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Potent Cytotoxicity of an Antihuman Transferrin Receptor-Ricin A-Chain Immunotoxin on Human Glioma Cells *in Vitro*¹

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ABSTRACT

The cytotoxic effects of an antihuman transferrin receptor monoclonal antibody-ricin A-chain conjugate (anti-TfR-A) immunotoxin on glioma cells were assessed *in vitro*. Five human glioma cell lines were studied; three were derived from surgical explants (MG-1, MG-2, MG-3) and two were well characterized established glioma cells (U-87 MG, U-373 MG). The C6 rat glioma line served as a nonhuman control. One of six lines (U-373) expressed glial fibrillary acidic protein, as assessed by immunohistochemistry. All five human lines expressed human transferrin receptor, as assessed by flow cytometry; no human transferrin receptor was demonstrable on rat C6 cells. Potent inhibition of protein synthesis was found after an 18-h incubation with anti-TfR-A. Fifty % inhibitory concentration (IC₅₀) values for human glioma cells ranged from 1.9×10^{-9} to 1.8×10^{-8} M. In contrast, no significant inhibition of leucine incorporation was observed when anti-TfR-A was tested on rat cells (IC₅₀ > 10^{-7} M) or when a control immunotoxin directed against carcinoembryonic antigen was substituted for anti-TfR-A on human glioma cells (IC₅₀ > 10^{-7} M). Coincubation with the carboxylic ionophore monensin (10^{-7} M) decreased the IC₅₀ of anti-TfR-A against human glioma lines from 16- to 842-fold (range, 7.0×10^{-12} to 1.5×10^{-10} M). In contrast, an IC₅₀ of > 10^{-7} M was obtained when C6 cells were incubated with anti-TfR-A and monensin. Anti-TfR-A immunotoxins potentiated by monensin are extremely potent *in vitro* cytotoxins for human glioma cells.

INTRODUCTION

The prognosis for patients with GBM,³ the most common primary brain neoplasm, remains poor despite intensive combined modality conventional treatments, including surgery, radiation, and chemotherapy (1); therefore, new strategies that could either supersede or complement current therapies are needed. Immunotherapy represents one alternative approach. Previously, immunotherapeutic treatment of brain tumors aimed to increase host antitumor defense mechanisms; patient immunization with glioma cells (2), administration of interferon (3), or, more recently, intratumoral delivery of interleukin 2 and lymphokine-activated killer cells (4) all represent examples of such a strategy. An alternative immunotherapy would utilize MoAbs directed against tumor-associated antigens to selectively deliver toxins into tumor cells. The administration of such ITs may result in potent and specific tumor cell killing.

In order to achieve selective effects using immunotoxins, antigens which are exclusively or differentially expressed on

tumor cells should be chosen as targets, so that adverse effects will be minimal. The optimal target, therefore, would be an antigen that is exclusively expressed on tumor cells; unfortunately, although a number of tumor membrane components, such as epidermal growth factor receptor (5, 6), appear to be present in higher numbers of glioma cells, a completely specific glioma membrane component has not yet been demonstrated. Nevertheless, antigens that are expressed in much higher concentrations on glioma cells, compared to normal neural tissues, might still make acceptable immunotherapeutic targets. One such candidate membrane component is the TfR, a cell surface glycoprotein whose expression on the cell surface is coordinately regulated with cell growth.

A number of observations suggest that TfR is differentially expressed in gliomas, compared to normal surrounding neural tissues. TfR immunostaining can be detected in virtually all primary brain tumors in amounts much higher than in normal surrounding tissues; furthermore, staining intensity tends to correlate with tumor anaplasia (7). In addition, TfR expression can often be demonstrated on human glioma lines *in vitro* (8-10). These observations suggest that high TfR expression is a frequent occurrence in gliomas and that this receptor might be a suitable target for IT therapy.

Previous studies using ITs constructed with anti-TfR murine MoAbs as carrier molecules have demonstrated potent effects on human glioma cells *in vitro* when attached to either whole ricin or modified diphtheria toxins (11, 12). However, both of these toxins present difficulties for clinical use. Whole ricin conjugates may be limited due to potential toxicity caused by nonspecific B-chain binding. ITs derived from diphtheria toxin could be hampered by neutralizing antibodies, due to the almost universal prior immunization of the adult population. These difficulties are avoided by using ricin A-chain-based immunotoxins, which may be more suitable for clinical use. Therefore, this study was conducted to investigate (a) the level of TfR expression on a number of glioma cell lines, (b) the cytotoxic effects of a ricin A-based IT directed against the TfR found on glioma cells, and (c) the enhancement of cytotoxicity by the known potentiator monensin.

MATERIALS AND METHODS

Chemicals. Monensin was purchased from Sigma Chemical Co. (St. Louis, MO). The ionophore was prepared as a 10^{-3} M stock solution in ethanol and diluted to an appropriate final concentration of 0.14 M NaCl, 0.01 M NaHPO₄, pH 7.4 (phosphate-buffered saline).

MoAbs and ITs. The 7D3 MoAb (IgG1), directed against the human TfR, was produced in mice by injection of cultured human leukemia CEM cells (13). The anti-carcinoembryonic antigen murine MoAb (IgG1) was a gift of Abbott Laboratories. Native ricin A-chain (Inland Laboratories and EY Laboratories) was disulfide-linked to anti-TfR and anti-carcinoembryonic antigen with the *N*-succinimidyl-3-(2-pyridylthio)propionate reagent, to produce immunotoxins (13).

Cell Lines. MG-1, MG-2, and MG-3 were established from primary explants of tumors removed from three male patients, 49, 58, and 61 years old, respectively. The pathological diagnosis of each tumor was

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³ The abbreviations used are: GBM, glioblastoma multiforme; anti-TfR-A, antihuman transferrin receptor monoclonal antibody-ricin A-chain conjugate; anti-CEA-A, anticarcinoembryonic antigen monoclonal antibody-ricin A-chain conjugate; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GFAP, glial fibrillary acidic protein; TfR, transferrin receptor; IT, immunotoxin; MoAb, monoclonal antibodies; MON, monensin; FCS, fetal calf serum; IC₅₀, 50% inhibitory concentration; CNS, central nervous system; MEM, minimum essential medium.

GBM. The primary tumors contained large numbers of TfR-positive cells, as assessed immunohistochemically (7). U-87 and U-373 were purchased from the American Type Culture Collection (Rockville, MD). C6 rat glioma cells were a gift of Dr. William Shapiro (Memorial Sloan Kettering Cancer Center, New York, NY). All cell lines were maintained by serial passage in monolayer culture, using RPMI 1640-10% FCS at 37°C in a humidified atmosphere of 5% CO₂.

Immunofluorescent Staining for Human TfR. Growing cultures were trypsinized and a trypan blue exclusion test was performed to ensure that >99% of the cells remained viable. Tumor cells (10⁶) were incubated for 1 h with 10 µg of either anti-TfR or MOPC21 (Sigma Immunochemicals, St. Louis, MO), a nonspecific murine monoclonal immunoglobulin which served as a control. The cells were washed twice in MEM and resuspended in 1 ml phosphate-buffered saline containing 1 µg fluorescein isothiocyanate-labeled goat anti-mouse antibody (Becton-Dickinson, San Jose, CA). After two further washes, the cells were fixed in 1% paraformaldehyde. Flow cytometric analysis was performed on a FACSCAN (BDIS, Mountain View, CA). The fluorescence intensity of 10,000 viable cells was measured.

GFAP Immunocytochemistry. In order to better characterize the tumor cell lines, immunostaining for the specific glial intermediate filament GFAP was performed. Tumor cells were grown on coverslips, fixed in acetone for 10 min, and incubated in 3% H₂O₂ with 1% bovine serum albumin for an additional 10 min. Slides were then incubated overnight in a solution containing murine anti-GFAP MoAb (Dakopatts Co., Glostrup, Denmark) diluted 1:2000 in 1% normal porcine serum in Tris buffer, at 4°C. After two washings in Tris-HCl buffer, slides were then incubated 1 h in a solution containing a 1:50 dilution of rabbit anti-mouse antibody (Dakopatts Co.), washed in Tris-HCl, and then reacted for 1 h with a 1:200 dilution of peroxidase antiperoxidase. After a final wash, the peroxidase reaction was developed with diaminobenzidine for 10 min, rinsed with water, and then counterstained with Harris hematoxylin.

Cytotoxicity Assays. For cytotoxicity assays, cells in 75-cm² flasks were treated with 1 ml 0.05% trypsin, 0.02% EDTA, for 2 min, after which the trypsin-EDTA solution was removed, leaving only a thin coating on the cells for an additional 8 min. Leucine-free MEM (10 ml) was added and cells were aspirated and expelled using a syringe and 22-gauge needle, after which they were seeded into microtiter plates (10⁵ cells/well) (Becton-Dickinson Labware, Oxnard, CA) containing leucine-free MEM, to a final volume of 200 µl/well. Additions of serial dilutions of either anti-TfR-A or control immunotoxin (10⁻⁷ to 10⁻¹³ M) were made in quadruplicate. Following an 18-h incubation at 37°C in 5% CO₂, the medium was removed and replaced with leucine-free MEM supplemented with 2 µCi/well [³H]leucine (New England Nuclear Corp., Boston, MA). Following an additional 2-h incubation, the medium was removed, the cells were treated with 0.05% trypsin, 0.02% EDTA (200 µl/well), for 30 min and collected onto glass fiber filters, and incorporation of [³H]leucine was measured by liquid scintillation counting of glass fiber discs.

To assess MON potentiation, similar experiments were carried out in the presence of 10⁻⁷ M levels of the ionophore.

The inhibitory effect of BCNU on cell growth was also evaluated, to compare the effects of ITs and chemotherapy on glioma cells. Glioma cells (5 × 10³) were seeded into 24-well plates containing RPMI 1640 supplemented with 10% FCS. Additions of serial dilutions of BCNU (10⁻⁴ to 10⁻⁸ M) were made in triplicate. After an 18-h incubation, the medium was decanted and replaced with RPMI 1640-10% FCS. Cells were trypsinized and counted 5 days later.

RESULTS

Flow cytometric analysis revealed that greater than 95% of cells from all five human glioma cell lines bound the anti-TfR MoAb (7D3), indicating the presence of cell surface human TfR (Table 1, Fig. 1a). In contrast, there was no difference between the binding of the specific anti-human TfR and control MOPC21 binding to the C6 rat glioma cell line

Table 1 GFAP and human TfR expression on glioma cell lines

The presence of GFAP was assessed *in vitro* by immunohistochemical means, as described in "Materials and Methods." -, no visualized cell immunostaining; +, greater than 90% of cells were immunostained. Mean fluorescence intensity compares fluorescence intensity on glioma cells after incubation with either anti-human TfR murine MoAb 7D3 or control murine MoAb MOPC-21.

Cell line	GFAP	Log mean fluorescence intensity with	
		Anti-TfR	MOPC-21
U-373	+	250	25
U-87	-	300	10
MG-1	-	190	4
MG-2	-	100	32
MG-3	-	90	15
C6	-	5	5

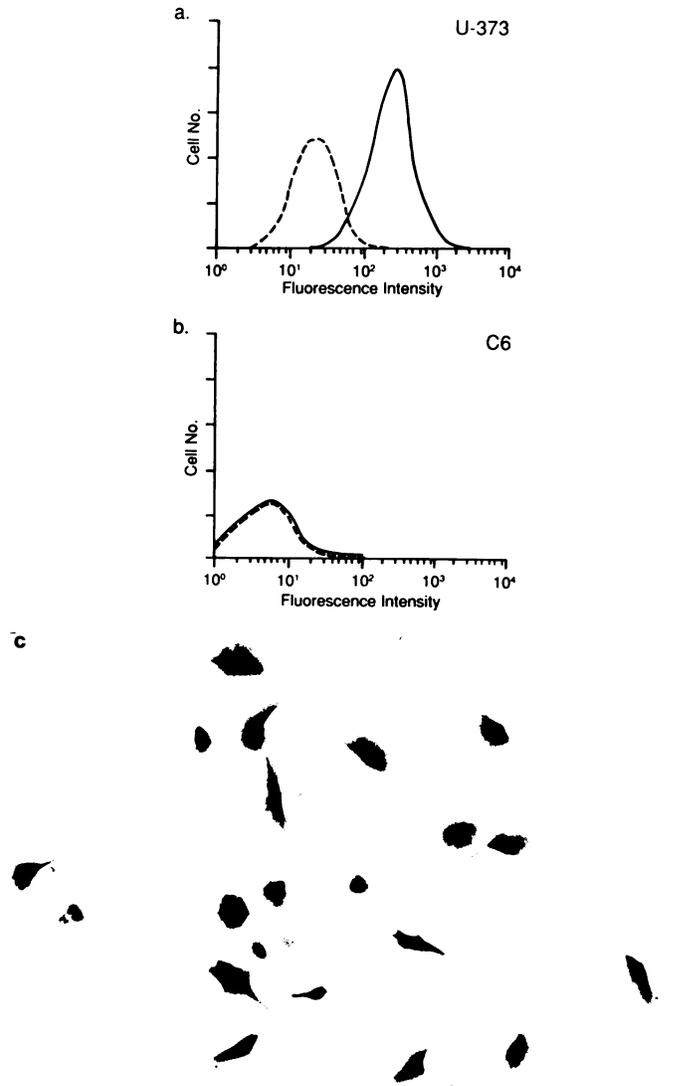


Fig. 1. Characterization of glioma lines. Results of flow cytometric analysis of human TfR cell surface expression in U-373 (a) and C6 rat glioma (b) lines. ---, control MOPC-21 MoAb; —, 7D3 anti-TfR MoAb. c, GFAP immunostaining of U-373 cells. Greater than 95% of cells are positively immunostained. × 300.

(Fig. 1b). GFAP cell immunostaining was present in only one (U-373 MG) of the six malignant cell glioma lines (Table 1, Fig. 1c).

Cytotoxic Effects of Anti-TfR-A IT on Glioma Cell Lines. The anti-TfR-A IT was a potent inhibitor of protein synthesis in all five human glioma cell lines tested, while the unmodified 7D3

anti-human TfR MoAb produced no effect (Table 2). The mean IC_{50} values obtained ranged from 1.9×10^{-9} to 1.8×10^{-8} M and were comparable for both the early passage cells and the established cell lines. This cytotoxicity was specific for the TfR, since the anti-CEA-A control IT was ineffective against human glioma cells ($IC_{50} > 10^{-7}$ M) (Fig. 2). Similarly, the human specific anti-TfR-A IT displayed negligible effects on rat C6 glioma cells ($IC_{50} > 10^{-7}$ M) (Fig. 3).

Potentialiation of IT Effects by MON. MON alone did not inhibit glioma cell protein synthesis at concentrations as high as 10^{-7} M. Coincubation of 10^{-7} M MON with anti-TfR-A, however, potentiated IT effects on all five glioma lines from 16- to 842-fold (Table 2) and reduced the mean IC_{50} levels to 1.5×10^{-11} to 7.0×10^{-12} M. By contrast, MON did not potentiate the effects of either the anti-TfR-A IT on rat C6 glioma cells (Fig. 3) or the anti-TfR-A IT on human glioma (Fig. 2).

The combined effects of anti-TfR-A IT plus monensin produced very effective cytotoxicity for human GBM cells. By comparison, BCNU, the standard clinical chemotherapeutic agent for GBM, was 100,000-fold less potent when tested on MG-3 cells under similar conditions (IC_{50} of BCNU was $4.0 \times$

Table 2 Effect of anti-TfR MoAb and anti-TfR-A IT on *in vitro* glioma cell [3H] leucine incorporation

Additions of serial dilutions of either 7D3 or anti-TfR-A IT (in the presence or absence of 10^{-7} M MON) were incubated with 100,000 glioma cells for 18 h, after which the medium was removed and replaced with leucine-free MEM, supplemented with $2 \mu Ci/well$ [3H]leucine, for 2 h. Incorporation of tritiated leucine was measured by liquid scintillation counting of glass fiber disks. Results represent mean IC_{50} values; figures in parentheses denote the number of times individual experiments were performed.

Cell line	Mean IC_{50} (M) ^a			Potentiating effect ^b
	Anti-TfR	Anti-TfR-A	Anti-TfR-A + MON (10^{-7} M)	
U-373	$>10^{-7}$ (2)	1.9×10^{-9} (4)	1.5×10^{-11} (4)	126
U-87	$>10^{-7}$ (2)	5.9×10^{-9} (4)	7.0×10^{-12} (4)	842
MG-1	ND ^c	1.8×10^{-8} (3)	7.0×10^{-11} (3)	257
MG-2	ND	2.4×10^{-9} (3)	1.5×10^{-10} (3)	16
MG-3	ND	1.8×10^{-9} (1)	4.1×10^{-11} (1)	44
C6	ND	$>10^{-7}$ (3)	$>10^{-7}$ (3)	

^a IC_{50} , concentration which resulted in 50% inhibition of protein synthesis.

^b Potentiating effect, potentiating effect of added MON (IC_{50} anti-TfR-A/ IC_{50} anti-TfR-A plus MON).

^c ND, experiment not performed.

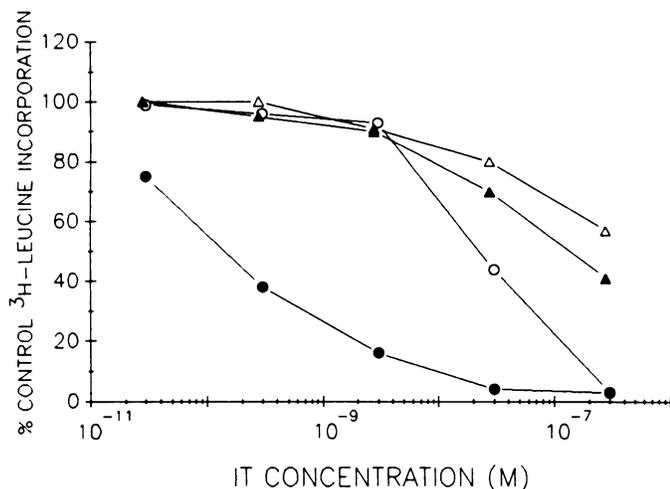


Fig. 2. Inhibition of [3H]leucine incorporation by MG-1 glioma cells after 18-h incubations with anti-TfR-A or an anti-CEA-A IT, with or without 10^{-7} M MON. \blacktriangle , anti-CEA-A IT; \triangle , anti-CEA-A IT in the presence of 10^{-7} M MON; \circ , anti-TfR-A IT; \bullet , anti-TfR-A IT in the presence of 10^{-7} M MON.

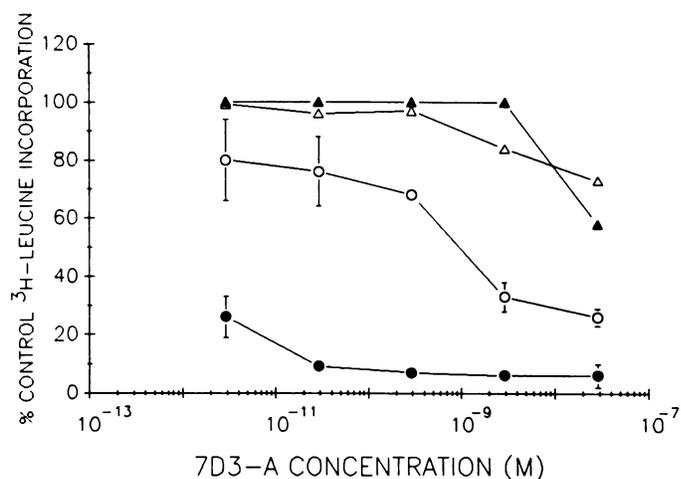


Fig. 3. Inhibition of [3H]leucine incorporation by U-87 and C6 rat glioma cells after an 18-h incubation with anti-TfR-A. \blacktriangle , C6 glioma cells; \triangle , C6 rat glioma cells plus 10^{-7} M MON; \circ , U-87 human glioma cells; \bullet , U-87 glioma cells in the presence of 10^{-7} M MON.

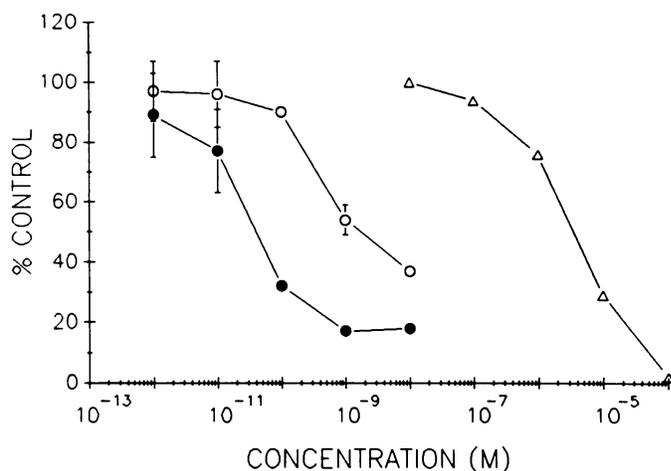


Fig. 4. Comparison of the effects of the chemotherapeutic agent BCNU (Δ) and of anti-TfR-A with (\bullet) and without (\circ) 10^{-7} M MON on MG-3 cells.

10^{-6} M versus 4.0×10^{-11} M for anti-TfR-A IT plus MON) (Fig. 4).

DISCUSSION

The TfR is a major immunodominant cell surface glycoprotein whose expression on the cell surface is coordinately regulated with cell growth (14–16). Proliferating cells express greater numbers of TfR compared to normal tissues, and very high TfR levels have been noted in certain tumors (16–18). Administration of MoAbs directed against TfR can inhibit cell growth, albeit modestly, in some tumor systems (19, 20). By conjugating anti-TfR MoAbs with an intracellular toxin, more potent antitumor effects have been obtained against a number of systemic human cancer lines both *in vitro* (21, 22) and *in vivo* (13, 23).

Recent evidence has indicated that malignant brain tumor cells express high numbers of TfR. Therefore, TfR expression has been noted on malignant glioma cell lines *in vitro*, utilizing immunohistochemistry, slot blot RNA analysis, and flow cytometry (8–10). Furthermore, immunohistochemical studies indicate high levels of TfR in tumor, as compared to normal brain tissue (7), and the amount of TfR detected by radio-

immunoassay is much greater in tumor than in surrounding brain (11). Such differential expression on malignant glial compared to normal cells suggests that this membrane component may be a suitable target for brain tumor immunotherapy.

Zovickian *et al.* (12) have demonstrated potent effects of TfR-targeted ITs on human GBM cells *in vitro*, utilizing an anti-TfR MoAb conjugated to whole ricin toxins; this effect was enhanced by cocubation of IT with MON. Subsequent studies from this group utilizing point-mutated diphtheria toxin confirmed the effectiveness of anti-TfR MoAbs as carrier molecules of intracellular toxins to brain tumor cells (11). A related approach to target brain tumor TfR has employed the use of human transferrin-ricin A-chain conjugates. Effective killing of a human glioma line was observed *in vitro* and the specific cytotoxicity of this conjugate was enhanced more than 100-fold in the presence of MON.

In the present study, human TfR expression was demonstrated on the five human glioma lines examined, by using flow cytometric techniques. The fact that targeting the TfR allowed adequate toxin internalization is supported by the cytotoxicity studies showing potent effects of anti-TfR-A IT on protein synthesis. By contrast, an anti-CEA-A [an antigen which is not expressed in human brain tumors (24)] IT resulted in no cytotoxicity, nor was anti-TfR-A effective against rat C6 glioma cells, which do not express human TfR as assessed by flow cytometry. Furthermore, the effects of anti-TfR-A were not well correlated with GFAP expression, cell passage number, or cell doubling times.

One method of potentiating ricin A-chain action *in vitro* makes use of the carboxylic ionophore MON, which accelerates ricin A-chain action by as yet unclear mechanisms (25). MON was a very effective potentiator of IT action in this study; therefore, the toxic potency of anti-TfR-A on glioma cells was increased on average 100-fold by cocubation with this ionophore. Compared with BCNU, the standard chemotherapy agent utilized in malignant brain tumor treatment, anti-TfR-A ITs plus monensin were 100,000 times more potent. Furthermore, the selective advantage of this IT on surface human glioma compared to control TfR-negative C6 cells is over 10,000-fold.

Although anti-TfR-A is an effective inhibitor of glioma cell protein synthesis *in vitro*, its therapeutic effectiveness *in vivo* may be restricted by the presence of TfR in normal brain. TfR has a wide CNS distribution in a pattern distinct from that of iron distribution (26–28). Although its CNS development and differentiation, especially myelination (28–30). TfR expression is especially prominent on cerebral oligodendrocytes and endothelial cells (31–33). The presence of CNS TfR raises the possibility of significant brain toxicity if an anti-TfR IT is utilized to treat malignant glioma. Nevertheless, even though antigen expression on normal cells is undesirable, anti-TfR ITs may still be therapeutically effective if the cell surface antigen concentration on normal cells is below that required for IT efficacy (34, 35). The observation that Tf-linked toxins have been administered intrathecally without undue toxicity to experimental animals (11) suggests, in fact, that toxicity may be minimal and that carrier molecules directed at TfR may be useful therapeutically, at least in meningeal malignancies.

Unfortunately, an intrathecal approach is unlikely to be efficacious in GBM because intratumoral penetration will be poor. A preferable route of administration might be via an intralésional or intratumoral route, such as has been clinically utilized recently with lymphokine-activated killer cells with or without

concomitant interleukin 2 (4, 36). This method of delivery would have the dual advantages of maximizing tumor penetration while minimizing normal tissue exposure. *In vivo* studies are in progress to examine this possibility.

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