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


Division of Tumor Immunology [M. L. G., L. M. N.], Dana-Farber Cancer Institute and Department of Medicine [M. L. G., L. M. N.], Harvard Medical School, Boston 02115, and ImmunoGen Inc., Cambridge [J. M. L., V. S. G., W. A. B.], Massachusetts 02139

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 IC₅₀ values as determined by in vitro cytotoxicity testing on cultured human cell lines, these lots differed in their in vivo toxicity when administered to patients. Thus, IC₅₀ values alone were not sufficient to predict in vivo toxicity. We report 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 in vivo cytotoxicity. Furthermore, we performed detailed cytotoxicity studies of the ricin species containing two and three covalently attached ligands, respectively. 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 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 concentration, 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-br² 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, is B-lineage restricted, and is detected early in B-cell ontogeny (4, 5). Ricin is a protein toxin comprised of two chains, an A-chain which is an N-glycosidase that acts at the 60S ribosomal subunit to inhibit protein synthesis, and a B-chain which serves the dual roles of mediating toxin binding to cells via galactose-terminated oligosaccharides and assisting in the translocation of the A-chain to the cytosol (6–8). 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 (9).

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2 To whom requests for reprints should be addressed, at Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02215.

3 The abbreviations used are: Anti-B4-br, Anti-B4-blocked ricin; IC₅₀, the molar inhibitory concentration for a toxin that leaves a surviving fraction of 0.37 (63% of cells are killed); LD₅₀, 50% lethal dose; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

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 [3,4,5-³H]leucine (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 enzymic modification of glycopolypeptides 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 l-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 immunoconjugate 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 immunoconjugate 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), P1.90.C001 (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 Immunonojugates. 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 Sepharose 6B (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 ε₉₀ 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 × 90 × 0.75 mm) of acrylamide prepared according to the method of Laemmli (14). Sample buffers containing 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.

Cytotoxicity Assays. The cytotoxicity (IC₅₀ and log kill) of samples of ricin, blocked ricin, and immunoconjugates 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 by 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 sheep anti-mouse IgG (Fc specific) and then assayed with either goat anti-mouse IgG immunoconjugated to alkaline phosphatase or with rabbit 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, hematocrit > 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 three 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 three patients were treated at the next dose level. Patients were eligible for treatment 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-rabbit 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

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μg/kg/day to 70 μg/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 μg/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 μg/kg/day for 7 days to 70 μg/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 μg/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_{50} values. Table 1 lists the IC_{50} 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_{50} values on Namalwa cells range from 10.3 × 10^{-3} to 45 × 10^{-3} fm for a 24-h exposure to Anti-B4-bR, a range of only 4-fold. Likewise, the IC_{50} values on Molt-4 cells range from 1.0 to 5.7 nm and were about 100-fold greater than the IC_{50} for Namalwa for each drug lot. All IC_{50} values were within the previously established range of cytotoxicities for clinical use (Namalwa: IC_{50} 6–60 × 10^{-3} nm, Molt-4: IC_{50} ≥ 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, B, C, D, and E (Table 1). In addition to the IC_{50}, 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, 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 ± 67.9 ng/ml obtained, using Lots A and B, and mean serum levels of 269 ± 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_{50}, 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_{50} alone did not adequately

Table 1 Characterization of lots used in continuous infusion clinical trial

<table>
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<th>Lot</th>
<th>Code</th>
<th>Specific cytotoxicity (Namalwa IC_{50} (nm))</th>
<th>Non-specific cytotoxicity (Molt-4 IC_{50} (nm))</th>
<th>Log kill Namalwa (5 nm Anti-B4-bR)</th>
<th>LD_{50} (μg/kg)</th>
<th>% of blocked ricin with 2 attached ligands</th>
<th>% of blocked ricin with 3 attached ligands</th>
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<td>76%</td>
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</table>
define and predict for cytotoxicity of Anti-B4-bR. Additionally, the toxicity of these lots was determined in Swiss albino mice. The LD<sub>50</sub> values for Lots A and B were 400 and 330 μg/kg, respectively, comparable to that of lots used in the previous bolus infusion trial. In contrast, LD<sub>50</sub> of Lots C, D, and E in mice ranged from 112 to 256 μg/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 Mr, 32,500) was converted into species of higher apparent Mr by reaction with the ligand (Mr, of the ligand is approximately 2500 (9)). The results of the gel were quantified by densitometry as shown in Fig. 2b. A band (apparent Mr, 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 Mr, 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 Mr, 30,000) and ricin A'-chain (a form of ricin A-chain, apparent Mr, 32,000, containing two N-linked oligosaccharides (19)). The intensity of the A-chain bands (Fig. 2a) remained constant in these reactions containing up to 20-fold molar excess of affinity ligand over ricin. At high concentrations of the reactive ligand, some of the ricin is cross-linked and appears as a broad band at apparent Mr, ~70,000.

Purified blocked ricin was fractionated as described in “Materials and Methods” in order to prepare samples of blocked 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 104-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). Indeed, the level of cytotoxicity relative to that of blocked ricin with two attached ligands (and 100 nM for blocked ricin species containing two or three covalently linked ligands). The binding of blocked ricin with three covalently linked ligands was further reduced by about 100-fold relative to native A-chain. Native A’-chain, and B-chain with three covalently linked ligands (M, 40,000). Other minor bands have not been characterized. (b), binding of ricin (a, O), blocked ricin containing two covalently linked ligands (a, O), and blocked ricin containing three covalently linked ligands (a, O), to Namalwa cells. See “Materials and Methods” for experimental details. Open symbols (O, A, D) indicate samples which were incubated in the presence of 100 mM lactose.

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 activity 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-µg 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 (a, O), blocked ricin containing two covalently linked ligands (a, O), and blocked ricin containing three covalently linked ligands (a, O), to Namalwa cells. See “Materials and Methods” for experimental details. Open symbols (O, A, D) indicate samples which were incubated in the presence of 100 mM lactose.

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-µg 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 (a, O), blocked ricin containing two covalently linked ligands (a, O), and blocked ricin containing three covalently linked ligands (a, O), to Namalwa cells. See “Materials and Methods” for experimental details. Open symbols (O, A, D) 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 blocking reagents was less than 5%. Analysis under reducing 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. (b), an 11% polyacrylamide gel run under reducing conditions. Samples in Lanes 1 through 4 are the same as in (a), except with twice the amount of protein per sample. The gels were calibrated with some of the marker proteins described in Fig. 2, except that an IgG marker (Mr, 160,000) also was included. The heavy (H) and light (L) chains of anti-B4 are indicated.
cytotoxicity against a CD19 antigen-positive cell line, Namalwa, and for their nonspecific cytotoxicity against a CD19 antigen-negative line, Molt-4. The IC\textsubscript{50} of the Anti-B4-bR made from blocked ricin having two attached ligands was about 14 × 10\textsuperscript{-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\textsubscript{50} of 63 × 10\textsuperscript{-3} nm (Fig. 6a) for Namalwa cells and 13 nm (Fig. 6b) for Molt-4 cells. The difference between the IC\textsubscript{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\textsuperscript{-5} to 10\textsuperscript{-6} after 24-h exposure of cells to 5 nm immunoconjugate, 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...
occurring frequently. During dose escalation, the toxicity increased as the dose increased. Because preclinical studies indicated that larger doses of Anti-B4-bR might be delivered by prolonged continuous infusion than by bolus injection and because in vitro cytotoxicity was enhanced with longer exposure times to the immunotoxin, we undertook a second Phase I trial in which patients received 7-day continuous infusions of Anti-B4-bR. In this trial, several different lots of immunotoxin were used, all possessing IC37 values which met the previously established product specifications.

In this report, five lots of immunotoxin used to treat patients 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 IC37 value of Anti-B4-bR to assess the cytotoxicity of the immunotoxin. This parameter (or the IC50) 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 IC37 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 IC37. We now demonstrate that the IC37 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 LD50, 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 \textit{in Vivo} Toxicity and Preclinical \textit{in Vitro} Parameters for the Immunotoxin Anti-B4-blocked Ricin
