Improved tumor-specific immunotoxins in the treatment of CNS and leptomeningeal neoplasia

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A novel antibody-toxin conjugate has been developed for use in cancer therapy. This report demonstrates that this new reagent selectively kills glioblastoma- and medulloblastoma-derived cell lines, medulloblastoma cells in primary culture, and cell lines derived from tumors commonly metastatic to the cerebrospinal fluid (CSF). Efficient killing of human tumor cells occurred at concentrations between $3.9 \times 10^{-13}$ M and $1.1 \times 10^{-10}$ M, whereas guinea pigs and rhesus monkeys tolerated intrathecal levels of $2 \times 10^{-9}$ M. Cerebrospinal fluid from normal humans and from brain-tumor patients does not inhibit the in vitro efficacy of this reagent.

The wide therapeutic window, extreme potency, and general applicability of this antibody-toxin conjugate against CSF-borne primary or metastatic tumors warrants clinical trials.

KEY WORDS - diphtheria toxin - immunotoxin - transferrin receptor - leptomeningeal neoplasia - brain neoplasm - cerebrospinal fluid

CURRENT treatments utilizing surgery, radiation therapy, and systemic chemotherapy have done little to alter the natural outcome of many malignant tumors of the central nervous system (CNS). Immunotoxins (protein toxins chemically linked to tumor-specific monoclonal antibodies or other ligands) offer potential advantage over more conventional forms of treatment by having higher tumor specificity.

Immunotoxins may be particularly efficacious for the treatment of neoplastic disease confined to compartments such as the peritoneum or intrathecal space. Direct delivery into the compartment avoids complications associated with systemic delivery and produces relatively high local concentrations, thereby achieving greater therapeutic effects. The cerebrospinal fluid (CSF) compartment may be amenable to this type of compartmentalized immunotoxin treatment. Zovickian and Youle examined the therapeutic effect of a monoclonal antibody-ricin immunotoxin, delivered directly into the CSF compartment in a guinea pig model of leptomeningeal neoplasia. Immunotoxin therapy extended survival, corresponding to a 2 to 5 log kill of tumor cells, without detectable toxicity.

Protein toxins used in the construction of immunotoxins have an A and B subunit. The A subunit catalyzes the inactivation of protein synthesis, resulting ultimately in cell death. The B subunit has two functions: it is responsible for toxin-binding to the cell surface, and it facilitates the translocation of the A chain across the membrane and into the cytosol, where the A chain acts to kill cells (Fig. 1). Previously, two general types of immunotoxins have been used. Immunotoxins made with the complete toxin molecule (A and B chains) have the complication of nonspecific killing mediated by the toxin B chain binding site. This can be avoided by eliminating the B chain and linking only the A chain to the antibody. However, A chain immunotoxins, although more specific, are much less toxic to tumor cells. In addition to a binding function, the B chain has an entry function, which facilitates the translocation of the A chain across the membrane and into the cytosol (Fig. 1). Since A chain immunotoxins lack the entry function of the B chain, they are less toxic than their intact toxin counterparts containing the complete B chain. An ideal toxin for immunotoxin construction would contain the A chain enzymatic function and the B chain translocation function, but not the B chain binding function.

We have recently described a new, genetically engineered toxin that offers these major advantages. This...
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**Fig. 1.** Diagrammatic representation of diphtheria toxin (DT), CRM 107, and DT A chain structure and function. Native DT is composed of an A and B subunit, with the A chain containing the enzymatic function and the B chain containing the binding and translocation functions. Two point mutations in CRM 107 inactivate the toxin binding function but leave the translocation and enzymatic functions intact. Toxin A chain contains only the enzymatic function.

Toxin, called CRM 107, is identical to diphtheria toxin (DT) except for two amino acid changes in the B chain. These two point mutations inactivate toxin binding 8000-fold but leave the translocation function intact. Immunotoxins made with CRM 107 therefore have the advantage of the lower nontarget-cell toxicity of A chain conjugates but retain a 10,000-fold increase in tumor-specific toxicity similar to that found for immunotoxins made with complete toxin molecules.

The tremendous potential of CRM 107 for use in immunotoxins together with the advantage of CSF compartmentalized delivery may result in effective treatment for tumors in the CNS. The transferrin receptor (TfR) has been chosen as the target for these immunotoxins. Expression of TfR increases during cell replication in order to fulfill the dividing cell's need for iron. Since tumor cells are dividing while normal cells in the CNS parenchyma are quiescent, the TfR should provide an effective target for immunotoxins. It has previously been shown that TfR's are expressed on human medulloblastoma and glioblastoma cells. This report demonstrates that transferrin (Tf) or an anti-Tf monoclonal antibody conjugated to CRM 107 has potential for effective treatment of medulloblastoma and breast leptomeningeal carcinomatosis.

**Materials and Methods**

**Established Cell Lines**

The SNB75 cell line was established by primary explants from a tumor removed from a 72-year-old woman with a bifrontal glioblastoma multiforme. SNB101 was also established by primary explants from a glioblastoma multiforme removed from the right parietal lobe of a 49-year-old man. SNB40 was derived from primary explants of a medulloblastoma surgically removed from the posterior fossa of an 8-year-old boy. At the time of this study, SNB75 was in its 48th passage, SNB101 in its 14th passage, and SNB40 in its 20th passage. U251 was in its 37th passage. TE671* is derived from a human medulloblastoma. All of these cell lines were maintained in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 μg/ml gentamicin.

Three cell lines from human breast cancers were examined. MCF-7, a breast adenocarcinoma-derived cell line,24 was maintained in DMEM containing the supplements described above plus 10 μg/ml insulin. ZR-75-1, a cell line derived from a malignant ascitic effusion of a patient with infiltrating ductal carcinoma,2 was maintained in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 20 μg/ml gentamicin, and 2 mM glutamine. T47D, a cell line derived from an infiltrating ductal carcinoma,13 was grown in RPMI 1640 medium as described above with the addition of 10 μg/ml insulin. All breast-derived cell lines were a gift from Dr. J. Greiner and Dr. J. Schlom.

**Primary Medulloblastoma Cultures**

Since established cell lines long adapted to culture conditions could conceivably possess Tf requirements and receptor levels different from those of the original cells, we established two primary medulloblastoma cultures and determined sensitivity to TF-CRM 107. The SNB104 cell line was established from biopsy of a midline cerebellar vermian mass in an 18-year-old man, and SNB105 was derived from a midline posterior fossa tumor in a 3-year-old girl. In both cases, fresh tumor was transported to the laboratory in Eagle's minimum essential medium (MEM) with 10% FCS. Tumors were mechanically and then enzymatically dissociated according to previously described techniques23 and cultured at a density of 1 to 5 × 10⁵ cells/ml in 75 sq cm tissue culture flasks. The culture medium consisted of Eagle's MEM with Earle's salts supplemented with 10% FCS, 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin (100,000 μg/liter), streptomycin (100 mg/liter), and Fungizone (amphotericin B, 0.25 mg/liter). Cultures attained 50% confluence within 10 to 14 days, at which time trypsinization, transfer to 96 well plates, and cytotoxicity testing were performed as described below.

**Synthesis and Purification of CRM 107 Immunotoxins**

CRM 107, isolated by Laird and Groman,14 was purified as described previously.12 Human Tf was loaded with iron according to the method of Shindelman, et al.23 Conjugation of Tf with CRM 107 was accomplished by first generating free sulhydryl groups on Tf with 2-iminomithiolane, which was dissolved in 0.8 boric acid (pH 8.5) and incubated with Tf in an 8:1 ratio.

* TE671 cell line obtained from American Type Culture Collection, Rockville, Maryland.
† Human transferrin obtained from Sigma Chemical Co., St. Louis, Missouri.
molar ratio. The modified Tf was kept at room temperature for 1 hour, then separated from free 2-imino-thiolane by gel filtration on a Sephadex G-25 gel filtration column equilibrated with phosphate-buffered saline (PBS). The bifunctional cross-linking agent, M-maleimido-benzoyl-N-hydroxysuccinimidy (MBS) ester, was used to link Tf to CRM 107; MBS was dissolved in dimethylformamide and added in five-fold molar excess to the toxin. The mixture was incubated for 30 minutes at room temperature, then separated by chromatography on a Sephadex G-25 gel filtration column. The MBS-conjugated toxin was mixed with thiolated Tf in 1:1.3 molar ratio and incubated for 3 hours at room temperature; the toxin-conjugate was then purified by gel filtration on a TSK-3000 high-performance liquid chromatography column. One-minute fractions were collected and individual fractions were tested for toxicity using protein synthesis inhibition. Peak fractions of the toxin conjugate were pooled and then A chain activity in this pooled peak was quantified using the elongation factor 2 (EF-2) adenosine diphosphate (ADP)-ribosylation assay. This pooled peak was used for all further experiments.

The anti-Tf monoclonal antibody, 454A12, was prepared as previously reported. It was linked to CRM 107 as described above. This antibody was also conjugated with recombinant ricin toxin A chain (RTA) as described by Bjorn, et al. ADP-Ribosylation Assay

The concentration of the CRM 107 immunotoxins was determined using an ADP-ribosylation assay. This assay measures the ability of the CRM 107 immunotoxins to catalyze the transfer of ADP-ribose from nicotinamide-adenine dinucleotide (NAD) to EF-2.

Elongation factor 2 was purified from rat liver cells following a procedure described previously. ADP-ribosylation was carried out in 80-μl reaction mixtures containing 40 μl 0.01 M Tris-HCl buffer, with 1.0 M dithiothreitol (pH 8.0), 20 μl EF-2, and 10 μl toxin sample. The reaction was initiated by the addition of 10 μl of phosphate-32-labeled NAD (1.2 μCi, specific activity 277 Ci/mmol, adjusted to 180 μM with cold NAD). Reaction mixtures were incubated at room temperature for 20 minutes and the reaction was stopped by the addition of 1 ml 10% trichloroacetic acid (TCA). The precipitate was washed once with 10% TCA, solubilized in 0.1 M NaOH, and counted.

The ADP-ribosylation activities of unknown samples were compared to values obtained from DT standards consisting of known concentrations of DT (values based on Lowry's protein determination using bovine serum albumin (BSA) as a standard). The background of the assay was determined by replacing the toxin with Tris-HCl buffer.

Protein Synthesis Assay

Inhibition of protein synthesis was used to assay the cytotoxic effects of the toxin-conjugate. Cells were trypsinized, washed in their regular growth medium, and dispersed in this medium into 96-well microtiter plates at a density of 5 × 10^4 cells per well. The cells were allowed to reattach and grow in the 96-well plate for 24 hours before the assay was performed. They were then washed twice with leucine-free RPMI medium containing 10 mM HEPES and 10 μg/ml gentamicin but without FCS, and the wells were refilled to a final volume of 100 μl. Toxin-conjugates, toxins alone, or control solutions were added to the wells in 11-μl aliquots and the cells were incubated at 37°C for 24 hours. At the end of this time, 20 μl of PBS containing 0.1 μCi of carbon-14 (14C)-labeled leucine was added, incubation continued for 1 hour, and the cells were harvested onto glass fiber filters using a PHD cell harvester. The filters were washed with water, dried, and counted. All cytotoxicity assays were performed two to five times in triplicate. The results were expressed as a percentage of 14C-leucine incorporation in mock-treated control cultures.

In Vivo Toxicity

Guinea Pig. To investigate the efficacy of intrathecal immunotoxin therapy for tumors of the CSF compartment, we determined the toxicity of DT or CRM 107 alone, or Tf-CRM 107 conjugate injected directly into the cisterna magna of Strain 2 guinea pigs. The animals were anesthetized with intraperitoneal ketamine (30 to 50 mg/kg). Toxin or conjugate, suspended in 100 μl PBS/0.2% BSA, was slowly injected percutaneously via a No. 25 needle into the cisterna magna. Injections were performed only after CSF was clearly visualized in the hub of the needle. Final concentrations achieved in the CSF were calculated based on a total guinea pig CSF volume of 500 μl. The length of survival was recorded as the number of days following injection until death. Body weight was also measured at designated intervals and compared to that of control animals injected with PBS alone.

Rhesus Monkey. A single dose of Tf-CRM 107 in 0.5 cc normal saline containing 0.2% human serum albumin was administered to two anesthetized adult rhesus monkeys by an occiput-C-1 puncture and gentle barbotage. This technique is known to effect rapid mixing of injectant with cisternal and ventricular CSF. Venous blood for chemistry and hematology panels and lumbar CSF for routine studies were obtained every other day for 1 week and weekly thereafter. The animals were weighed at the same time that blood and CSF were collected.

Results

Toxicity of CRM 107 Immunotoxins on Human Tumor-Derived Cell Lines

Diphtheria toxin or DT-like toxins such as CRM 107 inhibit protein synthesis. This process can be measured

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for an accurate in vitro assessment of the lethal effect of the toxin on cells.

Figure 2A shows representative dose-response curves of the Tf-CRM 107 conjugate on four medulloblastoma-derived cell lines. A steep dose-response inhibition of protein synthesis by Tf-CRM 107 was observed with all the cell lines. Protein synthesis in SNB40 cells was blocked by Tf-CRM 107 at a 50% inhibiting concentration (IC50) of 3.9 × 10^{-13} M (Table 1). TE671 cells and the primary medulloblastoma-derived cell line, SNB104, were also extremely sensitive to Tf-CRM 107 (IC50:2.5 × 10^{-12} M and 2.1 × 10^{-12} M, respectively). The IC50 of Tf-CRM 107 for the other primary medulloblastoma derived cell line, SNB105, was 1.1 × 10^{-10} M. The receptor specificity of the Tf-CRM 107 conjugate was demonstrated by the fact that excess free Tf blocked cell killing by the toxin conjugate (data not shown).

Figure 2B shows the results of similar experiments performed on the glioblastoma-derived cell lines. As observed with cells derived from medulloblastoma, Tf-CRM 107 exhibited potent killing with all the glioblastoma cells. The IC50 for SNB75 was 6.5 × 10^{-11} M, and the IC50 for SNB101 and U251 was 5.4 × 10^{-12} M and 2.6 × 10^{-12} M, respectively. Using a monoclonal antibody against the human TfR, 454A12, linked to CRM 107 in trials with two continuous medulloblastoma cell lines and three glioblastoma cell lines showed IC50 levels between 10^{-11} and 10^{-10} M, whereas, when this same conjugate was assayed on Vero cells, which lack the receptor, the IC50 level was 1 × 10^{-8} M. The therapeutic window between tumor and nontarget cells is therefore 100- to 1000-fold. CRM 107 and RTA were assayed as free toxins, as shown in Table 1. Both free toxins displayed similar toxicities and were 1000 to 10,000-fold less toxic than the 454A12-CRM 107 immunotoxin. The wide therapeutic windows between target and nontarget cells and between free toxin and immunotoxin demonstrate that the 454A12-CRM 107 is highly potent and specifically toxic to brain-tumor cells.

### TABLE 1

<table>
<thead>
<tr>
<th>Tumor Cells</th>
<th>IC50 values for CRM 107 and ricin A chain (RTA) immunotoxins*</th>
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<tr>
<td></td>
<td>TF-CRM 107</td>
</tr>
<tr>
<td>medulloblastoma</td>
<td></td>
</tr>
<tr>
<td>SNB40 (established)</td>
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<tr>
<td>TE671 (established)</td>
<td>2.1 × 10^{-12}</td>
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<tr>
<td>SNB104 (primary)</td>
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<tr>
<td>SNB105 (primary)</td>
<td>1.1 × 10^{-10}</td>
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<tr>
<td>glioblastoma</td>
<td></td>
</tr>
<tr>
<td>SNB75</td>
<td>6.5 × 10^{-11}</td>
</tr>
<tr>
<td>SNB101</td>
<td>5.4 × 10^{-12}</td>
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<tr>
<td>U251</td>
<td>2.6 × 10^{-12}</td>
</tr>
<tr>
<td>breast carcinoma</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>2.3 × 10^{-11}</td>
</tr>
<tr>
<td>T47D</td>
<td>1.1 × 10^{-12}</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>1.6 × 10^{-11}</td>
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* Immunotoxins and toxins were incubated with the cells for 24 hours followed by incubation of 1 hour with 14C-leucine. Cells were then harvested and concentrations of immunotoxin that inhibit protein synthesis by 50% of control values (IC50) were determined. Tf = transferrin.
FIG. 3. Comparison of the toxicity of 454A12 monoclonal antibody linked to CRM 107 or to ricin A chain. Cell lines derived from medulloblastoma (TE671 cell line, A), glioblastoma (U251 cell line, B), or breast carcinoma (T47D cell line, C) were incubated with 454A12 immunotoxins for 3 hours followed by a 1-hour pulse with 14C-leucine. Cells were harvested and protein synthesis in treated cells was expressed as a percentage of 14C-leucine incorporated into untreated control cells.

Toxicity of Anti-TfR Antibody Conjugated to Ricin A Chain and to CRM 107

The toxicity of the anti-TfR antibody, 454A12, conjugated to ricin A chain (RTA) or to CRM 107 was compared with Tf-CRM 107 on cell lines derived from medulloblastoma and glioblastoma (Table 1). Both the 454A12-RTA and the 454A12-CRM 107 immunotoxin displayed very similar toxicities when evaluated after 24 hours using the in vitro assay of protein synthesis described above. The IC_{50} observed for both of these immunotoxins on established medulloblastoma and glioblastoma cell lines ranged from approximately 10^{-10} to 10^{-11} M. Only one cell line, SNB101, displayed a significant difference in sensitivity to the two immunotoxins. For this cell line, 454A12-CRM 107 was approximately 10-fold more toxic than 454A12-RTA.

Three established breast-derived cell lines, MCF-7, T47D, and ZR-75-1, were assayed, since breast tumors most commonly lead to meningeal carcinomatosis (Table 1). These cells displayed approximately the same sensitivity to the immunotoxins as was found for the medulloblastoma or glioblastoma cells. In a 24-hour assay, the IC_{50} was similar for both 454A12-RTA and 454A12-CRM 107, ranging between 1 \times 10^{-10} M and 7 \times 10^{-11} M. Tf-CRM 107 was approximately 10- to 100-fold more toxic than the 454A12 immunotoxins on these breast cell lines.

Figure 3 demonstrates very different dose-response curves for 454A12-CRM 107 and 454A12-RTA after 3 hours of incubation with the immunotoxins on representative cell lines derived from medulloblastoma, glioblastoma, and breast carcinoma. The 454A12-CRM 107 displayed a steep dose-response curve for all three cell lines, with an IC_{50} between 1 \times 10^{-10} M and 6 \times 10^{-10} M. The RTA immunotoxin is less toxic, inhibiting protein synthesis by less than 30% in TE671 and U251 cells at concentrations greater than 10^{-7} M. 454A12-RTA is approximately 15-fold less toxic to T47D breast carcinoma cells than the CRM 107 immunotoxin.

Similar dose-response curves for 454A12-CRM 107 and 454A12-RTA were obtained in a 24-hour assay (Table 1), yet very different curves result from a 3-hour assay with the two toxin conjugates (Fig. 3). This indicates that large differences exist in the rate of cell killing by the two immunotoxins. Kinetic differences in the rate of killing by immunotoxins can be masked in an assay where inhibition of protein synthesis is measured over a long incubation period. The assay cannot detect cell killing beyond 90% of the input cells (10% of control protein synthesis). Immunotoxins with rapid killing may reach this level quickly and continue to kill additional logs of cells that are not detected in the assay. After long incubation times, the dose-response curves of immunotoxins with efficient killing rates may appear identical to those with less efficient rates of kill, since the assay does not reflect the additional log kill.

Maximum Tolerable Dose In Vivo

Guinea Pigs. To investigate the feasibility of intrathecal immunotoxin therapy for tumors in the CSF, the toxicity of DT, CRM 107, and Tf-CRM 107 was determined. Varying concentrations of the toxins were injected percutaneously into the cisterna magna of guinea pigs. The maximum safe dose (maximum dose where no significant weight loss was observed) of DT was between 3.2 \times 10^{-11} M and 3.2 \times 10^{-12} M (Table 2). Up to 100-fold higher doses of CRM 107 were tolerated without detectable toxicity. Therefore, the toxicity of CRM 107 in vivo is 1/100 that of DT, whereas the nonspecific toxicity of CRM 107 in vitro.
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<p>| TABLE 2 |
| Maximum tolerated dose in guinea pig cerebrospinal fluid* |</p>
<table>
<thead>
<tr>
<th>Toxin</th>
<th>Concentration</th>
</tr>
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<tr>
<td>diphtheria toxin</td>
<td>3.2 $\times 10^{-11}$ M to 3.2 $\times 10^{-12}$ M</td>
</tr>
<tr>
<td>CRM 107</td>
<td>3.2 $\times 10^{-10}$ M</td>
</tr>
<tr>
<td>TF-CRM 107</td>
<td>2 $\times 10^{-9}$ M</td>
</tr>
</tbody>
</table>

* Toxin alone or transferrin (TF)-CRM 107 conjugate was injected percutaneously into the cisterna magna of Strain 2 guinea pigs. The maximum dose consistent with survival is shown. No significant weight loss was observed in animals treated at these doses when compared with control animals.

is 1/10,000 that of DT. Furthermore, conjugation of CRM 107 to TF reduced the toxicity approximately 10-fold more ($2 \times 10^{-9}$ M). Inactivation of toxicity due to conjugation was previously observed with other immunotoxins.

Rhesus Monkeys. TF-CRM 107 was injected intrathecally into the cisterna magna of two adult rhesus monkeys. Assuming a volume of distribution of 6 cc, doses were administered to produce a CSF concentration of $3.3 \times 10^{-10}$ M and $2 \times 10^{-9}$ M. Neither dose of TF-CRM 107 caused apparent neurological illness, and both animals were alive 2 months after treatment. Weight loss was limited to less than 10% of baseline. At both dose levels, prominent fevers (> 39.5°C) occurred on Days 1 and 2 following treatment. Serum chemical levels, liver enzyme concentrations, renal function, and hematological parameters did not change. A CSF inflammatory response with pleocytosis, elevated protein levels, and normal glucose content was apparent for 48 hours but had largely resolved by 14 days after treatment.

The concentration of $2 \times 10^{-9}$ M, which was reached safely in vivo, was 20 to 5000 times greater than the IC$_{50}$ of TF-CRM 107 to all the medulloblastoma, glioblastoma, and breast cells assayed in culture.

Inhibiting Effects of Circulating Anti-DT Antibodies

A critical factor in the efficacy of any CRM 107 immunotoxin in man is the level of inactivating anti-DT immunoglobulin produced by intentional immunization with diphtheria toxoid. Since CRM 107 differs from DT in only two amino acids, it is expected that the majority of circulating antibodies would be cross-reactive with CRM 107. We therefore investigated the effect on DT toxicity of circulating levels of antibody in the serum and CSF.

As shown in Fig. 4A, most human sera contain significant titers of inactivating antibody. Further titration of the sera with higher levels of DT revealed approximately a 10,000-fold block by sera (data not shown). Serum samples from two donors, reportedly not intentionally immunized against DT, exhibited dose-response curves that closely paralleled the control DT curve; these samples serve as controls showing that human sera have no other effects on DT toxicity.

Low levels or total absence of inactivating antibody was found in the CSF (Fig. 4B) of normal volunteers. The CSF from a glioblastoma patient, a patient with breast cancer-related leptomeningeal carcinomatosis, and a patient with lymphomatous leptomeningitis was also tested (data not shown) and showed no inhibition of DT toxicity. The fact that CSF has only 0.2% to 0.4% of the immunoglobulin G levels found in serum is consistent with our results which also substantiate the belief that the CSF compartment is an immunologically privileged site.

Inhibition of TF-CRM 107 and 454A12-CRM 107 by Free Tf

It is reported that CSF has 14 µg/ml of circulating Tfn. Therefore, the effect of this concentration of free
that an anti-idiotype monoclonal antibody-ricin immunotoxin, delivered intrathecally, significantly extended survival in a guinea pig model of leptomeningeal neoplasia. The increased survival time, which corresponded to a median 2- to 3-log kill of tumor cells, occurred without detectable toxicity related to the immunotoxin.24

CRM 107 represents a significant advance in the design of toxins for use in immunotoxin therapy. This is a genetically modified form of DT, differing from native DT at amino acid positions 390 and 525.8 The toxin molecule consists of an A and B subunit. The A subunit enzymatically inactivates protein synthesis by transferring ADP-ribose to EF-2, thereby stopping the addition of amino acids to the growing polypeptide chain and thus killing the cell. The B subunit has two functions, facilitating both the binding of the toxin to the cell surface and the entry or translocation of the A subunit across the cell membrane into the cytosol where it functions. The advantage of CRM 107 is that the two amino acid changes in the toxin B chain inactivate toxin binding 8000-fold yet have no effect on the translocation function.9 Therefore, by linking CRM 107 to a specific binding moiety such as a tumor-specific monoclonal antibody, it is possible to target the full toxicity of the native toxin and still avoid the problems of nonspecific toxicity caused by toxin binding.

The advantages of CRM 107-based immunotoxins become apparent when compared with immunotoxins made with DT A chains alone. Colombatti, et al., compared the toxicity of native DT conjugated with a monoclonal antibody specific for the T lymphocyte antigen receptor with that of the DT A chain conjugated to the same antibody. It was found that A chain immunotoxins were 10,000-fold less toxic than those made with native DT. This reduction in toxicity reflects the loss of the B chain translocation function. CRM 107 retains the translocation function, and therefore, when linked to a new binding site, maintains the full potency of killing found in the native toxin but with the high cell-type specificity of A chain conjugates.10,12

Cell lines derived from medulloblastoma, glioblastoma, and breast carcinoma were chosen to assess the in vitro efficacy of CRM 107-based immunotoxins for treatment of tumors of the CSF compartment. These three types of tumors were selected because they represent examples of CNS tumors which are often difficult to treat with conventional forms of therapy and, in the case of breast carcinoma and medulloblastoma, frequently cause morbidity and mortality from leptomeningeal involvement. These in vitro results indicate that immunotoxin therapy offers certain advantages over existing treatments for these tumors.

Medulloblastoma comprises 20% of all brain tumors in children.3 Because of this tumor's high incidence of recurrence and propensity for dissemination through the CSF, these children require postoperative radiation therapy which usually impairs their intellectual and physical development.3,21 A more targeted treatment

**Discussion**

In this report, the potential of a new type of toxin, CRM 107, has been demonstrated for use in the construction of highly potent immunotoxins directed specifically at tumors of the CNS. Immunotoxins may be most effective for the regional treatment of disease confined to an anatomic compartment where transvascular delivery is not a problem and where relatively high local concentrations, and therefore greater therapeutic effect, may be achieved. It was previously shown
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such as immunotoxin therapy would be especially useful for this type of tumor. Intrathecal immunotoxin treatment of medulloblastoma has the added advantage of free access to the tumor cells within the CSF, avoiding the potential problem of limited penetration into the tumor mass.

Glioblastoma, the most common primary malignant brain tumor, is rapidly fatal. The best treatment currently available (surgery, radiation therapy, and systemic chemotherapy) results in median survival times of less than 1 year.27,28 Inasmuch as most treatment failures occur because of local recurrence of glioma,10 immunotoxins administered directly into the CSF or tumor to avoid delivery problems caused by the blood-brain barrier may provide additional tumor response and enhance log kill required for effective treatment of glioblastoma.

A variety of carcinomas, most commonly those involving the breast, metastasize to the leptomeninges. The incidence of meningeal carcinomatosis in breast cancer is believed to be rising due to improvements in the management of systemic disease.30 Diffuse secondary involvement of the leptomeninges without focal parenchymal involvement is occasionally reported.26,30 These patients would be particularly suited for immunotoxin therapy since the problems involved with penetration of a solid tumor mass by immunotoxins would be minimal.

This study compared the efficacy of immunotoxins made with CRM 107 and RTA on representative cell lines derived from medulloblastoma, glioblastoma, and breast carcinoma. A monoclonal antibody to the TIR, 454A12, was linked to each toxin and the in vitro toxicity was examined. Using a 24-hour inhibition of protein synthesis assay, both immunotoxins killed these cell lines at concentrations ranging between $10^{-10}$ and $10^{-11}$ M. However, it was found that CRM 107 immunotoxins kill at a much faster rate than the RTA immunotoxins. A shorter incubation time (3 hours) for the in vitro assay demonstrates these kinetic differences. In these assays, 454A12-CRM 107 is 10- to 1000-fold more toxic than 454A12-RTA. The CRM 107 conjugates displayed steep dose-response curves, again indicative of rapid killing. This increase in the rate of killing by CRM 107 immunotoxins compared to those made with RTA probably reflects the potentiating effect derived from the B chain entry function.

It is logical that an immunotoxin that kills at a faster rate will ultimately produce greater log kill than a slower-acting immunotoxin. Laurent, et al.,15 demonstrated this principle using a clonogenic assay. They found that immunotoxins with the fastest kinetics of killing also exhibited the greatest log kill of human lymphocytes. Our results predict that the in vivo efficacy of CRM 107 immunotoxins may surpass that of RTA immunotoxins.

CRM 107 immunotoxins offer another advantage over RTA immunotoxins. Most individuals are immunized against DT, as evidenced here by high titers of circulating neutralizing antibody. Therefore, any "leakage" of the CRM 107 immunotoxin from the CSF into the systemic vasculature would be quickly neutralized by circulating anti-DT immunoglobulin.

Unlike many monoclonal antibodies, Tf cross-reacts among species.1 Therefore, by linking Tf with CRM 107 we were able to evaluate the toxicity of the conjugate administered intrathecally in both guinea pigs and rhesus monkeys. Problems encountered in the periphery which would limit the efficacy of Tf-CRM 107 (that is, high levels of Tf and circulating anti-DT antibodies) do not appear to occur in the CSF. In rhesus monkeys, the highest dose of Tf-CRM 107 tested ($2 \times 10^{-9}$ M) was without toxicity. Continuing toxicity trials to test higher concentrations will determine the maximum tolerated dose. In guinea pigs, the maximum tolerated dose in the CSF was $2 \times 10^{-9}$ M. The concentration of Tf-CRM 107 required to kill 50% of the cells derived from medulloblastoma, glioblastoma, or breast carcinoma in vitro ranged from $1 \times 10^{-10}$ M to $4 \times 10^{-11}$ M. We were therefore able to achieve a concentration of the conjugate in the CSF that was from 20- to 5000-fold higher than the level effective in vitro without detectable animal toxicity.

CRM 107 conjugates represent a significant advance in immunotoxin efficacy. They combine a high degree of tumor specificity, the ultimate degree of potency (1 molecule/cell is sufficient to kill), with extremely rapid killing to produce a therapeutic window of up to 5000-fold. These factors, together with the advantages offered by compartmentalized treatment, demonstrate that CRM 107 immunotoxins have considerable potential for the treatment of leptomeningeal neoplasia.

Acknowledgments

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References


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