The role of the high mannose carbohydrate chains in the mechanism of action of ricin toxin was investigated. Ricin is taken up by two routes in macrophages, by binding to cell surface mannose receptors, or by binding of the ricin galactose receptor to cell surface glycoproteins. Removal of carbohydrate from ricin by periodate oxidation led to a large loss in toxicity via both routes of uptake by an effect on the B chain not due to a loss of galactose binding affinity. These data suggest that the carbohydrate chains of ricin B chain may be required for full toxicity. The pathway of uptake of ricin by the macrophage mannose receptor was found to differ in several respects from uptake via the galactose-specific pathway. Analysis of intoxication of macrophages by ricin in the presence of ammonium chloride suggested that mannose receptor bound ligand passes through acidic vesicles prior to translocation, unlike galactose bound ligand. Intoxication by ricin via galactose-specific uptake was potentiated by swainsonine but not by castanospermine, suggesting that ricin may be attacked by an endogenous mannosidase within the cell, and that ricin passes through either a lysosomal or a Golgi compartment prior to translocation.

The plant toxin ricin has recently been extensively employed in the synthesis of hybrid toxins targeted to specific cell types (1, 2). Ricin toxin is a 65,000 M, heterodimeric glycoprotein from castor beans (Ricinus communis) which binds to cell surface glycoproteins by virtue of the galactose binding property of its B chain (3-5). Following binding via the B chain, the toxin is taken up by the cell and, in a poorly understood process, the A chain is translocated into the cell cytoplasm. After initially binding to the cell surface, ricin is internalized by endocytosis leading to its accumulation within endocytic vesicles (3-5). Ricin A chain is then translocated from endocytic vesicles into the cytoplasm where it catalytically inactivates the 60S ribosomal subunit leading to the inhibition of cellular protein synthesis and, consequently, cell death. Hybrid toxins make use of the A chain which is inactive unless translocated into the cytoplasm following an initial binding interaction with the cell surface. By combining A chain with a ligand of specific binding activity (monoclonal antibody, lectin, etc.), a toxin of new specificity is produced.

A major drawback of these hybrid toxins is their relatively weak toxicity compared to intact ricin, often requiring large doses to intoxicate target cells and, consequently, often with poor specificity (1, 2). This problem is presumed to be due to poor internalization and/or translocation of the A chain into the cytoplasm. A variety of data suggests that ricin B chain may play a role in facilitating translocation by some process distinct from the initial cell surface binding event. The B chain has been shown to increase the rate of A chain entry into cells (6) and has been suggested to shuttle the internalized toxin to some endocytic vesicular pool where the translocation process takes place (7), but the issue remains unresolved.

The present study examines the role of the carbohydrate chains of ricin toxin in two routes of uptake: its previously characterized route of binding via the B chain to surface glycoproteins in L929 fibroblasts, and in a novel route of uptake as a ligand for the macrophage mannose receptor which is characterized herein. Ricin contains three high mannose oligosaccharide chains, two in the B chain (one GlcNAc2Man3 and one GlcNAc2Man4), and one in the A chain (GlcNAc3Man3) (3, 4). It has previously been reported (8, 9) that periodate treatment of ricin selectively oxidizes the terminal sugar moieties of its oligosaccharide chains, substantially decreasing the toxicity of ricin but having no effect on cell binding affinity or A chain activity as measured with in vitro methods. These investigators concluded that the mannose residues in ricin may play some role in the internalization of ricin. The work described here expands upon these findings by investigating the role of carbohydrate both in the galactose and mannose receptor-mediated uptake routes in L929 and macrophages. Removal of carbohydrate by periodate oxidation and glycosidase digestion was also performed to better characterize the role of carbohydrate in ricin intoxication. The two pathways of uptake of ricin in macrophages were characterized with respect to their sensitivity to ammonium chloride, swainsonine, and castanospermine. The data suggest that significant differences exist in the intracellular transport of ricin taken up via the two receptors and provides additional evidence that the carbohydrate of ricin may facilitate intoxication. In addition, highly purified ricin A chain was shown to act directly as a ligand for the mannose receptor, leading to intoxication of mannose receptor-bearing cells. Thus A chain and presumably A chain containing hybrid toxins may be toxic to the reticuloendothelial system in vivo.

**EXPERIMENTAL PROCEDURES**

Reagents—Castor beans (R. communis) were obtained from A. H. Hummer's Seed Company, St. Louis, MO. Carrier-free sodium $^{35}$S-methionine were obtained from Amersham. Swainsonine and castanospermine were obtained from Calbiochem.
Carbohydrate in Ricin-mediated Intoxication

Ricin Purification—Ricin toxin was prepared as previously described (10). Ricin A chain was isolated by binding 70 mg of ricin to a 1×30-cm galactosyl-Sepharose column followed by elution with 250 ml of 1 M 2-mercaptoethanol, 0.1 M Tris, pH 6.6 (11). The A chain was isolated by concentrating the 2-mercaptoethanol eluate using an Amicon PM-10 ultrafiltration membrane and cell, followed by removal of mercaptoethanol by chromatography with Sephadex G-25 immediately prior to use. Highly purified A chain (<0.02% ricin contamination; see below) was obtained by coupling a 1 ml galactosyl-Sepharose column in series below a 2.5×20-cm Sephadex G-25 column (to bind any residual ricin) and eluting with 20 mM Tes, 0.14 M NaCl, pH 7.4. A high flow rate (1 drop/s) was found to give the best yield of A chain; lower flow rates drastically reduced the yield by over 50% (data not shown). Ricin B chain was isolated as the best yield of A chain; lower flow rates drastically reduced the yield by over 50% (data not shown). Ricin A and B chains were recombined to form heterodimeric ricin by dialysis at pH 8.0 as previously described (12). No residual free subunits were found in the reaction mixture by dialysis with sodium dodecyl sulfate-polyacrylamide electrophoresis after dialysis.

Periodate Oxidation of Ricin—For isolation of periodate-treated ricin subunits, a total of 68 mg of ricin was oxidized. To 68 mg of ricin in 45 ml of 50 mM sodium acetate buffer, pH 4.5, was added 200 mg of sodium metaperiodate. The solution was stirred in a foil-wrapped beaker for 4 h at 4 °C. The reaction was stopped by the addition of 0.5 ml of ethylene glycol, followed by dialysis against 2 liters of 10 mM sodium phosphate, 0.2 M NaCl, pH 7.4 (8). Preparative material was removed by centrifugation for 10 min at 1500 × g, and the solution was stored at 4 °C in the presence of 0.02% azide. Periodate A chain was obtained from this material as described above for unmodified ricin. For determining the kinetics of inactivation of ricin by periodate, 1 ml aliquots were taken at hourly intervals, dialyzed, and assayed for mannose and cytokotoxic activity.

Cell Culture—L929 cells were maintained in RPMI 1640 culture media containing 5% bovine calf serum (Kansas City Biologicals). Rat bone marrow macrophages were prepared by culture of marrow cells in medium containing 5% bovine serum albumin (Kansas City Biologicals). The cells were lysed with 0.5 ml of 0.1 N trichloroacetic acid. The precipitated material was digested with 0.3 mg of sodium metaperiodate. The solution was stirred in a foil-wrapped beaker for 10 min at 1500 × g, and the mixture was allowed to react for 10 min at room temperature and then purified by dialysis, or for ricin by isolation on a 1 ml galactosylthiophenyl-Sepharose column, followed by elution with 0.1 M galactose and dialysis with phosphate-buffered saline.

Cytotoxicity Studies—The toxicity of ricin derivatives was determined by assaying for [35S]methionine incorporation in cell cultures treated with the toxins at various dilutions. Parallel cultures included 0.1 M lactose to prevent binding of the B chain to galactose sites, and/or 1 mg/ml yeast mannan to prevent binding to mannose receptors. Aliquots were allowed to react with the appropriate media. Following incubation for 2 h at 37 °C to allow cell adherence, various concentrations of sterile ricin were added. After incubation for 18 h at 37 °C, 2.5 μCi of carrier-free [35S]methionine was added to each well, and the cells were incubated an additional 3.5 h. The wells were gently washed with media to remove free [35S]methionine, and the cells were lysed with 0.5 ml of 0.1 N NaOH. Aliquots of the cell lysate (100 μl) were combined with 0.1 ml 20% trichloroacetic acid, 40 μl of 5 mg/ml bovine serum albumin, and 3 ml of 10% trichloroacetic acid. The precipitates were centrifuged for 10 min at 1500 × g and washed three times with 3 ml of 10% trichloroacetic acid. The precipitated material was digested with 0.3 ml NCS reagent (Amersham), neutralized with 10 μl of glacial acetic acid, mixed with 3 ml of PCS scintillation mixture (Amersham), and assayed in a liquid scintillation counter.

Preparation of Iodinated Proteins—Ricin, periodate-ricin, and β-gluconuridase were iodinated by the lactoperoxidase method (14). Briefly, 30 μg of toxin in 25 μl of phosphate-buffered saline was added to 1 μCi of carrier-free [125I]-NaI (Amersham, IMS-30, 100 mCi/μl). To this was added 200 μg/ml lactoperoxidase and 25 μl of 8.5×10−7 M H2O2. The mixture was stirred for 10 min at room temperature and then purified by dialysis, or for ricin by isolation on a 1×20-cm galactosylthiophenyl-Sepharose column, followed by elution with 0.1 M galactose and dialysis with phosphate-buffered saline.

Toxins were generally adjusted to a specific activity of 106 cpm/μg by addition of unlabeled toxin for binding and uptake studies.

Binding and Uptake of Ricin—Iodinated ricin, periodate-ricin, or β-gluconuridase were allowed to bind to 5×105 L929 or RRM macrophages in 0.4 ml of Hanks’ balanced salt solution, 10 mM HEPES, 0.1% bovine serum albumin in 24-well Linbro plates. The plates were placed on a rocker platform (Labsquake, Labindustries) and incubated for various periods of time (30 min–2 h, see figure legends). Duplicate 100-μl aliquots of media were then removed and assayed for remaining radioactivity to quantitate uptake. To differentiate bound versus internalized ricin, the cells were washed three times with ice-cold medium followed by the addition of 0.5 ml of 0.1 M lactose in medium to displace surface bound material. Lactose was found to rapidly displace surface bound material with a half-time of less than 15 s (data not shown). Lactose-washed cells were three times with regular medium and then lysed with 0.5 ml of 0.1 N NaOH to release internalized material. Duplicate 150-μl aliquots were assayed with a γ-counter.

Glycosidase Digestion—Ricin was digested with either endoglycocidase H as described (17) or with N-glycosidase F (N-Glycanase, Genzyme) using 2 μg of ricin in a total volume of 0.34 ml of 0.2 M Na phosphate, pH 6.6, 10 mM p-phenanthroline, and 10 units/ml enzyme, followed by dialysis against phosphate-buffered saline, and assayed for toxicity against L929 cells.

RESULTS

Toxicity of Ricin and Periodate-ricin—The inhibitory effect of ricin on protein synthesis in L929 fibroblasts and macrophages was assessed in the presence or absence of the inhibitors lactose and/or mannan (Fig. 1). Lactose binds to the galactose binding site of ricin B chain, and mannan binds to the macrophage mannose receptor, each acting as a competitive inhibitor of its respective receptor. Lactose was found to protect L929 cells from the toxic effect of ricin, as shown in Fig. 1A. Yeast mannan had no effect on the toxicity of ricin in L929 cells.

When RRM macrophages were treated with ricin, however, lactose failed to protect against intoxication (Fig. 1B). Only when both lactose and yeast mannose were present in the
media was any protection observed. Coupled with the above observation that mannan has no effect on ricin toxicity in L929 cells, this suggests that ricin can be internalized in macrophages either by the mannos-binding mannose receptor or by binding to surface glycoproteins containing galactose.

Effect of A Chain on Protein Synthesis in Macrophages and L929—The toxicity of A chain towards RBM macrophages and L929 cells was examined to determine whether the high mannos-binding of A chain allows it to act as a ligand for the mannose receptor and to be internalized and translocated. As shown in Fig. 2, mannan protected macrophages from A chain intoxication, shifting the dose response curve by more than two orders of magnitude, but lactose had no effect. L929 cells, which lack mannose receptors, were insensitive to A chain at concentrations as high as 2 μg/ml. This indicates the level of ricin contamination was below 0.02% in the A chain preparation, and the protective effect of mannan alone rules out ricin contamination as the cause of toxicity. Thus A chain itself can be taken up by the mannose receptor but is much less toxic than intact ricin taken up via the mannose receptor (ID50 of 10−5 g/ml for A chain versus 10−10 g/ml for ricin), presumably since translocation is much less efficient in the absence of the B chain.

Periodate Oxidation of the Carbohydrate of Ricin—To investigate the role of the carbohydrate chain in the intoxication of macrophages and L929 cells, ricin was treated with sodium metaperiodate which, at low pH, selectively oxidizes the carbohydrate chains leading to destruction of mannose residues. The time course of inactivation of ricin by periodate was determined by taking serial aliquots from the reaction mixture and assaying them for cytotoxicity toward L929 cells and mannos content. As illustrated in Table I, periodate treatment led to a rapid loss of both cytotoxic activity and mannos residues from ricin, with a total loss of detectable mannos after 3-4 h of treatment. Totally deglycosylated periodate-ricin was then assayed for toxicity toward both L cells and macrophages in parallel with native ricin. Fig. 3 illustrates that periodate-ricin differed from ricin in its toxicity in two respects. With both L929 cells and RBM macrophages, an approximate two orders of magnitude loss in activity was observed relative to intact ricin. Second, in both cell types lactose alone was sufficient to protect against intoxication. This suggests that the periodate oxidation destroyed the recognition unit on the ricin which had allowed it

![Graphs and diagrams](https://example.com/graphs.png)
to be taken up by the mannose receptor, and in addition, had led to a large loss in toxicity via the uptake route mediated by B chain binding observed in both cell types.

**Binding and Uptake of Ricin and Periodate-ricin**—To determine whether the loss of toxicity of periodate-ricin was due to an effect of the galactose binding site, binding studies were performed. The ability of $^{125}$I-ricin to bind to L929 cells was assessed in order to quantitate the binding affinities of ricin and periodate-ricin. Using a fixed concentration of $^{125}$I-ricin ($5.5 \times 10^6$ cpm/µg, $1.7 \times 10^6$ cpm/ml), binding reached equilibrium after 60 min at 4°C, and this binding was inhibited by 0.1 M lactose but not sucrose (Fig. 4A). Fig. 4B demonstrates that $^{125}$I-ricin binds in a saturable manner to L929 cells, using varying doses of ricin (adjusted to a specific activity of $10^6$ cpm/µg, 2-h incubation, 4°C).

In order to determine if periodate oxidation had affected ricin by the inactivation of the galactose binding sites, ricin and periodate-ricin were iodinated using lactoperoxidase and used in parallel in an equilibrium binding analysis with L929 cells. To determine the binding constants, the data were plotted using the method of Steck and Wallach (19) (Fig. 5A). At 4°C the total number of ricin binding sites were calculated to be $2.0 \times 10^7$, with an affinity constant ($K_T$) of $1.5 \times 10^6$ M$^{-1}$. Using periodate-ricin, the approximate number of binding sites per cell was $2.0 \times 10^7$, and the $K_T$ was $5.0 \times 10^6$ M$^{-1}$. Periodate oxidation, therefore, led to a reduction of galactose-specific binding affinity to 37% that of intact ricin at 4°C. A previous report (8) demonstrated a similar difference in the binding constant of ricin versus periodate-ricin determined at 5°C.

It has been reported that the binding affinity of ricin toxin is markedly temperature-dependent, varying from a $K_T$ of $11 \times 10^7$ at 0°C to $8 \times 10^6$ at 37°C (20). Because of this it was important to examine the binding properties of the two proteins at 37°C to determine if the difference in their binding affinity was similarly temperature-dependent. This binding experiment was performed using the same protocol as with the 4°C experiments, except that 0.2% Na azide was added to prevent endocytosis, and the incubation time reduced to 60 min. Uptake of the toxin into the cell was negligible under these conditions (data not shown). As demonstrated in Fig. 5B, the difference in binding constants was negligible at 37°C. Table II summarizes the results of binding analysis at 4 and 37°C. At physiologic temperatures, therefore, there is little difference in binding affinity between periodate-ricin and intact ricin, and thus the decrease in toxicity cannot be attributed to a change in binding affinity of the B chain for galactose.

**TABLE I**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ID$_{50}$ (µg/ml)</th>
<th>Mannose (mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>$3 \times 10^{-4}$</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td>2 h</td>
<td>$8 \times 10^{-4}$</td>
<td>0.2 ± 0.9</td>
</tr>
<tr>
<td>3 h</td>
<td>$1.4 \times 10^{-4}$</td>
<td>Undetectable</td>
</tr>
<tr>
<td>4 h</td>
<td>$2.5 \times 10^{-4}$</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Ricin</td>
<td>$5 \times 10^{-10}$</td>
<td>15.2 ± 2.6</td>
</tr>
</tbody>
</table>

* Hours of incubation with periodate, 4°C.

* Dose to produce 50% inhibition of protein synthesis of L929.

* Mannose content, determined enzymatically.

**TABLE II**

<table>
<thead>
<tr>
<th>Toxin</th>
<th>L929</th>
<th>Macrophage</th>
<th>4°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ricin</td>
<td>$10^{-9}$</td>
<td>$&lt;3 \times 10^{-11}$</td>
<td>$1.4 \times 10^6$</td>
<td>$3.8 \times 10^6$</td>
</tr>
<tr>
<td>Periodate-Ricin</td>
<td>$10^{-7}$</td>
<td>$2 \times 10^{-8}$</td>
<td>$7.6 \times 10^6$</td>
<td>$3.2 \times 10^6$</td>
</tr>
</tbody>
</table>

**Fig. 5.** Binding analysis of $^{125}$I-ricin and $^{125}$t-periodate-ricin. Increasing concentrations of iodinated toxin ($10^6$ cpm/µg) were added to L929 cultures as described in Fig. 4. Panel A. 4°C binding. After 3-h incubation at 4°C, aliquots were assayed for free counts as described. Panel B, 37°C binding. Toxins were allowed to bind for 90 min in the presence of 0.2% NaN$_3$ before assaying for free activity in the supernatant. The data were plotted using the method of Steck and Wallach (19).

**Assay of A Chain Activity by the Preparation of Hybrid Ricin (Periodate-A Chain-ss-B Chain)**—While previous investigators had determined that periodate oxidation had no effect on A chain activity in *in vitro* translation systems (8, 9), it was important to demonstrate that the A chain retained normal cytotoxic activity toward whole cells when delivered by a surface binding interaction. Since the isolated A and B chains undergo spontaneous and virtually stoichiometric recombination into intact toxin following dialysis (12), this method...
was used to detect any change in A chain activity due to the periodate oxidation. Isolated A chain from either intact ricin or periodate-oxidized ricin was recombined with purified ricin B chain to regenerate intact ricin or periodate-A-as-normal B chain. Each was then assayed for toxicity toward L929 cells. As illustrated in Fig. 6, both normal A + normal B and the periodate-A + normal B hybrid were equally toxic and as toxic as intact ricin itself. The periodate-ricin preparation from which the periodate-A chain was obtained was approximately 2 logs less toxic than the hybrids containing normal B chain. This implies that the periodate effect is localized to the B chain of ricin and that periodate A chain has normal cytotoxic activity when recombined with normal B chain. These data argue for the site of the periodate effect being in the B chain itself, ruling out both effects on the A chain ribosomal inactivation activity and on any other property of the A chain required for its translocation into the cytoplasm.

Uptake of \( ^{125}I \)-Ricin by RBM Macrophages—The binding and uptake of iodinated ricin to macrophages was studied as with L929 cells by adding increasing doses of ricin. Both the surface-bound (lactose displaceable, Fig. 7A) and internalized (Triton or NaOH releasable, Fig. 7B) toxin were quantitated (10^6 cpm/\( \mu \)g, 2 h, no azide). As illustrated in Fig. 7A, a considerable quantity (1-1.5 \( \mu \)g) of ricin was bound to the cell surface of macrophages following a 2-h incubation at 25 \(^\circ\)C (calculated from the radioactivity released by lactose treatment of the washed cells). This was inhibited by the presence of lactose in the medium, but not to a measurable extent by mannan, consistent with the large difference in the total number of mannose receptors versus lactose binding sites on macrophages (10^2 versus 2 x 10^5). The uptake of ligand into the cell was measured by washing and then lysing the cells (Fig. 7B). Again a large amount of ricin was taken up into the cell (>1 \( \mu \)g) either in the presence or absence of mannan. When lactose was added, the amount internalized dropped to less than 0.1 \( \mu \)g and was further inhibited by the presence of mannan plus lactose. The inhibitory effect of lactose was not due to hypertonicity of the medium, as 0.1 M sucrose was found to have no effect on uptake (data not shown). The small difference in uptake between the lactose group and lactose plus mannan may represent uptake by the mannose receptor and is of a magnitude consistent with the uptake of known ligands for the mannose receptor under similar conditions (15, 16). Thus, galactose-specific uptake accounts for a much larger mass of internalized toxin than does mannose receptor-mediated uptake, although the amount required to inhibit protein synthesis by 50% is identical for both routes of endocytosis (Fig. 1B; see below).

Competition of Ricin and Periodate-Ricin with \( ^{125}I \)-\( \beta \)-Gluconuridase for Uptake by the Mannose Receptor—The ability of ricin and periodate-ricin to compete with \( ^{125}I \)-\( \beta \)-glucuronidase (a ligand for the mannose receptor (15, 16)) for uptake in macrophages was examined in order to demonstrate an interaction of ricin with the mannose receptor. As shown in Fig. 8, ricin but not periodate-ricin markedly inhibited the uptake of \( ^{125}I \)-\( \beta \)-glucuronidase. The ID_{50} for ricin was approximately 1 \( \mu \)g/ml whereas for periodate-ricin it was over 100 \( \mu \)g/ml. This is consistent with ricin competing with \( \beta \)-glucuronidase for binding to the mannose receptor, periodate-ricin failing to do so because of the destruction of mannose residues. Since ricin exhibits a lag period before which A chain enters the cytoplasm, with protein synthesis inhibition beginning several hours after addition (3, 6), this effect is highly unlikely to be due to intoxication of the cells. Ricin and periodate-ricin were tested for an inhibitory effect on the uptake of heat-aggregated \( ^{125}I \)-rabbit IgG in macrophages and were found to have no effect, suggesting that ricin has no short term effect on the uptake of other ligands (data not shown). These data strongly imply that ricin competes with \( \beta \)-glucuronidase for uptake by the mannose receptor and confirm the results of earlier experiments demonstrating the potential for intoxication via this route.

Glycosidase Digestions—Ricin was digested with the glyco-
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FIG. 8. Inhibition of $^{125}$I-$\beta$-glucuronidase uptake by ricin and periodate-ricin. Interaction of ricin with the mannose receptor was determined by examining its ability to compete with $^{125}$I-$\beta$-glucuronidase, a characterized ligand of the mannose receptor (15), for uptake. Unlabeled ricin or periodate-ricin was added at several concentrations to compete with the radioactive ligand for uptake. $^{125}$I-$\beta$-Glucuronidase at a specific activity of $5 \times 10^8$ cpm/µg was added to $5 \times 10^7$ RBM macrophages at a final concentration of $1 \mu$g/ml, in the presence of several concentrations of ricin or periodate-ricin, in a final volume of 0.4 ml of 0.1 M lactose, HBSS, 10 mM HEPES, 0.1% bovine serum albumin. The cells were incubated at 37°C for 1 h, after which the cells were washed and internalized material released by lysis with 0.5 ml of 0.1 M NaOH, with duplicate 150-µl aliquots assayed in a γ-counter.

sidases endoglucohydrolase H and N-glycanase to supplement the results obtained using periodate oxidation; however, little mannoside could be removed (mannose content for Endo H-digested ricin = 10.4 ± 1.6 mol/mol; for N-glycanase = 12.2 ± 3.7 mol/mol, versus 16.4 ± 1.2 for intact ricin). Consistent with these results is a recent report indicating that the carbohydrate of the B chain is resistant to in vitro glycosidase digestion, suggesting that the B chain carbohydrate chains are relatively inaccessible to commercially available glycosidases (17).

Effect of Ammonium Chloride on the Toxicity of A Chain and Ricin in Macrophages—The effect of ammonium chloride, a lysosomal agent that raises the intracellular pH of acidic vesicles, was examined on the toxicity of A chain and ricin. Macrophages were treated with 10 mM ammonium chloride 30 min prior to adding toxin, and protein synthesis was determined 18 h later. As illustrated in Fig. 9, ammonium chloride protected macrophages against intoxication by A chain. It has previously been shown that NH$_4^+$ interferes with mannose receptor-mediated uptake by interfering with the dissociation of ligand from receptor, suggesting that mannose receptor bound ligand passes through an acidic intracellular compartment (21). The effect of ammonium chloride on ricin intoxication for the two uptake routes in macrophages was determined by performing the experiment in the presence of either mannan or lactose. As shown in Fig. 10A, when ricin is taken up via the mannose receptor route (i.e. in the presence of lactose), NH$_4^+$ protected the cells against intoxication. When ricin was taken up via its galactose binding site (mannan present to block mannose receptors, Fig. 10B), NH$_4^+$ was found to potentiate toxicity. This suggested a different route of intracellular transport for ricin bound to macrophages via the B chain versus being internalized by the mannose receptor.

Effect of Swainsonine and Castanospermine on the Toxicity of Ricin and A Chain—The effects on ricin toxicity by two drugs which interfere with processing of newly synthesized glycoproteins were investigated to better characterize the role of carbohydrate in the toxicity of ricin. Castanospermine blocks the endoplasmic reticulum α-glucosidase, an enzyme responsible for the first step in processing of the high mannose oligosaccharide (removal of glucose residues) following its transfer to newly synthesized proteins (22, 23). Swainsonine inhibits a later step of oligosaccharide processing by inhibiting Golgi α-mannosidase II. This leads to the accumulation of high mannosamine containing glycoproteins within the cell (18, 21), and has been shown to block mannose receptor-depend-
ent uptake of ligands probably by the occupancy of intracellular receptors by high mannose ligands (18).

The effects of the two drugs on A chain and ricin-mediated cytotoxicity were examined in macrophages and L929. As shown in Fig. 11, both swainsonine and castanospermine protected macrophages against intoxication by A chain, consistent with earlier reports of these drugs inhibiting uptake of ligands by mannose receptors (18). The effect of swainsonine on the two routes of ricin intoxication in macrophages was investigated, as shown in Fig. 12. When ricin was taken up via the mannose receptor, swainsonine was found to protect against intoxication similar to what was observed with A chain. Uptake of ricin via B chain binding, on the other hand, was found to be potentiated by swainsonine. The effects of castanospermine on the two routes of ricin intoxication was illustrated in Fig. 13. Castanospermine protected against mannose receptor-mediated intoxication similar to what was observed with swainsonine. It had no effect on B chain-mediated intoxication, unlike the effect seen with swainsonine. As with B chain-mediated uptake in macrophages, swainsonine was similarly found to potentiate the effects of ricin on L929 fibroblasts, whereas castanospermine had no effect (data not shown). To rule out an effect on the number of binding sites as contributory to the effects of swainsonine, binding of 125I-ricin to L929 cells pretreated with 1 µg/ml swainsonine for 2 h was performed as described above. Analysis of the binding data using the method of Steck and Wallach (19) yielded a value of 1.0 × 10^7 sites/cell for control L929 cells and 0.86 × 10^7 sites/cell for swainsonine-treated cells. Thus, no increase in the number of binding sites that might explain the potentiating effect of swainsonine was observed. Thus, whereas both swainsonine and castanospermine protected against mannose receptor-mediated uptake of ricin, B chain-mediated uptake was potentiated by swainsonine but totally unaffected by castanospermine.

**DISCUSSION**

These data demonstrate that both ricin toxin and ricin A chain can intoxicate macrophages following uptake by the mannose receptor. The presence of dual routes of uptake in macrophages (mannose receptor versus the B chain galactose

**Fig. 11.** The effect of swainsonine and castanospermine on ricin A chain-mediated intoxication of macrophages. To determine the effects of swainsonine and castanospermine on the susceptibility of cells to intoxication by ricin, 5 × 10^6 rat bone marrow macrophages were either pretreated for 2 h with 1 µg/ml swainsonine or for 6 h with 20 µg/ml castanospermine prior to adding the toxin (18). Various dilutions of ricin were added and the cultures assayed for [35S]methionine incorporation as described above. Both glycosylation inhibitors were allowed to remain in the media during the entire experiment and had no effect on protein synthesis. Protein synthesis was determined 18 h later.

**Fig. 12.** The effect of swainsonine on ricin toxicity via mannose receptor versus B chain-mediated uptake in rat bone marrow macrophages. Panel A, Mannose receptor uptake. Macrophages cultured with lactose were pretreated with 1 µg/ml swainsonine prior to addition of ricin and processed as described. Panel B, B chain-mediated uptake. Macrophages cultured with 1 mg/ml mannann either with or without swainsonine pretreatment.

**Fig. 13.** The effect of castanospermine on ricin toxicity via mannose receptor versus B chain-mediated uptake in rat bone marrow macrophages. Panel A, Mannose receptor-mediated uptake. Macrophages cultured with lactose were pretreated with 1 µg/ml castanospermine 6 h prior to the addition of ricin as described in Fig. 11. Panel B, B chain-mediated uptake. Macrophages cultured with mannann were treated with castanospermine as described above.
Carbohydrate in Ricin-mediated Intoxication

receptor) allowed an investigation of the role of carbohydrate in the process of intoxication. The effect of periodate oxidation on toxicity, the relative efficiency of intoxication by both routes of uptake, and the effects of various drugs on intoxication, were each examined.

Several observations support the hypothesis that the carbohydrate of ricin facilitates intoxication. Removal of mannose from ricin by periodate oxidation was found to lead to a loss of toxicity via uptake by both receptors. This effect was not due to a loss of either galactose binding affinity or A chain inactivation, but was functionally localized to the B chain. This was shown by recombining periodate-oxidized A chain with normal B chain, ruling out a subtle effect on the ability of A chain to be translocated. One explanation for this observation is that periodate oxidation altered the ability of the B chain to facilitate translocation of the A chain into the cytoplasm. It has been suggested that the B chain facilitates translocation of A chain. This conclusion is based mainly on the considerably greater toxicity of ricin versus hybrid toxins and the potentiation of toxicity of hybrids by B chain (3-5). In an attempt to further address this point, ricin was digested with glycosidases to remove carbohydrate. However, native ricin was resistant to in vitro enzyme treatment. The data therefore suggest a role for the oligosaccharide moieties of the B chain in translocation, either by some direct mechanism (binding to intracellular mannose binding sites?) or by stabilizing a domain within the B chain that facilitates translocation.

Uptake of ricin by the B chain appears to be less efficient than mannose receptor-mediated uptake in inducing intoxication (i.e. of leading to translocation of the A chain), based on a comparison of the data shown in Figs. 1 and 7. Fig. 1B illustrates that lactose has no effect on the toxicity of ricin towards macrophages, whereas lactose significantly decreases the uptake of ricin in these cells (Fig. 7). Although a much larger mass of ricin is internalized via galactose-mediated uptake than with mannose receptor uptake, most of the material taken up by the galactose binding appears unable to result in intoxication. The diversity of membrane proteins to which the B chain binds may mean that only a subpopulation of internalized ricin is destined for A chain translocation. Translocation of the A chain into the cytoplasm may take place within a specific pool of intracellular vesicles to which only certain classes of macromolecules have access.

The experiments with ammonium chloride also illustrate differences between the routes of uptake of ricin via the mannose receptor versus via B chain binding. Ammonium chloride was found to protect macrophages against A chain intoxication and against mannose receptor-mediated ricin intoxication in macrophages (i.e. ricin in the presence of lactose), suggesting that toxin internalized by the mannose receptor passes through an acid compartment prior to translocation. Toxicity via B chain binding to surface galactose glycoproteins (ricin in the presence of mannan), on the other hand, was potentiated by ammonium chloride. Potentiation has been previously seen with ricin using fibroblasts (3) and with a variety of hybrid toxins (1, 2). This effect can be interpreted as suggesting that translocation occurs in neutral vesicles. However, the result may also be attributed to protection against lysosomal degradation of internalized ricin. In sum, it is clear that ricin taken up by these two routes passes through distinct intracellular pathways prior to translocation, with mannose receptor-bound ricin apparently being carried through an acidified compartment, and galactose-bound ricin (or some component of it) moving through a neutral compartment.

The experiments with swainsonine and castanospermine further support the hypothesis that the carbohydrate of ricin in some way facilitates translocation of the A chain. Swainsonine and castanospermine inhibit the earlier steps in the trimming of high mannose glycoproteins (via inhibition of α-glucosidase) and α-mannosidase, respectively, both leading to the intracellular accumulation of high mannose oligosaccharides. Both drugs protected against mannose receptor-mediated toxicity of A chain and ricin in macrophages, consistent with previous observations that these drugs induce a loss of surface mannose receptors (18). The mechanism for the protection against A chain is apparently due to the loss of surface mannose receptors to which the toxin can bind, observed with both swainsonine and castanospermine (Fig. 11).

Perhaps more interesting is the comparative effect of the two drugs on B chain-mediated uptake of ricin. Swainsonine, which inhibits Golgi and lysosomal α-mannosidase II, was found to potentiate the toxicity of ricin following uptake by B chain binding, both in L929 and macrophages (but alone having no effect on protein synthesis). The possibility that this effect was secondary to the intracellular accumulation of high mannose oligosaccharides induced by swainsonine was investigated by comparing the effect of castanospermine, which blocks α-glucosidase activity and similarly causes accumulation of high mannose glycoproteins. Castanospermine was found to have no effect on the toxicity of ricin, even at high doses and with prolonged periods of preincubation. Two experiments appear to rule out an effect of swainsonine via accumulation of partially processed sugar chains. First, swainsonine had no effect on the number of ricin binding sites on L929 cells ("Results"). Second, tunicamycin pretreatment failed to protect against potentiation by swainsonine (data not shown). The potentiation by swainsonine is compatible with the hypothesis that swainsonine inhibits some intracellular mannosidases. This putative mannosidase may partially protect cells from ricin intoxication by degrading the carbohydrate of ricin in the Golgi apparatus or lysosomes. At least three distinct mannosidase enzymes have been characterized from rat liver (from lysosomes, Golgi, and cytosol), which may be inhibited by swainsonine (28-30). This degradation of the mannose chains in ricin could affect the toxicity of ricin is supported by the observations that periodate oxidation of ricin leads to a loss of toxicity.

These findings coupled with earlier ultrastructural data also suggest that translocation of the A chain may take place only after transit of internalized toxin through or in close proximity to the Golgi apparatus. Ricin has previously been shown to accumulate in the trans-Golgi of neurons using ricin-gold conjugates (24, 25). Colloidal gold-ricin (or immunotoxin) conjugates have been found to accumulate in tubular vesicles adjacent to the Golgi, relatively early after binding (20-60 min), with accumulation of ricin-gold conjugates within the lysosomes after several hours incubation (26, 27). This ultrastructural evidence demonstrates that ricin is transported to regions of the cell where mannosidase activity may be localized. Although it is impossible to strictly rule out subtle differential effects of swainsonine and castanospermine on the intracellular trafficking of macromolecules that could affect the toxicity or ricin, the data as a whole are most consistent with a direct effect of swainsonine on a mannosidase resulting in potentiation.

The demonstration of mannose receptor-mediated uptake of ricin toxin and A chain is consistent with earlier reports suggesting that ricin is taken up by the reticuloendothelial system. It has been shown that iodinated ricin accumulates in liver Kupffer cells (9). Histopathologic consequences of
rubicin intoxication include severe damage to both liver hepatocytes and Kupffer cells, the latter of which is consistent with mannose receptor-mediated uptake (31). Although these effects have been viewed mainly as a potential limitation to the in vivo use of immunotoxins containing A chain (2, 9), it also suggests the use of A chain as a tool for analyzing reticuloendothelial cell function through the selective depletion of mannose receptor bearing macrophages.

REFERENCES