In vitro efficacy of recombinant diphtheria toxin–murine interleukin-4 immunoconjugate on mouse glioblastoma and neuroblastoma cell lines and the additive effect of radiation

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Object. The prognosis for patients with primary malignant brain tumors is poor despite aggressive treatment, and tumor recurrence is common regardless of the chosen therapy. Although multimodal treatment does not provide a cure, it is necessary to determine which treatment modalities have the greatest cytotoxic effect and can potentially prolong survival. Immunotoxin therapy is a novel approach for the treatment of tumors, and it has been successfully used in the central nervous system. Because the interleukin (IL)-4 receptor is commonly expressed on brain tumor cells, the purpose of this study was to evaluate the cytotoxic effect of using a modified diphtheria toxin–murine IL-4 (DT₃⁹₀–mIL4) immunoconjugate for the treatment of murine brain tumor cell lines and to determine whether the addition of radiation therapy could potentiate the effect of this agent.

Methods. Spontaneous murine glioblastoma (SMA-560) and two neuroblastoma (Neuro-2a and NB41A3) cell lines were treated using DT₃⁹₀–mIL4 at different concentrations, and the anti–mouse IL-4 monoclonal antibody (11B11) was used for blocking its cytotoxicity. Other SMA-560 and Neuro-2a cell lines were treated using 500 cGy of radiation 3 hours before DT₃⁹₀–mIL4 treatment. Cytotoxicity was evaluated using a trypan blue viability assay.

The immunoconjugate exhibited a dose-dependent cytotoxic effect with 50% inhibitory concentration values of 0.56 × 10⁻⁹ M in SMA-560, 1.28 × 10⁻⁹ M in Neuro-2a, and 0.95 × 10⁻¹⁰ M in NB41A3 cell lines. The cytotoxicity of DT₃⁹₀–mIL4 was specifically blocked by an excess of 11B11. Cytotoxicity was additive when the DT₃⁹₀–mIL4 at different concentrations, and the anti–mouse IL-4 monoclonal antibody (11B11) was used for blocking its cytotoxicity. Other SMA-560 and Neuro-2a cell lines were treated using 500 cGy of radiation 3 hours before DT₃⁹₀–mIL4 treatment. Cytotoxicity was evaluated using a trypan blue viability assay.

Conclusions. These results indicate that the IL-4 receptor can be a target for diphtheria toxin fusion proteins and that radiation can potentiate the effects of DT₃⁹₀–mIL4. The development of multimodal approaches to treat malignant brain tumors with agents that have different mechanisms of action may be beneficial.

Key Words • glioblastoma • immunotoxin • interleukin-4 receptor • radiation therapy • neuroblastoma

Survival of patients with malignant glioma has been improved with the advent and use of adjuvant therapeutic modalities, but the prognosis for these patients remains poor. In addition to radiation therapy, which has improved patient survival, chemotherapy is usually included in the multidisciplinary treatment protocols and it offers some therapeutic benefit.

Immunotoxin therapy, developed as an adjuvant therapy, has shown promising results in vitro, in vivo, and in clinical trials. In patients with recurrent malignant brain tumors, regional therapy with the transferrin–crossreacting material 107 immunoconjugate has been shown to achieve at least a 50% reduction in tumor volume on magnetic resonance imaging in nine of 15 patients, including complete responses in two patients. The IL-4 receptor is commonly expressed on many malignant brain tumor cells but not on normal brain tissue, and in some studies fusion proteins directed against the IL-4 receptor have been reported to exert a potent tumor-killing effect. Although multidisciplinary protocols have been designed in which surgery, radiotherapy, and chemotherapy are combined, immunotoxin therapy has not yet been introduced into these regimens.

In the studies described herein, we developed a recombinant fusion protein DT₃⁹₀–mIL4 that was constructed by the fusion of the gene encoding for murine IL-4 to a gene encoding for truncated DT, which was devoid of its native binding domain, DT₃⁹₀. The DT₃⁹₀ encodes for the first 389 amino acids of DT. We examined the specific cytotoxic activity of DT₃⁹₀–mIL4 against murine glioblastoma and neuroblastoma cell lines and also determined the effect of combining DT₃⁹₀–mIL4 with radiation on these same murine cell lines.

MATERIALS AND METHODS

Tumor Cell Lines and Culture Conditions

The spontaneous murine glioblastoma cell line, SMA-560, was cultured in minimum essential medium with zinc option containing 10% FBS and split 1:15 every 3 days. Two murine neuroblastoma cell lines, Neuro-2a and

Abbreviations used in this paper: DT = diphtheria toxin; DT₃⁹₀–mGMCSF = DT₃⁹₀–murine granulocyte-macrophage–colony stimulating factor; DT₃⁹₀–mIL-4 = DT–murine interleukin-4; FBS = fetal bovine serum.
NB41A3, were used. The Neuro-2a cells were maintained in a minimum essential medium supplemented with 10% FBS and split 1:10 every 4 to 5 days. The NB41A3 cells were grown in Ham’s F10 medium supplemented with 15% horse serum and 2.5% FBS and split 1:10 every 4 to 5 days. The 3T3 murine fibroblasts were maintained in Dulbecco’s modified Eagle medium containing 10% bovine calf serum.

Construction of the Modified Diphtheria Toxin–Murine IL-4 Conjugate

A 1549-bp NcoI/XhoI DNA fragment was prepared by splice overlap extension encoding amino acids 1 to 389 of the DT gene positioned upstream of a fragment encoding amino acids 25 to 144 of murine IL-4. This $DT_{390}$-mIL4 hybrid gene was ligated into the NcoI/XhoI cloning sites in the pET21d plasmid, which was transformed into the Escherichia coli strain BL21(DE3). The fusion protein was expressed and purified by ion-exchange chromatography.

Cytotoxicity Assays and Radiation

The cytotoxicity assays of $DT_{390}$-mIL4 were performed at different concentrations. The cells were divided into a control group treated with phosphate-buffered saline, a 10$^{-8}$ M group (treated with $DT_{390}$-mIL4 at the concentration of 10$^{-8}$ M), a 10$^{-9}$ M group, a 10$^{-10}$ M group, and a 10$^{-11}$ M group. Each group was tested in triplicate. The cells were transferred into 24-well cell culture clusters with 0.1 $\times$ 10$^6$ cells per well. After incubation for 24 hours at 37°C, the tumor cells were treated with $DT_{390}$-mIL4. The viable cells were counted after 24, 48, and 72 hours of incubation by trypan blue exclusion. Percentage viability (that is, the percentage of the number of viable cells remaining in each group compared with that of the control group) was calculated.

To determine the specificity of $DT_{390}$-mIL4, the tumor cells were pretreated with 300 $\mu$g of 11B11 per well, and they were then treated with $DT_{390}$-mIL4 at 10$^{-8}$ M 1 hour later. Another group of cells were pretreated with 300 $\mu$g per well of irrelevant control A20-1.7 antibody (rat monoclonal antibody immunoglobulin G$_\text{m}$). The viable cells were counted after 24, 48, and 72 hours of incubation, and percentage of viable cells was calculated. For a control, the cytotoxicity of $DT_{390}$-mIL4 was tested on murine 3T3 fibroblasts, and the cytotoxic activity of another recombinant fusion toxin, $DT_{390}$-mGMCSF constructed previously was examined on SMA-560.

The combined effect of radiation treatment and $DT_{390}$-mIL4 was studied on the murine SMA-560 and Neuro-2a cell lines at two different $DT_{390}$-mIL4 concentrations: 10$^{-9}$ M and 10$^{-10}$ M. The tumor cells were divided into a phosphate-buffered saline–treated group, a $DT_{390}$-mIL4–treated group, a radiation-treated group (500-cGy dose), and a combined group that was treated with $DT_{390}$-mIL4 3 hours postirradiation. The viable cells were counted on the 2nd, 3rd, and 4th days after incubation, and the percentage of viable cells was calculated.

Statistical Analysis

Repeated-measures analysis of variance was used to determine the effects of treatment, the day, and the interaction of treatment and day on concentration. A p value of 0.05 or less was considered statistically significant. Tests of simple effects were produced to determine on which days treatment differed. We used t-tests to compare the differences between each active treatment and control group (for example, radiation compared with control; $DT_{390}$-mIL4 compared with control; and radiation and $DT_{390}$-mIL4 compared with control), and each single-agent treatment with the combination treatment (for example, radiation compared with radiation and $DT_{390}$-mIL4; $DT_{390}$-mIL4 compared with radiation and $DT_{390}$-mIL4). Both the simple effect tests and the t-tests were adjusted for multiple comparisons by using the method of Bonferroni.

Sources of Supplies and Equipment

The glioblastoma cell line was kindly provided by Dr. Bigner (Duke University Medical Center, Durham, NC). The minimum essential medium with zinc and the Dulbecco’s modified Eagle medium were obtained from Life Technologies, Inc. (Bethesda, MD). We acquired the Neuro-2a and NB41A3 neuroblastoma cell lines as well as the 3T3 murine fibroblasts from American Type Culture Collection (Rockville, MD). The pET21d plasmid and the E. coli strain (BL21(DE3)) were purchased from Novagen (Madison, WI). To determine tumor cell specificity, cells were pretreated with 11B11 acquired from Pharmingen (San Diego, CA), which also produced the rat monoclonal antibody, immunoglobulin G$_\text{m}$.

RESULTS

Cytotoxicity of $DT_{390}$-mIL4 on SMA-560 Cells

The percentage viability of SMA-560 cells on the 4th day after incubation was 10.4% at 10$^{-9}$ M, 46.7% at 10$^{-8}$ M, 60.2% at 10$^{-10}$ M, and 113.4% at 10$^{-11}$ M (Table 1). The survival curve shows that $DT_{390}$-mIL4 inhibited SMA-560 in a dose-dependent manner (Fig. 1 upper). When percentages of viable cells counted on the 3rd and 4th days were compared, the latter was higher than the former: 4.6% cells were viable on the 3rd day and 10.4% on the 4th day at 10$^{-8}$ M; 18.6% on the 3rd day and 46.7% on the 4th day at 10$^{-9}$ M; and 43.9% on 3rd day and 60.2% on the 4th day at 10$^{-10}$ M. The cytotoxicity of $DT_{390}$-mIL4 on SMA-560 was neutralized by an excess of 11B11 antibody, which reacted to the mouse IL-4 carrier ligand portion of the fusion protein (Fig. 1 lower).

<table>
<thead>
<tr>
<th>TABLE 1 Percentage of viable SMA-560 cells after treatment with $DT_{390}$-mIL4$^*$</th>
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<tbody>
<tr>
<td>$DT_{390}$-mIL4 Concentration</td>
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<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>10$^{-8}$ M</td>
</tr>
<tr>
<td>10$^{-9}$ M</td>
</tr>
<tr>
<td>10$^{-10}$ M</td>
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<tr>
<td>10$^{-11}$ M</td>
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</table>

* Values represent the mean percentage ± standard deviation.
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Cytotoxicity of DT$_{390}$-mIL4 on Neuro-2a Cells

The percentage viability on the 4th day was 6.2%, 63.3%, and 95% at 10$^{-8}$ M, 10$^{-9}$ M, and 10$^{-10}$ M concentration of DT$_{390}$-mIL4, respectively (Table 2). The survival curves show dose-dependent cytotoxic activity of DT$_{390}$-mIL4 (Fig. 2 upper) and a tendency for the percent age of viable cells to decrease over time. The cytotoxic activity of DT$_{390}$-mIL4 was blocked by an excess of 11B11 (Fig. 2 lower).

Cytotoxicity of DT$_{390}$-mIL4 on NB41A3 Cells

The percentage viability on the 4th day was 0% at 10$^{-8}$ M, 2.3% at 10$^{-9}$ M, and 56.2% at 10$^{-10}$ M (Table 3). The cytotoxicity shown in the survival curves was dose-dependent and time dependent at 10$^{-8}$ M and 10$^{-9}$ M. An excess of neutralizing anti–IL-4 antibody abolished the cytotoxic activity of DT$_{390}$-mIL4.

Effect of DT$_{390}$-mIL4 on Fibroblasts and Effect of DT$_{390}$-mGMCSF on SMA-560 Cells

The cytotoxic activity of DT$_{390}$-mIL4 on the 3T3 fibroblasts was tested at 10$^{-8}$ M. The survival curves showed no statistically significant cytotoxicity. The DT$_{390}$-mGMCSF did not exhibit a tumor-killing effect on SMA-560 cells. Taken together, these data indicate the specific, dose-dependent cytotoxic activity of DT$_{390}$-mIL4 on SMA-560, Neuro-2a, and NB41A3 cell lines.

Additive Effect of DT$_{390}$-mIL4 Combined With Radiation on SMA-560 Cells

At a concentration of 10$^{-9}$ M, the percentage of viable cells in the DT$_{390}$-mIL4–treated group was 78.8% on the 2nd day, 66.7% on the 3rd day, and 59.5% on the 4th day (Table 4). The percentage viabilities on the 3rd and 4th days represented statistically significant cytotoxicity. On the 3rd day, the percentage of viable cells was 70.2% in the radiation-treated group and 26.3% in the combined treatment group, and the differences were statistically significant. To evaluate the combined cytotoxic activity of DT$_{390}$-mIL4 and radiation therapy in the combined group, the relative percentage of the number of viable cells in the combined group compared with that of the DT$_{390}$-mIL4–treated group and the radiation-treated group was calculated. The relative percentage viability of the combined group was 39.4% for the DT$_{390}$-mIL4–treated group and 37.5% for the radiation-treated group. Both were statistically significant. On the 4th day of cell incubation, the relative percentage viability was 53.8% in the radiation-treated group, which was statistically significant (p < 0.05).

At a concentration of 10$^{-10}$ M, the percentages of viable cells in the DT$_{390}$-mIL4–treated group were 106.4% on the 2nd day, 89.6% on the 3rd day, and 94.6% on the 4th day (Table 4). The cytotoxicity did not have statistical significance. Taken together, the combination of 10$^{-9}$ M DT$_{390}$-mIL4 and radiation with a 500-cGy dose showed an additive cytotoxic effect on SMA-560 cells.

Additive Effect of DT$_{390}$-mIL4 and Radiation on Neuro-2a Cells

At a concentration of 10$^{-9}$ M, the percentage of viable cells in the DT$_{390}$-mIL4–treated group was 74.1% on the 2nd day, 89.6% on the 3rd day, and 94.6% on the 4th day (Table 5). The percentage of viable cells present on the 4th day represented statistically significant cytotoxicity. On the 4th day after cell incubation, the percentage of viable cells was 32.5% in the radiation-treated group and 16.2%
in the combination group; the relative percentage viability of the combination group was 25.6% for the DT390-mIL4-treated group and 50% for the radiation-treated group. All were statistically significant (p < 0.05).

At a concentration of 10^{-10} M, the percentage of viable cells in the DT390-mIL4-treated group was 83.8% on the 2nd day, 77.5% on the 3rd day, and 94.7% on the 4th day (Table 5). The cytotoxicity did not achieve statistical significance. Taken together, an additive cytotoxic effect of 10^{-9} M DT390-mIL4 and radiation with 500 cGy on Neuro-2a cells was demonstrated.

**DISCUSSION**

Despite aggressive therapeutic trials, primary malignant brain tumors are not yet curable. Many multidisciplinary protocols have been designed and applied clinically, and improvement in patient survival has been shown.22,25 Combining individual therapeutic modalities with different mechanisms of action has advantages; for example, tumor cells that escape from one treatment may succumb to another. Additionally, the toxic effects of treatment may be decreased by reducing the amount of the administered agent or extending the duration between administration sessions.

Total eradication of malignant brain tumor cells is not yet possible, and the main goal of treatment is to decrease the tumor burden to such a size that growth can be suppressed by the patient's immune system. Shapiro35,36 has described the concept of cytoreduction in the treatment of brain tumors. To elicit the clinical symptoms related to the mass effect, the number of tumor cells should reach 3 to 6 x 10^{10}. He determined that the surgical removal of less than 50% of a brain tumor will leave behind 1 to 5 x 10^{9} tumor cells. The tumor burden can be reduced to approximately 10^{7} cells with the addition of radiation therapy. It is hoped that subsequent administration of chemotherapeutic agents will further decrease the tumor burden so that the patient's immune system will recognize and react to the tumor.

Many protocols in which radiation therapy is combined with various anticancer drugs have been developed; however, the number of protocols that include immunotoxin treatment in humans is limited. Before including immunotoxins in multidisciplinary treatment protocols, it will be necessary to consider the mechanism of action of individual treatment modalities and to determine the advantage of immunotoxin-including multimodal therapy. Surgical debulking is the first step in the management of brain tumors, and this intervention can be used to determine a diagnosis and to reduce increased intracranial pressure, thus improving the quality of a patient's survival.26,7,24,31,42 Moreover, the hypoxic area of the tumor, where the tumor cells are not affected by radiation thera-

**TABLE 3**

<table>
<thead>
<tr>
<th>DT390-mIL4 Concentration</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
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<tr>
<td>10^{-8} M</td>
<td>22.5 + 10.6</td>
<td>1.7 + 2.7</td>
<td>0</td>
</tr>
<tr>
<td>10^{-9} M</td>
<td>62.8 + 17.7</td>
<td>45.6 + 5.4</td>
<td>2.3 + 4.8</td>
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<tr>
<td>10^{-10} M</td>
<td>74.7 + 16.1</td>
<td>81.2 + 10.4</td>
<td>56.2 + 13.8</td>
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</tbody>
</table>

* Values represent the mean percentage ± standard deviation.

**TABLE 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-9} DT390-mIL4</td>
<td>78.8</td>
<td>66.7*</td>
<td>59.5*</td>
</tr>
<tr>
<td>10^{-10} DT390-mIL4</td>
<td>106.4</td>
<td>89.6</td>
<td>94.6</td>
</tr>
</tbody>
</table>

* Statistically significant (p < 0.05).
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<table>
<thead>
<tr>
<th>TABLE 5</th>
<th>Percentage of viable Neuro-2a cells</th>
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<tr>
<td>Treatment</td>
<td>10$^{-10}$ DT$_{390}$-mIL4</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>DT$_{390}$-mIL4 radiation</td>
<td>74.1</td>
</tr>
<tr>
<td>combination</td>
<td>58.0*</td>
</tr>
<tr>
<td></td>
<td>39.7*</td>
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</table>

* Statistically significant (p < 0.05).

Radiation therapy is a well-accepted cornerstone of most treatment protocols for the management of malignant brain tumors. The main intracellular target for ionizing radiation is DNA. Ionizing radiation (x-ray or γ-ray) interacts with water molecules within the cell, producing hydroxyl radicals, which break DNA strands. The effect of radiation is dose-, oxygen-, and cell-cycle dependent. The cells in the mitotic phase are more sensitive to radiation therapy than those in the synthetic phase or resting phase. Measurements of oxygen tissue tension is also important for predicting the response to radiation, and hypoxic cells are known to be resistant to radiation. Tumor cells located at the periphery of a mass divide actively and receive a higher oxygen supply than those located in the necrotic center. These cells that remain after surgery can be targets for radiation treatment.

Chemotherapy is considered by many to be standard adjuvant treatment for patients with brain tumors, and numerous protocols that include anticancer drugs have been examined clinically. An extensive discussion of chemotherapy is beyond the scope of this paper. One of the main limitations in the use of anticancer drugs is that they usually demonstrate cytotoxic activity on tumor cells in the mitotic phase and that tumor cells in the resting phase may not respond to chemotherapy or radiotherapy. Although chemotherapeutic agents and radiation therapy are delivered to kill these cells, many remain alive because of drug resistance and DNA repair after radiation-induced damage.

Targeted fusion toxins, chimeric proteins that consist of the cytotoxic domain of the natural toxin and a carrier ligand such as a growth factor or monoclonal antibody, have been developed for the treatment of malignant central nervous system tumors, and the initial results have been promising. Fusion toxins are characterized by high specificity and great potency, and they exert cytotoxic activity through receptor-mediated endocytosis. The IL-4 receptor is an appropriate cell surface antigen for fusion toxins against malignant brain tumors because IL-4 receptors are expressed on many kinds of brain tumor cells but not on normal neural tissue. Interleukin-4 linked to Pseudomonas exotoxin has been shown to exhibit potent cytotoxicity in vitro and in vivo.

Diphtheria toxin is a bacterial toxin frequently used for making fusion toxins and has extreme potency such that one molecule of DT that reaches the cytosol will kill the cell. A mutant form of DT, crossreacting material 107, has been shown to exhibit potent cytotoxicity in vitro. In our study, it is not clear whether radiation, which is known to abolish the expression of high-affinity IL-4 receptors, affected the sensitivity of cells to DT$_{390}$-mIL4. In the U373 human glioblastoma cell line, IL-4 receptor expression was decreased 49.6%* and NB41A3 cell lines, respectively (data not shown), reflecting the lower potency than the agent noted above. Because rat cells lack functional DT receptors, they are 1000 to 10,000 times less sensitive to DT than human cells.

Our results are similar to those for DAB389-mIL4, which is composed of the enzymatically active and membrane translocation domains of DT fused to murine IL-4, against a murine mastocytoma cell line where the IC$_{50}$ was about 4 × 10$^{-10}$ M. Another factor that may influence the differential sensitivity of cells to DT-based immunoconjugates is the effect of radiation on the expression of IL-4 receptors. In the U373 human glioblastoma cell line, IL-4 receptor expression was decreased 49.6% one hour after irradiation with 500 cGy and returned to normal levels at 24 hours. In the Daoy medulloblastoma cell line, IL-4 receptor expression was decreased 61.4% one hour postradiation and later returned to normal (data not shown). In our study, it is not clear whether radiation, which is known to abolish the expression of high-affinity IL-4 receptors, affected the sensitivity of cells to DT$_{390}$-mIL4, which after binding to IL-4 receptors will enter the cytosol to cause cell death.

In conclusion, targeted toxins have high specificity and great potency. Their cytotoxic activity is cell-cycle independent, which provides one of the theoretical reasons for combining fusion toxin treatment with radiation therapy.

In this study, radiation therapy combined with DT$_{390}$-mIL4 exhibited statistically significant additive cytotoxic activity. Clinical trials in which fusion proteins are delivered during radiation treatment or combined with chemotherapeutic drugs are recommended.

Acknowledgments

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References


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