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Clinical Study

Pilot study of local autologous tumor infiltrating lymphocytes for the treatment of recurrent malignant gliomas

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Summary

A prospective pilot study was performed in order to assess the safety of treating recurrent malignant gliomas (MGs) with locally infused autologous tumor infiltrating lymphocytes (TILs) and recombinant interleukin-2 (rIL-2). Six patients were entered between June 27, 1994 and June 2, 1995 and followed until July 1, 1998. At surgery an Ommaya reservoir was placed for later infusion of TILs and rIL-2. Following surgery, autologous TILs were expanded *in vitro* in the presence of rIL-2 and infused on treatment days 1 and 14, with concurrent rIL-2 infusions performed three times each week for one month. Following completion of immunotherapy all patients were offered chemotherapy. Phenotypic analysis demonstrated TILs to be T-lymphocytes (87–99% CD3+). Of these, 4 of 6 cases (67%) phenotyped as cytotoxic/suppressor T-lymphocytes (CD8+) and 2 of 6 cases (33%) phenotyped as helper/inducer T-lymphocytes (CD4+). TILs demonstrated limited selective cytotoxicity, with dose dependent cytotoxicity against autologous tumor, allogenic tumor and long term MG cell lines.

There were no significant (Grade 3 or 4) complications. One patient developed transient low grade fevers, and 2 developed asymptomatic hydrocephalus. All patients developed transient and asymptomatic cerebral swelling, noted on the immediate post-treatment imaging studies.

At three and six month follow-up, 3 patients responded with partial response, 2 demonstrated stable disease and 1 patient progressed. At long term follow-up, 1 patient had a complete response (45 month follow-up), 2 had a partial response (48 and 47 month follow-up) and 3 patients expired as a result of progressive disease (at 12, 12 and 18 months following immunotherapy). A relationship between subsequent chemotherapy or extent of resection to outcome was not appparent but could not be excluded.

This pilot study demonstrated that locally infused autologous TILs and rIL-2 could be delivered without serious toxicity. Further studies are indicated to determine the safety and long term efficacy of TIL immunotherapy.

Introduction

Malignant gliomas (MGs) are the most common primary malignancies of the brain, comprising over 50% of cases and accounting for the majority of the 12,000 annual deaths due to primary malignancies of the central nervous system [6,8,24,34]. The treatment of intracranial MGs continues to be a significant challenge. Despite aggressive surgical treatment, radiation therapy, and chemotherapy, the median survival time for patients is 37–60 weeks, with a 90% patient mortality at 2 years [15].

Several studies have described the use of adoptive immunotherapy, using lymphokine activated killer (LAK) cells or tumor infiltrating lymphocytes (TILs), in the treatment of patients with extracranial tumors, such as metastatic melanoma and renal cell carcinoma [31,32,33]. LAK cells are peripheral blood lymphocytes nonspecifically activated by the T-cell growth factor, recombinant interleukin-2

(rIL-2). LAK cells demonstrate activity against a wide range of solid and hematopoietic tumors, and are not major histocompatability (MHC) antigen restricted. TILs are primarily cytotoxic T-lymphocytes (CTLs) which recognize proteolytically cleaved intracellular tumor antigen fragments (epitopes) which have become associated with specific MHC-I antigens on the cell surface [7,26]. The complex is recognized by the T-cell receptor (TCR) of specific T-lymphocytes [7,28]. TILs are relatively specific in terms of their cytotoxicity to tumors, due to the requirement for the presence of tumor antigen fragments associated with specific classes of MHC-I antigens, and therefore have potential advantages over LAK cells in terms of tumor specificity and cytotoxicity [7,22,28].

There is evidence supporting immunotherapy as a rational treatment modality for MGs. Circulating antibodies and CTLs, with specificity for autologous tumor, have been isolated from the peripheral blood of patients with MGs, despite the fact that systemic and local cellular immune function appears to be suppressed [3,26]. At least 2 studies have found the extent of lymphocytic infiltration within the perivascular spaces surrounding these tumors may correlate with improved prognosis [9,26]. The extent of mononuclear cell infiltration within the tumor parenchyma, however, does not appear to correlate with improved prognosis [9,26]. Several lines of evidence indicate that any potential immune response to MGs may be suppressed *in situ*. For example, MGs appear to secrete immunosuppressive factors, such as transforming growth factor (TGF)-β_2 and prostaglandin (PG)-E_2 and Interleukin (IL)-10, theoretically inhibiting both IL-2 dependent and IL-2 independent CTL activity [2,3]. Despite local immunosuppression, TILs and tumor stimulated-peripheral blood mononuclear cells (TS-PBMCs) have been successfully expanded from patients with MGs [20,25,27,36,37]. Miescher and his colleagues demonstrated that TILs isolated from patients with MGs demonstrated reduced proliferative responses to lectins phytohemagglutinin and concanavalin A [26]. However, limiting dilutional clonal analysis of TILs demonstrated cytotoxicity against autologous tumor [26]. TS-PBMCs, like TILs, appear to be primarily CTLs. In the one reported clinical trial using TS-PBMCs in the treatment of MGs, 2 of 5 patients treated with intratumoral injections of 10^8–10^9 cells, demonstrated greater than 50% reduction in tumor burden [20]. More recent clinical studies, using locally infused autologous LAK cells or systemically infused CTLs, have shown promise with low toxicity and 50% response rates at one year follow-up [16,28]. To date, however, we are not aware of any clinical trials using TILs to treat MGs in man.

This pilot study was therefore designed to investigate the safety of autologous intralesional TILs and rIL-2 in the treatment of recurrent MGs.

Methods

Clinical Study

This study was approved by the Advisory Board of the General Clinical Research Center and the Committee on the Rights of Human Subjects, at the University of North Carolina, Chapel Hill. Patients with recurrent MGs who required surgery for debulking, were eligible for entry into the study. All patients were at least 18 years of age. Patients with evidence of immunosuppression due to systemic illness and those with a Karnofsky Performance Status of less than 50 were excluded, as were patients who required more than 4 mg of decadron daily, at the time of treatment initiation. Tumor specimens were obtained at surgery from 6 recurrent MG patients, and an Ommaya reservoir catheter placed into the tumor cavity at the time of surgery. TIL cultures were then expanded as noted below, with successful expansion in the 6 consecutive patients selected for the pilot study. The patient's clinical and radiographic characteristics, as well as the histological classification of the patient's tumors are summarized in Table 1. TIL immunotherapy treatments were performed on an outpatient basis on the General Clinical Research Center (GCRC) at the University of North Carolina, Chapel Hill. Patients received intratumoral infusions of 1×10^9 TILs in 3–5 ml of preservative free sterile saline on treatment days 1 and 14. All patients also received intra-tumoral infusions of rIL-2 on every Monday, Wednesday and Friday for 1 month. rIL-2 was given on a dose escalation basis, with the first 3 patients receiving 1×10^5 IU/dose and the second 3 patients receiving 4×10^5 IU/dose. Prior to infusion all TIL samples were tested by gram stain and culture for bacterial, viral and fungal contamination. All radiographs [contrast enhanced magnetic resonance (MR) imaging and computerized tomography (CT)] were independently assessed by the neuroradiologist.

Because this was a preliminary pilot study designed to assess safety, the Advisory Board of the General Clinical Research Center and the Committee on the

Table 1. Patient characteristics

Patient	Age	Karnofsky	Location	Histology[1]	Prior treatment[2]	Resection
Case 1	35	90	L frontal	AA	Res, XRT, Cis Res, IV BCNU	Subtotal
Case 2	70	70	R frontoparietal	GBM	Res, XRT, BCNU	Gross total
Case 3	30	80	L temporal	AA	Res, XRT, Cis, Res, BCNU, PCZ	Gross total
Case 4	47	50	R temporal	GBM	Bx, XRT, Res, RaS, BCNU	Subtotal
Case 5	48	70	L temporal	AA	Res, XRT, Cis, Res, PCV	Subtotal
Case 6	41	90	L frontal	GBM	Res, XRT, Res, BCNU	Gross total

[1]AA: anaplastic astrocytoma; GBM: glioblastoma multiforme.
[2]Bx: biopsy; Res: resection; XRT: local radiation therapy; Cis: intraarterial cisplatin; PCZ: procarbazine; PCV: procarbazine, CCNU and vincristine; BCNU: 1,3-bis(2-chloroethyl)-1-nitrosourea; RaS: radiosurgery.

Rights of Human Subjects required that the option of adjuvant chemotherapy be offered to all patients undergoing immunotherapy. It was recommended that, pending the results of this pilot study, further studies could be designed with stand alone immunotherapy. No patients received chemotherapy during the 30 day period of TIL immunotherapy infusions.

Expansion of TILs

TILs were produced by *in vitro* expansion, essentially as a modification of methods previously described [19,37]. Culture media (CM) used in the production of TILs consisted of serum free AIM-V media (GIBC0-BRL, Grand Island, NY) supplemented with rIL-2 (Cetus Corporation, Emeryville, CA) at a concentration of 1000 Cetus units/ml (equivalent to 6000 IU/ml), 10 μg/ml of gentamycin and 50 μg/ml of streptomycin.

Tumor samples were washed in RPMI 1640 (GIBCO) and finely minced into 1 mm pieces, centrifuged at 200*g* for 8 min and resuspended at a concentration of 1 g (weight of original sample) per 40 ml of HANKS Balanced Salt Solution. The samples were enzymatically digested with 0.1 mg/ml of DNase, 2.5 U/ml hyaluronidase and 1.0 mg/ml collagenase. The resulting slurry was passed through a 70 μm filter, separated over Hypaque–Ficoll (GIBCO) and washed twice in RPMI 1640. A total of 1.5×10^7 cells were placed in 30 ml of CM (5×10^5 cells/ml) in 75 cm^2 culture flasks and incubated 37°C with 5% CO_2 for 7 days, then harvested, washed, supplemented with fresh media and recultured at a concentration of 5×10^5 lymphocytes/ml in CM. Cell media was enriched at every 3–5 days for 6 weeks, at which time the cells were harvested. Total cell counts were in excess of 3×10^9 cells.

Cell surface phenotyping

Lymphocyte phenotyping was performed on all TIL specimens by standard direct labeling with fluorescein isothiocyanate conjugated monoclonal antibody essentially as previously decribed [29]. Briefly, 1×10^6 TILs were stained with fluorescein isothiocyanate conjugated monoclonal antibodies. Cell phenotyping included CD3ε (epsilon unit of the TCR, pan T-cell marker), CD4 (helper/inducer T-cell marker), CD8 (suppressor/cytotoxic T-cell marker), CD19 (B-cell marker), CD14 (monocyte marker), CD56 (natural killer cell marker), HLA-DR (class II MHC antigen) and CD25 (IL-2 receptor). Murine isotypic immunoglobulins served as background controls for nonspecific immunofluorescence. Stained cells were counted on a FACS Scan flow cytometer. Cells were gated on a scattergram which plotted 90° light scatter against forward angle light scatter, with the entire viable cell population being analyzed.

Cytotoxicity assays

TIL cytotoxicity was determined by standard ^{51}Cr release microcytotoxicity assays, essentially as previously described [29]. Effector cells (TILs) were tested against target cells including: autologous tumor cells, SKMG-1 (long term MG cell line), allogenic short-passaged MG cell lines (tumor specimens recently resected at UNC), Raji cells (NK resistant, LAK cell sensitive), K562 cells (NK sensitive, LAK cell sensitive), and an adenocarcinoma cell line (HPAF-2).

Ommaya supernatants

Cells were periodically obtained from the Ommaya reservoirs prior to intratumoral rIL-2 and/or TIL

infusions. Trypan blue exclusion studies were performed to determine cell viability. Wright–Geimsa stained cytocentrifuge preparations were also prepared from patients at the time of the final rIL-2 infusion.

Toxicity and response to therapy

Toxicity was documented according to the National Cancer Institute Common Toxicity Criteria. The response to therapy was based on independent radiographic interpretations of the patients enhanced MR imaging studies by the neuroradiologist. Tumor burden was determined by estimation of the tumor volume based on the cross sectional area of the greatest area of gadolinium enhancement and the number of consecutive scan cuts of gadolinium enhancement (summation of cross sectional areas of tumor enhancement). Responses were defined as complete (no evidence of tumor on contrasted studies), partial (greater than 50% reduction in summation of cross sectional areas of tumor enhancement) or progressive (presence of a new lesion or an increase in the summation of cross sectional areas of the tumor).

Results

TIL production and proliferation

Trypan blue exclusion demonstrated greater than 95% cell viability, following 6 weeks of incubation. TILs continued to undergo log phase growth for at least 12 weeks. Microscopic examination of TIL in early incubation frequently showed TILs clustered around tumor cells (Figure 1A). Following 4–6 weeks of incubation all remnants of tumor cells were absent and only lymphoblasts, with minimal cytoplasm and large nuclei, were present (Figure 1B).

Ommaya supernatants

Cells were periodically obtained from the Ommaya reservoirs prior to intratumoral rIL-2 and/or TIL infusions. Trypan blue exclusion evaluation showed 95–100% cell viability and lymphocyte incubation studies demonstrated that the infused TILs remained viable during the 2 weeks between TIL infusions. In each of the 6 cases, Wright–Geimsa stained cytocentrifuge preparations from patients, at the time of the final rIL-2 infusion, exhibited a marked increase in the percentage of eosinophils present (Figure 1C).

TIL phenotyping

Greater than 95% of the TILs typed as T-cells (+ CD3) with and 66% of cases demonstrating primarily the suppressor/cytotoxic (CD8) phenotype (Cases 3–6) and 33% of cases expressing the helper/inducer (CD4) phenotype (Cases 1 and 2). In all but one case, TILs avidly expressed the IL-2 receptor (CD25), an early marker of lymphocyte activation. The exception was Case 1, with only 12% of cells expressing the IL-2 receptor. All TIL preparations demonstrated expression of HLA-DR, a late marker of lymphocyte activation. No TIL cultures contained a significant number of NK cells (CD56) or monocytes (CD14), confirming TILs were not phenotypically consistent with either natural killer cells or monocytes (Table 2).

TIL cytotoxicity

Cytotoxicity was noted against all glioma cell lines tested (Table 3). For cases in which autologous tumor could be cultured effectively (Cases 1, 2, 4 and 5), dose dependent cytotoxicity against autologous tumor was demonstrated. Cytotoxicity against autologous tumor was relatively low for Case 1 and relatively high in Cases 2, 4 and 5.

Nonspecific anti-glioma cytotoxicity was demonstrated by measuring the activity of TILs against allogenic tumor lines cultured in our laboratory and against SKMG-1, a long term malignant glioma cell line. Cytotoxicity against the SKMG-1 glioma cell line was relatively low for Cases 1, 4 and 5 and was significantly lower than the cytotoxicity measured against autologous glioma in Cases 4 and 5. In contrast the cytotoxicty of TILs against the adenocarcinoma cell line (HPAF-2) was inconsistent, being relatively low for Cases 2 and 5 and higher for Cases 3, 4 and 6. Autologous glioma cytotoxicty was much greater than HPAF-2 cytotoxicity for Cases 2 and 5.

Nonspecific LAK cytotoxicity was nearly absent, with the exception of Case 2. Natural killer cytotoxicity was insignificant for Cases 4–6, low for Cases 1 and 3, and significant for Case 2.

Case review

Case 1

The first patient was entered into the Pilot TIL immunotherapy study on June 27, 1994. The patient was a 35-year-old woman initially diagnosed, 2 years

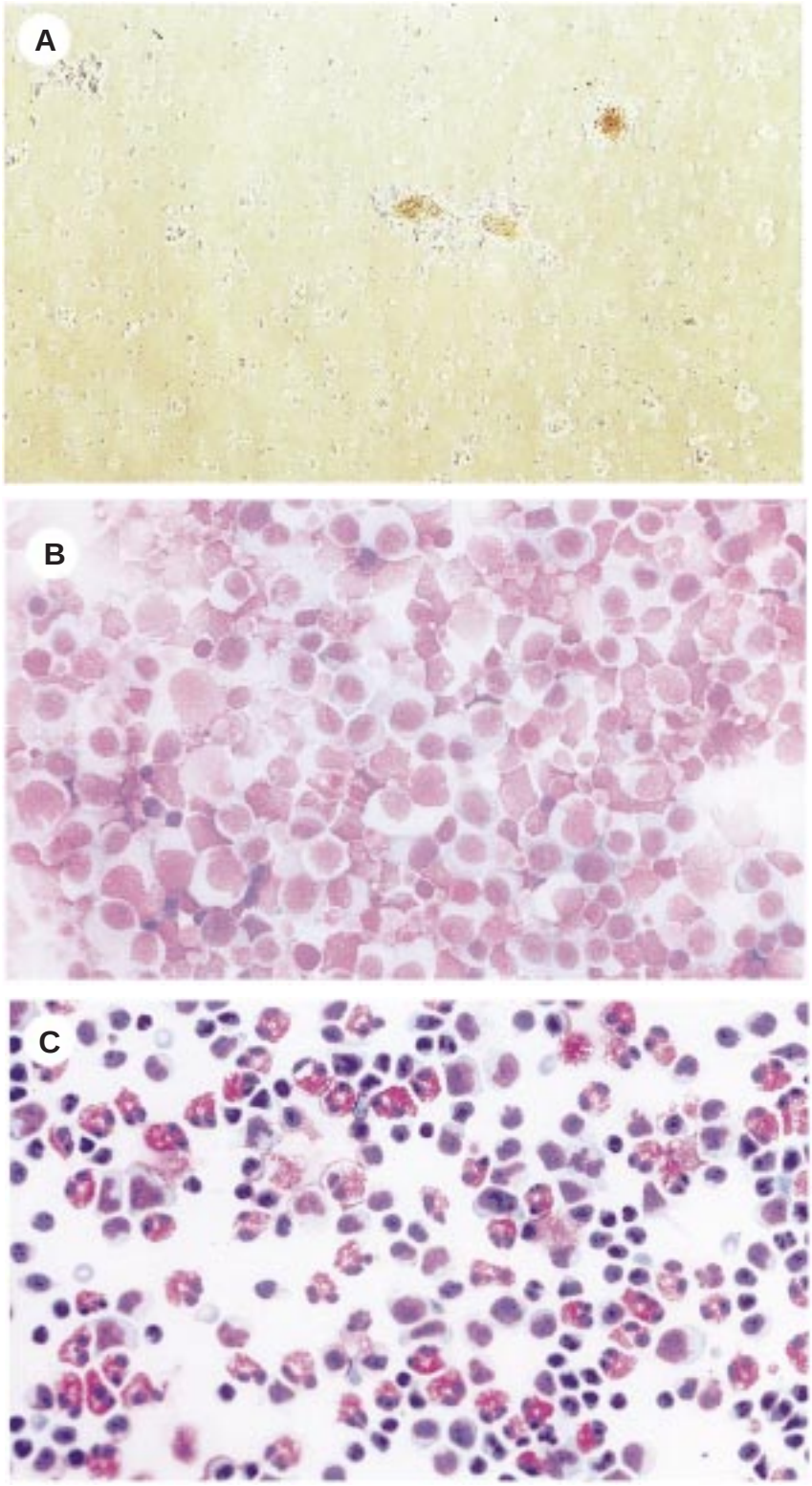

Figure 1. Photomicrographics of TILs. A: TILs surrounding tumor cells following 1 week of incubation in the presence of 1000 U/ml of rIL-2 (unstained at 200× magnification). B: TILs following 6 weeks of expansion with rIL-2, demonstrating lymphoblasts with large nuclei and scanty cytoplasm (Geimsa–Wright cytopreparation at 400× magnification). C: Eosinophilia present within tumor cavity fluid, obtained at the completion of TIL immunotherapy (Geimsa–Wright cytopreparation at 400× magnification).

Table 2. TIL[1] phenotyping by flow cytometric analysis[2]

Patient	% Total cells staining						
	CD3	CD4	CD8	CD14	CD25	CD56	HLA-DR
Case 1	99	99	2	2	12	3	83
Case 2	99	99	2	2	63	2	86
Case 3	99	2	99	1	40	18	98
Case 4	97	7	88	5	50	6	83
Case 5	98	4	95	2	65	1	60
Case 6	99	3	99	1	96	4	99

[1]TILs expanded with 1000 U/ml of rIL-2.
[2]Direct fluorescence flow cytometric analysis.

Table 3. Cytotoxicity of TILs[1]

Case	E/T[2] ratio	Autologous[3] (%)	Allogenic (%)	SKMG-1[4] (%)	HPAF-2[5] (%)	Raji (%)	K562 (%)
Case 1	50 : 1	4	ND[6]	10	ND	0	15
	100 : 1	17	ND	17	ND	0	23
Case 2	25 : 1	19	ND	18	0	12	32
	100 : 1	30	ND	42	5	26	55
	200 : 1	38	ND	59	7	38	62
Case 3	50 : 1	ND	ND	44	33	0	16
	100 : 1	ND	ND	43	36	0	15
Case 4	50 : 1	16	ND	10	22	0	0
	100 : 1	50	ND	12	18	0	0
Case 5	50 : 1	38	28	19	2	2	5
	100 : 1	64	39	29	3	2	8
Case 6	50 : 1	ND	ND	31	15	4	13
	100 : 1	ND	ND	37	25	4	11

[1] Cells expanded in 1000 U/ml of rIL-2. Incubation time of 5 weeks.
[2] E/T ratio: effector/target ratio.
[3] Autologous glioma cells from first passage of operative malignant glioma specimen (Case 3).
[4] Long term human malignant glioma cell line.
[5] Long term human adenocarcinoma cell line.
[6] ND: not done.

previously (December 4, 1992), with a left frontal anaplastic astrocytoma. She subsequently underwent 2 surgical resections, 6 courses of intraarterial cisplatin chemotherapy, with subsequent recurrence, and then progressed during intravenous carmustine (BCNU) chemotherapy based on MR imaging and MR spectroscopy. The patient was subsequently entered into the TIL immunotherapy trial and underwent a left frontal craniotomy with subtotal tumor resection and placement of a left frontal Ommaya reservoir (May 4, 1994). The pathological diagnosis was anaplastic astrocytoma. Following 6 weeks of TIL expansion, repeat MR imaging was performed, revealing midline extension of the tumor across the corpus callosum (Figure 2A). The patient received 3×10^8 autologous TILs, on treatment days 1 and 14. Intralesional rIL-2 was infused at a dose of 1×10^5 IU, three times each week for one month. At the completion of TIL immunotherapy computerized tomography (CT) demonstrated asymptomatic mild cerebral swelling which did not require treatment. No complications occurred and the patient was at her pretreatment neurological baseline following therapy. Following TIL immunotherapy the patient was placed on oral procarbazine (PCZ). Due to profound hematopoietic suppression and non-compliance, the patient never received more than 30% of her calculated dose. Oral PCZ was therefore discontinued after 3 months. Forty-eight months following TIL immunotherapy there was

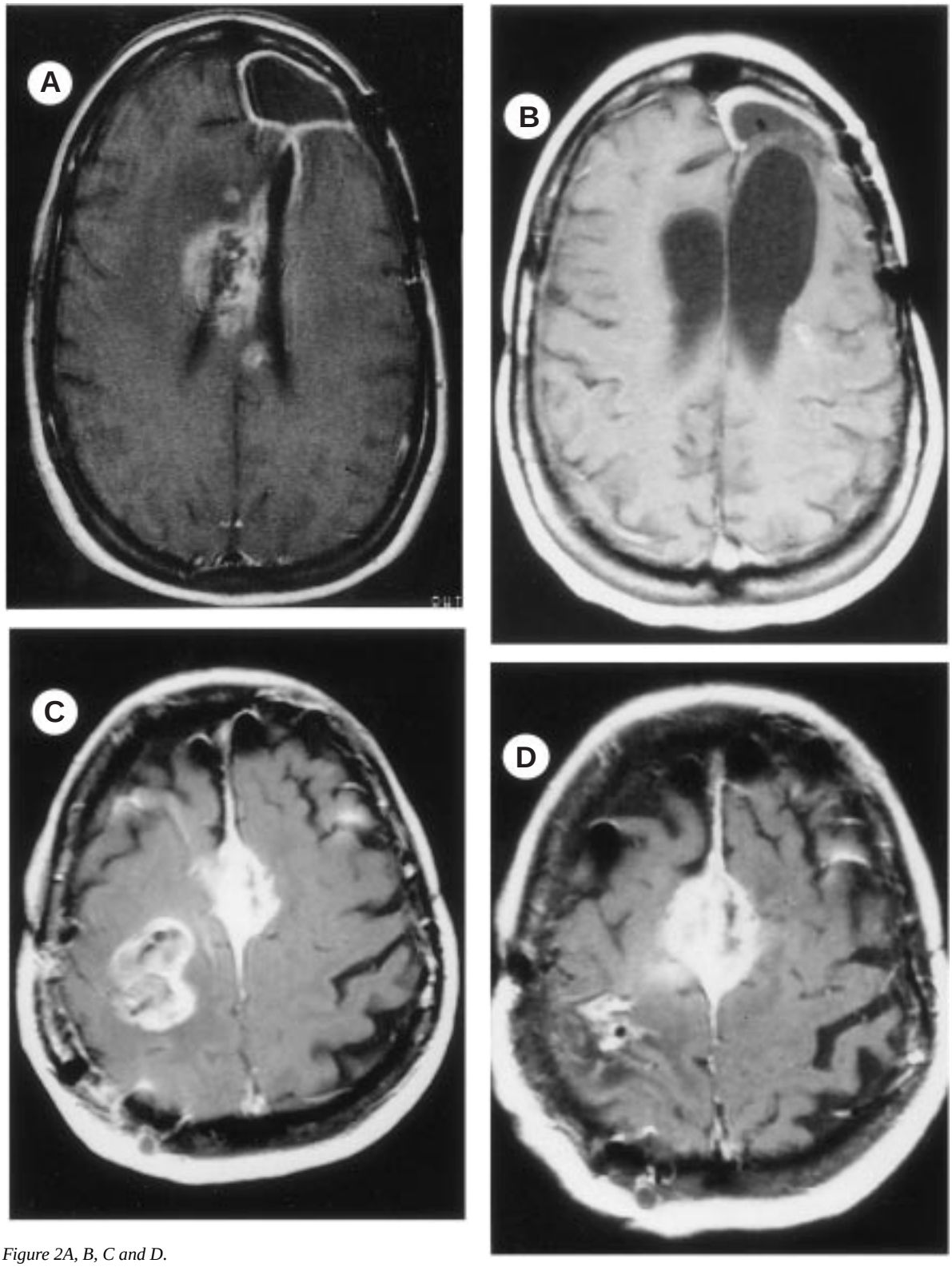

Figure 2A, B, C and D.

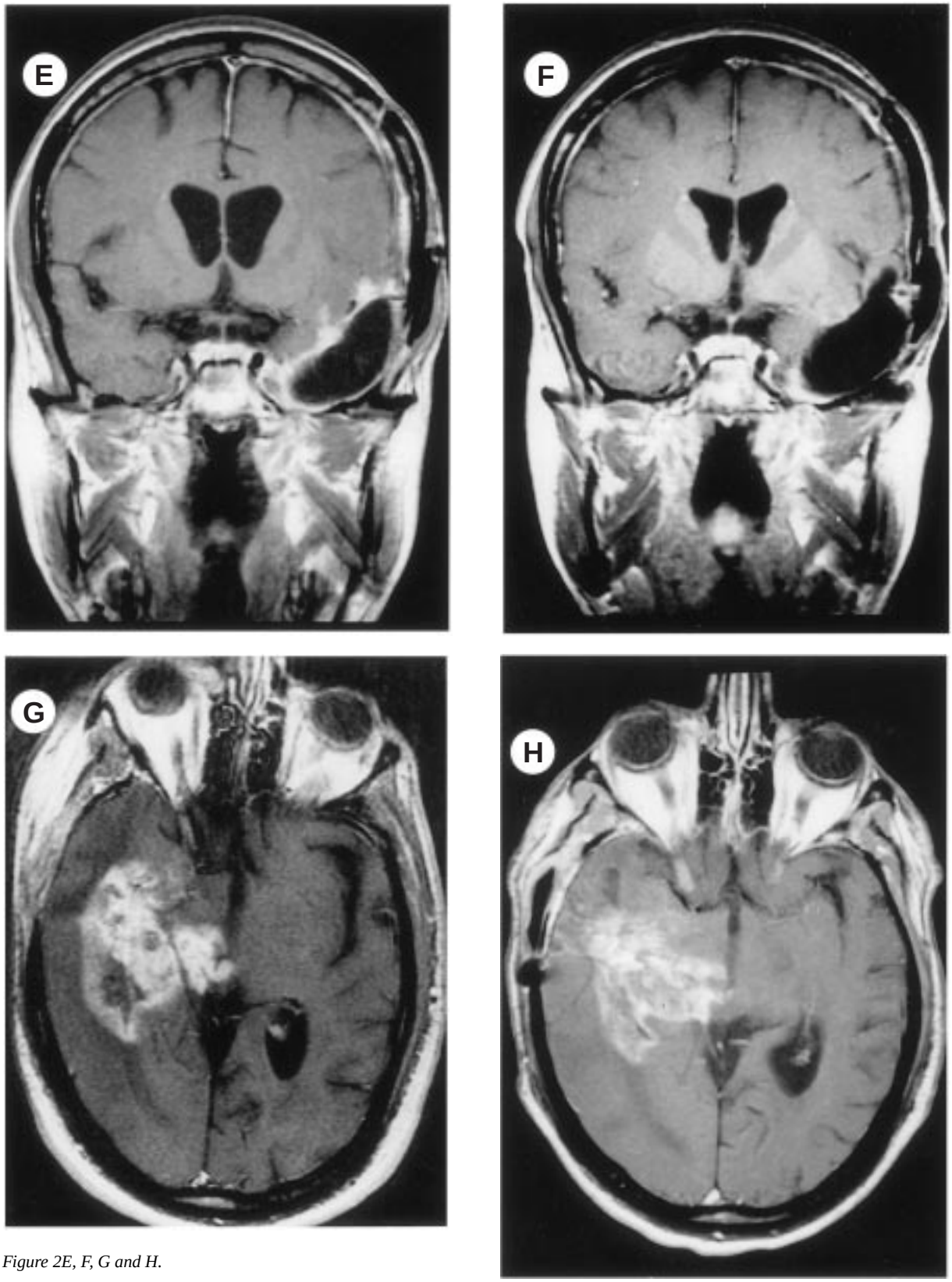

Figure 2E, F, G and H.

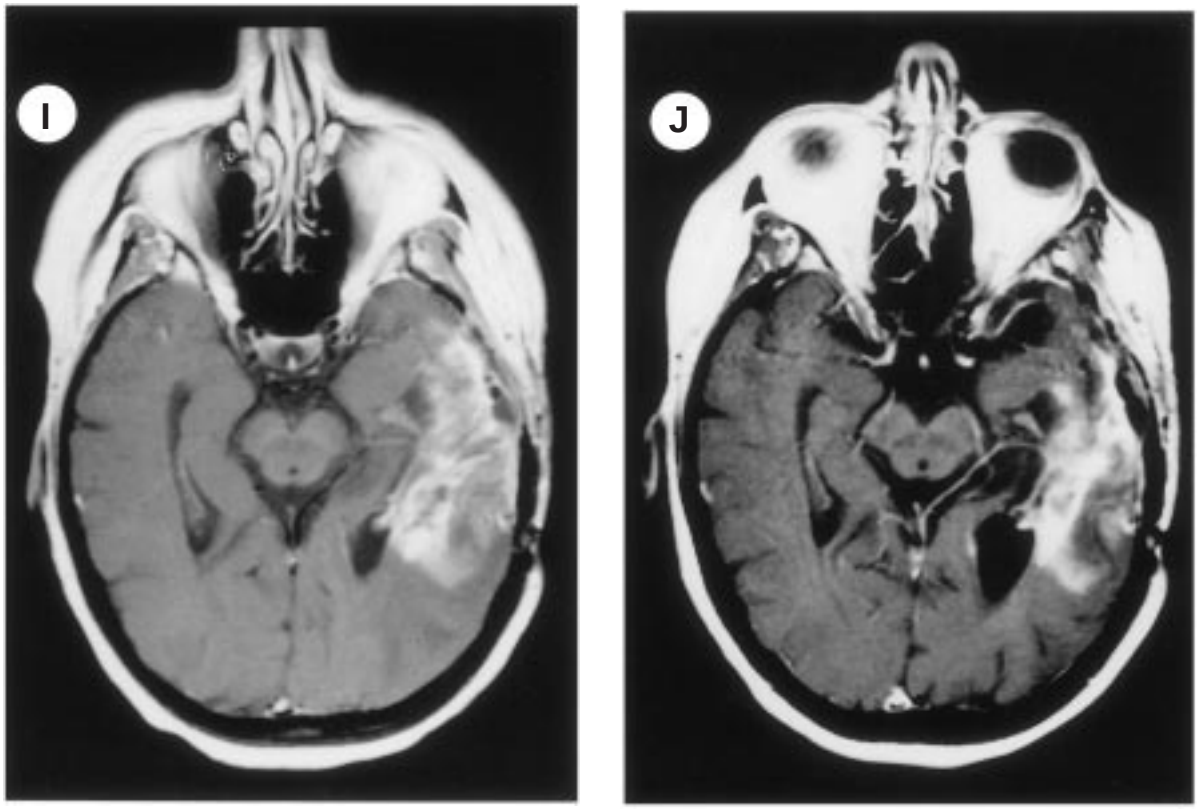

Figure 2I and J.

no evidence of periventricular or midline enhancement (Figure 2B). Six months following treatment the patient developed asymptomatic hydrocephalus (Figure 3A).

Case 2

The second patient entered the Pilot TIL immunotherapy study on August 4, 1994. This patient had previously undergone resection (March 17, 1994) and radiation therapy for a right frontal glioblastoma multiforme (GBM), followed by initiation of BCNU chemotherapy. Following the second course of chemotherapy the patient developed recurrence. The patient as then entered into the TIL immunotherapy study and underwent gross total resection and placement of an Ommaya reservoir. The pathological diagnosis was consistent with GBM. Following surgery, the patient began BCNU chemotherapy. Following 6 weeks of TIL expansion an MR imaging study was repeated (Figure 2C) and the patient subsequently received 2 infusions of 1×10^9 TILs and rIL-2 at the same dose and schedule as the previous patient. At the completion of TIL immunotherapy, CT imaging demonstrated asymptomatic mild cerebral swelling which did not require treatment. The patient requested and was allowed to complete 4 additional courses of BCNU chemotherapy following her immunotherapy treatments. At 47 months following treatment the patient demonstrated a 70% reduction in tumor burden based on MR imaging, representing a partial response to therapy (Figure 2D).

Case 3

A third patient underwent TIL immunotherapy beginning September 19, 1994, receiving 2 infusions of 1×10^9 TILs and rIL-2, at the same dose and schedule as the previous patient. This patient had previously been diagnosed with a left temporal anaplastic astrocytoma on April 8, 1993. Following radiation therapy and intraarterial cisplatin with subsequent recurrence, the patient was placed on BCNU, which was discontinued as a result of worsening pulmonary function. In July 1994 the patient was placed on PCZ, which

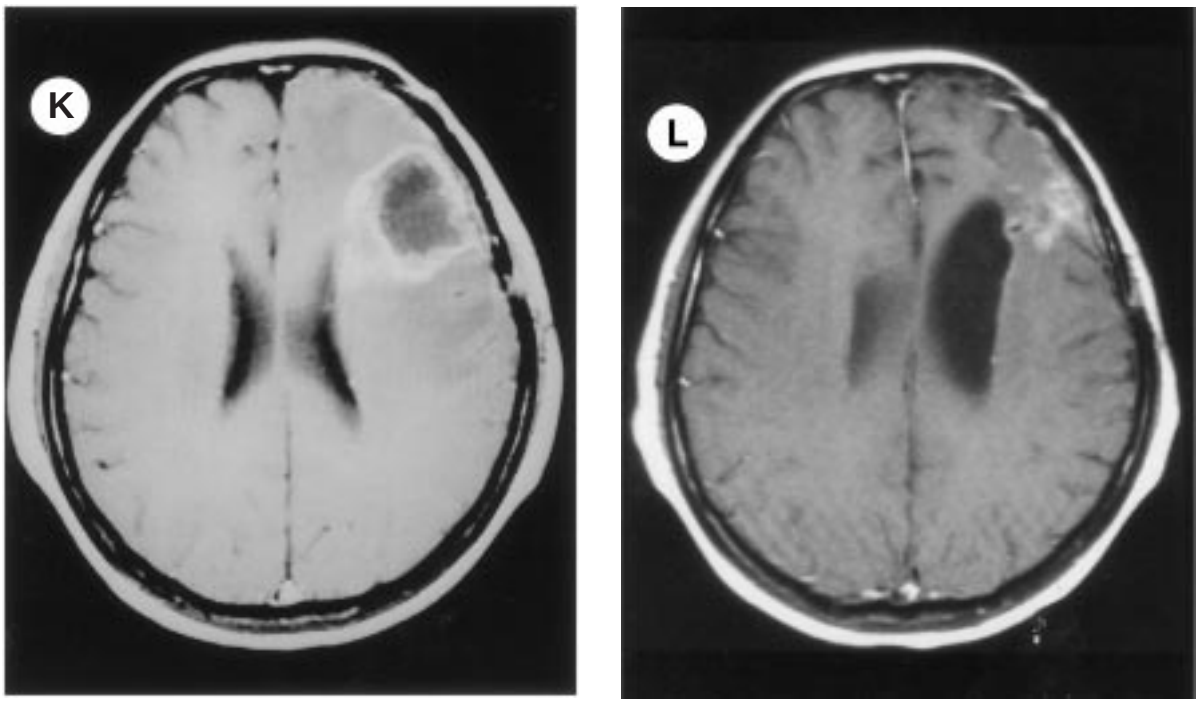

Figure 2. Gadolinium enhanced MR imaging studies just prior to the first TIL immunotherapy infusion and follow-up studies. All pre-immunotherapy MR imaging studies were performed 6 weeks following surgery, at which time TILs had been expanded *in vitro* and were available for infusion. A and B: Case 1, demonstrating partial response to therapy at 48 months. C and D: Case 2, demonstrating a partial response to therapy at 47 months. The centrally enhancing lesion is a previously diagnosed meningioma. E and F: Case 3, demonstrating a complete response to therapy at 45 months. G and H: Case 4, demonstrating progressive disease at 6 months. I and J: Case 5, patient with a partial response to treatment followed by recurrence at 6 months. K and L: Case 6, demonstrating a partial response to therapy at 6 months.

was discontinued when the patient developed a rash. Due to tumor progression the patient entered the TIL immunotherapy study and underwent repeat left temporal resection with placement of an Ommaya reservoir in July 1994. After 6 weeks of TIL expansion, an MR imaging study was repeated (Figure 2E). During and following TIL immunotherapy treatments the patient's neurological status was stable and the patient developed no clinical complications to therapy, other than transient low grade fevers following the second TIL infusion, which did not require treatment. At the completion of TIL immunotherapy, CT imaging demonstrated asymptomatic mild cerebral swelling which did not require teatment. The patient's 45 month post-treatment MR scan demonstrated a complete response (Figure 2F).

Case 4

The fourth patient underwent TIL immunotherapy on January 22, 1994. One year previously the patient had undergone a right temporal craniotomy with subtotal resection of a GBM. The patient underwent local fractionated radiation therapy and BCNU chemotherapy with subsequent radiosurgery, subsequently representing with recurrence. The patients entered the TIL immunotherapy study and a repeat right temporal craniotomy was performed with subtotal resection and placement of an Ommaya reservoir. After 6 weeks of TIL expansion an MR imaging study was performed (Figure 2G). The patient subsequently received 1×10^9 autologous TILs, on treatment days 1 and 14. Intralesional rIL-2 was infused at a dose of 2×10^5 IU, three times each week for one month. Following the completion of TIL immunotherapy, the patient was placed on PCZ. A CT scan following completion of TIL immunotherapy demonstrated mild cerebral swelling, which did not require treatment. The patient continued to progress following TIL immunotherapy (Figure 2H) and expired on January 10, 1995 (12 months following immunotherapy).

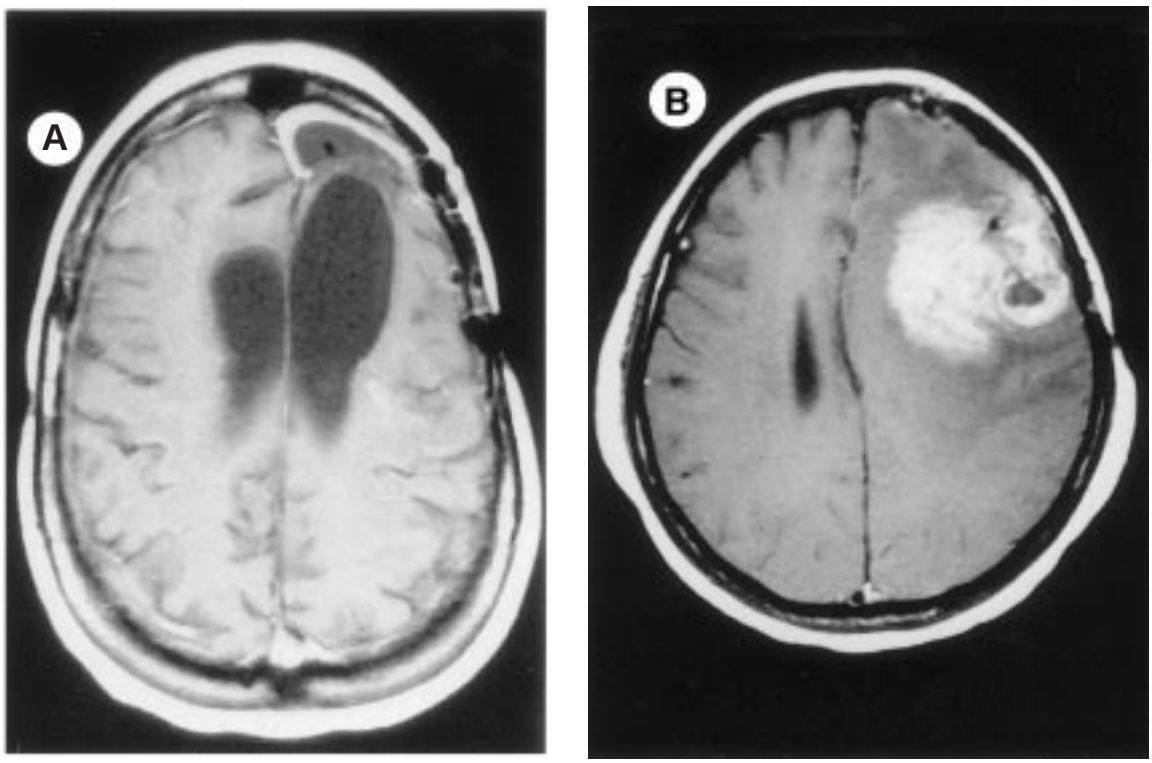

Figure 3. MR imaging studies of asymptomatic hydrocephalus (A) and transient cerebral swelling (B) at the completion of TIL immunotherapy.

Case 5

The fifth patient underwent TIL immunotherapy on March 3, 1995. The patient initially underwent a left temporo-parietal craniotomy with subtotal tumor resection of an anaplastic astrocytoma. The patient then underwent local fractionated radiation therapy and intraarterial cisplatin chemotherapy. The patient recurred and was placed on PCZ, which was discontinued due to the development of a rash. Due to recurrent tumor the patient subsequently underwent left temporal craniotomy with subtotal tumor resection and placement of an Ommaya reservoir. After 6 weeks of TIL expansion, an MR imaging study was repeated (Figure 2I). The patient then received 1×10^9 autologous TILs, on treatment days 1 and 14. Intralesional rIL-2 was infused at a dose of 2×10^5 IU, three times each week for one month. At the complection of TIL immunotherapy, CT imaging demonstrated asymptomatic mild cerebral swelling which did not require treatment. The patient initially demonstrated a partial response with subsequent recurrence, 6 months following treatment (Figure 2J). Intravenous BCNU was then initiated and later discontinued due to continued progression. The patient expired on April 10, 1996 from recurrent tumor (12 months following immunotherapy).

Case 6

The sixth patient underwent TIL immunotherapy on June 2, 1995. The patient was originally diagnosed with GBM in March 1994, following a left frontal craniotomy with gross total tumor resection. The patient underwent local fractionated radiation therapy and progressed on intravenous BCNU. Due to progressive disease the patient underwent a repeat left frontal craniotomy with subtotal tumor resection and placement of an Ommaya reservoir in March 1995. After 6 weeks of TIL expansion, an MR imaging study was performed (Figure 2K). MR imaging at the completion of TIL immunotherapy demonstrated asymptomatic cerebral swelling (Figure 3B). Following TIL immunotherapy the patient was placed on PCZ, but received only 50% of the calculated dose due to severe myelosuppression. The patient demonstrated asymptomatic

hydrocephalus and 50% tumor reduction at 6 months (Figure 2L) with subsequent progression. The patient expired from progressive tumor on December 2, 1996 (18 months following immunotherapy).

Toxicity and response to immunotherapy

There were no Grade 3 or 4 complications. One patient developed transient low grade fevers, which did not require treatment. Local asymptomatic cerebral hyperemia and swelling was seen on all the immediate post-treatment imaging studies. In no case did this require treatment with corticosteroids, as the patients were asymptomatic at the time these imaging studies were performed. In 2 cases the patients subsequently developed asymptomatic hydrocephalus (Cases 1 and 6). Toxicity and clinical outcomes are summarized in Table 4.

At 3 and 6 month follow-up, 3 patients responded with partial response, 2 demonstrated stable disease and 1 patient progressed. At long term follow-up, 1 patient had a complete response (45 month follow-up), 2 had a partial response (48 and 47 month follow-up) and 3 patients expired as a result of progressive disease (at 12, 12 and 18 months following immunotherapy).

Discussion

The reults of this study indicate that TILs can be safely infused locally with rIL-2 for the treatment of recurrent MGs. The use of locally infused TILs and IL-2 was based on laboratory studies of TIL cytotoxicity, animal studies of TIL immunotherapy for MGs and clinical studies of adoptive immunotherapy for the treatment of recurrent MGs [5,16,18,20,21,25,27,28,35,36]. *In vitro* studies have determined that TILs can be expanded from MGs [20,25,27,36,37]. These cells have been shown to be primarily MHC-I restricted cytotoxic T-cells and appear to be locally suppressed by glioma secretion of TGF-β2, PG-E_2 and IL-10 [2,3,14]. Although TILs for MGs appear to be suppressed and proliferate poorly, when stimulated by T-cell growth factors, such as IL-2 or anti-CD3, these cells demonstrate cytotoxicity against MGs [26].

Route of infusion

Numerous animal and clinical studies of adoptive immunotherapy for MGs indicate that both local and systemic routes of infusion can be used safely and possible effectively [5,16–18,20,25,28,35]. The method by which lymphocytes are expanded for subsequent treatment may have a significant impact on whether local or systemic treatment should be considered.

Lymphocytes expanded by exposure to irradiated tumor have been infused by either local or systemic routes. One method involves direct stimulation of autologous peripheral blood lymphocytes by irradiated cells, resulting in TS-PBMCs, which are essentially CTLs [20]. In one clinical trial, intratumoral injections of 10^8–10^9 TS-PBMCs resulted in 2 of 5 patients demonstrating greater than 50% reduction in tumor burden [20].

More recent studies involving tumor stimulated lymphocytes have depended on *in vivo* priming followed by *in vito* expansion. Essentially, irradiated lymphocytes can be injected near draining lymph nodes which, having an intact cyto-architecture, allows for optimal tumor antigen presentation by antigen presenting cells

Table 4. Clinical outcomes of TIL immunotherapy pilot study

Case	Sex	Age	Grade[1]	Complications[2]	Concurrent Rx[3]	Response[4]
Case 1	F	35	AA	TCS, asymptomatic hydrocephalus	PCZ[5]	PR, No progression: 48 months post-IT
Case 2	F	70	GBM	TCS	BCNU	PR, No progression: 47 months post-IT
Case 3	M	30	AA	TCS, fever	None	CR, No progression: 45 months post-IT
Case 4	M	47	GBM	TCS	PCZ[5]	PD, Expired: 12 months post-IT
Case 5	F	48	AA	TCS	PCV[5]	PR, Progressed: 6 months post-IT
Case 6	F	41	GBM	TCS, asymptomatic hydrocephalus	None	PR, Progressed: 6 months post-IT

[1]AA: anaplastic astrocytoma: GBM: glioblastoma multiforme.
[2]TCS: transient cerebral swelling; HA: headache.
[3]PCZ: procarbazine; BCNU: 1,3-bis(2-chloroethyl)-1-nitrosourea; PCV: procarbazine, CCNU and vincristine.
[4]PD: progressive disease; SD: stable disease; PR: >50% reduction; CR: complete response; Post-IT: post-immunotherapy.
[5]Received less than 50% of ideal drug dose secondary to complications, as noted in text.

[17,40]. These nodes can then be harvested for CTL expansion, resulting in highly specific CTL clones which, in animal studies, appear to effectively treat either centrally placed or peripherally placed MGs, following systemic infusion of these autologous CTLs [17,28,40]. A recent clincial study of CTLs produced by injecting irradiated autologous tumor intradermally and expanding T-cells from activated inguinal nodes, treated patients with systemic infusions, with 4 of 8 patients alive 1 year following treatment [28].

Previous studies of TILs in animal models with implanted MGs have supported the use of locally infussed TILs [17,35]. In one study TILs were expanded from tumors which were produced by subcutaneous glioma tumor injection. These TILs were found to eradicate systemically placed, but not centrally placed gliomas, suggesting that TIL immunotherapy might be more effective if infused locally [35]. These results led us to attempt to expand TILs from patients with MGs and to initiate a pilot study in which autologous TILs and rIL-2 were locally infused in the treatment of recurrent MGs.

Expansion of TILs and TIL cytotoxicity

In this study TILs were effectively expanded from tumor samples from 6 consecutive patients with MGs who qualified for this pilot study. Other studies in our laboratory indicate that TILs can be expanded from 70–80% of cases. In the cases described here up to 3×10^9 cells could be obtained from 5 to 15 of tumor tissue.

This study demonstrated that dose dependent autologous tumor cytotoxicity could be obtained in those cases in which autologous tumor was available for study (Cases 1, 2, 4 and 5). There was no significant LAK cell cytotoxicity or NK cell cytotoxicity, except for Case 2. This argues against nonspecific LAK or NK cell cytotoxicity as a mechanism for the tumor cytotoxicity demonstrated. The low level of LAK cell cytotoxicity activity is especially notable in view of the high doses of rIL-2 used during the *in vitro* expansion of the TILs. In addition, for both Cases 4 and 5 the degree of autologous cytotoxicity was also significantly greater than the cytotoxicity observed against the adenocarcinoma cell line (HPAF-2), the long term malignant glioma cell line (SKMG-1), or the allogenic malignant glioma cell lines. Although autologous tumor cytotoxicity was relatively high, there was minimal cytotoxicity against the adenocarcinoma cell lines in Cases 2, 4 and 5, suggesting possible differences in antigen recognition. Taken together, this data suggests minimal selective antigenic recognition by these TIL cultures, most notably in Cases 4 and 5. These findings are consistent with those of other investigators, and as discussed below, TIL cultures produced in this manner have been shown to contain subpopulations of CTLs with more specific autologous tumor activity [20,21,26,31–33].

Controversy regarding the optimal conditions for culturing TILs remains. TIL cultures with autologous tumor activity can be produced with up to 6000 IU/ml of rIL-2, though lower concentrations of rIL-2 are generally used in conjunction with limiting dilutional methods when attempting to clone CTLs with autologous tumor activity [17–21,26,28,30–33]. For example, in one recent study, 6000 IU/ml of rIL-2 was used to expand TILs with autologous tumor activity, with specific clones then being produced by limiting dilutional methods using lower concentrations of rIL-2 (120 IU/ml) [19]. In addition, investigators using lower concentrations of IL-2 generally use T-cell growth factors, such as anti-CD3, and/or T-cell mitogens (such as staphylococcal enterotoxin A), in order to sufficiently expand their T-cell populations for use in clinical studies, often requiring up to 10 cells [18,20,26,28,31,33]. In preclinical studies in our laboratory and others, it has been demonstrated that large numbers of TILs with autologous tumor activity and limited specificity can be expanded using concentrations of IL-2 of up to 6000 IU/ml, without requiring further expansion with potentially hazardous mitogens or staphylococcal enterotoxin A [17–21,26,30–33]. Current research in our laboratory involves the use of high concentrations of rIL-2 to produce TIL cultures, which will then be expanded with anti-CD3 and low concentrations of rIL-2 using limiting dilutional analysis methodology, in order to produce uniform CTL clones from TILs, potentially avoiding injections of irradiated tumor and the use of potentially dangerous mitogens or enterotoxins.

Another area of active investigation involves the expansion of TILs or CTLs by costimulation of CD28 and the TCR, an approach which may help to overcome both IL-2 dependent and IL-2 independent immunosuppression in situ [15].

TIL phenotyping

There was a slightly higher proportion of patients whose TIL populations were cytotoxic/suppressor

(CD8+) T-cells than helper/inducer (CD4+) T-cells. Previous studies of TIL expansion in patients with melanoma and renal cell carcinoma, have also demonstrated a preponderance of CD8+ lymphocytes [31–33]. Sawamura et al. demonstrated that early cultures of TILs from patients with MGs showed an equal proportion of CD4+ and CD8+ lymphocytes, whereas CD8+ lymphocytes were predominant following 3–4 weeks of incubation with rIL-2. Our data is consistent with these observations. It is known that CTLs recognize tumor antigens in association with MHC-I antigens, which have been shown to be present on the surface of unstimulated MGs, *in vitro* [2].

The significance of the 2 cases in which the TILs expressed the CD4+ phenotype is unclear. Previous studies have shown that the CD4+ phenotype can demonstrate cytotoxicity in addition to their helper/inducer function. Induction of the CD4+ phenotype generally requires the present of tumor antigens in association with MHC-II antigens which, at least *in vitro*, tend not to be presence on the surface of unstimulated MGs [2]. Although increased MHC-II expression can be induced by *in vitro* incubation of human malignant glioma cell lines with either TNF-α or INF-γ, incubation with rIL-2 is not effective in increasing MHC-II expression [2]. Nonetheless, the presence of cytotoxic CD4+ TIL cultures suggests that MHC-II antigens may be present in these cases or that the requirement of MHC-II expression was bypassed by the culture conditions. The fact that in most cases expansion of TILs and limiting dilution studies demonstrate CTLs with autologous tumor activity, indicates that some degree of antigen presenting cell activity, likely involving microglia expressing tumor epitopes in association with MHC-II antigens and important co-stimulatory molecules such as B7, may be present *in situ* despite the presence of immunosuppressive factors [36,37]. The use of rIL-2 in this study appears to have resulted in the expansion of this population of CTLs present within the tumor, while also allowing other CTLs present within the tumor to expand, thus resulting in autologous tumor activity, and in most cases, activity against other tumor lines. It is significant that nearly all the cells were T-lymphocytes and there was little evidence for nonspecific LAK cell activity, again supporting the argument that tumor stimulated T-cells may be present *in situ*, but not able to expand *in situ*, likely due to the presence of immunosuppressive factors.

Toxicity

There were no Grade 3 or 4 complications. The finding of transient cerebral swelling immediately following TIL immunotherapy has been previously published for this group of 6 patients [39]. In each case the imaging studies demonstrated increased nodular, irregular enhancement in the tumor bed, increased perilesional signal on T2-weighted images consistent with vasogenic edema, and increased mass effect upon adjacent structures. Follow-up MR imaging studies demonstrated resolution of this so-called asymptomatic 'flare' response in each case. The 'flare' response may represent transient cerebral edema and hyperemia (Figure 3A).

Two patients (Cases 1 and 6) developed asymptomatic hydrocephalus. Of note both of these patients had evidence of transient cerebral swelling and both had tumors within 2–5 mm of the ventricular wall. Although the ventricular ependymal wall was not violated during surgical resection in either case, it seems possible that the close proximity of the ventricular wall could result in either TILs or rIL-2 escaping into the cerebrospinal fluid. This finding should be kept in mind when interpreting the results of other immunotherapy protocols in the future.

This toxicity data demonstrates that TIL immunotherapy appears to be safe at the current dose of TILs and rIL-2. The asymptomatic complications did not require any alteration in treatment or discontinuation of immunotherapy. The presence of cerebral hyperemia and swelling (so called 'flare' response) in each case raises the question of possible symptomatic cerebral swelling at higher doses of rIL-2. In one recent study by Hayes et al., local infusions of LAK cells and rIL-2 demonstrated that rIL-2 doses of up to 1.2×10^6 IU could be safely tolerated in patients with recurrent MG [16]. A recent study of systemic CTL infusions and systemic rIL-2 infusion did result in a significant number of Grade I and Grade II complications, including fevers, chills, nausea/vomiting, hypotension, somnolence and confusion [28]. Of note, 5 of 10 patients treated with systemically delivered CTLs required corticosteroid treatment for headaches and progression of preexisting neurological symptoms. These toxicities, while not of long term consequence, appear to be more extensive than those seen with local autologous LAK cell infusion, local autologous TS-PBMC infusion or local autologous TIL infusion [5,16,18,20,25]. Further studies will be necessary to determine whether

systemic infusions of CTLs and rIL-2 are more or less toxic than treatments locally [16,28].

In situ *studies*

Lymphocytes removed at treatment day 14, just prior to the second infusion of TILs, were noted to be viable by trypan blue exclusion (greater than 90%). In this study it is not possible to determine whether these lymphocytes were recruited or were those infused on treatment day one. Of interest, there was a significant increase in the number of eosinophils present towards the end of treatment. This may be due to the effects of rIL-2, which has been shown to result in a relative eosinophilia in a small percentage of patients treated with systemic rIL-2 [16]. The significance of this finding is speculative, but questions of possible IgA or IgE mediated antibody dependent cell cytotoxicity, should be explored.

Extent of resection or chemotherapy treatment and response to TIL immunotherapy

Three of the 6 patients underwent gross total tumor resections (Cases 2, 3, 6). Of these 1 had a complete response (Case 3) and 2 had a partial responses (Cases 2, 6). Of those patients with subtotal resections, 2 had a partial response (Cases 1 and 5) and 1 had progressive disease (Case 4). No definitive statements can be made with respect to the relationship of the extent of resection to outcome in this small pilot study.

In this pilot study, it was a requirement of the Committee on the Rights of Human Subjects that the option of chemotherapy following immunotherapy be discussed and offered to all patients. The rational was based on the lack of clear evidence for the efficacy of adoptive immunotherapy and also based on the determination that mild immunosuppression with cyclophosphamide, during adoptive immunotherapy, may actually enhance the response *in vivo*, possibly be selective suppressor T-cell inhibition [21]. Four patients (Cases 1, 2, 4, 5) requested chemotherapy following TIL and rIL-2 infusions. Three of these patients received less than 50% of their calculated doses (Cases 1, 4 and 5) due to systemic toxicity. Of note, the patient with a complete response (Case 3), received no chemotherapy. There was no relationship between the administration of chemotherapy and either response or toxicity in this study, but the possible contribution of chemotherapy to patient response cannot be dismissed.

Conclusions

Effective treatments for MGs, the most common primary tumor of the brain, are not available. One novel approach to the treatment of these tumors is local adoptive immunotherapy with autologous TILs expanded from patient tumor specimens in the presence of rIL-2. Previous studies have determined that TILs demonstrate TCR mediated and MHC restricted cytotoxicity against autologous tumor. This form of adoptive immunotherapy has been utilized in the treatment of refractory melanoma and renal cell carcinoma.

In this pilot study we demonstrate that TILs can be effectively expanded, in the presence of rIL-2, from patients with MGs. These lymphocytes were found to variably express either the helper/inducer (CD4) or cytotoxic/suppressor (CD8) phenotypes and demonstrated partial selective cytotoxicity against autologous and allogenic malignant glioma cell lines. TILs at a dose of 1×10^9 cells/infusion and rIL-2 at a concentration of 4×10^5 IU/ml could be infused without significant toxicity. Further studies are indicated in order to determine the upper dosing limits at which rIL-2 and TILs can be safely infused, and to determine the therapeutic efficacy of TIL immunotherapy.

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