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Comparative cytotoxicity and pharmacokinetics of antimelanoma immunotoxins containing either natural or recombinant gelonin

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Abstract Immunotoxins are a class of targeted therapeutic agents under development by various research groups. The murine monoclonal antibody designated ZME-018 recognizes a high molecular weight glycoprotein present on most human melanoma cells and biopsy specimens and has been utilized for clinical imaging studies in patients with melanoma. The plant toxin gelonin is a ribosome-inactivating protein (RIP) with *n*-glycosidase activity similar to that of ricin A chain. In previous studies by our group, the gelonin toxin was sequenced, cloned and expressed in *E. coli*. The purified recombinant gelonin (RG) was found to have identical protein synthesis inhibitory activity to that of natural gelonin (NG). For comparative purposes, chemical conjugates of antibody ZME and either RG or NG were produced using the heterobifunctional cross-linking reagents SPDP and SMPT. The ZME-NG and ZME-RG immunotoxins were found to be 10^4- to 10^5-fold more cytotoxic to antigen-positive human melanoma cells than free toxin. NG toxin alone was cytotoxic to intact cells (IC_{50} = 100 n*M*) while RG was nontoxic to cells at doses up to 1 μ*M*. Both ZME-NG and ZME-RG immunoconjugates were nontoxic to antigen-negative (Me-180) cells. ZME-RG immunotoxins constructed with the more stable SMPT reagent were slightly more effective in culture than conjugates made with SPDP. Tissue distribution studies in tumor-bearing nude mice demonstrated that tumor uptake of the ZME-RG immunotoxin was similar to that of the intact ZME antibody with reduced distribution to normal organs compared to an immunoconjugate produced with NG. Pharmacokinetic studies showed that the terminal-phase plasma half-life of ZME-RG was similar to that of ZME itself (42 h vs 50 h) and almost threefold higher than that of ZME-NG (11.5 h). The area under the concentration curve (Cxt) for ZME-RG was 50% lower than that for ZME due to an increased apparent volume of distribution (Vd_a) but was almost tenfold higher than the Cxt for ZME-NG. These studies suggest that immunoconjugates comprising RG demonstrate identical in vitro cytotoxic effects to immunoconjugates produced with NG and immunotoxins with RG display improved in vivo pharmacodynamics and tissue distribution compared to immunotoxins containing NG.

Key words Immunotoxins · Gelonin · Melanoma · Recombinant toxins · Pharmacokinetics

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Introduction

The use of tumor-targeted therapy has been extensively investigated over the past two decades [2, 7, 13, 26]. Numerous extracellular targets have been identified and specific, high-affinity antibodies have been developed recognizing these epitopes using conventional hybridoma techniques. Advances in molecular biology have recently been applied to design and generate unique, high-affinity recombinant antibodies using the phage display approach [8, 11]. While there have been a variety of problems noted with the antibody targeting approach, there have also been some notable advances in the field primarily in the use of monoclonal antibodies (mAb) for radioimmunotherapy of solid tumors [6, 31, 32]. This area of research has led to effective therapy in some disease states [9, 33] and remains a potentially important avenue of research opportunity.

The advent of molecular approaches leading to the design and expression of novel, recombinant molecules has brought forward second- and third-generation constructs directed to the tumor cell surface and to the vasculature supporting tumor growth [10, 14, 27]. Growth

factor-fusion toxins and engineered antibody fragments also fused to toxins have been generated and tested in preclinical models over the past several years [12, 23]. In addition, various cytokine-fusion toxins such as IL-2/DT and EGF/DT are currently in clinical trials [5].

We recently described the complete amino acid sequence for the single-chain, ribosome-inhibiting plant toxin gelonin [19] and we have also developed a synthetic gene encoding the mature toxin and have expressed in *E. coli* biologically active, recombinant gelonin (RG) toxin. The availability of this novel recombinant protein adds to the currently small but growing list of cytotoxic effector molecules suitable for the development of targeted therapeutic agents. The purpose of the current study was to evaluate the in vitro and in vivo biological activity of an antimelanoma immunotoxin containing RG and compare its behavior to an immunotoxin containing natural gelonin (NG). In addition, we produced immunotoxins utilizing either the standard SPDP crosslinking reagent or a more stable reagent designated SMPT [28] to determine the optimal construct for eventual in vivo use. Finally, we examined the pharmacokinetics and tissue localization of immunotoxins containing both NG and RG in an effort to understand how immunotoxin construction details affect the overall in vivo performance of these agents.

Materials and methods

Materials

Antibody ZME-018 is a murine mAb which recognizes epitope "a" on the high molecular weight proteoglycan gp240 present on the cell surface of over 80% of human melanoma cell lines and biopsy specimens. This antibody was purified from ascites using salt fractionation and DEAE chromatography and was judged homogeneous by SDS-PAGE. SPDP reagent (*N*-syccinimidyl-3-[2-pyridyldithio]proprionate), SMPT reagent [4-succinimidyloxycarbonyl-2-methyl-2-(2-pyridyldithio)toluene], and 2-iminothiolane HCl were purchased from Pierce Chemical Co. (Rockford, Ill.). Sephacryl S-300 gel permeation resin and blue sepharose CL-6B resin were purchased from Pharmacia (Piscataway, N.J.). For estimation of the cell-free protein synthesis inhibitory activity of the toxin, a rabbit reticulocyte translation kit was utilized as purchased from Amersham (Arlington Heights, Ill.).

Modification of ZME-018 using SPDP

The details of the generation of conjugates of the antibody ZME-018 and the plant toxin gelonin have been published elsewhere [18]. Briefly, A stock solution of SPDP (6 mg/ml) in dry DMF was added to 1 ml of a PBS solution containing 1 mg ZME-018. SPDP was slowly added to a fivefold molar excess. Excess unreacted SPDP was removed from the sample by gel filtration chromatography on a Sephadex G-25 column. SPDP-derivatized antibody eluted at the void volume and these fractions were pooled and kept at 4 °C.

Modification of ZME-018 using SMPT

A stock solution of SMPT (5 mg/ml) in dry DMF was prepared. To a solution of 1 mg ZME-018, SMPT was added slowly with vortexing to a final concentration of a fourfold molar excess. The mixture was then incubated with stirring for 1.5 h at room temperature and then purified using a G-25 column equilibrated with 100 m*M* Na_2HPO_4 containing 0.5 m*M* EDTA (pH 7.0). Protein fractions were pooled and analyzed for protein content using the Bradford dye binding assay.

Coupling of SPDP-modified or SMPT-modified ZME-018 to either NG or RG

For these studies, NG toxin was extracted from the seeds of *Gelonium multiflorum* and purified to homogeneity utilizing the method of Stirpe et al. [24] with modifications [18]. We have previously demonstrated the complete amino acid sequence for gelonin and a designer gene encoding the mature gelonin protein [19]. RG was expressed under the control of an arabinose-induced promoter in the *E. coli* strain E-104 and purified from the culture supernatant by ion exchange and Blue Sepharose Cl-6B affinity chromatography as previously described for NG [24].

Purified gelonin (1 mg in PBS at 2 mg/ml) was added to triethanolamine hydrochloride (TEA/HCl) buffer to a final concentration of 60 m*M* TEA/HCl and adjusted to pH 8.0. EDTA was added to a concentration of 1 m*M*. A 2-iminothiolane stock solution (0.5 *M* in 0.5 *M* TEA/HCl, pH 8.0) was added to a final concentration of 1 m*M* and the solution was incubated for 90 min at 4 °C under nitrogen.

Excess 2-iminothiolane was removed by gel filtration on a column of Sephadex G-25 (1 × 24 cm) pre-equilibrated with 5 m*M* bis-tris acetate buffer, pH 5.8, containing 50 m*M* NaCl and 1 m*M* EDTA. Fractions were analyzed for protein content in 96-well microtiter plates using the Bradford dye binding assay. Gelonin eluted at the void volume (fractions 14–20). SPDP-modified antibody ZME was mixed with an equal weight of 2-iminothiolane modified gelonin. This proportion corresponded to a fivefold molar excess of gelonin as compared to antibody. The pH of the mixture was adjusted to 7.0 by the addition of 0.5 *M* TEA/HCl buffer (pH 8.0) and the mixture was incubated for 20 h at 4 °C under nitrogen. Iodoacetamide (0.1 *M* in H_2O) was added to a final concentration of 2 m*M* to block any remaining free sulfhydryl groups and incubation was continued for an additional hour at 25 °C.

For production of SMPT conjugates, 1 mg purified gelonin (either NG or RG) was first reduced by addition of DTT to final a concentration of 2 m*M* followed by incubation for 30 min at room temperature with occasional vortexing. Excess DTT was removed by gel filtration on a Sephadex G-25 column pre-equilibrated with 100 m*M* Na_2HPO_4 buffer containing 0.25 *M* NaCl at pH 7.5. Protein fractions were pooled and analyzed by the Bradford dye binding assay then SMPT-modified ZME-018 was mixed with an equal weight of reduced gelonin. The mixture was then incubated for 96 h at room temperature under nitrogen. Afterward, unreacted sites on the SMPT-modified antibody were treated with 0.2 m*M* cysteine and stored at 4 °C.

Purification of ZME-gelonin complexes

To remove low molecular weight products and nonconjugated gelonin, the reaction mixture was applied to a Sephacryl S-300 column (1.6 × 31 cm) previously equilibrated with PBS. Fractions (1.0 ml) were collected and 50-µl aliquots were analyzed for protein content using the Bio-Rad dye binding assay. To remove unconjugated ZME-018, the high molecular peak (fractions 28–41) from the S-300 column was applied to an affinity chromatography column of Blue Sepharose CL-6B (1 × 24 cm) pre-equilibrated with 10 m*M* phosphate buffer (pH 7.2) containing 0.1 *M* NaCl. After sample loading, the column was washed with 50 ml buffer to completely elute nonconjugated antibody. The column was eluted with a linear salt gradient of 0.1 to 2 *M* NaCl in 10 m*M* phosphate buffer, pH 7.2. Protein content of the eluted fractions was determined by the dye-binding assay described previously [18].

Cell culture methods

Cell lines were maintained in culture in complete medium at 37 °C under an atmosphere of 5% CO_2 in humidified air in an incubator. For assays with immunotoxins, cultures were washed, detached using versene and resuspended in complete medium at a density of 25×10^3 cells/ml. Aliquots (200 µl) were dispensed into 96-well microtiter plates and the cells were then allowed to adhere. After 24 h, the medium was replaced with medium containing different concentrations of either immunotoxin or gelonin. The cells were incubated for 72 h and analyzed for relative cell proliferation by crystal violet staining as previously described [18]. Values shown are the means of duplicate experiments performed in octuplicate. Human bladder carcinoma (T-24), human cervical carcinoma (ME-180) or human metastatic melanoma (A375M or AAB-527) cells were maintained in culture using minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum plus 100 µ*M* non-essential amino acids, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, vitamins and antibiotics. Cultured cells were routinely screened and found to be free of mycoplasma infection.

Iodination of gelonin and gelonin complexes

Briefly, 1 mCi of $Na^{125}I$ (NEN DuPont, specific activity 17.4 mCi/µg) was added to 20 µl of 0.1 *M* Na_2HPO_4, pH 7.4. The mixture was then added to a reaction vial coated with 10 µl of iodogen (previously dried in chloroform). The reaction was allowed to proceed for 15 min at room temperature. Unreacted radio-iodine was removed by chromatography on a Sephadex G-25 (PD-10) column (Pharmacia, Piscataway, N.J.). Incorporation of radiolabel into protein complex as measured by TCA precipitation was greater than 90%.

Animal model studies

Tissue distribution study

Athymic (nu/nu) mice were obtained at 4–6 weeks of age from Harlan Sprague Dawley, Indianapolis, Ind. The animals were maintained under specific pathogen-free conditions and were used at 6–8 weeks of age. Animals were injected subcutaneously (right flank) with 2×10^6 log-phase A375-M melanoma cells and tumors were allowed to establish for 3 weeks. MAbs and immunoconjugates were labeled with ^{125}I 24 h prior to injection. After examining the immunoreactivity of the antibody and immunoconjugate, mice were injected (i.v., tail vein) with 5 µCi of label and 10 µg of total protein in 200 µl normal saline. Mice were sacrificed by cervical dislocation 72 h following injection. Samples of blood, tumor, heart, lung, liver, spleen, kidney, intestine and muscle were removed, weighed and assayed for radioactivity in a Packard gamma counter (model 5360). The percentage of the injected dose (ID) of mAb per gram of tissue (%ID/g) in tumor and normal organs was calculated. Tumor to blood ratios were calculated by dividing the %ID/g mAb in tumor by the %ID/g mAb in the respective organ.

Pharmacokinetic studies

BALB/C mice at 4–6 weeks of age were injected with 0.3 µCi (5 µg) of labeled mAb ZME-018, ZME-rG or ZME-NG immunoconjugate. At 15, 30, 45, 60, 75, 90, 105, 120 and 240 min and 24 h after injection, two mice at each time-point were sacrificed by cervical dislocation. Blood samples were removed (chest cavity), weighed and counted to determine the total radioactivity in a gamma counter (Packard, model 5360). The blood samples were also centrifuged and plasma was decanted and counted to determine radioactivity. Results from plasma determination of radioactivity were analyzed by a least-squares nonlinear regression (RSTRIP, from MicroMath) program to determine pharmacokinetic parameters.

Results

We and other groups have previously noted that NG is relatively nontoxic to log-phase mammalian cells in vitro except at relatively high concentrations [24]. Comparison of RG and NG toxicity to intact cells (Fig. 1) showed that the RG was severalfold less toxic to intact cells than the parental NG. On the other hand, examination of these two molecules in a cell-free assay for the inhibition of ribosomal protein synthesis suggests that the *n*-glycosidic activity of both NG and RG is equivalent [19]. Thus, the nonspecific toxicity observed with NG but not with RG may be a consequence of toxin binding to cell-surface components recognizing carbohydrate sites on the natural molecule.

Immunotoxins composed of ZME and containing either RG or NG were produced, purified and tested against log-phase human melanoma (A375) cells in culture. As shown in Fig. 2, both immunotoxins displayed almost equivalent cytotoxicity against antigen-positive cells. Against antigen-negative (Me-180) cells, both ZME-NG and ZME-RG were not cytotoxic at doses up to 1 µ*M* (data not shown). A slightly higher percentage cytotoxicity was noted with ZME-RG than with ZME-NG at doses higher than 1 n*M*.

The heterobifunctional crosslinking reagent SMPT has been shown to create a more metabolically stable, hindered disulfide crosslink compared to the parental SPDP reagent when utilized to enable antibody-toxin conjugates [29]. Immunoconjugates of ZME-RG were produced utilizing either SMPT or SPDP to enable the crosslink. The in vitro cytotoxicity of both reagents was then compared at equivalent concentrations. As shown in Fig. 3, both immunotoxins showed substantial cyto-

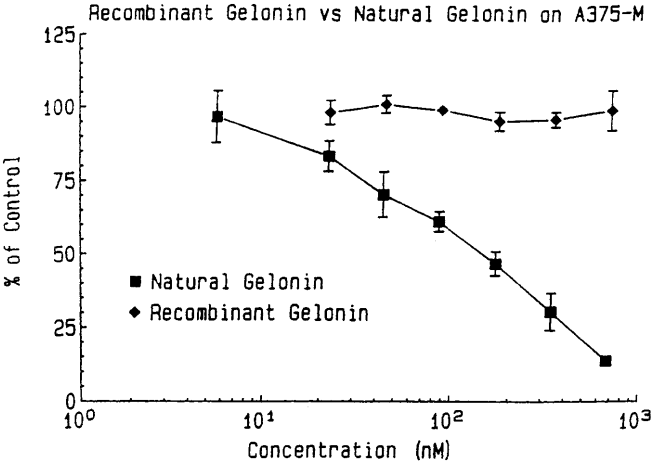

Fig. 1 Cytotoxicity of either recombinant gelonin (RG) or natural gelonin (NG) against human melanoma cells (A-375) in log-phase culture. Various doses of each molecule were added to 96-well culture plates containing approximately 5000 cells/well. The plates were incubated for a further 72 h at 37 °C under an atmosphere of 5% CO_2 in humidified air in an incubator. Remaining cells were assessed and compared to untreated control wells. Values shown are means ± SEM for one experiment performed in octuplicate

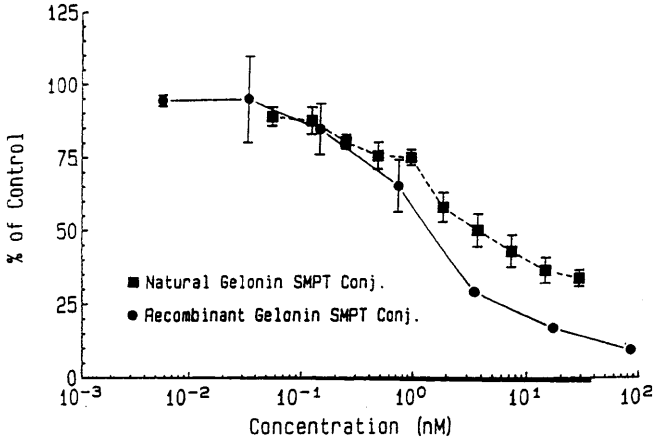

Fig. 2 Comparative cytotoxicity of immunotoxins containing either natural gelonin (NG) or recombinant gelonin (RG) against human melanoma cells (A-375) in log-phase culture. Doses of antimelanoma immunotoxins containing either NG or RG were added to 96-well culture plates containing approximately 5000 cells/well. The plates were incubated for a further 72 h at 37 °C under an atmosphere of 5% CO_2 in humidified air in an incubator. Remaining cells were assessed and compared to untreated control wells. Values shown are means ± SEM for one experiment performed in octuplicate

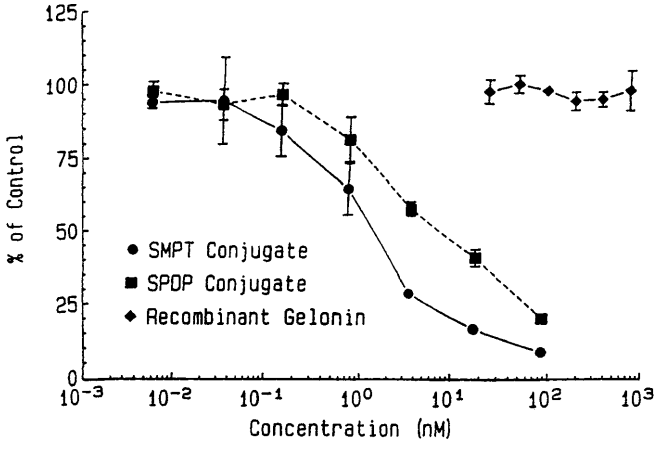

Fig. 3 Comparative cytotoxicity of antimelanoma immunotoxins containing recombinant gelonin (RG) but generated utilizing either SMPT or SPDP crosslinking reagents. Various doses of immunotoxins or RG (control) were added to 96-well culture plates containing approximately 5000 cells/well. The plates were incubated for a further 72 h at 37 °C under an atmosphere of 5% CO_2 in humidified air in an incubator. Remaining cells were assessed and compared to untreated control wells. Values shown are means ± SEM for one experiment performed in octuplicate

toxicity with IC_{50} values of 1–2.5 n*M*. Under these in vitro conditions, these results suggest that the more stable crosslinked immunotoxin displays equivalent if not slightly improved cytotoxicity compared to the immunotoxin produced with SPDP.

Both ZME-RG and ZME-NG immunotoxins created utilizing the SMPT reagent and native ZME antibody were radiolabeled and administered as an i.v. bolus to Balb/c mice. As shown in Fig. 4 and Table 1, all three

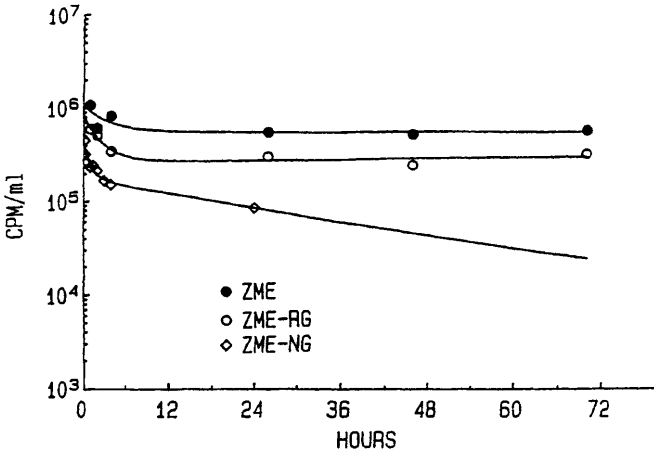

Fig. 4 Plasma clearance of ^{125}I labeled ZME antibody and immunotoxins containing either recombinant gelonin (RG) or natural gelonin (NG). Mice were injected (i.v., tail vein) and then sacrificed at various times after administration. Blood samples were obtained and counted as described in the Methods section. Values shown are the data points and the least squares, best-fit lines. Pharmacokinetic values for these data are presented in Table 1

Table 1 Pharmacokinetic summary. Pharmacokinetic studies of ZME, ZME-RG and ZME-NG demonstrate that the conjugate containing the recombinant toxin was cleared with a plasma half-life approaching that of native ZME antibody while the ZME-NG construct was cleared at a much faster rate. The area under the concentration curve (Cxt) for the recombinant toxin was sixfold greater than that of the immunoconjugate containing NG. Values shown are means ± SEM

Parameter	ZME	ZME-NG	ZME-RG
Half-life (h)	50.3 ± 4.6	11.5 ± 1.6	41.9 ± 5.2
Cp_0 (μCi/ml)	0.38 ± 0.12	0.38 ± 0.04	0.22 ± 0.04
Vd (ml)	2.86 ± 0.21	2.65 ± 0.32	4.9 ± 0.67
Cxt (μCi/ml × min)	27.6 ± 0.35	1.82 ± 0.19	13.4 ± 0.21
Clp (ml/kg × min)	1.81 ± 0.12	27.5 ± 3.6	3.73 ± 0.57

proteins cleared biphasically from plasma. The terminal phase half-life ($t_{1/2\ beta}$) of ZME-NG was the shortest of the three at 11.5 h while that of the ZME-RG immunotoxin and native ZME were similar at 42 and 50 h, respectively. Surprisingly, the immediate apparent volume of distribution (Vd_a) for the immunotoxin containing RG was almost twofold higher than that of the native ZME or the ZME-NG immunotoxin, suggesting significant adsorption of this immunotoxin at sites outside the vasculature. This accounts for a comparative 50% decrease in the area under the concentration curve (Cxt) of ZME-RG compared to that of the native ZME. On the other hand, the rapid clearance of the ZME-NG immunotoxin resulted in a Cxt approximately 10 times lower than that of the ZME-RG immunotoxin and almost 17 times lower than that of the native antibody.

At 72 h after administration, tissue distribution studies of ZME-RG and ZME-NG compared to ZME in tumor-bearing nude mice (Fig. 5) demonstrated significant tumor localization. The concentrations of each agent were found to be 3.7, 3.2 and 2.4%ID/g in tumor

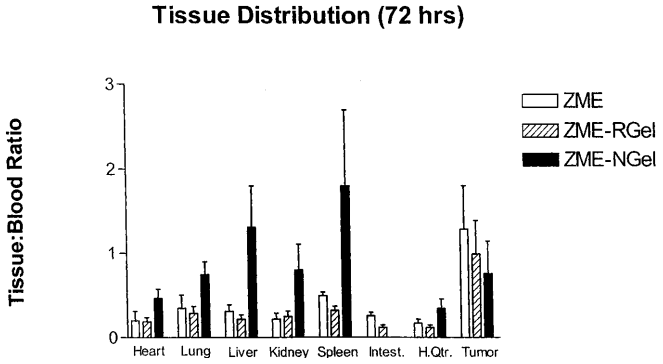

Fig. 5 Distribution of ^{125}I-labeled ZME antibody and immunotoxins containing either recombinant gelonin (RG) or natural gelonin (NG). Mice were injected (i.v., tail vein) and then sacrificed 72 h after administration. Samples of blood and other organs were obtained, weighed and counted. Values shown are means ± SEM for six mice per group

tissue for ZME, ZME-RG and ZME-NG, respectively. The concentrations of the ZME-NG construct were higher in spleen, liver, kidney, lung and heart than the concentrations of either ZME itself or the ZME-RG construct.

Discussion

Over the past several years, various immunotoxins have been developed which target both hematopoietic and solid tumors [22, 34]. Major problems associated with immunotoxin therapy include poor penetration of the agent into the tumor structure, rapid in vivo clearance of the construct by components of the reticuloendothelial system (RES), premature degradation of the immunotoxin, capillary leak syndrome leading to fluid imbalance and rapid onset of action of antitoxin antibodies limiting further drug administration [3, 30]. In an attempt to circumvent some of these problems, we have developed the unique recombinant type I ribosome-inhibiting toxin, RG. While ricin A chain (RTA) and gelonin are both known to act functionally by *n*-glycosidic cleavage of 28s rRNA, it was surprising to note that gelonin shares only 30% structural homology with the plant toxins RTA and trichosanthinin. Arias et al. [1] and Fong et al. [4] have also reported plant toxins with similar enzymatic activities but which differ markedly in their primary amino acid sequence. We are currently comparing a three-dimensional model of RTA with proposed models of RG in an attempt to more closely identify structure-activity relationships and functional domains within the RG molecule.

One of the persistent problems with immunotoxin constructs containing either RTA or *Pseudomonas* exotoxin (PE) has been the relatively rapid clearance in vivo compared to that of the original antibody. Studies with RTA as well as with other toxins [17, 21, 28] have demonstrated that the carbohydrate sites on the native molecule are, in part, responsible for the observed rapid clearance of RTA-based immunotoxins. Studies by Thorpe et al. [28] have demonstrated specificity of the in vivo recognition by the RES of plant carbohydrate structures. These studies clearly demonstrate that improved tissue localization and pharmacokinetics can be achieved by chemical modification of the carbohydrate residues on the plant toxin molecule. The generation of recombinant, nonglycosylated (i.e. *E. coli*-derived) toxins such as recombinant RTA have provided immunotoxin constructs with improved in vivo performance compared to the natural product [21]. Previous studies in our laboratory have demonstrated that immunotoxins containing NG appear to have in vivo clearance and tumor localization properties similar to those of the original antibody [15]. This occurs despite the observation that NG appears to contain a significant number of carbohydrate molecules [19]. The current study demonstrates that immunotoxins containing *E. coli*-derived nonglycosylated RG also have improved clearance and localization kinetics compared to NG while maintaining their in vitro cytotoxic effects. In addition, we demonstrated that free RG was even less toxic to intact mammalian cells than the NG. The cytotoxicity of free NG is probably due to nonspecific cellular uptake of the molecule probably mediated, in part, by binding of the carbohydrate residues to the cell surface.

The development of recombinant cell-targeted fusion constructs containing ribosome-inhibiting toxins such as gelonin has provided a new generation of simple, smaller and less-toxic molecules for preclinical and clinical investigation. However, for some therapeutic applications, chemical constructs of recombinant plant toxins and recombinant, intact antibodies is still an appropriate developmental approach. There are numerous studies still ongoing utililizing chemically produced immunotoxins [25]. Preclinical and clinical studies are also ongoing in our laboratory utilizing chemical constructs of RG and humanized antibodies targeting CD33 [16] and HER2/neu [20].

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