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Advances in Brief

Vaccination of Malignant Glioma Patients with Peptide-pulsed Dendritic Cells Elicits Systemic Cytotoxicity and Intracranial T-cell Infiltration[1]

John S. Yu, Christopher J. Wheeler, Paul M. Zeltzer, Han Ying, Divina N. Finger, Paul K. Lee, William H. Yong, Francesca Incardona, Reid C. Thompson, Mary S. Riedinger, Wenxuan Zhang, Robert M. Prins,[2] and Keith L. Black[3]

Maxine Dunitz Neurosurgical Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048 [J. S. Y., C. J. W., P. M. Z., H. Y., D. N. F., P. K. L., W. H. Y., F. I., R. C. T., M. S. R., W. Z., R. M. P., K. L. B.]; and Department of Neurological Surgery, University of California, Irvine, California 92868-3298 [J. S. Y., P. M. Z., R. C. T., K. L. B.]

Abstract

In this Phase I trial, patients' peripheral blood dendritic cells were pulsed with peptides eluted from the surface of autologous glioma cells. Three biweekly intradermal vaccinations of peptide-pulsed dendritic cells were administered to seven patients with glioblastoma multiforme and two patients with anaplastic astrocytoma. Dendritic cell vaccination elicited systemic cytotoxicity in four of seven tested patients. Robust intratumoral cytotoxic and memory T-cell infiltration was detected in two of four patients who underwent reoperation after vaccination. This Phase I study demonstrated the feasibility, safety, and bioactivity of an autologous peptide-pulsed dendritic cell vaccine for patients with malignant glioma.

Introduction

Despite aggressive multimodality therapy, the prognosis for patients with malignant glioma is poor. The current treatment of glioblastoma, GBM,[4] which consists of surgical resection followed by radiation therapy and/or chemotherapy, results in a median survival of less than 1 year (1). Glioblastoma cells are known to be poor antigen presenters to the immune system, in part because of down-regulation of B7 costimulatory molecules required for direct tumor cell activation of T cells (2, 3). To induce an antitumor immune response against glioblastomas, professional APCs may be needed to efficiently internalize, process, and present glioma antigens to T cells (4–6). In the present study, DCs were used for antigen presentation of glioma antigens to circumvent the inability of gliomas to directly induce a cytotoxic T-cell response. DCs are potent "professional" APCs. Recent studies have demonstrated the use of tumor peptide-pulsed DCs to successfully treat murine models of metastatic intracranial tumor (7) and glioma (8, 9). This report describes the response of patients with malignant gliomas to peptide-pulsed DC vaccination. In this Phase I trial, patients' peripheral blood stem cells were expanded *ex vivo* into DCs and pulsed with peptides eluted from the surface of cultured autologous brain tumor cells. Three biweekly intradermal vaccinations of peptide-pulsed DCs were administered to seven patients with GBM and two patients with anaplastic astrocytoma. Immunological monitoring end points were systemic cytotoxicity and, when clinically indicated, intracranial T-cell infiltration. Clinical end points were toxicity and survival. We speculated that DC vaccination would generate antigen-specific T cells. Indeed, DC vaccination elicited significant systemic T-cell cytotoxicity against autologous glioma tumor cells in four of seven patients. We sought to determine whether these cytotoxic T cells were trafficking to intracranial tumor. Two of four patients who underwent reoperation for new areas of gadolinium enhancement on magnetic resonance imaging (consistent with tumor, radiation necrosis, or inflammation) demonstrated a robust cytotoxic (CD8+) and memory (CD45RO+) T-cell infiltration in areas of intracranial tumor. This Phase I study demonstrates the feasibility, safety, and antitumor immune activity of an autologous peptide-pulsed DC vaccine for malignant glioma. Systemic cytotoxicity and intracranial cytotoxic T-cell infiltration were elicited in a subset of patients, and this biological activity may be associated with prolonged survival in patients with glioblastoma.

Materials and Methods

Patient Population. Inclusion criteria were a Karnofsky score of 60 or greater, lowest possible maintenance dose of glucocorticoid therapy, no allergy to components of the DCs, and normal baseline hematological parameters (within 1 week before first vaccination): hemoglobin >9.9 g/dl; total granulocyte count >1000/μl; platelet count >60,000/μl; BUN <30 mg/dl; creatinine <2 mg/dl; alkaline phosphatase; aspartate aminotransferase less than twice the upper limit of normal; and a prothrombin time and activated partial thromboplastin time no greater than 1.4 times control, unless therapeutically warranted.

Exclusion criteria included pregnancy, severe pulmonary, cardiac, or other systemic disease associated with an unacceptable anesthetic or operative risk, presence of an acute infection requiring active treatment, and history of an autoimmune disorder or prior history of other malignancies, excluding basal cell carcinoma and benign tumors. Patients were required to use a medically accepted form of birth control during the study.

There were nine patients enrolled in this Phase I study: five women and four men with an age range from 28 to 77 years of age (mean, 49 years; see Table 1). Patients with verified histological diagnoses of newly diagnosed anaplastic astrocytoma (two patients) or GBM (seven patients) were eligible for this study. After surgical resection of their tumor, patients were required to complete a course of external beam radiation therapy (standard dose, 4500 cGy to tumor with 3-cm margins, 1500 cGy boost to tumor bed). All of the patients were off steroids at the time of vaccination. The 7 study patients with GBM were included in a survival analysis and compared with 42 patients in the control group. Their mean age was 55.9 $\pm$ 14.5 years, and 50% were male. All of the patients had tumor pathology consistent with GBM, had undergone surgical resection at our institution within the past 2 years, and had completed a course of radiation therapy (60 Gy as above).

Autologous Tumor Culture. Tumor samples from surgical resection were processed for tissue culture by mincing with scissors and passing through metal meshes of decreasing pore size. The cell suspension was then plated onto tissue culture flasks and grown in DMEM/F10 (Irvine Scientific, Santa Ana, CA) plus 10% FCS (Irvine Scientific) and 1% penicillin/streptomycin (Life Technologies, Inc.). The adherent cell population was retained and passaged until 10^8 cells were generated.

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[2] Present address: Department of Anatomy, Medical College of Virginia, Richmond, Virginia 23298.

[3] To whom requests for reprints should be addressed, at 8631 West Third Street #800E, Los Angeles, CA 90048. Phone: (310) 423-0815; Fax: (310) 423-0810; E-mail: blackk@cshs.org.

[4] The abbreviations used are: GBM, glioblastoma multiforme; APC, antigen-presenting cells; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; DTH, delayed type hypersensitivity.

Table 1 *Patient characteristics*

Patient no.	Tumor pathology	Age	Prevaccine Rx	After vaccination Rx	Adverse events	Survival (days)	Time to progression (days)	Time of additional surgery	Intratumoral T cells	Peripheral CTL
1	AA[a]	28	Hydroxyurea	PolyIC		632				Negative
2	GBM	51		Reoperation		D381	259	264	Negative	Positive
3	GBM	48	Gliadel		Fever, nausea	618				
4	GBM	49		Reoperation, SRT		D463	216	224	Negative	Positive
5	AA	30		PolyIC	Lymph nodes	550				Positive
6	GBM	59		Reoperation, SRT		455	105	112	Positive	Positive
7	GBM	42				574				Pre-existing
8	GBM	55		Reoperation, SRT		315	74/222	81/232	Positive	Pre-existing
9	GBM	77				300				

[a] AA, anaplastic astrocytoma; Gliadel, intracranial 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; Poly IC, nonspecific immunotherapy; SRT, stereotactic radiation therapy; D, time of death.

Methods for Tumor Antigen PCR. Total tumor cell RNA was isolated using TriZol RNA isolation kit (Life Technologies, Inc.) from cultured autologous glioma cells from nine study patients. For cDNA synthesis, 10 μg of total RNA were digested with 2 μl of DNase I (Ambion) to remove genomic DNA. The RNA was transcribed with cDNA synthesis reagents (Life Technologies, Inc.) in total volume of 50 μl with the use of oligo(dT) (Life Technologies, Inc.). For PCR, 1 μl/50 μl of the synthesized cDNA was used with 10 pmol of primers, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, and 0.1 μl of Platinum Taq polymerase (Life Technologies, Inc.). Thermal cycler parameters included 5 min at 95°C initiation and 35 cycles involving denaturation at 95°C for 30 s, annealing at 58°C (for MAGE-1 and β-actin) or 52°C (for gp100 and TRP-2) for 30 s, and extension at 72°C for 1 min. Primers used for PCR are as follows: primers for MAGE-1, forward, 5′-GCCTGCTGCCCCTGACGAGAG-3′, reverse, 5′-AGGAGAGACCTAGGCAGGTG-3′; primers for gp100, forward, 5′-TGGCTCTTGGTCTCAGAAGA-3′, reverse, 5′-AGGTGCAGTGCTTATGACTT-3′; primers for TRP-2, forward, 5′-GAGGTGCGAGCCGACACAAG-3′, reverse, 5′-TCTGTACACACATCACACTC-3′; primers for β-actin, forward, 5′-AATCTGGCACCACACCTTCTAC-3′, reverse, 5′-CTTCTCCTTAATGTCACGCACG-3′.

Isolation of Tumor-specific MHC-I-associated Peptides. MHC-I-associated peptides were enriched through acid elution as described previously (10). Briefly, 10^8 glioma cells were removed from tissue culture plates by incubation with 2 mM EDTA at room temperature, followed by three washes with HBSS. The cells were resuspended in 10 ml of citrate-phosphate buffer (pH 3.2) to dissociate peptides from surface MHC-I, triturated gently, and centrifuged for 5 min at 1000 rpm. The supernatant was then collected and concentrated by passage through Sep Pak C18 columns, aliquoted, and frozen at −80°C. Before patient vaccination, tumor peptide was tested in the Limulus Amoebocyte Lysate assay (BioWhittaker Inc., Walkersville, MD) for endotoxin contamination, for aerobic, anaerobic, and fungal culture, and by Gram's stain before administration to the patient.

Preparation of Autologous DCs. Venous blood (20 ml) was drawn from patients on days −7, 7, and 21 (day 0 = first vaccination). The anticipated yield was 10^6 APCs/20 ml whole blood. In addition, 50 ml of blood was drawn on day −14 to obtain autologous serum (15 ml) for each of three APC cultures.

APCs were isolated from whole blood by Ficoll-Hypaque centrifugation, washed three times in PBS, and plated at a concentration of 5×10^6 cells/ml in complete medium consisting of RPMI 1640 (Life Technologies, Inc.) with 10% autologous heat-inactivated serum, 1% gentamicin, and 0.01 M HEPES buffer. After 2 h at 37°C, nonadherent cells were removed by washing with warm complete medium. To generate autologous DCs, adherent APCs were cultured in complete medium for 7 days in the presence of recombinant human GM-CSF (800 units/ml; clinical grade; Immunex Corp., Seattle, WA) and recombinant human IL-4 (500 units/ml; R&D Systems, Inc., Minneapolis, MN).

Preparation and Administration of Autologous DCs Pulsed with Tumor-specific MHC-I Peptides. On the days before each of the three DC vaccinations (days −1, 13, and 27), DC cultures were incubated with citrate-phosphate buffer [0.131 M Na_2HP0_4 (pH 3.0)] for 1 min to strip endogenous peptides from MHC-I molecules. Cells were then washed in RPMI 1640 with 10% autologous patient serum supplemented with 50 μg/ml autologous tumor-specific MHC-I peptides. The DCs were cultured overnight with these peptides on a tissue rotator to facilitate their interaction. Patients received 10^6 tumor-specific MHC-I peptide-pulsed DCs s.c. in 0.1–0.2 ml saline in the deltoid region. Three vaccinations at 2-week intervals were administered to each patient.

DC Phenotypic Evaluation. After 7-day maturation in GM-CSF and IL-4, DCs were harvested from flasks. Cells were resuspended in RPMI-10% human AB medium and irradiated with 2500 rad in a Cesium-source irradiator. The remaining cells were resuspended in PBS containing fetal bovine serum (2% v/v) and stained with anti-CD14 FITC, anti-HLA-DR phycoerythin for MHC Class II, and biotinylated anti-CD86 antibodies for B7–1(PharMingen, San Diego, CA). Species and isotype-matched monoclonal antibodies were used as controls.

DC Functional Assay. For the functional assay, irradiated DCs were resuspended in RPMI-10% human AB serum at 2×10^5 cells/ml. Allogeneic PBMC (10,000) were mixed with limiting dilutions of DCs. PHA (5 μg/ml) was added to separate PBMC cultures as a positive control. RPMI-10% AB medium alone was added to separate PBMC cultures as a negative control. All of the cultures were established in triplicate. PBMC under the above conditions were incubated for 6 days in a 37°C/5% CO_2 incubator. [^{3}H]thymidine (1 μCi/well) was added for the final 18 h. Cells were harvested with a 96-well harvester (Tomtec, Hamden, CT), and cpm were determined on a Microbeta 1450 Trilux liquid scintillation counter (Wallac, Gaithersburg, MD).

CTL Precursor Assay Method. For stimulation, 50 μl of irradiated tumor cells at 1×10^5/ml were plated in RPMI 1640 with 10% heat-inactivated autologous serum with autologous 100 ml of PBMC at 1×10^6/ml. IL-2 was added to give a final concentration of 1000 units/ml, and the plate was incubated at 37°C in 5% CO_2 for 14 days. Cultures were fed on day 3 by removing 50 μl of supernatant and replacing it with medium with IL-2 (4000 units/ml), and on day 7, 100 ml of supernatant was removed and replaced with l00 μl (10,000) autologous irradiated tumor cells at 1×10^5/ml. Autologous tumor cell targets were labeled for 48 h with [^{3}H]thymidine (2.5 μCi/ml). On day 14, targets were trypsinized, washed twice in HBSS, and resuspended at 10,000 cells/ml. Cells (100 μl) were transferred from the coculture plates and added to an empty 96-well plate. Labeled target cells (1×10^4/ml) were added on top of the stimulated PBMCs and resuspended in RPMI-10% human AB serum. The plate was centrifuged at $1000 \times g$ for 3 min and incubated at 37°C for 5 h. Maximum release was assessed in triplicate wells containing 10,000 target cells and 5% SDS in H_2O and DNase 1 (Roche, Indianapolis, IN) at 20 units/ml final concentration for 10 min. The cells were harvested with a cell harvester (Wallac), and cpm were determined on a Microbeta 1450 Trilux liquid scintillation counter (Wallac). The first two patients were analyzed using a CTL precursor assay to quantitate cytotoxic precursor frequency independent of *in vitro* stimulation effects. The inability to grow sufficient numbers of tumor cells to calculate precursor frequencies necessitated adoption of non-quantitative bulk CTL assays for the remainder of the patients.

Bulk Cytotoxic T-cell Assay. CTL activity was tested by JAM assay (11). Patients four through eight grew sufficient numbers of tumor cells (2×10^7 cells) to complete JAM assays and were evaluated. PBMC for pretreatment and after treatment time points were thawed, washed twice by centrifugation, and counted. The concentration was adjusted to 8×10^6/ml in RPMI 1640 medium containing heat-inactivated human AB serum (10%), nonessential amino acids (1%), penicillin/streptomycin (1%), and 1 M HEPES buffer (1%).

PBMC were stimulated with allogeneic PBMC (irradiated with 2,000 rad) or with autologous cultured tumor cells (irradiated with 11,000 rad; 1×10^6/ml) for 6 days in RPMI-5 and recombinant human IL-2 (20 units/ml). Tumor or allogeneic PHA blasts (1×10^5/ml) were labeled as targets in 5 μCi/ml

[^{3}H]thymidine for 48 h at 37°C in 5% CO_2. After incubation, target cells in maximum release wells were lysed with 5% SDS in H_2O and incubated in DNase 1 (Roche) at 20 units/ml final concentration for 10 min. PBMC (100 μl; maximum 1×10^7/ml for 100:1 E:T ratio) were added to targets (100 μl; 1×10^5/ml) at various E:T ratios for 6 h. Cells were harvested from plates with a 96-well harvester (Tomtec). CPM were determined using a Microbeta 1450 Trilux liquid scintillation counter (Wallac).

Immunohistochemistry. Serial 10-μm paraffin sections of surgical intracranial tumor specimen were stained with mouse antihuman monoclonal antibodies against CD8 (C8/144B clone M7103 at 1/25 dilution), CD45RO (OPD4 clone M0834 at 1/50 dilution), CD20 (L26 clone M0755 at 1/200 dilution), and CD56 (T199 clone M0852 at 1/10 dilution; DAKO Corp., Carpinteria, CA). Primary antibodies were detected using the biotin-peroxidase system (DAKO Corp.).

Statistical Analysis. Continuous variables were compared using Student's *t* test, and categorical variables were compared using χ^2 or Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Characterization of Autologous Tumor Cells. Patients' tumors were resected, and the surgical tissue was used to establish autologous glioma cell lines. Cells were passaged 7–12 times to obtain approximately 10^8 cells. MHC-I-associated peptides derived from these lines were enriched through acid elution. Karyotype analysis was performed on seven autologous tumor cultures from seven study patients. Six patients had glioblastoma, and one patient had anaplastic astrocytoma. At least 20 cells were analyzed from the cultures of each of these patients. Of the six glioblastoma patients, five patients had abnormal karyotype in all of the cells studied. Four of these karyotypes displayed gain of chromosome 7. One patient with glioblastoma and one patient with anaplastic astrocytoma demonstrated normal karyotypes. Cell lines from nine study patients were characterized using an established reverse transcriptase reaction to determine mRNA expression of tumor-associated antigens originally described on melanoma.

The β-actin primers were designed to detect genomic DNA contamination. The PCR product from cDNA is 394 bp. The PCR product from genomic DNA should be 835 bp. Fig. 1*A* suggests that the RNA preparation was free of genomic DNA contamination. Four out of nine patients expressed MAGE-1 in cultured glioma cells (Fig. 1*B*). Six out of nine patients demonstrated gp100 expression (Fig. 1*C*), and three patients demonstrated TRP-2 expression (Fig. 1*D*). In total, the cultured glioma cells of eight out of nine patients expressed at least one tumor-associated antigen. Immunohistochemical staining of autologous tumor culture cells revealed that in the four out of nine patients that were PCR positive for the MAGE-1 antigen, nearly every cell was immunopositive for the MAGE-1 protein (Data not shown).

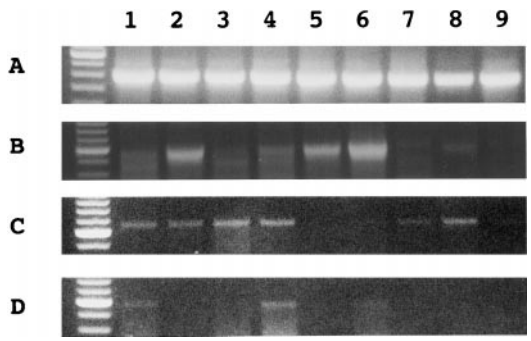

Fig. 1. Analysis of tumor-associated antigen mRNA in cultured tumor cells isolated from glioma patients. Total tumor cell RNA was isolated and reverse transcribed into cDNA. PCR was performed using gene-specific primers for *A*, β-actin; *B*, MAGE-1; *C*, GP-100; and *D*, TRP-2.

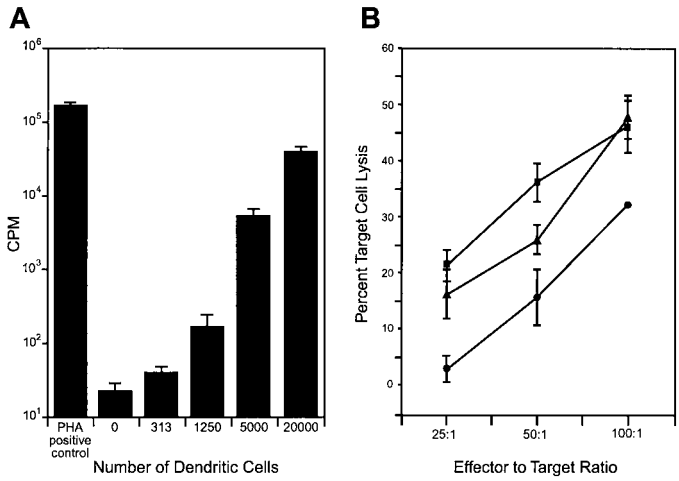

Fig. 2. *A*, DC allostimulation. Irradiated DCs at the indicated concentrations or PHA to 5 mg/ml were added to 5×10^5 allogeneic PBMC/ml in medium and incubated for 5 days. Proliferation was assessed by incorporation of [^{3}H]thymidine in PBMC after 18 h of additional incubation. The graph depicts DC activity from a patient before preparation of vaccine. *B*, peripheral cytotoxicity. PBMC were collected from patients before and after DC/tumor peptide administration and stimulated *in vitro* for 6 days with irradiated autologous tumor cells in the presence of 10 units/ml IL-2. CTL activity was determined by specific fragmentation of [^{3}H]-labeled DNA in autologous tumor targets at various E:T ratios using the JAM test. The graph depicts CTL activity from patient 5 before vaccination (●), as well as 6 weeks (▲) and 12 weeks (■) after the last of three DC/tumor peptide administrations.

Isolation and Characterization of DCs. Mononuclear cells were isolated by Ficoll gradient centrifugation and differentiated into DCs in the presence of IL-4 and GM-CSF. These professional APCs expressed high levels of MHC class II and of costimulatory molecule B7-1 and the absence of markers for mature monocytes (CD14; data not shown). In all of the patients, greater than 70% of the cells exhibited a DC phenotype: HLA-DR+, B7–1+, and CD14−. Allogeneic proliferation assays confirmed that the DCs isolated were functional (Fig. 2*A*) and sufficient numbers of functional DCs were isolated in all of the patients in this clinical trial (10^6 DCs/vaccination). These DCs were cocultured with autologous tumor peptide, washed, and administered to patients three times over the course of 6 weeks.

Safety of Autologous DC Administration. There were no serious adverse events or clinical or radiological evidence of autoimmune reaction in any patient (Table 1). Patient 3 developed a mild fever (100°F) lasting several hours after vaccination. The same patient also developed nausea and vomiting several weeks later. Patient 5 developed palpable supraclavicular, axillary, and inguinal lymph nodes 1 week after the first vaccination, which persisted for 2 months.

Development of Systemic Cytotoxicity. One goal of this clinical trial was to determine whether *ex vivo* maturation of DCs and exposure of DCs to a MHC-I-associated tumor antigen from autologous cultured glioma cells could induce an immune response against malignant gliomas. Cytotoxicity directed toward autologous tumor cells was assessed in PBMC from patients' prevaccination, 1 week after each vaccination, and 6 and 12 weeks after the last vaccination. Of seven patients that were evaluated, four demonstrated enhanced cytotoxic T-cell activity after DC vaccination. In all of the patients who developed cytotoxic T-cell activity, cytotoxicity was sustained through the last time point, 3 months after the last vaccination (Fig. 2*B*). In two patients (2 and 4), cytotoxicity developed 12 weeks after the third DC vaccination. Patients 7 and 8 demonstrated cytotoxicity before and after vaccination. Patient 1 had no discernible CTL activity before or after vaccination.

Intracranial Infiltration of T Cells after Vaccination. Four patients who developed apparent tumor progression as suggested by new

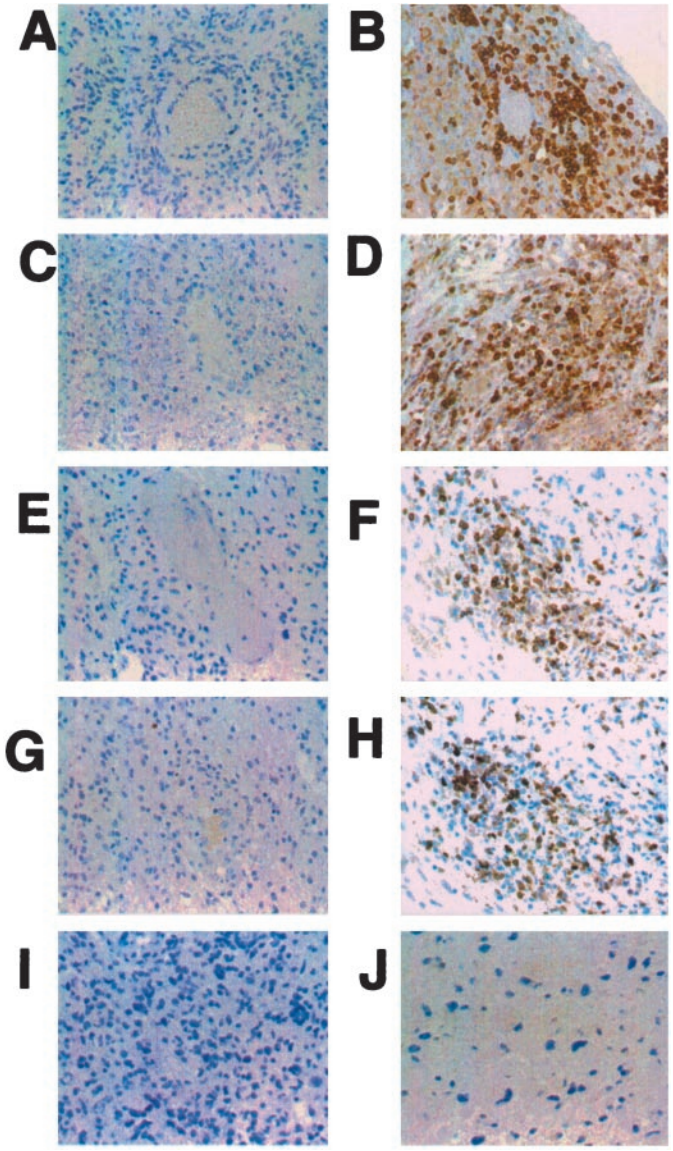

Fig. 3. Immunohistochemical characterization of infiltrating cells in intracranial tumor at first surgery, before vaccination (*left column*) and at reoperation, after vaccination (*right column*); *A*, CD45RO+ memory T-cell staining before vaccination in patient 8; *B*, CD45RO+ staining after vaccination in patient 8; *C*, patient 8 before vaccination CD8+ cells; *D*, patient 8 after vaccination CD8+ cells; *E*, patient 6 before vaccination CD45RO+ cells; *F*, patient 6 after vaccination CD45RO+ cells; *G*, patient 6 before vaccination CD8+ cells; *H*, patient 6 after vaccination CD8+ cells; *I*, control patient CD8+ cells at first operation; *J*, control patient CD8+ cells at reoperation. (Magnification × 400).

areas of gadolinium enhancement on magnetic resonance imaging scans underwent reoperation subsequent to the third DC vaccination (Table 1, patients 2, 4, 6, and 8). Two of these four patients developed a robust CD8+ cytotoxic and CD45RO+ memory T-cell infiltration in areas of tumor (patients 6 and 8) that was not apparent on tumor specimen obtained before vaccination (Fig. 3, *A-H*). There were increased numbers of CD4+ helper T cells after vaccination but significantly less than CD8+ T cells (data not shown). One of these patients underwent two reoperations, the first before vaccination and the second at 94 days after the first vaccination (patient 8). In this patient, the tumor specimen after the second reoperation (after vaccination) demonstrated massive intratumoral infiltration by CD8+ cytotoxic T cells and CD45RO+ memory T cells, as compared with tumor specimens before vaccination (Fig. 3, *A-D*). There were no CD20+ B cells or CD56+ NK cells detectable before or after vaccination in either patient (data not shown). In contrast, two other patients who underwent reoperation for a recurrent gadolinium-enhancing mass (patients 2 and 4) displayed few infiltrating CD45RO+, CD8+, CD4+, CD20+, or CD56+ cells in surgical specimens taken before or after vaccination (data not shown). Both of these patients died from tumor progression. Similarly, there was no increase in numbers of intratumoral lymphocytes in specimens from four non-vaccinated GBM patients who underwent re-resection. All of the four nonvaccinated patients demonstrated few CD45RO+ and CD8+ T cells from their first surgical specimen, which diminished in number in reoperation specimens (Fig. 3, *I* and *J*). There were very few CD20+ B cells in the first and second specimens, and no CD56+ NK cells were detected in either specimen. Thus, enhanced cytotoxic (CD8+) T-cell and memory (CD45RO+) T-cell infiltration appears to be a characteristic of a subset of vaccinated patients who undergo reoperation.

DC Vaccination Is Associated with Prolonged Survival. A further objective of this study was to determine the clinical response to DC vaccination. There were 7 patients in the study group and 42 patients in the control group with newly diagnosed GBM treated at our institution. Control patients underwent craniotomy by the same surgeons as the study group and external beam radiation therapy of 60 Gy with or without chemotherapy. They met all of the inclusion criteria of the DC immunotherapy trial including Karnofsky score but were treated before the trial opening (within 2 years) or chose not to participate. There were no statistically significant differences between the study and control groups for age (54.4 ± 11.3 years, study group, *versus* 55.9 ± 14.5 years, control group; $P = 0.79$), gender (42% male, study group, *versus* 50% male, control group; $P = 0.77$), and percentage of patients with gross total removal of gadolinium-enhancing tumor (71%, study group, *versus* 58%, control group; $P = 0.68$). The median survival times for the study and control groups were 455 and 257 days, respectively.

Discussion

Previous immunotherapeutic treatments for brain tumors have focused on passive, adoptive, and nonspecific strategies and have yielded unclear benefits (12). The active immunotherapy strategy using DC vaccinations reported here could be more effective against malignant glioma than previous strategies. Active immunotherapy requires the administration of an antigenic target. MHC-I-associated peptides were eluted from cultured autologous glioma cells. Cultured autologous tumor cells were characterized for three tumor-associated antigens: TRP-1, MAGE-1, and gp100. Melanocytes and glial cells are embryologically derived from neural ectoderm. Malignant transformed counterparts have been shown to share common antigens. Melanoma-associated antigen mRNA (13) and protein (14) have been described on glioma cells. Eight out of nine cell lines derived from study patients demonstrated expression of at least one of these tumor-associated antigens. The expression of tumor-associated antigens on autologous tumor cell cultures suggests that these lines are derived from glioma rather than from normal neural cells or fibroblasts, because these tissues rarely or never express melanoma-associated antigens (13). Abnormal karyotype was seen in five of seven cultured cell lines from study patients, further suggesting that these cells were tumor cells. The frequent expression of these tumor-associated antigens on glioma-derived cell lines points to potential antigenic targets on these tumors.

One goal of this clinical trial was to determine whether *ex vivo* maturation of DCs and exposure of DCs to a MHC-I-associated tumor antigen from autologous cultured glioma cells could induce an immune response against glioma tumor cells. That four of seven tested

patients in this pilot trial have shown cytotoxic T cell-mediated immunological responses after peptide-pulsed DC vaccination supports the role of DC vaccination in generating specific immunity. The relationship between peripheral CTL activity and tumor rejection has not been unequivocally established by the present or by previous trials (15–18).

It was not known whether peripheral cytotoxic T cells would reach their intracranial target after DC vaccination. In some immunotherapy trials, systemic cytotoxic responses have failed to prevent tumor growth in the central nervous system (19, 20). We observed a dramatic intratumoral infiltration of CD45RO+ memory and CD8+ cytotoxic T cells after vaccination in two of four patients who had undergone reoperation for gadolinium enhancement on magnetic resonance imaging which is suggestive of recurrent tumor, whereas nonvaccinated patients exhibited no similar infiltration. Thus, we demonstrate that DC vaccination can induce intra-glioma T-cell infiltration despite the immunologically privileged status of the central nervous system. All of the four patients that underwent reoperation demonstrated either preexisting or new systemic cytotoxicity *in vitro*. The study of tumor expression of immunosuppressive cytokines or cell surface markers may elucidate factors that differentiate patients who will respond to systemic cytotoxicity.

The presence of CD8+ T cells and absence of CD20+ B cells in intracranial tumor of vaccinated patients are consistent with the initiation of a Th1- rather than a Th2-mediated response by peptide-pulsed DCs, although other alternatives include preferential migration/survival of T cells. Intracranial CD8+ infiltration has been associated with vaccination in active immunotherapy models in mice (9, 21). The robust intratumoral infiltration of CD45RO+ T cells suggests vaccine-mediated induction of activated T cells with specific homing and/or *in situ* expansion properties. In a DC immunotherapy trial for melanoma, CD45RO+ memory T cells were shown to strongly infiltrate DTH sites, as well as metastases in regression. In addition, significant CD8+ infiltration was noted in regressing metastases. In a separate clinical trial, vaccination of renal cell carcinoma patients with tumor cell-DC hybrids demonstrated DTH sites that were infiltrated by CD8+ cells. Therefore, the intracranial cellular response of patients with malignant glioma mirrors the peripheral response noted in regressing melanoma tumors and in DTH sites of renal cell carcinoma patients after DC vaccination. The absence of both B and NK cell tumor infiltration in the present trial suggests that such effects are relatively limited to the T-cell compartment. This suggests that DCs in this study may preferentially activate antigen-specific T cells, rather than NK cells, as described in distinct *in vitro* studies (22).

DC vaccination of patients with glioma appears to be safe and not associated with autoimmunity. In this Phase I trial, DC vaccinations were well tolerated. Mild toxicities included transient fever, nausea, and vomiting in one patient and the development of palpable lymph nodes in another patient. To date, with a median follow-up of 455 days, no clinical or radiological features of autoimmune disease were detected in our patients. Clinical trials using DC immunotherapy have been published for four other cancers: lymphoma (15); melanoma (16); prostate cancer (17); and renal cell carcinoma (18). In these DC vaccine studies, mild fever and swelling of an injected lymph node lasting 1–2 days have been reported (16). No other significant adverse events including autoimmunity were reported in trials using DC immunotherapy (15–18) or in animal models of brain tumor therapy with DC vaccination (7–9). Thus, DC vaccination to tumor has not been associated with significant autoimmunity. On the other hand, induction of lethal experimental allergic encephalomyelitis has been described in primates and guinea pigs after vaccination with human glioblastoma tissue (23). Continued evaluation will be necessary to determine if autoimmunity develops from cross-reactivity between brain tumor antigens and normal neural antigens.

Patients with GBM treated with DC vaccination appear to have prolonged survival compared with patients who have had conventional treatment. When study patients were compared with GBM patients who underwent craniotomy with radiation and chemotherapy at our institution, there appeared to be a prolongation in survival of study patients. Although there was no difference in these groups with respect to age, gender, or degree of resection, a selection bias could not be ruled out. Although the number of patients in the study group was small, the survival suggests that this form of active immunotherapy may be a promising approach.

Study patients with glioblastoma were divided into Radiation Therapy Oncology Group (RTOG) recursive partitioning classes (24). This technique allows comparison of series data based on clinical and histological stratification parameters. Three patients with GBM in the study were stratified in RTOG Class III with <50 years of age and Karnofsky performance scores of ≥ 90. These patients had a median survival of 19.1 months compared with RTOG data of 18 months for this class of 175 patients. Four patients with GBM in the study were stratified in RTOG Class V with >50 years of age, radiotherapy of $>$ 54Gy, and the inability to return to work. This group of patients had a median survival of 11.6 months compared with the RTOG class V patients ($n = 395$) with a median survival of 9 months. Because of the small number of study patients in each of these classes, statistical analysis could not be performed. The patients in this study, however, exceeded the expected survival according to this analysis. Despite the prolongation of survival in this analysis as well as in the survival analysis compared with GBM patients treated at our institution, the potential for patient selection bias will only be eliminated by a randomized trial.

The association of intracranial CD8+ T-cell infiltration and survival has been established in an active immunotherapy model of intracranial metastasis (25). Active immunotherapy with DCs or tumor vaccines for experimental intracranial glioma have demonstrated an association of CD8+ intratumoral infiltration and prolonged survival (9, 21). The enhanced median survival of patients enrolled in this study suggests that tumor-specific T-cell activation, infiltration, and/or function may prolong the survival of glioma patients. Phase II studies using DC immunotherapy are under way to further elucidate the effects of DC vaccination in patients with malignant gliomas and to confirm its effect on survival. This immunotherapy strategy appears promising as an approach to successfully induce an antitumor immune response and to increase survival in patients with gliomas.

References

1. Fine, H. A., Dear, K. B., Loeffler, J. S., Black, P. M., and Canellos, G. P. Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults. Cancer (Phila.), *71:* 2585–2597, 1993.
2. Satoh, J., Lee, Y. B., and Kim, S. U. T cell costimulatory molecules B7–1 (CD80) and B7–2 (CD86) are expressed in human microglia but not in astrocytes in culture. Brain Res., *704:* 95–96, 1995.
3. Lampson, L. A., Wen, P., Roman, V. A., Morris, J. H., and Sarid, J. Disseminating tumor cells and their interactions with leukocytes visualized in the brain. Cancer Res., *52:* 1018–1025, 1992.
4. Constant, S., Sant'Angelo, D., Paqualini, T., Taylor, T., Levin, D., Flavell, R., and Bottomly, K. Peptide and protein antigens require distinct antigen-presenting cell subjects for the priming of CD4+ T cells. J. Immunol., *154:* 4915–4923, 1995.
5. Levin, D., Constant, S., Pasqualini, T., Flavell, R., and Bottlomly, K. Role of dendritic cells in the priming of CD4+ lymphocytes to peptide antigen *in vivo*. J. Immunol., *151:* 6742–6748, 1993.
6. Steinman, R. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol., *9:* 271–996. 1991.
7. Ashley, D. M., Faiola, B., Nair, S., Hale, L. P., Bigner, D. D., and Gilboa, E. Bone marrow-generated dendritic cells pulsed with tumor extracts or tumor RNA induced antitumor immunity against central nervous system tumors. J. Exp. Med., *186:* 1177–1182, 1997.

8. Siesjo, P., Visse, E., and Sjogren, H. O. Cure of established, intracerebral rat gliomas induced by therapeutic immunizations with tumor cells and purified APC or adjuvant IFN-γ treatment. J. Immunother. Emphas. Tumor Immunol., *19:* 334–45, 1996.
9. Liau, L. M., Black, K. L., Prins, R. M., Skys, S. N., Dipatre, P. L., Cloughesy, T. F., Becker, D. P., and Bronstein, J. M. Treatment of intracranial gliomas with bone marrow-derived dendritic cells pulsed with tumor antigens. J. Neurosurg., *90:* 1115–1124, 1999.
10. Zitvogel, L., Mayordomo, J., Tjandrawan, T., DeLeo, A. B., Clarke, M. R., Lotze, M. T., and Storkus, W. J. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. J. Exp. Med., *183:* 87–97, 1996.
11. Matzinger, P. A simple assay for DNA fragmentation and cell death. J. Immunol. Methods, *145:* 185–192, 1991.
12. Zeltzer, P. M., Moilanen, B., Yu, J. S., and Black, K. L. Immunotherapy of malignant brain tumors in children and adults. Child's Nerv. Syst., *15:* 514–528, 1999.
13. Chi, D. D., Merchant, R. E., Rand, R., Conrad, A. J., Garrison, D., Turner, R., Morton, D. L., and Hooh, D. S. Molecular detection of tumor-association antigens shared by human cutaneous melanomas and gliomas. Am. J. Pathol., *150:* 2143–2152, 1997.
14. Kuramoto, T. Detection of MAGE-1 tumor antigen in brain tumor. Kurume Med. J., *44:* 43–51, 1997.
15. Hsu, F., Benike, C., Fagnoni, F., Liles, T. M., Czerwinski, D., Taidi, B., Engleman, E. G., and Levy, R. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat. Med., *2:* 52–58, 1996.
16. Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med., *4:* 328–332, 1998.
17. Tjoa, B. A., Simmons, S. J., Bowes, V. A., Radge, J., Rogers, M., Elgamal, A., Kenny, G. M., Cobb, O. E., Ireton, R. C., Troychak, M. J., Sallgaller, M. L., Boynton, A. L., and Murphy, G. P. Evaluation of Phase I/II clinical trials in prostate cancer with dendritic cells and PSMA peptides. Prostate, *36:* 39–44, 1998.
18. Kugler, A., Stuhler, G., Walden, P., Zoller, G., Zobywalski, A., Brossart, P., Trefzer, U., Ullrich, S., Muller, C., Becker, V., Gross, A., Hemmerlein, B., Kanz, L., Muller, G., and Ringert, R. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat. Med., *6:* 332–336, 2000.
19. Grooms, G. A., Eliber, F. R., and Morton, D. L. Failure of adjuvant immunotherapy to prevent central nervous system metastases in malignant melanoma patients. J. Surg. Oncol., *9:* 147–153, 1977.
20. Mitchell, M. S. Relapse in the central nervous system in melanoma patients successfully treated with biomodulators. J. Clin. Oncol., *7:* 1701–1709, 1989.
21. Herrlinger, U., Kramm, C. M., Johnston, K. M., Louis, D. N., Finkelstein, D., Reznikoff, G., Dranoff, G., Breakefield, X. O., and Yu, J. S. Vaccination for experimental gliomas using granulocyte-macrophage colony-stimulating factor-transduced tumor cells. Cancer Gene Ther., *4:* 345–352, 1997.
22. Fernandez, N. C., Lozier, A., Flament, C., Ricciardi-Castagnoli, P., Benet, D., Suter, M., Perricaudet, M., Turzt, T., Maraskovsky, E., and Zitvogel, L. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune response *in vivo*. Nat. Med., *5:* 405–411, 1999.
23. Bigner, D. D., Pitts, O. M., and Wikstrand, C. J. Induction of lethal experimental allergic encephalomyelitis in nonhuman primates and guinea pigs with human glioblastoma multiforme tissue. J. Neurosurg., *55:* 32–42, 1981.
24. Curran, W. J., Scott, C. B., Horton, J., Nelson, J. S., Weinstein, A. S., Fischbach, A. J., Chang, C. H., Rotman, M., Asbell, S. O., Krisch, R. E., and Nelson, D. F. Recursive partitioning analysis of prognostic factors in three radiation therapy oncology group malignant glioma trials. J. Natl. Cancer Inst. (Bethesda), *85:* 704–710, 1993.
25. Sampson, J. H., Archer, G. E., Ashley, D. M., Fuchs, H. E., Hale, L. P., Dranoff, G., and Bigner, D. D. Subcutaneous vaccination with irradiated, cytokine-producing tumor cells stimulates CD8+ cell-mediated immunity against tumors located in the "immunologically privileged" central nervous system. Proc. Natl. Acad. Sci. USA, *93:* 10399–10404, 1996.