose-binding sites by reaction with affinity ligands, followed by purification by affinity chromatography as described previously (9). The immunon conjugate between anti-B4 and blocked ricin was prepared and purified as described previously (3), and was formulated for clinical use in sterile phosphate-buffered saline (Dulbecco's; Sigma Chemical Co., St. Louis, MO) containing human serum albumin (1 mg/ml). The immunon conjugate was characterized and analyzed as described previously (3, 9). There was no detectable contamination by ricin as assessed by polyacrylamide/sodium dodecyl sulfate gel electrophoresis stained with silver stain (detection limit < 0.1%). Lots O19L.003 (A), P1.90C.001 (B), P1.90I.003 (C), P1.90.K005 (D), and P1.91B.001 (E) were used in the clinical trial described in this paper.

Analytical Scale Preparation of Species of Blocked Ricin Having Two or Three Covalently-linked Affinity Ligands, and Preparation of Their Immunon conjugates. Purified blocked ricin, which contained a mixture of species having two or three affinity ligands covalently linked to the B-chain as described previously (3, 9), could be fractionated on an analytical scale by cation exchange chromatography. Purified blocked ricin (100 mg) was applied to a column (20-ml bed volume) of S-Sepharose (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM sodium acetate buffer, pH 4.0. The column was developed with 50 mM sodium acetate buffer of increasing pH (pH 4 to 6.8). Analysis of fractions by polyacrylamide/sodium dodecyl sulfate gel electrophoresis showed that blocked ricin containing three covalently linked ligands eluted earlier from the column than blocked ricin containing only two ligands. Fractions eluting between pH 5.2 and 5.5 (about 10% of the total protein) predominantly contained blocked ricin with three ligands covalently linked to the B-chain, while fractions eluting at pH values above 6.0 (about 20% of the total protein) predominantly contained blocked ricin with two covalently linked ligands. These fractions were pooled separately. (The remainder of the protein contained a mixture of both species eluting approximately between pH 5.5 and 6.0.) The fractionated blocked ricin species were then passed through a galactose affinity column made from acid-treated Sepharose CL-4B and equilibrated in 10 mM potassium phosphate buffer, pH 6.8, containing 145 mM NaCl (13). The two different species of blocked ricin were conjugated to anti-B4 following the procedures already established (3).

Measurement of Protein Concentration. Concentrations of solutions of purified proteins were determined from their absorbance at 280 nm, assuming ε1% values of 14.0 for IgG, 11.8 for ricin and blocked ricin, and 13.4 for antibody-blocked ricin conjugates (3).

Polyacrylamide Gel Electrophoresis. Samples of protein were analyzed by polyacrylamide/sodium dodecyl sulfate gel electrophoresis in gel slabs (145 x 95 x 0.75 mm) of acrylamide prepared according to the method described (14). Sample buffers contained 2-mercaptoethanol (2%, v/v) for reducing conditions, or iodoacetamide (10 mg/ml) for nonreducing conditions. Following staining with Coomassie brilliant blue R-250, gels were scanned by using an LKB Ultrascan 2202 laser densitometer.

Assay for Protein Synthesis in Cell-free System. The activity of ricin A-chain in blocked ricin and in conjugates was determined as previously described (15, 16).

Binding Assays for Ricin and Blocked Ricin. Namalwa cells (2 x 10⁶ cells/sample) were incubated for 30 min at 0°C with various concentrations of ricin in AB-medium, then with rabbit anti-ricin immunoglobulin, and finally with fluorescein-labeled goat-anti-ricin IgG immunoglobulin, with a buffer wash between each incubation. Treated cells were then fixed with 1% formaldehyde in 10 mM potassium phosphate buffer, pH 7.2, containing 145 mM NaCl and analyzed on a flow cytometer (FACSScan, Becton-Dickinson, Mountain View, CA). The rabbit anti-ricin immunoglobulin was shown in an enzyme-linked immunosorbent assay to bind ricin and blocked ricin equally well.

Competition Binding Assay for Antibody. A competition binding assay was performed by incubating Namalwa cells with fluorescein-labeled anti-B4 (about 4 nm) mixed with various concentrations of competing test material (antibody or conjugate), and analyzing the labeled cells on a flow cytometer as described previously (3).

Cytotoxicity Assays. The cytotoxicity (IC₅₀ and log kill) of samples of ricin, blocked ricin, and immunon conjugates were determined for cultured cells by incubating the test samples in growth medium together with the cells for 24 h at 37°C. The treated cells were then washed and placed into fresh medium, and the surviving fraction determined by a direct cytotoxicity assay (growth back-extrapolation assay) as described previously (17, 18).

Preclinical in vivo toxicity was assessed by an LD₅₀ test in Swiss albino CD-1 mice.

Pharmacology. Anti-B4-bR concentration in serum was determined using 2 independent enzyme-linked immunosorbent assay methods. The two enzyme-linked immunosorbent assays were sandwich assays in which the anti-B4-bR conjugate was captured on plates coated with a sheep anti-mouse IgG (Fc specific) and then assayed with either goat anti-mouse IgG immunoglobulin conjugated to alkaline phosphatase or with rabbit anti-ricin immunoglobulin followed by goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase. A signal amplification method (Bethesda Research Laboratories) was used that allowed the use of highly diluted serum samples and reduced the nonspecific background signals.

Patient Selection. Patients were eligible for this study if they had non-T-cell acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, or B-cell non-Hodgkin's lymphoma which had relapsed from conventional primary or salvage chemotherapy regimens, and demonstrated failure to respond to any regimens of known therapeutic benefit. Tumor cells from all patients were required to demonstrate reactivity with the anti-B4 or anti-B1 monoclonal antibody, since it previously has been demonstrated that tumor cells which express the B1 antigen also express the B4 antigen (4). Tumor cell reactivity with the anti-B4 antibody could only be determined in patients for whom fresh or frozen tissue was available for analysis, while reactivity with the B1 antigen could be assessed by using fixed tissue. Patients were required to have an Eastern Cooperative Oncology Group performance status of 0–2, an expected survival of more than 2 months, and at least 3 weeks since any prior chemotherapy, radiation therapy, or major surgery. All patients were required to have a total bilirubin < 2.0 mg/dl, SGOT < 90 IU, and creatinine < 2.0 mg/dl. At protocol entry, patients had a WBC > 3000/mm³, hemoglobin > 25%, and platelets > 100,000/mm³. Patients with hematological parameters outside of this range were eligible for protocol entry if their cytopenias were considered secondary to bone marrow involvement by their neoplasm. All patients were without clinically significant cardiac or pulmonary symptomatology. Several patients underwent lumbar puncture prior to therapy to rule out the presence of lymphomatous meningitis, which would preclude their enrollment on this protocol. All patients signed an informed consent form approved by the Institutional Review Board of the Dana-Farber Cancer Institute.

Study Design. The study was designed to gradually escalate the administered dose of Anti-B4-bR until Grade 3 National Cancer Institute Common Toxicity Criteria toxicity was reached. The dose limiting toxicity was defined as the Grade 3 toxicity which resulted in cessation of dose escalation. At least 3 patients underwent therapy at each dose. If there was no demonstrable Grade 3 toxicity at a given dose level for all three patients, both during the infusion and 4 weeks thereafter, at least 3 patients were treated at the next dose level. Patients were eligible for retreatment at the same dose every 28 days if they continued to meet protocol eligibility requirements and failed to develop human anti-mouse antibody or human anti-ricin antibody. Eligible patients were admitted to the Dana-Farber Cancer Institute and received a continuous infusion of Anti-B4-bR via a central venous line for 7 consecutive days. The complete Phase I dose escalation trial involved 43 patients who were treated at doses of Anti-B4-bR ranging from 10
\( \mu g/kg/day \) to \( 70 \mu g/kg/day \) for 7 days. This paper will report cohorts of patients who received different lots of drug at doses of 50, 60, and \( 70 \mu g/kg/day \) for 7 days (patient numbers 119-143). All patients received allopurinol, 100 mg p.o. 3 times/day starting 48 h prior to treatment.

RESULTS

Patients Included in This Report. Forty-three patients with relapsed and refractory B-cell neoplasms were treated on this trial with constant infusions of Anti-B4-bR at doses ranging from \( 10 \mu g/kg/day \) for 7 days to \( 70 \mu g/kg/day \) for 7 days. For the purposes of this report, 24 patients who were treated with doses of Anti-B4-bR at 50, 60, and \( 70 \mu g/kg/day \) will be considered. The complete dose escalation characteristics of the entire trial, including the patient characteristics, pharmacokinetics, toxicities, and response rates will be described in a forthcoming report.

In Vitro Cytotoxicity of Anti-B4-bR blocked Ricin. Five different lots of Anti-B4-bR were used in this trial and are designated Lots A, B, C, D, and E (Table 1). Their cytotoxicity was assessed by using the growth back-extrapolation assay to determine IC\(_{37}\) values. Table 1 lists the IC\(_{37}\) values for the Lots A to E as tested on the B4-expressing Namalwa cell line and the B4-negative Molt-4 cell line. The IC\(_{37}\) values on Namalwa cells range from \( 10.3 \times 10^{-3} \) to \( 45 \times 10^{-3} \) nm for a 24-h exposure to Anti-B4-bR, a range of only 4-fold. Likewise, the IC\(_{37}\) values on Molt-4 cells range from 1.0 to 5.7 nm and were about 100-fold greater than the IC\(_{37}\) for Namalwa for each drug lot. All IC\(_{37}\) values were within the previously established range of cytotoxicities for clinical use (Namalwa: IC\(_{37}\) 6–60 x \( 10^{-3} \) nm, Molt-4: IC\(_{37}\) \( \geq 1 \) nm).

Treatment of Patients with Anti-B4-bR. Fig. 1A shows the elevations in SGOT and SGPT (expressed as the number of times each parameter is elevated above the upper limit of normal) that developed as patients received Anti-B4-bR from Lots A and B with the dose escalated from 50 \( \mu g/kg/day \) for 7 days up to \( 70 \mu g/kg/day \) for 7 days. All patients tolerated the full 7-day course of therapy, developing 5- to 10-fold elevations in hepatic transaminases. Remarkably, the SGOT and SGPT values did not increase in proportion to the administered dose, in contrast to our previous experience with bolus injections of Anti-B4-bR, a range of only 4-fold. Likewise, the IC\(_{37}\) values on Molt-4 cells range from 1.0 to 5.7 nm and were about 100-fold greater than the IC\(_{37}\) for Namalwa for each drug lot. All IC\(_{37}\) values were within the previously established range of cytotoxicities for clinical use (Namalwa: IC\(_{37}\) 6–60 x \( 10^{-3} \) nm, Molt-4: IC\(_{37}\) \( \geq 1 \) nm).

Table 1 Characterization of lots used in continuous infusion clinical trial

<table>
<thead>
<tr>
<th>Lot</th>
<th>Code</th>
<th>Specific cytotoxicity (Namalwa) IC(_{37}) (nm)</th>
<th>Non-specific cytotoxicity (Molt-4) IC(_{37}) (nm)</th>
<th>Log kill Namalwa (5 nm Anti-B4-bR)</th>
<th>LD(_{50}) (( \mu g/kg ))</th>
<th>% of blocked ricin with 2 attached ligands</th>
<th>% of blocked ricin with 3 attached ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>O19L.003</td>
<td>A</td>
<td>45 x ( 10^{-3} )</td>
<td>3.2</td>
<td>2.7</td>
<td>400</td>
<td>51</td>
<td>49</td>
</tr>
<tr>
<td>P1.90C.001</td>
<td>B</td>
<td>45 x ( 10^{-3} )</td>
<td>5.7</td>
<td>1.1</td>
<td>330</td>
<td>28</td>
<td>72</td>
</tr>
<tr>
<td>P1.90K.001</td>
<td>C</td>
<td>19 x ( 10^{-3} )</td>
<td>1</td>
<td>3</td>
<td>112</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>P1.90K.005</td>
<td>D</td>
<td>10.3 x ( 10^{-3} )</td>
<td>1.7</td>
<td>&gt;5</td>
<td>252</td>
<td>62</td>
<td>38</td>
</tr>
<tr>
<td>P1.91B.001</td>
<td>E</td>
<td>15.7 x ( 10^{-3} )</td>
<td>2.3</td>
<td>4</td>
<td>256</td>
<td>76</td>
<td>24</td>
</tr>
</tbody>
</table>

Through E were used. Fig. 1B shows that at any given dose of Anti-B4-bR, the elevation in transaminases was greater in patients treated with the Lots C, D, and E. Likewise, when the effect of different lots of Anti-B4-bR on platelet counts was examined, a similar difference was observed between Lots A and B and the latter three lots (data not shown).

Serum levels of Anti-B4-bR were determined to ascertain whether differences between the levels achieved after administering each lot of drug could explain the difference in elevation of serum transaminases. However, a comparison of patients treated at the maximum tolerated dose failed to show a significant difference in Anti-B4-bR serum levels, with mean serum levels of \( 165 \pm 67.9 \) ng/ml obtained, using Lots A and B, and mean serum levels of \( 269 \pm 31.0 \) ng/ml obtained, using Lots C through E (\( P = 0.09 \), one-sided test). Thus, the serum levels of Anti-B4-bR in the two groups were comparable and did not appear to account entirely for the observed difference in hepatotoxicity.

This prompted further in vitro characterization of the different lots of immunotoxin (Table 1). In addition to the IC\(_{37}\), the log cell kills for Namalwa cells at 5 nm concentration of Anti-B4-bR (a saturating concentration of the conjugate for the B4 antigen) were measured. Lots A and B were able to kill 2.7 and 1.1 logs of cells, respectively, while Lots C, D, and E, as well as previous lots of Anti-B4-bR used in the bolus infusion trial, demonstrated a log kill ranging from 3 logs to greater than 5 logs of cells. This indicated that IC\(_{37}\) alone did not adequately
define and predict for cytotoxicity of Anti-B4-bR. Additionally, the toxicity of these lots was determined in Swiss albino mice. The LD$_{50}$ values for Lots A and B were 400 and 330 $\mu$g/kg, respectively, comparable to that of lots used in the previous bolus infusion trial. In contrast, LD$_{50}$ of Lots C, D, and E in mice ranged from 112 to 256 $\mu$g/kg, lower than that of Lots A and B.

Blocked ricin was previously defined as the modified ricin species that can no longer bind to a column of immobilized asialofetuin, and that had retained the full catalytic activity of the A-chain (9). We were aware that blocked ricin consisted of two different species, one with three attached ligands and one with two attached ligands, as previously reported (3, 9). Therefore, we next examined the lots of Anti-B4-bR to look for changes in the ratio of the two species of blocked ricin that may have occurred during manufacturing of the lots. The relative proportion of blocked ricin containing two and three attached ligands that were present in each of the lots is shown in Table 1. Lots C, D, and E differed from Lots A and B in that they all contained at least 60% of the species with two covalently attached ligands. Notably, in the bolus infusion trial, lots of Anti-B4-bR also contained a percentage of blocked ricin with two ligands that was similar to that of Lots C, D, and E. This observation prompted us to study the two species of blocked ricin in an effort to further characterize the difference between Lots A and B, and Lots C, D, and E.

Characterization of Two Species of Blocked Ricin. Native ricin was reacted with increasing concentrations of the reactive affinity ligand and the reaction products of the "blocking reaction" were analyzed by polyacrylamide/sodium dodecyl sulfate gel electrophoresis under reducing conditions (Fig. 2a). Native B-chain [apparent $M_r$, 32,500] was converted into species of higher apparent $M_r$ by reaction with the ligand ($M_r$ of the ligand is approximately 2500 (9)]. The results of the gel were quantified by densitometry as shown in Fig. 2b. A band (apparent $M_r$, 35,000) corresponding to covalent modification of the B-chain with one molecule of ligand reached a maximum at a 4-fold molar excess of ligand in the reaction mixture and thereafter declined in yield, while a band (apparent $M_r$, 37,500) corresponding to covalent modification of B-chain with two reactive ligands reached a maximum (50% of total B-chain) at about a 14-fold molar excess of ligand. A band corresponding to B-chain modified with three ligands was first apparent at about 6-fold excess of ligand in the reaction mixture and increased to about 25% of the total B-chain with a 20-fold excess of ligand. A band corresponding to B-chain modified with four ligands has not been observed, even with the use of prolonged reaction times or higher concentrations of reactive affinity ligand. Different ratios of the species of blocked ricin were produced, depending upon the ligand concentration in the reaction mixture. The specificity of the reaction of the affinity ligand with B-chain has been demonstrated previously (9) and is shown in Fig. 2a by the lack of modification of ricin A-chain (apparent $M_r$, 30,000) and ricin A'-chain (a form of ricin A-chain, apparent $M_r$, 32,000, containing two $N$-linked oligosaccharides (19)]. The intensity of the A-chain bands (Fig. 2a) remained constant in the different reaction mixtures was as follows: Lane 1, 0.5; Lanes 2 and 3, 1.0; Lanes 4 and 5, 2.0; Lanes 6 and 7, 4.0; Lane 8, 6.0; Lane 9, 8.0; Lane 10, 10; Lane 11, 12; Lane 12, 14; Lane 13, 16; Lane 14, 18; and Lane 15, 20 mol of ligand/mol of ricin. Lane 16 contains native ricin. The band corresponding to A-chain ($M_r$, 30,000) is indicated on the right ordinate of (a), as is the band corresponding to the A'-chain (19) ($M_r$, 32,500) and B-chain ($M_r$, 37,500) which comigrate in this gel. Bands corresponding to the covalent reaction of B-chain with one ($M_r$, 35,000), two ($M_r$, 37,500), or three affinity ligands ($M_r$, 40,000) are also indicated. At high concentrations of reactive ligand, some of the protein is cross-linked (20% of the protein) and appears as a broad band at apparent $M_r$, ~70,000. The gel was calibrated with the marker proteins carbonic anhydrase ($M_r$, 29,000), ovalbumin ($M_r$, 45,000), bovine serum albumin ($M_r$, 68,000), phosphorylase b ($M_r$, 93,000), and $\beta$-galactosidase ($M_r$, 116,000). (b) shows the proportion of ricin B-chain species in each of the bands containing 0 (A), 1 (O), 2 (A), or 3 (O) covalently linked ligands as quantified by densitometry of the gel shown in (a). The A'-chain was estimated to comprise about 25% of the total staining of this band, as judged from the residual staining in this band in Lane 15 of (a).

Ricin highly enriched for the species containing two covalently linked ligands and the species containing three ligands attached to the B-chain, Fig. 3a (nonreducing conditions) and Fig. 3b (reducing conditions) show the polyacrylamide/sodium dodecyl sulfate gel electrophoresis analysis of blocked ricin with two covalently bound ligands (Lane 2) and the species with three ligands (Lane 3). Under reducing conditions, the gels show that each of the two different species of blocked ricin were about 90% free of the other.

The binding of blocked ricin having two covalently linked ligands was reduced by about 100-fold relative to that of native ricin on Namalwa cells (Fig. 3c). The binding of this species of blocked ricin could be further reduced by including 100 mM lactose in the incubation with cells, suggesting that this species
of blocked ricin had some residual low affinity for cell surface galactose residues. The binding of blocked ricin with three covalently attached ligands was further reduced by about 100-fold relative to that of blocked ricin with two attached ligands (and by about 10^4-fold relative to native ricin). Indeed, the level of binding was only just detectable at the highest concentration tested (0.4 mg/ml), and was not affected by 100 mM lactose. The results of cytotoxicity experiments on Namalwa cells are consistent with the binding experiments, with IC50 values of 6.0 and 100 nM for blocked ricin species containing two or three covalently linked ligands, respectively (that is, 1,500-fold and 25,000-fold less cytotoxic than native ricin, respectively).

Analytical Scale Preparation of Conjugates of Anti-B4 Antibody with Blocked Ricin Species Having Two or Three Covalently Linked Ligands. Immunoconjugates were made between anti-B4 and the two different species of blocked ricin using methods described previously (3). Fig. 4 shows the analysis of these conjugates by polyacrylamide/sodium dodecyl sulfate gel electrophoresis under nonreducing (Fig. 4a) and reducing (Fig. 4b) conditions. The major species in both preparations of immunoconjugate (Fig. 4a, Lanes 2 and 3) had a mobility corresponding to one antibody molecule linked to one molecule of blocked ricin. Any nonconjugated antibody or nonconjugates containing blocked ricin having either two or three covalently linked ligands, respectively (that is, 1,500-fold and 25,000-fold less cytotoxic than native ricin, respectively).

The avidity of conjugates for the CD19 antigen was tested in a competitive binding assay as shown in Fig. 5b. The two conjugates of anti-B4 made from each of the species of blocked ricin were similar in their ability to compete with fluorescein-labeled anti-B4 in this assay. Both immunoconjugates required a concentration about 2-fold higher than that of native anti-B4 to effect a similar degree of competition.

In Vitro Cytotoxicity of Anti-B4 Immunoconjugates. Immunoconjugates containing blocked ricin having either two or three covalently linked ligands were tested for their specific

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Fig. 3. Analysis of purified blocked ricin species containing two or three covalently linked ligands by polyacrylamide/sodium dodecyl sulfate gel electrophoresis, and for binding activity to cells. Purified blocked ricin was fractionated as described under "Materials and Methods." (a), samples (1 μg) of native ricin (Lane 1), blocked ricin containing two covalently linked ligands (Lane 2), and blocked ricin containing three covalently linked ligands (Lane 3), were analyzed on an 11% (w/v) polyacrylamide gel under nonreducing conditions. (b), same as (a) except that 3-nm samples were analyzed under reducing conditions. The gels were calibrated with the same marker proteins as in Fig. 2, with the addition of myosin (M, 205,000). Under reducing conditions, Lane 2 shows three major bands corresponding to native A-chain (M, 30,000), native A'-chain (M, 32,000), and B-chain with two covalently linked ligands (M, 37,500), while Lane 3 shows three major bands corresponding to native A-chain, native A'-chain, and B-chain with three covalently attached ligands (M, 40,000). Other minor bands have not been characterized. (c), binding of ricin (O, A), blocked ricin containing two covalently linked ligands (•, A), and blocked ricin containing three covalently linked ligands (△, A), to Namalwa cells. See "Materials and Methods" for experimental details. Open symbols (O, A, △) indicate samples which were incubated in the presence of 100 mM lactose.

Fig. 4. Analysis by polyacrylamide/sodium dodecyl sulfate gel electrophoresis of conjugates of anti-B4 antibody with the blocked ricin species having two or three covalently linked ligands. (a), a 5-10% polyacrylamide gradient gel run under nonreducing conditions with the following samples: Lane 1, anti-B4 antibody (1 μg); Lane 2, purified conjugate between anti-B4 and blocked ricin having two covalently linked ligands (2 μg); Lane 3, purified conjugate between anti-B4 and blocked ricin having three covalently linked ligands (1.5 μg); Lane 4, native ricin (1 μg). The predominant band seen in both Lanes 2 and 3 has an apparent Mr of about 225,000 which would correspond in mobility to one antibody molecule linked to one molecule of blocked ricin. Any nonconjugated antibody or nonconjugates containing blocked ricin was less than 5%. Analysis under reducing conditions (Fig. 4b, Lanes 2 and 3) showed bands for the heavy and light chains of anti-B4 and for the two ricin A-chain species at Mr 30,000 and 32,000, but no bands for the blocked B-chain species having two or three covalently linked ligands (Fig. 4b, Lanes 2 and 3) since the blocked B-chain is linked to the antibody by a noncleavable linkage (3).

The gel analysis of the blocked ricin species and the immunoconjugates suggests that the A-chain was not modified by the affinity ligand. Fig. 5a shows that the activity of the A-chain in both species of immunoconjugate was indistinguishable from that of the A-chain derived from native ricin in its ability to inhibit protein synthesis in a cell-free system from rabbit reticulocytes.
cytotoxicity against a CD19 antigen-positive cell line, Namalwa, and for their nonspecific cytotoxicity against a CD19 antigen-negative line, Molt-4. The IC_{50} of the Anti-B4-bR made from blocked ricin having two attached ligands was about $14 \times 10^{-3}$ nM for Namalwa cells (Fig. 6a) and 1.8 nM for Molt-4 cells (Fig. 6b), indicating that the conjugate had a specificity for the CD19 antigen-positive cells about 130-fold greater than for the antigen-negative cells. The conjugate made from blocked ricin having three covalently linked ligands was about 5-fold less cytotoxic on both CD19 antigen-positive and antigen-negative cells, with an IC_{50} of $63 \times 10^{-3}$ nM (Fig. 6a) for Namalwa cells and 13 nM (Fig. 6b) for Molt-4 cells. The difference between the IC_{50} values of the conjugate for the antigen-positive and antigen-negative cells was about 200-fold. The nonspecific cytotoxicity of each conjugate on Molt-4 cells was the same as that achieved by their respective blocked ricin species (result not shown).

Cytotoxicity experiments on Namalwa cells were extended to higher concentrations of the conjugates in order to determine the lowest surviving fraction that can be achieved when the surface antigens are saturated with conjugate. The results of these assays (Fig. 6c) demonstrate a dramatic difference in the maximum cell kill that can be achieved with the conjugates made with the two different species of blocked ricin. The conjugate made with blocked ricin having only two covalently linked ligands left a very low surviving fraction of $10^{-5}$ to $10^{-6}$ after 24-h exposure of cells to 5 nM immun conjugate, while the conjugate with blocked ricin having three covalently attached ligands left a surviving fraction of only 0.1.

**DISCUSSION**

Anti-B4-blocked ricin is an immunotoxin directed against cells expressing the CD19 antigen and has been administered clinically by both daily bolus infusion and 7-day continuous infusion in clinical trials. In an initial clinical report, we demonstrated that Anti-B4-bR could be safely administered to patients with relapsed and refractory B-cell neoplasms by daily bolus infusions for 5 consecutive days with tolerable, reversible toxicities (10). The maximum tolerated dose was defined by Grade 3 hepatotoxicity, with transient platelet reductions
at and above the maximal tolerated dose of 50 μg/kg/day for 7 days are described. In contrast to the bolus infusion trial, initially no progressive increase in serum transaminases was observed with increasing Anti-B4-bR dose in patients who were treated with Lots A and B at doses of up to 70 μg/kg/day for 7 days. However, Grade 4 elevations in serum transaminases were seen in patients treated with Lots C, D, and E at doses at or above 50 μg/kg/day. This led us to expand the preclinical testing of Anti-B4-bR in order to further characterize the immunotoxin and to establish additional preclinical screening parameters to ascertain characteristics of lots which might predict in vivo specific and nonspecific toxicity.

At the outset of this trial, we used the IC_{37} value of Anti-B4-bR to assess the cytotoxicity of the immunotoxin. This parameter (or the IC_{50}) has been used to assess the cytotoxicity of many immunotoxins used clinically to date and was the parameter used in testing lots for our previous bolus trial (10, 20, 21). However, small variations in the IC_{37} value, which may lie within the accuracy of the measurement, may be accompanied by large variations in the maximal log cell kill. Indeed, the log cell kill in Lots A and B was less than 3 logs, while the log cell kill for the latter three lots was greater than or equal to 3 logs despite relatively small differences in the IC_{37}. We now demonstrate that the IC_{37} values, while suitable to evaluate potency, are not sufficient to predict in vivo toxicity for Anti-B4-bR, and have introduced the degree of cell kill at saturating drug concentrations (5 nm) as an additional in vitro parameter.

Despite the additional information gained from these screening tests, the difference between the drug lots remained unclear from a biochemical standpoint. Previously, we had defined blocked ricin as the species of modified ricin that was not retained by an affinity column, but we recognized that it was composed of about equal amounts of two species, one with two covalently attached affinity ligands and one with three covalently attached ligands (3). We therefore speculated that a variation in the ratio of these two species of blocked ricin in the conjugate might have contributed to the observed difference in cytotoxicity between Lots A and B and Lots C, D, and E. This led us to the development of analytical methods that allowed the preparation of blocked ricin comprised of only one species for in vitro cytotoxicity analysis. This testing revealed that Anti-B4-bR made with blocked ricin having two attached ligands is capable of depleting 5 logs of cells in a cytotoxicity assay, while Anti-B4-bR comprised of blocked ricin with three ligands can deplete only 1 log of cells. Based on this analysis, we established that all lots of Anti-B4-bR for clinical use must contain blocked ricin that is composed of these two species at a fixed ratio.

Our results demonstrate that the immunoconjugate containing blocked ricin with two ligands was a much more potent cytotoxin than the immunoconjugate containing blocked ricin having three covalently linked ligands. However, the increased potency is associated with increased nonspecific toxicity. Although the conjugate containing blocked ricin with two ligands was 4 logs more potent than the conjugate made from blocked ricin with three ligands, the ricin A-chains of each conjugate were equally active. Moreover, the binding avidities of the conjugated antibodies were identical, so that at saturating concentrations of immunotoxin, the same number of molecules of the conjugates were bound to the CD19 antigen. These results may suggest that residual galactose binding is necessary for efficient translocation of the A-chain into the cytoplasm of the target cell, since there is a correlation between the specific and nonspecific cytotoxicity of the immunotoxin and the level of residual binding of the blocked ricin to cells that can be inhibited by lactose. Such an interpretation is consistent with the experiments of Youle et al. (22) which suggest that the galactose binding and A-chain translocation activity of ricin cannot be completely separated.

Another explanation also is consistent with the experimental evidence. When three ligands are covalently linked to the B-chain, one of the ligands could interfere with the interaction of the membrane of the targeted cell with a putative functional site on ricin B-chain that enables membrane translocation of the ricin A-chain. This may be achieved by nonspecific steric hindrance of this possible functional site by covalent attachment of the third ligand to the B-chain. Alternatively, the third amino group with which the affinity ligand reacts may be part of that functional site and may no longer be effective after the attachment of a third ligand.

A review of the drug lots used in this clinical trial has indicated the importance of using additional parameters in the preclinical drug evaluation. Log cell kill at antigen-saturating concentration, murine LD_{50}, and biochemical analysis of the composition of blocked ricin have been shown to be additional considerations for establishing the potential efficacy and safety of lots of Anti-B4-bR. Following our recognition of these additional parameters, we were able to complete the continuous infusion clinical trial and achieve a maximum tolerated dose with drug lots which met this more rigorous set of preclinical standards.

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REFERENCES


Correlation between in Vivo Toxicity and Preclinical in Vitro Parameters for the Immunotoxin Anti-B4-blocked Ricin


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