Effect of Chemical Deglycosylation of Ricin A Chain on the in Vivo Fate and Cytotoxic Activity of an Immunotoxin Composed of Ricin A Chain and Anti-Thy 1.1 Antibody

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ABSTRACT

The carbohydrate present on ricin A chain causes ricin A chain immunotoxins to be cleared rapidly in animals by the reticuloendothelial system. In an effort to overcome this problem we destroyed the carbohydrate on ricin A chain by treating it with a mixture of sodium metaperiodate and sodium cyanoborohydride and then linked the "deglycosylated" A chain to monoclonal anti-Thy 1.1 antibody. The deglycosylation procedure did not affect the ability of the A chain component of the immunotoxin to inhibit protein synthesis in a cell-free system or the capacity of the immunotoxin to inhibit protein synthesis in Thy-1.1 positive lymphoma cells in vitro.

Immunotoxins prepared with deglycosylated A chain were cleared from the bloodstream of mice more slowly than native ricin A chain immunotoxins. The difference in the blood clearance rates of the two immunotoxins could be accounted for by a decreased entrapment of the deglycosylated ricin A chain immunotoxin by the liver. Both immunotoxins broke down in vivo with the appearance of free antibody in the bloodstream. The site of cleavage of the immunotoxin was possibly the liver because immunotoxins taken up by liver were metabolized further suggesting that dissociation of the A chain from the antibody had occurred. The immunotoxins taken up by the liver were metabolized further and the acid insoluble radioactive metabolites gradually accumulated in the stomach, thyroid, and salivary gland.

The deglycosylated ricin A chain immunotoxin should be a more effective antitumor agent in vivo because it is cleared from the blood more slowly and so has greater opportunity to localize within the tumor target.

INTRODUCTION

Novel antitumor agents have been synthesized in several laboratories by linking the A chain of ricin and other toxins to antibodies against tumor associated antigens. These reagents (called "immunotoxins") bind to antigens on the tumor cell surface via the antibody moiety. The A chain then enters the cell and kills it by inactivating its ribosomes.

The therapeutic activity of IT-A$^2$ in tumor-bearing animals is undermined by their rapid clearance from the bloodstream. Only about 1% of the injected dose of IT-A remains in the blood 24 h after i.v. administration as compared with 30% for an equivalent dose of native antibody (1-3). The rapid clearance of the IT-A is due primarily to the recognition of mannose or fucose residues on the A chain component by cells with mannose or fucose receptors in the liver and other organs. The evidence for this comes from several reports that ricin (4, 5), ricin A chain (6, 7), and IT-As (8) are all taken up by liver cells in vitro and in vivo through a route that can be antagonized by mannose and fucose-terminating glycoproteins and saccharides. In the rat, the liver cells which take up ricin A chain are in the nonparenchymal cell fraction (6) and are probably reticuloendothelial cells (9). In the mouse, both the parenchymal and nonparenchymal cell fractions appear to be involved (7).

Ricin A chain is heterogenous with respect to carbohydrate composition. About two-thirds of the A chain molecules have one complex oligosaccharide of monosaccharide composition (GlcNAc$_3$(Xyl)$_1$(Fuc)$_1$(Man)$_3$)$_{2}$ whereas the remaining one-third have a high mannose type oligosaccharide (GlcNAc$_2$(Man)$_{4}$) in addition to the complex unit (10). The two forms of the A chain have molecular weights of 30,000 and 32,000 and are known as A$_1$ and A$_2$ chains respectively (11). The high mannose oligosaccharide in the A$_1$ chain contains a trimannosidic core which is recognized with high affinity by hepatic reticuloendothelial cells (12). The complex oligosaccharide present on the A$_1$ and A$_2$ chains lacks a trimannosidic core and so probably binds to reticuloendothelial cells less avidly. It has, however, a fucose residue which may be recognized by hepatic parenchymal (13, 14) and nonparenchymal cells (15).

We recently developed a chemical method for modifying the carbohydrate on ricin that prevents the recognition of the toxin by liver cells in vitro (5) and in vivo (4). The procedure is to treat the toxin at low pH with a mixture of sodium metaperiodate and sodium cyanoborohydride. The fucose and most of the mannose residues present on the A chain are modified by the procedure; the N-acetylgalactosamine and most of the xylose remain unaffected (4, 16).

In the present study we investigated the effect of deglycosylation on the in vivo fate and cytotoxic activity of an anti-Thy-1.1-racin A chain immunotoxin. Deglycosylation greatly decreased the liver entrapment and prolonged the blood half-life of the IT-A while not reducing its ability to kill Thy-1.1-expressing tumor cells in vitro.

MATERIALS AND METHODS

Materials

Untoasted castor bean cake was a gift from Croda Premier Oils, Ltd., Hull, United Kingdom.

The hybridoma cell line, MRC OX7, secreting a mouse IgGl anti-Thy-1.1 antibody was kindly provided by Dr. A. F. Williams (MRC Cellular Immunology Unit, University of Oxford). The antibody was purified from the blood and ascitic fluid of hybridoma-bearing mice as described by Mason and Williams (17).

Anti-ricin antibody (IgG) was purified from the blood of sheep which had been immunized with ricin toxoid. The procedures were the same as those we used previously to prepare sheep anti-abrin antibody.$^3$ Rabbit anti-mouse IgG was obtained from Miles, Ltd., Slough, England.

The Thy-1.1$^+$ lymphoma cell line, AKR-A, was obtained from Prof.

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$^2$ The abbreviations used are: IT-A, ricin A chain immunotoxin; IT-dg.A, deglycosylated ricin A chain immunotoxin; PBS, phosphate buffered saline (170 mM NaCl, 3.4 mM KC1-12 mM Na$_2$HPO$_4$-1.8 mM KH$_2$PO$_4$, pH 7.2); TCA, trichloroacetic acid; IC, 50% inhibitory concentration; SDS, sodium dodecyl sulfate.
I. MacLennan (Department of Experimental Pathology, Birmingham University, England). The Thy-1.2 lymphoma cell line, EL4, was acquired from Dr. F. Spencer (Institute of Cancer Research, Sutton, London, United Kingdom).

The culture medium RPMI 1640 and fetal calf serum were purchased from Gibco-Biocult, Ltd., Paisley, Scotland.

Sodium metaperiodate and sodium cyanoborohydride were purchased from Aldrich Chemical Co., Gillingham, England, and were used without further purification. Both chemicals were stored in desiccators in the dark. 2-Iminothiolane hydrochloride was purchased from Sigma Chemical Co., Poole, Dorset, United Kingdom. NaI (IMS30), NaI (121I) (IBS3), l-[4,5-3H]leucine (TRK170) and l-[U-14C]leucine (CFB 67) were purchased from Amersham International, Amersham, England. The IODO-GEN reagent for protein iodination was from Pierce, Ltd., Chester, England.

Methods

Purification of Ricin A Chain. Ricin was purified from an aqueous extract of defatted castor bean cake by (NH4)2SO4 precipitation, affinity chromatography on Sepharose 4B, and gel filtration on Sephacryl S-200 essentially as described by Nicolson and Blaustein (18). The toxin was split by reduction into its component chains and the A chain purified as described previously (19).

Preparation of Deglycosylated Ricin A Chain. Ricin was treated with a mixture of 10 mM sodium metaperiodate and 20 mM sodium cyanoborohydride at pH 3.5 for 1 h at 4°C as described previously (4). The A chain was then separated from the deglycosylated toxin and purified by the same method as for native A chain (19). Deglycosylation of ricin A chain does not affect the amino acid composition of the protein or reduce its ability to inhibit protein synthesis in a cell-free system (4). The deglycosylated A chain ran as a single component (Mr, 32,000) when electrophoresed on 10% polyacrylamide gels in SDS. The carbohydrate composition of the deglycosylated A chain has been described previously (16).

Preparation of Immunotoxins. Ricin A chain and deglycosylated ricin A chain were coupled by a disulfide bond to OX7 antibody using the 2-iminothiolane reagent.3 The IT-As and IT-dg.As used in the biological tests were those that eluted from the final gel filtration columns iminothiolane reagent.3 The IT-As and IT-dg.As used in the biological studies (16).

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Preparation of Immunotoxins. Ricin A chain and deglycosylated ricin A chain were coupled by a disulfide bond to OX7 antibody using the 2-iminothiolane reagent.3 The IT-As and IT-dg.As used in the biological tests were those that eluted from the final gel filtration columns (Sephacryl S-200) with a molecular weight corresponding to 180,000-200,000. They contained antibody and A chain in the ratio of 1:1.4 as previously (19).

Binding of radioactivity to the cells. At the end of the experiment, the radioactivity in the plasma samples containing 8,000 cpm each was decreased by the removal of 5-10% polyacrylamide gradient gels (1 mm thick) containing 1% SDS. The gels were dried and autoradiographed using Kodak SB5 film with an exposure period of 3 days at room temperature. The autoradiographs were scanned with a Joyce Loebel Chromoscan 3 gel scanner and the area under the immunotoxin or antibody peak was divided by the total area under the peaks to determine the proportion of the radioactivity in the plasma that corresponded to either intact immunotoxin or antibody. Calibration experiments had previously shown that the area under each peak was directly proportional to the cpm it contained. Clearances were expressed as a percentage of the injected dose assuming that the mice had a blood volume of 2.18 ml/25 g body weight (21).

Control experiments the IT-A or IT-dg.A was replaced with 10 µg of radioiodinated native OX7 antibody or OX7 antibody which had been thiolyzed by treatment with 2-iminothiolane followed by reduction with dithiothreitol.

Blood Clearance Measurements. The purified IT-As or IT-dg.As were iodinated with carrier-free 125I and OX7 antibody with carrier-free 131I to a specific activity of 1 x 10^6 cpm/mg using the IODO-GEN reagent. Groups of three adult male BALB/c mice weighing about 25 g were injected i.v. of 10 µg of 125I-radiolabeled IT-A or IT-dg.A and 10 µg of 131I-radiolabeled antibody both containing about 1 x 10^6 cpm. After various time intervals the mice were anesthetized, a blood sample was taken and the animals were perfused with PBS containing heparin (1 USP unit/ml) as described by Brown et al., (22). This procedure removed 95% of the blood from the mice as judged from the radioactivity in the perfusate compared with that in the blood at the time of death. Various organs were then removed, weighed, and their radioactivity (125I and 131I) was measured in a gamma counter. The radioactivity measurements in the 125I channel were corrected for cross-over from the 131I radioactivity and measurements were corrected for decay assuming half-lives of 60 days for 125I and 8.1 days for 131I. The radioactivity remaining in the carcass was also determined by counting in the gamma counter. Results are expressed as the percentage of the injected dose found in the particular tissue.

After radioactivity had been measured, certain tissues were homogenized in heparinized PBS using a tissue grinder. The homogenates were centrifuged (10,000 x g) to remove tissue debris and 200-µl aliquots of the supernatant were mixed with equal volumes of 20% (w/v) TCA or with 10 µg of sheep anti-ricin IgG or rabbit anti-mouse IgG linked to CNBr-sepharose. One h later the supernatant was centrifuged (10,000 x g), the pellets were washed once with PBS, and the percentage of radioactivity precipitated by each treatment was determined by counting the pellets and supernatants in a gamma counter. Control experiments were performed to determine the efficiency of the precipitation procedures. This was done by adding a known amount of purified 125I-labeled IT-A or IT-dg.A to blood or to excised nonradioactive tissues before homogenization, and the percentage of the radioactivity precipitable by 10% TCA, anti-ricin IgG, or anti-mouse IgG was determined as described above. Both TCA and the anti-mouse IgG precipitated greater than 95% of the radioactivity associated with the IT-A or IT-dg.A. In contrast, the anti-ricin IgG only precipitated 80% of the radioactivity. This suggests that in a portion of the IT-As and IT-dg.As the antibody masks antigenic determinants on the A chain that are recognized by the anti-ricin antibody. To correct for the inability of the anti-ricin IgG to precipitate the IT-A and IT-dg.A completely, the percentage of the radioactivity precipitated by the anti-ricin IgG was multiplied by a factor of 1.2.

Inhibition of Protein Synthesis in Cells in Tissue Culture. The ability of the A chains or the immunotoxins to inhibit protein synthesis by AER-A cells or EL4 cells was determined in [3H]leucine incorporation assays as described previously (23).

RESULTS

Blood Clearance. In Fig. 1 are shown autoradiographs of SDS gels of blood samples from mice that had been given injections i.e. of IT-A or IT-dg.A at various earlier time periods. The IT-
after injections, respectively. (M, 150,000); lane 2, immunotoxin (M, 180,000) before injection. Lanes 3-10, nated IT-A (a) or IT-dg.A (*). Lanes 1 and //, radioiodinated native OX7 antibody of plasma samples taken from mice at various times after injection of radioiodi-

plasma samples taken from a mouse 10 min, and 2, 4, 8, 24, 48, 72, and 120 h was cleared very rapidly (7).

The amounts of intact IT-A plus its released antibody (•) or IT-dg.A plus its

released antibody (A) are shown. The contribution that instability has to the
time points that corresponded to intact IT-A (D), IT-dg.A (A), or OX7-antibody

to that of the intact antibody molecule (150,000) (Fig. 1). At

to release a component with a molecular weight corresponding

to the intact antibody molecule (150,000) (Fig. 1). At

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The amounts of IT-A or IT-dg.A remaining intact in the blood at various times after injection are shown in Fig. 2. The
clearance curves were biphasic having an initial rapid α phase followed by a slower β phase. During the α phase IT-A was removed from the bloodstream much more rapidly and to a greater extent than the IT-dg.A. One-half of the injected dose of the IT-A had disappeared from the bloodstream after only 15 min whereas it took 80 min for one-half of the IT-dg.A to disappear. After 8 h, when the α phase appeared to be complete, 4.3% of the IT-A remained in the bloodstream as compared with 18.4% of the IT-dg.A. Thereafter both IT-A and IT-dg.A cleared at a similar rate with half-lives of about 9.5 h.

Native OX7 antibody disappeared from the bloodstream more slowly than either the IT-A or IT-dg.A (Fig. 2) both in the α phase (tα = 5.4 h) and the β phase (tβ = 190 h). THiolation of the OX7-antibody with 2-iminothiolane before injection did not affect the clearance rate of the antibody (results not shown).

It was possible that the breakdown of the IT-dg.A to free antibody (and, presumably, A chain) was the main reason for its having a shorter in vivo half-life than the native antibody. To assess this possibility, the amounts of antibody released from the IT-A or IT-dg.A at the various time points were quantified and added to the amounts of intact immunotoxin remaining in the bloodstream (Fig. 2). Approximately one-half of the accelerated disappearance of the IT-dg.A (and one-fifth that of the IT-A) could be accounted for by instability. Thus there appears to remain a further mechanism of clearance that is due to neither the recognition of carbohydrate on the A chain nor to instability of the IT-A or IT-dg.A. This further clearance was not due to adsorption of IT-A or IT-dg.A to blood cells because no radioactivity was associated with the cell pellet when blood samples were collected and centrifuged.

Tissue Localization. The tissue distribution of 125I-labeled IT-A and IT-dg.A at various times after injection into mice is shown in Fig. 3. The tissues had been perfused before excision to remove radioactivity present in the blood. As seen in the Fig. 3 the rapid disappearance of the IT-A from the bloodstream was due primarily to its entrapment by the liver and less of the IT-dg.A was trapped by this organ. Approximately 30% of the radioactivity associated with the IT-A had localized in the liver 10 min after injection as compared with just 10% of the IT-dg.A. Nevertheless the amount of IT-dg.A entrapped in the liver was significantly greater (P < 0.05) than that of free antibody showing that deglycosylation did not abolish liver uptake.

The spleen was the only other tissue which, within 10 min of injection, accumulated more of the IT-A than the IT-dg.A or the antibody. Its contribution to clearance was small, however, compared with that of the liver.

The disappearance of radioactivity from the liver and spleen was accompanied by its appearance in the stomach and thyroid (Fig. 3) and to a lesser extent in the salivary gland (result not shown). Radioactivity in these tissues reached a maximum after about 4 h and the amount present varied in the following order: IT-A > IT-dg.A > antibody.

Metabolism. The radioactivity detected in the blood and tissues above could have been associated with either the intact protein or its metabolites. A series of experiments was therefore performed to determine how much of the radioactivity could be precipitated by TCA, antiricin or anti-mouse IgG. The results obtained for blood and liver are shown in Fig. 4.

Ten min after injection of either IT-A or IT-dg.A, 90–95% of the radioactivity in the blood was precipitable by TCA, anti-mouse IgG and antiricin (Fig. 4a). Thereafter, a decreasing proportion of the radioactivity could be precipitated by antiricin although it could still be precipitated by TCA or with anti-

A or IT-dg.A preparations that were injected contained a single major component with a molecular weight of 180,000 consisting of one molecule of antibody and one molecule of A chain. Both the IT-A and IT-dg.A broke down slowly after injection to release a component with a molecular weight corresponding to that of the intact antibody molecule (150,000) (Fig. 1). At later time points minor components having molecular weights of about 210,000 and 100,000 were also seen on all the SDS gels. No component corresponding in molecular weight to A chain (30,000) was seen at any time point probably because it was cleared very rapidly (7).

Fig. 1. Instability of IT-A and IT-dg.A in mice. Autoradiographs of SDS gels of plasma samples taken from mice at various times after injection of radioiodi-

nated IT-A (a) or IT-dg.A (b). Lanes 1 and 11, radioiodinated native OX7 antibody (M, 150,000); lane 2, immunotoxin (M, 180,000) before injection. Lanes 3–10, plasma samples taken from a mouse 10 min, and 2, 4, 8, 24, 48, 72, and 120 h after injections, respectively.

Fig. 2. Blood clearance rates of IT-A or IT-dg.A. Mice were given injections i.v. of radiiodinated OX7-antibody, IT-A, or IT-dg.A, and blood samples were taken at various periods of time later. Percentage of injected dose at the various time points that corresponded to intact IT-A (●), IT-dg.A (△), or OX7-antibody (□) was determined by scanning autoradiographs of SDS gels. Both immunotoxins (M, 180,000) split up in the bloodstream to release free antibody (M, 150,000). The amounts of intact IT-A plus its released antibody (●) or IT-dg.A plus its released antibody (△) are shown. The contribution that instability has to the clearance of the immunotoxins is represented by the difference between the solid and broken lines. Points, geometric mean ± 1 SD (bars), of the results obtained in three mice.

% of blood

Time after injection (hours)
be calculated from the radioactivity precipitable by antiricin. After 10 min and 1, 4, and 24 h, 54.1, 19.8, 9.1, and 1.0% of the IT-A and 79.3, 46.5, 25.3, and 5.2% of the IT-dg.A remained. These values are in good agreement with those of Fig. 2.

The IT-A taken up by the liver appeared to be rapidly split into its components and metabolized (Fig. 4b). Ten min after injection, 70% of the radioactivity in the liver could be precipitated by anti-mouse IgG whereas only 35% could be precipitated by antiricin, suggesting that the A chain had been released from the antibody. Thereafter, a progressively smaller proportion of the IT-A could be precipitated by TCA or by either of the antibodies showing that it had been digested to low molecular weight metabolites. The IT-dg.A also became unreactive with antiricin faster than with anti-mouse IgG but the appearance of non-TCA-precipitable radioactivity was much slower than with the IT-A. The carbohydrate present on ricin A chain therefore appears to accelerate digestion of the IT-A possibly by causing it to be internalized via degradative pathways. The radioactivity localizing in the spleen showed a similar pattern of precipitation with TCA and with the two antibodies to that found in the liver (results not shown).

Less than 10% of the radioactivity that localized in the stomach and salivary gland after injection of either the IT-A or IT-dg.A could be precipitated by TCA or the antibodies at any time point. The radioactivity present in these tissues must therefore be in the form of low molecular weight fragments or as free radioactive iodide. In contrast, the majority of the radioactivity in the thyroid could be precipitated by TCA but not by the antibodies suggesting that free radioactive iodide had been assimilated into thyroglobulin in the thyroid.

Excretion. The IT-A was metabolized and excreted more rapidly than the IT-dg.A. After 24 h, less than 20% of the IT-A or its radiolabeled metabolites remained in the mice as compared with 40% of the IT-dg.A. The OX7 antibody was excreted more slowly than either IT-A or IT-dg.A; 75% of the injected dose remained in the mice after 24 h. The rate of excretion of the IT-A, IT-dg.A, and the antibody thus reflected the rate at which they were cleared from the bloodstream.

Toxicity to Thy-1.1+ and Thy-1.1− Cells in Tissue Culture. The IT-A and IT-dg.A were equally toxic to the Thy-1.1+ lymphoma cell line, AKR-A (Fig. 5). Both IT-A and IT-dg.A reduced the [3H]leucine incorporated by the cells by 50% at a concentration (IC50) of 1.1−1.2 × 10^-12 M. The IT-A and IT-

mouse IgG. This trend is probably explained by the release of free antibody from the IT-A and IT-dg.A as was seen in Fig. 1. Free A chain would not have been detected because it is rapidly cleared (7) and because only 10% of the radioactivity in the immunotoxins was associated with the A chain. The amounts of intact IT-A or IT-dg.A remaining in the blood could therefore...
PROPERTIES OF IT-dg.A

dg.A were approximately 30-fold more toxic to AKR-A cells than was ricin (IC<sub>50</sub> = 3 × 10<sup>-11</sup> m) and 100,000-fold more toxic than were the purified A chains (IC<sub>50</sub> = 0.8–1.0 × 10<sup>-7</sup> m). The cytotoxicity of the IT-A and IT-dg.A was specific. Neither IT-A or IT-dg.A reduced protein synthesis in the Thy-1.2<sup>+</sup> lymphoma cell line EL4 at concentrations up to 3 × 10<sup>-8</sup> M (results not shown). Treatment of the lymphoma cell lines with OX7 antibody alone at 10<sup>-7</sup> M had no effect on [<sup>3</sup>H]leucine incorporation.

DISCUSSION

In agreement with previous studies (1-3) the IT-A rapidly disappeared from the bloodstream of mice. Chemical deglycosylation of the A chain substantially retarded the blood clearance of the IT-A. Twenty-four h after injection 8% of the IT-dg.A remained in the bloodstream compared with just 1% of the IT-A. The blood clearance rate of the IT-dg.A was almost identical to that of an OX7 immunotoxin prepared using abrin A chain which does not have oligosaccharide side chains (24). Furthermore, coinjection of a IT-A with excess yeast mannan, a polysaccharide containing terminal mannose residues, has been shown by Bourrie et al. (8) to slow the blood clearance rate of the IT-A to approximately that seen with the IT-dg.A in the present study. The deglycosylation procedure therefore appears to have eliminated the contribution that the A chain-associated sugar residues make to IT-A clearance.

The slower blood clearance rate of the IT-dg.A was primarily due to its decreased entrapment by the liver. It is likely that entrapment was reduced both in the parenchymal and nonparenchymal fractions of the liver. This is supported by our findings that free ricin A chain localized to both parenchymal and nonparenchymal cells in mice in equal amounts and that deglycosylation greatly reduced uptake into both cell fractions (7). The mannose and fucose residues present on the ricin A chain probably cause the binding to the two cell fractions. The nonparenchymal cells (i.e., Kupffer and sinusoidal endothelial cells) recognize mannose-terminating carbohydrate (25–27) while the parenchymal cells recognize α1-3-linked fucose (13, 14). Chemical deglycosylation destroys all of the terminal and most of the remaining mannose residues and all the fucose. Hepatic uptake of IT-A by either recognition pathway would thus be abolished.

The IT-A was rapidly metabolized in the liver and became unreactive with antiricin faster than it did with anti-mouse IgG, suggesting that release of A chain from the antibody had occurred. This was followed by the degradation of the IT-A to low molecular weight, non-TCA-precipitable material. These low molecular weight metabolites were released into the bloodstream and taken up by the stomach and thyroid, the two major sites of iodide entrapment (22, 28). A similar release into the bloodstream of low molecular weight components, identified predominantly as free iodide, was found by Worrell et al. (1) after injection of a radioiodinated IT-A into rats. The IT-A thus behaved in vivo like other glycoproteins which bind to carbohydrareceptors in the liver and which are rapidly internalized, transported to the lysosomes, metabolized, and released as acid-insoluble metabolites (22, 29–31). In contrast the IT-dg.A underwent a much slower rate of degradation in the liver. The radioactivity persisted in TCA and anti-mouse IgG precipitable form for as long as did the native antibody. However, reactivity with antiricin was lost more rapidly, again suggesting that the IT-dg.A was split into free antibody and A chain in this organ.

In previous reports there has been controversy concerning the breakdown of immunotoxins in the bloodstream. Some authors favor lability (1, 32) while others disagree (3, 8, 33). In the present study, both IT-A and IT-dg.A were found to break down progressively, yielding free antibody in the blood. This was shown both by the SDS gels and by the finding that the immunotoxins present in the bloodstream gradually became unreactive with antiricin but retained reactivity with anti-mouse IgG. Little or no breakdown was seen when either IT-A or IT-dg.A were incubated for 24 h at 37°C with mouse plasma in vitro, suggesting they are cleared as they circulate through the organs or tissues of the mouse. The site of cleavage is possibly the liver, as discussed above. The liver manufactures glutathione and releases it into the bloodstream (34). It is therefore possible that the glutathione reduces the disulfide bond in the IT-A and IT-dg.A as they pass in the blood through the hepatic vein.

Deglycosylation of A chain did not reduce the ability of the IT-A to kill Thy-1.1<sup>+</sup> AKR-A lymphoma cells in tissue culture. This agrees with our previous finding that deglycosylated ricin A chain coupled to anti-human immunoglobulin antibody was almost as effective at killing Daudi lymphoblastoid cells as an immunotoxin prepared with the native A chain (16). We have since made IT-dg.As from antibodies of other specificities and all have had unaltered cytotoxic activity against target cells.

In conclusion, the IT-dg.A had a longer half-life in vivo, decreased hepatic clearance, and unaltered cytotoxicity to target cells. Such IT-dg.As should be more effective antitumor agents in vivo. Indeed, Bourrie et al. (8) have found that the antitumor activity of an IT-A was markedly enhanced when its clearance was blocked by coadministering excess mannan with the IT-A. It is this result that we would expect to emulate using a deglycosylated ricin A chain immunotoxin.

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