Potent and specific killing of human malignant brain tumor cells by an anti-transferrin receptor antibody-ricin immunotoxin

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Immunotoxins are hybrid molecules which combine the exquisite selectivity of monoclonal antibodies with the potent toxicity of protein toxins. An immunotoxin was constructed by linking a murine monoclonal antibody against the human transferrin receptor (TR) to the plant toxin, ricin.

The cytotoxic activity of the anti-TR-ricin immunotoxin was tested in vitro and demonstrated highly potent and cell type-specific killing of cells derived from human glioblastoma, medulloblastoma, and leukemia. The anti-TR-ricin immunotoxin killed more than 50% of "target" cells at a concentration of $5.6 \times 10^{-13}$ M after an 18-hour incubation with the ionophore, monensin. This potency exceeds that of any other anti-TR immunotoxin reported in the literature. When the activity of the anti-TR-ricin immunotoxin against "target" tumor-derived cells was compared with the immunotoxin's activity against "non-target" cells, it could be predicted that a selective toxicity of anti-TR-ricin immunotoxin between tumor cells and normal brain was more than 150- to 1380-fold. Solid-phase indirect radioimmunoassay techniques were used to demonstrate significantly higher levels of TR in the glioblastoma- and medulloblastoma-derived cell lines, as well as in surgical tissue samples of medulloblastoma and glioblastoma, as compared to normal brain.

Immunotoxins targeted to the TR may possess sufficient specificity to be of therapeutic importance, particularly to treat neoplastic disease of the central nervous system involving compartments (such as intrathecal, intraventricular, or cystic) where delivery of immunotoxins to tumor would not require transvascular transport.

KEY WORDS • immunotoxin • transferrin receptor • ricin • brain neoplasm • immunotherapy • monoclonal antibody

AGGRESSIVE treatment with surgery, radiation therapy, and chemotherapy has done little to improve the dismal prognosis for patients with primary malignant brain tumors. Inability to direct these modalities specifically to tumor results in unacceptable and dose-limiting side effects and restricts their therapeutic efficacy.

The advent of monoclonal antibody technology has generated renewed enthusiasm for Paul Ehrlich's original concept of using tissue-specific "carriers" to deliver "therapeutically active groups" selectively to neoplastic tissues. A new class of cell-type selective cytotoxic reagents has now been constructed which consists of a monoclonal antibody linked to a naturally occurring toxin. Such hybrid molecules have been termed "immunotoxins" and combine the exquisite cell-type selectivity of monoclonal antibodies with the extraordinary potency of protein toxins.

While monoclonal antibodies have been raised against a wide spectrum of antigens including human neuroectodermal tumor- and human glioma-associated antigens, the inability to identify so-called "tumor-specific antigens," which would be expressed by all cells of a tumor but not by normal cells, limits the clinical application of immunotoxins. This problem is compounded in malignant gliomas, in which biological heterogeneity includes variable expression of antigens both among and within tumors and, possibly, with time.

The transferrin receptor (TR) is a transmembrane glycoprotein which mediates cellular uptake of iron. Proliferating cells express much larger numbers of TR's than resting cells, and some tumors selectively express very high levels of TR's compared to normal tissues. The abundance of TR's on certain malignant tumors raises the possibility of using monoclonal
antibodies against the human TR to target anti-TR-toxin hybrids, or immunotoxins, to human neoplasms.

Ricin (MW 62,000) is an extremely powerful protein toxin which is purified from castor bean seeds. The toxin consists of two subunits joined by a disulfide bond. The A-chain subunit is a 30,000-dalton enzyme which catalytically inactivates the 60S subunit of ribosomes, thus blocking protein synthesis and killing cells. The B-chain subunit binds the ricin molecule to cell-surface galactose-containing receptors (present on most eucaryotic cells) and facilitates transport of the A chain into the cytosol. This ubiquitous binding of ricin to cells can be prevented by co-incubating the toxin with lactose, which blocks the galactose-binding site of the B chain and thus blocks the ordinary route of ricin’s binding and killing.

Several anti-TR immunotoxins have been investigated. A new approach using an intact ricin-anti-TR immunotoxin is described, and the first report of any immunotoxin selectively toxic to human malignant brain-tumor cells is presented. It is suggested that anti-TR-ricin immunotoxins may offer a new modality for treatment of malignant tumors of the central nervous system (CNS), with particular application to intrathecal, intraventricular, and cystic neoplastic disease of the CNS.

Materials and Methods

Toxin

Ricin was purified from seeds of Ricinus communis by elution from Sepharose columns with N-acetylglactosamine, as described by Nicolson, et al.

Monoclonal Antibody Against Human Transferrin Receptor

Mouse HB21 hybridoma cells which secrete a monoclonal antibody (5E9) reactive to the human TR were propagated in BALB/C mice and ascites was collected. The anti-human TR monoclonal antibody, 5E9, was purified from the ascites using anion exchange high-performance liquid chromatography (HPLC) on a diethylaminoethyl (DEAE)-5PW column, as described by Gemski, et al.

After dialysis against phosphate-buffered saline (PBS: 10 mM NaH2PO4, 150 mM NaCl, pH 7.4), the antibody was concentrated by dialyzing against sucrose. High-performance gel filtration on a TSK 3000 SW column§ and solid-phase indirect radioimmunooassay using a K562 cell supernatant preparation (see below) were used to confirm homogeneity of the separated monoclonal antibody. Final concentration of antibody was 2.1 mg/ml. As described by Haynes, et al., the anti-human TR monoclonal antibody 5E9 is of the immunoglobulin (IgG) subclass.

Synthesis and Purification of Anti-TR-Ricin Immunotoxin

The bifunctional cross-linking agent, m-maleimido-benzoyl-N-hydroxysuccinimidyl (MBS) ester, was used to synthesize a hybrid between ricin and the mouse IgG, monoclonal antibody 5E9 against the human TR. Via the active ester, MBS reacts with primary amino groups of ricin resulting in a protein-coupled maleimide residue. The maleimide reacts with a free sulphydryl group on the antibody, forming a thioether linkage between the two proteins. The thioether bond is a stable, nonreducible linkage between IgG and toxin.

The antibody was prepared by adding 11 μl of 1 M dithiothreitol (DTT) in PBS to 0.4 mg (in 100 μl) of antibody. This mixture was incubated at room temperature for 30 minutes to reduce the antibody. The antibody-DTT mixture was then applied to a G-25, PD-10 column which was equilibrated with PBS to remove DTT from reduced antibody.

Ricin, 3.4 mg in PBS (176 μl), was mixed with 15 μl of dimethylformamide containing 0.056 mg of MBS. This mixture was incubated at room temperature for 20 minutes and then added to freshly reduced antibody. The resulting mixture was incubated for 3 hours at room temperature, at which time N-ethylmaleimide was added to stop the conjugation, and the mixture was refrigerated.

The following day, 300 μl of the mixture was run over a TSK 3000 SW HPLC column with 0.1 M sodium phosphate buffer (pH 7.0) to remove excess ricin, and the peak fraction containing antibody and immunotoxin was collected. This fraction was applied to a Sepharose 4B column. After the column was flushed with cold PBS to remove the antibody, 0.01 M α-lactose was added to elute the immunotoxin. The final concentration of collected immunotoxin was 49.1 μg/ml.

Cell Lines

The SNB75 cell line was established by primary explants from tumor removed in 1980 from a 72-year-old woman with a bifrontal mass. The pathological diagnosis was glioblastoma multiforme. The SNB40 cell line was established by primary explants from a tumor surgically removed from the posterior fossa of an 8-year-old boy in 1982. The pathological diagnosis was medulloblastoma. Each of these cell lines has been grown in monolayer culture in plastic tissue culture flasks in D/F media (equal parts Dulbecco’s minimum essential medium and Ham’s F12) containing 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin.

*MBS obtained from Pierce Chemical Co., Rockford, Illinois.

* All media and media supplements were purchased from Gibco Laboratories, Grand Island, New York.
tomycin (100 µg/ml) at 37°C in a 5% CO₂ atmosphere and passed at confluence with 1X trypsin. At the time of this study, SNB75 was in its 40th passage, and SNB40 was in its 10th passage. Trimonthly mycoplasma testing of these cell lines has yielded consistently negative results.

The K562 cell line is an established line derived from human erythroleukemia, and has been found to contain about 1.6 x 10⁶ TR sites/cell. Cells were grown in suspension in RPMI 1640 media with 10% FCS, 2% glutamine, 1 mM sodium pyruvate, and 10 µg/ml gentamicin. The WEHI-7.1 cell line is an established mouse lymphoma line. Cells have been cultured in suspension in RPMI 1640 media supplemented with 10% FCS and 10 µg/ml gentamicin.

**Surgical Tissue Samples**

Fresh samples of tumor tissue and normal brain were obtained from surgical specimens. All samples were immediately placed on dry ice and stored at −70°C. All three “normal brain” samples were taken from specimens of temporal lobe removed from patients with intractable epilepsy. Histological evaluation of the surgical specimens showed no evidence of neoplasia.

**Assay for Presence of TR on Cell Lines and Surgical Tissue Samples**

Cultured cells were harvested using a rubber policeman† and washed in PBS containing 0.1 mM phenylmethyl-sulfonyl fluoride and 1.0 mM epsilon-amino-caproic acid. Surgical tissue samples were finely minced in PBS containing the protease inhibitors noted above. Harvested cells and minced tissue samples were then homogenized using a polytron‡ at maximum speed for 1 minute. The homogenates were then centrifuged at 1000 G for 15 minutes. The supernatants were further clarified by centrifugation at 10,000 G for 15 minutes, and the supernatant preparations were collected. All steps were performed at 4°C. Protein concentrations were determined by the method of Lowry, et al., using bovine serum albumin (BSA) as a standard.

Presence of TR was determined by solid-phase indirect radioimmunoassay. Supernatant preparations of cell lines and surgical tissue samples were dried in duplicate (10 µg in 50 µl PBS per well) in 96-well polystyrene microtiter plates.§ To minimize nonspecific protein absorption, microtiter wells were treated with 150 µl of 0.2% BSA and incubated for 1 hour at 37°C. The BSA was removed and anti-human TR monoclonal antibody 5E9 (50 µl) was added. An irrelevant antibody of the same subclass and protein concentration was also tested to measure nonspecific binding. After incubation for 1½ hours at room temperature, unbound antibody was removed by washing with PBS containing 0.2% BSA and Tween 40. Iodine-125 (125I)-labeled sheep anti-mouse F(ab')2 antibody fragment was then added (75,000 cpm in 50 µl) as the secondary antibody. Following further incubation at room temperature for 1½ hours, the wells were washed; and bound secondary antibody was detected by cutting individual wells from the plate and measuring the radioactivity in a gamma counter.

**Cytotoxicity Bioassays**

Inhibition of protein synthesis was used to assay the cytotoxic effect of anti-TR-ricin immunotoxin and ricin in SNB75, SNB40, K562, and WEHI-7.1 cells. The SNB75 and SNB40 cells were suspended with trypsin and, after washing, 40,000 cells were plated per well in 100 µl of leucine-free RPMI medium containing 10% FCS in 96-well microtiter plates. After overnight preincubation at 37°C in 5% CO₂, designated amounts of anti-TR-ricin immunotoxin or ricin were added to wells in 11 µl of medium. Lactose was added to appropriate wells in aliquots of 4 µl to yield a 20-mM concentration. Monensin was added to appropriate wells in 10 µl of medium to yield a 50-nM concentration. Excess monoclonal antibody 5E9 was added to appropriate wells in aliquots of 5 µl to yield a 100-µg/ml concentration. Cells were then incubated for 18 hours, after which 20 µl of medium containing 0.1 µCi of carbon-14 (14C)-labeled leucine was added for 3 to 4 hours. Cells were then harvested into glass fiber filters with a PHD cell harvester; washed with water, dried, and counted in a beta counter. All cytotoxicity assays were performed two to four times in duplicate. Results were expressed as a percentage of incorporation in the control-treated cultures.

Cytotoxicity assays using K562 and WEHI-7.1 cells were carried out in a similar manner except that: 1) cells were dispensed into 96-well microtiter plates after washing without trypsinization; 2) no overnight preincubation was performed; 3) cells were incubated with test reagents and controls for 3 to 4 hours; and 4) cells were incubated with 14C-leucine for 1 hour before harvesting. Again, all assays were performed two to four times in duplicate, and results were expressed as a percentage of incorporation in mock-treated cultures.

Lactose alone at a 20-mM concentration had no effect on protein synthesis. Both monensin at a 50-nM concentration and monoclonal antibody 5E9 at a 100-

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† Policeman manufactured by Costar, Cambridge, Massachusetts.
‡ Polytron manufactured by Brinkman Instruments, Westbury, New York.
§ Microtiter plates manufactured by Flow Laboratories, McLean, Virginia.

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µg/ml concentration inhibited protein synthesis by approximately 10% in our assays.

Results

Reactivity of Anti-TR Monoclonal Antibody 5E9 With Human Primary Malignant Brain Tumor-Derived Cell Lines

Solid-phase indirect radioimmunoassay techniques (as described in Materials and Methods) were used to test reactivity of the anti-TR monoclonal antibody 5E9 with human primary malignant brain tumor-derived cell lines, as well as against a human leukemia cell line (K562) which is known to possess very large numbers of TR (Table 1).30 Values expressed are the number of times above background activity, where background is reactivity of antibody with WEHI-7.1 (a mouse lymphoma cell line which, accordingly, does not express the human TR). For each cell line, Table 1 compares the level of reactivity with the anti-TR monoclonal antibody 5E9 and the level of reactivity with an irrelevant antibody of the same IgG subclass. The data show that the anti-TR monoclonal antibody 5E9 reacted both with SNB75, a human glioblastoma-derived cell line, and with SNB40, a human medulloblastoma-derived cell line, at levels even greater than obtained with K562 cells, which contain approximately 1.6 × 10^5 TR sites/cell.30

Immunotoxin Cytotoxic Activity Against Human Glioblastoma-Derived and Human Medulloblastoma-Derived Cell Lines

Human Glioblastoma-Derived Cells. Figure 1A shows a representative dose-response curve of the anti-TR-ricin immunotoxin on SNB75 cells derived from a human glioblastoma and found on radioimmunoassay to express high levels of TR's. There is a steep dose-response inhibition of protein synthesis by the anti-TR-ricin immunotoxin, with 50% inhibition of protein synthesis at an anti-TR-ricin hybrid concentration (IC_{50}) of 6.8 × 10^{-12} M. Lactose (20 mM) which competitively blocks ricin's binding to cells and thus blocks non-cell-type specific or non-antibody-mediated binding of the immunotoxin, inhibited the anti-TR-ricin hybrid's toxicity only 25-fold. In contrast, Fig. 1B shows that the presence of lactose blocked toxicity of ricin 400-fold. Thus, in the presence of lactose, SNB75 cells were more sensitive to the anti-TR-ricin immunotoxin (IC_{50} = 1.7 × 10^{-10} M) than to native ricin (IC_{50} = 2.8 × 10^{-10} M).

Monensin is a carboxylic ionophore which alters pH in endosomes and disrupts intracellular vesicle traffic, presumably increasing the rate of transport of immunotoxins from within intracellular vesicles to the cytosol. Figure 1C shows that, when 50 nM monensin was added to the media, the dose-response curve shifted to 1000-fold greater potency (IC_{50} = 5.6 × 10^{-11} M). In contrast, toxicity of ricin alone was increased only sixfold in the presence of monensin (Fig. 1D). Therefore, in the presence of lactose and monensin, the anti-TR-ricin immunotoxin was more potent than native ricin.

The differential effects of lactose and monensin on the cytotoxicity of immunotoxin compared to ricin are two measures of cell type-specific killing of SNB75 cells. The clearest measure of specific toxicity is the demonstration that excess monoclonal antibody 5E9 blocks cell killing by the immunotoxin. The SNB75 cells were incubated 1) with the anti-TR-ricin hybrid, lactose, and monensin, and 2) with ricin and lactose, in the presence of excess free anti-TR monoclonal antibody 5E9 (100 µg/ml). As shown in Table 2, excess monoclonal antibody blocked the anti-TR-ricin immunotoxin's toxicity by at least 90% but had no significant effect on ricin's toxicity. This result confirms that the high potency of the anti-TR-ricin immunotoxin is selectively mediated by the anti-TR monoclonal antibody portion of the hybrid.

Human Medulloblastoma-Derived Cells. Figure 2 shows results of similar experiments performed with SNB40 cells, derived from a human medulloblastoma and also found on radioimmunoassay to express a high level of TR's. Figure 2A shows that, as observed with SNB75 cells, the anti-TR-ricin immunotoxin killed SNB40 cells in a steep dose-response fashion, killing 50% of the cells at an IC_{50} of 6 × 10^{-12} M. Addition of lactose, which blocks non-antibody-mediated killing, decreased activity of the anti-TR-ricin immunotoxin only 20-fold but blocked toxicity of ricin alone approximately 400-fold (Fig. 2B). Therefore, when nonspecific binding is blocked by lactose, SNB40 cells were greater than six times more sensitive to the immunotoxin than to ricin. Presence of the ionophore monensin markedly potentiated cytotoxicity of the anti-TR-ricin immunotoxin (110 times, Fig. 2C) but had little effect on ricin's killing (Fig. 2D). Thus, in the presence of lactose and monensin, the anti-TR-ricin immunotoxin was more potent than native ricin.

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<td>SNB75</td>
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<td>K562</td>
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* TR = transferrin receptor; MoAb = monoclonal antibody. Reactivity values are determined by radioimmunoassay and are expressed as the number of times above the control level, where control is reactivity of the antibody with WEHI-7.1 (a mouse lymphoma cell line which, accordingly, does not express human TR) (approximately 200 cpm). The irrelevant antibody is an immunoglobulin G of the same subclass and concentration as MoAb 5E9.

The K562 cell line is known to have approximately 1.6 × 10^5 TR sites/cell.
FIG. 1. Cytotoxic effect of anti-transferrin receptor (TR)-ricin immunotoxin versus ricin on human glioblastoma-derived (SNB75) cells. SNB75 cells in media alone or in media supplemented with 20 mM lactose or lactose plus 50 nM monensin or excess anti-human TR monoclonal antibody 5E9 (100 μg/ml) received the designated concentrations of anti-TR-ricin immunotoxin or ricin. After 18 hours' incubation at 37°C, 0.1 μCi of 14C-leucine was added, and cells were further incubated for 3 to 4 hours. Cells from duplicate cultures were then harvested and assayed for incorporation of radioactivity. Protein synthesis in treated cells is expressed as a percentage of 14C-leucine incorporated into untreated control cells.

Panel A: Toxicity dose-response curve for anti-TR-ricin immunotoxin in the absence (open circles) and the presence (closed circles) of lactose.

Panel B: Dose-response curve for ricin in the absence (open circles) and the presence (closed circles) of lactose.


Panel D: Toxicity dose-response curve for ricin in the presence (open squares) of monensin. Closed circles: points on the toxicity dose-response curve for ricin in the presence of lactose.

potent than ricin. The data shown in Table 2 confirm that antibody binding is required for immunotoxin activity, in that the presence of excess monoclonal antibody blocked cell killing by immunotoxin 88% but had no effect on ricin's toxicity.

**Immunotoxin Cytotoxic Activity Against Human Leukemia-Derived Cells**

Despite the impact of preventive therapy, CNS relapse remains a significant cause of treatment failure in leukemia. The anti-TR-ricin immunotoxin and ricin alone were assayed for inhibition of protein synthesis in K562 cells, a human leukemia cell line which is known to express TR. Figure 3A shows that within 4 hours the anti-TR-ricin immunotoxin killed 50% of K562 cells at a concentration (IC50) of 4.1 × 10⁻¹¹ M, making it more potent than native ricin (Fig. 3B). Furthermore, while lactose blocked cell killing by the anti-TR-ricin hybrid only sevenfold (Fig. 3A), lactose blocked cell killing by ricin alone over 50-fold (Fig. 3B).

Figure 3C and D show that, as we observed with the other “target” cells tested, the ionophore monensin significantly increased the immunotoxin’s toxicity on K562 cells compared to its small effect on killing by ricin alone.

**Immunotoxin Cytotoxic Activity Against a Mouse Lymphoma-Derived Cell Line**

A critical measure of antibody-mediated killing is the differential toxicity of the anti-TR-ricin immunotoxin between antigen-positive or “target” cells and antigen-negative or “non-target” cells. As WEHI-7.1 is an established mouse lymphoma cell line, WEHI-7.1 cells can be used as “non-target” cells, in that they do not express...
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**Fig. 2.** Cytotoxic effect of anti-transferrin receptor (TR)-ricin immunotoxin versus ricin on human medulloblastoma-derived (SNB40) cells. Cytotoxicity bioassays were performed, and the results are expressed as described in Fig. 1 and in Materials and Methods. **Panel A:** Toxicity dose-response curve for anti-TR-ricin immunotoxin in the absence (open circles) and the presence (closed circles) of lactose. **Panel B:** Dose-response curve for ricin in the absence (open circles) and the presence (closed circles) of lactose. **Panel C:** Enhanced toxicity of anti-TR-ricin immunotoxin in the presence (open squares) of monensin. Closed circles: points on the toxicity dose-response curve for anti-TR-ricin immunotoxin in the presence of lactose. **Panel D:** Toxicity dose-response curve for ricin in the presence (open squares) of monensin. Closed circles: points on the toxicity dose-response curve for ricin in the presence of lactose.

The human TR and should not be sensitive to antibody-mediated killing by the anti-human-TR-ricin immunotoxin.

We assayed WEHI-7.1 cells for their sensitivity to the anti-TR-ricin immunotoxin in the presence of lactose and monensin (conditions that block non-antibody-mediated cell killing and maximize cell-type specific killing by the anti-TR-ricin immunotoxin) and compared their sensitivity to that of “target” cells. As shown in Fig. 4, even at concentrations of anti-TR-ricin immunotoxin 10,000 times greater than that necessary to kill 60% of “target” SNB75 cells after 18 hours’ incubation, the anti-TR-ricin immunotoxin had no measurable toxicity on the “non-target” WEHI-7.1 cells after 4 hours’ incubation. To show that this differential toxicity of immunotoxin between “target” and “non-target” cells did not merely reflect inherent resistance of WEHI-7.1 cells to ricin, sensitivity of SNB75 cells to ricin alone was compared with sensitivity of WEHI-7.1 cells to ricin alone. The inset in Fig. 4 shows that WEHI-

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SNB75 Cells</th>
<th>SNB40 Cells</th>
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<tbody>
<tr>
<td>anti-TR-ricin immunotoxin alone</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>plus excess MoAb</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>ricin alone</td>
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<tr>
<td>plus excess MoAb</td>
<td>94</td>
<td>106</td>
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* Toxicity is IC₅₀ of immunotoxin or ricin with excess MoAb 5E9 for each cell line, expressed as percent of control, where control is IC₅₀ of immunotoxin or ricin without excess antibody 5E9. TR = transferrin receptor; MoAb = monoclonal antibody. SNB75 (glioblastoma-derived) and SNB40 (medulloblastoma-derived) cells were incubated for 18 hours with various concentrations of anti-TR-ricin immunotoxin or ricin, as described in Materials and Methods, with and without excess MoAb 5E9 (100 µg/ml). Cells were then pulsed for 3 hours with ¹⁴C-leucine, and 50% protein synthesis inhibition IC₅₀ was determined.
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Fig. 3. Cytotoxic effect of anti-transferrin receptor (TR)-ricin immunotoxin versus ricin on human leukemia-derived (K562) cells (known to have approximately $1.6 \times 10^4$ TR sites/cell). Cytotoxicity assays were performed, and the results are expressed as described in Fig. 1 and in Materials and Methods. Panel A: Toxicity dose-response curve for anti-TR-ricin immunotoxin in the absence (open circles) and the presence (closed circles) of lactose. Panel B: Dose-response curve for ricin in the absence (open circles) and the presence (closed circles) of lactose. Panel C: Enhanced activity of anti-TR-ricin immunotoxin in the presence (open squares) of monensin. Panel D: Toxicity dose-response curve for ricin in the presence (open squares) of monensin. Closed circles indicate points on the toxicity dose-response curve for anti-TR-ricin immunotoxin in the presence of lactose. Closed circles indicate points on the toxicity dose-response curve for ricin in the presence of lactose.

7.1 cells were 240 times less sensitive to ricin, under our assay conditions, than were SNB75 cells. An “index of specificity,” which is corrected for the lower sensitivity of WEHI-7.1 cells to ricin, can be calculated by dividing the differential sensitivity of SNB75 (“target”) and WEHI-7.1 (“non-target”) cells to the anti-TR-ricin immunotoxin (at least 8500-fold) by the differential sensitivity of SNB75 and WEHI-7.1 cells to ricin (240-fold). This calculation yields a “corrected index of specificity” of more than 35-fold.

Anti-TR-Ricin Immunotoxin Versus Ricin Toxicity on “Target” Versus “Non-Target” Cells

Figure 5 compares the cytotoxicity of anti-TR-ricin immunotoxin and native ricin on SNB75 and SNB40 cells. Anti-TR-ricin immunotoxin achieved greater than 90% killing of “target” cells at concentrations at which native ricin showed no toxicity. When this 25- to 230-fold greater toxicity of immunotoxin compared to ricin on “target” cells is combined with the approximately sixfold less toxicity of immunotoxin compared to ricin on “non-target” (WEHI-7.1) cells (data not shown), an overall selective toxicity of anti-TR-ricin immunotoxin between “target” tumor cells and normal brain of more than 150- to 1380-fold can be predicted. This conclusion assumes only that normal brain has the same sensitivity to ricin as brain-tumor cells.

Reactivity of Anti-TR Monoclonal Antibody 5E9 With Human Primary Malignant Brain Tumors and Normal Brain

Solid-phase indirect radioimmunoassay techniques (as described in Materials and Methods) were used to test the anti-TR monoclonal antibody 5E9 for reactivity with a number of surgical tissue specimens, including normal brain, and for reactivity with known antigen-positive K562 cells for comparison (Table 3). Values are expressed as the number of times above background.
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![Graph showing the comparison of the cytotoxic effect of anti-transferrin receptor (TR)-ricin immunotoxin on "target" (SNB75) cells versus "non-target" (WEHI-7.1) cells.](image)

**FIG. 4.** Comparison of the cytotoxic effect of anti-transferrin receptor (TR)-ricin immunotoxin on "target" (SNB75) cells (circles, 18 hours' incubation) versus "non-target" (WEHI-7.1) cells (triangles, 4 hours' incubation). Cytotoxicity bioassays were performed, and the results are expressed as described in Materials and Methods. **Inset** compares inherent sensitivity to ricin of SNB75 cells (circles) with that of WEHI-7.1 cells (triangles).

activity with BSA. Table 3 compares for each specimen its level of reactivity with the anti-TR monoclonal antibody 5E9 and its level of reactivity with an irrelevant antibody of the same IgG subclass. The data show that none of the three samples of normal brain reacted detectably with the anti-TR monoclonal antibody 5E9. In contrast, all three of the medulloblastoma samples showed levels of reactivity with the anti-TR monoclonal antibody 5E9 that were comparable to or greater than reactivity of the K562 cell line. One of the two glioblastoma multiforme specimens also showed specific reactivity with the anti-TR monoclonal antibody 5E9.

**Discussion**

Highly potent and cell type-specific killing of human glioblastoma-derived and medulloblastoma-derived cells, as well as human leukemia-derived cells, has been demonstrated with a molecular hybrid, or immunotoxin, consisting of a monoclonal antibody against the human TR covalently joined to the protein toxin ricin. Five criteria were used to show that killing by the anti-TR-ricin immunotoxin was cell type-specific in all cell lines tested. 1) Lactose, which blocks the galactose-binding site of the ricin B chain (via which ricin binds to cells), inhibited cell killing by ricin 20 to 25 times more in the glioblastoma-derived and medulloblastoma-derived cells, and 10 times more in the leukemia-derived cells, than it inhibited cell killing by the anti-TR-ricin immunotoxin. 2) Monensin, a carboxylic ionophore that disrupts intracellular vesicle traffic and facilitates delivery of immunotoxin to the cytosol, markedly potentiated the cytotoxicity of the anti-TR-ricin immunotoxin (up to three orders of magnitude in the glioblastoma-derived cells) but had minimal effect on ricin alone. 3) Presence of excess anti-TR monoclonal antibody 5E9, which competes for antibody-binding sites with the anti-TR-ricin immunotoxin and thus provides the clearest measure of antibody-mediated specific cytotoxicity, inhibited cell killing by the anti-TR-ricin immunotoxin by 90% but had no effect on killing by ricin. 4) At concentrations of anti-TR-ricin immunotoxin more than 10,000 times greater than the concentration necessary to kill "target" tumor cells after 18 hours' incubation, the anti-TR-ricin immunotoxin showed no activity after 4 hours' incubation against a "non-target" cell line that did not possess human TR.
FIG. 5. Comparison of the cytotoxic effect of anti-transferrin receptor (TR)-ricin immunotoxin versus ricin under conditions of maximal "target cell" selectivity. Glioblastoma-derived (SNB75) cells (squares) and medulloblastoma-derived (SNB40) cells (circles) were assayed for protein synthesis (as described in Materials and Methods) in the presence of designated concentrations of anti-TR-ricin immunotoxin (broken line) or ricin (solid line) in media supplemented with 20 mM lactose and 50 nM monensin.

TABLE 3
Reactivity of anti-human TR MoAb 5E9 with human primary malignant brain tumors and normal brain*

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<th>Reactivity of Irrelevant Antibody</th>
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<td>K562†</td>
<td>4.1</td>
<td>2.1</td>
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* TR = transferrin receptor; MoAb = monoclonal antibody. Reactivity values are determined by radioimmunoassay and are expressed as the number of times above the control level, where control is reactivity of bovine serum albumin (approximately 200 cpm). The irrelevant antibody is an immunoglobulin G of the same subclass and concentration as MoAb 5E9.

† K562 is a human erythroleukemia cell line known to have approximately 1.6 x 10⁷ TR sites/cell.

Immunotoxin between tumor cells and normal brain exceeding 150- to 1380-fold.

Solid-phase radioimmunoassay techniques were used to demonstrate that the "target" cell lines all possessed significantly higher levels of TR than did a control "non-target" cell line. Finally, based on samples of tumor tissue obtained at surgery, all three medulloblastomas tested and one of two glioblastomas tested expressed levels of TR comparable to or higher than levels in known TR-containing K562 cells. In contrast, all three samples of normal brain tested did not express significant levels of TR. These results support the hypothesis that immunotoxins targeted to human TR and prepared with whole ricin under conditions that inhibit non-antibody-mediated cell killing may provide powerful tumor-specific therapy for the treatment of certain neoplasms involving the CNS.

Other investigators have prepared immunotoxins using anti-TR monoclonal antibodies. Trowbridge and Domingo tested activity of an anti-TR-ricin A chain conjugate against human T-cell leukemia and human melanoma cells, and showed IC₅₀ values of 3 x 10⁻¹¹ M and 3.5 x 10⁻⁹ M, respectively. Fitzgerald, et al., have tested cytotoxicity of anti-TR 5E9 hybrids prepared with Pseudomonas exotoxin against a variety of "target" cell lines (including human epidermal carcinoma, breast carcinoma, and ovarian carcinoma) and have demonstrated IC₅₀ values under optimal conditions in the range of 10⁻¹⁰ M. Akiyama, et al., achieved an IC₅₀ of 9.5 x 10⁻¹³ M after incubating epidermal carcinoma cells for 7 to 10 days with an anti-TR 5E9-Pseudomonas exotoxin hybrid in the presence of a potentiating agent. Finally, Ramakrishnan and Houston constructed an immunotoxin composed of the anti-TR monoclonal antibody 5E9 and pokeweed antiviral protein and, under their best conditions, achieved an IC₅₀ of 5.6 x 10⁻¹² M against human T-cell leukemia cells. Using the same anti-TR monoclonal antibody 5E9, but linking it to intact ricin, we achieved an IC₅₀ of 5.6 x 10⁻¹² M against human T-cell leukemia cells. The potency of the anti-TR-ricin immunotoxin under our conditions thus exceeds that of any other anti-TR immunotoxin described in the literature.

Immunotoxins directed against the human TR possess special properties which may overcome many of the problems anticipated with clinical application of immunotoxins. Most cells do not express detectable levels of TR and, while the level of TR in normal human brain has not been extensively examined, our results suggest that normal brain tissue expresses levels of TR significantly lower than those present in malignant brain-tumor tissue. Accordingly, in some cases,
Immunotoxin for killing human malignant brain-tumor cells

TR may be used as a marker to distinguish tumor cells from normal brain tissue. Additionally, because transferrin, the major serum iron-transport protein, enters cells via receptor-mediated endocytosis, and because anti-TR monoclonal antibodies appear to enter cells via the same internalization pathway, the TR should facilitate internalization of bound anti-TR-toxin hybrid into the cell. In contrast, most other "anti-tumor" monoclonal antibodies, such as those directed against extracellular matrix antigens, are not internalized and, hence, immunotoxins prepared with them will likely be less potent. Lysosomal agents such as chloroquine, carboxylic ionophores such as monensin (as utilized in this study), and calcium channel-blocking agents such as verapamil and diltiazem allow easier access of immunotoxins to the cytosol, and thus can potentiate the toxicity of anti-TR immunotoxins. Furthermore, because iron is essential for tumor growth, it is not likely that tumor cells would be able to escape anti-TR-mediated cell killing either by antigenic heterogeneity, antigenic modulation, or genetic loss of TR's. Finally, although TR's are expressed on some proliferating normal cells and on normal tissues with high iron requirements (such as placenta and maturing erythroid cells), Trowbridge found no evidence of acute toxicity to normal tissues in mice with administration of a rat anti-mouse TR monoclonal antibody. Moreover, it has been demonstrated that early hematopoietic progenitors and stem cells in murine bone marrow are relatively insensitive to an anti-TR monoclonal antibody-ricin A chain immunotoxin that was active against tumor cells in vivo.

Other properties of immunotoxins in general make them especially attractive as potential clinical agents in the treatment of malignant brain tumors. Their extreme potency (a single molecule of ricin in the cytosol is sufficient to kill a cell) means that immunotoxins can kill "target" tumor cells at concentrations well below concentrations required by conventional chemotherapeutic agents. Immunotoxins kill "target" cells independently of deoxyribonucleic acid (DNA) synthesis and thus kill quiescent cells, in contrast to most chemotherapeutic drugs which kill only dividing cells. Finally, the cytotoxicity of immunotoxins is not affected by conditions of hypoxia, a major factor responsible for resistance of tumor cells to radiation therapy and chemotherapy.

Certain properties of immunotoxins, however, pose special problems in their application to cancer therapy. While immunotoxins constructed with the whole ricin molecule display much greater potency than do immunotoxins prepared with the ricin A subunit, intact ricin hybrids require the presence of lactose to block endogenous binding of the ricin B chain to generate cell type-specific or antibody-mediated killing in vitro. Modification of the ricin B chain binding site by chemical or genetic engineering techniques may provide other ways to block nonspecific binding and yet still preserve potency. Another potential problem concerns the mounting of an immune response by the patient to administered mouse monoclonal antibodies and to administered ricin. The response to monoclonal antibody-toxin conjugates in humans, however, is not known, and use of human monoclonal antibodies or less immunogenic Fab or F(ab)2 fragments may reduce this potential problem. Furthermore, patients who are immunosuppressed because of their underlying disease or because of immunosuppressive chemotherapy may have an attenuated anti-immunotoxin immune response, and the first dose of immunotoxin therapy (before patient antibodies arise) may have a significant therapeutic effect. Finally, to the extent that the CNS is partially an "immunologically privileged site," immunotoxins targeted to tumors within the CNS may be protected from a patient's immune response.

Perhaps the greatest problem in the clinical application of immunotoxins concerns their access to target brain-tumor tissue. The large size of immunotoxins may limit their diffusion from the vascular system and poses a particularly difficult problem in the case of parenchymal brain tumors in those areas where an intact blood-brain barrier may further exclude immunotoxins from target tissue. Variable blood flow within brain tumors may also limit access of immunotoxins. Blood-brain barrier disruption may enhance immunotoxin delivery to intracerebral tumors but there is little information available describing accessibility of immunotoxins or antibodies to human intracerebral tumors.

We believe that, insofar as problems of delivery and clearance presently stand as obstacles to the use of immunotoxins in the treatment of parenchymal brain tumors, immunotoxins may be particularly efficacious in the treatment of CNS neoplastic disease involving compartments — intrathecal, intraventricular, or cystic — where access to target tumor tissue will not depend upon transvascular transport. Our preliminary evidence for high levels of TR's in medulloblastomas, together with work by others demonstrating TR expression in 30 of 38 cases of carcinoma, five of five germ-cell tumors, and a variety of leukemias (all of which display a propensity for spread within the CSF compartment), further support a possible role for anti-TR-ricin immunotoxins in the treatment of leptomeningeal neoplastic disease. Work in progress using a guinea pig model of leptomeningeal neoplasia has demonstrated that a monoclonal antibody-ricin immunotoxin delivered intrathecally specifically kills tumor cells and produces extended survival in tumor-bearing animals (unpublished data, 1987).

Although the TR is clearly not a "tumor-specific" antigen, it is well established that certain tumors selectively express an abundance of TR's relative to normal tissue. Using an intact ricin-anti-human TR immunotoxin, we have achieved highly potent killing of cells derived from human medulloblastoma, glioblastoma, and leukemia in tissue culture at a level of selectivity.
causing death of "target" tumor cells but not "non-target" cells. This differential toxicity may be sufficient to kill "target" tumor cells selectively in vivo, particularly in those clinical situations (such as intrathecal, intraventricular, and cystic neoplastic disease) where access to tumor does not rely upon transvascular delivery of immunotoxins. These results, along with our finding that nearly all of the surgically obtained malignant brain tumors that we tested showed high levels of TR's relative to normal brain, suggest that immunotoxins prepared with intact ricin and directed against the human TR may serve as potent and specific agents in the treatment of a wide range of these devastating tumors.

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