Modification of the Binding Site(s) of Lectins by an Affinity Column Carrying an Activated Galactose-Terminated Ligand†

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ABSTRACT: An affinity column approach is described, aimed at the modification of the galactose binding site(s) of ricin in an effort to block the binding of ricin to cells. The affinity column was prepared by linking *N*-(2′-mercaptoethyl)lactamine to pyridyldithio-activated polyacrylamide beads. The linker between the ligand and the solid support thus contained a disulfide bond and an unmodified terminal galactose moiety. The amino group of the ligand was allowed to react with the bifunctional cross-linking reagent 2,4-dichloro-6-methoxytriazine. The lectin was then allowed to bind to the galactose functions on the activated column at pH 7.0, prior to raising the pH to 8.6 to initiate the cross-linking reaction between the ligand and the lectin. Lectin that was not covalently linked to the functionalized galactose residues on the column was eluted with galactose or lactose. Finally, the covalent ligand–lectin complexes were released from the solid support by reducing the disulfide bond between the ligand and the support. The affinity column was used in this way to modify the galactose binding site(s) of ricin. Upon release from the affinity column, blocked ricin was purified from unmodified ricin by affinity chromatography on columns of immobilized asialofetuin (a ligand to which ricin binds very tightly). The sulfhydryl group formed by cleavage of the ligand–ricin complex from the column was labeled with [^{3}H]-*N*-ethylmaleimide to provide evidence that one blocking ligand was linked per ricin molecule. The blocked ricin and a conjugate of the blocked ricin with the monoclonal antibody J5 were toxic for cultures of Namalwa cells in vitro. Although both of these materials were 10-fold less toxic than native ricin itself, the toxicity of the conjugate was only eliminated completely by the presence of excess unconjugated antibody in the presence of galactose or lactose. Our results are consistent with the notion that ricin has two separate binding sites for galactose, only one of which is modified by the activated ligand of the affinity column.

The toxic lectin ricin has attracted much attention recently because of its potential application in cancer therapy as a component of immunotoxins (Thorpe, 1985; Vitetta & Uhr, 1985; Frankel et al., 1986; Pastan et al., 1986). Ricin consists of two subunits, the A-chain and B-chain, which are linked by a single disulfide bond (Olsnes & Pihl, 1982). The A-chain is an enzyme that inactivates the 60S subunit of eucaryotic ribosomes, and the B-chain binds to galactose-terminated oligosaccharides that are ubiquitous on eucaryotic cell surfaces.

Immunotoxins made from intact ricin are potent cytotoxic agents, but such conjugates lack cell specificity because they can bind to nontarget cells through the B-chain of ricin. This problem was eliminated by preparing immunotoxins containing only the A-chain of ricin, but such conjugates were found to show varying cytotoxicities and to be less potent than immunotoxins that incorporated intact ricin (Thorpe, 1985). These observations led to the suggestion that the B-chain of ricin not only is responsible for the binding to the cell surface but also participates in the transport of either the entire toxin or the A-chain alone across the cell membrane (Youle et al., 1979, 1981, 1982; Vitetta et al., 1983).

An effective way of reducing the nonspecific binding and toxicity of intact ricin conjugates in vitro is by the use of high concentrations of free galactose or lactose [see, for example, Houston and Nowinski (1981) and Vallera et al. (1983)]. This method is now successfully applied for the removal of specific cell populations with antibody–ricin conjugates during ex vivo bone marrow treatment (Vallera et al., 1982; Filipovich et al., 1984). The association between ricin and galactose or lactose is of low affinity, which makes it difficult for this approach to be used in vivo to block the high nonspecific toxicity of ricin. However, the method has been tried successfully against a human solid tumor in a nude mouse system, where the ricin conjugate was injected intratumorally in a solution containing lactose and at the same time the animals received iv injections of solutions of lactose (Weil-Hillman et al., 1985, 1987).

Several groups have used the method of affinity labeling to achieve the permanent blocking of the binding capacity of ricin. In the most successful attempt reported, Houston (1983) used a derivative of the low-affinity ligand galactose ($K_a \approx 10^4$ M^{-1}) that could be converted photochemically into a nitrene. Up to 100% of the lectin was said to be altered so as to reduce its binding to agarose, and the modified ricin was 280 times less toxic to intact cells than the unmodified material. The extent of nonspecific labeling was not determined but was probably substantial, given the low affinity of the ligand and the reactivity of the photogenerated species. Baenziger and Fiete (1982) used an analogous derivative of an oligosaccharide derived from fetuin. The choice of this ligand was based on the observation (Baenziger & Fiete, 1979) that a number of glycopeptides from fetuin gave much higher association constants for ricin than do simple monosaccharides. Several of these glycopeptides contain two or three terminal galactose residues in bi- or triantenary structures. In contrast to the report of Houston (1983), Baenziger and Fiete (1982) observed only 1–2% incorporation of the ligand, and the toxicity of the preparation was not determined.

The conflicting results obtained with photoaffinity reagents prompted us to seek an alternative approach for preparing blocked ricin. Equilibrium binding studies (Houston &

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Dooley, 1982; Frênoy, 1986) together with the low-resolution crystal structure (Villafranca & Robertus, 1981) show that ricin binds two galactose molecules at two distinct sites. We therefore attempted to block ricin with the help of an affinity column, on the basis that, considering the low association constant of galactose (on the order of 10^4 M^{-1}; Olsnes & Pihl, 1982), ricin was only retained by an affinity column containing galactose because of the interaction of the lectin with *two* galactose ligands. Following ricin absorption, covalent cross-linking of the ligands to the bound ricin and release of the ligand–ricin complex from the column matrix could therefore afford ricin having *both* of its binding sites blocked.

The ultimate goal of this work was to incorporate affinity-blocked ricin into immunotoxins in the hope of achieving high cytotoxicity and high target specificity. This assumes that the B-chain transport function, which facilitates the translocation of A-chain, can be separated from the galactose recognition function, so that transport is not affected by the permanent blocking of the galactose binding sites. Youle, Neville, and their colleagues have addressed this question using a variety of conjugates of ricin: (a) with a mannose 6-phosphate terminated oligosaccharide (Youle et al., 1979), (b) with a monoclonal antibody (Youle & Neville, 1980), and (c) between a chemically modified ricin that had been treated with *N*-acetylimidazole to block the binding of galactose by the B-chain and a mannose 6-phosphate terminated oligosaccharide (Youle et al., 1981). From toxicity studies using cultured cells expressing the appropriate receptors, these workers concluded that conjugates involving whole ricin must use galactose-containing receptors of the cell to reach the cytosol efficiently and to attain high cytotoxicity (Youle et al., 1981). They also suggested that the two functions of the B-chain, recognition and transport, *cannot* be separated from one another. In contrast, Thorpe and his collaborators (Thorpe et al., 1984) blocked the binding of ricin to cells by linking the toxin to a monoclonal antibody using a short linker and found that the conjugate showed high selective toxicity for antigen-bearing cells that could not be reduced by exogenous lactose. The toxicity of this conjugate with whole ricin was much higher than the toxicity of the corresponding conjugate with ricin A-chain alone, suggesting that the property of the B-chain that facilitates the membrane penetration is independent of the galactose recognition function.

Materials and Methods

Materials

Ricin, which is ricin D according to the nomenclature of Wei and Koh (1978), was purchased from Sigma Chemical Co. (St. Louis, MO). Aminoethylpolyacrylamide P-150 was supplied by Bio-Rad (Richmond, CA). [^{3}H]-*N*-Ethylmaleimide (53.3 Ci/mmol), which was diluted to a specific radioactivity of 533 mCi/mmol with nonradiolabeled *N*-ethylmaleimide, and a rabbit reticulocyte lysate system for cell-free protein synthesis, which included L-[3,4,5-^{3}H]leucine, were obtained from New England Nuclear (Boston, MA). Betafluor scintillation cocktail was from National Diagnostic (Somerville, NJ). The murine monoclonal anti common acute lymphoblastic leukemia antigen (CALLA) antibody J5 (Ritz et al., 1980) was prepared and purified as described by Lambert et al. (1985). Succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) and carbonyldiimidazole-activated TSK beads (Reacti-Gel HW-65F) were purchased from Pierce Chemical Co. (Rockford, IL). 3-(2-Pyridyldithio)propionic acid was synthesized as described by Carlsson et al. (1978), and asialofetuin–TSK was prepared according to the method of Bethell et al. (1979) using asialofetuin type II purchased from Sigma.

Methods

Synthesis of N-(2′-Mercaptoethyl)lactamine (**1**). To a stirred solution of α-lactose (9.72 g, 13.5 mmol as the monohydrate) in water (36 mL) was added cystamine diacetic acid salt (3.68 g, 13.5 mmol) and a solution of sodium cyanoborohydride (1.70 g, 27.0 mmol) in methanol (72 mL). The pH was adjusted to 6.2 with acetic acid, and the solution was stirred at room temperature.

After 36 h, methanol was removed by evaporation, the pH of the residual aqueous solution was adjusted to 5.0 with acetic acid, and the solution was applied to a column (360 mL) of (carboxymethyl)cellulose (CM-cellulose) equilibrated in 2 mM pyridinium acetate buffer, pH 5.0. The column was washed with 2 column volumes of the equilibrating buffer, and the bound material was eluted with 0.1 M aqueous ammonia. The pooled ammonia fractions were evaporated to dryness, redissolved in water (200 mL), and treated with dithioerythritol (2.0 g, 13.5 mmol) for 1 h at room temperature. The resulting mixture of *N*-(2′-mercaptoethyl)lactamine (**1**) and cysteamine was adsorbed onto a column (370 mL) of CM-cellulose in 10 mM triethylammonium bicarbonate, pH 7, containing 1 mM dithioerythritol (DTE). The column was washed with 500 mL of the same buffer and then developed with a linear gradient (1 L + 1 L) of 10–100 mM triethylammonium bicarbonate, pH 7, containing 1 mM dithioerythritol. Analysis by TLC (butanol:acetic acid:water, 5:2:3) showed the material eluting between 1.3 and 1.9 L to be pure **1**, containing less than 1% dithioerythritol. Repeated evaporation from water and lyophilization gave a sample of **1** (containing a small quantity of the corresponding disulfide) as a white powder (3.85 g, 35%): ^{1}H NMR (D_2O) δ 2.8 (2 H, m, CH_2SH), 3.2 (4 H, m, CH_2NHR), 3.8 [12 H, m, CH(OH), CH(OR), and CH_2OH], 4.5 [1 H, m, CH(OR)$_2$]; MS (FAB, glycerol matrix) carboxamidomethyl derivative of **1** obtained from the reaction of **1** with iodoacetamide, m/z 461 (MH^+).

Preparation of the Affinity Column (**3**). Residual carboxyl groups on aminoethylpolyacrylamide P-150 were capped by reaction with a water-soluble carbodiimide and aqueous ammonia as described by Inman (1974). To a suspension of capped aminoethylpolyacrylamide P-150 (30 mL of packed beads) in 0.1 M NaCl (10 mL) was added 3-(2-pyridyldithio)propionic acid (1.01 g, 5 mmol) in dimethylformamide (10 mL) and water (1 mL). The pH was adjusted to 4.7 with dilute hydrochloric acid, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC·HCl) (1.15 g, 6 mmol) in water (5 mL) was added, and the suspension was shaken gently. After 7 h, an additional 766 mg (4 mmol) of EDC·HCl was added and the pH readjusted to 4.7 with hydrochloric acid. The suspension was shaken for 4 days, and the beads were then washed extensively with 0.2 M NaCl. To ensure complete removal of excess 3-(2-pyridyldithio)propionic acid, the beads were suspended in 0.1 M hydrochloric acid and extracted with ethyl acetate (5 mL). Finally, they were washed with 100 mM sodium phosphate buffer, pH 7.0, until a portion of the supernate, when treated with excess dithioerythritol, gave an absorbance at 343 nm of less than 0.1 AU, indicating a low concentration of 2-pyridinethione.

The concentration of linked disulfide groups was determined by treatment of a portion (0.25 mL) of the beads suspended in 100 mM $NaHCO_3$ (5 mL) with dithioerythritol (154 mg, 1 mmol) at room temperature. After 30 min, the beads were washed with 0.2 M NaCl until the washings gave an absorbance of less than 0.01 AU at 343 nm. The amount of 2-

pyridinethione liberated was then determined by measuring the absorbance of the pooled washings at 343 nm and corresponded to a concentration of 38 μmol/mL of packed beads.

The pyridyldithio-activated polyacrylamide P-150 (**2**, 27 mL) was suspended in 100 mM $NaHCO_3$ (50 mL) and treated with excess methyl chloroformate (4.8 g, 5.2 mmol) at room temperature. After 5 min, the excess reagent was removed by extraction with ethyl acetate (2 × 16 mL), and the beads were then washed with 100 mM sodium phosphate buffer, pH 7.0.

Immediately prior to the coupling reaction, a sample of *N*-(2′-mercaptoethyl)lactamine (**1**, 1.5 g, 3.7 mmol) in water (10 mL) at pH 8 was treated with dithioerythritol (154 mg, 1 mmol) at room temperature for 1 h in order to reduce any of the corresponding disulfide present. The pH was then lowered to 5.2 with acetic acid, and the solution was applied to a column (100 mL) of CM-cellulose in 2 mM pyridinium acetate buffer, pH 5.2. The column was washed with the same buffer until all dithioerythritol had been removed (200 mL) followed by 0.1 M hydrochloric acid to elute **1** in the free sulfhydryl form. The solution containing the liberated **1** was assayed for thiol content with Ellman's reagent (Ellman, 1959) and was found to contain 3.2 mmol of thiol (85% recovery). This solution (50 mL, 3.2 mmol) was adjusted to pH 5.0 with dilute sodium hydroxide solution and then added to the pyridyldithio-activated polyacrylamide P-150 (27 mL). The resulting suspension had pH 6.5 and was shaken at room temperature for 15 h. The beads were then washed with 0.2 M NaCl by repeated centrifugation/decantation until the washings gave an absorbance at 343 nm of less than 0.05 AU. The concentration of 2-pyridinethione liberated in the reaction was determined by measuring the absorbance of the pooled washings (50 mL) at 343 nm and corresponded to quantitative disulfide exchange, resulting in a concentration of bound **1** of 38 mM.

Preparation of Blocked Ricin. The affinity gel **3** (10 mL) in 100 mM $NaHCO_3$ (10 mL) was treated with a solution of 2,4-dichloro-6-methoxytriazine (360 mg, 2 mmol; Dudley et al., 1951) in dioxane (10 mL), vortexed for 1 min, and then extracted with ether (4 mL). The gel was then equilibrated with 100 mM sodium phosphate buffer, pH 7.0, and packed into a column.

A solution of ricin (7.5 mL of 1.9 mg/mL in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl) was applied to a column (16 mL), which was then washed with 3 column volumes of 50 mM triethanolamine hydrochloride, pH 8.6. After 24 h, ricin that was bound, but not covalently cross-linked, was removed by eluting with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.5 M galactose (3 column volumes). The ricin retained by covalent linkage was released at 4 °C by five consecutive treatments (5 × 10 min) of the gel with single column volumes of 0.1 M sodium phosphate buffer, pH 7.0, containing 10 mM dithioerythritol. This resulted in the release of blocked ricin in 28% yield (4.0 mg). Analysis by polyacrylamide/sodium dodecyl sulfate gel electrophoresis (Laemmli, 1970) showed that approximately 90% of the released protein was nonreduced ricin with the remainder being separated A-chain and B-chain (data not shown).

In order to remove low molecular weight thiols, such as dithioerythritol and *N*-(2′-mercaptoethyl)lactamine, the solution of blocked ricin was applied to a column (1.5 mL) of concanavalin A–agarose equilibrated in 100 mM sodium phosphate buffer, pH 6.0. The column was washed first with the same buffer containing 10 mM lactose and 0.1 mM dithioerythritol (30 mL) and then with the pH 6.0 buffer again (30 mL). Elution of the column with the above buffer containing 1 M methyl α-D-mannopyranoside (30 mL) yielded a solution containing 3.0 mg of protein, corresponding to a 75% recovery from the concanavalin A–agarose column.

Assay for Protein Synthesis in Cell-Free System. This was performed as described in detail by Lambert et al. (1985).

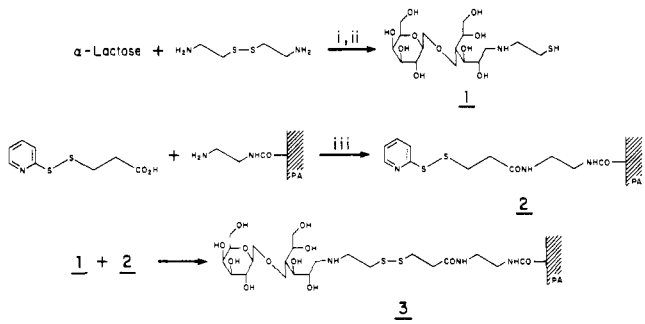
Preparation of J5-Blocked Ricin Conjugate. To a solution of J5 in 100 mM sodium phosphate buffer, pH 7.0 (1.5 mL at 1.0 mg/mL), was added SMCC (6 μL of a 10 mM solution in dioxane) to give a final concentration of 40 μM SMCC. After incubation at 30 °C for 30 min, the reaction was terminated by passage of the solution through a column of Sephadex G-25 (superfine) equilibrated in the above buffer. Fractions containing the antibody were pooled and added to a solution of blocked ricin (150–200 μg in 12 mL of 100 mM sodium phosphate buffer, pH 6.0, containing 1 M methyl α-D-mannopyranoside), and the pH of the reaction solution was immediately adjusted to 7 with 1 M NaOH. After 1 h, a portion of the reaction mixture was removed for analysis by polyacrylamide/sodium dodecyl sulfate gel electrophoresis (5–12.5% gradient gel), which showed that approximately 50% of the blocked ricin had reacted with antibody.

The reaction solution (14.1 mL) was then passed through a column (0.2 mL) of asialofetuin–TSK at 4 °C. The column was washed with 100 mM sodium phosphate buffer, pH 7.0, and the eluate was applied at 4 °C to a column (2 mL) of protein A–Sepharose CL-4B. The column was washed with the above buffer (20 mL) and then eluted with 0.1 M acetic acid containing 150 mM NaCl (4.5 mL) into 1 M sodium bicarbonate (0.5 mL). This final eluate was allowed to warm to room temperature and was then applied to a column (0.4 mL) of concanavalin A–agarose. The column was washed with 100 mM sodium phosphate buffer, pH 6.0 (3 mL), and eluted with the same buffer containing 1 M methyl α-D-mannopyranoside (4 mL). Analysis by polyacrylamide/sodium dodecyl sulfate gel electrophoresis (5–12.5% gradient gel) showed that a substantial purification of conjugate from free antibody had been effected, leaving an approximately 1:1 mixture of J5 and J5-blocked ricin (about 30 μg in 4 mL) and some material of higher molecular weight.

Cytotoxicity Testing. Immediately after the preparation of blocked ricin, solutions of samples for cytotoxicity testing were adjusted to pH 7.0 with 0.2 M Na_2HPO_4, and iodoacetamide was added to a final concentration of 2 mM. The solutions (2 mL) were then passed slowly through columns (0.2 mL) of asialofetuin–TSK, to remove partially modified or unmodified ricin. The testing of blocked ricin and J5-blocked ricin on cultured cells was then performed as described in detail by Goldmacher et al. (1985).

^{3}H-Labeled Blocked Ricin. To a solution of blocked ricin prepared as described above (6 mL, 150 μg/mL) in 1.0 M methyl α-D-mannopyranoside, pH 6.0, was added 0.2 M Na_2HPO_4 (0.2 mL) to raise the pH to 7.0. An approximately 10-fold molar excess of [^{3}H]-*N*-ethylmaleimide was then added (5×10^{-4} M solution in dry pentane, 320 μL), and the reaction mixture was shaken vigorously. The phases were then allowed to separate, and the organic layer was evaporated with a gentle stream of dry nitrogen. The aqueous phase was incubated at ambient temperature overnight. To ensure separation of intact blocked ricin from its component A- and B-chains, samples were first denatured at 100 °C for 10 min in the presence of 1% sodium dodecyl sulfate and then chromatographed on a Zorbax GF-250 gel filtration column (9.4 × 300 mm) equilibrated in 50 mM sodium phosphate buffer, pH 6.0,

Scheme I: Preparation of the Polyacrylamide Affinity Gel Containing Lactamine[a]



[a] i = $NaCNBH_3$, pH 6.2. ii = dithioerythritol, CM-cellulose chromatography. iii = EDC·HCl, pH 4.7.

containing 0.1% sodium dodecyl sulfate. A Gilson Model HPX pump operated at 0.5 mL/min and a Gilson Holochrome detector set at 280 nm were used.

Determination of the Specific Radioactivity of [^3H]-N-Ethylmaleimide. The specific radioactivity of the [^{3}H]-*N*-ethylmaleimide was determined under conditions identical with those used for measuring samples of labeled blocked ricin. To prepare an analogous chemical species in the same buffer, a known amount of [^{3}H]-*N*-ethylmaleimide was reacted with excess 2-mercaptoethanol in 100 mM sodium phosphate buffer, pH 7.0, and the solution was then diluted with an equal volume of aqueous 0.2% (w/v) sodium dodecyl sulfate. Aliquots of this solution were then subjected to scintillation counting.

Amino Acid Analysis. Samples of ^{3}H-blocked ricin were hydrolyzed under vacuum in 6 M hydrochloric acid for 24 h at 110 °C. Analysis was performed either on a Beckman 6300 amino acid analyzer or on a Liquimat IV analyzer, using in both cases the "pico-tag" method for detection.

The protein concentration of ricin analyzed as a standard was determined after buffer exchange on a column of Bio-Gel P-6 by measuring the absorbance at 280 nm assuming an $E^{1\%}_{1cm}$ value of 11.0 (quoted by the manufacturer).

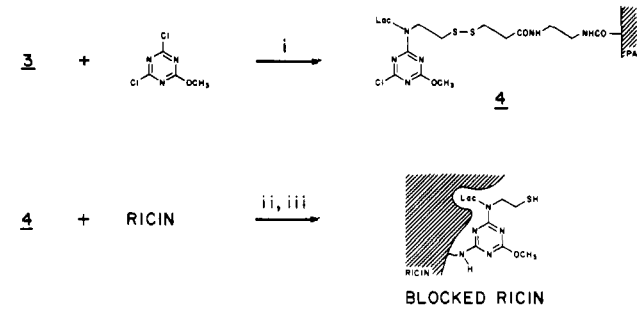
Scintillation Counting. Samples of equal volume (100 μL) of purified ^{3}H-blocked ricin or of [^{3}H]-*N*-ethylmaleimide were mixed with 4 mL of liquid scintillation fluid (Biofluor; New England Nuclear), and the clear solutions were counted on a Packard Tri-Carb Model 4530 scintillation counter.

Results

To obtain specific blockage of the galactose binding sites of ricin, the desired protein–ligand interaction was established on an affinity column. By allowing covalent attachment to proceed only with column-bound ricin, we reasoned that introduction of the ligand would occur specifically, possibly labeling the galactose binding sites exclusively. Our reasoning was based on the following points. Ricin bound specifically by the column is likely retained by virtue of binding simultaneously to two immobilized galactose-terminated ligands, yielding a high-affinity interaction that limits the diffusion of the ricin. The diffusion of the ligand is eliminated by its immobilization, and, therefore, we expected that even at millimolar concentrations of bound ligand there would be very limited nonspecific interaction. The chemical steps by which the affinity column was prepared are outlined in Scheme I, the synthetic details for which are given under Methods.

Polyacrylamide beads bearing 30–60 μmol of disulfide-linked sugar per mL of packed beads could be prepared. At bound ligand concentrations above 10 mM, a maximum binding capacity of about 1 mg of ricin per mL of packed beads was reached. With gels having higher levels of ligand,

Scheme II: Activation of the Affinity Gel 3 with 2,4-Dichloro-6-methoxytriazine and Preparation of Blocked Ricin Using the Activated Gel 4[a]



[a] Lac is used as an abbreviation for 1-deoxylactitol. i = 100 mM $NaHCO_3$–dioxane, 1:1 v/v. ii = pH 8.6, 24 h. iii = 10 mM dithioerythritol, pH 7.0.

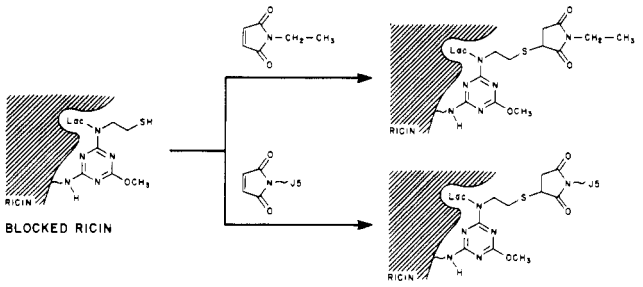
this capacity was not increased. The interaction of the ricin with the support-bound lactose derivative was specific, since the lectin could be eluted quantitatively with solutions of 0.5 M galactose.

Immediately prior to application of ricin to the gel, the ligands were allowed to react with the cross-linking reagent 2,4-dichloro-6-methoxytriazine (Scheme II). After washing, a solution of ricin was applied to the column at pH 7, and after extensive washing of the column with phosphate buffer, pH 7, to remove excess ricin, cross-linking was effected by raising the pH of the wash solution to 8.6.

To release the covalently bound ricin from the column, reaction conditions had to be found to cleave only the disulfide bond between the ligand and the solid support and not the interchain disulfide bond of the ricin. The four *intra*chain disulfide bonds, which are all located in the B-chain, were of no concern, since it is known that these bonds survive such severe treatments as 5% (v/v) 2-mercaptoethanol [see, e.g., Olsnes and Pihl (1973) and Fulton et al. (1986)]. Initially, we used 200 mM dithioerythritol, pH 7.0, because preliminary experiments had shown that this treatment released 80% of the ligand from an affinity column in 30 min at room temperature. Further, Lappi and co-workers (Lappi et al., 1978) had reported that the interchain disulfide bond of ricin is stable to solutions of dithioerythritol at concentrations up to 0.5 M. However, this treatment, while quantitatively releasing the covalently bound ricin from the column, led to complete cleavage of the interchain disulfide bond in the blocked ricin. In an attempt to establish conditions for the release of *intact* blocked ricin, a range of concentrations of dithioerythritol in solutions of different pH values was evaluated. The best yields of intact blocked ricin (30–40%, 12 experiments) were obtained with 10 mM dithioerythritol, pH 7.0, for 5 times 10 min at 4 °C. Analysis by polyacrylamide/sodium dodecyl sulfate gel electrophoresis under nonreducing conditions showed that the released material was intact ricin, containing only traces of the separated A- and B-chains (data not shown). Once released from the support, the interchain disulfide bond of the modified ricin was stable at 4 °C for at least 24 h in 10 mM dithioerythritol, pH 7.0. The increase in sensitivity toward reduction by dithioerythritol when ricin is bound to the polyacrylamide beads may suggest conformational changes that expose or strain the interchain disulfide bond. It may be relevant that Lappi et al. (1978) have reported an increased sensitivity of the interchain bond of ricin upon denaturation in sodium dodecyl sulfate.

An important feature of the design of the column ligand was the incorporation of a nascent sulfhydryl group that could be used as a specific locus on the blocked ricin for conjugation

Scheme III: Reaction of Blocked Ricin with [^{3}H]-*N*-Ethylmaleimide or with the Monoclonal Antibody J5 That Had Been Modified with Succinimidyl 4-(*N*-Maleimidomethyl)cyclohexane-1-carboxylate To Introduce an Average of 0.9 Maleimido Group per Antibody Molecule



with antibody. It was, therefore, necessary to remove all contaminating thiol compounds, such as dithioerythritol and *N*-(2′-mercaptoethyl)lactamine (**1**) from the blocked ricin preparation. A column of concanavalin A–agarose, which binds ricin through its α-1,4-mannopyranoside residues, was used, and contaminating thiol compounds were removed by extensive washing. The blocked ricin was then desorbed by using 1 M methyl α-D-mannopyranoside. The affinity chromatography procedure with concanavalin A–agarose was chosen over gel filtration because it shortened the length of time during which the blocked ricin containing a free sulfhydryl group was in buffer solutions with no reducing thiol compounds and, therefore, lowered the chance of disulfide bond formation between two blocked ricin molecules.

Samples of purified blocked ricin for in vitro cytotoxicity experiments were allowed to react with iodoacetamide to cap the free sulfhydryl group. Samples to be labeled with radioactive *N*-ethylmaleimide or those destined for conjugation to antibody were used without further modification in the buffer containing the methyl α-D-mannopyranoside eluant.

To ensure that the preparation of blocked ricin was free of any unmodified or partially modified ricin, samples were passed through a column of TSK-bound asialofetuin, a protein that binds to ricin with an association constant of at least 10^8 M^{-1} (Olsnes & Pihl, 1982). Unmodified ricin is efficiently adsorbed by such columns (data not shown). The size of the column was determined from the known capacity of the beads for native ricin and was always at least twice the volume required to bind the amount of ricin equivalent to the blocked ricin sample. The protein in the blocked ricin sample that did not bind to the column (about 90%) was considered blocked, because it has been shown that ricin binds as tightly to asialofetuin as it does to cell surfaces (Sandvig et al., 1978).

The purified blocked ricin was then tested for its ability to inhibit protein synthesis in a cell-free translation system. Figure 1 shows that the A-chain from blocked ricin inhibits protein synthesis in a rabbit reticulocyte lysate system to the same extent as the A-chain derived from native ricin, thus establishing that the procedure used in blocking the binding sites has no effect on the enzymatic activity of the A-chain and suggesting that the covalent modification is confined to the B-chain.

Conjugation to the monoclonal anti-CALLA antibody J5 (Scheme III) was performed by adding blocked ricin bearing the pendant sulfhydryl group to an excess of J5 that had been modified with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate so as to introduce an average of 0.9 maleimido groups per antibody molecule. After the conjugation reaction was complete, the product solution was passed through an asialofetuin–TSK column to remove traces of unblocked ricin or modified ricin that still could bind to its

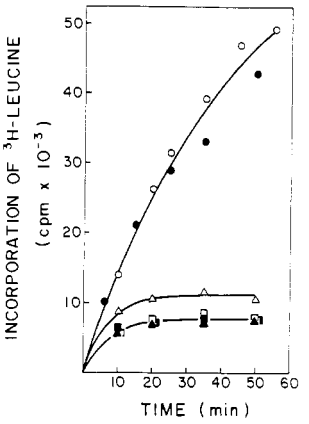


FIGURE 1: Time course of protein synthesis in the presence of reduced ricin or reduced blocked ricin. Protein synthesis was measured in a cell-free system derived from rabbit reticulocytes by the incorporation of [^{3}H]leucine into protein precipitable by trichloroacetic acid as described in Lambert et al. (1985). Samples of ricin or blocked ricin were diluted to 4 μg/mL of A-chain in 10 mM sodium phosphate buffer, pH 7.4, containing NaCl (20 mM), bovine serum albumin (0.2 mg/mL), and 2-mercaptoethanol (1% v/v) and incubated at room temperature for 3 h to effect reduction. The solutions were then further diluted to 0.01–0.04 μg of A-chain/mL, and samples (1 μL) containing 10–40 ng of A-chain were added to assay mixtures (total volume 27 μL) as described previously (Lambert et al., 1985). At various times, the incorporation of [^{3}H]leucine into protein was measured in 3-μL samples as described previously (Lambert et al., 1985). (●) Control assay with 1 μL of pH 7.4 buffer without 2-mercaptoethanol; (○) control assay with 1 μL of pH 7.4 buffer with 2-mercaptoethanol; (△, ▲) assays that contained 10 and 30 pg of ricin A-chain, respectively; (□, ■) assays that contained 20 and 40 pg of A-chain from blocked ricin, respectively.

natural ligand. Next, nonconjugated blocked ricin and methyl α-D-mannopyranoside were removed by use of a column of staphylococcal protein A–Sepharose CL-4B, which binds both the murine monoclonal antibody J5 and the immunoconjugate. Finally, the conjugate was separated from most of the free antibody by chromatography on a column of concanavalin A–agarose. Elution with a solution containing 1 M methyl α-D-mannopyranoside gave an antibody-blocked ricin conjugate that was contaminated with about 30% of free antibody. This contamination was due to the heterogeneity in glycosylation of the monoclonal antibody J5. It was found that up to 5% of the antibody in our J5 preparation bound specifically to a column of immobilized concanavalin A. The degree of purification achieved in each step is illustrated in Figure 2.

The samples of blocked ricin and their J5 antibody conjugates were then tested for cytotoxicity against the human lymphoblastoid cell line Namalwa. These cells express approximately 60 000 common acute lymphoblastic leukemia antigens (CALLA) for the monoclonal antibody J5 (Goldmacher et al., 1986) on the surface of each cell. We speculated that blocked ricin should have a toxicity toward intact cells in the range of the cytotoxicity of ricin A-chain alone, which is 10 000–100 000-fold lower than that of native ricin. However, the blocked ricin was only about 20 times less toxic than native ricin (Figure 3), indicating that a substantial proportion of the binding capability of ricin remained. In agreement with this view, the toxicity was reduced 1000-fold by the addition of 30 mM lactose to the cell cultures (Figure 3b). Linking of the blocked ricin to the anti-CALLA antibody J5 increased its toxicity only slightly for the CALLA-positive Namalwa cells (Figure 3b) but decreased its toxicity for CALLA-negative Molt 4 cells 5–10-fold (data not shown). [Because the concentrations used for toxicity testing were well below the concentration of antibody necessary to saturate all the antigenic sites on the cell surface (Goldmacher et al., 1986), the

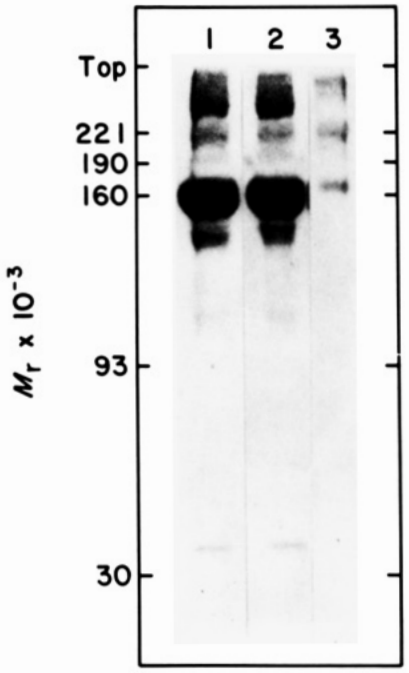


FIGURE 2: Polyacrylamide/sodium dodecyl sulfate gel analysis of the purification of a J5-blocked ricin conjugate on a 5–12.5% (w/v) gradient gel under nonreducing conditions. (Lanes 1 and 2) Product mixture from the conjugation reaction and after purification through a column of protein A–Sepharose CL-4B, respectively. The heavy bands at M_r 160000 correspond to free antibody J5, the bands at approximately M_r 220000 to antibody-blocked ricin conjugate, and the bands at approximately M_r 300000 to antibody dimer. The latter bands and the bands of M_r 140000–150000 are usually seen when lanes contain heavy loads of succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate modified antibody. (Lane 3) Conjugation mixture after further purification through a column of concanavalin A–agarose. This lane shows bands for antibody (M_r 160000), antibody-blocked ricin conjugate (M_r 220000), and high molecular weight species (M_r >300000) composed of more than one antibody and blocked ricin molecules. The calibration of M_r is from the mobility of conjugates of J5 with two molecules of gelonin (221000) and one molecule of gelonin (190000), J5 (160000), phosphorylase *b* (93000), and carbonic anhydrase (30000).

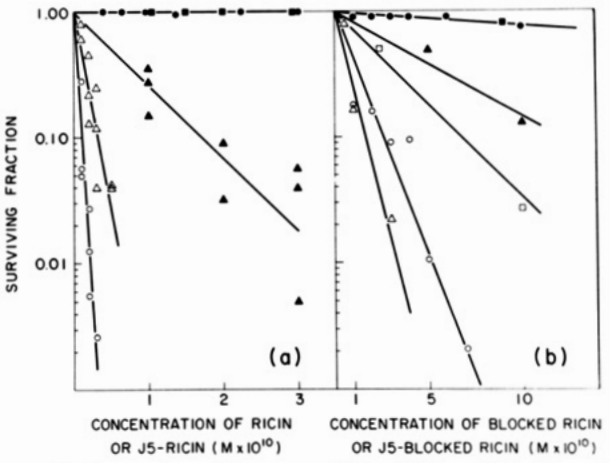


FIGURE 3: Comparison of the cytotoxicities of ricin and J5–ricin with the cytotoxicities of blocked ricin and J5-blocked ricin. The concentration of immunotoxin was determined from the measurements of A_{280}, assuming a ratio for antibody to ricin of 1:1 and correcting for the presence of 30% free antibody. Namalwa cells were exposed to the toxins at 37 °C for 24 h, washed, and placed into fresh medium for the determination of the surviving fractions as described in detail in Goldmacher et al. (1985). (a) Toxicity of ricin and J5–ricin. Ricin without (○) and with (●) lactose (30 mM); J5–ricin without (△) and with (▲) lactose (30 mM); (■) J5–ricin in the presence of J5 (1 μM) and lactose (30 mM). (b) Toxicity of blocked ricin and J5-blocked ricin. Blocked ricin without (○) and with (●) lactose (30 mM); J5-blocked ricin without (△) and with (▲) lactose (30 mM); J5-blocked ricin in the presence of J5 (1 μM) without (□) and with (■) lactose (30 mM).

residual free antibody in the conjugate preparation does not significantly affect the values observed for the cytotoxicity of the conjugate.] As expected from this result, an identical 5–10-fold decrease in toxicity relative to blocked ricin was observed on CALLA-positive cells, when the toxicity of the conjugate was measured in the presence of unconjugated J5 antibody to compete with the binding through the antibody portion of the conjugate (Figure 3b). [This reduction is probably due to steric interference by the antibody with the residual binding of the blocked ricin to the cell surface, as has been suggested by Thorpe and collaborators (Thorpe, 1985) for antibody–ricin conjugates with short linkers.] This finding indicates that the blocked ricin component of the conjugate still binds to the cell surface. Consistent with this finding, only the simultaneous addition of free antibody and of lactose could protect the antigen-bearing cells from the toxicity of the conjugate (Figure 3b). But despite the residual binding of the blocked ricin to the cell surface, the above results indicate that the J5 conjugate of this blocked ricin preparation shows a 10-fold antigen-specific toxicity.

The results described above suggested that our blocked ricin preparation had only suffered an incomplete blockage of its binding sites. Specifically, it seemed possible that we had blocked only one of the two binding sites for galactose residues and that the remaining free binding site was sufficient to bind ricin to cell-surface oligosaccharides and to cause the observed cytotoxicity. This prompted us to determine the number of ligands bound covalently in the blocked ricin. Accordingly, blocked ricin was labeled with radioactive *N*-ethylmaleimide of known specific radioactivity (Scheme III). On the basis that *N*-ethylmaleimide under these conditions only reacts with free sulfhydryl groups [see, e.g., Ji (1983)], blocked ricin should only incorporate radiolabel through the reaction of the sulfhydryl groups of covalently bound blocking ligands. Any nonspecific incorporation of label could be determined by reacting native ricin with labeled *N*-ethylmaleimide under the same reaction conditions. The amount of radioactivity specifically incorporated can therefore be directly related to the number of covalently linked ligands.

To establish the molar ratio of covalent ligand to ricin by this method of labeling, it is essential to have samples of pure labeled blocked ricin and to remove the small amounts of contaminating A- and B-chain. Labeled samples were therefore first denatured in sodium dodecyl sulfate in order to destroy the strong noncovalent interaction between the A- and B-chains of ricin (Lappi et al., 1978) and then subjected to gel filtration on a Zorbax GF-250 column in buffers containing 0.1% sodium dodecyl sulfate. Two protein peaks eluted as monitored by the absorbance at 280 nm (see Figure 4). Polyacrylamide/sodium dodecyl sulfate gel electrophoresis under nonreducing conditions showed that the first protein peak consisted of intact labeled blocked ricin and that the second peak contained a mixture of the two chains of ricin. The purified labeled samples were then analyzed quantitatively for radioactivity with a liquid scintillation counter and for ricin by amino acid analysis. The amino acid analyses were performed in parallel with samples of known amounts of the same ricin preparation that had been used in the blocking reaction. The amount of protein in the labeled samples was calculated by comparing independently the amounts of five amino acids (aspartic acid, glutamic acid, leucine, alanine, and arginine) to their respective amounts in the samples of native ricin. The average from these five determinations was taken as the protein

Table I

(A) Quantitation of Ricin and Blocked Ricin by Amino Acid Analysis

amino acid	ricin standard[a] amt of amino acid (nmol)	labeled ricin amt of amino acid (nmol)	labeled ricin calcd amt of ricin (nmol)	blocked ricin experiment 1[b] amt of amino acid (nmol)	blocked ricin experiment 1[b] calcd amt of ricin (nmol)	blocked ricin experiment 2[b] amt of amino acid (nmol)	blocked ricin experiment 2[b] calcd amt of ricin (nmol)
Asp	4.79	2.85	0.063	1.75	0.039	0.87	0.019
Glu	4.40	2.18	0.053	1.56	0.038	0.82	0.020
Leu	3.70	1.94	0.056	1.20	0.034	0.70	0.020
Ala	3.80	1.72	0.048	1.21	0.039	0.68	0.019
Arg	2.88	1.31	0.048	0.97	0.036	0.50	0.018

(B) Incorporation of [^{3}H]-*N*-Ethylmaleimide into Ricin and Blocked Ricin

	ricin	blocked ricin experiment 1[b]	blocked ricin experiment 2[b]
cpm measured[c] (sample size in parentheses)	6.04×10^3 (50 μL)	1.84×10^4 (30 μL)	3.73×10^4 (100 μL)
cpm calcd for sample used in amino acid analysis	2.72×10^4[d]	4.09×10^4[e]	2.48×10^4[e]
[^{3}H]-*N*-ethylmaleimido groups in amino acid analysis sample[f] (nmol)	4.9×10^{-2}	7.34×10^{-2}	4.46×10^{-2}
amt of ricin in amino acid analysis sample[g] (nmol)	5.4×10^{-2}	3.7×10^{-2}	1.9×10^{-2}
[^{3}H]-*N*-ethylmaleimido groups incorporated per ricin molecule	0.91	1.98	2.35
[^{3}H]-*N*-ethylmaleimido groups specifically incorporated into blocked ricin		1.07	1.44

[a] The amino acid analysis sample of the ricin standard contained 0.106 nmol of ricin as calculated from the absorbance at 280 nm of a standard solution assuming an $E^{1\%}_{1cm}$ of 11.0 and M_r of 60000. [b] Experiment 1 and experiment 2 were performed with independently prepared blocked ricin samples, starting with two different batches of affinity column. [c] All measurements of radioactivity are average values from at least two measurements, which deviated not more than 5% from each other. [d] A sample of 450 μL was hydrolyzed, and half the amount was used in the amino acid analysis. [e] Samples of 100 μL were hydrolyzed, and two-thirds of the amounts were used in the amino acid analysis. [f] The [^{3}H]-*N*-ethylmaleimide had a specific radioactivity of 5.57×10^5 cpm/nmol (see Methods for its determination), which corresponds to a counting efficiency of 50%. [g] The indicated numbers are averages derived from the corresponding five independently calculated values listed in (A).

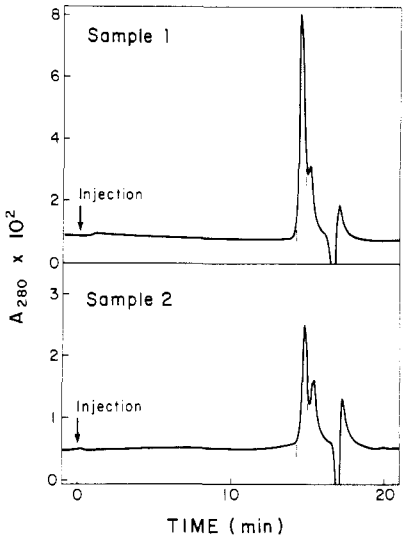


FIGURE 4: Separation of denatured labeled blocked ricin from its A-chain and B-chain by HPLC on a Zorbax GF-250 gel filtration column equilibrated in 50 mM sodium phosphate buffer, pH 6, containing 0.1% sodium dodecyl sulfate. Chromatography was monitored by measuring the absorbance of the eluant at 280 nm, and the traces obtained for the two independently prepared labeled blocked ricin samples described in Table I are shown. Peaks eluting at 14.8 min are blocked ricin and were collected between the vertical bars. The smaller peaks eluting at 15.5 min were shown by polyacrylamide/sodium dodecyl sulfate gel electrophoresis to consist of equal amounts of A- and B-chain.

concentration (see Table I). This method, which does not rely on the published amino acid composition of ricin, avoids the problems associated with variations in the amino acid composition of ricin preparations of different isotoxin compositions. It is, however, assumed that all isotoxins in the ricin preparation react to the same extent with a ligand directed toward the binding sites.

From the study of two independent samples of labeled blocked ricin, it was found that one sample had 1.98 maleimido groups per ricin molecule and the second one had 2.35 maleimido groups. Similarly, it was shown that native ricin incorporates 0.91 maleimido group per molecule under analogous conditions. [This latter result was not unexpected because it was known that sodium dodecyl sulfate exposes the otherwise inaccessible sulfhydryl group in the A-chain portion of intact ricin (Cawley & Houston, 1978), and this modification probably occurred after denaturation.] The blocked ricin samples contained, therefore, between 1.07 and 1.44 free sulfhydryl groups more than native ricin.

The above data support the notion that we have linked one ligand (with its sulfhydryl group) to one binding site of ricin. (The introduction of apparently 1.44 extra sulfhydryl groups per ricin molecule in the second sample can be explained by contamination of this sample with 20% of reduced A- and B-chain rather than by the nonspecific incorporation of ligand into the protein. As can be seen in Figure 4, the sample of blocked ricin from the second experiment contained a higher proportion of the reduced protein and the separation was less complete. Due to the small amount of material available, attempts at further purification were unsuccessful.)

Discussion

Three features were important for the ligand on the affinity column: first, an unchanged terminal galactose moiety was needed for the specific interaction with ricin; second, a functional group was required for cross-linking the ligand to ricin in a temporally controllable way; and, third, a functional group was needed to link the ligand to the solid support in a way that allowed the release of the ligand–ricin complex without affecting the protein. A ligand that meets all three criteria is *N*-(2′-mercaptoethyl)lactamine (**1**).

The bifunctional cross-linking reagent 2,4-dichloro-6-methoxytriazine has two labile chlorines that can be replaced successively by amines under mild conditions (Kay & Crook, 1967). This reagent allows quantitative modification of the ligands to be followed by the binding of protein, prior to initiating the cross-linking of ligand and protein by raising the pH. The two newly formed covalent bonds are stable. Thus, Lang et al. (1977) have shown that [^{14}C]alanyltriazinylagarose

loses less than 1% of its alanine residues when suspended in buffers between pH 5.8 and 9.6 at ambient temperature over a period of 1 month.

A disulfide bond was chosen to link the ligand to the solid support. Following the reductive release of the blocked ricin from the affinity column, the newly formed sulfhydryl group could be readily used in making a conjugate with an appropriately modified antibody. Further modification of the ricin–ligand complex before conjugation to antibody was thus avoided, and the site of attachment of the antibody was confined to the B-chain. It was therefore expected that the toxicity of the A-chain would not be compromised.

The use of an affinity column procedure has the further advantage that noncovalently bound (non-cross-linked) ricin can be removed by washing the column with buffers containing 0.5 M galactose. This treatment ensures that the release of the ligands from the beads with dithioerythritol causes the release only of ricin that is covalently modified. If covalent cross-linking of ligand and ricin occurs with the desired specificity, the released material will be exclusively labeled at the galactose binding sites.

The results of toxicity tests on cultured Namalwa cells indicated that the blocked ricin samples were still capable of binding to cell-surface receptors, seemingly contradicting the results from the binding tests on the columns of asialofetuin–TSK. If the 20-fold decrease in toxicity that was observed between ricin and our samples of blocked ricin can be equated with an equal 20-fold decrease in the binding of blocked ricin to cells, one can calculate from the association constants of ricin binding to cells [$K_a = (1-5) \times 10^7$ M^{-1}; Olsnes & Pihl, 1982] or of ricin binding to the asialoglycopeptide isolated from fetuin ($K_a = 1.4 \times 10^7$ M^{-1}; Baenziger & Fiete, 1979) that the *blocked* ricin samples would have association constants of the order of 10^6 M^{-1}. Since adsorption of protein to affinity columns, however, usually requires association constants that are larger than 10^6 M^{-1} (Scopes, 1982), it is not surprising that these blocked ricin samples are not retained on asialofetuin columns. This is particularly true when one considers that the blocked ricin samples were passed in relatively large volumes of buffer through proportionately small asialofetuin columns.

The fact that it is possible to form a covalent complex between a natural galactose-containing ligand and ricin and at the same time to interfere only marginally with the binding capacity of ricin for galactose-terminated receptors on cell surfaces suggested strongly that we had blocked only one site of the two binding sites for galactose-terminated ligands. The number of ligands bound per molecule of blocked ricin was therefore determined by exploiting the sulfhydryl group on the ligand after release of the ricin–ligand complex from the solid support. Reaction of the ligand–ricin complex with the reagent [^{3}H]-*N*-ethylmaleimide was expected to establish directly the number of ligands bound per ricin molecule. This analysis showed that we had indeed introduced one extra sulfhydryl group, and therefore one ligand, into each blocked ricin molecule. This result does not necessarily invalidate our assumption that ricin binds through two ligands to the affinity column. Covalent cross-linking of the ligand requires the presence of a nearby amino group on the protein, and one might argue that only one binding site has an amino group in the right position. However, the possibility that ricin binds through only one galactose-terminated ligand on the column despite the low association constant of free galactose of about 10^4 M^{-1} cannot be dismissed, because it is known that the nonspecific interaction between a protein and a hydrophobic linker arm can permit the use of ligands in affinity columns having low intrinsic association constants (Scopes, 1982).

The results from the cytotoxicity tests with this preparation of blocked ricin showed only a 20-fold reduction in toxicity compared to that of native ricin. This result, taken together with the evidence that we have incorporated only one ligand per ricin molecule, is consistent with the notion that ricin has two independent binding sites with similar binding constants for galactose-terminated ligands. Houston and Dooley (1982) have also suggested, from equilibrium dialysis binding experiments, that the two galactose binding sites on ricin have very similar affinities. Frênoy (1986), in a more recent study, has indicated that the second molecule of galactose is bound with a 3–4-fold lower affinity.

Despite the only modest reduction in the cytotoxicity of the blocked ricin, conjugates of this toxin with the monoclonal antibody J5 were prepared. Thorpe and collaborators (Thorpe et al., 1984) have shown that conjugation of an antibody to ricin can obstruct the galactose binding sites of the toxin and thus reduce cytotoxicity. We reasoned that by linking the antibody to the free sulfhydryl group of the bound ligand the antibody might be positioned close to the second binding site and further decrease the binding and cytotoxicity. We therefore prepared the conjugate between the monoclonal antibody J5, which binds to the common acute lymphoblastic leukemic antigen (CALLA), and blocked ricin prepared as described here. The affinity-purified conjugate was highly toxic to CALLA-positive cells. However, the toxicity was only slightly affected by the addition of excess antibody and was only eliminated completely by coincubation with excess antibody and lactose. The addition of lactose alone also diminished the toxicity. Together, these results indicate that the conjugate still binds to the cell surface via the incompletely blocked ricin. The presence of the antibody in the conjugate seems therefore not to interfere significantly with the binding of the blocked ricin. This suggests that the two independent binding sites of ricin are far apart from each other, so that antibody linked near one binding site does not affect binding to the second site.

During the revision of this paper, high-resolution X-ray structures of the B-chain of ricin (Rutenber et al., 1987) and of whole ricin (Montfort et al., 1987) have been published. In this excellent work, Robertus and his colleagues show and describe that the two galactose binding sites of ricin are located at the extreme ends of the B-chain some 75 Å apart from each other. Our interpretation of our results fits well this model for the structure of ricin determined by X-ray crystallography.

The affinity column approach described in this paper may be useful for labeling nucleophilic groups near the carbohydrate binding sites of lectins. The covalently bound ligand is easily radiolabeled, which enables the site(s) of reaction to be identified by the isolation and analysis of radiolabeled peptides. In the specific case of ricin, this information may be of value toward efforts in altering the specificity of binding of the B-chain by site-directed mutagenesis of cDNA clones (Lamb et al., 1985). This affinity column approach, however, does not result in complete blocking of the ability of ricin to bind to cells, and so this approach is not useful, at present, for making highly specific immunotoxins from ricin. We are currently developing a new method to achieve the complete blocking of the binding of ricin to cell surfaces that, we anticipate, will lead to a blocked ricin reagent that will prove useful in making immunotoxins.

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