T Cell Adoptive Immunotherapy of Newly Diagnosed Gliomas[1]

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ABSTRACT

Patients with newly diagnosed gliomas were treated with adoptive transfer of *ex vivo* activated T lymphocytes, derived from lymph nodes (LNs) draining autologous tumor vaccines, to determine the long-term toxicity of this treatment. Twelve consecutive patients were enrolled: 2 with grade II astrocytoma, 4 with anaplastic gliomas, and 6 with glioblastoma multiforme. Patients were injected intradermally with short-term cultured autologous irradiated tumor cells, admixed with granulocyte macrophage colony-stimulating factor, to stimulate draining LNs. The LN cells were activated with staphylococcal enterotoxin A for 48 h and then cultured in medium containing interleukin 2 for an additional 6–8 days and subsequently transferred i.v. to the patients. The number of cells obtained from the LNs ranged from 9×10^7 to 1.1×10^9, and the median cell proliferation was 41-fold. The dose of T cells infused ranged from 0.6 to 5.5×10^{10} with a median of 1.1×10^{10}, the majority of which were CD 4$^+$ (mean, 71%). The entire treatment was performed as outpatient therapy and was associated with a toxicity of grade 2 or less, consisting mainly of fever, nausea, and myalgias during the first 24 h. There were no indications of late adverse events from this treatment even among three patients with follow-up greater than 2 years post T cell transfer. Moreover, four patients demonstrated partial regression of residual tumor. This Phase I clinical trial of adoptive immunotherapy for patients with newly diagnosed malignant gliomas demonstrates feasibility, lack of long-term toxicity, and several objective clinical responses.

INTRODUCTION

The treatment of malignant gliomas consists principally of surgery and radiation therapy (1, 2). Chemotherapy provides substantial therapeutic benefit for particular subsets of malignant gliomas, for example, oligodendrogliomas harboring chromosome 1p deletions and to a lesser extent anaplastic astrocytomas (3, 4). However, the use of chemotherapy for grade II astrocytomas is controversial, because of the slow natural progression of the disease weighed against the potential for short term toxicity associated with chemotherapy (5). More importantly, chemotherapy has demonstrated only a modest beneficial effect on median survival or 18-month survival for the most aggressive form of glioma, glioblastoma multiforme (6). As such, there is considerable interest in developing additional therapeutic options, particularly immunotherapy, for gliomas.

Considerable evidence indicates that tumor-reactive T cells arise in cancer patients but are not effective because of tumor-induced functional defects (7–10). We have used animal models to develop approaches to the generation of therapeutically effective T cells from tumor-bearing hosts. These preclinical studies demonstrated that LNs[3] draining progressively growing tumors are the optimal source of T cells that are sensitized to specific tumor antigens (11). Analogous to the situation in cancer patients, the sensitized LN T cells are not able to prevent progressive tumor growth. However, a key observation was that *ex vivo* activation of tumor-draining LN cells with anti-CD3 mAb or bacterial superantigens induces potent effector function (11, 12). Systemic adoptive transfer of these cells mediates immunologically specific regression of established tumors in many anatomical sites including the brain (13, 14). Importantly, the activated tumor-draining LN T cells avidly infiltrate tumors, even in immunoprivileged sites, such as the central nervous system, and this process is critical to their function (15, 16).

The conceptual and operational validity of this approach to immunotherapy has recently been tested in clinical trials for recurrent high-grade gliomas and for metastatic renal cell carcinoma (17, 18). The clinical trial for recurrent gliomas established the lack of short-term (less than 30 days) toxicity of this treatment and demonstrated radiographic regression lasting more than 4 months in 3 of 10 treated patients. However, the rapid course of progression of disease in these patients prevented an analysis of longer-term toxicity. Theoretically, strong immune reactions to glial antigens could lead to autoimmune neurological disease, with a prolonged time course greater than 30 days. Moreover, the use of whole tumor cell vaccines as well as the method used to activate the LN cells induces polyclonal T cell expansion that could potentially generate T cells with reactivity against autoantigens presented on both normal and

malignant brain cells. In light of these important considerations, we investigated adoptive immunotherapy in patients with newly diagnosed gliomas after surgery and radiation therapy. In this clinical setting, there are fewer baseline neurological deficits to confound an analysis of neurological toxicity, and the typical course of the disease permits a longer follow-up period. We observed no signs of autoimmune disease or neurological toxicity during extended follow-up in these patients, including several with radiographic tumor regression.

PATIENTS AND METHODS

Patients. Eligible patients had pathologically confirmed glioma after the maximal obtainable surgical resection. Pathological grading of tumors was performed according to the Berger and Vogel modification of the Ringhertz grading system. Six of the patients had glioblastoma multiforme, one had anaplastic astrocytoma, one had mixed oligodendroglioma-astrocytoma, one had anaplastic ependymoma, one had anaplastic oligodendroglioma, and two had grade II astrocytoma. Except for two patients with grade II astrocytoma, all patients received standard external beam radiation therapy. The study was approved by the institutional review board and written informed consent was obtained from all patients. Eligibility criteria included a performance status of 0 to 1 in the Eastern Cooperative Oncology Group scale, clinical stability without concurrent corticosteriod treatment, white blood count greater that $2 \times 10^3/\mu l$, platelet count greater than $10^5/\mu l$, blood urea nitrogen <25 mg/dl, creatinine <1.8 mg/dl, aspartate aminotransferase less than two times normal upper limit, total bilirubin <3 mg/dl, and negative serology for hepatitis B virus and human immunodeficiency virus. Pregnant or lactating women were excluded, as were patients with active or unexplained febrile illness or collagen vascular disease.

Tumor Vaccination. Fresh tumor samples were transported under sterile conditions to a dedicated tissue culture facility and then were minced and suspended in 40 ml of HBSS containing 4 mg DNase I, 40 mg of collagenase type IV, and 100 units of hyaluronidase type V (all from Sigma Chemical Co., St. Louis, MO) at room temperature for 3 h. The single cell suspension was filtered through no. 100 nylon mesh, washed twice in Hanks' buffered salt solution, and added to fibronectin-coated tissue culture flasks. Cells were cultured in media consisting of DMEM (84%), X-VIVO 15 (10%) (Bio-Whittaker Inc., Walkersville, MD), human AB serum (5%) (Sigma), G5 supplement (1%) (Life Technologies, Inc., Grand Island, NY), and hydrocortisone (10 μg/ml). Cultured tumor cells within the first three passages were harvested and used directly or in some cases cryopreserved in a liquid nitrogen tank until time of use. The median duration of tumor cell culture was 5 weeks (range, 2–14 weeks). Tumor cells were irradiated (25 Gy) and suspended in 0.6 ml of PBS containing 500 μg of GM-CSF (Sargramostim, Immunex Corp., Seattle, WA). The vaccine was given intradermally as a split dose (0.3 ml) on each upper thigh. GM-CSF (500 μg) was administered in a split dose to both vaccine sites daily for 3 additional days.

Analysis of EGFR in Cultured Tumor Cell Lines. Cell extracts prepared from cultured tumor cells containing 15 μg of protein were separated electrophoretically by 6% SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and blocked with 5% nonfat dried milk in PBS containing 0.1% Tween 20. Anti-EGFR (Sigma) was allowed to react for 2 h at room temperature followed by peroxidase conjugated anti-sheep IgG. Antibody complexes were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Ex Vivo **Activation of LN Cells.** Inguinal LNs that were palpably enlarged were surgically removed 8–10 days after vaccination and transported under sterile conditions to the tissue culture facility. LNs were mechanically dissociated to obtain a single cell suspension and were activated in X-VIVO 15 containing 10% human AB serum and 50 ng/ml SEA (Sigma) in 24-well tissue culture plates at 4×10^6 cells/well at 37°C, 5% CO_2 in a humidified incubator. Two days later, the cells were harvested, washed, and resuspended at $1–2 \times 10^4$/ml in X-VIVO 15 with IL-2 (60 IU/ml). Cells for patient 12 were additionally stimulated overnight on day 8 of culture on tissue culture flasks coated with OKT3 (Ortho Biotech, Raritan, NJ) followed by culture in X-VIVO 15 with IL-2 for 7 days to generate a greater number of cells. T cells were harvested from culture using a modified blood cell separator (CS3000, Baxter, Deerfield, IL) and were suspended in 250–300 ml of 0.9% NaCl containing 5% dextrose, 1.25% human albumin, and IL-2 (60 IU/ml). An aliquot of cells was stained directly with conjugated mAb against CD3, CD4, CD8, and CD25 (Becton Dickinson, Sunnyvale CA) and analyzed by flow cytometry.

T Cell Transfer. Patients received a single oral dose of cyclophosphamide (10 mg/kg) between 24 and 48 h prior to T cell infusion. They received acetaminophen (650 mg), and diphenhydramine (50 mg) 30 min before cell transfer. The T cells were infused through a peripheral vein over a period of 1-h in the outpatient clinic, and the patients were monitored for an additional 3 h.

Toxicity and Response Criteria. Toxicity was monitored according to the National Cancer Institute Common Toxicity Criteria. Neurological exam and history for autoimmune symptoms were performed at follow-up visits. Time to progression was defined as the interval between surgical resection and radiographic evidence of tumor progression.

RESULTS

Patient Characteristics. Twelve consecutive patients with newly diagnosed gliomas were enrolled on this Phase I clinical trial of adoptive immunotherapy. The characteristics of the patients are listed in Table 1 and are typical of the clinical spectrum of gliomas. There were two patients with pathological grade II gliomas who were offered adoptive immunotherapy. They were considered by additional clinical criteria to be at greater risk of early recurrence because of incomplete resection of tumor and a high number of mitotically active nuclei detected by immunohistochemical staining of tumor samples (19). Four of the patients had anaplastic gliomas, one each of the following: mixed oligodendroglioma-astrocytoma, ependymoma, oligodendroglioma, and astrocytoma. There were six patients with glioblastoma multiforme that clustered into two distinct age categories, with three <50 and three >68 years old. Except for the two patients with grade II gliomas, all other patients received

Table 1 Patient characteristics, treatment dose, and response to immunotherapy

Patient no.	Age/sex	Diagnosis	Cell dose	Response	TTP (mo.)[a]
1	29/F	Grade II astrocytoma[b]	1.8×10^{10}	SD	>29
2	37/M	Grade II astrocytoma[b]	5.5×10^{10}	SD	>21
3	32/F	Anapl. mixed[b]	1.4×10^{10}	PR	17
4	49/M	Anapl. oligodendroglioma	1.9×10^{10}	NE	>29
5	46/M	Anapl. ependymoma[b]	1.0×10^{10}	PR	11
6	69/M	Anapl. astrocytoma	0.6×10^{10}	PD	7
7	43/M	Glioblastoma multiforme[b]	3.0×10^{10}	PR	14
8	45/F	Glioblastoma multiforme[b]	1.0×10^{10}	PR	>29
9	50/M	Glioblastoma multiforme[b]	1.7×10^{10}	PD	14
10	68/M	Glioblastoma multiforme	1.1×10^{10}	PD	7
11	78/F	Glioblastoma multiforme	0.8×10^{10}	PD	8
12	69/M	Glioblastoma multiforme[b]	1.1×10^{10}	PD	7

[a] TTP, time to progression from diagnosis (in months). SD, stable disease (>1 year from T cell transfer); PR, partial response; NE, not evaluable (due to absence of gross residual disease); PD, progressive disease; Anapl., anaplastic.

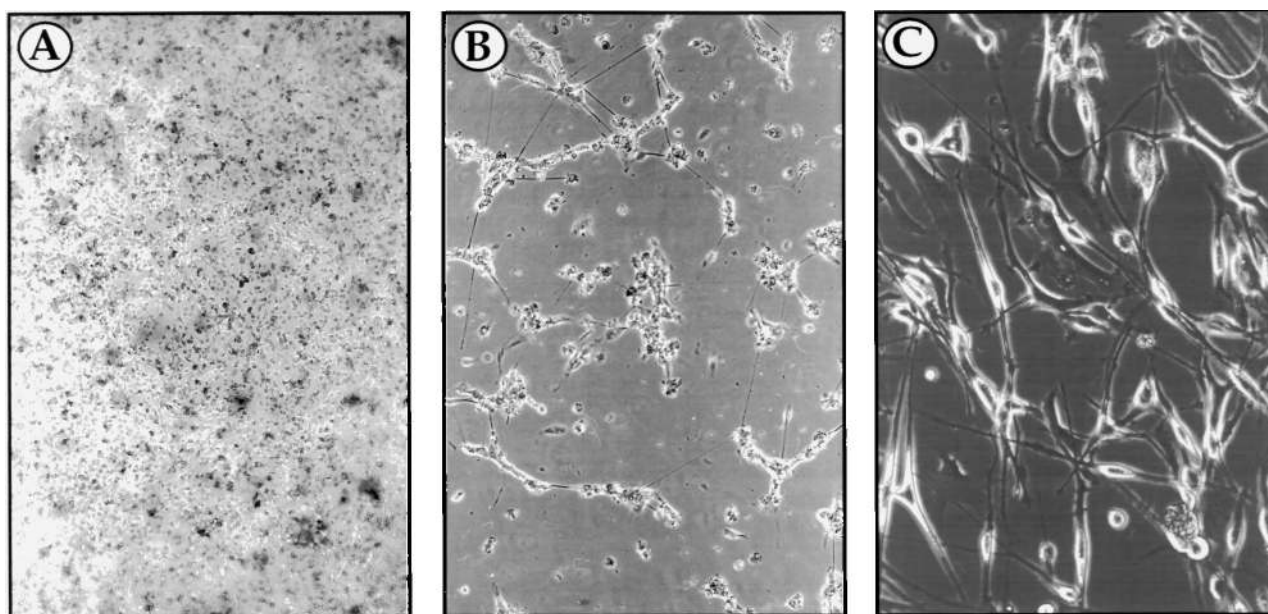[b] Gross residual disease at the time of immunotherapy.

Fig. 1 Photomicrographs of glioblastoma multiforme tumor cell culture. *A,* single cell preparation 24 h after enzymatic dissociation of tumor specimen. (×40). *B,* tumor culture at 48 h after removal of nonadherent debris (×40). *C,* tumor cells 10 days after initiation of culture (×200).

a standard course of external beam radiotherapy. All patients were on concurrent anticonvulsant therapy but were not taking corticosteroids at the time of the immunotherapy or at any time thereafter until they were considered off study because of tumor progression. Thus, all evaluations for toxicity and treatment response were performed in the absence of corticosteroid effects. The median time between the completion of radiation therapy and the initiation of immunotherapy was 8 weeks (range, 4–21 weeks). All patients had a baseline MRI scan at least 1 month after completion of radiation therapy, prior to starting the immunotherapy. As indicated in Table 1, eight of the patients had gross residual tumor at the initiation of immunotherapy.

Characterization of the Autologous Tumor Vaccine. A sample of tumor was obtained at the time of surgery and was prepared as a single cell suspension. For many of these tumors

there was a considerable amount of necrotic debris, RBCs, and dead cell contamination, as is readily apparent in Fig. 1*A*. The tumor cells became adherent to the fibronectin-coated tissue culture flasks, and much of the debris could be washed away after 2 days of culture (Fig. 1*B*). The tumor cells proliferated and were subsequently established as short-term cell lines (Fig. 1*C*). They were used to prepare the vaccine within the first three passages. The establishment of short-term glioma cell lines is successful for approximately 80% of all of the samples that we have attempted, and the primary factors that decrease the efficiency of establishing cell lines are small sample size (fewer than 6×10^6 viable cells at the start of culture) or tumors treated with prior radiation and chemotherapy (17). For the patients in this study with newly diagnosed tumors, autologous tumor cell lines were established for all patients. The cultured tumor line was supplemented with fresh-frozen tumor for the vaccine for
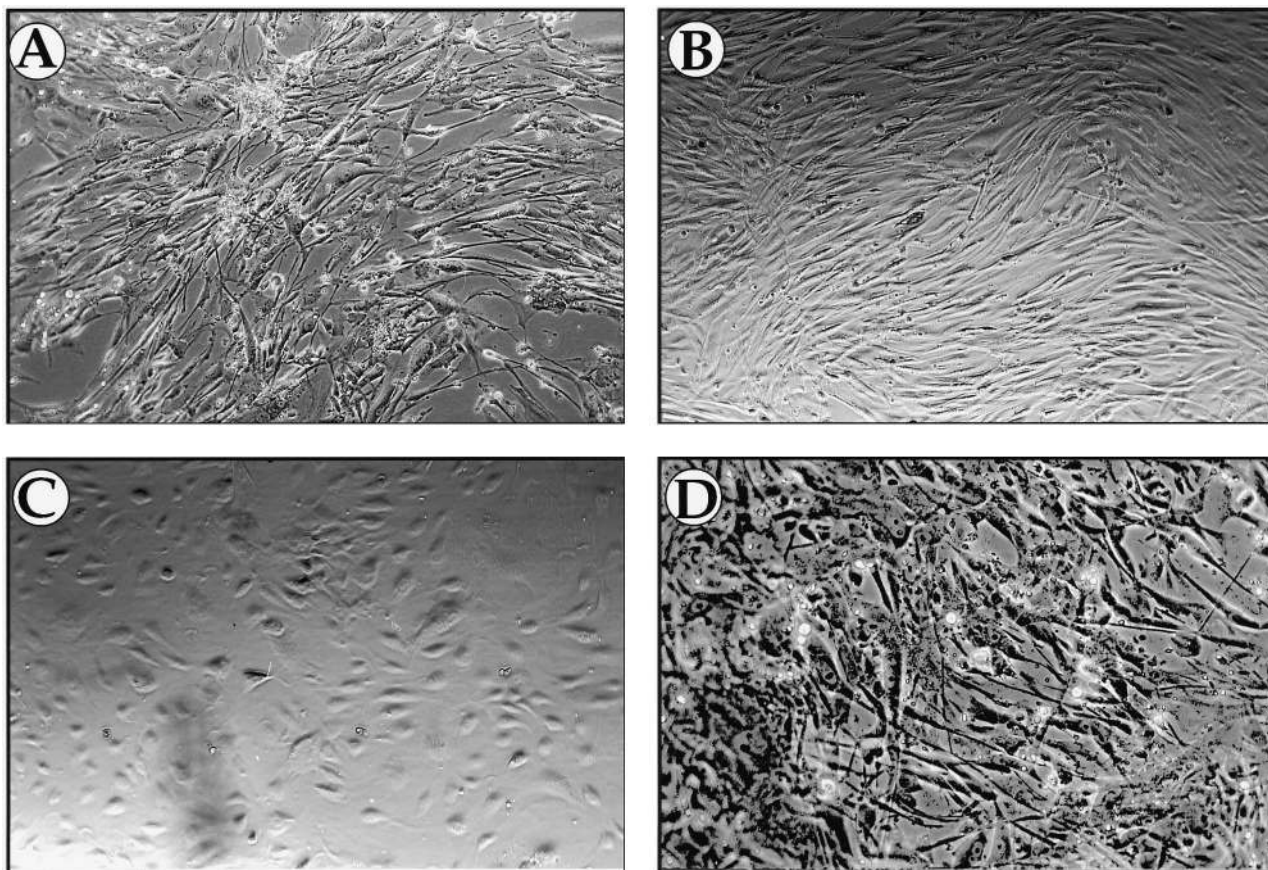
Fig. 2 Morphological heterogeneity of glioblastoma multiforme cell lines. *A-D,* cell lines established from four patients with glioblastoma multiforme. *A* and *B,* ×100; *C* and *D,* ×200.

patient 10 because of a low viability of this sample when the cryopreserved cultured tumor cells were thawed.

The morphology of the tumor cell lines was heterogeneous, and four representative glioblastoma cell lines are depicted in Fig. 2. Although variability was observed between different patient samples, the cell morphology for each cell line was generally uniform and unchanging. Occasionally, during the initial stages of culture, cells with two types of distinct morphology could be observed but one type would rapidly come to predominate. FACS analysis was performed on eight of the tumor cell lines, and they all expressed MHC class I molecules at variable levels, but none expressed MHC class II. Tumor cells often express cell adhesion molecules that are thought to aid in metastatic spread. We observed expression of CD54 (ICAM-1) in five of the eight tumor lines tested. The percentage of cells that expressed CD54 was typically 20–40%, and the pattern of CD54 expression showed a broad distribution rather than discrete subpopulations of positive and negative cells. Although CD44 was not specifically analyzed for these tumor lines, we have previously observed its expression on all of the cultured tumor lines tested ($n > 10$).

Brain tissue is highly vascular, and glioblastoma multiforme is characterized by extensive vascular proliferation presumably related to high expression of angiogenic factors such as vascular endothelial growth factor. It was anticipated that vascular endothelial cells could be present in the initial tumor preparation and that they could possibly propagate, although the tumor culture conditions were not optimized for the growth of normal nonmalignant endothelial cells. However, FACS analysis did not detect CD31 positive cells in the glioma tumor lines. Apparently, these culture conditions do not favor the selective outgrowth of vascular endothelial cells.

De novo malignant gliomas frequently express the EGFR at elevated levels and a significant fraction express an isoform containing an internal deletion of exons 2–7 (20–22). As demonstrated in Fig. 3, expression of EGFR was increased in four of six of the cultured glioma cell lines that were tested, whereas two tumor cell lines derived from renal cell carcinoma were negative. Moreover, one of the two glioma cell lines that was negative for EGFR, as well as one that was positive for EGFR, expressed glial fibrillary acidic protein. Thus, short-term cell lines derived from surgical specimens of gliomas reliably proliferated and gave rise to cells with the characteristics of malignant gliomas under these culture conditions.

Generation of Activated T Lymphocytes. A vaccine consisting of irradiated autologous tumor cells mixed with GM-CSF was administered as an intradermal injection bilaterally on the anterior thigh approximately 5 cm distal to the inguinal
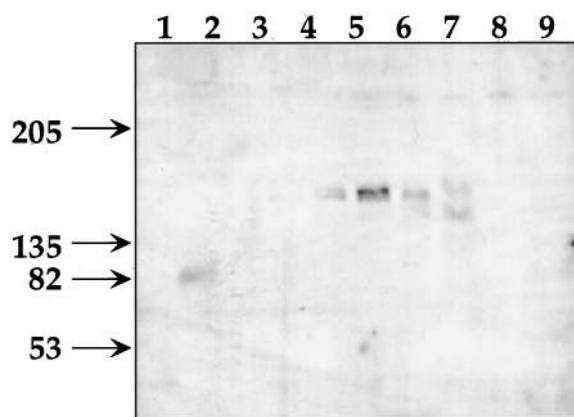
Fig. 3 Presence of EGFR in cultured glioma cell lines. Cell extracts subjected to SDS-PAGE and Western blot with anti-human EGFR. *Lane 1,* molecular weight markers; *Lane 2,* patient 7; *Lane 3,* patient 11; *Lane 4,* patient 8; *Lane 5,* patient 5; *Lane 6,* patient 4; *Lane 7,* patient 3; *Lanes 8 and 9,* renal cell carcinoma cell lines.

crease. This site was chosen because of its proximity to draining inguinal LNs. GM-CSF was injected directly into the vaccine site daily for an additional 3 days. Typically, the vaccine sites developed 5–10 mm of induration with a halo of erythema 4–10 cm in diameter but were not ulcerated or painful. Enlarged inguinal LNs were surgically removed 8–10 days later. As demonstrated in Table 2, the number of cells obtained varied considerably between patients, with more than 10-fold difference between patient 2 and patient 6. The majority of cells in the LNs were T lymphocytes (average, 70%; SE ± 2.3) and $CD4^+$ T cells predominated over $CD8^+$ T cells (averages, 56 *versus* 9%).

The LN cells were activated with SEA for 2 days. SEA is a bacterial superantigen that binds to MHC class II molecules and Vβ regions of the T-cell receptor (23). It provides a strong mitogenic stimulus to both $CD4^+$ and $CD8^+$ T cells that is independent of their antigen specificity. Although superantigens are characterized by the selective activation of T cells with particular Vβ phenotypes, SEA has the ability to bind and activate >80% of all human T cells. The cell numbers usually did not increase during the 2-day SEA activation, and they typically decreased to 60–80% of the input number. However, the activated cells underwent blast transformation and formed large multicellular aggregates. The SEA-treated cells were washed and subsequently cultured at low concentrations (1–2.5×10^4 cells/ml) in gas-permeable bags in the presence of a low concentration of IL-2 (60 IU/ml). The number of cells usually reached a maximum at days 8–9 of *ex vivo* culture, and as demonstrated in Table 2, there was a median of 41-fold expansion in the total cell number. At the end of the *ex vivo* activation, virtually 100% of the cells were T-cell receptor positive T cells, and the relative percentages of $CD4^+$ and $CD8^+$ cells were 71 and 27%, respectively. In most cultures, nearly all of the T cells expressed the activation marker, CD25. The SEA/IL-2 activation brought the total cell number to greater than 10^{10} for 10 of the 12 patients; however, the cells for patient 12 were stimulated for a second cycle with anti-CD3 mAb and IL-2 to promote additional proliferation.

Toxicity and Clinical Outcome. The T cell infusions were administered in the outpatient clinic and were well tolerated with the following toxicity noted in the first 24 h; grade 1 fevers in most patients, grade 2 rigors in patients 2 and 7, and grade 1 vomiting in patient 4. Two patients (patients 2 and 7) reported grade 2 mood alteration consisting of anxiety/agitation within the first 60 days after T cell infusion. Two patients (patients 2 and 3) reported an increase in seizure frequency but without a change in seizure characteristics. Seizures increased for patient 3 within the first 2 months after T cell transfer and for patient 2 approximately 8 months later. These seizures were controlled with minor adjustments in anticonvulsant medication. Several patients reported mild memory loss, but this finding was not appreciably different from what is commonly observed in patients with malignant brain tumors after radiation. There were no new radiographic abnormalities observed in the brain on follow-up MRI scans after the T cell transfer except for those directly related to tumor growth at the time of tumor recurrence. Thus, adoptive immunotherapy was well tolerated in this group of patients with newly diagnosed gliomas, and three patients have been followed for more than 2 years from diagnosis with no evidence of significant toxicity. In addition, no patient has demonstrated any signs of tumor growth at the vaccination site.

Eight of the patients had gross residual disease at the time of adoptive immunotherapy as indicated in Table 1 and could be evaluated for radiographic evidence of tumor regression. The other four patients did not have measurable residual disease. Four patients, patients 3, 5, 7, and 8, had partial regression of residual tumor. Interestingly, for the two patients with glioblastoma multiforme, the tumor regression was not apparent at the initial 1 month follow-up but developed over a period of several months. Fig. 4 shows equivalent coronal sections of gadolinium enhanced MRIs obtained 1 (*A*), 3 (*B*), 5 (*C*), and 8 (*D*) months post-T cell transfer in patient 7. Serial MRI scans for patient 8 in Fig. 5 demonstrate the extensive tumor present at diagnosis (*A*), residual tumor after surgery and radiation therapy (*B*), and minimal change 1 month post-T cell transfer (*C*). However, subsequent MRI scans demonstrated a decrease in enhancement, and the most recent study is more than 2 years from diagnosis (Fig. 5*D*). It is unusual for patients with residual glioblastoma multiforme to exhibit a delayed response to radiation therapy alone suggesting that the T cells contributed to these responses. Two patients with grade II astrocytoma, patients 1 and 2, have had stable residual tumor for greater than 27 and 20 months, respectively. Patient 4 did not have detectable residual disease at the time of T cell transfer, preventing a direct assessment of *in vivo* antitumor response. However, this patient demonstrated a large delayed type hypersensitivity-like reaction at the site of injection of irradiated autologous tumor and GM-CSF performed 15 months after the T cell transfer (Fig. 6).

DISCUSSION

There are several aspects to the biology and treatment of malignant gliomas that make immunotherapy theoretically attractive. In preclinical models of adoptive immunotherapy, the disease burden is a major factor influencing treatment efficacy. For malignant gliomas, frequently a state of minimal residual disease is achieved through surgery and local

Table 2 Characteristics of T cells used for immunotherapy

Patient no.	Diagnosis	LN cell (no.)	Proliferation SEA/IL-2	%CD4/%CD8, initial	%CD4/%CD8, final	%CD25, final
1	Grade II astrocytoma	10×10^8	$36 \times$	66/11	ND[a]	97
2	Grade II astrocytoma	11×10^8	$51 \times$	55/9	66/35	89
3	Anapl. mixed	4.9×10^8	$30 \times$	42/14	59/40	73
4	Anapl. oligodendroglioma	3.3×10^8	$62 \times$	63/8	64/33	ND
5	Anapl. ependymoma	1.4×10^8	$75 \times$	55/10	74/24	88
6	Anapl. astrocytoma	0.9×10^8	$61 \times$	56/6	62/22	58
7	Glioblastoma multiforme	6.5×10^8	$54 \times$	49/6	76/25	96
8	Glioblastoma multiforme	2.8×10^8	$40 \times$	45/12	70/29	96
9	Glioblastoma multiforme	2.9×10^8	$33 \times$	64/7	78/18	99
10	Glioblastoma multiforme	2.9×10^8	$41 \times$	62/8	77/17	94
11	Glioblastoma multiforme	2.3×10^8	$42 \times$	ND	81/20	98
12	Glioblastoma multiforme	1.6×10^8	$19 \times$[b]	58/7	72/31	99

[a] ND, not done; Anapl., anaplastic.
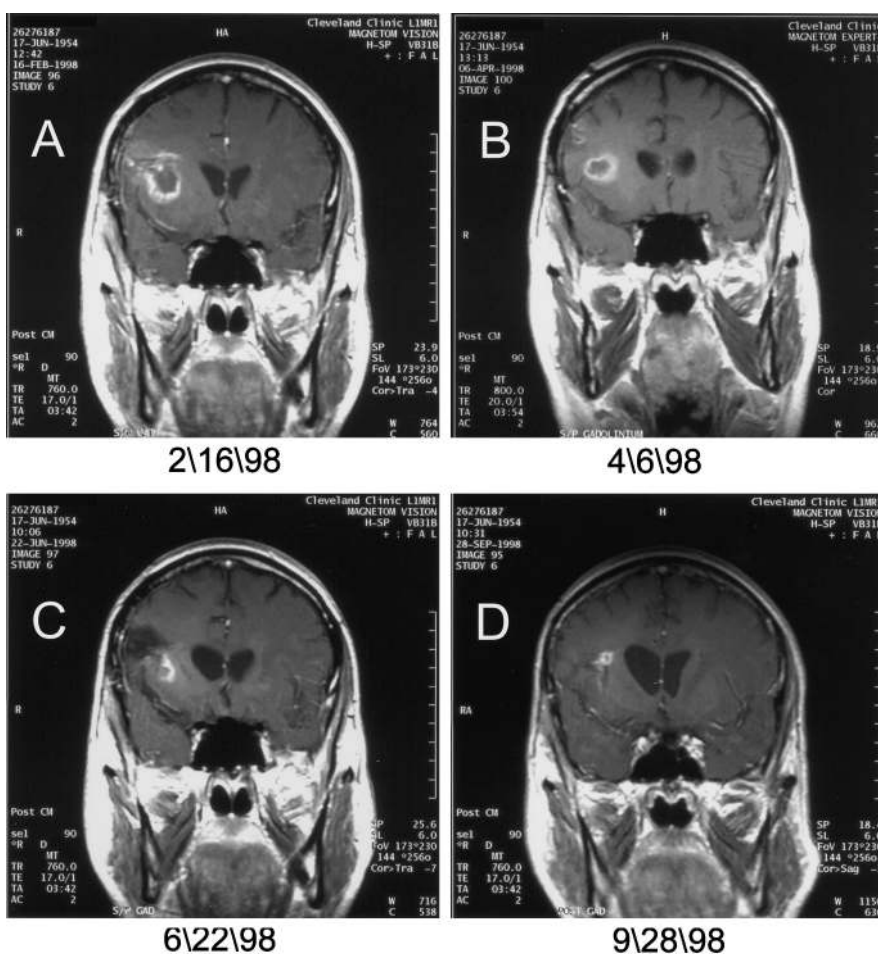[b] Final proliferation after additional OKT3 stimulation and IL-2 expansion was $71\times$.

Fig. 4 Serial gadolinium enhanced MRI scans for patient 7. *A,* 1 month after T cell transfer; *B,* 3 months after T cell transfer; *C,* 5 months after T cell transfer; *D,* 8 months after T cell transfer.

radiotherapy. Innovative approaches to the local delivery of chemotherapeutic agents may prove beneficial in further reducing the burden of residual disease while avoiding the immunosuppression that is associated with systemic chemotherapy (24). Thus, it may be feasible to incorporate immu-

notherapy in a form of multimodality therapy for malignant gliomas. Several approaches to stimulate a therapeutic antitumor immune response against gliomas are in development and clinical testing (25–27). The protocol described here was designed to incorporate principles of the antitumor immune
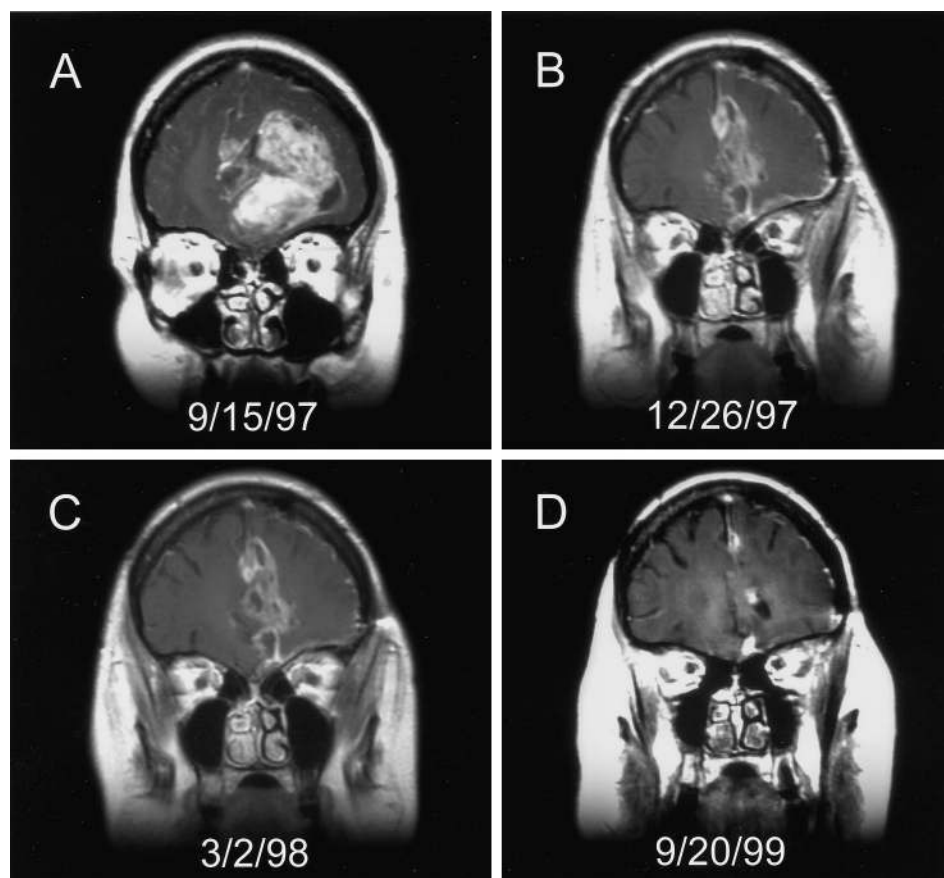
Fig. 5 Serial gadolinium enhanced MRI scans for patient 8. *A,* presurgery; *B,* after surgical resection of the tumor and radiation therapy but before adoptive immunotherapy; *C,* 1 month after T cell transfer; *D,* 15 months after T cell transfer.

response that were established through extensive testing in animal tumor models (11–14).

An important feature of this protocol is the use of autologous short-term cultured tumor cells as the immunogen. The advantages of using autologous tumor cells are that unique tumor antigens, multiple antigens, and both MHC class I and class II epitopes are available for presentation to the immune system. Arguably, the host immune system already has access to intact tumor cells in cancer patients; however, the central nervous system environment, tumor stroma, and immunosuppressive molecules released by the tumor (such as IL-10 and transforming growth factor-β) have all been demonstrated to inhibit the generation of a therapeutic immune response and protect tumor cells from apoptosis (28–32). The milieu at the intradermal vaccination site is more favorable for stimulating a cellular immune response, and GM-CSF has been demonstrated to be an effective adjuvant (33). Whole tumor cell vaccines contrast with the strategy of using reagents consisting of single tumor associated antigens. Broadly expressed tumor associated antigens have been defined for several types of malignancies. MHC class I restricted epitopes for some of these tumor antigens have been defined at a molecular level, and corresponding peptide reagents have advanced to clinical testing, yet it has not been determined whether MHC class I peptides from tumor associated antigens alone will be sufficient to produce a clinically beneficial immune response to human cancers (34).

Recently, approaches to using peptides that potentially bind to MHC class II molecules have also been demonstrated to give rise to immune responses in cancer patients (35). Other approaches to tumor immunotherapy use tumor lysates as a potential source of multiple MHC class I and class II epitopes (36). There is considerable evidence indicating that $CD4^+$ T cells are extremely important for achieving a therapeutic antitumor immune response (37, 38). In particular, our preclinical studies also suggest a critical role for $CD4^+$ T cells for the treatment of intracranial tumors by adoptive immunotherapy (13, 39). Several MHC class II restricted melanoma antigens identified by their stimulation of tumor infiltrating lymphocyte clones have recently been identified (40, 41). However, it is not yet clear whether the dominant MHC class II epitopes will be broadly shared or unique. It is important to note that although the glioma tumor lines from these patients do not express MHC class II molecules, they could still serve as an adequate source of such antigens to specialized APCs. It is likely that APCs are the vehicle for presentation of tumor antigens to T cells in the draining LN through the process of cross-priming (42–44). In this respect, the use of GM-CSF as an immune adjuvant would result in attracting a large number of APCs to the vaccination site. Several clinical trials have incorporated GM-CSF transduced tumor cell vaccines in an active immunotherapy strategy and have demonstrated sensitization of tumor-reactive T cells

Fig. 6 Delayed type hypersensitivity reaction at the site of injection of 2×10^7 autologous irradiated tumor cells admixed with 500 μg of GM-CSF at 48 h for patient 4 performed 15 months after T cell transfer.

(45–48). It is thus possible that host cells in addition to those activated *ex vivo* contributed to the biological activity observed.

Another strong consideration favoring the use of short-term cultured tumor cells for the vaccine is the ability to eliminate soluble antigens while purifying intact tumor cells. It is readily apparent from Fig. 1 that the short-term culture removed a significant amount of soluble necrotic debris in addition to amplifying the number of viable tumor cells that could be used for vaccination. Numerous preclinical experiments have established the superiority of whole cell tumor vaccines over soluble cell lysates for the generation of a cell mediated anti-tumor response. Future developments may eventually lead to vaccine reagents derived from specialized cellular components of tumor cells or methods to load APCs with relevant tumor antigens. Currently, however, irradiated autologous tumor cells established in short-term culture represent a reliable and appropriate immunogen.

The vaccination procedure and *ex vivo* activation gave rise to predominantly CD4$^+$ T cells (71%) as shown in Table 2. In our previous studies, we used two cycles of *ex vivo* activation that required a longer culture period and often gave rise to predominantly CD8$^+$ T cells (17). In addition, other strategies

of glioma immunotherapy have used predominantly CD8$^+$ T cells (25–27). Many approaches to tumor immunotherapy have focused on the CD8$^+$ T cell effector functions. This is conceptually based on the observation that most tumor cells express MHC class I but not class II molecules. However, typically there are MHC class II positive APCs within solid tumors that have the potential to stimulate CD4$^+$ T cells. Our murine intracranial tumor models have demonstrated that CD4$^+$ tumor-reactive T cells can eradicate an MHC class I positive but class II negative tumor in the complete absence of CD8$^+$ T cells, highlighting the importance of interactions between CD4$^+$ T cells and MHC class II expressing tumor-associated macrophages (39). Because the mechanism of tumor rejection *in vivo* is not completely defined and may well depend on CD4$^+$ T cells and MHC class II APCs, it is not yet clear what type of *in vitro* assay would provide an optimal surrogate marker of an effective immune response.

The design of this Phase I clinical trial, in terms of small sample size and no randomization, coupled with differences in the typical time to progression dependent on the tumor grade and cell type, does not permit a statistically valid assessment of the therapeutic efficacy. However, based on the lack of toxicity and the encouraging clinical course for several patients, we have initiated a Phase II clinical study in patients with newly diagnosed glioblastoma multiforme and anaplastic astrocytoma designed to evaluate therapeutic efficacy. In addition, we have begun to investigate whether performing booster vaccinations with autologous irradiated tumor cells, starting several months after the adoptive immunotherapy, increases the magnitude or duration of the antitumor activity of the transferred T cells. Frequently, a complete surgical resection of gross disease is achieved for patients with gliomas. This does not permit tumor regression to be used as the criterion for treatment efficacy; however, the antitumor response in such cases can be established by comparison of the time to progression to an appropriate control group. This type of analysis of treatment efficacy is appropriate for a disease such as glioblastoma multiforme, in which the time to progression is relatively uniform with current therapy. Moreover, a state of minimal residual disease is also theoretically desirable for immunotherapy in which an extensive tumor burden may impede therapeutic responses.

ACKNOWLEDGMENTS

REFERENCES

1. Walker, M. D., Alexander, E., and Hunt, W. E. Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas: a cooperative trial. J. Neurosurg., *49:* 333–343, 1978.

2. Walker, M. D., Green, S. B., Byar, D. P., Alexander, E. J., Batzdorf, U., Hunt, W. E., MacCarty, C. S., Mahaley, M. S. J., Mealy, J. J., Owens, G., Ransahoff, J. I., Robertson, J. T., Shapiro, W. R., Smith, K. R. J., Wilson, C. B., and Strike, T. A. Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant gliomas after surgery. N. Engl. J. Med., *303:* 1323–1329, 1980.

3. Levin, V. A., Silver, P., Hannigan, J., Wara, W. M., Gutin, P. H., Davis, R. L., and Wilson, C. B. Superiority of post-radiotherapy adjuvant chemotherapy with CCNU, procarbazine, and vincristine (PCV) over BCNU for anaplastic gliomas: NCOG 6G61 final report. Int. J. Radiat. Oncol. Biol. Phys., *18:* 321–324, 1990.

4. Cairncross, J. G., Ueki, K., Zlatescu, M. C., Lisle, D. K., Finkelstein, D. M., Hammond, R. R., Silver, J. S., Stark, P. C., Macdonald, D. R., Ino, Y., Ramsay, D. A., and Louis, D. N. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. J. Natl. Cancer Inst., *90:* 1473–1479, 1998.

5. Levin, V. A. Controversies in the treatment of low-grade astrocytomas and oligodendrogliomas. Curr. Opin. Oncol., *8:* 175–177, 1996.

6. DeAngelis, L. M., Burger, P. C., Green, S. B., and Cairncross, J. G. Malignant glioma: who benefits from adjuvant chemotherapy? Ann. Neurol., *44:* 691–695, 1998.

7. Mizoguchi, H., O'Shea, J. J., Longo, D. L., Loeffler, C. M., McVicar, D. W., and Ochoa, A. C. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. Science (Washington DC), *258:* 1795–1798, 1992.

8. Loeffler, C. M., Smyth, M. J., Longo, D. L., Kopp, W. C., Harvey, L. K., Trible, H. R., Tase, J. E., Urba, W. J., Leonard, A. S., Young, H. A., and Ochoa, A. C. Immunoregulation in cancer-bearing hosts: down-regulation of gene expression and cytotoxic function in CD8$^+$ T cells. J. Immunol., *149:* 949–956, 1992.

9. Finke, J. H., Zea, A. H., Stanley, J., Longo, D. L., Mizoguchi, H., Tubbs, R. R., Wiltrout, R. H., O'Shea, J. J., Kudoh, S., Klein, E., Bukowski, R. M., and Ochoa, A. C. Loss of T-cell receptor ζ chain and p56^{lck} in T-cells infiltrating human renal cell carcinoma. Cancer Res., *53:* 5613–5616, 1993.

10. Lee, P. P., Yee, C., Savage, P. A., Fong, L., Brockstedt, D., Weber, J. S., Johnson, E., Swetter, S., Thompson, J., Greenberg, P. D., Roederer, M., and Davis, M. M. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat. Med., *5:* 677–685, 1999.

11. Yoshizawa, H., Chang, A. E., and Shu, S. Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. J. Immunol., *147:* 729–737, 1991.

12. Shu, S., Krinock, R. A., Matsumura, T., Sussman, J. J., Fox, B. A., Chang, A. E., and Terman, D. S. Stimulation of tumor-draining lymph node cells with superantigenic staphylococcal toxins leads to the generation of tumor-specific effector T cells. J. Immunol., *152:* 1277–1288, 1994.

13. Inoue, M., Plautz, G. E., and Shu, S. Treatment of intracranial tumors by systemic transfer of superantigen-activated tumor-draining lymph node T cells. Cancer Res., *56:* 4702–4708, 1996.

14. Plautz, G. E., Touhalisky, J. E., and Shu, S. Treatment of murine gliomas by adoptive transfer of ex-vivo activated tumor-draining lymph node cells. Cell. Immunol., *178:* 101–107, 1997.

15. Kjærgaard, J., and Shu, S. Tumor infiltration by adoptively transferred T cells is independent of immunologic specificity but requires down-regulation of L-selectin expression. J. Immunol., *163:* 751–759, 1999.

16. Mukai, S., Kjærgaard, J., Shu, S., and Plautz, G. E. Infiltration of tumors by systemically transferred tumor-reactive T lymphocytes is required for antitumor efficacy. Cancer Res., *59:* 5245–5249, 1999.

17. Plautz, G. E., Barnett, G. H., Miller, D. W., Cohen, B. H., Prayson, R. A., Krauss, J. C., Luciano, M., Kangisser, D., and Shu, S. Systemic T cell adoptive immunotherapy of malignant gliomas. J. Neurosurg., *88:* 42–51, 1998.

18. Plautz, G. E., Bukowski, R. M., Novick, A. C., Klein, E. A., Kursh, E. D., Olencki, T. E., Yetman, R. J., Pienkny, A., Sandstrom, K., and Shu, S. T cell adoptive immunotherapy of metastatic renal cell carcinoma. Urology, *54:* 617–624, 1999.

19. McKeever, P. E. Strawderman, M. S., Yamini, B., Mikhail, A. A., and Blavis, M. MIB-1 proliferation index predicts survival among patients with grade II astrocytoma. J. Neuropathol. Exp. Neurol., *57:* 931–936, 1988.

20. Nishikawa, R., Ji, X. D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavanee, W. K., and Huang, H. J. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumoriganicity. Proc. Natl. Acad. Sci. USA., *91:* 7727–7731, 1994.

21. Diedrich, U., Lucius, J., Baron, E., Behnke, J., Pabst, B., and Zoll, B. Distribution of epidermal growth factor receptor gene amplification in brain tumors and correlation to prognosis. J. Neurol., *242:* 683–688, 1995.

22. Rasheed, B. K., Wiltshire, R. N., Bigner, S. H., and Bigner, D. D. Molecular pathogenesis of malignant gliomas. Curr. Opin. Oncol., *11:* 162–167, 1999.

23. Marrack, P., and Kappler, J. The staphyloccal enterotoxins and their relatives. Science (Washington DC)., *248:* 705–711, 1990.

24. Brem, H., Piantadosi, S., Burger, P. C., Walker, M., Selker, R., Vick, N. A., Black, K., Sisti, M., Brem, S., Mohr, G., Muller, P., Marawetz, R., and Schold, S. C. Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. Lancet, *345:* 1008–1012, 1995.

25. Merchant, R. E., Ellison, M. D., and Young, H. F. Immunotherapy for malignant glioma using human recombinant interleukin-2 and activated autologous lymphocytes. J. Neurooncol., *8:* 173–188, 1990.

26. Kruse, C. A., Cepeda, L., Owens, B., Johnson, S. D., Stears, J., and Lillehei, K. O. Treatment of recurrent glioma with intracavitary alloreactive cytotoxic T lymphocytes and interleukin-2. Cancer Immonol. Immunother., *45:* 77–87, 1997.

27. Jaeckle, K. A. Immunotherapy of malignant gliomas. Semin. Oncol., *21:* 249–259, 1994.

28. Head, J. R., and Griffin, W. S. T. Functional capacity of solid tissue implants in the brain: evidence for immunological privilege. Proc. R. Soc., *224:* 375–387, 1985.

29. Castelli, M. G., Chiabrando, C., Fanelli, R., Martelli, I., Butti, G., Gaetani, P., and Panletti, P. Prostaglandin and thromboxane synthesis by human intracranial tumors. Cancer Res., *49:* 1505–1508, 1989.

30. Maxwell, M., Galanpoulos, T., Neville-Golden, J., and Antoniades, H. N. Effect of the expression of transforming growth factor-β2 in primary human glioblastomas on immunosuppression and loss of immune surveillance. J. Neurosurg., *76:* 799–804, 1992.

31. Huettner, C., Paulus, W., and Roggendorf, W. Messenger RNA expression of the immunosuppressive cytokine IL-10 in human gliomas. Am. J. Pathol., *146:* 317–322, 1995.

32. Uhm, J. H., Dooley, N. P., Kryitsis, A. P., Rao, J. S., and Gladson, C. L. Vitronectin, a glioma-derived extracellular matrix protein, protects tumor cells from apoptotic death. Clin. Cancer Res., *5:* 1587–1594, 1999.

33. Disis, M. L., Bernhard, H., Shiota, F. M., Hand, S. L., Gralow, J. R., and Cheever, M. A. GM-CSF: an effective adjuvant for protein and peptide based vaccines. Blood, *88:* 202–210, 1996.

34. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwartz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H., and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., *4:* 321–327, 1998.

35. Disis, M. L., Grabstein, K. H., Sleath, P. R., and Cheever, M. A. Generation of immunity to the HER-2/*neu* oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin. Cancer Res., *5:* 1289–1297, 1999.

36. 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:* 328–335, 1998.

37. Cohen, P. A., Cohen, P. J., Rosenberg, S. A., and Mule, J. J. CD4+ T cells from mice immunized to syngeneic sarcomas regonize distinct, non-shared tumor antigens. Cancer Res., *54:* 1055–1058, 1994.

38. Greenberg, P. D. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv. Immunol., *49:* 281–355. 1991.

39. Kagamu, H., and Shu, S. Purification of L-selectin^low cells promotes the generation of highly potent CD4 antitumor effector T lymphocytes. J. Immunol., *160:* 3444–3452, 1998.

40. Wang, R., Wang, X., and Rosenberg, S. A. Identification of a novel major histocompatibility complex class II-restricted tumor antigen resulting from a chromosomal rearrangement recognized by CD4+ T cells. J. Exp. Med., *189:* 1659–1667, 1999.

41. Wang, R., Wang, X., Atwood, A. C., Topalian, S. L., and Rosenberg, S. A. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science (Washington DC), *284:* 1351–1354, 1999.

42. Bevan, M. J. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J. Exp. Med., *143:* 1283–1288, 1976.

43. Bevan, M. J. Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during *in vivo* priming. J. Immunol., *117:* 2233–2238, 1976.

44. Huang, A. Y. C., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D., and Levitsky, H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science (Washington DC), *264:* 961–965, 1994.

45. Dranoff, G., Soiffer, R., Lynch, T., Mihm, M., Jung, K., Kolesar K., Liebster, L., Lam, P., Duda, R., Mentzer, S., Singer, S., Tanabe, K., Johnson, R., Sober, A., Bhan, A., Clift, S., Cohen, L., Parry, G., Rokovich, J., Richards, L., Drayer, J., Berns, A., and Mulligan, R. C. A Phase I study of vaccination with autologous irradiated melanoma cells engineered to secrete human granulocyte-macrophage colony stimulating factor. Hum. Gene Ther., *8:* 111–123, 1997.

46. Simons, J. W., Jaffee, E. M., Weber, C. E., Levitsky, H. I., Nelson, W. G., Carducci, M. A., Lazenby, A. J., Cohen, L. K., Finn, C. C., Clift, S. M., Havola, K. M., Beck, L. A., Leiferman, K. M., Owens, A. J., Jr., Piantadosi, S., Dranoff, G., Mulligan, R. C., Pardolll, D. M., and Marshall, F. F. Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by *ex vivo* granulocyte-macrophage colony-stimulating factor gene transfer. Cancer Res., *57:* 1537–1546, 1997.

47. Soiffer, R., Lynch, T., Mihm, M., Jung, K., Rhuda, C., Schmollinger, J. C., Hodi, F. S., Liebster, L., Lam, P., Mentzer, S., Singer, S., Tanabe, K. K., Cosimi, A. B., Duda, R., Sober, A., Bhan, A., Daley, J., Neuberg, D., Parry, G., Rokouch, J., Richards, L., Drayer, J., Berns, A., Clift, S., Dranoff, G., *et al.* Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. Proc. Natl. Acad. Sci. USA., *95:* 13141–13146, 1998.

48. Simons, J. W., Mikhak, B., Chang, J. F., DeMarzo, AM, Carducci, M. A., Lim, M., Weber, C. E., Baccala, A. A., Goemann, M. A., Clift, S. M., Ando, D. G., Levitsky, H. I., Cohen, L. K., Sanda, M. G., Mulligan, R. C., Partin, A. W., Carter, H. B., Piantadosi, S., Marshall, F. F., and Nelson, W. G.. Induction of iimmunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. Cancer Res., *59:* 5160–5168, 1999.