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

IV. Cytotoxic Effect of an Anti-T11-Gelonin Immunotoxin

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Abstract

The cytotoxic effect of a lymphocyte-specific immunotoxin formed by disulfide conjugation of an anti-T11 monoclonal antibody with the ribosome-inactivating protein gelonin was assessed in vitro on peripheral blood T cells and in vivo on splenic and lymph node T cells of macaque monkeys. This immunotoxin was cytotoxic to proliferating peripheral blood T cells in vitro as measured by both direct and indirect assays. Two sequential intravenous infusions into macaque monkeys achieved plasma concentrations of immunotoxin far in excess of those shown to be cytotoxic for cultured T cells and coated all T cells in lymph nodes and spleen with intact immunotoxin for four days. However, the cytotoxic effect of the immunotoxin on T cells in vivo was considerably less than that predicted by the in vitro studies. Further experiments suggested that the state of activation of the targeted T cell population in vivo, or the appearance of anti-immunotoxin antibodies, which occurred in all infused monkeys, might attenuate immunotoxin-mediated cell killing in vivo. These studies illustrate the significant differences between the action of immunotoxin conjugates in vitro, and those seen when these conjugates are utilized as therapeutic agents in vivo.

Introduction

Conjugates of monoclonal antibodies with ribosome-inactivating proteins (immunotoxins) hold promise as cytotoxic therapeutic agents of unprecedented efficacy and selectivity. The production and testing of a number of these compounds which recognize surface structures of specific normal or tumor cell populations is currently underway (reviewed in 1–5). In vitro studies with cultured cells have shown that some of these immunotoxin conjugates can kill the specific cell population recognized by the constituent monoclonal antibody, but are relatively nontoxic to cells which do not bind that antibody (1–5). While these findings are encouraging, considerably less experimental data are available regarding the systemic toxicity and the efficacy of these compounds when used in vivo. Such information gained through in vivo animal studies is essential for rationally planning the clinical application of these agents.

The nonhuman primate provides a unique experimental model for the in vivo study of immunotoxin conjugates. A conservation of certain blood cell surface antigens in primates has been well established (6–10). Thus, leukocyte-specific immunotoxins that are of potential therapeutic utility in man can be studied in nonhuman primates.

We have been examining immunotoxins formed by disulfide linkage of T lymphocyte-specific monoclonal antibodies to single chain ribosome-inactivating proteins. We have established the in vivo safety of these compounds in monkeys, as well as their circulatory stability and the kinetics of their clearance (11, 12). The dosage and method of administration necessary to allow penetration and selective delivery of these immunotoxins to target cell populations within secondary lymphoid organs have also been established (13).

In the present experiments, we have assessed the cytotoxic effect of one of these lymphocyte-specific immunotoxins, anti-T11-gelonin. We have compared the cytotoxicity of this immunotoxin on the target T cell population when administered in vivo in macaque monkeys with the cytotoxicity for cultured peripheral blood T cells. Intravenous infusions of this immunotoxin resulted in selective delivery of intact conjugate to the target cell population and cytotoxicity was demonstrated. However, this cytotoxic effect in vivo was less marked than that observed for this same compound in vitro on cultured cells.

Methods

Animals. The monkeys used in this study were adult *Macaca fascicularis* (cynomolgus) and ranged in weight from 3 to 6 kg. They were maintained in accordance with the guidelines of the Committee on Animals for the Harvard Medical School and the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. [NIH] 85-23, revised 1985).

Preparation of disulfide-linked immunotoxin conjugates. The production and purification of the monoclonal antibody used in the preparation of the immunotoxin conjugate, anti-$T11_{1A}$ (hereafter referred to as anti-T11), and the purification of the ribosome-inactivating protein gelonin (M_r 30,500) have been described previously (11, 14, 15). Disulfide conjugation between antibody and toxin was effected using *N*-succinimidyl 3-(2-pyridyldithio)propionate and 2-iminothiolane HCl, and the resulting immunotoxin conjugate was purified as described earlier (12, 15).

Culturing of normal PBL. Peripheral blood mononuclear cells were obtained from healthy human donors or from healthy cynomolgus monkeys, and isolated by routine density gradient centrifugation. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and Hepes buffer (10 mM) (hereafter referred

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to as growth medium), or in growth medium which was additionally supplemented with recombinant human IL-2 (100 U/ml; Biogen, Cambridge, MA, or 3 U/ml; E. I. DuPont de Nemours and Co., Wilmington, DE). Mitogen supplemented growth medium contained PHA (0.25 μg/ml; Burroughs Wellcome Laboratories, Greenville, NC) for human cells, or concanavalin A (Con A) (12.5 μg/ml; Difco, Detroit, MI) for monkey cells. All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Thymidine incorporation (indirect) cytotoxicity assay. PBL from cynomolgus monkeys (10^6/ml) were incubated for 3 d in Con A-supplemented growth medium. Cells were then washed and resuspended in IL-2 supplemented growth medium and maintained in this medium in asynchronous exponential growth for 2–4 more days by diluting daily to a concentration of 2–4 × 10^5 cells/ml. Cytotoxicity assays on dividing T cells were performed in 96 well, round bottom polystyrene plates. Triplicate wells of 5 × 10^4 cells were cultured in 0.2 ml of IL-2 supplemented growth medium which also contained serial dilutions of gelonin, monoclonal antibody, or immunotoxin. Cultures were incubated for 3 or 4 d and pulse labeled during the last 4 h or, in some experiments, during the last 18 h with 1 μCi [^{3}H]thymidine per well. Cytotoxicity assays on resting PBL were performed in snap top polystyrene tubes containing culture volumes of 1.0 to 1.5 ml. Cells (5 × 10^5/ml) were incubated in growth medium containing serially diluted gelonin, monoclonal antibody, or immunotoxin. After 1–4 d of incubation, cells were washed three times and resuspended in the same volume of fresh Con A supplemented growth medium and plated onto 96 well round bottom polystyrene microtiter plates. Triplicate cultures (0.2 ml/well) were incubated an additional 4 d and pulse labeled during the last 18 h of incubation with 1 μCi [^{3}H]thymidine per well.

The incorporation of [^{3}H]thymidine into cells was quantified by routine automated cell harvesting and standard liquid scintillography. Experiments assessing immunotoxin cytotoxicity by [^{3}H]thymidine incorporation were performed four times and experiments assessing cytotoxicity of gelonin and antibody were performed at least twice. To estimate the cytotoxicity in a quantitative manner, the concentration of gelonin or immunotoxin that caused 50% inhibition of [^{3}H]-thymidine incorporation (ID_{50}) was calculated.

Growth back-extrapolation (direct) cytotoxicity assay. Freshly isolated PBL from humans or from cynomolgus monkeys were resuspended (10^6 cells/ml) in growth medium containing PHA or Con A, respectively, and maintained in culture for 2 d. After activation, cells were washed and resuspended in IL-2 supplemented growth medium. Immediately after activation, or on ensuing days, activated cells were exposed to immunotoxin, antibody alone, gelonin alone, or irrelevant immunotoxin for 24 h, after which the cells were washed and diluted in IL-2 supplemented growth medium daily to the concentration of 5 × 10^5 cells/ml for human PBL, or 1.5 × 10^6 cells/ml for monkey PBL. Cell counts were performed daily on a Coulter counter (Coulter Electronics, Hialeah, FL) and growth curves were established as described previously (16), from which ID_{50} values were determined.

Alternatively, to evaluate the toxicity on resting PBL, cells in some cultures were similarly exposed to immunotoxin for 24 h before mitogen activation. These cells were then activated and maintained as above in IL-2 supplemented growth medium. Surviving fractions of cells and ID_{50} were determined as described above.

Protocol for the anti-T11-gelonin infusions. Cynomolgus monkeys were sedated with ketamine HCl throughout the infusion of the immunotoxin conjugate. Infusions of 5 mg/kg of anti-T11-gelonin were delivered intravenously in a 20–30 ml volume over 3–4 h. Each animal received two such infusions 48 h apart (on day 0 and on day 2).

Blood samples were obtained before the start of each infusion, 2 h after the end of each infusion, and daily thereafter. Axillary or inguinal lymph node biopsies were obtained on days 1–3, and 5 to evaluate the delivery to, and persistence of immunotoxin on the surface of T cells in secondary lymphoid organs. Mesenteric lymph node and splenic biopsies were obtained at laparotomy under general anesthesia 1–3 wk before infusions, and on days 4, 9, and 16 to evaluate morphologic and functional changes in target cells following immunotoxin infusions. Animals designated as controls received the same sedation and handling as the infused animals. Blood sampling and spleen and lymph node biopsies were done on these animals in parallel with the monkeys that received immunotoxin.

Routine histologic and immunohistologic studies. Tissue samples from spleen and lymph node biopsies were fixed with formalin, sectioned, and stained with hematoxylin and eosin for routine microscopic examination. Another biopsy sample of the same tissues was snap frozen, sectioned and stained for the presence of mouse Ig and for the presence of gelonin on cell surfaces using the immunoperoxidase techniques described previously (13). In serial sections from each tissue, the amount of staining with and without the in vitro addition of immunotoxin conjugate was compared. This allowed us to estimate the number of cells with antibody and gelonin bound to their surface as well as estimate the number of potential target lymphocytes in that tissue.

RIA for mouse Ig and gelonin. A solid-phase RIA, described previously, was used to quantify mouse Ig and gelonin present in plasma following infusions and in gel filtration column eluates (13).

Isolation of mononuclear cells from tissue. Suspensions of mononuclear cells from spleen or lymph nodes were obtained by teasing biopsy specimens with forceps, or passing tissue through a size 40 stainless steel mesh. Occasionally, samples were obtained by fine needle aspiration of lymph node or spleen in situ. All splenocyte suspensions were further purified by density gradient centrifugation. Contaminating RBC were lysed by brief suspension in 0.17 M NH_4Cl.

Assay for in vivo toxicity of the immunotoxin for splenic and lymph node mononuclear cells. The proliferative response of splenic and lymph node mononuclear cells following stimulation with two mitogens and in a mixed lymphocyte reaction (MLR)[1] before and after infusion of anti-T11-gelonin was used as an index for cytotoxicity of the immunotoxin. Cells (10^5) were cultured in 0.2 ml of growth medium. Each culture well, which was plated in triplicate, contained medium without mitogens or the indicated quantity of one of the following: Con A, PHA, or mitomycin-treated xenogeneic stimulator B cells. Lymphocytes were cultured for 4 d, then pulse labeled and the proliferative response quantified by measuring [^{3}H]thymidine incorporation into cells.

Analysis of immunotoxin stability after incubation in normal monkey serum. Immunotoxin (100 μg/ml) was incubated in normal monkey serum, or in Hepes-buffered RPMI 1640 medium containing 0.1% BSA at 37°C for 12 h and then frozen in liquid nitrogen. Samples (0.75 ml) were thawed and immediately analyzed by gel filtration on a column (46 cm × 1.0 cm) of Sephacryl S-300 equilibrated in PBS containing 0.05% sodium azide. The column was run at 6 ml/h collecting 1-ml fractions. The gelonin concentration in each fraction was measured by RIA. Samples of anti-T11-gelonin and unconjugated gelonin were applied to the column for calibration and their elution positions were determined both by ultraviolet absorption at 280 nm and by RIA for gelonin.

Assay for ability of normal monkey serum to block the cytotoxicity of immunotoxin in vitro. Immunotoxin (100 μg/ml) was incubated at 37°C in either normal monkey serum, or in Hepes-buffered RPMI 1640 medium containing 0.1% BSA, for 2, 12, or 24 h. After incubation, immunotoxin mixtures were serially diluted and the cytotoxicity of the incubated immunotoxin was determined using the previously described indirect cytotoxicity assay. As a control, the cytotoxicity of untreated immunotoxin was assessed in the same assay. Also, the cytotoxicity of immunotoxin was assessed in samples that were mixed with ice-cold monkey serum, or with ice-cold medium containing 0.1% BSA, assayed immediately for cytotoxicity without any preincubation at 37°C (0 h).

RIA for detecting monkey anti-mouse Ig antibodies. Monoclonal anti-T11 antibody (5 μg/ml) was adsorbed onto flexible 96 well polyvinyl chloride plates overnight at 4°C. Nonspecific binding sites on the

1. *Abbreviations used in this paper:* MLR, mixed lymphocyte reaction.

plates were blocked with PBS containing 1% BSA for an additional hour. Aliquots (50 μl) of diluted test plasma were applied to each well, incubated at room temperature for 2 h, and washed with PBS. To detect anti-mouse Ig antibodies of the IgG class, radiolabeled ^{125}I-monoclonal anti-human IgG (provided by Dr. Victor Raso, Dana Farber Cancer Institute) was incubated in each well for 2 h at room temperature. This antibody cross-reacts with monkey IgG, but not with murine IgG. Antibodies of the IgM class were detected similarly using radiolabeled goat anti-human IgM ($Fc_{5\mu}$) (Jackson ImmunoResearch Laboratories, Inc., Avondale, PA). Plates were washed with PBS and radioactivity retained in each well was quantified on a gamma counter. Titration endpoint for each sample was determined as the highest dilution that retained counts per minute greater than the mean plus 3 SD of 10 dilutions of negative control plasma.

Results

In vitro toxicity of anti-T11-gelonin conjugate for normal monkey and human PBL. We have previously shown that anti-T11-gelonin inhibits the growth in vitro of the virus-immortalized monkey T lymphocyte line 1022 (12). These studies were done using an indirect cytotoxicity assay that measured the inhibition of [^{3}H]thymidine incorporation into cellular DNA. We sought to determine whether such a conjugate exhibits similar toxicity in vitro for normal human and monkey T lymphocytes.

Inhibition of [^{3}H]thymidine incorporation is an assay that is widely used for measuring the cytotoxicity of compounds on cultures of growing cells. In addition, this type of assay was convenient for assessing cytotoxicity of immunotoxin conjugates in vivo. However, while this assay allows estimation of ID_{50} values that are useful for the comparison of the toxicity of different compounds, it is indirect since it does not give a value for the true surviving fraction of cells, but rather a value for the amount of radiolabeled thymidine incorporated into cellular DNA. In order to determine the surviving fraction of lymphocytes in culture directly, we utilized the "growth back-extrapolation" assay (16), which we adapted for PBL. We then compared the results of the indirect, thymidine incorporation assay with those from the direct assay.

Normal PBL can be activated and then maintained in culture in an expohential phase of growth for ~ 7 d in the presence of IL-2. A typical growth curve for human PBL activated with PHA, and then cultured in IL-2 supplemented growth medium is shown in Fig. 1 *A*. After a brief lag in cell number for 2–3 d, cells proliferate exponentially for the following 6–7 d with a doubling time of 16–18 h. The proliferating cells are T lymphoblasts, and within 2–3 d of exponential growth these cells make up the vast majority of the cultured population (17, 18). We were able to use a direct proliferation cytotoxicity assay on PBL by quantifying cell number during this period of exponential growth.

A cytotoxicity assay where human PBL were first activated with PHA, then cultured for 24 h in medium containing anti-T11-gelonin, followed by culture in medium alone is shown in Fig. 1 *B*. The 24-h exposure of the cells to anti-T11-gelonin decreased their capacity to proliferate. The ID_{50} of anti-T11-gelonin for human PBL (24-h exposure to immunotoxin, added after a 2-d activation with PHA) was 0.5 nM as estimated from these data. When human PBL were exposed to the immunotoxin for 24 h on day 4 after activation, the proliferative capacity was similarly affected (ID_{50} 0.4 nM, data not shown). Unconjugated anti-T11 antibody or unconjugated gelonin was not cytotoxic at the concentration of 0.1 μM. Irrelevant immunotoxins directed against human B cells (anti-B1-gelonin) (15) or murine specific determinants (anti-Thy-1.2-gelonin) (19) were not cytotoxic at 50 nM concentrations.

This direct assay was also used to assess the cytotoxicity of anti-T11-gelonin for cynomolgus monkey PBL. The data illustrated in Fig. 2 compares the cytotoxicity of this immunotoxin for human and monkey PBL. In two independent experiments where Con A-activated monkey PBL were exposed for 24 h to the immunotoxin on day 3 after activation, the ID_{50} of anti-T11-gelonin was 0.7 nM for these cells. Thus, anti-T11-gelonin was toxic at comparable concentrations for

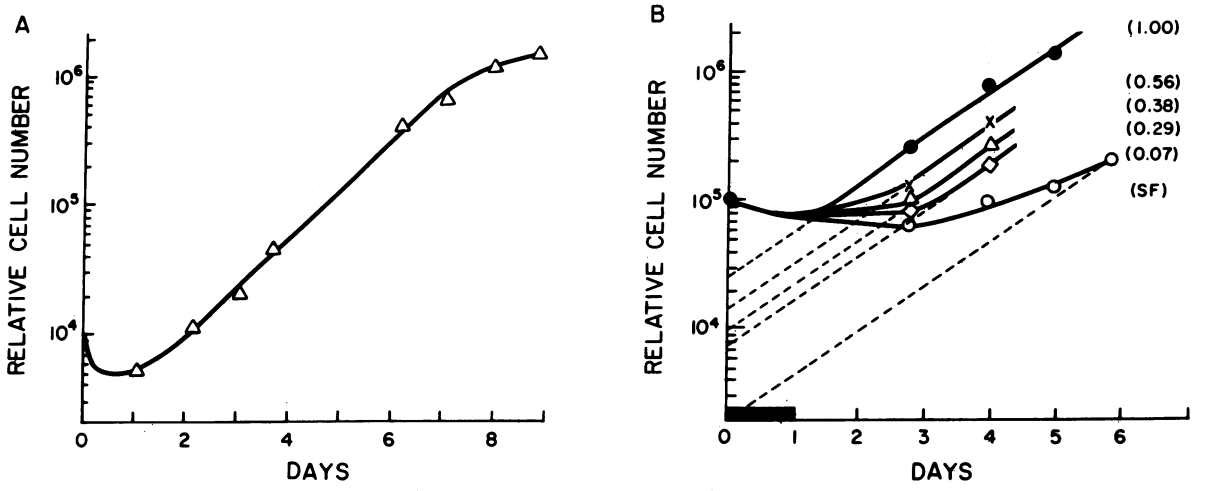

Figure 1. (*A*) A typical growth curve for human PBL cultured in IL-2 supplemented growth medium after activation with PHA for 2 d. Cultures were diluted daily to the concentration of 5×10^5 cells/ml. (*B*) Growth curves for human PBL similarly cultured in IL-2 supplemented growth medium after PHA activation. Cells were exposed to the following concentrations of anti-T11-gelonin for a 24-h period, following the 2-d PHA activation: 0 nM (control), (●); 0.3 nM, (×); 0.6 nM (△); 1.0 nM (◇); 3.0 nM, (○). An estimate of the number of proliferating cells at the end of the treatment period was made by back extrapolating the exponential growth curves. Relative surviving fractions (SF) were determined as the ratios of the number of surviving cells in treated cultures to the number of cells in control cultures.

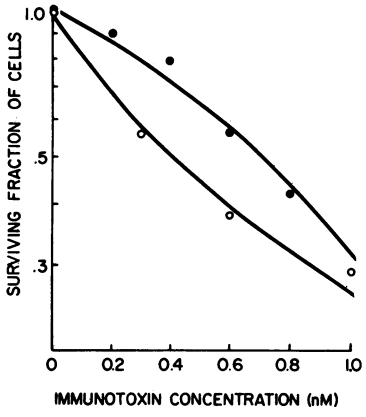

Figure 2. Cytotoxicity of anti-T11-gelonin on human and monkey PBL as determined by a growth back-extrapolation assay. Surviving fractions of cultured human PBL (○) or monkey PBL (●), determined as shown in Fig. 1 *B*, when exposed to varying concentrations of anti-T11-gelonin.

human and monkey T cells. Irrelevant immunotoxin (anti-B1-gelonin) was not cytotoxic to monkey cells at a 50-nM concentration.

We also assessed the cytotoxicity of anti-T11-gelonin for monkey PBL by the direct assay and compared these results with the cytotoxicity as measured by the indirect, thymidine incorporation assay. When measured by thymidine incorporation, this immunotoxin gave an ID_{50} of 0.4 nM, which agrees well with the ID_{50} of 0.7 nM as determined by the direct assay. Therefore, these two assay systems estimate in a comparable fashion the cytotoxicity of this immunotoxin on monkey PBL.

In vivo cytotoxicity of anti-T11-gelonin in cynomolgus monkeys. Previous in vivo experiments with this conjugate in the macaque monkey showed that a single 5-mg/kg infusion resulted in coating of T cells in the lymph node and spleen with intact immunotoxin for 48 h (13). Based on these results, we elected to administer two infusions of anti-T11-gelonin at a dose of 5 mg/kg, 48 h apart, to achieve a continuous 3–4-d exposure of T cells to the immunotoxin. In the present experiments, infusions of this immunotoxin resulted in peak plasma concentrations of mouse Ig of 55–69 μg/ml (0.3–0.4 μM) and peak gelonin concentrations of 5.8–6.5 μg/ml (0.19–0.22 μM) when measured 2 h after the end of each infusion.

All three monkeys tolerated these infusions without evident toxic complications. Significant changes in serum electrolytes, liver enzyme concentrations, liver function tests, and renal function tests were never observed. The histologic examination of a liver biopsy taken from one monkey on day 9 revealed no pathologic changes. Changes in routine peripheral blood cell counts were monitored before and after immunotoxin infusions. All infused animals had a marked neutrophilic leukocytosis after the first but not the second immunotoxin infusion. In addition, a decrease in circulating peripheral blood lymphocytes was noted after each infusion. However, a similar pattern of neutrophilia and lymphopenia was observed in control monkeys that received the same anesthesia, restraint, and surgery, but did not receive immunotoxin. Therefore, it is unlikely that these hematologic changes were entirely an effect of the immunotoxin; rather, they may, in part, have been a physiological response to stress.

To evaluate the persistence of immunotoxin coating of target cells after infusions, biopsies of peripheral or mesenteric lymph nodes were performed on days 1 through 5 and on day 9 postinfusion. These biopsies were stained for mouse Ig and gelonin on cell surface membranes using an immunoperoxidase technique. These results are shown in Fig. 3. Gelonin was detectable on T cells in biopsies taken from lymph nodes on days 1 through 4. T cells in lymph node biopsies performed on day 5 stained only faintly for toxin while biopsies taken on day 9 lacked visible staining for gelonin on cell surfaces. Mouse Ig was found to be present on lymph node T cells on postinfusion days 1 through 5, but was absent on day 9 (data not shown). Similar coating of T cells in the spleen was observed on day 4 postinfusion. The in vitro addition of immunotoxin to tissue sections before immunoperoxidase staining did not increase the number of cells that stained as positive for either mouse Ig or toxin, and did not increase the intensity of that staining in biopsies obtained on days 1–4. Thus, these intravenous infusions appeared to result in binding of immunotoxin to all binding sites on potential target cells and resulted in at least a 4-d exposure of the target cell population to gelonin.

The cytotoxic effect of in vivo administered immunotoxin on the target cell population was evaluated by routine histologic examination of spleen and lymph nodes. In addition, specific proliferation assays were performed on lymphocytes obtained from spleen and lymph node before and after immunotoxin infusion. Tissues were obtained for these studies before infusion and on days 4, 9, and 16. Mesenteric lymph nodes were utilized in these studies since they were less likely than peripheral lymph nodes to develop reactive changes due to surgery.

Examination of spleen and lymph node biopsies performed 4, 9, and 16 d after infusion showed no evidence of lymphoid necrosis or cell death. The only change that was observed consistently was a mild to moderate neutrophilic infiltration on days 4 and 9 that was distributed uniformly throughout the lymph node and spleen. However, a similar neutrophilic infiltrate was sometimes observed in spleen and lymph node biopsies from control monkeys as well. Some biopsies obtained from infused monkeys at day 9 showed moderate to marked follicular hyperplasia. This hyperplasia was more pronounced on biopsies taken on day 16. There was, therefore, no histologic evidence of T cell damage or death.

Lymphocytes were also isolated from spleen and lymph node for functional analysis. The proliferative response of these cells to the T cell mitogens PHA and Con A, and to xenogeneic B lymphocytes, was assessed in these cell populations before, and 4 and 9 d after immunotoxin infusions (Tables I–III). Proliferative responses of splenocytes to T cell mitogens and xenogeneic stimulator cells were suppressed in two of three animals (experiments 1 and 3) 4 d after immunotoxin administration. By 9 d after infusion, values had either returned to normal or were significantly higher than day 4 values, suggesting that they were returning to the normal level. Lymph node mononuclear cells exhibited more variability in their response to mitogenic stimulation. However, a similar pattern of transient loss of T cell blastogenic responsiveness was not observed.

Thus, plasma concentrations of immunotoxin were achieved that clearly resulted in a coating of T cells in spleen and lymph nodes with toxin and, based upon the results of in vitro studies, should have been sufficient to exert toxic effects on those cells. Nevertheless, histologic and functional effects on the T lymphocytes in vivo after immunotoxin delivery were minimal.

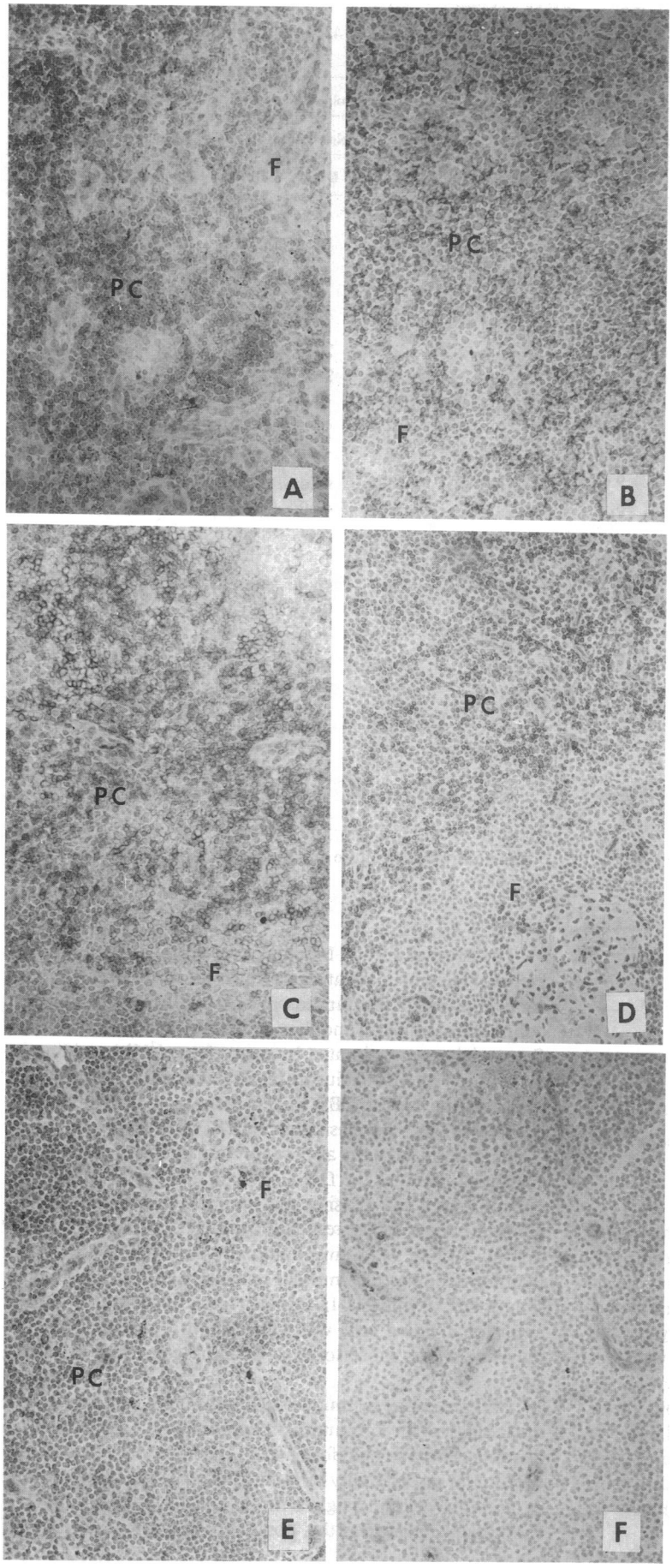

Figure 3. Sections of lymph nodes taken from monkeys that received two intravenous infusions of anti-T11-gelonin (on day 0 and day 2). Sections were developed for the presence of gelonin on cell surfaces with rabbit anti-gelonin serum followed by biotinylated anti-rabbit Ig, then avidin-biotin-peroxidase complex and diaminobenzidine with 0.03% H_2O_2. (*A*) day 1, 1 d after the first 5-mg/kg infusion; (*B*) day 2, 2 d after the first infusion; (*C*) day 3, 1 d after the second 5-mg/kg infusion; (*D*) day 4, 2 d following second infusion; (*E*) day 5, 3 d after second infusion; (*F*) day 9, 7 d after second infusion. F, follicle. PC, paracortex. × 230.

Table I. Proliferation of Monkey Lymphocytes in Response to Con A

	Proliferative response to mitogen ($\times 10^3$ cpm)*					
	Splenocytes			Lymph node lymphocytes		
Con A	Day 0	Day 4	Day 9	Day 0	Day 4	Day 9
μg/ml						
Experiment 1						
Infused						
0	0.3	0.4	0.2	0.2	0.2	0.2
6.25	56.5	10.2	5.9	1.5	2.5	0.6
12.50	75.5	30.9	17.8	6.8	4.4	3.9
25.00	113.5	10.0	28.8	6.4	2.3	13.4
Control						
0	—	0.3	0.4	—	0.2	0.1
6.25	—	30.7	11.7	—	1.9	2.1
12.50	—	41.4	30.0	—	13.7	9.6
25.00	—	35.6	53.2	—	19.5	26.1
Experiment 2						
Infused						
0	0.9	1.7	2.5	0.3	0.2	0.2
6.25	96.2	59.3	78.6	33.4	22.4	67.0
12.50	78.7	67.5	76.6	80.8	25.2	71.6
25.00	37.7	28.5	37.0	55.1	1.3	19.9
Control						
0	1.0	2.5	4.0	0.2	0.2	0.3
6.25	50.3	55.7	40.9	28.3	22.3	58.8
12.50	66.8	81.1	64.5	60.2	25.6	65.2
25.00	39.0	40.7	43.5	56.7	4.7	29.5
Experiment 3						
Infused						
0	4.1	0.4	1.7	—	—	—
6.25	68.5	17.0	57.4	—	—	—
12.50	103.7	36.1	81.0	—	—	—
25.00	85.8	23.9	33.2	—	—	—
Control						
0	1.8	3.7	2.3	—	—	—
6.25	93.9	120.9	161.0	—	—	—
12.50	115.5	153.8	147.0	—	—	—
25.00	75.2	124.4	64.9	—	—	—

* Mean of triplicate culture groups of [^{3}H]thymidine incorporation per well.

Table II. Proliferation of Monkey Lymphocytes in Response to PHA

	Proliferative response to mitogen ($\times 10^3$ cpm)*					
	Splenocytes			Lymph node lymphocytes		
PHA	Day 0	Day 4	Day 9	Day 0	Day 4	Day 9
μg/ml						
Experiment 2						
Infused						
0	0.8	2.2	2.2	0.2	0.3	0.3
0.50	20.2	19.8	37.6	7.9	11.8	10.0
1.00	26.7	20.0	38.1	11.8	12.3	11.1
2.00	30.5	28.2	41.8	15.5	15.3	19.5
Control						
0	0.7	2.6	5.0	0.2	0.8	0.4
0.50	34.9	34.4	52.1	12.9	28.4	16.9
1.00	38.9	43.4	56.5	15.4	31.9	17.0
2.00	34.6	46.0	57.7	21.2	33.4	21.9
Experiment 3						
Infused						
0	4.7	0.5	1.2	—	—	—
0.50	63.1	12.7	35.4	—	—	—
1.00	73.6	15.7	40.5	—	—	—
2.00	76.5	17.6	55.3	—	—	—
Control						
0	2.3	4.8	1.9	—	—	—
0.50	46.5	59.2	49.7	—	—	—
1.00	54.3	62.4	65.3	—	—	—
2.00	47.5	59.7	68.2	—	—	—

* Mean of triplicate culture groups of [^{3}H]thymidine incorporation per well.

Toxicity of anti-T11-gelonin in vitro on resting and proliferating cells. We sought to explain why this immunotoxin, which was markedly toxic to cells in vitro, caused only modest functional impairment of T lymphocytes following immunotoxin delivery in vivo. One difference between the targeted cell populations in vivo and those previously studied in vitro was their state of activation. The targeted cell in the in vivo infusion experiments was largely a nonactivated, nonproliferating T cell in the lymph nodes or spleen. The targeted cell in the in vitro growth back-extrapolation assay was a lectin activated, rapidly proliferating T cell. We, therefore, performed in vitro experiments to compare the cytotoxicity of this conjugate on resting and dividing target cell populations.

The effect of anti-T11-gelonin on resting monkey PBL was first evaluated by incubating freshly isolated PBL with varying concentrations of immunotoxin, washing them to remove unbound immunotoxin, and then activating the cells with Con A. Cytotoxicity was estimated by quantifying [^{3}H]thymidine incorporation into DNA during the subsequent lectin activation. Exposing the resting PBL to this immunotoxin caused suppression of DNA synthesis with an ID_{50} of $\sim$ 10 nM (Fig. 4 *A*).

This immunotoxin also suppressed the incorporation of [^{3}H]thymidine into the DNA of lectin activated PBL populations in which cell division was maintained with IL-2 (Fig. 4 *B*). Comparison of the results shown in Fig. 4 (*A* and *B*) show that 30–50-fold more immunotoxin was required for resting than for dividing cells in order to achieve equivalent suppression of DNA synthesis. In this assay, the ID_{50} of the immunotoxin for dividing cells was 0.2–0.3 nM. Therefore, this immunotoxin was more toxic to dividing cells than to resting cells.

Different assay techniques had to be employed to evaluate the toxicity of an immunotoxin compound on resting and on dividing cells. We therefore had to consider that the apparent differential susceptibility of resting and dividing cells to killing by anti-T11-gelonin might simply reflect differences in assay conditions. To address this possibility, the cytotoxic effect of

Table III. Proliferation of Monkey Lymphocytes in an MLR

Stimulator cells/well	Proliferative response to stimulator cells (×10³ cpm)*					
	Splenocytes			Lymph node lymphocytes		
	Day 0	Day 4	Day 9	Day 0	Day 4	Day 9
Experiment 1						
Infused						
0	0.4	0.2	—	—	—	—
50,000	4.5	2.3	—	—	—	—
100,000	7.3	5.5	—	—	—	—
Control						
0	—	0.3	—	—	—	—
50,000	—	2.3	—	—	—	—
100,000	—	4.5	—	—	—	—
Experiment 2						
Infused						
0	0.9	1.6	2.0	0.4	0.5	0.4
50,000	11.5	4.8	11.6	4.0	17.3	4.1
100,000	18.7	10.3	21.4	7.6	26.9	12.1
Control						
0	1.6	5.0	1.7	0.4	1.0	0.9
50,000	7.6	9.8	20.1	2.7	16.7	9.4
100,000	9.9	14.0	20.1	2.7	25.2	12.7
Experiment 3						
Infused						
0	3.9	0.8	1.3	—	—	—
50,000	16.0	5.6	6.2	—	—	—
100,000	28.7	9.1	8.4	—	—	—
Control						
0	2.6	5.5	1.6	—	—	—
50,000	16.3	21.2	14.0	—	—	—
100,000	17.1	29.1	14.4	—	—	—

* Mean of triplicate culture groups of [³H]thymidine incorporation per well.

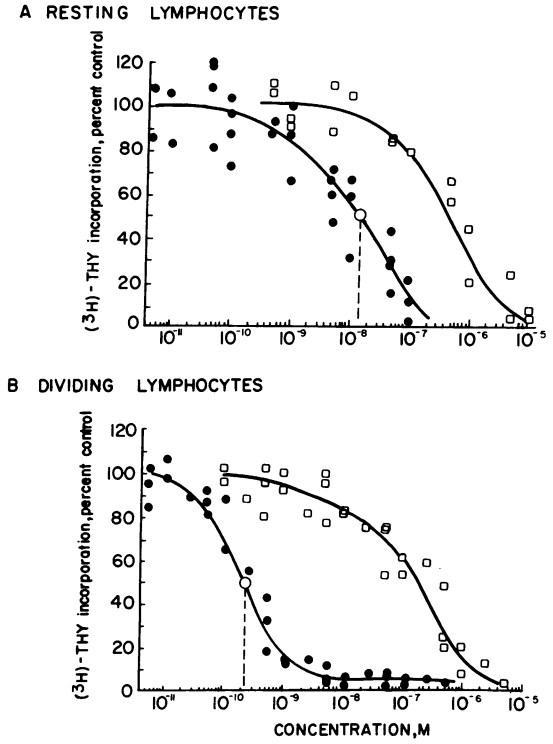

Figure 4. Cytotoxicity of anti-T11-gelonin on resting and proliferating monkey lymphocytes. (*A*) Freshly isolated resting PBL were exposed to varying concentrations of anti-T11-gelonin (●), or gelonin (□) for 3 d, washed and then activated with Con A. Cytotoxicity was estimated by measuring suppression of [³H]thymidine incorporation after 3 d of activation with lectin. (*B*) PBL were activated with Con A for 3 d, washed, and then grown in IL-2 supplemented growth medium. Cultures were exposed to varying concentrations of anti-T11-gelonin (●), or gelonin (□) during exponential cell growth. Cytotoxicity was estimated by measuring suppression of [³H]thymidine incorporation after 3 d of growth with immunotoxin.

gelonin on both resting and dividing cell populations was measured. This single-chain toxin does possess biological activity in its unconjugated form at high concentrations (20). The results shown in Fig. 4 (*A* and *B*) demonstrate that significantly higher concentrations of gelonin (100–1,000-fold) than immunotoxin were required to cause comparable suppression of DNA synthesis in monkey lymphocytes. However, the measured toxicity of gelonin was similar on both resting and dividing PBL. These results suggest that the slightly differing assay systems are indeed comparable in assessing the cytotoxic effect of a nonspecific nontargeted toxin.

In control experiments, monoclonal anti-T11 alone was nontoxic up to a concentration of 1 μM in both systems. This observation was also confirmed using a direct, growth back-extrapolation assay.

Experiments with human PBL were consistent with results obtained from monkey PBL. Anti-T11-gelonin was significantly less toxic (ID_{50}, 4 nM) for nonactivated than for activated human PBL (data not shown).

Effects of normal monkey serum on cytotoxicity and stability of immunotoxin. Our previous studies had shown that a significant portion of infused immunotoxin remains intact in the circulation. However, the functional concentration of immunotoxin is significantly less than might be predicted based upon the immunochemical measurement of the amount of circulating immunotoxin. We, therefore, considered the possibility that normal monkey serum might have the ability to inactivate immunotoxin either by rapid cleavage of the disulfide linkage between the antibody and the toxin, or through some other enzymatic or chemical alteration of conjugates. In these experiments, we first determined the amount of immunotoxin cleavage that was due simply to the exposure of immunotoxin to normal monkey serum. To accomplish this, immunotoxin was incubated for 12 h at 37°C with either normal monkey serum or with buffered medium which contained 0.1% BSA. These samples were then submitted to gel filtration on a column that had been calibrated to determine the elution position of intact conjugate and of unconjugated gelonin. The gelonin concentration in the eluted fractions from these samples is shown in Fig. 5. In the sample in which immunotoxin was incubated with medium containing 0.1% BSA, < 20% of

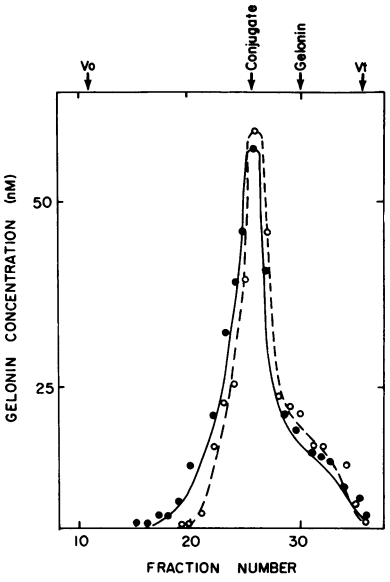

Figure 5. Analysis by gel filtration of the effect of incubation of immunotoxin in normal monkey serum. Anti-T11-gelonin conjugate (100 μg/ml) was incubated for 12 h at 37°C in either buffered medium containing 0.1% BSA (○) or in normal monkey serum (●). Samples were gel filtered on a column of Sephacryl S-300 equilibrated in PBS. Gelonin concentration in the fractions was measured by RIA. The elution positions for intact conjugate and for unconjugated gelonin are as shown. V_o and V_t are the void volume and total column volume.

the measured gelonin corresponded to unconjugated, cleaved gelonin. No more unconjugated gelonin was found in the immunotoxin sample incubated for 12 h in monkey serum. Thus, incubating the immunotoxin with monkey serum alone does not cause significant cleavage of the disulfide linkage.

To determine if monkey serum might attenuate the cytotoxicity of this conjugate by some other mechanism, immunotoxin was again incubated at 37°C with normal monkey serum, or with buffered medium containing 0.1% BSA for 0, 2, 12, or 24 h. Then, the cytotoxicity of immunotoxin incubated in this fashion was compared with that of untreated immunotoxin. As summarized in Table IV, the loss of cytotoxicity by immunotoxin after incubation with monkey serum was small. When compared with untreated immunotoxin, or with the immunotoxin incubated with 0.1% BSA in buffered medium, there was about a twofold increase in ID_{50} concentration when immunotoxin was incubated with monkey serum for periods of up to 24 h. Moreover, the minor attenuation of cytotoxicity

Table IV. Effect of Incubation with Normal Monkey Serum on the Cytotoxicity of Immunotoxin

	Immunotoxin ID_{50} concentration (*nM*)*			
	Hours of incubation			
	0	2	12	24
Immunotoxin in monkey serum	0.68±0.20	0.84±0.34	0.94±0.07	0.97±0.28
Immunotoxin in 0.1% BSA	0.42±0.14	0.48±0.14	0.47±0.09	0.38±0.05
Untreated immunotoxin	0.40±0.12	0.45±0.11	0.42±0.04	0.41±0.08

* Mean of three experiments±SD.

seen following incubation with monkey serum was not time dependent over the period examined. Any change in potency of the immunotoxin occurred within the first 2 h of incubation. Therefore, incubation with monkey serum had only a minor effect on the stability and cytotoxicity of immunotoxin.

Development of anti-mouse Ig antibodies. The delivery of immunotoxins to targeted cells in vivo also differed from experiments in vitro in that the infused individuals may develop an immune response to the immunotoxin that might interfere with the cytotoxic activity of that material. We, therefore, assessed the development of antibodies directed against the infused immunotoxin in monkeys. The time course of the appearance of anti-mouse Ig antibodies of the IgG and IgM class as determined by radioimmunoassay is shown in Table V. Anti-mouse Ig antibodies of the IgG class were evident in all animals by 9 d postinfusion. Antibodies of the IgM class were detectable as early as 3 d postinfusion.

Discussion

In our previous studies utilizing the nonhuman primate model, we have examined the effect of three monoclonal anti-T11 antibodies on the circulating T cell pool. When administered intravenously, all three antibodies resulted in a coating of circulating T cells, modulation of T11 from the cell surface, and a transient clearance of T cells from the circulation (11). However, significant differences existed between each antibody in its ability to effect these changes on circulating T cells. One of these monoclonal anti-T11 antibodies was chosen for study as an antibody-toxin conjugate and was coupled to the ribosome-inactivating proteins gelonin or saporin by a disulfide linkage. When intravenously infused into monkeys, these anti-T cell immunotoxins retained functional activity in the circulation and were sufficiently stable to permit the selective delivery of toxin to the surface of all T cells in the lymph nodes and spleen (12, 13).

In the present experiments, the cytotoxicity of the same anti-T11-gelonin immunotoxin was evaluated both in vitro and in vivo. When studied in normal cultured T lymphocytes derived from peripheral blood, immunotoxin concentrations far lower than those reached in the plasma of infused monkeys caused near total suppression of mitogen induced proliferation. Suppression of proliferation of cultured monkey splenocytes was also seen in the presence of similar concentrations of immunotoxin (ID_{50}, 0.5 nM).

To evaluate cytotoxicity in vivo, macaque monkeys received two consecutive infusions of immunotoxin. This method of delivery permitted 4 d of continuous coating of the target T cells with detectable levels of intact immunotoxin. In two of three experiments, modest, transient suppression of splenic lymphocyte responsiveness was observed. Histologic evidence of T lymphocyte death in lymph nodes or spleen was never seen. Therefore, the magnitude of the cytotoxic effect of this T cell immunotoxin in vivo was considerably less than expected based on the in vitro studies.

This method of in vivo administration allowed binding of intact immunotoxin to all target lymphocytes within the lymph nodes and spleen. While previous studies with this immunotoxin have shown that the disulfide linkage between antibody and toxin is slowly cleaved in the circulation, a significant fraction of immunotoxin does remain in the conjugated

Table V. Antibody Titer to Mouse Immunoglobulin after Infusion of Immunotoxin into Monkeys

Days after first infusion	Experiment 1		Experiment 2		Experiment 3	
	IgM	IgG	IgM	IgG	IgM	IgG
0	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4
1	—	—	—	—	—	—
2	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4
3	—	—	1:8	<1:4	1:16	<1:4
4	1:128	<1:4	1:128	<1:4	1:16	<1:4
5	—	—	1:256	<1:4	—	—
6	—	—	—	—	—	—
7	—	—	—	—	1:64	<1:4
8	—	—	—	—	1:256	1:8
9	—	1:16,384	1:8,192	1:4,096	1:2,048	1:128

form for three days (12). Furthermore, the intensity of immunoperoxidase staining of lymph node sections for surface bound gelonin was not increased by the in vitro addition of immunotoxin to tissue sections. Therefore, it is unlikely that higher plasma concentrations of immunotoxin or different regimens of administration would have facilitated additional binding or enhanced toxicity in vivo.

This series of experiments, which examines the in vivo effects of lymphocyte specific monoclonal antibodies and immunotoxins in macaque monkeys, constitutes the first detailed report of monoclonal antibody conjugates used as a parenteral therapeutic agent in higher primates. These experiments demonstrate the effectiveness of murine monoclonal antibodies as a vehicle to deliver potent cytotoxic agents to specific lymphocyte subpopulations. However, the marginal cytotoxic effect of this agent in vivo was inconsistent with its marked cytotoxicity in in vitro lymphocyte cultures. A number of factors may contribute to this difference.

The present studies do suggest that the state of activation of the target cell may affect its susceptibility to killing by this immunotoxin. Resting lymphocytes of both human and monkey were significantly more resistent to killing by immunotoxin than were cultures of proliferating cells. One plausible explanation for this difference may relate to differing densities of antigen on the surface of activated cells. An enhanced expression of the 50-kD glycoprotein T11 cell surface structure occurs after T lymphocyte activation (14, 21). The resulting increase in immunotoxin binding to the surfaces of activated T cells might increase its specific toxicity to those cells. In addition, the trafficking of surface molecules to endosomes may be enhanced in proliferating cells. Thus, the T11 immunotoxin complex may be internalized more rapidly in the dividing than in the resting lymphocyte. Other intracellular events in dividing cells might also favor immunotoxin release from endosomes to the cytoplasm or accelerate the rate of free toxin liberation by disulfide bond cleavage. Finally, proliferating cells might be more susceptible to the gelonin-induced inhibition of protein synthesis than resting cells due to the need to synthesize more protein. However, this enhanced susceptibility of dividing cells to killing by immunotoxin may provide a useful increase in therapeutic index when immunotoxins are used to kill selectively rapidly proliferating cell populations.

Our previous studies indicated that the stability and circulatory clearance of this immunotoxin are adequate to permit its use as a parenteral therapeutic agent. Two hours after a single infusion of immunotoxin, 60% of the total remains in the circulation and 90% of that in the circulation remains intact (12). The half-life for elimination of the immunotoxin was 4–6 h (13). Furthermore, data presented here, and data shown previously (13), indicate that this immunotoxin retains its ability to selectively bind to the target cell population when delivered by intravenous infusion. However, the functional concentration of immunotoxin in the plasma of infused animals was only one-tenth of the actual measured concentration of intact conjugate (12, 13). We, therefore, considered the possibility that serum might act to functionally inactivate immunotoxin in vivo. The present studies, however, show that exposure of immunotoxin to serum causes neither cleavage nor functional inactivation to an extent which would explain the absence of a functional effect in this in vivo trial. Experiments are currently underway to quantify and characterize further the loss of immunotoxin function in the plasma of infused monkeys. We also considered other explanations for the absence of toxicity of these conjugates. The effect of any infused immunotoxin might be significantly blunted by the appearance of "neutralizing" antibodies. In the present experiments, anti-mouse Ig antibodies of the IgG class were detectable in the circulation by 9 d postinfusion. Antibodies of the IgM class were detected by 3 to 4 d postinfusion. Furthermore, preliminary studies indicate that immune sera from monkeys previously infused with this immunotoxin are able to block the cytotoxic effect of immunotoxin in an in vitro assay. While these antibody responses may be occurring too late to account for the absence of in vivo efficacy, the appearance of "neutralizing" antibodies may limit the temporal window during which these agents can be expected to exert their cytotoxic effect on the target cell population. Attempts to suppress the host's humoral response to infused murine monoclonal antibody through immunosuppressive drugs or radiation have had varied success (22, 23). Sequential infusions with immunotoxins that bind to the same cell surface structure, but whose monoclonal antibody moiety is either not recognized as xenogeneic or is isotypically or idiotypically variant, and therefore not immediately "neutralized" by circulating antibodies, might serve to expand this window of potential treatment time.

The absence of cytotoxic effect of this compound following in vivo delivery appears to be due either to a relative lack of toxicity of this immunotoxin conjugate for lymphocytes in vivo or to a host-induced attenuation of toxicity, and not due to compound instability or an inability to deliver immunotoxin to target cells. These problems of attenuated cytotoxicity may be overcome by chemical modifications to enhance toxin delivery to its site of action, or through the use of more potent toxins, or through the modification of existing toxins to improve their ability to kill. However, the present studies continue to underscore the feasibility and utility of immunotoxin conjugates as powerful, new therapeutic agents, as well as the important role of nonhuman primates in testing these compounds.

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