In Vivo Administration of Lymphocyte-specific Monoclonal Antibodies in Nonhuman Primates

In Vivo Stability of Disulfide-linked Immunotoxin Conjugates

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Abstract

The stability in vivo and circulatory clearance of immunotoxins were assessed in rhesus monkeys. The immunotoxins studied were T cell–specific monoclonal anti-T11 antibodies conjugated by disulfide linkage to ribosome-inactivating toxins. Intact immunotoxin was detectable in the circulation of the monkeys following a single intravenous infusion. This was demonstrated by quantitative flow-cytometric analysis, gel-filtration, and sodium dodecyl sulfate–gel electrophoresis. This intact conjugate was shown to be functional in the plasma of the infused animals in an in vitro cytotoxicity assay. However, a number of factors contributed to bring the level of circulating immunotoxin to a less than optimal level. When conjugated to a ribosome-inactivating toxin, the antibody was cleared more rapidly than was the native antibody. Furthermore, following infusion, some breakdown of the conjugate occurred, resulting in the generation of detectable levels of circulating free antibody. The present data indicate the feasibility of using immunotoxins as therapeutic tools in man.

Introduction

Monoclonal antibodies have already proved to be useful therapeutic agents in man. Anti-T lymphocyte-specific monoclonal antibodies have been used successfully in treating episodes of renal transplant rejection (1–3) and in the elimination of mature T lymphocytes from donor cell populations in bone marrow transplantation (4, 5). The exquisite specificity of these reagents suggests that they should also facilitate the targeting of toxins and drugs to restricted cell populations in man (6). Work has already begun in a number of laboratories to prepare and test such immunotoxin conjugates (7, 8).

A number of problems will require clarification before such conjugates can be rationally used in therapeutic trials in man. The in vivo stability of various chemical linkages must be assessed, the circulatory clearance and metabolic fate of these conjugates must be determined, and the optimal dosing schedules for delivery of conjugates to target cells must be established. The nonhuman primate provides an ideal experimental model to address these issues. Man and nonhuman primate species share many physiologic properties. We and others have also recently demonstrated a remarkable conservation of their cell surface antigens on bone marrow–derived elements (9–13). Thus, an antibody-toxin or antibody-drug conjugate with specificity for a restricted lymphocyte population in man can be studied in an experimental animal in which the structure recognized by that antibody is conserved. This provides a much more rational system for studying these conjugates than using, for example, the commonly employed nude mouse.

We have initiated a series of studies in the rhesus monkey on the in vivo use of monoclonal antibodies as therapeutic agents. In these studies we have selected for investigation antibodies which recognize the erythrocyte-rosette receptor (T11), a structure present on all mature T lymphocytes. In this work we have gained considerable experience in using a series of different monoclonal anti-T11 antibodies in the monkey (14). In the present studies we have explored the use of monoclonal anti-T11 antibodies chemically linked to the ribosome-inactivating toxins gelonin and saporin, which are potent inhibitors of protein synthesis in cell-free mammalian in vitro systems. Because these toxins are incapable of binding to cells, they are not toxic in vitro to intact cells (15). Gelonin and saporin, however, become cytotoxic when linked to antibodies, such as anti-T11, that bind to cell surface antigens (16–19).

It is clear from in vitro studies that the nature of the chemical linkage between a monoclonal antibody and a toxin can profoundly alter the action of that toxin on a target cell population. For example, while a conjugate of an antibody and gelonin is cytotoxic in vitro when these proteins are linked by cleavable disulfide bonds, a similar antibody-gelonin conjugate demonstrates little in vitro cytotoxicity when those proteins are linked by a noncleavable thioether bond (16). The ideal linkage between a monoclonal antibody and toxin or drug is one which will remain intact in the circulation of the individual, but will be cleaved either on the surface membrane of the target cell or in an internal compartment of that cell.

In the present studies we have assessed the circulatory clearance and stability in vivo in the rhesus monkey of T cell–specific monoclonal anti-T11 antibodies conjugated by disulfide linkage to ribosomal toxins. We have found that significant amounts of such conjugates remain intact and functional in the monkey after infusion.

Methods

Animals. The monkeys used in this study were adult *Macaca mulatta* (rhesus) and ranged in weight from 4 to 10 kg. They were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the National Research Council.

Materials. 2-iminothiolane HCl, *N*-succinimidyl 3-(2-pyridyldi-

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thio)propionate (SPDP)[1] and 1,3,4,6-tetrachloro-3*a*, 6*a*-diphenylglycoluril (Iodo-gen) were purchased from Pierce Chemical Co., Rockford, IL. Protein A–Sepharose CL-4B and 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol (bis-tris) were obtained from Sigma Chemical Co., St. Louis, MO. [Methyl-^{3}H]thymidine (2 Ci/mmol) and Na ^{125}I (carrier free, 100 mCi/ml in 0.1 M NaOH) were purchased from Amersham Corp., Arlington Heights, IL. Goat anti-mouse IgG was obtained from Jackson Immuno Research Laboratories, Inc., Avondale, PA. Fluorescein-labeled goat anti-mouse Ig (G/M-FITC) and fluorescein-labeled goat anti-rabbit Ig (G/R-FITC) were from Meloy Laboratories, Springfield, VA. Rabbit anti-gelonin and rabbit anti-saporin antiserum were prepared by injection of the purified proteins in complete Freund's adjuvant into New Zealand White rabbits (16).

Murine monoclonal antibody anti-T11$_{1A}$. Anti-T11$_{1A}$ is a monoclonal antibody of isotype IgG1 that reacts with the sheep erythrocyte rosette receptor, a determinant found on all resting human T cells (20). This antibody reacts with the homologous structure on T cells from other primate species, including *M. mulatta* (10). The antibody was produced by hybridoma cells grown as ascites tumors in pristane-primed BALB/c mice. It was purified by precipitation with $(NH_4)_2$ SO_4, ion-exchange chromatography using carboxymethyl cellulose (CM-52, Whatman, Inc., Clifton, NJ), and by gel filtration through Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) as described previously (14, 16). The antibody was judged pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and by isoelectrofocusing using polyacrylamide gel plates (Ampholine; pH range, 3.5–9.5) purchased from LKB Instruments, Inc. (Gaithersburg, MD) (16). The purified antibody was sterilized by filtration through Millex-GV filters (0.22 μm; Millipore/Continental Water Systems, Bedford, MA), and was tested for the presence of endotoxin using the Limulus Amebocyte Lysate Test (Microbiological Associates, Walkersville, MD).

Ribosome-inactivating proteins. Seeds from *Gelonium multiflorum* were from United Chemical and Allied Products, Calcutta, India, and were obtained through the Meer Corp., North Bergen, NJ. Gelonin (M_r 30,500) was purified as described previously (15). Seeds of *Saponaria officinalis* were purchased from Germania Seed Co., Chicago, IL, and the major ribosome-inactivating protein, termed here saporin (M_r 29,500), was purified from the seeds by the method of Stirpe et al. (21).

Preparation and purification of disulfide-linked conjugates between anti-T11$_{1A}$ and the ribosome-inactivating proteins. Anti-T11$_{1A}$ (1 mg/ml) in 100 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM) was mixed with SPDP (60 μM) added from a freshly made stock solution (10 mM) in ethanol. The mixture was incubated at 30°C for 30 min and then dialyzed against the pH 7.0 buffer to remove excess reagent. About 2.3 dithiopyridyl groups were incorporated per molecule of antibody, measured as described previously (22).

Gelonin (2 mg/ml) in 60 mM triethanolamine/HCl buffer, pH 8.0, containing EDTA (1 mM) was treated with 2-iminothiolane (1 mM) at 0°C for 90 min under argon, following the procedure described previously (23, 24). Excess reagent was removed by gel filtration at 4°C on a column of Sephadex G-25 (fine) equilibrated with 5 mM bis-tris/acetate buffer, pH 5.8, containing NaCl (50 mM) and EDTA (1 mM). About 0.6–0.7 sulfhydryl groups were added per gelonin molecule as determined by the method of Ellman (25). Saporin was modified with 2-iminothiolane using exactly the same conditions.

Conjugation of anti-T11$_{1A}$ and ribosome-inactivating proteins was effected by mixing the modified anti-T11$_{1A}$ in the above pH 7.0 buffer with an equal weight (equivalent to a fivefold molar excess) of the modified gelonin or the modified saporin in the above pH 5.8 buffer (16). The pH of the mixture was then raised to 7.0 by addition of 0.5 M triethanolamine/HCl buffer, pH 8.0, and the conjugation reaction allowed to proceed at 4°C for 20 h under argon.

The immunotoxin conjugates were purified from nonconjugated antibody, nonconjugated ribosome-inactivating proteins, and aggregates of high molecular weight by gel filtration through a column of Sephacryl S-300 followed by ion-exchange chromatography with carboxymethyl cellulose (CM-52, Whatman, Inc.) using conditions that are described in detail elsewhere (16).

The polyacrylamide/dodecyl sulfate gel illustrated in Fig. 1 *A* shows that the purified conjugate between anti-T11$_{1A}$ and gelonin (lane *5*) contained antibody linked to one (M_r 190,500) or two (M_r 221,000) molecules of gelonin and that the mixture did not contain nonconjugated antibody (M_r 160,000) or nonconjugated gelonin. The immunotoxin that contained saporin was similarly pure (results not shown). The yield of immunotoxin was generally ~40 mg of purified conjugate, starting from 100 mg of anti-T11$_{1A}$.

The immunotoxin preparations were passed through a 2-ml column (for 40 mg of protein in 40 ml of phosphate-buffered saline [PBS]) of Detoxigel (Pierce Chemical Co.) at 4°C as a precaution to minimize endotoxin levels. The conjugates were then sterilized by passage through a 0.22-μm filtration membrane (Millex-GV) and tested for the presence of endotoxin using the Limulus Amebocyte Lysate test. Both the binding activity of the antibody and the ribosome-inactivating activity of the toxins were not damaged by the conjugation reactions (16).

Preparation of radiolabeled immunotoxins from radioiodinated gelonin and radioiodinated saporin. Each ribosome-inactivating protein (0.12 mg in 0.12 ml) in 50 mM triethanolamine/HCl buffer, pH 7.25, containing NaCl (100 mM) was radioiodinated by the Iodo-gen method

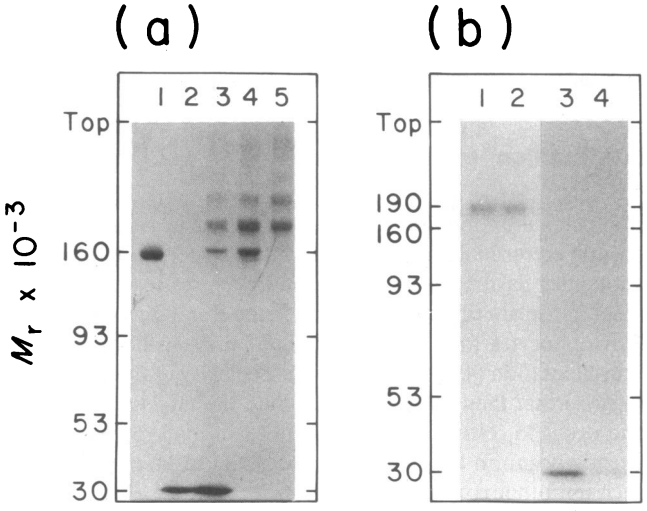

Figure 1. Analysis of purification of anti-T11$_{1A}$–gelonin by SDS-PAGE. *A*, 5–10% (wt/vol) polyacrylamide gradient gel run under nonreducing conditions; lane *1*, anti-T11$_{1A}$; lane *2*, gelonin; lane *3*, conjugation reaction mixture containing nonconjugated gelonin, nonconjugated antibody, and bands corresponding to antibody conjugated to one (M_r, 190,000) and to two (M_r, 221,000) molecules of gelonin; lane *4*, conjugation mixture after purification through gel filtration on Sephacryl S-300; lane *5*, purified conjugate after further purification by carboxymethyl cellulose ion-exchange chromatography. *B*, radioautograph of gels such as that shown in *A* run with anti-T11$_{1A}$–toxin conjugates that were made with ^{125}I-labeled gelonin (lanes *1* and *3*) or with ^{125}I-labeled saporin (lanes *2* and *4*). Lanes *1* and *2* (containing ~4×10^3 cpm) were run under nonreducing conditions, while lanes *3* and *4* (containing ~2,400 and 700 cpm, respectively) were run under reducing conditions. Radioautography was for 36 h (lanes *1* and *2*) or 72 h (lanes *3* and *4*). The calibration of M_r was from the mobility of IgG (160,000), phosphorylase b (93,000), glutamate dehydrogenase (53,000), and carbonic anhydrase (30,000).

1. *Abbreviations used in this paper:* bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; FACS, fluorescence-activated cell sorter; G/M-FITC, fluorescein-labeled goat anti-mouse Ig; G/R-FITC, fluorescein-labeled goat anti-rabbit Ig; Iodo-gen, 1,3,4,6-tetrachloro-3*a*, 6*a*-diphenylglycoluril; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)-propionate; T11, erythrocyte-rosette receptor.

of Fraker and Speck (26), using 1 mCi of Na ^{125}I and 1.5 μg of Iodo-gen in each reaction. The mixtures were incubated at 0°C for 20 min and then the proteins were separated from excess iodine by two successive gel filtration steps (Sephadex G-25 superfine) with an incubation at 25°C for 20 min with nonradiolabeled NaI (10 mM) in between each step. The columns (4.5 × 1.2 cm) were equilibrated with 60 mM triethanolamine/HCl buffer, pH 8.0, containing EDTA (1 mM). The specific radioactivity of the gelonin was 1.0×10^7 cpm/μg, and that of the saporin was 0.9×10^7 cpm/μg.

Each of the radiolabeled ribosome-inactivating proteins (0.1 mg in 0.6 ml) was modified with 2-iminothiolane (1.0 mM). The modified toxins were each conjugated with modified anti-T11$_{1A}$ (0.65 mg) as described above. The conjugates were purified from excess nonconjugated ribosome-inactivating protein by gel filtration through Sephacryl S-300 (48 × 1.0 cm) equilibrated in pyrogen-free PBS. The peak fraction of each immunotoxin was passed through a column (0.2 ml) of Detoxigel and stored at 4°C until its use in vivo within 48 h of preparation. A sample of each conjugate was analyzed by SDS-PAGE under reducing and nonreducing conditions. Fig. 1 *B* shows the location of the radioactive protein by radioautography. Most of the radiolabeled protein (>90%) comigrates with the molecular weight of a conjugate (M_r 190,500) in the lanes run under nonreducing conditions (lanes *1* and *2*). However, under reducing conditions, the radioactivity comigrates with the bands corresponding to gelonin or saporin. Samples containing 2.2×10^7 cpm of anti-T11$_{1A}$-gelonin and 2.1×10^7 cpm of anti-T11$_{1A}$-saporin were used as trace labels in vivo as described below.

Protocol for anti-T11 and anti-T11-toxin infusions. Rhesus monkeys were sedated with ketamine throughout the infusion of the monoclonal antibodies and the antibody-toxin conjugates. Antibody or antibody-toxin conjugate was delivered intravenously by continuous infusion over a 4-h period in a 20-ml volume. The trace-radiolabeled antibody-toxin conjugates were delivered as a single intravenous bolus immediately following the infusions of 5 mg/kg of the unlabeled conjugates. Heparinized blood samples were obtained prior to the start of the infusions, at 2 h into the infusions, 30 min and 2 h after the infusions, and daily thereafter. By using heparinized blood, we were able to do studies on both isolated peripheral blood lymphocytes (PBL) and plasma from the same samples. We therefore have utilized plasma rather than serum in these experiments. Total urine collections were obtained every 24 h for 6 d following the infusions with the trace-radiolabeled conjugates. Samples were assayed for ^{125}I-radioactivity in plasma and urine to determine the rate of clearance and excretion of the radiolabeled toxin.

Radioimmunoassay (RIA) for mouse Ig. Goat anti-mouse IgG was adsorbed for 2 h at 4°C onto flexible polyvinyl microtiter plates (Becton Dickinson & Co., Oxnard, CA). Wells were then incubated with 1% bovine serum albumin (BSA) in PBS for 1 h to block nonspecific binding sites on the plastic. Equal volumes of test plasma from the infused monkeys and a mouse monoclonal ^{125}I-IgG were incubated in these wells for 1 h at room temperature. The wells were washed extensively, cut from the plate, and counted in an automated gamma counter.

Measurement of anti-T11$_{1A}$-toxin in rhesus plasma by cell staining and flow cytometry. PBL were prepared from heparinized venous blood of normal human volunteers by density gradient centrifugation using a 9% Ficoll (Sigma Chemical Co.)/34% sodium diatrizoate (Sterling Drug, New York) solution having a specific gravity of 1.076 g/ml. The cells were treated with 0.15 M NH_4Cl to lyse erythrocytes and washed with Hanks' balanced salt solution. Serial threefold dilutions of each rhesus plasma sample were prepared and used to stain 1×10^6 cell aliquots of normal human PBL. The stained samples were then incubated with G/M-FITC or incubated with the rabbit anti-gelonin or the rabbit anti-saporin antibody followed by G/R-FITC. These samples were then analyzed on an Epics V cell sorter (Coulter Electronics, Hialeah, FL). The titer of anti-T11$_{1A}$ or toxin in the plasma of an experimental animal at any time point is expressed as the reciprocal of the lowest dilution of plasma that can maximally stain normal PBL.

SDS-PAGE. Protein purification and cross-linking reactions were analyzed by SDS-PAGE in gel slabs (145 × 90 × 0.75 mm) cast with acrylamide gradients (5–10% wt/vol) prepared by the method of Laemmli (27). Sample buffers for gels run under nonreducing conditions contained 10 mg/ml iodoacetamide (24). Samples of plasma (50 μl) containing up to 5,000 cpm of radioiodinated anti-T11$_{1A}$ conjugates were mixed with 50 μl of 0.2 M iodoacetamide (24) and then lyophilized. The dry pellets were dissolved in 150 μl of 35 mM Tris/HCl buffer, pH 6.8, containing SDS (4% wt/vol) and urea (8 M) and heated at 100°C for 5 min. The samples were then submitted to electrophoresis on gel slabs (145 × 90 × 1.5 mm) as described above.

In vitro cytotoxicity assay. Anti-T11$_{1A}$ conjugates were tested for cytotoxicity using the cell line 1022 created by the in vitro immortalization of cotton-top tamarin splenocytes by *Herpesvirus ateles* (28). Cells were grown at 37°C in a 5% CO_2 humidified atmosphere in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with heat-inactivated (56°C for 30 min) fetal calf serum (10%) (Flow Laboratories, McLean, VA), L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). The cells were maintained in asynchronous exponential growth by dilution twice per week to maintain a concentration of $1–2 \times 10^5$ cells/ml. The cytotoxicity assay was performed in 96-well (flat bottom) polystyrene microtiter plates (Microtest III; Becton-Dickinson & Co.) with 5×10^4 cells in each well. Equal volumes of medium containing serial dilutions of the immunotoxins or plasma samples being tested for the presence of immunotoxins were added to each well to a total volume of 0.2 ml, and the cells were incubated for 3 d. The cells were then pulsed for 2 h with [^{3}H]thymidine (0.8 μCi/well), harvested, and lysed onto glass fiber discs using a PHD cell harvester (Cambridge Technology Inc., Cambridge, MA). The radioactivity that was retained on the filters after washing with water and ethanol was measured in 2 ml of Betafluor (National Diagnostics, Inc., Somerville, NJ) using a Tri-Carb 4530 scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). All wells were set up in triplicate and each experiment was repeated twice. Values of ID_{50} were estimated as the concentration of immunotoxin that caused 50% inhibition of [^{3}H]thymidine incorporation.

Results

Clearance of anti-T11$_{1A}$ from the circulation following infusion of conjugated and nonconjugated antibody. A solid phase RIA was used to assess plasma levels of nonconjugated monoclonal antibody in monkeys at various intervals following the infusions. Anti-T11$_{1A}$ was detectable in the monkey plasma for only 24 h following a single infusion at a dose of 0.2 mg/kg, reaching a peak concentration of 8 μg/ml of mouse IgG (Fig. 2 *A*). Following single infusions at a dose of 2 mg/kg, anti-T11$_{1A}$ reached a peak plasma concentration of 65–90 μg/ml of mouse IgG and was detectable in the plasma for at least 7 d (Fig. 2 *B*).

The plasma concentrations of anti-T11$_{1A}$ were then assessed by measuring the mouse Ig in the plasma following similarly performed infusions of disulfide-linked antibody-toxin conjugates in rhesus monkeys. Lower peak plasma levels of the infused antibody could be attained following infusion of these conjugates than were achieved through infusion of an equivalent dose of monoclonal antibody alone. Peak plasma levels of only 10–20 μg/ml of mouse IgG were achieved following 1 mg/kg infusions of disulfide-linked anti-T11$_{1A}$-gelonin and anti-T11$_{1A}$-saporin (Fig. 2 *C*). Infusions of 5 mg/kg anti-T11$_{1A}$-gelonin and anti-T11$_{1A}$-saporin resulted in peak plasma levels of 70–90 μg/ml of mouse IgG (Fig. 2 *D*), essentially equivalent concentrations to those achieved with 2 mg/kg infusions of anti-T11$_{1A}$ alone.

Clearance of radiolabeled toxin from the circulation following infusion of anti-T11$_{1A}$ conjugated to radioiodinated toxin. Samples of plasma were taken from monkeys at fixed intervals following the infusion of anti-T11$_{1A}$ conjugated to trace-radiolabeled toxin. Fig. 3 *A* shows that the amount of circulating radioactivity falls to about 60% of that infused after only 2 h, and to about 13% by 1 d following infusion of the radiolabeled ge-

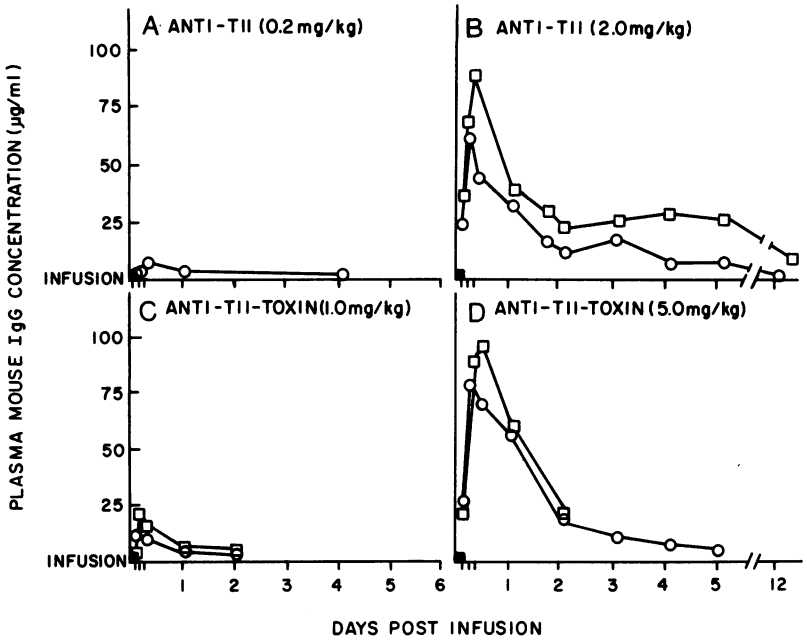

Figure 2. Plasma concentration of anti-$T11_{1A}$ following infusions of antibody alone or immunotoxins into rhesus monkeys. Plasma concentrations, determined by RIA, are shown at regular intervals after infusion of (*A*) anti-$T11_{1A}$ at a dose of 0.2 mg/kg, (*B*) anti-$T11_{1A}$ at a dose of 2.0 mg/kg (○ and □ represent values from separate experiments), (*C*) anti-$T11_{1A}$ conjugated to gelonin (○) and saporin (□) delivered at a dose of 1.0 mg/kg, and (*D*) anti-$T11_{1A}$ conjugated to gelonin (○), and saporin (□) delivered at a dose of 5.0 mg/kg.

lonin–antibody conjugate. It takes more than 3 d for the injected radioactivity in the circulation to fall to below 1% of that infused. Parallel studies done using a conjugate made with ^{125}I-saporin gave similar results (data not shown).

Comparison of data shown in Fig. 3 *A* with that shown in Fig. 2 *D* suggests that the plasma concentration of conjugate, as measured by the ^{125}I-trace, falls more rapidly than the plasma concentration of anti-$T11_{1A}$ as measured by RIA. While the plasma concentration of labeled toxin falls to 13% of the total infused material by 1 d following injection of immunotoxin, mouse IgG reached a similar relative plasma concentration by 4 d after infusion. These findings suggest that the toxin may be metabolized at a faster rate than the antibody. Since there is no nonconjugated toxin or antibody in the initial immunotoxin preparation, we infer that the toxin may be cleaved from the antibody and cleared from the circulation at a faster rate than the clearance of the antibody.

Total urine collections were made daily following infusions of the radiolabeled conjugates and samples were counted for radioactivity. The amount of the radiolabel excreted by one rhesus monkey as a proportion of the total amount injected is shown in Fig. 3 *B*. After 6 d, 79% of the injected radioactivity can be accounted for by excretion in the urine. None of the radioactivity in the urine could be precipitated by TCA, showing that the radioactivity in the urine was not contained in protein, but rather in molecules of low molecular weight.

The apparent disparity between the rate of clearance of mouse IgG and that of radiolabeled toxin following infusion of the immunotoxins suggested that these conjugates may not be completely stable in the circulation of the infused monkeys. Mono-

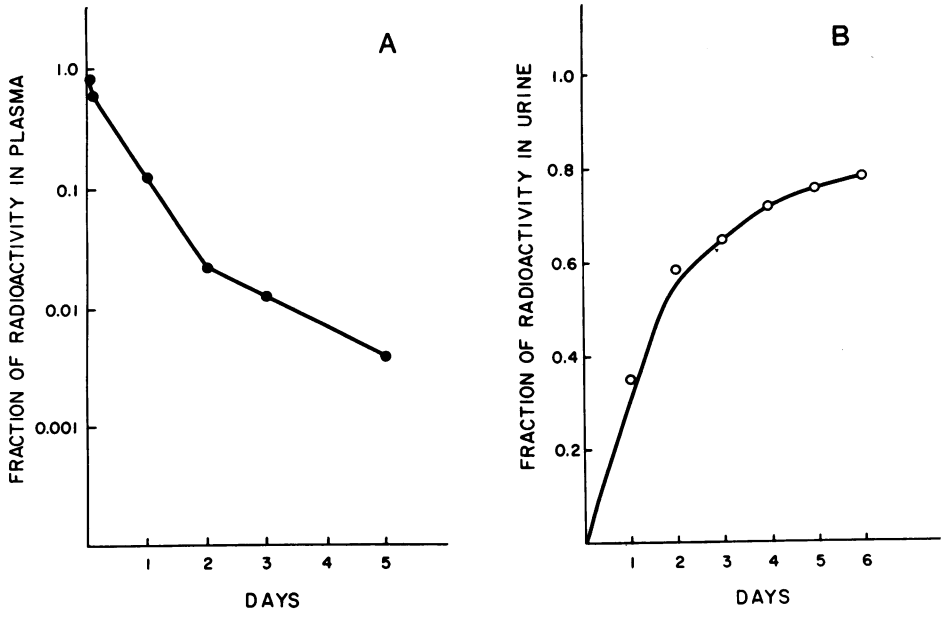

Figure 3. Rate of plasma clearance and urine excretion of ^{125}I after administration of anti-$T11_{1A}$–gelonin conjugate that was prepared from radiolabeled gelonin. The radiolabeled conjugate (2.2×10^7 cpm) was injected into a 4-kg animal as detailed in Methods. (*A*) Samples (1 ml) of plasma were counted for radioactivity and the fraction of the initial radioactivity that remained in the plasma was calculated assuming a plasma volume of 43 ml/kg (29). (*B*) Samples (1 ml) of daily total urine collections were counted for radioactivity and the cumulative fraction of the initial radioactivity that was excreted in the urine was calculated.

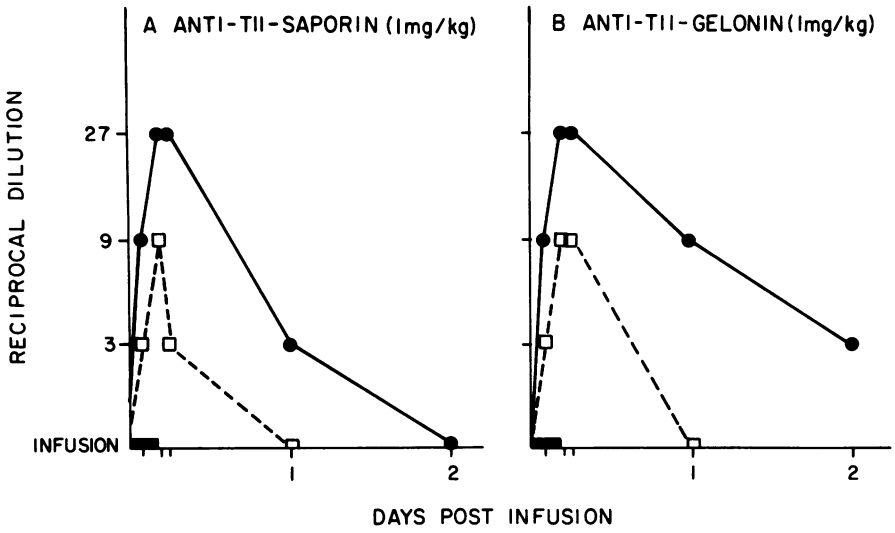

Figure 4. Relative concentrations of anti-$T11_{1A}$ and toxin in the plasma of rhesus monkeys after infusion of immunotoxins. Serial threefold dilutions of plasma taken from monkeys at regular intervals following immunotoxin infusion were used to stain normal human PBL. These stained cell populations were developed with either G/M-FITC (●) or rabbit anti-gelonin or rabbit anti-saporin followed by G/R-FITC (□) and analyzed by flow cytometry. The sample is plotted versus the reciprocal of the last dilution of that plasma which demonstrates maximal staining under these conditions.

clonal antibody conjugated by disulfide linkage to gelonin was shown to remain at least 70% intact after a 2-d incubation in vitro at 37°C in rhesus monkey plasma, as determined by quantitative flow-cytometric analysis (data not shown). The stability of the anti-$T11_{1A}$-toxin conjugates in the plasma of the rhesus monkeys following infusion was then assessed using three different approaches: indirect immunofluorescence and fluorescence-activated cell sorter (FACS) analysis, gel-filtration, and SDS-PAGE.

Stability of disulfide-linked conjugates in the plasma assessed by indirect immunofluorescence and FACS analysis. Normal human PBL were incubated with serial threefold dilutions of plasma samples taken from rhesus monkeys at regular intervals following infusion with the anti-$T11_{1A}$-toxin conjugates. If a significant amount of the conjugate remains intact in the plasma after infusion, the normal human PBL should have both mouse Ig and toxin bound to their surface membranes after that incubation. The toxins do not bind to a cell surface unless they are covalently linked to a molecule that can bind to cells. The normal human PBL, following incubation with plasma from the infused monkeys, were therefore developed with either G/M-FITC or rabbit anti-toxin antibody followed by G/R-FITC. These parallel samples were then analyzed by flow cytometry. As shown in Fig. 4, both mouse Ig and toxin could be readily detected on the surface membrane of the PBL. The level of detectable mouse Ig and toxin in the serum did not, however, appear to fall in a parallel fashion over time after the infusion. Rather, the titer of the toxin appeared to fall somewhat more rapidly than that of the antibody. However, these data indicated that at least some of the anti-$T11_{1A}$ in the plasma of these monkeys had toxin still linked to it. Since this approach did not lend itself to precise quantitation, and would not detect any free toxin which may exist in the plasma of these animals as a result of the cleavage of the linker in the circulation, other techniques were employed to determine the stability of the disulfide-linked antibody-toxin conjugates. In these experiments, conjugates were utilized which were radiolabeled in the toxin portion of the immunotoxin.

Stability of the disulfide-linked conjugates circulating in plasma assessed by biochemical analysis of the trace-radiolabeled conjugate. Plasma samples from monkeys, following inoculation with anti-$T11_{1A}$ conjugated to ^{125}I-labeled toxin, were submitted to gel filtration on a column of Sephacryl S-300 equilibrated in PBS. The column was calibrated with samples of purified anti-$T11_{1A}$-toxin conjugate (M_r ~190,000) and samples of purified gelonin and saporin (M_r about 30,000) before and after the series of runs with the samples of monkey plasma. Their elution positions are indicated in Fig. 5 *A* and *D*. Fig. 5 *A* shows that, at 30 min following delivery of the conjugate prepared with ^{125}I-gelonin, more than 90% of the radioactivity in the plasma was found to fractionate with the molecular weight of intact conjugate. There was a small peak of radioactivity having the molecular weight of free gelonin that accounts for only 6% of the radioactivity in the sample, while 1–2% of the radioactivity was found in molecules of low molecular weight that eluted at one column volume. 2 h after delivery of the radiolabeled conjugate into the monkey, 90% of the radioactivity in the plasma was still found to fractionate as intact conjugate (Fig. 5 *B*) with virtually no free ^{125}I-gelonin detectable. About 6% of the radioactivity at this time was found in the fraction of low molecular weight. In the analysis of the samples taken after 1, 2, 3, and 5 d (Fig. 5 *C* to *F*), the proportion of the radioactivity in each sample found to fractionate as intact conjugate progressively declined to about 40–50%, the balance being made up of the fraction of low molecular weight. At no time point was there more than a trace of free ^{125}I-gelonin.

The data shown in Fig. 3 *A* can be replotted in light of the results shown in Fig. 5 *A* to *F*. In Fig. 5 *G*, the fraction of initial radioactivity in plasma that was found in intact conjugate and the fraction found degraded in molecules of small molecular weight were plotted separately against the time after infusion. After 1 d, 9% of the initial radiolabeled conjugate was still in the circulation, and this falls to <1% by day three.

Samples of plasma taken at different times following infusion were also analyzed by SDS-PAGE. Fig. 6 shows an example of a radioautograph of such a gel. The result demonstrates that the ^{125}I-labeled gelonin was still covalently bonded to a protein that has the molecular weight of IgG, and is, therefore, likely to be intact anti-$T11_{1A}$-^{125}I-gelonin conjugate. Thus, the ^{125}I-label that fractionated at a M_r of ~190,000 upon gel filtration under nondenaturing conditions (Fig. 5 *A* to *F*) was from intact disulfide-linked radiolabeled conjugate. Parallel studies performed on plasma samples from monkeys following infusion of a conjugate

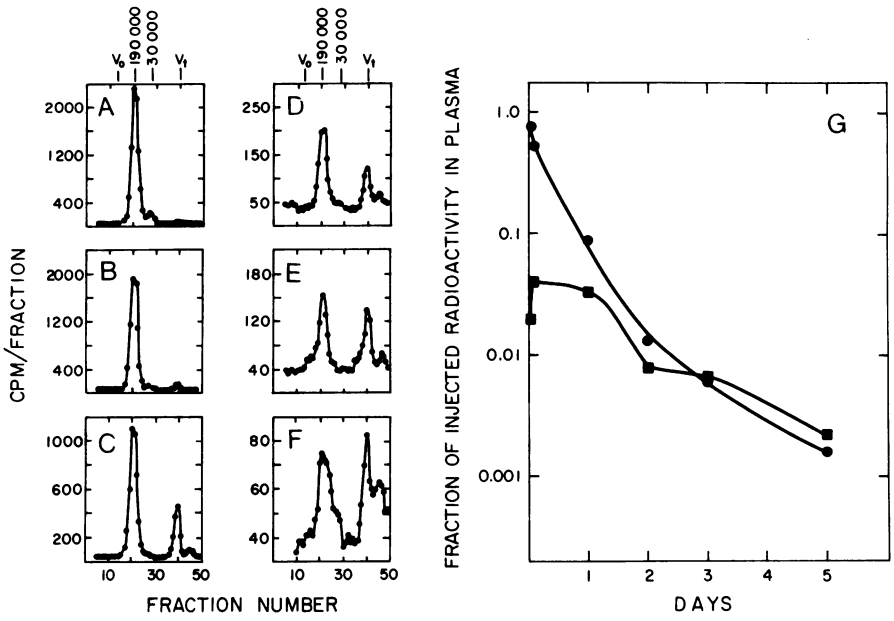

Figure 5. Analysis by gel filtration of plasma from an animal infused with anti-$T11_{1A}$-gelonin that contained radioiodinated gelonin. (*A* to *F*) Samples of plasma (0.4–1.0 ml containing 500–16,000 cpm) were submitted to gel filtration on a column of Sephacryl S-300 (50 × 1.0 cm) equilibrated in PBS. Each run was pumped at 0.25 ml/min using a Pharmacia FPLC pump, and 1.0-ml fractions were collected and counted for radioactivity. V_0 (void volume) was calibrated with blue dextran; 190,000 was the elution position of purified anti-$T11_{1A}$-toxin conjugate (M_r ~190,000); 30,000 was the elution position of the purified toxins (M_r ~30,000); V_t was the total volume of the column. (*G*) The fraction of the initial radioactivity in plasma that was found to be intact conjugate (●), and the fraction of the initial radioactivity found to be in molecules of small molecular weight (■), were calculated by combining the data in *A* to *F* with the data in Fig. 3 *A*.

made with ^{125}I-saporin gave similar results (data not shown) to those shown in Figs. 5 and 6 for the conjugate made with ^{125}I-gelonin.

In vitro cytotoxicity of the circulating immunotoxin conjugates. The in vitro cytotoxicity of plasma samples from rhesus monkeys following infusion with immunotoxins was assessed to determine the functional integrity of these conjugates in the animals. The cytotoxicity of the conjugates was tested in vitro on the 1022 cell line, an *H. ateles* immortalized cotton-top tamarin T cell line which expresses the T11 antigen. The incorporation of [^{3}H]thymidine, a reflection of the rate of DNA replication of this line, was measured in the presence of the conjugates. Incorporation of [^{3}H]thymidine falls when the cells are incubated in the presence of toxic concentrations of immunotoxin. The conjugates of anti-$T11_{1A}$ with saporin and with gelonin were potent inhibitors of the growth of 1022 cells (Fig. 7 *A*), while saporin and gelonin themselves were nontoxic up to a concentration of 10^{-7} M. The antibody alone was also nontoxic. The ID_{50} of both conjugates was 4×10^{-10} M.

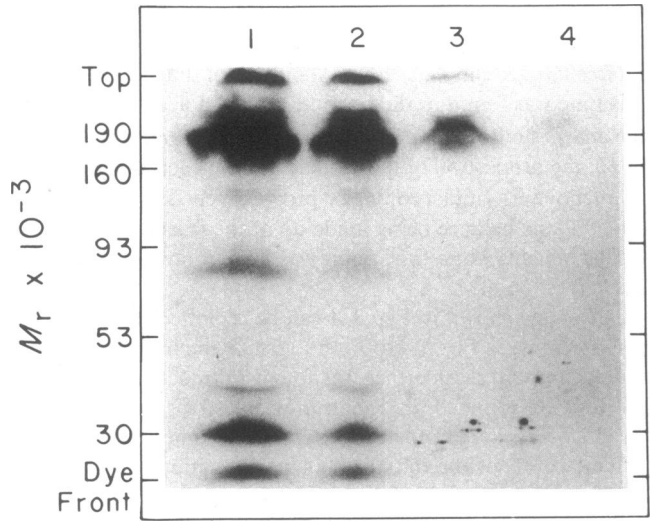

Figure 6. SDS-PAGE analysis of plasma containing ^{125}I-gelonin conjugated to anti-$T11_{1A}$. Samples were run on a 5–10% (wt/vol) polyacrylamide gradient gel as described in Methods. The samples were taken 30 min (lane *1*), 2 h (lane *2*), 1 d (lane *3*) and 2 d (lane *4*) after completion of the infusion of the immunotoxin. The gel was dried and submitted to radioautography using XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) using intensifying screens for 2 or 4 d at −70°C. The calibration of M_r was from the mobility of intact purified anti-$T11_{1A}$-gelonin conjugate (190,000), IgG (160,000), phosphorylase b (93,000), aldolase (40,000), and carbonic anhydrase (30,000).

In order to determine the functional concentration of anti-$T11_{1A}$-saporin and of anti-$T11_{1A}$-gelonin in the plasma of rhesus monkeys following infusion of these conjugates, samples of the monkeys' plasma were serially diluted in medium and the same cytotoxicity assay was performed (Fig. 7 *B*). Plasma of monkeys taken 2 h following infusion of these immunotoxins at a dose of 5 mg/kg was cytotoxic to the 1022 cells even when diluted greater than 100-fold in medium. These samples caused 50% inhibition of DNA replication (ID_{50}) when diluted to 0.039:1 (vol/vol) (anti-$T11_{1A}$-saporin) and to 0.11:1 (vol/vol) (anti-$T11_{1A}$-gelonin) (these values are averages of two independent experiments; each datum point done in triplicate). In contrast, plasma from these same animals taken before the infusions and plasma from animals 2 h after infusion of immunotoxins at a dose of 1 mg/kg were nontoxic when diluted 1:5 vol/vol or more with medium (Fig. 7 *B*). Comparing the ID_{50} values for immunotoxins with the ID_{50} values of the monkey plasma containing immunotoxins, we estimated that functional concentrations of immunotoxins in the monkey plasma at 2 h after infusion at a dose of 5 mg/kg were 1×10^{-8} M and 4×10^{-9} M for anti-$T11_{1A}$-saporin and anti-$T11_{1A}$-gelonin, respectively.

Determination of the concentration of anti-$T11_{1A}$ in these same plasma aliquots was performed using two different assay techniques: an RIA (see Fig. 2 *D*) and quantitative FACS analysis comparing the staining of 1022 cells using serial dilutions of the plasma samples or using serial dilutions of the purified conjugates. Both approaches yielded similar results, demonstrating 5×10^{-7} M (80 μg/ml) concentrations of antibody in the plasma samples. Thus, higher concentrations of antibody than functional conjugates were present in these plasma samples taken 2 h following infusion.

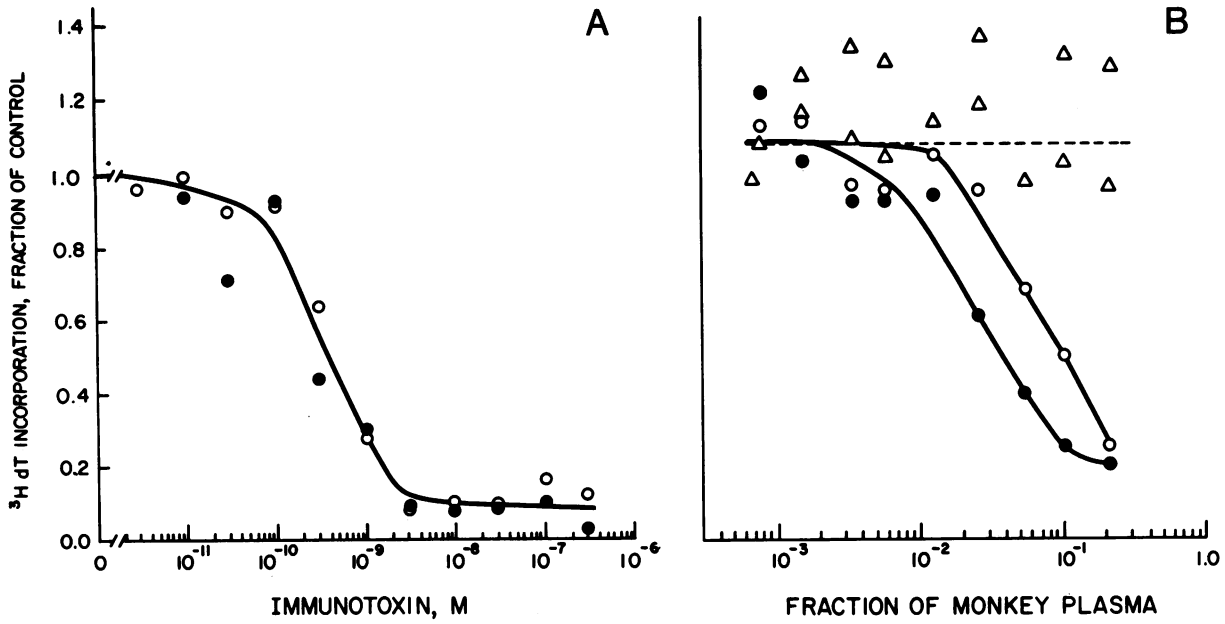

Figure 7. (*A*) cytotoxicity of immunotoxins on the 1022 cell line. Inhibition of DNA synthesis as measured by the inhibition of the incorporation of [^{3}H]thymidine relative to controls was used as a measure of cytotoxicity as described in Methods. Cells were incubated 3 d at 37°C in the presence of anti-T11$_{1A}$-saporin (●) or anti-T11$_{1A}$-gelonin (○). Each point is an average result of two independent experiments, each done in triplicate. (*B*) cytotoxicity of plasma samples on the 1022 cell line. Cells were incubated 3 d at 37°C in growth medium containing (△) monkey plasma which did not contain immunotoxins (prior to infusion), (●) monkey plasma 2 h after infusion of 5 mg/kg anti-T11$_{1A}$-saporin, and (○) monkey plasma 2 h after infusion of 5 mg/kg anti-T11$_{1A}$-gelonin. Each point is an average result of two independent experiments, each done in triplicate.

Discussion

In initiating studies in nonhuman primates, we previously examined the effects of three different monoclonal anti-T11 antibodies on the circulating T cell pool in the rhesus monkey (14). Single infusions of these antibodies at a dose of 2 mg/kg resulted in the coating of circulating T lymphocytes with antibody, the modulation of T11 off the T cell surface, and the transient clearance of T cells from the circulation. Yet, a significant variation was seen in the extent to which these changes occurred with these different antibodies. A useful monoclonal antibody for studying the utility of antibodies in targeting toxins to specific cell populations in vivo is one which is readily detected in vivo on the cell population to which it binds. We therefore selected anti-T11$_{1A}$ for use in the present studies. Of the three anti-T11 reagents examined in the rhesus monkey, anti-T11$_{1A}$ was the most readily detected in coating the monkey's circulating T lymphocytes. When compared with the other anti-T11 antibodies examined, anti-T11$_{1A}$ was most rapidly cleared from the serum, least efficient in eliminating T cells from the circulation, and caused an intermediate degree of modulation of the T11 antigen from the T lymphocyte surface membrane in vitro (14).

In the present studies we have shown that detectable concentrations of disulfide-linked antibody-toxin conjugate remain intact in the circulation of the rhesus monkey following a single intravenous infusion. This was directly demonstrated using three different techniques: quantitative flow-cytometric analysis, gel-filtration, and SDS-PAGE. Moreover, this intact conjugate was shown to be functional in the plasma of the infused animals in an in vitro cytotoxicity assay. In fact, the plasma concentration of functional conjugate at 2 h following the 5 mg/kg infusions was estimated in vitro to be about 10^{-8} M. The radiolabeled tracer conjugate injected at the end of the 4-h infusion showed that intact immunotoxin levels take 24 h to decline to about 10% of the initial level, and 3 d to decline to less than 1%. Thus, the concentration of immunotoxin remains above 10^{-10} M for about 3 d. Since the ID_{50} for this conjugate in the 3-d in vitro assay is 4×10^{-10} M (Fig. 7 *A*), this evidence suggests that the concentration of immunotoxin in the plasma remains above a level toxic for target cells for 2–3 d after infusion.

These studies, however, do demonstrate that a number of factors are contributing to bring the level of functional circulating conjugate to a less than optimal level. In repeated experiments, we have found that infusion of two to three times the quantity of antibody–toxin conjugates is needed to achieve the same peak plasma concentration of antibody which can be reached when unconjugated antibody is delivered to the monkey. Thus, when conjugated to a toxin, presumably by any linkage which will be stable in the circulation, the antibody is cleared more rapidly than is the native antibody. It is likely that any derivatized antibody will be cleared more rapidly than native antibody.

Furthermore, a number of experiments in these studies indicate that some of the monoclonal antibody circulating in these animals is no longer conjugated to toxin. The plasma concentration of ^{125}I-labeled toxin falls to approximately one-tenth of that initially infused by 1 d after inoculation, while the concentration of mouse Ig, as assessed by RIA, does not reach that same relative concentration until 4 d after inoculation. When assayed in parallel using indirect immunofluorescence and FACS analysis, the plasma titer of toxin falls more rapidly than that of the antibody. Finally, the molar concentration of antibody detectable in the plasma of the infused animals is almost 10-fold higher than the concentration of functional immunotoxin. Thus, there appears to be some breakdown of the conjugate following infusion. Further studies will be needed to determine whether the quite rapid generation of free mouse Ig in the monkey following infusion of the conjugate results from a simple

cleavage of the disulfide linkage or from an instability of the gelonin protein itself unrelated to the disulfide linkage.

Our data demonstrating the stability of the disulfide linked antibody–toxin conjugates and the attainable plasma levels of conjugates suggest that we should be able to deliver intact immunotoxin to target T cells in both the circulation and secondary lymphoid organs. In fact, preliminary studies indicate that we can detect substantial quantities of gelonin and saporin on splenic and lymph node T cells following immunotoxin infusion. Moreover, in these preliminary experiments, the monkeys have tolerated the infusions needed to achieve such targeting of toxins without significant untoward effects. These findings indicate the feasibility of using immunotoxins as therapeutic tools and demonstrate important facts concerning the metabolic fate of these conjugates, which will facilitate their rational use as a treatment modality.

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