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Preclinical Development of a Recombinant Toxin Containing Circularly Permuted Interleukin 4 and Truncated *Pseudomonas* Exotoxin for Therapy of Malignant Astrocytoma[1]

Raj K. Puri,[2] Dave S. Hoon, Pamela Leland, Phil Snoy, Robert W. Rand, Ira Pastan, and Robert J. Kreitman

Laboratory of Molecular Tumor Biology, Division of Cellular and Gene Therapies [R. K. P., P. L.], and Division of Veterinary Services [P. S.], Center for Biologics Evaluation and Research, Food and Drug Administration, NIH, Bethesda, Maryland 20892; Saint John's Hospital and Health Center, John Wayne Cancer Institute, Division of Molecular and Cellular Immunology and Neuro-Oncology, Santa Monica, California 90404 [D. S. H., R. W. R.]; and Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, NIH, Bethesda, Maryland 20892 [I. P., R. J. K.]

ABSTRACT

Effective treatment is lacking for malignant glioblastoma/astrocytoma. We have identified interleukin-4 receptors (IL-4R) on human malignant astrocytoma. We demonstrate that 16 of 21 surgical samples of high-grade astrocytoma and glioblastoma but not normal brain tissues expressed IL-4R as assessed by reverse transcriptase PCR. We further demonstrate that human malignant astrocytoma cell lines express high-affinity IL-4R. Using a chimeric protein composed of circularly permuted IL-4 and a truncated form of *Pseudomonas* exotoxin A, we observed that this toxin (IL4(38-37)-PE38KDEL) is highly cytotoxic to IL-4R-bearing glioblastoma cells. Compared with a previously reported IL4-PE chimeric protein (IL-PE4E), IL4(38-37)-PE38KDEL bound with higher affinity and was 3–30-fold more cytotoxic to glioblastoma cell lines. Upon intrathecal administration in monkeys, high cerebrospinal fluid IL4(38-37)-PE38KDEL levels were achieved using 2- and 6-μg/kg doses without any central nervous system or other abnormalities. IL4(38-37)-PE38KDEL levels were not detectable in the serum of any monkey studied. When IL4(38-37)-PE38KDEL was injected into the right frontal cortex of rats, localized necrosis was observed at 1000-ng/ml doses but not at ≤100-ng/ml doses. We conclude that by localized administration, nontoxic levels of IL4(38-37)-PE38KDEL can be achieved, which may have significant cytotoxic activity against malignant astrocytoma.

INTRODUCTION

We have reported previously that a variety of solid cancer cells overexpress high-affinity IL-4Rs[3] (1, 2). These receptors are functional because IL-4 is able to cause signal transduction, inhibit tumor cell growth, and up-regulate major histocompatibility antigens and intercellular adhesion molecule-1 (3–12). IL-4R are also expressed, although in low numbers, in normal immune cells such as T cells, B cells, monocytes, other blood cells such as eosinophils and basophils, and fibroblasts and endothelial cells (1, 2). The significance of the overexpression of IL-4R on epithelial cancer cells and the similarities and differences between IL-4R in cancer cells and immune cells is not completely clear. We have demonstrated that the common γ chain of the IL-4R that is expressed on immune cells (13, 14) is not expressed on human solid cancer cell lines examined (11, 15, 16). While studies on the structure and function of IL-4R on cancer cells are ongoing, we have exploited the overexpression of IL-4R on cancer cells by targeting with a cytotoxic chimeric protein composed of IL-4 and PE (17–21).

We have reported that human brain cancer cell lines express high numbers of IL-4R (22). Using a recombinant chimeric cytotoxin composed of IL4 and a mutated form of PE (termed IL4-PE^{4E}), we found that this toxin is highly toxic to brain tumor cells (22). We have subsequently produced a circularly permuted IL4 toxin, IL4(38-37)-PE38KDEL, which contains amino acids 38–129 of IL-4 fused via a peptide linker to amino acids 1–37, which are in turn fused to amino acids 353–364 and 381–608 of PE, with KDEL at positions 609–612. IL4(38-37)-PE38KDEL was more cytotoxic than native IL4 toxins on human Burkitt's lymphoma and other tumor cell lines (23–25) and showed better antitumor activity *in vivo* using a human epidermoid carcinoma xenograft model (24). However, it is not known whether IL4(38-37)-PE38KDEL is more cytotoxic to human brain tumor cells. It is also not known whether fresh human glioblastoma samples and normal brain tissues also express IL-4R *in situ*. Also, importantly, it is not known to what extent IL4(38-37)-PE38KDEL internalizes nonspecifically into normal brain.

Malignant astrocytoma comprise the third leading cause of cancer-related deaths in the United States in adolescents and adults between the ages of 15 and 34 (26). Conventional therapies including surgery, radiation therapy, and chemotherapy have not significantly changed the prognosis for patients with malignant astrocytoma. Innovative techniques including gene therapy are being explored to treat these cancers (27). However, at present this mode of therapy is very cumbersome, expensive, and may not be suitable for locally disseminated glioblastoma. Additional modes of therapy are needed to alter the prognosis of astrocytoma/glioblastoma.

In the present study, we have examined the expression of IL-4R in fresh malignant astrocytoma (including entities such as anaplastic astrocytoma, astrocytoma grade III, and glioblastoma multiforme) samples obtained from patients undergoing surgical resection, normal brain tissues obtained postmortem, and glioblastoma cell lines. We have tested the sensitivity of five glioblastoma cell lines to IL4(38-37)-PE38KDEL. In addition, *in vivo* toxicology and pharmacokinetics studies in rats and in cynomolgus monkeys were undertaken. Human IL-4 is a primate-specific cytokine. It has been shown to have many effects in cynomolgus monkeys when administered systemically (28, 29). In addition, it has also been shown that human IL-4 binds to monkey COS-7 cells (Refs. 2 and 13) and gibbon ape leukemia MLA 144 cells (Refs. 2 and 13). Thus, because human IL-4 can bind to monkey cells (13) and not to rat cells, the use of these animal models will provide assessment of specific and nonspecific toxicity of IL4 toxin, respectively. IL4(38-37)-PE38KDEL, therefore, was administered intrathecally in monkeys for maximum exposure of the brain to IL4 toxin to assess direct toxicity, or intracerebrally in rats to examine nonspecific toxicity of IL4(38-37)-PE38KDEL. Our data support pursuing the use of IL4(38-37)-PE38KDEL for treatment of malignant astrocytoma in patients who have failed all conventional therapies.

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[2] To whom requests for reprints should be addressed, at Laboratory of Molecular Tumor Biology, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, NIH Building 29B, Room 2NN10, 29 Lincoln Drive MSC 4555, Bethesda, MD 20892.Phone: (301) 827-0471; Fax: (301) 827-0449; E-mail: puri@a1.cber.fda.gov.

[3] The abbreviations used are: IL-4R, interleukin-4 receptor; PE, *Pseudomonas* exotoxin A; cpIL4-PE, circular permuted IL-4 *Pseudomonas* exotoxin (IL4(38-37)-PE38KDEL); HSA, human serum albumin; IC_{50}, 50% inhibitory concentration of protein synthesis; EC_{50}, 50% inhibitory competitor; CSF, cerebrospinal fluid, RT, reverse transcriptase; KDEL, represents amino acids lysine, aspartate, glutamate, and leucine, respectively.

MATERIALS AND METHODS

Recombinant Cytokines and Toxins. Recombinant IL-4 was provided by Schering-Plough Research Institute (Kenilworth, NJ). Recombinant IL4(38-37)-PE38KDEL (termed cpIL4-PE), IL4-PE^{4E}, and IL4-PE38KDEL were produced and purified to >95% homogeneity as described previously (18, 19, 23, 24). In the native IL4 toxin IL4-PE38KDEL, IL-4 was not circularly permuted, and COOH terminus of IL-4 is connected to NH_2 terminus of the mutated form of the *Pseudomonas* exotoxin molecule PE38KDEL. In the circular permuted toxin IL4(38-37)-PE38KDEL, the amino acids 38–129 of IL-4 were connected by a linker peptide (GGNGG) to amino acids 1–37, which were then connected with the mutated toxin molecule PE38KDEL (24).

Cell Lines. Glioblastoma cells lines (U251, A172, U373, T98G, SN19, U87, U118, and CCF-STTG1) were obtained from the American Type Culture Collection (Rockville, MD), and glioma cell line RC 1913 and meningioma line RC 1909 were obtained from Dr. R. Merchant (Virginia Commonwealth University, Richmond, VA). All of these cell lines were cultured in complete media composed of RPMI 1640 and 10% heat-inactivated FCS. In some cases, medium was supplemented with 3 mM glutamine and 50 μg/ml gentamicin or penicillin and streptomycin. These adherent cell lines were routinely passaged every 4–5 days. Cell lines for RT-PCR were harvested when 75% confluent.

Tumor and Normal Brain Tissue Specimens. Tumor samples were obtained from patients undergoing surgical resection for astrocytoma/glioblastoma (specimens obtained form Dr. R. Merchant). The pathology was verified by histology. Tumor samples were snap-frozen and stored at −80°C until used for RNA isolation. Normal brain tissues from six autopsy patients (3 males and 3 females) were obtained ≤20 h postmortem.

Animals. Female feral cynomolgus monkeys (*Macaca fasicularis*) between 2 and 7 years old weighing between 2 and 4 kg were selected for this study. These monkeys were fed certified primate diet twice daily.

These animals were individually housed at Corning Hazelton (Vienna, VA) and quarantined for a minimum of 4 weeks before entering in the study. Only healthy animals, based on physical examination, clinical laboratory tests, and other appropriate methods of evaluation standardized by Corning Hazelton, were enrolled.

Rats (Harlan Sprague Dawley) between 6 and 8 weeks old weighing 365 ± 24 g (mean ± SD) were obtained from Charles River Laboratories, Inc. These animals were housed at Corning Hazelton and fed Purina-certified rodent chow ad libitum. Monkey and rat toxicology studies were performed at Corning Hazelton under good laboratory practices under the direction of Drs. Dan Dalgard and Daniel J. Minnema, respectively.

Protein Synthesis Inhibition Assay. The cytotoxic activity of IL4 toxins was tested as described previously by determining inhibition of protein synthesis (17). Typically, 10^4 glioma cells were cultured in leucine-free medium with or without various concentrations of IL4 toxins for 20–22 h at 37°C. Then, 1 μCi of [^{3}H]leucine (NEN Research Products, Wilmington, DE) was added to each well, and cells were incubated for an additional 4 h. Cells were harvested, and radioactivity incorporated into cells was measured by a Beta plate counter (Wallac-LKB, Gaithersburg, MD).

^{125}I-labeled IL-4 Binding and Displacement Assay. IL-4 was iodinated with Iodo-Gen reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. The specific activity of radiolabeled IL-4 ranged between 31.5 and 212 μCi/μg. The IL-4 binding assay was performed by a previously described technique (3, 15). Briefly, tumor cells were harvested after brief incubation with Versene (BioWhittaker, Walkersville, MD), washed three times in HBSS, and resuspended in binding buffer (RPMI 1640 plus 1 mM HEPES and 0.2% human serum albumin). For the displacement assay, A172 cells (1×10^6/100 μl) were incubated at 4°C with ^{125}I-labeled IL-4 (100–200 pM) with or without increasing concentrations of unlabeled IL-4, IL4-PE^{4E}, or IL4(38-37)-PE38KDEL. For binding assays, cells were incubated with various concentrations of ^{125}I-labeled IL-4 with or without 200-fold molar excess of unlabeled IL-4. After a 2-h incubation, cell-bound radioligand was separated from unbound by centrifugation through a phthalate oil gradient, and radioactivity was determined with a gamma counter (Wallac). The number of receptors and binding affinities were determined as described previously (3).

RT-PCR and Southern Blot Analysis. The RT-PCR and Southern blot analysis were performed as described previously (9). Total RNA was isolated using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH) from cell lines and frozen tumor specimens following the manufacturer's instructions. All RNA extraction procedures were carried out in a designated laminar flow hood under sterile conditions. The concentration and purity of total RNA was determined by spectrophotometric analysis. RNA extraction, RT-PCR, and post-RT-PCR studies were carried out in separate designated rooms. One μg of total RNA was used in the RT-PCR assay. RT-PCR and Southern blot analysis was performed as described previously (9). RT-PCR conditions were as follows; 95°C for 5 min, 1 cycle; 95°C for 1 min, 65°C for 1 min; 65°C for 1 min, 30–35 cycles; and 72°C for 10 min for the final primer extension sequence. RT-PCR primers for IL-4R were: 5′ primer 5′-ATGGGGTGGCTT-TGCTCTGGG-3′ and 3′ primer 5′-ACCTTCCCGAGGAAGTTCGGG-3′. The IL-4R RT-PCR cDNA product is 345 bp. A 100-bp DNA ladder (Life Technologies, Gaithersburg, MD) was used as a base-pair reference marker.

***In Vivo* Toxicology and Pharmacokinetics Study in Cynomolgus Monkey.** IL4(38-37)-PE38KDEL was injected intracisternally every other day for 3 days in three cynomolgus monkeys at escalating doses (1 monkey/dose). IL4(38-37)-PE38KDEL was diluted in 0.2% HSA-PBS and injected at 0 (control), 2 (low), or 6 μg/kg dose levels. The animals were anesthetized with thiopental, the area over cisterna magna was prepared for aseptic injection, and 0.1 ml of CSF was removed. The needle was left at its place, and the syringe was replaced with IL4(38-37)-PE38KDEL, which was injected on days 1, 3, and 5. CSF samples (0.1 ml) were withdrawn 2 h after injection on days 3 and 5 and approximately 24 h after the first and second dose. In addition, CSF samples (0.5 ml) were withdrawn on days 8, 15, and 22. Blood was drawn once before treatment and on days 3, 5, 8, 15, and 22.

CSF and serum samples were assayed for IL4 toxin levels. The toxicities were evaluated by physical and clinical exams, hematology, and serum chemistry. The control monkey was later injected at a 6-μg/kg dose level. Thus, the dose levels of IL4(38-37)-PE38KDEL examined were 0, 2, and 6 μg/kg given intracisternally every other day for three doses.

***In Vivo* Toxicology Study in Rats.** To determine potential toxicity and/or neuropathology of IL4(38-37)-PE38KDEL, a cohort of three rats/group was given a single intracerebral injection of IL4(38-37)-PE38KDEL. A log-escalating dose of IL4(38-37)-PE38KDEL was injected in each group of animals. One μl of either 0, 0.1, 1, 10, 100, or 1000 μg/ml IL4(38-37)-PE38KDEL in 0.2% HSA-PBS was injected in right frontal cortical hemisphere on day 1. This concentration of drug corresponded to 0, 0.275, 2.75, 27.5, 275, and 2750 ng/kg body weight, respectively. The rats were anesthetized and placed in a stereotaxic unit, scalp-incised, and a small burr hole was placed over frontal cortex on the right side. A microsyringe with a needle was lowered into the right side of the frontal cortex. The IL4(38-37)-PE38KDEL was injected over a 10-min interval at a rate approximately 0.1 μl/min. The needle and syringe were left in place for 5 min after the injection of IL4(38-37)-PE38KDEL.

At 72 h after injection of IL4(38-37)-PE38KDEL, rats were weighed, anesthetized with sodium phenobarbital, and perfused with 10% neutral-buffered formalin and heparinized physiological saline. The brains were then removed and stored in 10% neutral-buffered formalin until histopathological studies were performed.

RESULTS

Inhibition of ^{125}I-labeled IL-4 Binding by IL4 Toxins on a Glioblastoma Cell Line. We have demonstrated previously that a chimeric protein composed of IL-4 and a mutated form of PE (IL4-PE^{4E}) was highly cytotoxic to many human brain tumor cell lines (22). However, concentration of IL4-PE^{4E} to displace ^{125}I-labeled IL-4 binding to 50% was up to 100-fold higher compared with IL-4 in human renal cell carcinoma cell lines (18). We have subsequently produced a circular permuted IL4-PE (IL4(38-37)-PE38KDEL; Refs. 23 and 24) that displaced ^{125}I-labeled IL-4 at much lower concentrations compared with IL4-PE^{4E} on A172 human glioblastoma cell line (Fig. 1). For example, EC_{50} for IL4-PE^{4E} binding was ~5.5 nM compared with ~0.35 nM using IL4(38-37)-PE38KDEL. These data suggest that IL4(38-37)-PE38KDEL binds to IL-4R with 16-fold higher affinity than the native IL4 toxin IL4-PE^{4E}.

Cytotoxicity of IL4(38-37)-PE38KDEL. Because IL4(38-37)-PE38KDEL bound ~10-16-fold better than the noncircularly permuted molecules IL4-PE38KDEL (24) or IL4-PE^{4E} (Fig. 1), we next

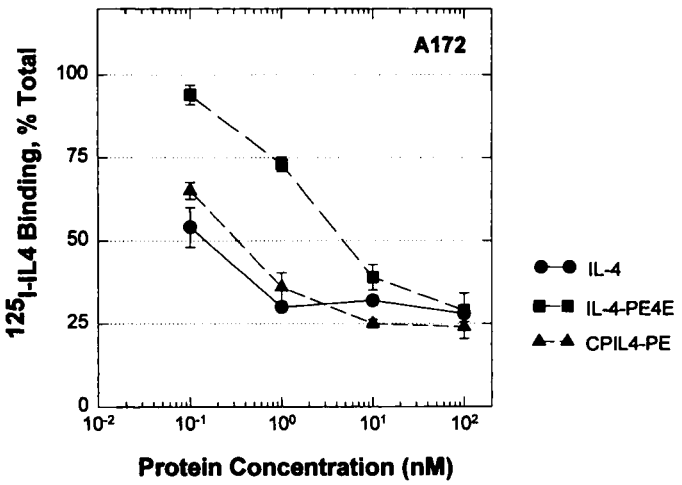

Fig. 1. Displacement of binding of ^{125}I-labeled IL-4 by IL4(38-37)-PE38KDEL to IL-4R. A172 glioblastoma cells were incubated at 4°C with 200 pM [^{125}I]IL-4 and various concentrations of IL-4, IL4(38-37)-PE38KDEL, IL4-PE38KDEL, or IL4-PE^{4E}. After 2 h, cells were centrifuged through a mixture of phthalate oils, and cell pellets were counted in a gamma counter. The data points shown are the mean of duplicate determinations. A total of 1566 ± 47 cpm (mean ± SD) bound to 1 × 10^6 A172 cells. *Bars*, SD. Deviations are larger than the size of point symbols.

evaluated whether IL4(38-37)-PE38KDEL is also more cytotoxic to glioblastoma cells. Cytotoxicity was determined by measuring inhibition of protein synthesis determined by the incorporation of [^{3}H]leucine. We have shown previously that the inhibition of protein synthesis directly correlates with the cell death (22). Cells were treated with various concentrations of either IL4(38-37)-PE38KDEL, IL4-PE38KDEL, or IL4-PE^{4E} for 20 h, and the level of protein synthesis was determined. As seen in Fig. 2 and reported previously (22), human glioblastoma cell lines were sensitive to the cytotoxic effect of IL4-PE^{4E}. The IC_{50} for IL4-PE^{4E} in glioblastoma cell lines ranged from approximately 5 to 40 ng/ml (approximately 60–480 pM; Table 1). However, IC_{50}s were 3–28-fold lower when cytotoxicity was determined in the presence of IL4(38-37)-PE38KDEL. Noncircular permuted IL4-PE (IL4-PE38KDEL) was the least effective in cytotoxic assays (Table 1). Excess IL-4 neutralized the cytotoxic activity of IL4(38-37)-PE38KDEL, indicating that these effects are mediated through the IL-4R (data not shown).

IL-4R Expression on Glioblastoma/Malignant Astrocytoma Specimens. We have reported previously that human brain tumor cell lines expressed high-affinity IL-4R (22). Using binding studies, we demonstrate that glioblastoma cell lines express high numbers of high-affinity (K_d ~ 100 pM) IL-4R (Fig. 3). By Northern analysis, all four glioma cell lines examined expressed mRNA for the IL-4R (data not shown).

To further determine whether specimens of glioblastoma/malignant astrocytoma also expressed IL-4R *in situ*, we analyzed tissue specimens from 21 patients undergoing surgical resection for either initial surgical debulking or resection of astrocytoma or glioblastoma. Normal brain tissues obtained from six individuals within 24 h postmortem were also analyzed. Tumor samples and normal brain tissues were analyzed for IL-4R mRNA expression by RT-PCR after total RNA was extracted from frozen tumor and brain tissues. The results on 21 tumor specimens and six normal brain tissues are shown Table 2. Sixteen of 21 specimens of malignant astrocytomas and glioblastomas expressed IL-4R (76% positive) as assessed under highly stringent RT-PCR plus Southern blot conditions. One sample was weakly positive. All six samples of normal brain tissues (samples 1–5 were obtained from frontal cortex, and sample 6 was from temporal lobe cortex) were negative for IL-4R expression except specimen 4, which expressed a very faint band for IL-4R.

Fig. 4 shows Southern blot analysis from representative samples. *Lane 2* corresponded to sample 16 in the table, *Lane 3* to sample 17, *Lane 4* to sample 2, *Lane 5* to sample 6, *Lane 6* to sample 4, *Lanes 7* and *14* to IL-4 cDNA standard, *Lane 8* to sample 5, *Lane 9* to sample 9, and *Lane 10* to sample 18. *Lanes 11–13* corresponded to glioblastoma cell lines U87, U118, and a MAT melanoma cell line, respectively.

RT-PCR analysis was also performed on six brain tumor (CCF-STTGI, RC1909, RC1913, SW1088, U87, and U118) cell lines. All six cell lines expressed a specific band for IL-4R mRNA by RT-PCR plus Southern blot analysis (data not shown).

Pharmacokinetics of IL4(38-37)-PE38KDEL in Monkeys after Intrathecal Administration. We used intrathecal administration of IL4(38-37)-PE38KDEL in monkeys as a toxicology model for intratumor injection of patients, because in both cases the drug would form a fluid space bound by normal brain. In addition, intrathecal administration will provide maximum exposure of the brain to high concentrations of IL4 toxin. Because human IL-4 is primate specific cytokine, produces many effects in cynomolgus monkeys (28, 29),

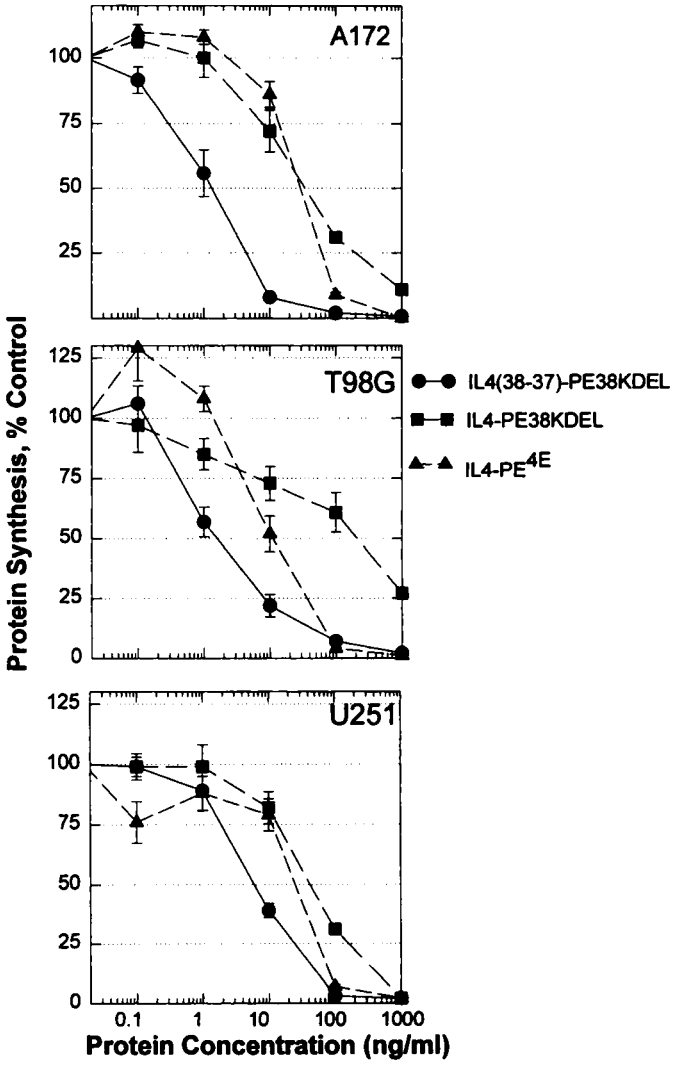

Fig. 2. Comparison of cytotoxicity mediated by IL4-PE^{4E} and IL4(38-37)-PE38KDEL. A172 (*A*), T98G (*B*), or U251 (*C*) glioma cells (1 × 10^4) were cultured with various concentrations of IL4(38-37)-PE38KDEL, IL4-PE38KDEL control, or IL4-PE^{4E}. Protein synthesis was measured after 20 h of culture by incorporation of [^{3}H]leucine (1 μCi for an additional 4 h) as described in "Materials and Methods." The results are presented as mean percentage of control of untreated cells from quadruplicate determinations. Mean total cpm ± SD incorporated in untreated A172 cells was 103,818 ± 5,872; in T98G cells it was 27,293 ± 4,070; and in U251 cells it was 34,027 ± 4,211. *Bars*, SD.

Table 1 *Comparison of cytoxicities of cpIL4-PE IL4(38-37)-PE38KDEL versus IL4-PE38KDEL and IL4-PE^{4E} on human glioblastoma cell lines*

Cells (1×10^4) were cultured with IL4 toxins for 20 h at 37°C, pulsed with 1 μCi of [^{3}H]leucine, and further incubated for 4 h. Cells were harvested and counted as described in "Materials and Methods."

Cells	IC_{50}[a] (ng/ml) IL4(38-37)-PE38KDEL	IL4-PE38KDEL	IL4-PE^{4E}
U251	6.5 ± 1.3[b]	100 ± 35	33.8 ± 3.1
A172	1, 0.5	5, 40	15, 30
U373	2, 5	125, 260	18, 50
T98G	1, 2	18, 60	5, 5
SN19	9, 10	175, 290	40, 40

[a] IC_{50}, the concentration of IL4 toxin at which 50% inhibition of protein synthesis is observed compared to untreated cells.

[b] The values are presented as mean ± SEM of four experiments performed in quadruplicate. However, individual IC_{50}s are shown for other tumor cell lines from two independent experiments performed in quadruplicate. In some cases, statistical comparisons were performed by Student's *t* test. For U251 cells, the difference of IC_{50}s between IL4(38-37)-PE38KDEL and IL4-PE38KDEL was statistically significant ($P < 0.04$), and between IL4(38-37)-PE38KDEL and IL4-PE^{4E} it was highly significant ($P < 0.0002$). Similarly, the difference between IL4(38-37)-PE38KDEL and IL4-PE^{4E} was significant in T98G and SN19 cells at $P < 0.02$ and $P < 0.0003$, respectively. The other differences were not statistically different because of a low number of experiments, but within the experiments, differences were statistically significant.

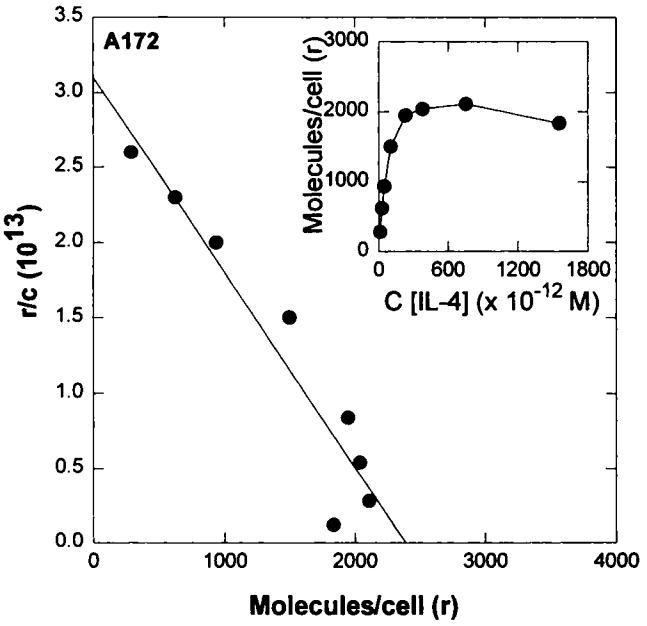

Fig. 3. IL-4R expression on glioblastoma cell lines as determined by IL-4 binding assay. Scatchard analysis of ^{125}I-labeled IL-4 binding to A172 glioblastoma cell line was determined from the binding data (shown in *inset*). For this experiment, single-cell suspensions of tumor cells were incubated for 2 h with increasing concentrations of ^{125}I-labeled IL-4 at 4°C. Nonspecific binding was determined by parallel incubations with 200-fold excess of unlabeled IL-4. Bound radioactivity was determined as described in "Materials and Methods."

and can bind to monkey fibroblast (13) and gibbon ape leukemia cell line IL-4R (2, 13), this model allowed us to evaluate the pharmacological and toxicological consequences of IL4(38-37)-PE38KDEL binding to normal brain, and whether this binding is specific or nonspecific for the IL-4R.

To determine the CSF levels of IL4(38-37)-PE38KDEL after intrathecal administration, three cynomolgus monkeys were dosed on days 1, 3, and 5 with 0.2% HSA-PBS containing 0, 2, or 6 μg/kg of IL4(38-37)-PE38KDEL. The CSF samples were drawn at seven different time points. Serum samples were also collected 2 h after dosing on days 3 and 5 at each dose level. These samples were used to determine IL4(38-37)-PE38KDEL levels. Dilutions of these samples were tested in cytotoxicity assays, and levels were determined using a standard curve generated from purified IL4(38-37)-PE38KDEL. As shown in Table 3, a significant CSF level of IL4(38-37)-PE38KDEL was achieved after administration of the drug. Because the CSF volume is approximately 1 ml/kg, the levels at 2 h were >15% of peak values expected if the drug were to immediately distribute throughout the CSF. Doses were cleared rapidly in the CSF, because at 24 h after injection <1% of the drug remained. There was a linear relationship between 2-h CSF level and dose of IL4(38-37)-PE38KDEL ($r^2 = 0.96$, data not shown). Serum samples from both monkeys at

Table 2 *IL-4 receptor expression by human glioblastoma and astrocytoma specimens*

IL-4R expression was determined by RT-PCR and Southern blot analysis as described in "Materials and Methods." +, specific IL-4R band. All samples were β-actin positive by RT-PCR analysis as detected by ethidium bromide-stained gel electrophoresis using β-actin specific primers. IL-4R-positive cDNA used was from the human gastric cancer cell line HTB-135 (ATCC) and melanoma cell line MAT (John Wayne Cancer Institute).

Specimen no.	IL-4R expression
Glioblastomas	
1	+
2	+/−[a]
3	+
4	+
5	+
6	+
7	+
8	+
9	+
10	−
11	−
12	−
13	+
14	+
15	+
16	+
17	+
18	+
19	+
Malignant astrocytomas	
1	−
2	+
Control	
1 (HTB-135)	+
2 (MAT)	+
Normal brain tissues[b]	
1	−
2	−
3	−
4	+/−[a]
5	−
6	−

[a] Faint band not detected by RT-PCR but detected by Southern blot analysis (+/−); two separate specimens examined.

[b] Normal brain tissue samples 1–5 were obtained from frontal cortex of the brain, and the sixth sample was from temporal lobe of the cortex.

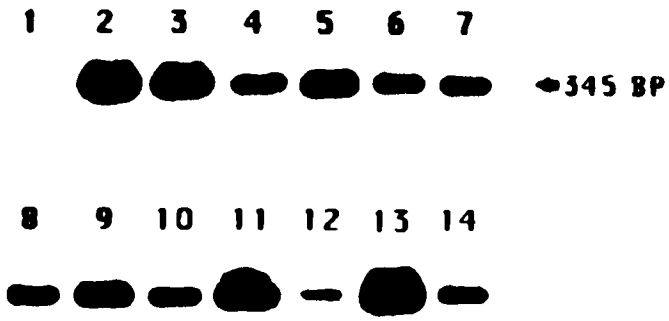

Fig. 4. Southern blot analysis of IL-4R expression in glioblastoma specimens and cell lines. Representative examples of glioblastoma specimens, cell lines, and a positive control (MAT melanoma cell line) were analyzed by RT-PCR plus Southern blot analysis. *Lane 1* represents a negative control (RT-PCR reagents plus primers without cell RNA), *Lane 2* corresponds to specimen 16 in the table, *Lane 3* to specimen 17, *Lane 4* to specimen 2, *Lane 5* to specimen 6, *Lane 6* to specimen 4, *Lanes 7* and *14* to IL-4 cDNA standard, *Lane 8* to specimen 5, *Lane 9* to specimen 9, and *Lane 10* to specimen 18. *Lanes 11–13* corresponded to glioblastoma cell lines U87, U118, and a MAT melanoma cell line, respectively. Southern blot hybridized cDNA product shown is 345 bp.

Table 3 *IL4(38-37)-PE38KDEL levels in CSF and serum of cynomolgus monkeys*

	IL4(38-37)-PE38KDEL levels (ng/ml) at doses[a]		
	Control	2 μg/kg	6 μg/kg
CSF			
Day 1, preinjection	<2	<2	<2
Day 2, 24 h post-1st injection	<2	<2	<2
Day 3, preinjection	<2	<2	<2
Day 3, 2 h post-2nd injection	<2	428 ± 44	680 ± 192
Day 4, 24 h post-2nd injection	<2	<2	<2
Day 5, preinjection	<2	<2	<2
Day 5, 2 h post-3rd injection	<2	323 ± 13	1417 ± 540
Serum			
Day 3, 2 h post-2nd injection	<2	<2	<2
Day 5, 2 h post-3rd injection	<2	<2	<2

[a] IL4(38-37)-PE38KDEL levels (ng/ml) were determined by cytotoxicity assay using Daudi Burkitt's lymphoma cell line. The concentrations in samples were determined using a standard curve generated from purified IL4(38-37)-PE38KDEL. Results are expressed in terms of mean ± SD of three simultaneous experiments.

both dose levels investigated did not show any detectable levels of IL4(38-37)-PE38KDEL.

Toxicity of IL4(38-37)-PE38KDEL in Monkeys. The same monkeys used for pharmacokinetics studies were also followed for any signs, symptoms, and clinical value changes as an indicator of toxicity. No distinct changes in hematology and serum chemistry were observed in any of the monkeys studied (data not shown), which was consistent with the absence of IL4(38-37)-PE38KDEL in the serum. Creatinine phosphokinase BB band, which can be a sensitive enzyme indicator for brain injury, was not elevated in any of the monkeys examined (data not shown).

Toxicity of IL4(38-37)-PE38KDEL in Rats. In developing IL4(38-37)-PE38KDEL for intratumoral injection, our next goal was to determine what concentration of toxin would cause necrosis of normal brain by nonspecific internalization. Therefore, we used rats whose IL-4 receptors do not bind human IL-4. Six groups of rats were injected into the frontal cortex with various doses of IL4(38-37)-PE38KDEL. The animals were examined once daily for obvious indications of toxic effects for 3 days. Then the animals were sacrificed, and their brain tissues were examined microscopically (Fig. 5). All animals survived until the terminal necropsy. No abnormalities in behavior were noted, and the mean body weights did not change for all groups. There were no remarkable gross pathology findings noted at necropsy. No IL4(38-37)-PE38KDEL-induced histopathological changes were observed at concentrations ≤100 μg/ml (Fig. 5*A* and photomicrograph not shown). Microscopic evaluation revealed necrosis of the right cortical hemisphere (*arrow*) at the injection site in group 6 [2750 ng/kg; 1000 μg/ml IL4(38-37)-PE38KDEL] rats (Fig. 5*B*).

DISCUSSION

In the present study, we demonstrate that 76% of malignant astrocytoma biopsies obtained from patients undergoing surgical resection are positive for the IL-4R expression. All glioblastoma cell lines examined were positive for the expression of high-affinity IL-4R by binding studies and RT-PCR plus Southern blot analyses. The PCR data corroborated with the binding results demonstrating surface expression of IL-4R protein on malignant astrocytoma cells. In contrast to tumors, five normal brain tissues obtained from six individuals were found negative, and one sample was found weakly positive, for the IL-4R mRNA. These results were verified by cytotoxicity assays, in which IL4(38-37)-PE38KDEL inhibited protein synthesis of IL-4R-positive glioma cells in a dose-dependent manner, which was blocked by excess of IL-4. Altogether, these studies indicate that IL-4R-ligand complex on glioma cells is internalized.

We have reported previously that a chimeric protein composed of IL-4 and a mutated PE molecule (IL4-PE^{4E}) is cytotoxic to brain tumor cells; however, as shown in this study, IL4-PE^{4E} bound to IL-4R in glioma cells with 37-fold less affinity than native IL-4 (Fig. 1). Recently, we have produced a circular permuted IL4 toxin, IL4(38-37)-PE38KDEL, in which the native the COOH terminus is available to bind to IL-4R, and a truncated form of PE is attached at position 38 of the IL-4 molecule. As shown in Fig. 1, IL4(38-37)-PE38KDEL displaced ^{125}I-labeled IL-4 binding at a 16-fold lower concentration compared with IL4-PE^{4E}. Consequently, glioblastoma cells were more sensitive (3–30-fold) to IL4(38-37)-PE38KDEL compared with IL4-PE^{4E} (Fig. 2). These results show that, as binding affinity of IL4 toxin to IL-4R is increased, the cytotoxicity of IL4 toxin is also increased. This is a desirable characteristic of a chimeric molecule that is expected to result in a more effective targeting agent with no increase in nonspecific toxicity. Our results further confirm previous conclusions that the COOH terminus of the IL-4 molecule is required for receptor binding (20, 21, 30–32) and binds best when not blocked with a toxin protein.

To further develop IL4(38-37)-PE38KDEL for clinical studies, we performed preclinical experiments in monkeys and rats to determine toxicity and pharmacokinetics. The route of administration of IL4(38-37)-PE38KDEL was chosen to ensure maximum exposure of brain tissue to assess possible toxicity. Monkey serves a good model be-

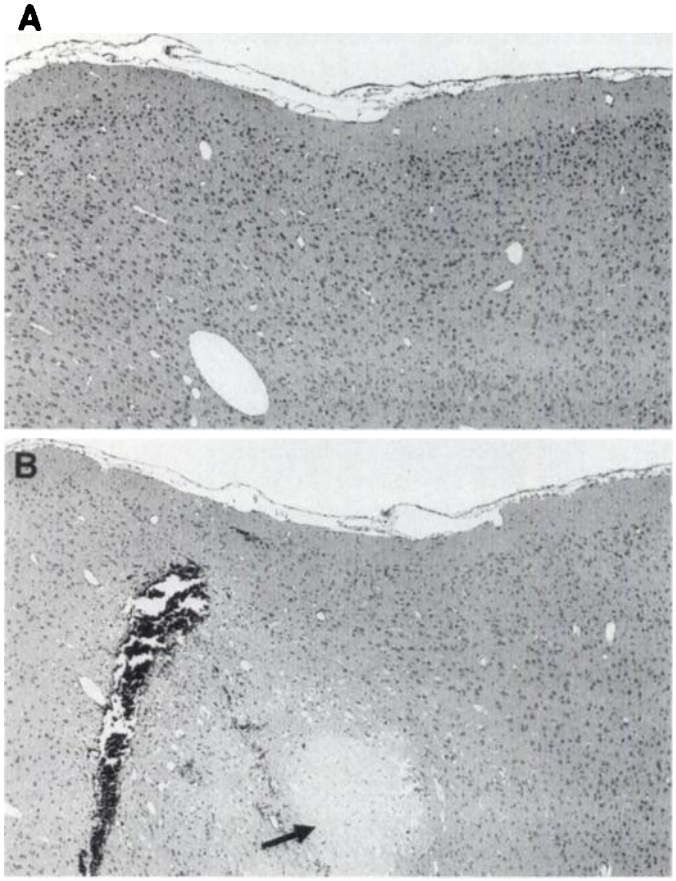

Fig. 5. H&E staining of rat brain injected with IL4(38-37)-PE38KDEL. One μl of various doses of IL4(38-37)-PE38KDEL in 0.2% HSA-PBS was injected in right frontal cortical hemisphere on day 1. At 72 h postinjection of IL4(38-37)-PE38KDEL, rats were anesthetized with sodium phenobarbital and perfused with 10% neutral-buffered formalin and heparinized physiological saline. The brains were then removed and stored in 10% neutral-buffered formalin until histopathological studies were performed. *A*, photomicrograph represents right cortical hemisphere of rat brain injected with IL4(38-37)-PE38KDEL at a dosage of 100 μg/ml (*A*) or 1000 μg/ml (*B*). *B* shows necrosis and hemorrhage (*arrow*). Both micrographs shown are ×13.2.

cause it has been shown that monkey cells can bind human IL-4 (13), and human IL4 toxin is cytotoxic to monkey COS-7 cells.[4] At doses up to 6 μg/kg, no toxicity was observed, although very high CSF levels of IL4(38-37)-PE38KDEL were achieved. The CSF protein levels were within normal limits, and no inflammatory cells were observed (results not shown).

No systemic toxicity was observed when toxin was administered by the intrathecal route in monkeys, as evidenced by normal hematological or serum chemistry tests, and as expected by the absence of drug in the serum. In addition, we have recently observed that IL4(38-37)-PE38KDEL is not toxic to resting human T cells, B cells, promonocytic cell lines, and resting and activated human bone marrow-derived cells, including highly purified activated CD34+ cells (25).

The direct administration of IL4(38-37)-PE38KDEL in cerebral hemispheres of rats did not produce any gross abnormality. The rationale to do this experiment was to determine nonspecific toxicity via its nonspecific internalization into normal brain. This is valuable information to obtain, because if human brain has no IL-4R, the only toxicity of IL4(38-37)-PE38KDEL would be mediated through this pathway. Microscopically, the brain tissue of animals receiving only the highest dose of toxin (1000 μg/ml) showed drug-induced tissue necrosis, whereas no necrosis was observed at any of the lower doses. These data suggest that IL4(38-37)-PE38KDEL internalizes nonspecifically into normal brain cells at 1000 μg/ml but not at 100 μg/ml.

It is of interest that only ~10-100 ng/ml IL4(38-37)-PE38KDEL were sufficient to kill >95% of glioblastoma cells in tissue culture. By intrathecal administration, one can achieve approximately 17-fold higher concentration using 6 μg/kg doses and approximately 3–4-fold higher concentration using 2 μg/kg doses of IL4(38-37)-PE38KDEL compared with *in vitro* dose to kill >95% of cells without causing any pathology. However, it is not known whether this level of drug can be achieved within the layers of glioblastoma to cause tumor regression. Our previous experiments using the A431 xenograft model suggested that complete regressions of s.c. growing tumors can be achieved after i.v. administration of IL4(38-37)-PE38KDEL (24). Thus, IL4 toxin is able to penetrate within the tumor layers. On the basis of *in vitro* and animal studies (24), it is predicted that sufficient levels may be achieved to penetrate tumor layers for therapeutic efficacy by administration of only 1–2 μg/kg doses of IL4(38-37)-PE38KDEL. However, further studies are necessary to determine antitumor activity of IL4(38-37)-PE38KDEL against glioblastoma *in vivo*.

In summary, circular permuted IL4 toxin, IL4(38-37)-PE38KDEL, may be a useful drug for the treatment of human glioblastoma and malignant astrocytoma because a majority of human gliomas express IL-4R. Because normal human brain tissues appear not to express significant amounts of IL-4R, it is predicted that IL4(38-37)-PE38KDEL will not be harmful to normal brain tissues when administered by intrathecal route at doses up to 6 μg/kg. Our preclinical data would support a Phase I trial of direct administration of IL4(38-37)-PE38KDEL into the tumor bed by the stereotactic route or into the tumor bed postsurgical resection followed by additional infusions through an Ommaya reservoir placed at the time of surgical resection of recurrent glioblastoma.

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