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The Galactose-Binding Sites of the Cytotoxic Lectin Ricin Can Be Chemically Blocked in High Yield with Reactive Ligands Prepared by Chemical Modification of Glycopeptides Containing Triantennary N-Linked Oligosaccharides†

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ABSTRACT: A glycopeptide containing a triantennary N-linked oligosaccharide from fetuin was modified by a series of chemical and enzymic reactions to afford a reagent that contained a terminal residue of 6-(*N*-methylamino)-6-deoxy-D-galactose on one branch of the triantennary structure and terminal galactose residues on the other two branches. Binding assays and gel filtration experiments showed that this modified glycopeptide could bind to the sugar-binding sites of ricin. The ligand was activated at the 6-(*N*-methylamino)-6-deoxy-D-galactose residue by reaction with cyanuric chloride. The resulting dichlorotriazine derivative of the ligand reacts with ricin, forming a stable covalent linkage. The reaction was confined to the B-chain and was inhibited by lactose. Bovine serum albumin and ovalbumin were not modified by the activated ligand under similar conditions, and we conclude, therefore, that the reaction of the ligand with ricin B-chain was dependent upon specific binding to sugar-binding sites. Ricin that had its galactose-binding sites blocked by the covalent reaction with the activated ligand was purified by affinity chromatography. The major species in this fraction was found to contain 2 covalently linked ligands per ricin B-chain, while a minor species contained 3 ligands per B-chain. The cytotoxicity of blocked ricin was at least 1000-fold less than that of native ricin for cultured cells in vitro, even though the activity of the A-chain in a cell-free system was equal to that from native ricin. Modified ricin that contained only 1 covalently linked ligand was also purified. This fraction retained an ability to bind to galactose affinity columns, although with a lower affinity than ricin, and was only 5- to 20-fold less cytotoxic than native ricin.

Ricin, a toxic lectin isolated from castor beans (*Ricinus communis* beans), is a glycoprotein that consists of two nonidentical subunits, the A-chain (M_r 30500) and the B-chain (M_r 32000), that are linked by a single disulfide bond (Yoshitake et al., 1978; Funatsu et al., 1979; Olsnes & Pihl, 1982). The A-chain is a specific *N*-glycosidase that inactivates the 60S subunit of eukaryotic ribosomes by hydrolyzing the adenine–ribose bond of residue 4324 of the 23S rRNA (Endo et al., 1987; Endo & Tsurugi, 1987). The B-chain has lectin activity and binds to galactose and galactose-terminated oligosaccharides (Olsnes & Pihl, 1973). Following binding to cell surfaces, ricin is internalized (Sandvig & Olsnes, 1982; Youle & Colombatti, 1987) and ultimately the A-chain (or the entire toxin) is transported across a membrane into the cytosol, where free A-chain inactivates ribosomes, ultimately causing cell death. The details of the events that occur between binding of ricin to the cell surface via the B-chain and the inactivation of ribosomes by the A-chain are not known.

The B-chain of ricin has long been known to have two binding sites for galactose[1] or a galactoside (Villafranca &

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Robertus, 1981; Houston & Dooley, 1982). The X-ray crystallographic structure of ricin has recently been determined at 2.8 Å (Montfort et al., 1987), and the model shows that the B-chain folds into two separate domains. Each domain can bind a molecule of lactose at sites separated by 75 Å (Rutenber et al., 1987). The affinity of these binding sites for galactose (or lactose) is quite low (K_a about 2×10^4 M^{-1}), requiring concentrations of mono- or disaccharide in the millimolar range for saturation (Frênoy, 1986; Houston & Dooley, 1982; Zentz et al., 1978). Some results suggest that the two sites have similar affinity for galactose or galactosides (Houston & Dooley, 1982) while other work suggests that the second site has a somewhat lower affinity [$K_a \sim 10^3$ M^{-1} estimated from the results of Frênoy (1986)].

Complex oligosaccharides such as the triantennary N-linked oligosaccharides from fetuin (Baenziger & Fiete, 1979a; Nilsson et al., 1979; Takasaki & Kobata, 1986; Townsend et al., 1986; Green et al., 1988; Bendiak et al., 1989) bind to ricin with much higher affinity than do simple sugars, with K_a values $\geq 10^7$ M^{-1} (Baenziger & Fiete, 1979b). Little is known about the nature of the binding sites of ricin for complex oligosaccharides, and the stoichiometry of binding has not been measured. One simple explanation for the 1000-fold higher affinity for a complex oligosaccharide, namely, that one galactose residue of the oligosaccharide occupies each of the two galactose-binding sites of the ricin B-chain, thereby multiplying the interaction at each site into a high affinity for the complex ligand, seems unlikely in view of the fact that the two binding sites are about 75 Å apart on opposite ends of the B-chain.

The interaction of complex multivalent galactose-containing ligands with another lectin, namely, the mammalian hepatic galactose/*N*-acetylgalactosamine lectin, has been studied in more detail (Lee et al., 1982, 1983, 1984; Townsend et al., 1986; Hardy et al., 1985). They conclude that properly clustered galactose residues bind to a lattice of galactose-binding sites, which results in a very high binding affinity ($K_a \sim 10^7$–10^9 M^{-1}) compared with those of monovalent sugars ($K_a \sim 10^3$–10^4 M^{-1}). Considering ricin B-chain, the X-ray crystallographic model shows that each of the two sugar-binding domains contains three homologous units, each about 40 amino acids in length, that appear to be derived from an ancient galactose-binding unit that is also found in discoidin I, a galactose-binding protein from *Dictyostelium discoideum* (Robertus & Ready, 1981; Rutenber et al., 1987). Thus, one might, therefore, consider that ricin B-chain consists of a lattice of 2 galactose-binding sites of measurable affinity (one from each domain) and 4 other weaker sugar-binding sites that together are capable of binding complex galactose-containing oligosaccharides with high affinity.

Ricin has a potential application in cancer therapy as a component of immunotoxins (Vitetta & Uhr, 1985; Frankel et al., 1986; Vitetta et al., 1987; Blättler et al., 1989). However, conjugates of intact ricin lack cell specificity because they can also bind to cells through the B-chain. Thus, there has been great interest in developing methods to efficiently and permanently block the sugar-binding sites of ricin. Chemical modification of functional groups of ricin and other lectins has been used in attempts to destroy the sugar-binding sites of these proteins, for example, by acetylation (Sandvig et al., 1978; Rice et al., 1975; Vancurova et al., 1976; Youle et al., 1981) and by oxidation (Vitetta, 1986). Affinity labeling by photochemically activated derivatives of monosaccharides (Beppu et al., 1975; Fraser et al., 1976; Thomas, 1977; Houston, 1983; Kohnken & Berger, 1987) or complex glycopeptides (Baenziger & Fiete, 1982; Lee & Lee, 1986) has also been tried. The affinity of the galactose-binding sites of ricin for cell surface oligosaccharides has also been decreased by nonspecific steric hindrance achieved by cross-linking ricin to an antibody (Thorpe et al., 1984). However, none of these approaches have been completely successful, owing variously to poor yields, high random modification, and/or incomplete blockage of the sugar-binding sites of the lectins.

In previous work, we used an affinity column carrying an activated galactose-terminated ligand in an attempt to completely and specifically block the binding sites of ricin (Moroney et al., 1987). However, this method resulted in the blockade of only one of the galactose-binding sites, and the product of the modification was only about 20-fold less toxic than native ricin itself, indicating that the modified ricin could still bind to cells (Moroney et al., 1987). In the work described in this paper, we have developed an affinity labeling reagent based on glycopeptides containing triantennary N-linked oligosaccharides from fetuin. Covalent linking of at least two complex oligosaccharide moieties results in blocking of both sugar-binding sites of ricin. The cytotoxicity of the blocked ricin is reduced about 1000-fold, which is the level of cytotoxicity attained by incubating cells with native ricin in the presence of high concentrations of lactose. The approach described in this paper for preparing a reactive affinity ligand for covalent modification of the oligosaccharide-binding sites of ricin may also provide a valuable tool for the study of the binding sites of other lectins.

Experimental Procedures

Materials

Ricin, which is ricin D according to the nomenclature of Wei and Koh (1978), was from Sigma Chemical Co. (St. Louis, MO). Fetuin (type IV, from fetal calf serum), asialofetuin (type II), Pronase (type XIV, from *Streptomyces griseus*), galactose oxidase (EC 1.1.3.9; from *Dactylium dendroides*), neuraminidase (EC 3.2.1.18; type X, from *Clostridium perfringens*), catalase (EC 1.11.1.6; from bovine liver), horseradish peroxidase (EC 1.11.1.17; type VI), bovine serum albumin, creatine phosphokinase (EC 2.7.3.2.), creatine phosphate, and mRNA (rabbit globin) were also purchased from Sigma Chemical Co. Carboxypeptidase Y (EC 3.4.16.1; from yeast) and leucine aminopeptidase (EC 3.4.11.1; from pig kidney) were obtained from Boehringer Mannheim (Indianapolis, IN). Lactose–polyacrylamide beads, carbonyldiimidazole-activated TSK beads (Reacti-gel HW-65F), Iodogen,[2] and 2-iminothiolane hydrochloride were purchased from Pierce Chemical Co. (Rockford, IL). Methylamine (40% w/w) and 1-*O*-methyl-β-D-galactoside[1] were from Aldrich Chemical Co. (Milwaukee, WI). $NaCNBH_3$ was also from Aldrich and was recrystallized before use by the method of Borch et al. (1971) to ensure that it was free of $NaBH_4$. Cyanuric chloride was purchased from Sigma and was stored under vacuum over Drierite at ambient temperature. A rabbit reticulocyte lysate system for cell-free protein synthesis, which included L-[3,4,5-^{3}H]leucine (specific radioactivity, 146.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [^{14}C]Methylamine hydrochloride (59 mCi/mmol) and iodo[1-^{14}C]acetamide (53 mCi/mmol) were from Amersham

[1] Abbreviations: All galactose or galactose derivatives mentioned in this paper are in the *pyranose* form. Glucosamine and galactosamine are abbreviations for 2-amino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-galactopyranose, respectively. 6-Aldehydo-β-D-galactoside is also termed D-galactohexodialdo-1,5-β-pyranoside.

[2] Iodogen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril.

International (Arlington Heights, IL) and, before use, were diluted with the respective nonradioactive compounds to the specific radioactivities indicated in the text. $Na^{125}I$ (carrier free; 100 mCi/mL) was also obtained from Amersham. Asialofetuin–TSK beads (immobilized asialofetuin) were prepared according to the method of Bethell et al. (1979).

Hazardous Procedures. Solutions contaminated with ricin were treated with 1% (v/v) household bleach for 1 h at ambient temperature, which inactivates ricin.

Methods

Assay Methods. Hexoses were assayed by the phenol/H_2SO_4 method of Ashwell (1966). Glycopeptides containing N-linked oligosaccharides were quantified by the hexose assay using lactose as a standard, and assuming 6 mol of hexoses/mol of glycopeptide (Baenziger & Fiete, 1979a; Nilsson et al., 1979; Takasaki & Kobata, 1986). Sialic acid was measured by the method of Warren (1959). Bound sialic acid was released from complex oligosaccharides by hydrolysis in 50 mM H_2SO_4 at 80 °C for 60 min. Reducing groups of sugars were measured by the procedure of Somogyi (1952) using galactose as a standard, except that the copper carbonate tartrate reagent was incubated with the sugar at 100 °C for 80 min. Sulfhydryl groups were quantified spectrophotometrically with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959), and primary amines were determined by reaction with 2,4,6-trinitrobenzenesulfonic acid (Habeeb, 1966), or by reaction with ninhydrin (Hirs, 1967). The concentration of purified proteins was determined from their absorption at 280 nm assuming $E^{1\%}_{1cm}$ values of 4.1 for fetuin and asialofetuin (Spiro, 1976) and 11.8 for ricin (Olsnes, 1978). Galactose oxidase was assayed immediately before each use with 3-methoxybenzyl alcohol as the substrate (Kwiatkowski & Kosman, 1973; Tressel & Kosman, 1980). TLC was performed on silica gel coated glass plates (0.25 mm, Analtech, Newark, DE) with two different solvent systems: solvent A, 1:1 (v/v) methanol/ethyl acetate; solvent B, 5:5:1:3 (v/v) pyridine/ethyl acetate/acetic acid/water. Carbohydrates were visualized by heating the TLC plates after spraying with 15% (v/v) sulfuric acid in 50% (v/v) ethanol, and carbonyl functions were detected by using 0.4% (w/v) 2,4-dinitrophenylhydrazine in 2 M HCl (Lee & Lee, 1986). The radioactivity of aqueous samples, or of precipitated proteins collected onto glass fiber disks (Whatman; GF-C, 2.4 cm), was measured by scintillation counting with Biofluor (NEN, Boston, MA) or Betafluor (National Diagnostics, Somerville, NJ) scintillation fluids, respectively, and a Packard Tri-Carb Model 4530 scintillation counter. The efficiency of counting for ^{14}C and ^{3}H was 90.5% and 25%, respectively.

Preparation and Purification of Glycopeptides Containing N-Glycosidically Linked Triantennary Oligosaccharides from Bovine Fetuin. Fetuin (5 g) was subjected to two cycles of proteolysis with Pronase (50 and 15 mg for each cycle) using conditions described previously for proteolysis and for purification by gel filtration on Bio-Gel P-6 (Baenziger & Fiete, 1979a; Lee et al., 1983). The resulting glycopeptide fraction was then treated with leucine aminopeptidase and carboxypeptidase Y (5 units of each) as described by Lee et al. (1983). The glycopeptide fractions containing N-linked oligosaccharides were then submitted to anion-exchange chromatography on a column (200 mL) of DEAE-cellulose (Whatman, DE-52) equilibrated in 2 mM pyridinium acetate buffer, pH 5.4, yielding two major peaks (FI and FII) as reported earlier (Baenziger & Fiete, 1979). The yields of FI and FII in a typical preparation were 207 and 218 mg, respectively, for a total of 143 μmol in these fractions (assuming an average M_r of 2974; Baenziger & Fiete, 1979a).

Preparation of Affinity Ligand **7**. The route for the preparation of affinity ligand **7**, starting from glycopeptide containing N-linked oligosaccharide, is drawn in symbols in Scheme I. Both FI and FII were used as starting material with very similar results, and so they are not further distinguished in the following description of the methods. All drying steps for concentration were done by rotary evaporation at 35 °C under reduced pressure.

Partial Removal of Sialic Acid from Glycopeptide Containing N-Linked Oligosaccharide by Neuraminidase. Glycopeptide **1** (50 μmol) was dissolved in 50 mM sodium acetate buffer, pH 5.0 (3 mL), containing NaCl (150 mM) and $CaCl_2$ (10 mM), and the pH was readjusted to 5.0 with the addition of NaOH (1.0 M solution). Neuraminidase (0.25 units) was added and the solution was incubated at 37 °C for 1.5 h. Under these conditions, 30–40% of the sialic acid was released from the glycopeptide. The reaction was stopped by purifying the glycopeptide by gel filtration on a column (45 cm × 1.5 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated with 0.1 M pyridinium acetate buffer, pH 5.4. The partially desialylated glycopeptide mixture was then fractionated by ion-exchange chromatography on a column (30 mL) of DEAE-cellulose that was equilibrated with 2 mM pyridinium acetate buffer, pH 5.4, and developed with a gradient of buffer (2–400 mM). Glycopeptide species separate on the basis of sialic acid content. The pool containing monodesialylated glycopeptide **2** (sialic acid:hexose ratio about 1:3) was obtained in 37% yield (18.5 μmol).

Reaction of Monodesialylated Glycopeptide **2** *with 2-Iminothiolane.* Glycopeptide **2** (18.5 μmol) was dissolved in 0.2 M sodium borate buffer, pH 10.0 (0.5 mL), containing EDTA (5 mM), and if necessary, the pH was readjusted to 10.0 with 1 M NaOH. 2-Iminothiolane hydrochloride [0.05 mL of a freshly prepared 1.0 M solution in a 1:1 (v/v) mixture of 0.2 M sodium borate buffer, pH 10.0, and 2.0 M NaOH] was added and the solution incubated at 25 °C for 30 min. Four further additions of freshly prepared solutions of 2-iminothiolane were made at 30-min intervals. One hour after the final addition, excess reagent and its hydrolysis products were removed by gel filtration on a column (45 cm × 1.5 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated in 0.1 M pyridinium acetate buffer, pH 5.4. Fractions containing glycopeptide were pooled, dried by rotary evaporation, and then dissolved in 0.1 M triethanolamine hydrochloride buffer, pH 8.0 (2.0 mL), containing EDTA (10 mM). The pH was adjusted to 8.0 with 1 M NaOH, and solid dithioerythritol was added to give a 0.5 M solution. The mixture was incubated at 37 °C for 2 h and then submitted to gel filtration on a column (45 cm × 1.5 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated in 0.1 M pyridinium acetate buffer, pH 5.4, containing EDTA (5 mM). The overall recovery of glycopeptide (**3** plus any unreacted **2**) was 100% according to hexose assay and contained 0.76 sulfhydryl group per molecule (Ellman, 1959), indicating that the reaction of **2** with 2-iminothiolane was incomplete.

Formation of Disulfide-Linked Glycopeptide Dimer **4**. In order to purify **3** from any unreacted **2**, the glycopeptide **3** was allowed to form disulfide-linked dimers. First, the glycopeptide was exchanged into 0.1 M *N*-ethylmorpholine–acetic acid buffer, pH 7.5, by gel filtration through a column (45 cm × 1.5 cm) of Bio-Gel P-6 (200–400 mesh). Oxidation of **3** (18.5 μmol of oligosaccharide in 25 mL) was effected by shaking in air at 25 °C for 20 h, after which only 0.5 μmol of sulfhydryl groups was detectable (96% oxidized). In other experiments, more rapid oxidation (5 min) was achieved by adding 0.1 mM

$CuSO_4$ and 0.05 mM *o*-phenanthroline. The reaction mixture was concentrated by rotary evaporation, and **4** was purified by gel filtration through a column (51 cm × 1.5 cm) of Bio-Gel P-30 (100–200 mesh) equilibrated in 0.1 M sodium phosphate buffer, pH 7.0. The dimeric compound **4** (yield equivalent to 12.1 μmol of monomer) was resolved on the column from monomeric glycopeptide containing the unreacted **2**. Fractions containing **4** were pooled for the next step of synthesis.

Oxidation of Glycopeptide Dimer **4** *and Reductive Amination To Form Glycopeptide* **5**. **4** (equivalent to 10.7 μmol of monomer) in 0.1 M sodium phosphate buffer, pH 7.0 (15 mL), was placed in a glass vial that had been rinsed with water after being treated for 24 h with the pH 7.0 buffer containing bovine serum albumin (1 mg/mL). Bovine serum albumin (0.2 mg/mL) was added to the solution of **4**, which was then treated with horseradish peroxidase [10 μg/mL; see Tressel and Kosman (1980)], catalase (1 μg/mL), and galactose oxidase (11 units as defined by Sigma Chemical Co., St. Louis, MO) and incubated at 30 °C for 30 h, at which time 9.5 μmol of reducing groups was measured (Somogyi, 1952). The reaction mixture was purified by gel filtration on a column (51 cm × 1.5 cm) of Bio-Gel P-30 (100–200 mesh) equilibrated in 0.05 M pyridinium acetate buffer, pH 5.4. The hexose-containing fractions were pooled and dried by rotary evaporation. The dry oligosaccharide was dissolved in water (0.6 mL), and to this solution was added a methylamine–acetic acid solution, pH 6.5 (3.0 mL), that had been made by adjusting the pH of an ice-cold solution of 40% (w/w) aqueous methylamine to pH 6.5 by slowly adding glacial acetic acid. Immediately, a freshly prepared aqueous solution of 1.0 M $NaCNBH_3$ (0.24 mL) was added and the reaction solution was incubated at 40 °C for 3.5 h. The reaction mixture was then applied to a column (45 cm × 1.5 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated in 100 mM acetic acid containing pyridine (7 mM). Fractions that contained the glycopeptide **5** were located by the hexose assay, pooled, dried by rotary evaporation, and then lyophilized from water (yield, 8.3 μmol monomer equivalent).

Preparation of Desialylated Glycopeptide **6**. **5** (8.3 μmol of monomer) was dissolved in 50 mM sodium acetate buffer, pH 5.0 (1.65 mL), containing NaCl (150 mM) and $CaCl_2$ (10 mM). Neuraminidase (0.38 unit) was then added and the solution incubated at 37 °C for 24 h. A second addition of enzyme (0.38 unit) was made and incubation continued for a further 24 h to complete the release of sialic acid. The reaction mixture was then applied to a column (45 cm × 1.5 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated with 0.1 M pyridinium acetate buffer, pH 5.4, and fractions were assayed for hexoses and sialic acids. The fractions containing **6** (8.2 μmol of monomer) were pooled, dried by rotary evaporation, and then lyophilized from water. The liberated sialic acid, eluting later from the column, was quantified at 17.9 μmol, corresponding to about 2 mol of sialic acid released per mole of glycopeptide monomer.

Reduction and Alkylation of **6** *To Form Affinity Ligand* **7**. **6** (equivalent to 2.1 μmol of monomer) was dissolved in 160 mM triethanolamine hydrochloride buffer, pH 8.0 (2 mL). The solution was degassed and equilibrated with an atmosphere of N_2. A freshly prepared aqueous solution of 100 mM dithioerythritol (50 μL) was added and the reaction solution stirred at 25 °C under N_2 for 1 h. [^{14}C]Iodoacetamide (1 μCi/μmol; 15.63 μmol in 0.75 mL of water) was then added to the reaction solution, and stirring was continued at 25 °C under N_2 in the dark for 16 h. 2-Mercaptoethanol (10 μL) was added to react with the remaining radiolabeled iodoacetamide, and affinity ligand **7** was purified from the reaction mixture by gel filtration on a column (45 cm × 1.5 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated in 100 mM pyridinium acetate buffer, pH 5.4. Pooled oligosaccharide-containing fractions were assayed by the hexose assay (yield of **7**: 1.9 μmol, 90%) and for incorporated radioactivity. The specific radioactivity could be calculated as 1.7×10^6 cpm/μmol. This value is consistent with the expected value (1.9×10^6 cpm/μmol) calculated from the specific radioactivity of the [^{14}C]iodoacetamide sample (2.22×10^6 dpm/μmol), the counting efficiency (90.5%), and the sulfhydryl group content of **6** following reduction with dithioerythritol (0.94 sulfhydryl group/monomer). The pool of **7**[3] in 100 mM pyridinium acetate buffer, pH 5.4, was dried by rotary evaporation and then lyophilized from water.

Synthesis of 1-O-Methyl 6-Aldehydo-β-D-galactoside. 1-*O*-Methyl β-D-galactoside (0.5 g, 2.58 mmol) was oxidized by using galactose oxidase (450 units) and catalase (1 mg) as described by Lee and Lee (1986). A portion of the resulting monosaccharide mixture (equivalent to 0.35 μmol of starting material) was dissolved in 95% ethanol (0.4 mL) for purification by gel filtration on a column (165 cm × 1.5 cm) of Sephadex LH-20 in 95% ethanol as described previously (Lee et al., 1982). Two hexose-containing peaks were identified, eluting at 80% and 88% of the column volume. The first peak (26.7 mg, 0.139 mmol, 40% yield) contained most of the reducing equivalents, and by TLC (solvent A) it was identified as the faster moving spot in the original reaction mixture (R_f 0.75), while the second peak was predominantly the starting material (R_f 0.68). In the hexose assay using lactose as a standard, the purified 1-*O*-methyl 6-aldehydo-β-D-galactoside gave a color yield at 490 nm of only 0.39 hexose unit, whereas values for 1-*O*-methyl β-D-galactoside were 1.0.

Synthesis of 1-O-methyl 6-(N-Methylamino)-6-deoxy-β-D-galactoside. A portion of the above galactose oxidase reaction mixture (1.20 mmol of reducing equivalent) in 10 mM sodium phosphate buffer, pH 6.5 (19 mL), was dried and then dissolved in water (10 mL). A solution of methylamine–acetic acid, pH 6.5 (3.08 mL; prepared as described above), was mixed with $NaCNBH_3$ (126 mg, 2 mmol) and then added to the solution of galactoside. The reaction mixture was incubated at 40 °C, and after 90 min, a further 126 mg (2 mmol) of $NaCNBH_3$ was added and incubation continued for a further 4 h. TLC (solvent A) showed the disappearance of the spot (R_f 0.75) that stained with dinitrophenylhdyrazine (see previous section) and the appearance of a new spot (R_f 0.1) visualized with sulfuric acid. The reaction mixture was treated with Dowex-1 (350-mL bed volume; 200–400 mesh, OH^- form), and the resulting alkaline solution was carefully

[3] The structure of the oligosaccharide moiety of **7**, illustrated diagrammatically in Scheme I, was supported by oligosaccharide analysis of samples performed by Oxford Glycosystems (Abingdon, Oxon, England). The monosaccharide composition of **7** determined by GLC/MS analysis was consistent with the structure of **7** represented in Scheme I. 1-*O*-Methyl 6-(*N*-methylamino)-6-deoxy-β-D-galactoside prepared by us (see Methods) was used by Oxford Glycosystems as a standard for this moiety. Oxford Glycosystems also performed "size-profile" analysis using Bio-Gel P-4 gel filtration in combination with specific exoglycosidase digestions of the oligosaccharide released from **7** by hydrazinolysis/re-N-acetylation and radiolabeled with alkaline [^{3}H]$NaBH_4$. This analytical technique, described by Parekh et al. (1989), confirmed a triantennary structure with a terminal galactose residue on each of two branches (releasable by β-galactosidase) and an altered structure at the terminal of a third branch (antenna) that was not releasable by β-galactosidase treatment. We infer that the altered structure that "caps" one of the antennae of the oligosaccharide is the N-acetylated derivative of the 6-(methylamino)galactose seen in the analysis of monosaccharide composition.

evaporated to dryness to remove the excess methylamine. The amino sugar was then purified from the residue by cation-exchange chromatography on a column (1 L) of (carboxymethyl)cellulose (Whatman, CM-52) equilibrated in 2 mM pyridinium acetate buffer, pH 5.4, and developed with a gradient (2–170 mM) of pyridinium acetate buffer, pH 5.4 (total volume, 8 L), followed by further purification by gel filtration on a column (165 cm × 1.5 cm) of Sephadex LH-20 equilibrated in 95% ethanol. One symmetrical hexose-containing peak which eluted at 60% of the column volume showed one spot (R_f 0.29) on TLC in solvent B (265 mg, 0.993 mmol, 47% yield, as the acetate salt). The purified 1-*O*-methyl 6-(*N*-methylamino)-6-deoxy-β-D-galactoside gave a color yield at 490 nm of only 0.22 hexose unit in the hexose assay (relative to a lactose standard). The purified amino sugar gave no reaction with 2,4,6-trinitrobenzenesulfonic acid (Habeeb, 1959), as expected for a secondary amine, and only a low value for color yield (ϵ_{560nm} = 50 M^{-1} cm^{-1}) by reaction with ninhydrin.

^1H NMR Spectroscopy (300 MHz, D_2O). The spectrum of 1-*O*-methyl-β-D-galactoside shows signals at chemical shifts in accordance with published values (Lemieux & Bock, 1983), with complete resolution of the signals only for H-1 (δ 4.18; d, 8 Hz), H-4 (δ 3.78; d, 3 Hz), and OCH_3 (δ 3.43; s). The signals for the other five carbon-bound protons are located in a multiplet between δ 3.45 and δ 3.70. The spectrum of 1-*O*-methyl 6-aldehydo-β-D-galactoside contains resolved signals at δ 5.00 (1 H, d, 7 Hz), δ 4.18 (1 H, d, 8 Hz), δ 3.94 (1 H, d, 3 Hz), and δ 3.44 (3 H, s). Peaks for three more protons are part of a multiplet between δ 3.25 and δ 3.55. No signal for an aldehydo proton is present. This spectrum is consistent with 1-*O*-methyl 6-aldehydo-β-D-galactoside in the fully hydrated form, with H-6 having a chemical shift of δ 5.00. 1-*O*-Methyl 6-(*N*-methylamino)-6-deoxy-β-D-galactoside was analyzed as the corresponding acetate salt. The spectrum contains signals at δ 4.18 (1 H, d, 8 Hz), δ 3.75–δ 3.8 (2 H, m), δ 3.49 (1 H, d, 10 Hz), δ 3.40 (3 H, s) δ 3.0–δ 3.37 (5 H, m), δ 2.55 (3 H, m), and δ 1.74 (3 H, s).

Amino Acid and Amino Sugar Analysis. Samples of glycopeptides (5–10 nmol) were hydrolyzed under vacuum in 6 M HCl at 100 °C for 24 h for analysis of amino acids. Acid hydrolysis of glycosidic bonds in samples of glycopeptides was done in 2 M trifluoroacetic acid in evacuated sealed tubes for 2–6 h (Hardy et al., 1988), or by hydrolysis in 3 M HCl under vacuum for 6 h (Fanger & Smyth, 1970), incubating all sample tubes in a heating block at 100 °C. Analysis was performed on a Beckman Model 6300 amino acid analyzer by M. Ehrhardt, Medical Research Building, Brigham and Women's Hospital, Boston. The elution position of 6-(*N*-methylamino)-6-deoxy-β-D-galactoside is distinct from those of glucosamine and galactosamine, although the sensitivity of detection is about 50-fold lower. Fractions (100 μL; 0.2 min) were collected from some runs for quantifying radioactivity.

Activation of Affinity Ligand **7**. Affinity ligand **7** (1 μmol) was dissolved in 0.5 M triethanolamine hydrochloride buffer, pH 8.0 (0.35 mL), and then dioxane (0.7 mL) was added to the solution. Freshly prepared 1 M cyanuric chloride in dry dioxane (40 μL) was added to the solution of **7** while continuously vortexing for 1 min, after which glacial acetic acid (0.2 mL) was added and vortexing continued for a further 10 s. The mixture was applied to a column (45 cm × 1 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated with 20 mM NaH_2PO_4·HCl buffer, pH 3.0. Fractions containing the activated ligand **8** (see Scheme II) were located by hexose assay and then pooled. Spectrophotometric analysis showed **8** to have an absorption peak at 242 nm with ϵ_{242} of 15 400 M^{-1} cm^{-1}, assuming a complete reaction of **7** with cyanuric chloride. This value is similar to that of a model compound, 2,4-dichloro-6-(diethylamino)triazine, synthesized in our laboratory, having ϵ_{242} of 19 680 M^{-1} cm^{-1}. The solution of **8** was used without further purification.

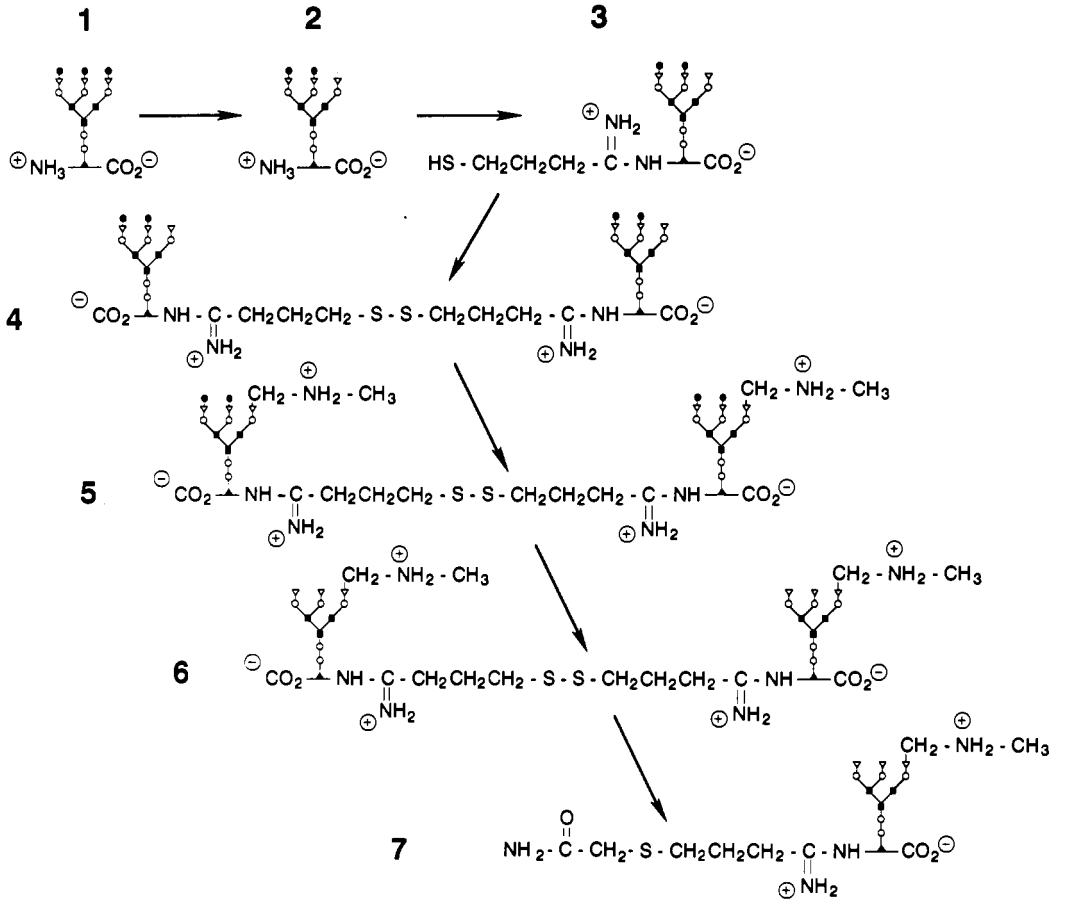
Preparation of Blocked Ricin. Ricin (2 mg/mL) in 50 mM triethanolamine hydrochloride buffer, pH 8.0, was mixed with a volume of **8** (0.1–0.2 mM) in 20 mM NaH_2PO_4·HCl buffer, pH 3.0. In different experiments, the molar ratio of ricin:**8** was varied from about 1:1 to 1:9. The pH of the solution was then adjusted to 8.0 by addition of 1.0 M triethanolamine while stirring gently. The solution was incubated for 24 h (48 h in some experiments) at 25 °C, and then 0.1 volume of 1.0 M ethanolamine hydrochloride, pH 8.0, was added and incubation continued for a further 24 h to block any remaining reactive groups. Reaction mixtures were then acidified with 1% (v/v) acetic acid and submitted to gel filtration on a column (column volume ≥ 10 × sample volume) of Bio-Gel P-60 (100–200 mesh) equilibrated in 0.1 M acetic acid, containing NaCl (0.145 M) and lactose (0.25 M) at 25 °C. Fractions containing protein were combined and dialyzed exhaustively against 10 mM potassium phosphate buffer, pH 6.8, containing NaCl (145 mM). The dialyzed protein was then passed through a column (1 mL of column volume/mg of ricin) of immobilized lactose (binding capacity, 4 mg of ricin/mL of gel) equilibrated in the above pH 6.8 buffer at 4 °C. Fractions which contained protein that had passed through the column without retention were combined, then concentrated to 3–5 mg/mL of protein by using an immersible ultrafiltration membrane (Millipore, CX-10), and finally applied to a column (1 mL of column volume/mL of protein solution) of immobilized asialofetuin (asialofetuin–TSK) equilibrated in the above phosphate buffer at 4 °C. The protein that did not bind to asialofetuin was collected and considered to be blocked ricin (see Results for further details). Yields of purified blocked ricin were 20–25% from reaction mixtures containing 4 mol of **8**/mol of ricin incubated at room tempertaure for 48 h.

Assay for Protein Synthesis in Cell-Free System. This was performed as described previously (Lambert et al., 1985; Lambert & Blättler, 1988).

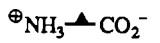
Cytotoxicity Testing. The cytotoxicity of samples of ricin and blocked ricin for cultured cells (Namalwa, ATCC CRL 1432) was determined according to assays for cytotoxicity previously described by Goldmacher et al. (1985, 1986b).

Assay of the Ability of Ligands To Inhibit Binding of Ricin to Asialofetuin. Ricin (0.5 mg) was radiolabeled with $Na^{125}I$ (1 mCi) using Iodogen as described by Goldmacher et al. (1986a) for antibodies, except that the two gel filtration steps used Bio-Gel P-6 (200–400 mesh) and the ricin was incubated in 10 mM NaI at room temperature for 30 min prior to the second gel filtration step. Precipitation of a sample of the radiolabeled ricin with trichloroacetic acid showed that ≥97% of the ^{125}I was covalently bound to the protein. Solutions of different ligands at various concentrations were prepared in 50 mM triethanolamine hydrochloride buffer, pH 7.25, containing NaCl (100 mM) and bovine serum albumin (2 mg/mL), and were mixed with asialofetuin–TSK beads (2 μL of packed beads for 0.45 mL of ligand solution) and then left in 1-mL polypropylene tubes at 4 °C. After 24 h, radioiodinated ricin (22 000 cpm; 11 ng in 50 μL of the pH 7.25 buffer containing albumin) was added to each of the tubes, which were then rotated end over end (6 rpm) at 4 °C for 24 h. The beads were then quickly separated by centrifugation (Eppendorf for 20 s), and the radioactivities contained in 400 μL

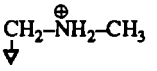
Scheme I: Steps in the Preparation of Affinity Ligand **7** from Triantennary N-Linked Glycopeptide **1** Containing Triantennary N-Linked Oligosaccharides[a]



[a] Symbols: ● indicates sialic acid; ▽ indicates galactose; ○ indicates *N*-acetylglucosamine; ■ indicates mannose;



indicates asparagine or short peptide;



indicates 6-(*N*-methylamino)-6-deoxy-β-D-galactose. For simplicity, only one branch of the three different branches is shown desialylated in structures **2**–**7**. It is likely that all three isomeric structures exist in the preparation of affinity ligand **7** (see text for further details).

of each clear supernate, and in each 100 μL of remaining supernate containing the pelleted beads, were determined. From these measurements, the radioactivity associated with the beads could be calculated.

Polyacrylamide Gel Electrophoresis. Samples of protein (0.5–5 μg) were analyzed by polyacrylamide/sodium dodecyl sulfate gel electrophoresis in gel slabs (145 × 90 × 0.75 mm) of 11% (w/v) acrylamide prepared according to Laemmli (1970). Sample buffers contained 2-mercaptoethanol (2% v/v) for reducing conditions or iodoacetamide (10 mg/mL) for nonreducing conditions. Radioautography of dried gels was performed as described previously by using Kodak X-OMAT-AR film (Kenny et al., 1979).

Results

Exhaustive proteolytic digestion of fetuin generated highly acidic glycopeptides containing triantennary N-linked oligosaccharides that were fractionated by anion-exchange chromatography under acidic conditions into two major species originally labeled FI and FII by Baenziger and Fiete (1979a). The composition of FI and FII with respect to hexose units, sialic acids, amino groups, amino acids, and amino sugars was consistent with the many previous results of structural analysis of glycopeptides containing triantennary N-linked oligosaccharides from fetuin (Baenziger & Fiete, 1979a; Nilsson et al., 1979; Takasaki & Kobata, 1986; Townsend et al., 1986; Yet et al., 1988). For the experiments with ricin described here, either of the two major fractions of glycopeptide can be used with very similar results. They are, therefore, not further distinguished in the following description of the results and are referred to as glycopeptide **1**.

The preparation of the modified glycopeptide **7** by chemical and enzymic modification of glycopeptide **1** from fetuin is illustrated in Scheme I. The key step in the sequence of reactions is the modification of one of the three galactose residues, which is achieved by purifying monodesialylated triantennary glycopeptide **2** by anion-exchange chromatography from a partial hydrolysis reaction catalyzed by neuraminidase and then specifically oxidizing the single terminal galactose residue with galactose oxidase. The remaining sialic acids serve to protect the other galactose residues from the action of galactose oxidase. It is expected that glycopeptide **2** will be a mixture of isomers since the neuraminidase (*C. perfringens*) used for partial desialylation can remove all sialic acid from **1** with longer incubation times (see Methods). Furthermore, it has been shown that the enzyme exhibits only

about a 2-fold difference in the relative rates of cleavage of the α-(2–3) and α-(2–6) glycosidic linkages (Schauer, 1982), the linkages found in the oligosaccharides from fetuin (Baenziger & Fiete, 1979a).

The α-amino group of glycopeptide **2** was first blocked by reaction with 2-iminothiolane before generating the aldehydo group on the exposed terminal galactose residue. The choice of 2-iminothiolane as the blocking group provided the means to purify the modified glycopeptide **3** from any unreacted **2** by forming a disulfide-linked dimer **4** which could be separated from monomeric glycopeptide by gel filtration over Bio-Gel P-30. The thiol introduced into the glycopeptide also provided a convenient site for attaching a radiolabel in the last step of the preparation of **7** using [^{14}C]iodoacetamide.

Treatment of **4** with galactose oxidase generated a single aldehydo group on one branch (antenna) of the triantennary glycopeptide unit, which was then reductively aminated with methylamine and $NaCNBH_3$. A reaction done by using the conditions described under Methods with 0.1 μmol (monomer equivalent) of oxidized **4** and [^{14}C]methylamine (specific radioactivity, 14500 cpm/μmol) showed the incorporation of 1363 cpm of radiolabel into the purified glycopeptide, which corresponds to an incorporation of 0.09 μmol of methylamine (0.9 group per molecule of monomer). In a control experiment under the same reaction conditions, no label was incorporated into **4** which had not been treated with galactose oxidase. This result indicated that there was a virtual quantitative conversion of the terminal galactoside residues of **4** to 6-(*N*-methylamino)-6-deoxy-β-D-galactoside moieties in **5**. **5** was also shown to contain no reducing groups.

In order to further characterize product **5** of the sequential oxidation and reductive amination reactions, 1-*O*-methyl β-D-galactoside was subjected to an analogous reaction sequence generating 1-*O*-methyl 6-aldehydo-β-D-galactoside after oxidation and 1-*O*-methyl 6-(*N*-methylamino)-6-deoxy-β-D-galactoside after both reactions. These compounds were purified and their identities confirmed by chemical analysis and NMR spectroscopy. 1-*O*-Methyl 6-(*N*-methylamino)-6-deoxy-β-D-galactoside then served as a standard for further analysis of **5** that was prepared from oxidized **4** and [^{14}C]-methylamine.

Samples of labeled **5** and the amino sugar standard were hydrolyzed, and the reaction products were analyzed by TLC in solvent B. The radiolabel migrated as a single spot that comigrated with the standard sample (result not shown). Hydrolyzed samples (6 h in 2 M trifluoroacetic acid at 100 °C in vacuo) of labeled **5** together with the standard were then further analyzed by amino acid analysis. In an analysis of a hydrolysate of a mixture of radiolabeled **5** (5.7 nmol) and a sufficient amount of 1-*O*-methyl 6-(*N*-methylamino)-6-deoxy-β-D-galactoside (57.0 nmol) to show a color peak for the 6-(*N*-methylamino)-6-deoxy-D-galactose, radiolabel was only found in fractions eluting at the position of 6-(*N*-methylamino)-6-deoxy-D-galactose, although the recovery of radioactivity was only about 50%. Further experiments showed that 1-*O*-methyl 6-(*N*-methylamino)-6-deoxy-β-D-galactoside was not stable during acid hydrolysis. Indeed, 6-(*N*-methylamino)-6-deoxy-β-D-galactose was completely destroyed by using conditions under which glucosamine showed recoveries of about 90%.

A competition binding assay was performed in order to judge whether the binding of **7** to ricin is altered compared with that of desialylated **1**. Figure 1 shows that **7** and asialo-**1** are indistinguishable in their ability to inhibit the binding of ricin to asialofetuin that is immobilized on TSK beads.

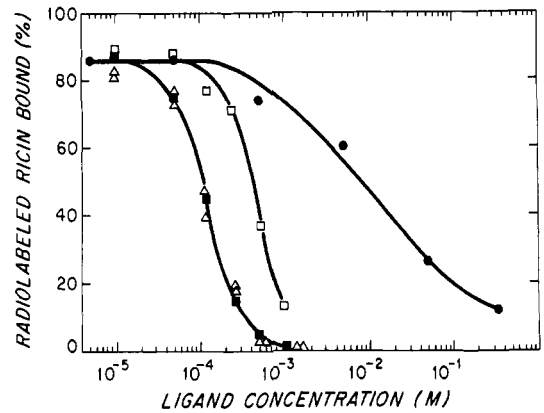


FIGURE 1: Binding of ricin to immobilized asialofetuin in the presence of glycopeptides or lactose. Two microliters of asialofetuin-TSK beads (measured with a positive displacement micropipet from a packed slurry) was added to 0.5 mL of 50 mM triethanolamine hydrochloride buffer, pH 7.25, containing NaCl (100 mM), bovine serum albumin (2 mg/mL), radioiodinated ricin (3.4×10^{-10} M containing 18 700 cpm of I^{125}-labeled ricin), and various concentrations of the following ligands: (●) lactose; (□) glycopeptide **1**; (■) glycopeptide **1** that was completely desialylated with neuraminidase; (△) glycopeptide **7**.

Glycopeptide **1** (fully sialylated) requires a 4-fold higher concentration to effect a similar degree of inhibition of ricin binding to the same beads. This finding is similar to results obtained by Baenziger and Fiete (1979b), who reported that the association constant between ricin and asialo triantennary N-linked oligosaccharides is 3- to 4-fold greater than that for the fully sialylated glycopeptides.

Gel filtration of a mixture of radiolabeled **7** (1.65 nmol) with a molar excess of ricin (0.95 mg, 15 nmol, in 0.5 mL) on a column of Bio-Gel P-60 (19 cm × 0.8 cm) at pH 7.2 showed that all the ligand **7** comigrated with ricin at 4 °C. Control experiments showed no association between bovine serum albumin (which has the same elution volume as ricin) and **7**. We conclude that if the preparation of **7** is composed of a mixture of isomers (see the footnote to Scheme I), then all can bind to the galactose-binding sites of ricin with an affinity sufficiently high so that dissociation at 4 °C is negligible during gel filtration under these conditions.

The amino group in **7** was reacted with the cross-linking agent cyanuric chloride to give the dichlorotriazine derivative **8** (Scheme II). The activated glycopeptide ligand **8** was then mixed with ricin to test whether it would bind to ricin and covalently react with a nucleophilic group (probably an amino group) of the protein as illustrated in Scheme II. For these experiments, labeled **7** was prepared by using [^{14}C]iodoacetamide and the reaction of labeled **8** with ricin was monitored by measuring the incorporation of radioactivity into protein.

In the first experiments, the influence of pH on the rate and extent of this reaction was examined by using an excess of ricin over labeled **8**. The results of one such experiment are shown in Figure 2a. The initial reaction rate increases with increasing pH over the pH range 6–9, while no reaction is detectable at pH 3. However, at pH 9, there was no further incorporation of label after about 2 h, at which time about 30% of the radiolabeled **8** had reacted with ricin. It is likely that competing base-catalyzed hydrolysis to form **9** (Scheme II) limits the extent of reaction of **8** with ricin. The single chlorine substituent of **9** will be much less reactive than a chlorine substituent of **8** (Kay & Crook, 1967; Smith & Lenhoff, 1974). Further experiments were performed at pH 8.0, and a time course of the extent of the reaction of ricin at room temperature with different concentrations of **8** is shown in Figure 2b. At concentrations of **8** of up to a 4.5-fold molar

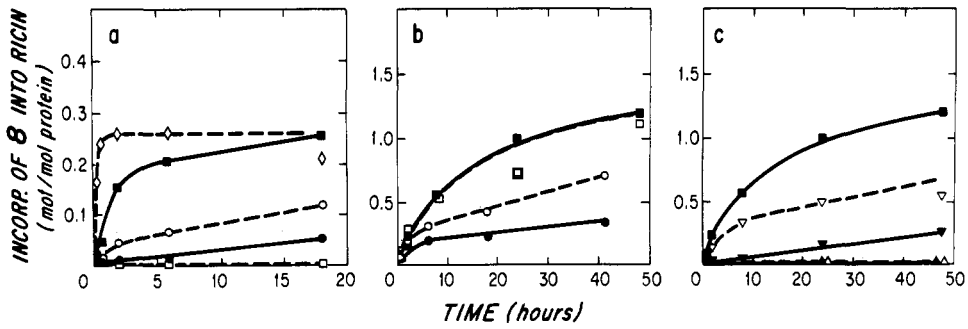
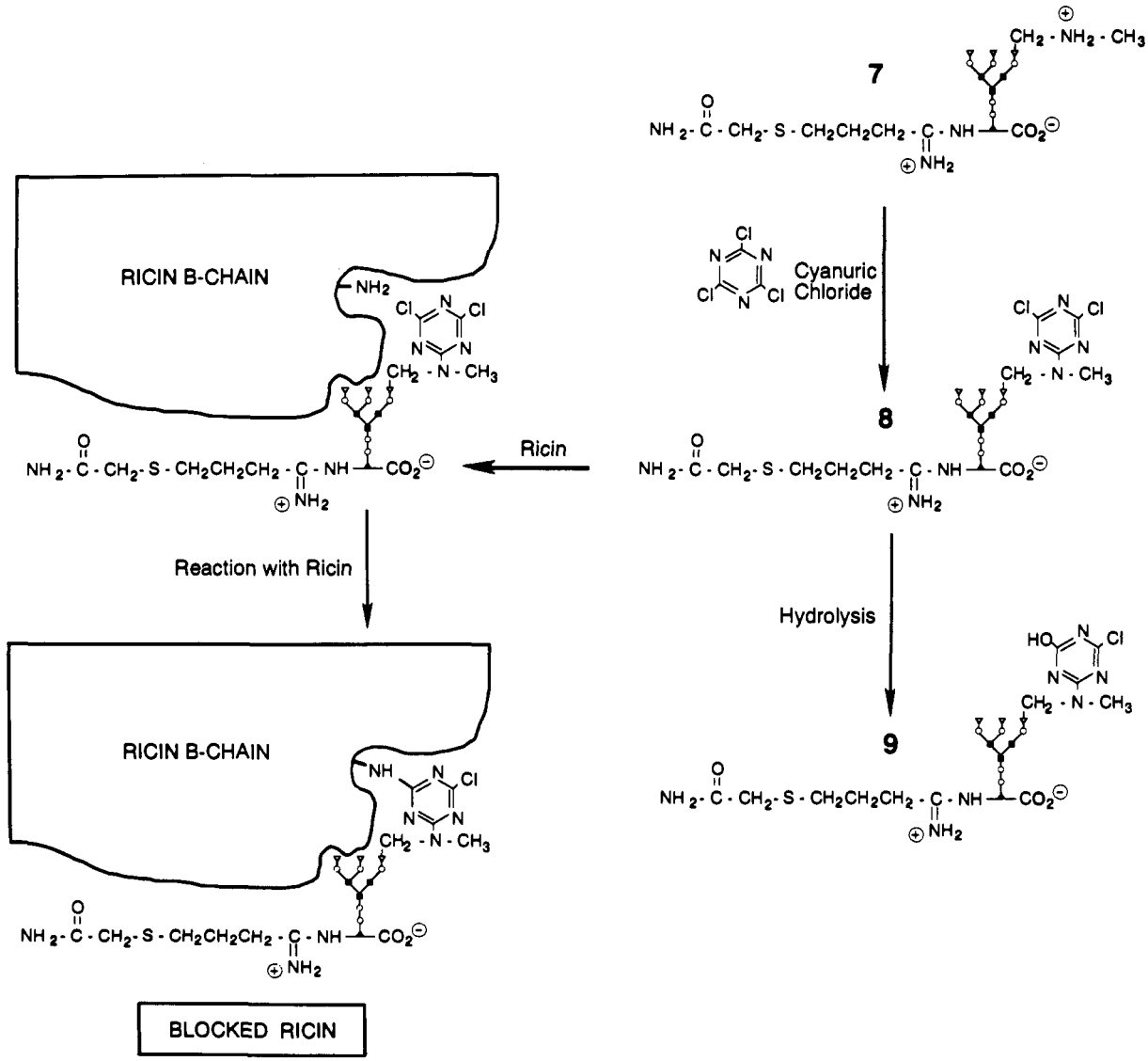


FIGURE 2: Time course of incorporation of ^{14}C-labeled **8** into ricin. Panel a: Reaction of ricin (0.57 mg/mL) with radiolabeled **8** (7.5 μM) at 25 °C in triethanolamine/phosphate buffers of various pH values that were made by titrating 50 mM NaH_2PO_4·HCl buffer, pH 3.0, to the desired pH with 1.0 M triethanolamine. Samples (0.2 mL) were taken at various time points, and the protein was precipitated with 10% (w/v) trichloroacetic acid for measurement of the radioactivity incorporated into protein as described under Methods. The specific radioactivity of **8** was 1.7×10^6 cpm/μmol). The pH values during the incubation of the various samples were pH 3.0 (---□---), pH 6.0 (—●—), pH 7.0 (---○---), pH 8.0 (—■—), and pH 9.0 (---◊---). Panel b: Reaction of ricin (0.19 mg/mL) with radiolabeled **8** (1.7×10^6 cpm/μmol) at pH 8.0 and 25 °C using ratios of 0.9 (●), 1.9 (○), 4.5 (■), and 9.0 (□) mol of **8**/mol or ricin. Other reaction conditions were as described under Methods. Samples (85 μL) were taken at various time points for measuring radioactivity incorporated into protein as described in panel a. Panel c: Reaction of ricin (0.19 mg/mL) with a 4.5-fold molar excess of **8** (13 μM) in the presence of 1 mM asialo N-linked oligosaccharide **1** (▼), 100 mM lactose (▽), or with no addition (■; identical with panel a). Other reaction conditions were as in panel b. In two other experiments, ricin was replaced by bovine serum albumin (▲) or by ovalbumin (△), each at 0.19 mg/mL.

Scheme II: Reaction of Glycopeptide Ligand **7** with Cyanuric Chloride, and Subsequent Reaction of Activated Glycopeptide **8** with Ricin To Form Blocked Ricin[a]



[a] The symbols used here to represent the N-linked oligosaccharide are the same as those used in Scheme I.

excess over the concentration of ricin, about 1.2 molecules of the ligand were incorporated per molecule of ricin after 40 h. Higher concentrations of ligand **8** (9-fold molar excess) neither increased the rate of its reaction with ricin nor the extent of

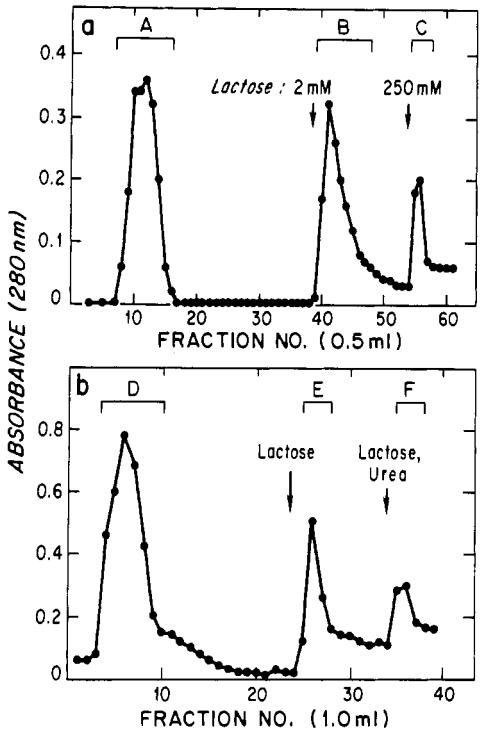


FIGURE 3: Purification of blocked ricin using affinity columns following reaction of ricin with **8**. Ricin was incubated at 25 °C with a 4-fold molar excess of **8** for 48 h and then purified from noncovalently bound **8** by gel filtration through Bio-Gel P-60 as described under Methods. Panel a: Modified ricin (2 mg in 3 mL) was applied to a column (2 mL) of lactose–polyacrylamide beads equilibrated with 10 mM potassium phosphate buffer, pH 6.8, containing NaCl (145 mM) at 4 °C. The flow rate was 1 mL/h. For other details, see Methods. Lactose at 2 or 250 mM was included in the elution buffer, where indicated. The yield of fraction A was 43% of the protein loaded onto this column. Panel b: Partially purified blocked ricin (4.0 mg in 4 mL) after passage through a column of lactose–polyacrylamide (equivalent to fraction A, panel a) was applied to a column (5 mL) of asialofetuin–TSK beads equilibrated in 10 mM potassium phosphate buffer, pH 6.8, containing NaCl (145 mM) at 4 °C. The flow rate was 2.5 mL/h. Where indicated, lactose (250 mM), or lactose (250 mM) and urea (7.0 M), was included in the elution buffer. The yield of fraction D was 53% of the material loaded onto this column. For other details, see Methods.

incorporation of ligand into ricin. There was no reaction of labeled **8** with either bovine serum albumin or ovalbumin at pH 8.0, proteins which would not be expected to bind **8** (Figure 2c). Also, asialo-**1** (1 mM) strongly inhibited the reaction of **8** (13 μM) with ricin, while lactose (100 mM) partially inhibited this reaction (Figure 2c). These results suggest that **8** was bound specifically to ricin at carbohydrate-binding sites prior to any covalent reaction of **8** with ricin and that the affinity of this interaction is greater than that of a simple disaccharide such as lactose. The results shown in Figure 2 also suggest that the reaction of **8** with ricin did not plateau at one ligand **8** per ricin molecule.

If **8** had, indeed, reacted with the carbohydrate-binding sites of ricin, thereby blocking such sites, we reasoned that we could purify the modified ricin from native ricin by affinity chromatography. Affinity chromatography works only at its best in the absence of competing ligand. It was important, therefore, to remove any noncovalently bound **8** or its hydrolysis byproduct **9** (Scheme II) from the reaction mixture prior to chromatography. Complete separation of noncovalently bound glycopeptide from ricin was achieved by gel filtration at 25 °C in acetate buffer, pH 3.0, in the presence of lactose. Lactose was removed from the protein by dialysis, and the protein was then applied to a column of lactose–polyacrylamide beads sufficient to bind 100% of the protein loaded if it were native ricin. Typically, three fractions were generated, as shown in Figure 3a. Fraction A was not retained by the column, while fractions B and C could be eluted by including 2 and 250 mM lactose, respectively, in the elution buffer. The yield of fraction A was 43% of the protein loaded onto this column (from a reaction mixture containing 4 mol of **8**/mol of ricin).

Table I: Purification of Blocked Ricin following Reaction of Ricin with ^{14}C-Labeled **8**[a]

step	total protein (mg)	total radiolabel bound (cpm)[b]	mol of affinity ligand/mol of ricin[d]
reaction mixture	3.79	(243 012)[c]	(2.4)[c]
after gel filtration, P-60 column	3.20	90 850	1.0
after lactose–polyacrylamide column[e]	1.63	66 700	1.5
after asialofetuin–TSK column[f]	0.63	34 856	2.1

[a] For details of modification of ricin with **8** (2.4-fold molar excess) and purification of blocked ricin, see Experimental Procedures. [b] Specific radioactivity of **8** was 1.7×10^6 cpm/μmol. [c] Numbers in parentheses include both covalently bound and noncovalently bound **8** (total **8** in reaction mixture). [d] M_r of ricin is taken as 62 500 (Olsnes & Pihl, 1982). [e] Equivalent to fraction A (see Figure 3a). [f] Equivalent to fraction D (see Figure 3b).

Fraction A was further separated by chromatography through a column of immobilized asialofetuin into three subfractions as shown in Figure 3b. Fraction D was not retained by the column, while fraction E could be eluted by including lactose in the elution buffer, and fraction F was obtained by including lactose and 7.0 M urea in the elution buffer. The latter conditions are those required for completely eluting native ricin from an asialofetuin–TSK column (result not shown). Fraction D (53% yield over the step) was considered to be blocked ricin owing to its inability to bind to the oligosaccharide moieties of asialofetuin. The overall yield of blocked ricin was 23% from reaction mixtures containing **8** in a 4-fold (mol/mol) excess over ricin.

The content of ligand **8** in blocked ricin was estimated by measuring the incorporation of radiolabeled **8** (Table I). A 2.4-fold excess of radiolabeled **8** over ricin was used in the experiment shown in Table I. The final preparation of purified blocked ricin contained 2.1 molecules of **8** per molecule of ricin.

Polyacrylamide/sodium dodecyl sulfate gel electrophoresis was used to analyze the purified blocked ricin (Figure 4) and the other fractions obtained during its purification (refer to Figure 3). Analysis under nonreducing conditions shows that blocked ricin (fraction D) migrates as a doublet of apparent M_r 66 000–69 000 (Figure 4a, lane 4), while ricin itself migrates as a doublet of apparent M_r 60 000–61 500 (lane 5) relative to the markers in this gel. Fractions B and E show bands that migrate at an intermediate apparent M_r (Figure 4a, lanes 3 and 1, respectively), while fraction C appears to be similar to ricin (Figure 4a, lane 2). Under reducing conditions (Figure 4b), native ricin shows three bands (lane 5) corresponding to the monoglycosylated A-chain with apparent M_r 30 000, the diglycosylated A′-chain with apparent M_r 32 000 [10–20% of the total amount of A-chain: see Foxwell et al. (1985) and Fulton et al. (1986) for a description of the differently glycosylated forms of ricin A-chain], and the B-chain with apparent M_r 32 500. Analysis of blocked ricin (fraction D, Figure 3) shows that the band corresponding to native B-chain is completely absent (lane 4), while a predominant new band of apparent M_r 37 500 with a weak band of apparent M_r 40 000 can be seen in addition to the band

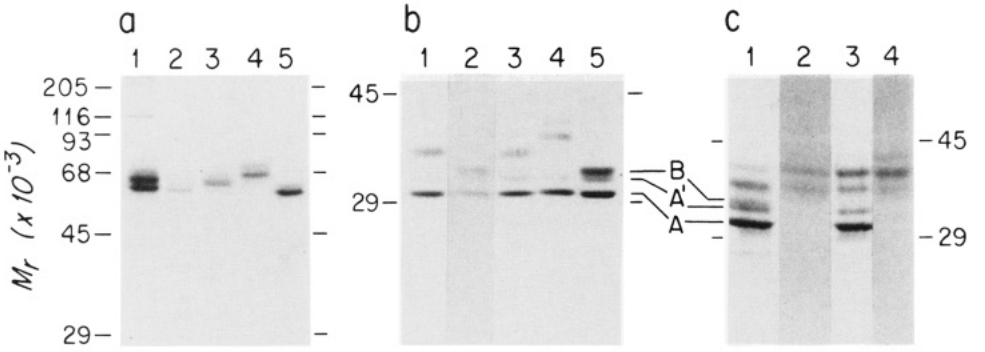


FIGURE 4: Polyacrylamide/sodium dodecyl sulfate gel analysis of ricin and blocked ricin. Samples of ricin, samples of purified blocked ricin, and samples taken during purification of blocked ricin (see Figure 3) were analyzed on 11% (w/v) polyacrylamide gels. Panel a: Electrophoresis under nonreducing conditions. Lane 1, fraction E (1.5 μg; see Figure 3); lane 2, fraction C (0.2 μg; see Figure 3); lane 3, fraction B (0.5 μg; see Figure 3); lane 4, blocked ricin (0.5 μg of fraction D; see Figure 3); lane 5, ricin (0.5 μg). Panel b: Electrophoresis under reducing conditions with 1–1.5 μg of protein. The samples were the same as in lanes 1–5 of panel a. Panel c: Electrophoresis under reducing conditions of the reaction mixtures after reaction between ricin and radiolabeled **8** at pH 8.0 under conditions described under Methods (also see Figure 2). Lanes 1 and 3 show Coomassie blue stained gels of the reaction mixtures which contained 2.3 and 4.5 mol of **8**/mol of ricin, respectively. Lanes 2 and 4 show radioautographs (30 days exposure) of the dried gels shown in lanes 1 and 3, respectively. The gels were calibrated with the marker proteins (M_r) carbonic anhydrase (29 000), ovalbumin (45 000), bovine serum albumin (68 000), phosphorylase *b* (93 000), β-galactosidase (116 000), and myosin (205 000). The bands corresponding to the A-chain, A′-chain, and B-chain of ricin (Olsnes & Pihl, 1982) are indicated in panels b and c.

corresponding to native A-chain and the weak band corresponding to A′-chain. We conclude that it is likely that the new bands of apparent M_r 37 500 and 40 000 are the result of covalent modification of B-chain (apparent M_r 32 500) with two or three molecules of **8** [calculated M_r 2422, from the data of Baenziger and Fiete (1979a)], respectively. Fractions B and E (Figure 3) also lack a band corresponding to native B-chain (lanes 3 and 1, respectively), while they contain a predominant new band of apparent M_r 35 000, which likely results from the covalent modification of B-chain with one molecule of **8**. Fraction C (Figure 3) shows bands corresponding to native A-chain and B-chain (lane 2), indicating that this fraction is mainly nonmodified ricin (A′-chain cannot be seen at the low loading used for this lane).

Figure 4c shows an analysis by polyacrylamide/sodium dodecyl sulfate gels under reducing conditions of unfractionated mixtures of the reaction between ricin and a 2.3-fold excess of radiolabeled ligand **8** (lanes 1 and 2) and a 4.5-fold excess of **8** (lanes 3 and 4). Lanes 1 and 3 are pictures of the gel stained with Coomassie blue, and lanes 2 and 4 are pictures of the respective radioautographs. This comparative analysis shows that radiolabeled **8** was only incorporated into the new bands, of apparent M_r 35 000, 37 500 (lanes 2 and 4), and 40 000 (lane 4), while no radiolabel was detected in the bands corresponding to A-chain, A′-chain, or nonmodified B-chain. [A small amount of nonmodified B-chain remains in the ricin treated with a 2.3-fold excess of **8** (lane 1), while it is undetectable in the ricin treated with a 4.5-fold excess of **8** (lane 3).] The predominant new band in the ricin treated with the lower concentration of **8** migrates at apparent M_r 35 000 (lane 1). In the ricin treated with a 4.5-fold excess of **8**, the predominant new band is that of apparent M_r 37 500 (lane 3), while a weak band of apparent M_r 40 000 is only readily seen in the radioautograph of the gel (lane 4).

The evidence cited above suggests that blocked ricin can result from the covalent reaction of ricin B-chain with at least two molecules of **8**, which together contain four terminal galactose residues. A small proportion of blocked ricin also appears to contain three molecules of **8** covalently linked to the B-chain. When a sample of blocked ricin was applied to a column of immobilized ricin (on agarose beads) in phosphate-buffered saline, ≥92% of the blocked ricin passed through the column and showed the same gel pattern as the

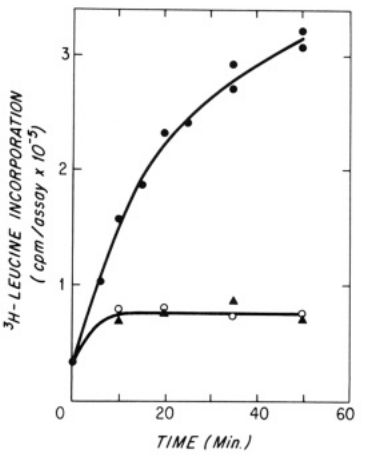


FIGURE 5: Time course of the inhibition of protein synthesis in the presence of reduced ricin or reduced blocked ricin. Protein synthesis was measured in a cell-free system derived from rabbit reticulocytes by the incorporation of [^{3}H]leucine into protein precipitable by trichloroacetic acid as described by Lambert et al. (1985). Samples of ricin or blocked ricin were diluted to 0.2 μg/mL 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 37 °C for 2 h to effect reduction. The solutions were further diluted to 0.02 μg/mL of A-chain, and samples (1 μL) containing 20 ng of A-chain were added to assay mixtures (total volume 27 μL) as described previously (Lambert et al., 1985; Lambert & Blättler 1988). The symbols represent the following experiments: (●) control assay; assays containing 20 pg of A-chain (○) from native ricin and (▲) from blocked ricin.

starting material (result not shown). A sample of an IgM antibody, used as a control glycoprotein having terminal galactose residues (Olsnes and Pihl, 1982), was 100% bound by the column. We thus conclude that most, if not all, of the galactose residues of the covalently bound ligands of the blocked ricin are unavailable to interact with a second ricin molecule.

The analysis of blocked ricin by polyacrylamide/sodium dodecyl sulfate gel electrophoresis suggested that the A-chain of ricin was not modified by **8**. Figure 5 shows that the A-chain from blocked ricin was indistinguishable from the A-chain of native ricin in its ability to inhibit protein synthesis in a cell-free system from rabbit reticulocytes. These assays have a range of sensitivity of about 20-fold, and a 2-fold loss

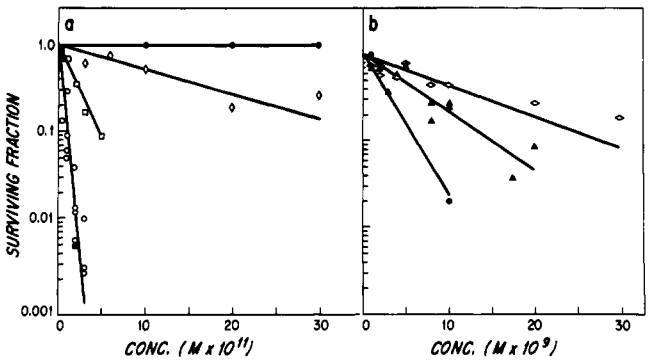


FIGURE 6: Cytotoxicity of ricin and blocked ricin for the human lymphoid cell line Namalwa. Samples of ricin, of purified blocked ricin (fraction D), and of protein fractions taken during purification of blocked ricin (see Figure 3) were incubated with cells at 37 °C for 24 h. The cells were then washed and placed into fresh medium for the determination of the surviving fractions by a direct cytotoxicity assay as described by Goldmacher et al. (1985). Panel a: Cytotoxicity of ricin and of fractions taken during the purification of blocked ricin as shown in Figure 3. (○) Ricin; (□) reaction mixutre of ricin and **8** (4 mol of **8**/mol of ricin); (◇) fraction A from purification through lactose–polyacrylamide; (■) fraction C, elution of bound protein from lactose–polyacrylamide with 250 mM lactose; (●) ricin in the presence of 30 mM lactose. Panel b: (●) Ricin in the presence of 30 mM lactose; (▲) purified blocked ricin; (◇) blocked ricin in the presence of 30 mM lactose.

in ability to inactivate ribosomes can be easily measured (Lambert & Blättler, 1988). This result shows that the activity of the A-chain is unaffected by the covalent modification of the B-chain.

Samples of blocked ricin and of various fractions of ricin taken during the purification of blocked ricin (refer to Figure 3) were tested for their cytotoxicity toward the human lymphoblastoid cell line, Namalwa. Ricin is highly cytotoxic, with an IC_{37}[4] of 4×10^{-12} M after a 24-h exposure of the cells to the toxin (Figure 6a). Upon incubation of ricin with **8** and then separation of the protein from noncovalently bound ligand, the cytotoxicity of the treated ricin was decreased by about 5-fold (Figure 6a). Purification of the reaction mixture through a column of immobilized lactose resulted in a fraction A that was 38-fold less cytotoxic than native ricin (Figure 6a). Further purification of fraction A through a column of immobilized asialofetuin gave blocked ricin (fraction D) which had an IC_{37} of 7×10^{-9} M, a value that shows that blocked ricin is more than 1000-fold less cytotoxic than native ricin (Figure 6b). Of the other fractions that were obtained by purification of the treated ricin through immobilized lactose and immobilized asialofetuin, fraction B and fraction E were only 5- to 20-fold less cytotoxic than native ricin (result not shown), while fraction C was equally as cytotoxic as ricin (Figure 6a). Fraction F, which was eluted with 7.0 M urea, was, following dialysis, not cytotoxic even though polyacrylamide/sodium dodecyl sulfate gels showed bands corresponding to ricin (Figure 4) and is, therefore, presumed to be denatured ricin.

Lactose (30 mM) reduced the cytotoxicity of native ricin by about 1000-fold (Figure 6), the IC_{37} being about 3×10^{-9} M. Blocked ricin with an IC_{37} of 7×10^{-9} M was slightly less cytotoxic than ricin in the presence of lactose, indicating that the covalent blocking of the sugar-binding sites of ricin B-chain was at least as efficient at abrogating the cytotoxicity of ricin as 30 mM lactose. Nevertheless, the cytotoxicity of blocked ricin was decreased by coincubation with 30 mM lactose by a factor of about 2-fold (IC_{37} of 12×10^{-9} M; Figure 6b). Another disaccharide, maltose (30 mM), had no effect on the cytotoxicity of blocked ricin (result not shown). The small effect of lactose suggests that blocked ricin has a residual weak interaction with cell surface carbohydrate moieties containing terminal galactose residues or that the preparation contains a small fraction which contains this weak binding activity for cells. Repurification of blocked ricin through a column of immobilized asialofetuin did not further reduce the cytotoxicity of blocked ricin, indicating that any residual galactose-binding activity of a fraction of the preparation must be of low affinity.

The properties of blocked ricin did not change in samples stored sterile at 4 °C for more than 1 year at pH 7.2, when analyzed by polyacrylamide/dodecyl sulfate gel electrophoresis and by in vitro cytotoxicity assays. We conclude that the covalent bonds formed between ricin and ligand **8** are stable: even a small release of native ricin would be detected by cytotoxicity assays.

Discussion

We have prepared a derivative (**8**) of glycopeptides containing triantennary N-linked oligosaccharides that contains a single reactive electrophilic group on one branch (antenna) of the ligand, with the aim of providing an affinity-labeling reagent for galactose-specific lectins. On the basis of binding studies by Baenziger and Fiete (1979b), who showed that the presence of one sialic acid on a complex oligosaccharide had only a small effect on its association with ricin as compared with the completely desialylated ligand, we reasoned that one branch of a triantennary ligand could be altered while still preserving the high-affinity interaction with ricin B-chain. Enzymic oxidation of terminal galactose residues affords a mild (pH 7.0; 30 °C) and highly specific method for modifying oligosaccharides at a specific site (Schweizer et al., 1982; Lee & Fortes, 1985; Lee & Lee, 1986), and this reaction was utilized in the preparation of the modified glycopeptide **7**. We show here that converting one galactose moiety of a glycopeptide containing a triantennary oligosaccharide to a residue of 6-(*N*-methylamino)-6-deoxy-D-galactose has no effect on its ability to block the binding of ricin to asialofetuin.

We also reasoned that, due to the high affinity of the interaction between ricin and asialo triantennary oligosaccharides (Baenziger & Fiete, 1979b), the sugar-binding sites of ricin B-chain could be saturated at low concentrations of a reactive ligand derived from such complex oligosaccharides or glycopeptides. We expected, therefore, to be able to achieve a specific reaction between such a reagent and the sugar-binding sites of ricin under conditions where there would be little or no nonspecific reaction.

The choice of chemical function for activating **7** was an important factor for efficient cross-linking of the ligands to ricin while minimizing any nonspecific reactivity of the affinity-labeling reagent. Previous experiments to covalently block the sugar-binding sites of ricin using ligands of high affinity derived from glycopeptides containing triantennary N-linked oligosaccharides used a photochemically activatable functional group to allow the temporal control of the cross-linking reaction (Baenziger & Fiete, 1982). Lee and Lee (1986) also prepared a triantennary glycopeptide ligand containing a photoactivatable group in order to affinity label the galactose/*N*-acetylgalactosamine-specific lectin from mammalian liver. However, the photochemical cross-linking of these affinity reagents to the lectins was inefficient, as is typical for photoaffinity labeling (Knowles, 1972).

[4] IC_{37}, inhibitory concentration that leaves a surviving fraction of 0.37. According to the one-hit/one-kill model, 37% ($1/e \times 100$) of the treated cells survive when the number of lethal hits received by a population of cells is equal to the number of cells in the population.

The cross-linking reagent used in our experiments, cyanuric chloride, contains three reactive chlorines which have characteristics of reactivity that allow the temporal control of the cross-linking between **7** and ricin by controlling the pH of the reaction solution (Kay & Crook, 1967; Moroney et al., 1988). First, cyanuric chloride is reacted with **7** to form the dichlorotriazine **8** which can be purified from the excess cross-linking reagent at low pH. Then, the purified glycopeptide ligand **8** (Scheme II) reacts efficiently with ricin at pH 8 to form blocked ricin.

The blocked ricin (defined as ricin that can no longer bind to a column of immobilized asialofetuin) can be purified by affinity chromatography. Analysis by polyacrylamide/sodium dodecyl sulfate gel electrophoresis shows that the modification is confined to the B-chain of ricin and indicates that most of the B-chain contains two covalently linked ligands while a small amount of B-chain contains three covalently linked ligands. We could not detect any nonspecific reaction of **8** with proteins that are not lectins using the same reaction conditions as used for ricin, and we conclude, therefore, that the covalent reaction of **8** with ricin may occur only after **8** has bound to the sugar-binding sites of the ricin B-chain. The newly formed covalent bonds are very stable, as discussed previously (Lang et al., 1977; Moroney et al., 1987).

How does ricin B-chain bind **8** and react with two or three molecules of a glycopeptide ligand containing a triantennary N-linked oligosaccharide? The X-ray crystallographic structure for ricin B-chain shows two domains, each of which contains one galactose- (lactose-) binding site (Montfort et al., 1987; Rutenber et al., 1987). However, *each* domain can be divided into four peptide loops, three of which (α, β, and γ) are structurally homologous with each other, with their counterparts in the second domain, and also with a peptide loop found in the galactose-binding protein from *Dictyostelium discoideum* (Robertus & Ready, 1984). In the crystal, lactose is bound to loop α in domain 1 and loop γ in domain 2 (Rutenber et al., 1987). We speculate that the other loops, the β and γ loops of domain 1 and the α and β loops of domain 2, still retain an ability to interact with galactose, albeit weakly. The strongest interactions with complex ligands are likely to be provided by the sites in each domain that can bind lactose in the ricin crystals, each binding one galactose-terminated antenna of a triantennary ligand. The other loops, two in each domain, may then contribute weak binding with the galactose residues of the other two antennae of each triantennary N-linked oligosaccharide moiety that multiply together to create a high-affinity interaction ($K_a = 1.4 \times 10^7$ M^{-1}; Baenziger & Fiete, 1979b) between each of two complex N-linked oligosaccharide ligands and each *oligosaccharide* binding site of the ricin B-chain.

If we speculate that the B-chain of ricin has binding sites of varying affinity for each of the six terminal galactose residues contained in two triantennary asialoglycopeptide ligands, then this speculation can provide a possible explanation for why some B-chain in blocked ricin can be identified as having three covalently linked ligands. It should be noted that the activated ligand **8** only has 2 native galactose moieties and 1 *altered* galactose residue. It may be, therefore, that sometimes the altered galactose residue(s) of the ligand(s) reacts (react) with a nucleophile(s) on the ricin molecule without blocking one of the putative binding sites, thus allowing binding (and subsequent covalent reaction) of a third molecule of **8** to the B-chain. One would have to assume that there are alternative nucleophilic sites capable of reacting with **8**. Experiments are in progress to evaluate this point further, including identifying the sites of reaction between **8** and the ricin B-chain by purifying peptides covalently linked to **8**.

Blocked ricin is more than 1000-fold less cytotoxic than native ricin toward the Namalwa cell line and is about 2-fold less cytotoxic than native ricin assayed in the presence of 30 mM lactose. However, the activity of the A-chain from blocked ricin is, as expected, indistinguishable from that of native ricin A-chain in inactivating ribosomes in a cell-free system. These results can be explained by the notion that the covalent modification of the B-chain with 2 (and 3) molecules of **8** prevents binding of the blocked ricin to the cell surface. However, lactose still has a slight effect on the cytotoxicity of blocked ricin, further reducing it by about 2-fold. It may be that blocked ricin has a residual weak binding affinity for galactose residues on cell surfaces, perhaps as a consequence of the flexibility of the large blocking ligands. Alternatively, it must be considered that blocked ricin, despite the affinity chromatography, contains up to 0.05% native ricin and, therefore, the intrinsic cytotoxicity of blocked ricin would be the cytotoxicity measured in the presence of lactose. The intrinsic cytotoxicity of ricin A-chain was only firmly established once high-affinity antibodies were used in an immunoaffinity step in its purification scheme (Fulton et al., 1986), and perhaps the intrinsic cytotoxicity of blocked ricin will only be firmly established by similar techniques. It is also possible that the small effect of lactose on the cytotoxicity of blocked ricin is a nonspecific effect of the disaccharide on liquid pinocytosis or endocytosis of the cells; indeed, 100 mM lactose exhibits some cytotoxicity toward Namalwa cells, showing that high concentrations of sugar can have effects on cells.

During purification of blocked ricin, fractions of ricin were obtained where the B-chain had reacted with only one molecule of **8**. These fractions, which bound to columns of immobilized lactose or asialofetuin, were only 5- to 20-fold less cytotoxic than ricin. This result is similar to previous results where *one* galactose-binding site was blocked by covalent attachment of a lactose derivative (Moroney et al., 1987). These results suggest that only one of the galactose-binding sites is necessary for cytotoxicity. The small decrease in cytotoxicity can easily be accounted for by the lower affinity for cells of ricin having only one free oligosaccharide-binding site.

The use of ricin as a component of immunotoxins has elicited great interest as a potential anticancer agent (Vitetta & Uhr, 1985; Frankel et al., 1986; Vitetta et al., 1987; Blättler et al., 1989). Immunotoxins made from native ricin are potent cytotoxic agents, but they are not specific owing to their ability to bind nontarget cells through the B-chain of ricin. Immunotoxins made from ricin A-chain are specific but are generally far less potent than native ricin (Thorpe, 1985; Blättler et al., 1989). Blocked ricin may be the ideal candidate as an agent for antibody-mediated delivery to target cells. Preliminary results with conjugates made from blocked ricin and monoclonal antibodies indicate that such immunoconjugates are able to kill the targeted cells in vitro as efficiently as native ricin (J. M. Lambert, W. A. Blättler, and V. S. Goldmacher, unpublished work), which suggests that the membrane penetration function of ricin that enables the A-chain to enter the cytoplasm is independent of the galactose recognition function of the B-chain.

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Cloning and Expression of Complementary DNAs for Multiple Members of the Human Cytochrome P450IIC Subfamily[†,‡]

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ABSTRACT: The present study characterizes the profile of cDNAs from the human P450IIC subfamily in a library from one individual, and it describes three new members of this subfamily (IIC17, IIC18, and IIC19) isolated from two human cDNA libraries. cDNA libraries were constructed from two human livers which differed phenotypically in the hepatic content of P450 HLx (IIC8). The library from the phenotypically low HLx individual was screened by using a cDNA for rat liver P450IIC13 and an oligonucleotide probe for human IIC8. One clone, 254c, was isolated which clearly represents a new member of the human P450IIC subfamily (IIC17). This clone lacked the first 358 nucleotides at the N-terminus but was only 91% homologous in its nucleic acid sequence to IIC9 and 79% homologous to IIC8. Near-full-length clones for IIC9 were also isolated from this library, but no clones for IIC8 were found. Northern blots indicated that the mRNA for IIC8 was low or absent in this individual. A second cDNA library (from a liver phenotypically high in HLx) was then screened. Eighty-three essentially full-length (>1.8 kb) clones belonging to the IIC subfamily were isolated from this library. These include full-length clones for two additional new members of the IIC subfamily. Clones 29c and 6b appear to be allelic variants (IIC18), differing by one nucleotide (one amino acid change) in the coding region. Clone 11a represents a full-length clone for a third new P450 (IIC19). Both IIC18 and IIC19 are most homologous to IIC17 (87% and 95%, respectively). Full-length clones were isolated for two allelic variants of IIC9 (clones 25 and 65) which differed by three nucleotides, resulting in one amino acid difference. Hybridization analysis and partial sequencing indicated that of the 83 clones in this library, 50 were 1 of the 2 allelic variants of IIC9, 29 were IIC8, 3 were the 2 allelic variants of IIC18, and 1 was IIC19. cDNAs for IIC9, IIC18, and IIC19 were expressed in COS-1 cells. Antibody to IIC9 recognized the proteins in cells transfected with cDNAs for IIC9; however, neither antibody to IIC9 or IIC8 recognized the other two cytochromes, suggesting that these cytochromes differ immunochemically from IIC8 and IIC9. COS-1 cells transfected with IIC18 showed an increase in metabolism of mephenytoin, but no metabolism of mephenytoin was detected in COS-1 cells transfected with IIC9 or IIC19.

Cytochrome P450[1] enzymes are important in the oxidative metabolism of both endogenous substrates and xenobiotics. Genetic polymorphisms of P450 enzymes result in distinct subpopulations which differ in their ability to perform particular drug biotransformation reactions. We have recently found that a polymorphism in rat P450g (IIC13) in outbred rats is due to the presence of a few single base mutations in the mRNA resulting in simple amino acid changes (Yeowell et al., 1990). Human P450 HLx, which is immunochemically

[†] A preliminary report of a portion of this work was presented at the 81st Annual Meeting of the American Society for Biochemistry and Molecular Biology (Romkes et al., 1990).
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05326.
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[1] We have used the recommended P450 gene nomenclature system (Nebert et al., 1991) throughout this paper. All new P450IIC sequences were submitted to Dr. Nebert for inclusion in the 1991 update to this nomenclature system, and the numbering system is consistent with this update. Abbreviations: SSC, standard sodium citrate; SDS, sodium dodecyl sulfate.