

## The antileukemic efficacy of an immunotoxin composed of a monoclonal anti-Thy-1 antibody disulfide linked to the ribosome-inactivating protein gelonin\*

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**Summary.** We prepared an immunoconjugate consisting of a monoclonal antibody recognizing the Thy-1 antigen and the ribosome-inactivating protein gelonin linked by a disulfide bond. This immunotoxin preparation was judged to contain less than 5% free antibody or gelonin. It was highly toxic *in vitro* in an antigen-specific fashion to the Thy-1 expressing RADA leukemia of A/J mice. The  $IC_{50}$  of this preparation on RADA *in vitro* was  $10^{-12}M$ , while the  $IC_{50}$  on the Thy-1 negative S1509a fibrosarcoma of A/J mice was  $10^{-7}M$ . The toxicity of this immunoconjugate was also measured in a direct proliferation assay and it was found that a 4-h exposure and a 24-h exposure of RADA cells to a 1 nM concentration of immunotoxin killed 90% and 99.9% of cells, respectively. Furthermore, efficacy *in vitro* was not due to the intrinsic susceptibility of RADA cells to this type of immunotoxin, as one prepared with gelonin and an antibody recognizing the TL<sup>a</sup> determinant on this leukemia had no efficacy *in vitro*. Clearance of the anti-Thy-1-gelonin immunoconjugate from the circulation of A/J mice after *i.v.* injection was rapid, especially during the first 8 h after injection, possibly because of binding to Thy-1 expressing tissue. Delivery of immunoconjugate to ascitic tumor *in vivo* was substantially better if the immunoconjugate was given by *i.p.* injection, rather than by the *i.v.* route. When given either *i.v.* or *i.p.* at the time of *i.p.* tumor inoculation *in vivo*, the anti-Thy-1-gelonin immunotoxin showed potency in an antigen-specific fashion; while this immunoconjugate prolonged survival and frequently cured RADA-inoculated mice, neither anti-Thy-1 antibody, gelonin, a combination of the two, nor immunotoxin of irrelevant specificity had any significant effect on survival. Anti-Thy-1-gelonin also had no effect on survival of A/J mice inoculated *i.p.* with S1509a. Furthermore, it was determined that a single *i.p.* dose of anti-Thy-1-gelonin killed 90% to 99% cells *in vivo*, and that the immunoconjugate was about as effective in this model as either adriamycin or cytoxan.

### Introduction

The use of protein toxins conjugated to monoclonal antibodies (MCA) to derive antineoplastic agents has been a

subject of intense research in the last decade [38]. The aim of such work is to produce more tumor-specific, and therefore more efficacious and less toxic reagents than are currently available for the treatment of neoplasia. Recent studies have focused on the conjugation of MCA to single chain polypeptide toxins, either derived from double chain toxins, such as ricin-A [30, 39, 43, 44, 45, 50, 57, 59, 61] or abrin-A [27], or prepared in native form such as gelonin [33, 54, 55], saporin [33, 56] and pokeweed antiviral protein [33, 43]. These immunoconjugates have shown efficacy against tumor cells *in vitro* and, less frequently, against tumor *in vivo* in animals [27, 30, 38, 43, 50, 54–57, 59, 61]. In addition, small drugs currently used for the treatment of neoplasia have been conjugated to MCA or polyclonal antisera and have shown some efficacy against animal tumors *in vivo* [1, 13, 40, 41, 46, 47, 51]. A large number of these animal tumor models have been human xenografts in congenitally athymic nude mice. While it is important to demonstrate the efficacy of a candidate immunotoxin against a human tumor *in vivo*, results of trials in human xenograft systems can sometimes be difficult to interpret since athymic mice readily reject large numbers of xenogeneic cells without treatment [18] and because MCA alone is frequently efficacious in these systems [23, 24, 36, 48, 52]. The advantages of using a syngeneic murine tumor model to test candidate immunotoxins are the rapidity and reproducibility of tumor development and the relative difficulty in achieving a cure because of the low numbers of cells required for a successful tumor growth [56, 59]. Murine leukemias and lymphomas have been used by various groups as model systems for the study of immunotoxin efficacy, and success has been reported in some cases [13, 43, 51, 55, 56, 59]. In this paper we report the development of an immunotoxin consisting of a conjugate of MCA to the Thy-1 antigen and the ribosome-inactivating protein gelonin, and its efficacy *in vitro* and *in vivo* in a syngeneic host against the murine thymic leukemia RADA. We found this immunotoxin to be as toxic as intact ricin to RADA cells *in vitro* in a wholly antigen-specific fashion. Furthermore, treatment of RADA-bearing mice with this immunotoxin produced cures in some mice.

### Materials and methods

#### Antibodies

The M549 MCA (RAT IgG<sub>2a</sub>, anti-Thy-1) was a gift from Dr. Tim Springer, Dana-Farber Cancer Institute. This an-

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tibody recognizes both the Thy1.1 and Thy1.2 alleles [12]. HDTLG (mouse IgG<sub>2a</sub>, anti-TL<sup>a</sup>) was a gift from Dr. E. Stockert, Memorial Sloan Kettering Cancer Center. 7T4.7E10 (mouse IgG<sub>2a</sub> anti-T11<sub>1C5</sub>, [37]) was a gift from Dr. E. Reinherz, Dana-Farber Cancer Institute. 5E9 (mouse IgG<sub>1</sub> anti-human transferrin receptor) was obtained from the American Type Culture Collection (ATCC HB21). M549 was grown as ascites in nude mice, the other antibodies were grown as ascites tumors in BALB/C mice and were purified as described previously [33, 34, 49]. Antibodies were judged pure by polyacrylamide sodium dodecyl sulfate (SDS) gel electrophoresis and by isoelectrofocusing gels.

#### Animals

A/J mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. Swiss nude outbred females were purchased from Taconic Farms. Animals were maintained in accordance with guidelines of the Committee on Animal Care of the Dana-Farber Cancer Institute and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. [NIH] 78-23 revised 1978).

#### Purification of M549

The anti-Thy-1 antibody was purified by precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 50% saturation. The precipitated protein was dissolved in 0.1 M potassium phosphate buffer, pH 7.5, and then dialysed into 20 mM Tris/HCl buffer, pH 7.5, containing NaN<sub>3</sub> (0.4 mM) before being applied to a column (30 ml bed volume for 200 mg protein) of DEAE-cellulose (DE-52; Whatman Inc., Clifton, NJ) that had been equilibrated in the same buffer. Fractions containing anti-Thy-1, which was not bound by the column, were concentrated and submitted to gel filtration on a column (95 cm × 2.6 cm) of Sephacryl S-300 (Pharmacia, Piscataway, NJ) equilibrated in 10 mM potassium phosphate buffer, pH 7.2, containing NaCl (145 mM). The purified antibody was sterilized by filtration through 0.22 μm filters (Millex-GV, Millipore Corp., Bedford, Mass.) and stored at -70 °C. The antibody was judged pure by polyacrylamide/SDS gel electrophoresis and by isoelectrofocusing using PAG plates (Ampholine pH range 3.5-9.5, from LKB, Gaithersburg, Md.) as described previously [33].

#### Other reagents

2-Iminoethiolane, *N*-succinimidyl 3-(2-pyridyldithio)propionate and Iodo-gen were from Pierce Chemical Co., Rockford, Ill. Carrier-free Na<sup>125</sup>I (100 mCi/ml) was obtained from Amersham Corp., Arlington Heights, Ill. The ribosome-inactivating protein gelonin was purified from the seeds of *Gelonium multiflorum* by the method of Stirpe et al. [53]. Seeds were from United Chemical and Allied Products, Calcutta, India, and were obtained through Mr. R. Parisen, Meer Corporation, North Bergen, NJ.

#### Preparation and purification of a disulfide-linked conjugate between anti-Thy-1 and gelonin

Anti-Thy-1 (120 mg) was dialysed into 5 mM sodium phosphate buffer, pH 6.5, containing NaCl (33 mM) and NaN<sub>3</sub> (0.4 mM), and then passed through a column (12 ml bed volume) of CM-cellulose (CM-52; Whatman) that was

equilibrated in the same buffer. Most of the antibody (103 mg) did not bind to the column under these conditions and this fraction was used in the conjugation reaction. It was expected that some of the purified antibody would bind to the column since it is well established that antibodies are heterogeneous with respect to charged species [2, 9, 33].

Conjugation of anti-Thy-1 and gelonin was performed using methods described in detail elsewhere [33, 34]. Briefly, anti-Thy-1 (100 mg at 1 mg/ml) in 100 mM sodium phosphate buffer, pH 7.0, containing EDTA (0.5 mM) was treated with *N*-succinimidyl 3-(2-pyridyldithio)propionate (60 μM) at 30 °C for 30 min, and then excess reagent was removed by dialysis. Gelonin (100 mg at 2 mg/ml) in 60 mM triethanolamine/HCl buffer, pH 8.0, containing EDTA (1 mM) was treated with 2-iminoethiolane (1 mM) at 0 °C for 90 min under an atmosphere of nitrogen, and then excess reagent was removed by gel filtration at 4 °C on a column of Sephadex G-25 (fine) equilibrated with 5 mM Tris acetate buffer, pH 5.8, containing NaCl (50 mM) and EDTA (1 mM). Conjugation was effected by mixing the modified antibody (100 mg) with a fivefold molar excess of modified gelonin (100 mg) and adjusting the pH of the mixture to 7.0 by addition of 0.5 M triethanolamine/HCl buffer, pH 8.0. The reaction was allowed to proceed at 4 °C for 20 h under nitrogen, after which iodoacetamide (2 mM) was added to alkylate any remaining free sulfhydryl groups.

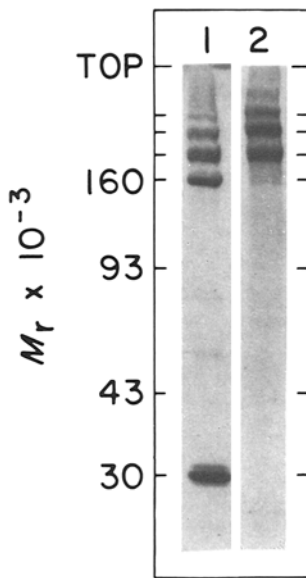
Disulfide-linked anti-Thy-1 gelonin conjugates were purified from the reaction mixture by concentrating the mixture by ultrafiltration and then passing it through a column (95 cm × 2.6 cm) of Sephacryl S-300 equilibrated with 10 mM potassium phosphate buffer, pH 7.2, containing NaCl (145 mM). Fractions containing conjugate, which does not resolve from antibody by gel filtration, were pooled and dialysed into 5 mM sodium phosphate buffer, pH 6.5, containing NaCl (33 mM) and NaN<sub>3</sub> (0.4 mM) and then fractionated on a column of CM-cellulose equilibrated in the pH 6.5 buffer. Free antibody passed through this column, as described above, while the conjugate was bound by the CM-cellulose and subsequently eluted by buffer containing 1.0 M NaCl. The purified conjugate was finally submitted to gel filtration on a column (95 cm × 2.6 cm) of Sephacryl S-300 equilibrated with 10 mM potassium phosphate buffer, pH 7.2, containing NaCl (145 mM), and then sterilized by filtration (0.22 μm filters) and stored at -70 °C. The final yield from 100 mg of antibody was 40 mg of conjugate that was free of nonconjugated antibody, nonconjugated gelonin and aggregates of high molecular weight, as shown in Fig. 1.

#### Polyacrylamide gel electrophoresis

Protein purification and conjugation reactions were analyzed by polyacrylamide/SDS gel electrophoresis in gel slabs (145 × 90 × 0.75 mm) cast with acrylamide gradients (5%-10% wt/vol) prepared by the methods of Laemmli [32]. Sample buffers for gels run under nonreducing conditions contained 10 mg/ml iodoacetamide [33].

#### Cell lines

The RADA cell line was a generous gift from Dr. E. Stockert, Memorial Sloan Kettering Cancer Center, NY. It



**Fig. 1.** Analysis of the purification of anti-Thy-1-gelonin by polyacrylamide/sodium dodecyl sulfate gel electrophoresis. Polyacrylamide gradient gels (5%–10% wt/vol) were run under nonreducing conditions and stained with Coomassie blue R-250. *Lane 1*, conjugation reaction mixture showing bands corresponding to gelonin (molecular weight,  $M_r$ , 30,500), nonconjugated antibody ( $M_r$ , 160,000), and bands corresponding to antibody conjugated to one ( $M_r$ , 190,500), two ( $M_r$ , 221,000) and three ( $M_r$ , 251,500) molecules of gelonin. *Lane 2*, purified conjugate. The calibration of  $M_r$  was from the mobility of IgG (160,000), phosphorylase b (93,000), ovalbumin (43,000) and carbonic anhydrase (30,000)

is a thymic leukemia of strain A mice (phenotype THY-1.2 + TL<sup>a</sup> + L3T4 + Lyt2.2–). The S1509a cell line is a methylcholanthrene-induced fibrosarcoma of strain A mice, which expresses none of the above antigens [21]. Both lines were maintained in tissue culture in RPMI 1640 medium supplemented with 10% preselected fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone.

#### Cytotoxicity assays

**(A) Inhibition of <sup>3</sup>H-thymidine incorporation into cellular DNA.** RADA or S1509a cells ( $1 \times 10^4$ ) were cultured in a volume of 0.2 ml in the presence of various reagents (see text) in the standard culture medium for 24 h in 96-well flat bottomed plates (Falcon). They were pulsed with 1 µCi of <sup>3</sup>H-thymidine for the last 4 h, then the cells were harvested on glass fiber filters on a PhD cell harvester and <sup>3</sup>H-thymidine incorporation was determined by scintillation counting. Results are expressed as a percentage of control (<sup>3</sup>H-thymidine incorporation in the absence of any reagents). The <sup>3</sup>H-thymidine incorporation in control wells ranged from 50,000 to 100,000 cpm. The IC<sub>50</sub> was determined to be the concentration at which 50% inhibition of the maximal proliferative response occurred.

**(B) Clonogenic assay.** After treatment with a toxin, RADA cells were washed with warm (37 °C) growth medium and plated into multiwell (96-wells per plate) flat-bottomed plates in 0.2 ml of growth medium/well. A total of 500 or more cells were plated at a cell density of 3 to 50 cells/well (control cells) or 3 to  $2 \times 10^4$  cells/well (treated cells). Plat-

ing efficiency (PE) of cells was about 0.1 to 0.3 and did not depend on the density of plated cells per well. Cells were maintained for 14 days at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> and within this interval of time colonies of about 10<sup>5</sup> cells were formed. These colonies were scored under a light microscope. Poisson distribution was used for the determination of PE [17]:

$$PE = \frac{-\ln(\text{fraction of empty wells})}{\text{average number of cells plated per well}}$$

The surviving fraction was determined as the ratio of the PE of a treated culture to the PE of the control culture.

**(C) Determination of surviving fractions in lymphoblastoid cell lines by back extrapolation of cell proliferation curves.** This assay (the so called “growth back extrapolation” assay [19]) is based on the assumption that dead cells, or cells that are committed to die, gradually cease to proliferate, while survivors of a toxic treatment continue to proliferate. Cell cultures that had been exposed to a toxin were centrifuged, washed once with warm growth medium and resuspended in fresh growth medium at a density about  $2 \times 10^5$  cells/ml. It was essential that the medium used for washings and resuspending of cells be warm, because even a brief (30 min) exposure to cold (0 °C) medium caused a growth delay. The cells were then incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> and counted daily using a Coulter counter. The cultures were diluted with fresh medium to  $3 \times 10^5$  cells/ml when they reached a density above  $6 \times 10^5$  cells/ml. The daily increase in the cell density was used to calculate an increase of the number of cells in the culture assuming no dilution. An estimate of the number of surviving cells was made by extrapolation of exponential growth curves of treated cultures to the end of the treatment period. Then surviving fractions were calculated as a ratio of the extrapolated number of surviving cells in treated culture to the number of cells in the control culture.

#### Clearance studies

Studies on circulatory clearance were performed essentially as previously described [49]. Purified anti-Thy-1 or anti-Thy-1-gelonin conjugate was iodinated with Na<sup>125</sup>I (Amersham) using the Iodo-gen technique [15]. Between  $5 \times 10^5$  and  $1 \times 10^6$  cpm of iodinated antibody or conjugate was injected in a carrier solution of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) via the tail vein, and 50 µl samples of blood were taken from the retroorbital plexus at various times thereafter, added to 50 µl of water and the proteins immediately precipitated with 1 ml of ice-cold 12.5% (w/v) trichloroacetic acid (TCA) for 30 min. The precipitate was collected and counted in a gamma counter. The percent remaining TCA precipitable counts per minute were calculated assuming a total blood volume of 10% of body weight, 2 ml in most cases.

Studies of binding of antibody or conjugate to tumor cells in vivo were performed as follows: <sup>125</sup>I-labeled MCA or conjugate was injected i.v. or i.p. into mice bearing ascitic tumor. In most cases, mice had received  $5 \times 10^6$  RADA or S1509a cells i.p. 7 days prior to the experiment and had visible ascites that could be tapped. A known amount of unlabeled antibody or conjugate was added to the radiolabeled preparation. At various times after injec-

tion 150–250  $\mu$ l of ascites was tapped and the total cell number was determined using a hemocytometer in the presence of acetic acid and trypan blue; greater than 95% of the cells were viable tumor cells. These cells were extensively washed at 4 °C and then incubated in 0.5 ml of water for 30 min at 4 °C, and the proteins precipitated with an equal volume of ice-cold 12.5% TCA and 50  $\mu$ l of PBS 1% BSA for an additional 30 min. The resulting precipitate was then counted in a gamma-counter. The number of molecules of antibody or conjugate bound per cell was then determined. Total TCA precipitable counts per minute was converted to total number of molecules, after determining the specific activity of the injected preparation. This number of molecules was then divided by the cell count. The number of anti-Thy-1 and anti-Thy-1-gelonin binding sites in RADA grown both in vitro and in vivo has been previously determined to be  $5 \times 10^5$  to  $9 \times 10^5$  per cell (unpublished data).

#### Efficacy of immunotoxin in vivo

A/J mice were inoculated i.p. with  $5 \times 10^4$  RADA or S1509a cells grown in vitro, except as indicated. This represented between  $10^2$  and  $10^3$  lethal doses of cells for both tumors. On the day of tumor inoculation, mice received either i.v. or i.p., varying doses of the anti-Thy-1-gelonin, antibody, gelonin, a mixture of antibody and gelonin, or immunoconjugate of irrelevant specificity, as indicated in *Results*. The endpoint used to determine efficacy was time to death after inoculation. The inoculation of  $5 \times 10^4$  RADA cells invariably resulted in death by 26 days (mean,  $16.9 \pm 2$  days, 50 mice, 6 different experiments). Furthermore, those mice inoculated with a cell number near the  $LD_{50}$  (which varied from less than 50 to about 200 cells) were always either dead by day 28 or survived for greater than 3 months. Therefore, mice surviving to 60 days without evidence of tumor on gross pathologic examination were considered to be long-term survivors and were sacrificed.

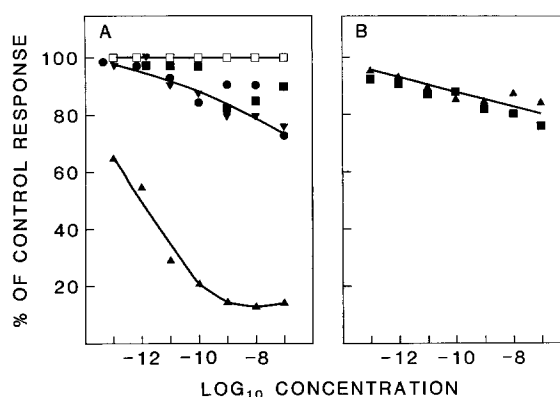
#### Statistical analysis

Survival medians were estimated by the method of Kaplan and Meier [29]. Differences in the distribution of survival times were compared between groups using a logrank test [10]. A nonparametric trend test for survival by dose was performed by ranking dose groups in ascending order corresponding to increasing dose. Cox regression analysis [11] was performed to evaluate the association between survival time distribution and dose intensity rank.

## Results

#### In vitro toxicity of the anti-Thy-1-gelonin conjugate

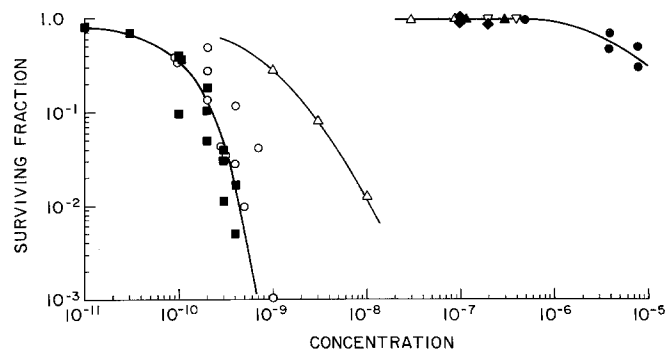
The toxicity of the anti-Thy-1-gelonin immunoconjugate was initially determined in an indirect cell proliferation assay using  $^3$ H-thymidine incorporation into DNA [33]. On RADA cells, the immunoconjugate showed an  $IC_{50}$  of about  $10^{-12}$  M, whereas anti-Thy-1, gelonin, a mixture of the two, or a similarly prepared immunoconjugate of irrelevant specificity (7T4-7E10-gelonin) showed an  $IC_{50}$  of greater than  $10^{-7}$  M (Fig. 2A). A similar  $IC_{50}$  (greater than  $10^{-7}$  M) was measured for the immunoconjugate on the Thy-1-negative A/J fibrosarcoma, S1509a (Fig. 2B). These data indicated at least 5 logarithms of specificity of the immunoconjugate. The anti-Thy-1-gelonin immunoconjugate



**Fig. 2.** Inhibition of proliferation of tumor cells by immunotoxin. RADA and S1509a cells were cultured in the presence of anti-Thy-1-gelonin ( $\blacktriangle$ ) anti-Thy-1 + gelonin ( $\blacktriangledown$ ), gelonin ( $\blacksquare$ ), anti-Thy-1 ( $\bullet$ ) or 7T4-7E10-gelonin ( $\square$ ), for 24 h, at the indicated concentrations and pulsed with  $^3$ H-thymidine for the last 4 h, as described in *Materials and methods*

also showed potent inhibitory effects in vitro on murine T cell responses, including the concanavalin A proliferative response, and the allospecific proliferative and cytolytic T cell response (not shown), as has been demonstrated for other anti-Thy-1 immunoconjugates [7, 8, 14, 25, 56, 58].

However, this type of assay using thymidine incorporation cannot accurately determine the actual percentage of cells killed or, conversely, the surviving fraction (S. F.) of cells after exposure to immunoconjugate. This was determined by the more accurate and sensitive direct cell proliferation assays, the “growth back extrapolation” assay and the clonogenic assay. The toxicity data obtained by these two assays were in accord, and therefore used interchangeably here. In these assays, anti-Thy-1-gelonin was as toxic for cultured RADA cells as ricin (Fig. 3). In sharp contrast, nonconjugated gelonin was about  $10^5$ -fold less toxic for these cells under similar conditions and anti-Thy-1 antibody, HDTLG-gelonin, HDTLG antibody, or 5E9-gelonin (which does not bind to the murine transferrin receptor) were also at least  $10^4$ -fold less toxic for RADA cells than anti-Thy-1-gelonin. Thus, the relative specificity of this immunoconjugate was identical in the indirect and di-



**Fig. 3.** Estimation of surviving fraction of cells by “growth back extrapolation” and clonogenic assays. RADA cells were exposed to the indicated concentrations of anti-Thy-1-gelonin ( $\circ$ ), ricin ( $\blacksquare$ ), gelonin ( $\bullet$ ), anti-Thy-1 ( $\blacktriangle$ ), HDTLG ( $\nabla$ ), or HDTLG-gelonin ( $\blacklozenge$ ) for 24 h, or to anti-Thy-1-gelonin for 4 h ( $\triangle$ ). Cells were then processed and the surviving fraction estimated, as described in *Materials and methods*

rect cell proliferation assay. Furthermore, the latter two assays indicated a S. F. of at least  $10^{-3}$  after a 24 h exposure to  $10^{-9}$  M anti-Thy-1-gelolin and a S. F. of  $10^{-1}$  even after only 4 h of exposure to the same concentration.

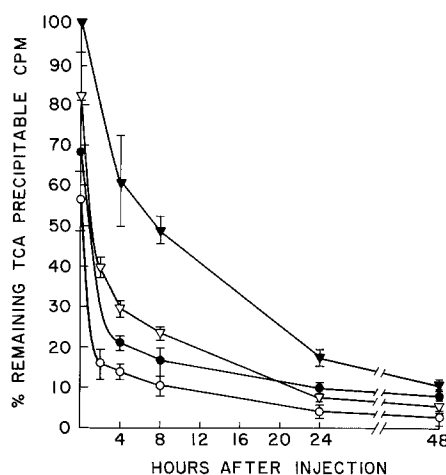
#### Pharmacokinetics and toxicity in vivo

The circulatory clearance of anti-Thy-1 and anti-Thy-1-gelolin was determined in A/J mice as described in *Materials and methods*. Both MCA and immunoconjugates were rapidly cleared from the circulation of A/J mice, but immunoconjugate was more rapidly cleared than MCA. Moreover, the co-injection of large amounts of unlabeled MCA or unlabeled immunoconjugate prolonged the circulatory clearance of each (Fig. 3). These findings are in accord with previous data which showed a rapid circulatory clearance in mice of antibodies or conjugates which recognized murine determinants, and a prolongation of clearance times when large amounts of unlabeled material are co-injected with the radiolabeled material [49].

The  $LD_{50}$  in mice of the anti-Thy-1-gelolin conjugate was about 2.5 mg/kg (see Fig. 8A), a figure approximately one order of magnitude lower than the  $LD_{50}$  in mice for identically prepared immunoconjugates of anti-human T cell specificity [49]. This higher toxicity may reflect the specificity of the anti-Thy-1-gelolin immunoconjugate for murine Thy-1 expressing T cells. In fact, splenocytes of mice injected i.v. with the conjugate at a dose of approximately one-half the  $LD_{50}$  showed transient, but modest decreases in vitro in concanavalin A proliferative and allospecific cytolytic T cell responses (not shown).

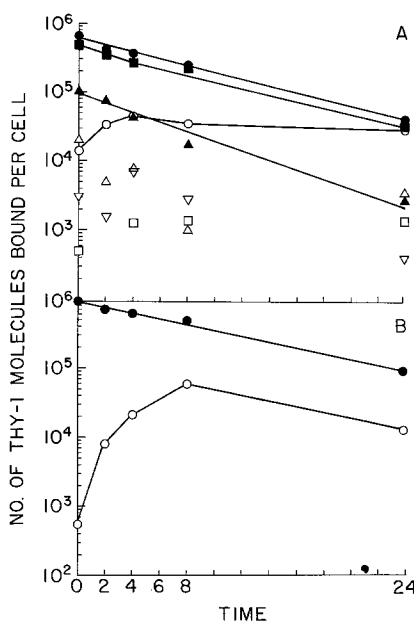
#### In vivo delivery of immunoconjugate

Since the number of Thy-1 binding sites per RADA cell in vitro or in vivo has been determined to be  $5 \times 10^5$  to  $9 \times 10^5$ , it was possible to determine how many sites were occupied by antibody or immunoconjugate after in vivo administration, as described in "Materials and methods". The ability to deliver immunoconjugate in vivo to tumor-bearing mice was studied. A/J mice which had been inoculated i.p. with  $5 \times 10^6$  tumor cells 7 days earlier were used. At this time, visible ascites containing greater than 95% viable tumor cells was present. These tumor-bearing mice were given between 1 mg/kg and 10 mg/kg (approx. 20–200  $\mu$ g) of  $^{125}$ I-labeled antibody or immunoconjugate, either i.p. or i.v., the ascites was tapped at various times afterward, and the number of molecules of antibody or immunoconjugate bound per tumor cell determined as described in "Materials and methods" (Fig. 5A and 5B). Several points are apparent. First, 10 mg/kg of either antibody or immunoconjugate clearly saturated all Thy-1 sites on RADA cells for several hours when given i.p., i.e., directly into the ascites. However, the apparent number of molecules bound decreased steadily with time after a single dose with a  $t_{1/2}$  of about 6 h for both MCA or immunoconjugate. Secondly, this same dose of MCA or immunoconjugate when given i.v. resulted in low levels of binding, although the level of binding after 24 h was similar for either i.p. or i.v. injection. Lower doses of antibody given i.p. resulted predictably in lower initial binding, but a roughly similar rate of decrease in number of molecules bound. Antigen specificity of delivery was demonstrated by the lack of binding of an irrelevant antibody (5E9, anti-transferrin receptor, [49]) to RADA in vivo and the lack of binding of anti-Thy-1 to S1509a in vivo. Finally, the



**Fig. 4.** Clearance of anti-Thy-1 and anti-Thy-1-gelolin from the blood of A/J mice; A/J mice were injected i.v. with  $^{125}$ I-labeled anti-Thy-1 (●),  $^{125}$ I-labeled anti-Thy-1-gelolin (○),  $^{125}$ I-anti-Thy-1, and 200  $\mu$ g unlabeled anti-Thy-1 (▼),  $^{125}$ I-anti-Thy-1-gelolin and 200  $\mu$ g unlabeled anti-Thy-1-gelolin (▽). Blood samples were obtained and processed at various times after injection, as described in *Materials and methods*. Each point represents at least three determinations  $\pm$  SD

amount of immunoconjugate bound to cells appeared to be similar to the amount of antibody bound when given i.p., but somewhat less when given i.v., which is not surprising in view of the more rapid circulatory clearance of immunoconjugate (Fig. 4). Binding of MCA or immunoconjugate to nodular tumor after i.v. injection was low (data not shown).

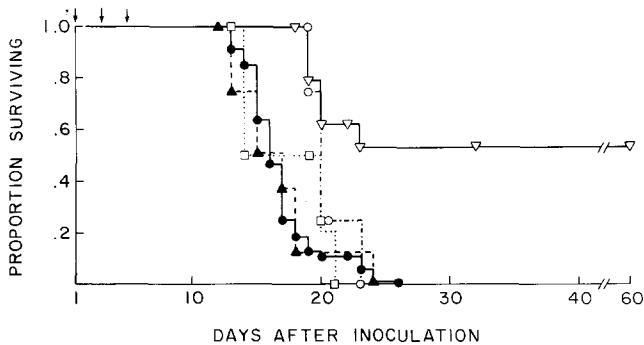


**Fig. 5A and 5B.** Binding of anti-Thy-1 and anti-Thy-1-gelolin to ascitic tumor in vivo. **A** RADA-bearing A/J mice were inoculated with  $^{125}$ I-anti-Thy-1 i.p. at 10 mg/kg (●), 5 mg/kg (■), or 1 mg/kg (▲),  $^{125}$ I-anti-Thy-1 i.v. at 10 mg/kg (○),  $^{125}$ I-5E9 i.v. at 10 mg/kg (□),  $^{125}$ I-5E9 i.p. at 10 mg/kg (△), or S1509a-bearing mice were inoculated with  $^{125}$ I-anti-Thy-1 i.p. at 10 mg/kg (▽). **B** RADA-bearing A/J mice were inoculated with  $^{125}$ I-labeled-anti-Thy-1-gelolin i.p. at 10 mg/kg (●) or i.v. at 10 mg/kg (○). Ascites was tapped and the cells processed as described in *Materials and methods*

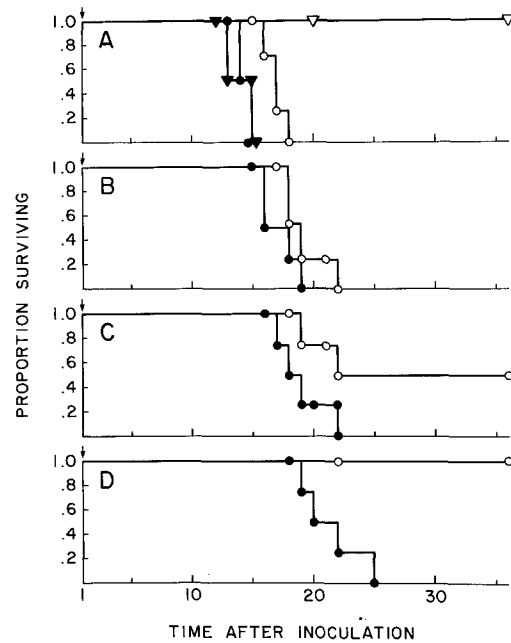
### Antitumor efficacy of immunoconjugate in vivo

The antitumor efficacy of the anti-Thy-1-gelonin immunoconjugate was evaluated in vivo. A/J mice were given  $5 \times 10^4$  RADA cells ( $10^2$ – $10^3$  lethal doses) i. p. on day 1 and then given  $10 \mu\text{g}$  (approximately  $0.5 \text{ mg/kg}$ ) of anti-Thy-1-gelonin, equivalent amounts of anti-Thy-1, gelonin, an admixture of equivalent amounts of anti-Thy-1 and gelonin, an immunoconjugate of irrelevant specificity (5E9-gelonin, [49]), HDTLG-gelonin, or PBS on days 1, 3, and 5 (Fig. 6). Only those mice receiving the anti-Thy-1-gelonin immunoconjugate showed any significant prolongation of survival indicating both the antigen specificity and in vitro predictability of immunoconjugate efficacy (anti-Thy-1-gelonin vs PBS,  $P < 0.0001$ ; anti-Thy-1-gelonin vs anti-Thy-1 + gelonin,  $P = 0.0001$ ; all other groups vs PBS N. S.). A/J mice given S1509a i. p. also showed no prolongation of survival when treated with anti-Thy-1-gelonin (not shown). Furthermore, neither anti-Thy-1 alone nor an admixture of anti-Thy-1 + gelonin had any effect at this dose on the survival of mice inoculated with  $5 \times 10^2$ , or  $5 \times 10^3$  RADA cells (not shown). Thus, the efficacy of immunoconjugate cannot be partially attributed to an antibody effect, as has been seen in some systems [3, 5, 56]. In the experiment shown, about 50% of mice treated with anti-Thy-1-gelonin were considered long-term survivors (L. S. F.), but this number varied from 30% to 100% using this treatment protocol.

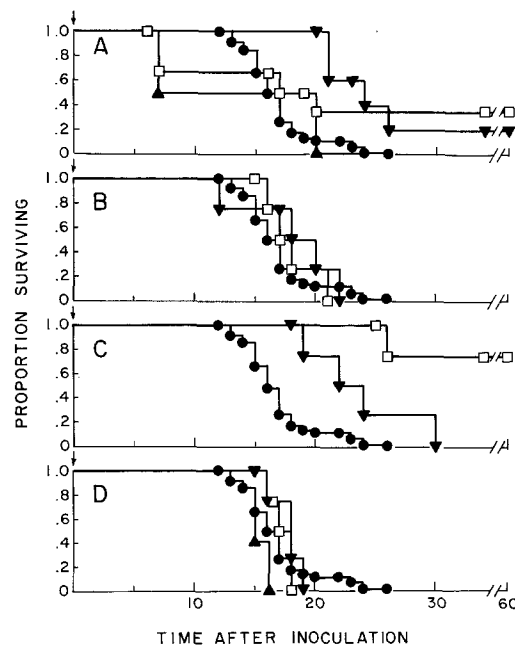
The S. F. of RADA in vivo after a single dose of immunoconjugate was determined by giving  $10 \mu\text{g}$  of immunoconjugate on day 1 to A/J mice who had received between  $5 \times 10^1$  and  $5 \times 10^4$  RADA cells i. p. (Fig. 7). Since the  $\text{LD}_{50}$  in this experiment was less than 50 cells (Fig. 7D), it is evident that a S. F. of no greater than 0.1, resulted from a single dose of immunoconjugate; there were 50% L. S. F. in animals given  $5 \times 10^2$  cells and immunoconjugate, and 0% L. S. F. in animals given  $5 \times 10^1$  cells and an admixture of anti-Thy-1 + gelonin. Furthermore, since there were 100% L. S. F. in animals given  $5 \times 10^4$  cells and  $10 \mu\text{g}$  of immunoconjugates three times, The S. F. must actually have been between 0.1 and 0.01 after a single dose; Since RADA has a doubling time of 18 h (unpublished data),



**Fig. 6.** Efficacy of anti-Thy-1-gelonin in vivo. A/J mice were inoculated with  $5 \times 10^4$  RADA i. p. on day 1 and received  $10 \mu\text{g}$  of anti-Thy-1-gelonin, 5E9-gelonin, or HDTLG-gelonin, or  $8 \mu\text{g}$  Thy-1 and  $2 \mu\text{g}$  gelonin or  $0.25 \text{ ml}$  of phosphate-buffered saline (PBS) i. p. on days 1, 3, and 5. Neither anti-Thy-1 alone nor gelonin alone had any effect different from PBS (not shown). (●) – PBS; (▼) – anti-Thy-1-gelonin; (▲) – anti-Thy-1 + gelonin; (□) – 5E9-gelonin; (○) – HDTLG-gelonin



**Fig. 7.** Determination of surviving fraction after immunotoxin treatment in vivo. A/J mice were inoculated with  $5 \times 10^4$  RADA i. p. (A),  $5 \times 10^3$  RADA i. p. (B),  $5 \times 10^2$  RADA i. p. (C) or  $5 \times 10^1$  RADA i. p. (D) on day 1, and were given either  $10 \mu\text{g}$  of anti-Thy-1-gelonin (○) or  $8 \mu\text{g}$  of anti-Thy-1 and  $2 \mu\text{g}$  of gelonin (●) i. p. on day 1 or  $10 \mu\text{g}$  of anti-Thy-1-gelonin i. p. (▼) or  $8 \mu\text{g}$  of anti-Thy-1 and  $2 \mu\text{g}$  of gelonin (▼) i. p. on days 1, 3, and 5. Survival of mice given anti-Thy-1 + gelonin was not different from mice given PBS (not shown)



**Fig. 8.** Comparison of anti-Thy-1-gelonin with adriamycin and cytoxin in vivo. A/J mice were injected with  $5 \times 10^4$  RADA cells i. p. on day 1 and received i. p. (A) PBS (●), anti-Thy-1-gelonin,  $10 \mu\text{g}$  (▼), anti-Thy-1-gelonin,  $25 \mu\text{g}$  (□), anti-Thy-1-gelonin,  $50 \mu\text{g}$  (▲); (B) PBS (●), cytoxin,  $10 \mu\text{g}$  (▼), cytoxin  $100 \mu\text{g}$  (□); (C) PBS (●), adriamycin  $10 \mu\text{g}$  (▼), adriamycin  $100 \mu\text{g}$  (□); (D) PBS (●), anti-Thy-1  $8 \mu\text{g}$  + gelonin  $2 \mu\text{g}$  (▼), anti-Thy-1  $40 \mu\text{g}$  + gelonin  $10 \mu\text{g}$  (□), or 7T4-7E10-gelonin  $50 \mu\text{g}$  (▲), all on day 1

about 800 cells would survive after three doses spaced apart by 48 h, if there were a S. F. of 0.1 after each dose, whereas a S. F. of 0.01 would result in 8 cells surviving after such treatment, below the LD<sub>50</sub> of less than 50 cells.

The efficacy of a single dose of anti-Thy-1-gelonin immunoconjugate was compared to the efficacy of a single dose of the known antineoplastic agents, adriamycin and cytoxan. Varying doses of these three reagents were given on day 1 to A/J mice bearing  $5 \times 10^4$  RADA (Fig. 8). In these experiments, both the tumor inoculum and reagent were given i.p., but i.v. inoculation of either anti-Thy-1-gelonin or adriamycin showed similar effects. It was evident that below an LD<sub>50</sub> of about 2.5 mg/kg (Fig. 8A) immunoconjugate was nearly as effective as adriamycin and more effective than cytoxan in prolonging survival and in producing L. S. F. (anti-Thy-1-gelonin 10  $\mu$ g vs PBS  $P = 0.001$ ; anti-Thy-1-gelonin 25  $\mu$ g vs PBS  $P = 0.005$ ; anti-Thy-1-gelonin 10  $\mu$ g vs anti-Thy-1 + gelonin, 10  $\mu$ g  $P = 0.003$ ; anti-Thy-1 + gelonin 10  $\mu$ g, or 100  $\mu$ g vs PBS N. S., adriamycin 10  $\mu$ g vs PBS  $P = 0.009$ ; adriamycin 100  $\mu$ g vs PBS  $P = 0.0001$ ; Cox regression trend analysis, anti-Thy-1-gelonin, 0–100  $\mu$ g  $P = 0.003$ ; adriamycin 0–100  $\mu$ g  $P = 0.0003$ ; cytoxan 0–100  $\mu$ g N. S.).

## Discussion

In this paper, we presented a study of the *in vitro* efficacy, *in vivo* pharmacokinetics, tumor delivery, and antitumor efficacy of an immunoconjugate consisting of a rat MCA recognizing the Thy-1 determinant linked by a disulfide bond to the ribosome-inactivating protein, gelonin. We studied the efficacy of this reagent against a murine leukemia in its natural, syngeneic host, and compared its potency to the potency of two currently used antineoplastic agents in the same model system. A variety of murine tumor model systems have been used for the evaluation of immunotoxin efficacy, but among the best nonxenograft model systems are those murine leukemias and lymphomas which bear the pan-T cell antigen Thy-1. The Thy-1 antigen appears to be a particularly useful target for immunotoxins because immunoconjugates recognizing this antigen are very toxic *in vitro* for either normal or tumor cells bearing the antigen [7, 8, 14, 25, 28, 55, 56, 58]. Several models using Thy-1 positive murine leukemias and lymphomas have been previously used to evaluate immunotoxin efficacy *in vivo* [28, 43, 55, 56]. In two of these studies, both an anti-Thy1.1-gelonin and an anti-Thy1.1-saporin immunoconjugate were shown to have potent antitumor effects on the Thy1.1 positive AKR-A lymphoma, including temporary regression of visible ascites in the case of the anti-Thy-1-saporin conjugate. However, both tumor models were essentially allografts in immunodeficient and nonsyngeneic hosts, which may have contributed to the effect of anti-Thy1.1 antibody alone in the case of the anti-Thy1.1-saporin conjugate. In using the A strain leukemia RADA in its natural, syngeneic, and immunocompetent host, the A/J mouse, we wished to determine if this type of immunotoxin could be effective under conditions which might more closely imitate the clinical situation. In addition, our antibody recognized determinants on normal A/J tissue, i.e., the Thy-1 expressing T cells, which was not the situation in the three previously described models; in all cases, an anti-Thy1.1 immunoconjugate was directed

against Thy1.1 lymphoma in a Thy1.2 host. Thus, the RADA-A/J model also duplicated the expected clinical situation where a candidate immunotoxin may cross-react with normal tissues [60].

Our laboratories have chosen to work on gelonin, a ribosome-inactivating protein derived from the seeds of *Gelonium multiflorum* [53], because it requires fewer precautions in its preparation than ricin A, the most commonly used toxin, and because it is more stable [4, 53]. In addition, contamination with free ricin, a potential problem of ricin-A conjugates [16], does not occur with native single chain toxins such as gelonin. Previous antibody-gelonin conjugates prepared by our group have shown a high degree of stability, retention of binding avidity compared to the original antibody, and potent toxicity and specificity *in vitro*. Furthermore, these immunoconjugates can be prepared with a high degree of purity, that is, free of unconjugated antibody or gelonin [33]. We also showed that these types of conjugates circulate for days and retain biological activity in both nonhuman primates and in the mouse [34, 49].

Importantly, the most potent immunoconjugate of this type in our hands to date has been the anti-Thy-1-gelonin conjugate, which is confirmation of previously cited work. In addition to demonstrating great specificity (greater than 5 logarithms of concentration), it produced a S. F. of  $10^{-3}$  or less in a 24-h exposure in the 1 nM range, and showed significant toxicity to RADA cells even after a short time exposure. We have also shown this conjugate to be highly toxic to other Thy-1-bearing murine tumors. The reason for its high toxicity is still unclear, but may relate to the extraordinarily high expression of Thy-1 antigen on RADA as well as other Thy-1 expressing murine tumors;  $5 \times 10^5$  or more binding sites are present per cell, a number about tenfold higher than many other surface antigens we have studied, including HDTLG in the mouse, and CALLA, T11, and the transferrin receptor in human tumors [20]. The efficiency and mechanism of internalization of the Thy-1 antigen when bound by antibody, or its putative ligand, may also explain the extraordinary efficacy of the anti-Thy-1-gelonin conjugate. Work of other groups has suggested that this antigen may be involved in a crucial way in murine T cell activation [22, 31, 35], and we have found that activation antigens [37] in human tumor systems are promising candidates for targeting by immunotoxin. The high potency of the anti-Thy-1-gelonin immunotoxin also does not reflect an intrinsic susceptibility of the RADA cell line; a gelonin conjugate of another antibody which recognized a surface structure on this tumor, i.e. TL<sup>A</sup>/HDTLG was nontoxic (Fig. 3). The phenomenon of marked differences in potency between immunoconjugates of this type (i.e., of the gelonin or ricin-A type) recognizing different antigens is well-known as is the demonstration of a difference in potency between immunoconjugates recognizing different determinants on the same cell [6, 7, 33, 42]. Efficiency of internalization of immunoconjugate has been a proposed explanation for these differences. This may be relevant in selecting candidate antibody conjugates for the treatment of human tumors *in vivo*.

*In vivo*, a rapid clearance of anti-Thy-1 and anti-Thy-1-gelonin conjugates from the A/J circulation was observed, which confirms previous findings of our group, in which antibodies which recognized determinants on normal murine tissue had much shorter circulation times than

those which did not [49]. The ability to prolong the circulatory time with large amounts of antibodies or immunoconjugate is also confirmation of previous work using other MCA [49]. Furthermore, the pharmacokinetic importance of a cross-reactivity for MCA or conjugate between a tumor surface determinant and normal tissue determinants is emphasized by our *in vivo* binding studies; doses of either antibody or conjugate which saturated Thy-1-binding sites in ascitic tumor *in vivo* when given directly into the peritoneal cavity, failed to do so when given *i.v.*; presumably the large number of normal Thy-1 expressing cells encountered by *i.v.* administered antibody effectively competed for binding prior to localization in the ascitic tumor. Indeed, binding of  $^{125}\text{I}$ -Thy-1 antibody to Thy-1 expressing cells in spleen, lymph nodes, and other sites after *i.v.* administration has been previously demonstrated [26]. Furthermore, the lower binding levels seen with immunoconjugate as compared to antibody after *i.v.* administration probably reflected a more rapid circulatory clearance of the conjugate.

Despite these pharmacokinetic drawbacks, a single *i.p.* dose of 0.5 mg/kg of anti-Thy-1-gelonin killed between 90% and 99% of RADA cells in a syngeneic, immunocompetent host in an antigen-specific fashion; up to 99.99% of cells were killed after three doses. Neither anti-Thy-1 antibody alone or in combination with gelonin had any potency against tumor even at very low levels of tumor inocula (50 cells), indicating that none of the *in vivo* efficacy of this immunotoxin could be attributed to an antibody-mediated effect. Furthermore, the modest immunosuppressive effects observed after *in vivo* administration probably had little effect on antitumor efficacy. If anything, a potentiation of tumor growth through interference with the host's antitumor response would be expected, and, in fact, no effect on S1509a growth was observed.

Finally, *in vivo* efficacy correlated well with *in vitro* toxicity, and anti-Thy-1-gelonin compared favorably with adriamycin or cytoxan in its efficacy. It is of interest, however, that a S. F. of 0.001 or less was observed *in vitro* after exposure to 1 nM anti-Thy-1-gelonin, while a S. F. no less than 0.01 was observed *in vivo*; this probably reflected the length of time to which RADA cells *in vivo* are exposed to toxic levels of immunotoxin. Efficacy *in vitro* may be greater because exposure of tumor cells to immunotoxin is likely to be more ideal than under *in vivo* conditions; competition for binding by determinants on normal tissue may at least partially explain the lower efficacy observed *in vivo*. In addition, the inability to give more than 50  $\mu\text{g}$  of this immunoconjugate without producing toxicity (Fig. 8A), may preclude the demonstration of greater efficacy *in vivo*. Indeed, the observed  $\text{LD}_{50}$  of the anti-Thy-1-gelonin conjugate is tenfold less than that observed for identically prepared conjugates of anti-human cell specificity [49], which may also be a reflection of the specificity of this conjugate for normal murine tissues. One might predict that a model system in which the candidate determinant on a tumor was not present on normal tissues would, in fact, allow the demonstration of greater efficacy *in vivo*; such a syngeneic model system is under study.

Several further issues are currently being addressed. First, demonstration of efficacy of this type of conjugate against human tumors *in vivo* in a nude mouse-xenograft model is of interest prior to initiating clinical studies. Second, the demonstration of a synergistic effect of immuno-

toxin and currently available chemotherapeutic agents would be of interest. Both such studies are currently underway and show great promise in this regard.

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