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Clinical Study

Therapy of recurrent high grade gliomas with surgery, and autologous mitogen activated IL-2 stimulated killer (MAK) Lymphocytes: I. Enhancement of MAK lytic activity and cytokine production by PHA and clinical use of PHA

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Abstract

Nineteen patients with recurrent high grade gliomas were treated in a phase I/II trial with aggressive debulking of the tumor, mitogen activated IL-2 stimulated peripheral blood lymphocytes (MAK cells), and rIL-2. Phytohemagglutin (PHA) was introduced into the tumor site in 16 patients prior to implanting MAK cells and IL-2 in an attempt to trigger more effective lysis of the tumor *in vivo*. *In vitro* both TNF bioactivity and cytolytic activity of long term cultured MAK (LMAK) cells were dramatically enhanced by adding PHA to the cultures of these activated PBL. Three of eleven patients (27%) had a decrease in size of the enhancing lesion on CT and/or MRI. Seven (37%) patients clinically improved. Median survival after therapy was 30 weeks. PHA was shown to be safe *in vivo* and more effective than IL-2 triggering enhanced effector function *in vitro*.

Introduction

Lymphocytes can be activated *in vitro* with Interleukin-2 (IL-2) to produce cells that can kill tumor cells in a non-major histocompatibility restricted (MHC) manner, and secrete lymphokines *in vitro* [1–4]. There are several types of lymphokine-activated effector cells: A) Natural Killer (NK)-like cells derived lymphokine-activated killer (LAK) cells [2, 3], B) non-MHC restricted T killers (mitogen activated killer (MAK) cells [1, 3–5], and C) tumor infiltrating lymphocytes (TIL's) [6, 7]. These activated lymphocytes when administered systemically along with IL-2 have been employed to treat metastatic melanoma, sarcomas, adenocarcinomas, and implanted gliomas, melan-

oma, and sarcomas in animal models. This type of immunotherapy is effective in patients with melanoma and renal cell carcinoma although the response rate is in the range of 20–35% [8–14].

Patients with high grade gliomas have a poor prognosis despite therapy with surgery, radiation and chemotherapy [15–17]. An approach to the therapy of high grade gliomas is to combine surgical debulking with local implantation of activated lymphocytes and IL-2. Theoretically, the implanted lymphoid cells could induce the destruction of the remaining glioma either directly via direct tumor cell lysis or indirectly via the production of cytokines. An animal model has demonstrated that LAK cells can prolong the survival of rats when implanted with an aggressive glioma [18]. Five pre-
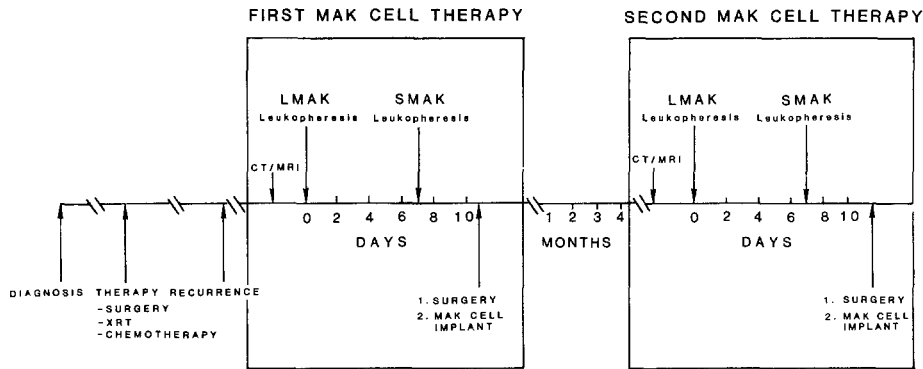
Fig. 1. Summary of leukapheresis and surgery schedule.

vious phase I or I/II studies have treated patients with recurrent high grade gliomas with activated peripheral bloodlymphocytes [18–22]. Two of these studies have reported improvement in survival more than would be expected from surgery alone [19, 22].

This phase I/II study reports the results of treating high grade glioma patients with surgery, and activated lymphocytes that are stimulated *in vivo* with IL-2 alone or IL-2 combined with phytohemagglutinin (PHA). We describe a new approach to trigger more effective cytotoxic function of MAK cells with PHA. Preliminary clinical data are presented examining the role of PHA as part of the immunotherapy protocol to treat recurrent high grade gliomas.

Materials and methods

Patient selection and protocol design

Patients were eligible for this trial if they met the following criteria: 1) age 18 or older; 2) histologic grade III or IV glioma (Daumas-Duport/Burger Classification [23, 24]); 3) Karnofsky score greater or equal to 60; 4) recurrent tumor of sufficient size and location to warrant surgery; 5) exhausted conventional therapeutic alternatives; and, 6) adequate hematologic status to allow leukapheresis.

The current protocol calls for surgical debulking of the glioma followed by implantation of activated lymphocytes (MAK cells) combined with rIL-2 (RO-23-6019) (Hoffman-LaRoche, Nutley, NJ)

(see Fig. 1). If the patient survived four months, each patient underwent a second round of therapy with surgery, MAK cells and rIL-2. At entry into the study the patients undergo a complete physical examination, appropriate blood work and computed tomography (CT) or magnetic resonance imaging (MRI) of the brain. Leukocytes were collected from the patients either by venipuncture or leukapheresis. Leukapheresis was done using the Fenwal CS-3000 Blood Cell Separator. Approximately 10^9 cells were collected from leukapheresis of 1–2.5 liters of blood. These cells were stimulated for 48 hours with PHA and cultured in IL-2 (RO-23-6019) for approximately 10 days, as described initially by Ingram *et al.* [19] and further characterized by Yamamoto *et al.* [4]. A second leukapheresis was done 3–4 days before surgery to obtain 10^9 cells. These cells were cultured with PHA and rIL-2, as described below, for 3–4 days before implantation into the surgical cavity. We refer to long-term cultures (LMAK cells) as cells that are cultured *in vitro* for 10 days or longer; short term cultures (SMAK cells) as cells that are stimulated *in vitro* for 3–4 days.

At the time of surgery, aggressive surgical debulking of the tumor mass was done. The LMAK and SMAK cells were combined and 10^5 units of rIL-2 added to the cells. The cells and rIL-2 were implanted into the surgical defect and the defect was closed. CT and/or MRI scans (with contrast) done preoperatively, were compared with scans done 40–70 days postoperatively to evaluate change in tumor size after surgery and immunotherapy. Clinical response to therapy, and survival

duration were also employed to asses the toxicity and efficacy of the surgery and immunotherapy.

There are three arms to the current protocol. In the first arm of the protocol the patients are treated with surgery, MAK cells and rIL-2 (as just described). In the second arm of the protocol, patients are treated at the time of the first surgery with MAK and rIL-2, but at the second surgery the patients are treated with MAK, rIL-2 after flooding surgical site with PHA. In the third arm of the protocol, at both the first and second surgery the patient is treated with MAK cells, and rIL-2 after flooding the surgical site with PHA. The PHA was administered by dissolving 3 mg PHA HA17 (Wellcome Diagnostics, Temple Hill Dartford, England) in 10 ml physiological saline and placing the PHA in the surgical defect to coat the tumor cavity for 15 minutes. The residual PHA containing fluid was aspirated and the SMAK and LMAK cells, and IL-2 implanted. All patients were operated upon by the same neurosurgeons. This phase I/II protocol has been approved by the F.D.A. (BBIND2774-G) and the Tustin Medical Center Institutional Review Board.

Mononuclear cell collection

Patients peripheral blood leukocytes (PBL) were collected by venipuncture or leukapheresis. Sixteen patients PBL were collected by leukapheresis. Three patients (# 1, 2, 11) PBL and normal volunteer PBL were separated from whole blood. Peripheral blood (300–500 ml) were drawn into a blood bag unit containing anticoagulant CPD solution. The blood was diluted 1:1 in Hanks balanced salt solution (HBSS) (Gibco, Grand Island, NY). Thirty five ml of the diluted blood is layered onto 15 ml of Histopaque 1.077 (Sigma, St, Louis, Mo) to separate leukocytes from red blood cells. Leukapheresis is done with the Fenwal CS-3000 Blood Cell Separator (Fenwal, Baxter Healthcare Corp., Deerfield, Ill). Approximately 10^9 cells are collected from the pheresis of 1–2.5 liters of blood. Further elimination of red blood cells (RBC) and platelet contamination is accomplished by separation on 1.077 Histopaque. The mononuclear cells are

washed three times with HBSS followed by centrifugation at $500 \times G$ for 10 min to remove the remaining Histopaque.

Generating mitogen simulated, lymphokine-activated killer (MAK) cells

Human peripheral blood mononuclear cells (PBM) are cultured for 48 hours in Aim-V (Gibco) supplemented with 2% heat-inactivated fetal bovine serum (FBS) (Gibco), 0.4 ug/ml PHA-P (Sigma) and 200 units recombinant human interleukin-2 (rIL-2, RO-23-6019) (Hoffman-LaRoche, Nutley, NJ). These cells are cultured at a density of 2×10^6 cells/ml in either T-150 cm^2 tissue culture flasks or 3-liter PL-732 Fenwall tissue culture flasks. After 48 hours at 37°C, the cells are centrifuged to remove the media and PHA, the cells are counted, and viability determined using trypan blue dye exclusion. The cells are then resuspended in fresh Aim-V containing 200 units/ml rIL-2 at a concentration of 0.5×10^6 cells/ml.

Each time the cultures are expanded and transferred to new flasks, a 3 ml sample of the culture is used to test for sterility. One ml each of the culture is added to either Septi-Chek-TB or Septi-Chek-Thioglycollate (Roche Diagnostic Systems, Nutley, NJ). One ml of the culture is centrifuged to remove the media and the pellet is resuspended in 3 ml of RPMI 1640 (Gibco) with 10% FBS but no antibiotics. The test samples are incubated at 37°C and checked daily microscopically for signs of contamination.

Target cells for the ^{51}Cr release assay

Two continuous human cell lines are employed for target cells in these studies: A) U373MG, a glioblastoma cell line obtained from American Type Culture Collection Rockville, Md) and B) K562, a NK sensitive erythroleukemia cell line. The U373 is as adherent cell line and the K562 is a nonadherent cell line. Both are passed regularly and maintained in RPMI 1640 with 10% Fetal bovine serum (FBS) at 37°C.

Cytolytic ^{51}Cr release assay

Cytolytic assays employing non-adherent target cells K562 are performed in 96 well round-bottomed assay plates, whereas cytolytic assays for adherent U373 MG cells are performed in 96 well flat-bottomed assay plates as described by Yamamoto *et al.* [4]. Radiolabeling of the non-adherent target cells is accomplished by the addition of 20 ul of ^{51}Cr with a specific activity of 183 mCi/mg (ICN, Irvine, CA), at a concentration of 5 mCi/ml to 10^6 cells in 1 ml of serum free media for 1–2 hours at 37° C. After 1–2 hours at 37° C, the cells are washed 3 times (300 × G for 5 min) with cold RPMI-10% FBS. A constant number of labeled target cells (2×10^4 in 20 ul) is added to assay plate wells with variable numbers of effector cells. Radiolabeling of adherent target cells is accomplished by the addition of 25 ul of ^{51}Cr to 1.5×10^6 cells in 10 ml of RPMI-10% FBS . The radiolabeled cell suspension is then dispensed 100 μl/well in the flat-bottom assay plate. After incubation for 24 hours at 37° C, the labeled cells are washed with 37° C RPMI-1640 to remove free isotope. Effector cells are added to the target cells at various effector-to-target-cell ratios. Both non-adherent and adherent target cell cultures are incubated at 37° C for 4 hours. The release of ^{51}Cr is measured by the uptake of cell-free supernatants with Titertek Supernatant Collection System (Skatron, Norway) and quantified in an automated Biogamma counter (Beckman, Fullerton, CA). The total ^{51}Cr releasable (90–95% of total counts) is determined by lysing the cells with 100 μl of 3% (w/v) sodium dodecyl sulfate solution. Spontaneous ^{51}Cr release is 1–2% per hour. Percent lysis is determined by the following formula:

$$\frac{(\text{Experimental Release}) - (\text{Spontaneous Release})}{(\text{Total Release}) - (\text{Spontaneous Release})}$$

$\times 100 =$ Specific Lysis

Phenotype analysis

Cell surface markers on cultured and fresh lymphoid cells are analyzed by using monoclonal anti-bodies: Leu-4 (CD3, Pan T), Leu-3 (CD4, helper/inducer), Leu-2 (CD8, cytotoxic/suppressor), and Leu-11 (CD16, NK) (Beckton Dickinson, San Jose, CA). The monoclonal antibodies are either fluorescein (FITC) or phycoerythrin (PE) conjugated. Lymphocytes are incubated with desired monoclonal antibodies (according to the manufacturer's recommendation) for 30 minutes on ice and washed twice with PBS by centrifugation for 5 minutes at 1600 rpm. Lymphocytes are fixed in a 0.5% paraformaldehyde solution and stored at 4° C until analysis. The lymphocytes then are analyzed by using FACScan (Becton Dickinson, San Jose, CA). Cells are gated on forward vs. side scatter to obtain a uniform distribution and the green (FL1) or red (FL2) fluorescence is measured by analyzing the cells stained with FITC or PE labeled antibodies, respectively. Wherever appropriate, the dual color analysis is performed. Cells stained with isotypic control antibodies are used to set markers for background fluorescence. Data are expressed as percent positive cells for corresponding cell surface antigen.

Tumor necrosis factor (TNF-alpha)/lymphotoxin (LT, TNF-beta) bioassay

This TNF/LT bioassay is a microassay described in detail by Jeffes *et al.* [25]. Briefly, L-929 target cells were plated at a concentration of 20,000 cells/well in 0.1 ml of RPMI-3% in 96 well flat-bottomed microtiter plates (Corning, Corning, N.Y.). The cells were treated with 0.5 ug/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo.) and incubated for 24 hours in a 37° C humidified CO_2 incubator (95% air, 5% CO_2). After 24 hours of incubation, both NaF (at a final concentration of 2 mM/well) and tenfold serial dilutions of recombinant human LT or TNF were added to each well. The L-929 target cells were incubated for an additional 24 hours in a humidified CO_2 incubator. After incubation, the media was aspirated and the plate was stained with 0.2 ml of a 2% crystal violet solution (40% ethanol and 60% H_2O) for 5 minutes at room temperature. Excess dye was rinsed from the microtiter plate with distilled water. The crystal

violet was dissolved from the cells with 0.1 ml of acidified alcohol (methanol, 150 mM HCI). Microtiter plates were read by a Titertek Multiscan plate reader equipped with a 580 nm interference filter. Percent lysis was determined by the following formula:

$$1 - \frac{(O.D, \text{ of LT or TNF treated wells})}{(O.D. \text{ of non-treated wells})} \times$$

(100) = percent lysis

Results

Clinical characteristics of the study population

The clinical characteristics of the 19 patients entered into the study are summarized in Table 1. The mean age in the patients in this study was 45 years. There were 16 males and 3 females; 14 grade IV gliomas, and five grade III gliomas. The mean tumor volume of the main tumor site was measured radiographically; eight patients had tumors smaller than 90 cm^3, 5 patients had intermediate sized tumors, and one patient had a very large (> 700 cm^3) tumor. Mean Karnofsky at entry into the protocol was 72.

Characteristics of the MAK cells implanted

The mean number of MAK cells implanted after surgical debulking of the gliomas was 20×10^9 and ranged from 2.2×10^9 to 58×10^9 (Table 2). These MAK cells were composed on average of 92% LMAK cells (cell cultured in vitro > 10 days) and 8% SMAK cells (cells cultured in vitro < 3–4 days). Based on the measured phenotypes of SMAK and LMAK, and the cell number of each implanted, we estimate that 91% of the implanted MAK cells were CD3$^+$ T cells. The MAK effector cells phenotype data is shown in Table 2. The mean percentage of cells having the following cell surface markers are: 1) For LMAK – 92% CD3$^+$, 34%

Table 1. Patient history

Patient Number	Sex	Age	Tumor location	Tumor grade	Prior* treatment	Tumor size (cm³) Pre-MAK I⁺⁺	
						CT	MRI
1	M	57	Left Occipital	IV	RT	56	ND
2	M	56	Left Parietal	III/IV	RT,CT	110	ND
3	M	58	Left Parietal	IV	RT	60	ND
4	M	57	Left Front Parietal	III/IV	RT,CT	69	ND
5	M	36	Left Frontal	III/IV	RT,CT	157	222
6	M	26	Right Parietal Occipital	III/IV	ND	ND	10
7	F	31	Left Parietal	III	RT	12	19
8	M	36	Right Occipital	III/IV	RT,CT	721	864
9	M	55	Left Front Parietal	III/IV	RT,CT	110	142
10	M	42	Right Temporal	IV	RT	27	46
11	F	38	Left Temporal Parietal	IV	RT	91	140
12	M	54	Right Temporal	II/III	RT	ND	ND
13	M	38	Left Frontal Temporal	II/III	RT	231	184
14	M	36	Left Frontal	IV	RT,CT	ND	218
15	M	28	Left Frontal	IV	ND	ND	154
16	M	39	Left Frontal	II/III	RT,CT	ND	237
17	F	63	Left Parietal Occipital	III	RT	67	91
18	M	66	Right Temporal Parietal	IV	RT	ND	372
19	M	38	Right Temporal Parietal	IV	ND	88	63

* CT = Chemotherapy RT = Radiation Therapy ND = Not Done
⁺⁺ CT = Computerized Tomography; MRI = Magnetic Resonance Imaging; prior to therapy.

CD4$^+$, 39% CD8$^+$, 21% CD16$^+$ and 73% HLA-Dr$^+$; 2) For SMAK – 80% CD3$^+$, 42% CD4$^+$, 36% CD8$^+$, 13% CD16$^+$, and 45% HLA-Dr$^+$. The data in Table 2 show that our first 5 cultures had high levels of CD 16$^+$ cells, and that all but one culture since then has less than 6% CD16$^+$ cells. Although the data are not shown, considerable variation patient to patient in percentages of CD4$^+$ and CD8$^+$ cells was noted despite identical culture conditions employed to stimulate the peripheral blood lymphocytes.

Lytic activity of unstimulated PBL, SMAK, LMAK assayed on K562 and the glioma, U373 target cells

The unstimulated PBL and MAK effectors were extensively tested *in vitro* for their ability to lyse the NK sensitive K562 and NK resistant U373 (glioma) tumor target cells. Representative data demonstrating lytic activity of PBL, SMAK, and LMAK cells assayed at a 25:1 effector to target ratio in a 4 hour ^{51}Cr assay is shown in Fig. 2. The patients lytic activity was categorized on K562 and U373 as 'good' if it had mediated > 60% lysis, 'marginal' if it had mediated > 30%–60% lysis, and 'poor' if it had mediated < 30% lysis. The data in Fig. 2A demonstrates that 18% of patients have PBL that are already good effectors on K562 target cells before stimulation *in vitro*. After stimulation for 3–5 days *in vitro* with PHA and rIL-2 (see material and methods) to generate SMAK effectors, 76% of patients have good effectors on *in vitro* testing on K562. After stimulation *in vitro* for 10 days to generate LMAK effectors, 63% of the patients had good effector on *in vitro* testing. Thus, SMAK and

Table 2. Composition of MAK cells implanted in glioma patients

| Patient number | Number of cells implanted X10^9 | | | | | | Phenotype of MAK at surgery I | | | |
| | Surgery$^+$ | | % SMAK^{++} | | % LMAK^{+++} | | SMAK (%) | | LMAK (%) | |
	I	II	I	II	I	II	CD3$^+$	CD16$^+$	CD3$^+$	CD16$^+$
1	4.1	24.0	0	5	100	95	81	13	95	25
2	2.2	42.8	10	0	90	100	84	37	77	40
3	6.3	26.0	27	7	73	93	73	26	97	75
4	12.0	20.5	8	4	92	96	81	24	96	62
5	12.0	22.0	2	3	98	97	87	40	97	88
6	58.0	35.0	1	3	99	97	80	9	97	3
7	20.0	47.0	7	1	93	99	80	0	98	4
8	6.3	ND	52	ND	48	ND	64	0	90	3
9	16.0	5.0	2	2	98	98	84	8	93	5
10	13.6	ND	2	ND	98	ND	76	30	98	1
11	10.0	22.0	2	7	98	93	89	5	ND	ND
12	20.0	ND	4	ND	96	ND	85	6	94	3
13	16.8	ND	1	ND	99	ND	ND	ND	ND	ND
14	50.0	35.0	4	2	96	98	ND	1	ND	1
15	24.0	35.0	1	12	99	88	ND	2	ND	1
16	30.0	3.5	1	2	99	98	ND	0	ND	0
17	4.0	8.4	11	7	89	93	ND	1	ND	1
18	10.0	ND	10	ND	90	ND	ND	ND	ND	3
19	10.0	10.0	1	4	99	96	ND	6	ND	0

$^+$ = Total number of SMAK + LMAK implanted at time of surgery + 10^9. Surgery I and Surgery II indicated by I and II below the relevant column.
$^{++}$ = The % of implanted cells that were SMAK. I & II Indicate Surgery I and Surgery II.
$^{+++}$ = The % of implanted cells that were LMAK at Surgery I and Surgery II.
ND = Not Done.

LMAK effectors have similar distribution of effector function when assayed on K562 target cells. Furthermore, both SMAK and LMAK kill K562 better than unstimulated PBL. Surprisingly, 18% of the patients had unstimulated PBL with good effector function (> 60% lysis at a 25:1 E:T ratio) when assayed on K562. Normal individuals PBL tested and analyzed in this manner show few (< 5% of normal volunteers) that could kill this well at an E:T of 25:1. This suggests that lymphocytes from some patients are already activated *in vivo* prior to stimulation *in vitro*.

The data in Fig. 2b show the distribution of the patients effector cell lytic function assayed on U373 glioma target cells at an E:T of 25:1. Seven percent of patients have unstimulated PBL that are good effectors assayed on U373 targets. After stimulation for 3–5 days, 47% of the patients effectors are good killers (SMAK) when assayed on U373. Only 11% of the patients generate good killers (LMAK) assayed on U373 after 10 days of activation and proliferation driven by rIL-2 *in vitro*. LMAK cells are better effectors than unstimulated PBL when assayed on U373 target cells (Good + Marginal Categories: Unstimulated PBL-14% vs LMAK-72%). Thus, unstimulated PBL in general kill U373 glioma poorly. LMAK and SMAK effectors from glioma patients kill K562 equally well, but LMAK are slightly less effective killer lymphocytes when compared with SMAK when assayed on U373.

Enhancing lytic activity of MAK cells with PHA

Since LMAK effectors did not kill optimally *in vitro*, we were interested in ways to enhance the *in vitro* lytic activity of these effector cells. Because it has been demonstrated that PHA enhances *in vitro* lytic activity of PBL [26], PHA was tested for its ability to enhance the cytotoxic activity of LMAK cells against U373 glioma target cells. Standard 4 hour ^{51}Cr assays were performed with LMAK lymphocytes obtained from the glioma patients in the presence and absence of 1 ug/ml PHA. The distribution of patients LMAK killing activity on U373 at an 25:1 E:T ratio and how it is influenced by
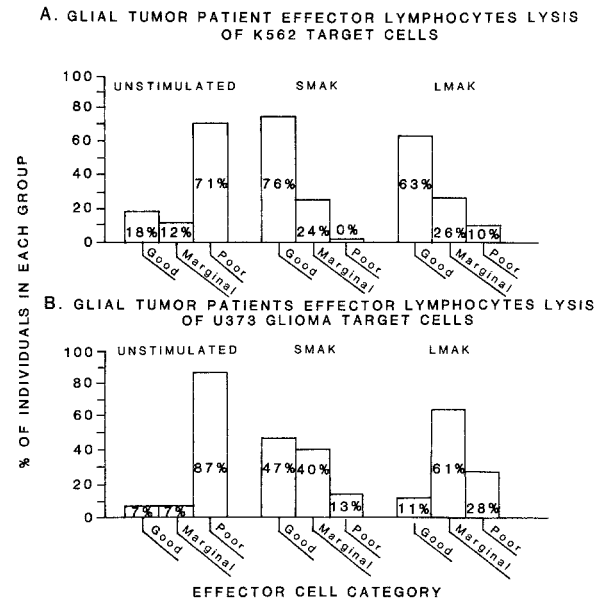

Fig. 2. Effector function of glial tumor patients lymphocytes prior to stimulation (unstimulated), after 3–4 days of culture (SMAK), and after 10 days or more of culture (LMAK). See Materials and methods for details on SMAK and LMAK generation. Effector function measured at an E:T of 25:1, and lytic activity was categorized as 'good' if it had mediated > 60% lysis, 'marginal' if it had mediated 30–60% lysis, and 'poor' if it had mediated < 30% lysis. The percentage of patients in each effector category is indicated. (A) Effector function assayed on K562 target cells. (Number of patients tested: Unstimulated N = 17, SMAK N = 17, LMAK N = 19). (B) Effector function assayed on U373 glioma target cells. (Unstimulated N = 15, SMAK N = 15, LMAK N = 18).

PHA is shown in Fig. 3. As expected, including PHA in the killing reaction enhanced lysis of LMAK cells. Good effector function (> 60% lysis) increased from 11% of the patients without PHA to 75% of the patients with added PHA. The number of patients with poor effectors decreased from 28% without PHA to 0% with PHA. The distribution shows a clear shift toward better effector function.

To further examine the influence of added mitogens on the lytic function of LMAK cells, we examined the effect of rIL-2 and PHA on six LMAK samples in parallel. LMAK were obtained from six normal donors. Three cytolytic assays were done with each sample of LMAK lymphocytes. LMAK effectors were assayed on U373 alone, on U373 in the presence of 200 μ/ml rIL-2, and on U373 in the presence 1 μg/ml PHA. The data obtained in these

LMAK EFFECTORS-U373 TARGETS

A. (−) PHA n=18

B. (+) PHA n=12

% OF INDIVIDUALS IN EACH GROUP
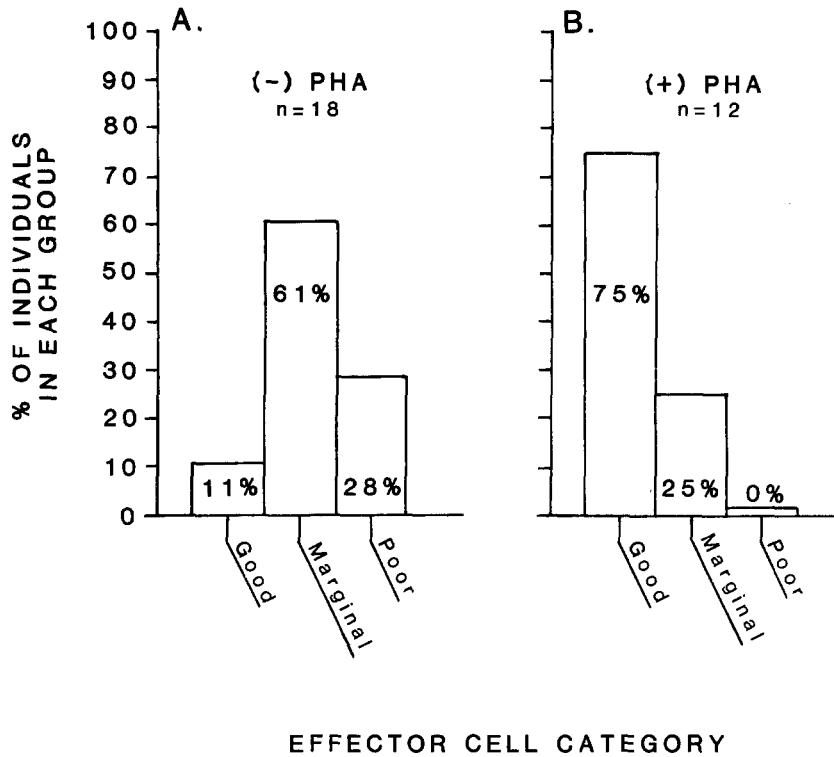
EFFECTOR CELL CATEGORY

Fig. 3. PHA enhances of LMAK effector function assayed on U373 glioma target cells. (A) Distribution of LMAK lytic activity measured at an E:T of 25:1 without any additions. (B) Distributions of LMAK lytic activity in the presence of 1 μg/ml of PHA during the assay.

experiments is shown in Fig. 4. The data in Fig. 4a demonstrate that IL-2 did not reproducibly enhance *in vitro* lytic activity of LMAK. For example, LMAK killing activity measured on U373 of sample A changed little with added IL-2, while preparations C & E lytic activity was inhibited by the added IL-2. PHA added to the killing reaction reproducibly enhanced the lytic activity of all the LMAK preparations assayed on U373. LMAK preparations A & B changed from poor effectors to marginal effectors, while LMAK preparation D changed from a poor effector to a very good effector. Compared to rIL-2, PHA even enhanced lytic activity in the LMAK preparations which IL-2 induced significant depression in lytic activity. Although the data are not presented, similar results were seen with SMAK preparations. Based upon

the significant and reproducible enhancements of LMAK effector function, in 2 arms of the prolocol the tumor bed of patients was treated with PHA prior to implantation of IL-2 and MAK cells.

Enhanced LMAK cytokine production stimulated by mitogens

It has been demonstrated that mitogens not only enhance the lytic activity of PBL, but they enhance the secretion of cytokines [26, 27]. These cytokines might play an important role in the induction of local anti-tumor responses. We examined whether the mitogens rIL-2, and PHA could trigger the release of cytokines from LMAK cells. To examine this question, LMAK cells were established in 1 ml

cultures and incubated alone in tissue culture media or with 200 μ/ml rIL-2, 1 μg/ml PHA, or PHA+ IL-2. After 24 hours, the supernatants were collected and assayed at various dilutions on L-929 target cells in a bioassay which detects TNF and LT. The data from a representative experiment are shown in Fig. 5. The unstimulated LMAK do not produce any detectable LT or TNF bioactivity *in vitro* after a 24 hour incubation. IL-2 stimulated very low levels of LT/TNF. PHA, alone or combined with IL-2, stimulated large amounts of LT/TNF activity. Thus, PHA and IL-2 induced the release of LT/TNF activity from activated LMAK cells, however PHA induced significantly more cytokine than did IL-2.

Effect of corticosteroid treatment on the generation of MAK cells

Previous authors have had difficulty generating effectors which could kill tumor target cells [21]. Many of the patients that did not generate lytically active effector lymphocytes were on higher doses of corticosteroids. This suggested corticosteroid use to control cerebral edema inhibited generation of lytically active lymphocytes. However, we observed no statistically significant (P < .05) relationship between the dose of corticosteroid employed at the time of leukapheresis and the ability to generate effective cytotoxic effector cells assayed on K562 or U373 target cells (data not shown).

Survival after surgery and immunotherapy

The survival after surgery and adoptive immunotherapy with MAK cells and 10^5 u rIL-2 is presented in Table 3 and Table 4. In this analysis, all patients have been in the study for a minimum of six months. When the data from all the patients are combined, 6/19 (32%) were alive at the time of the analysis. Two patients died of causes not directly related to tumor growth. Patient's # 1 died of pulmonary emboli. Patient # 10 died of pneumonia.
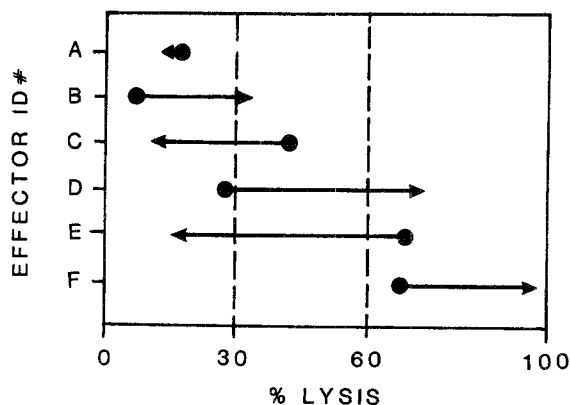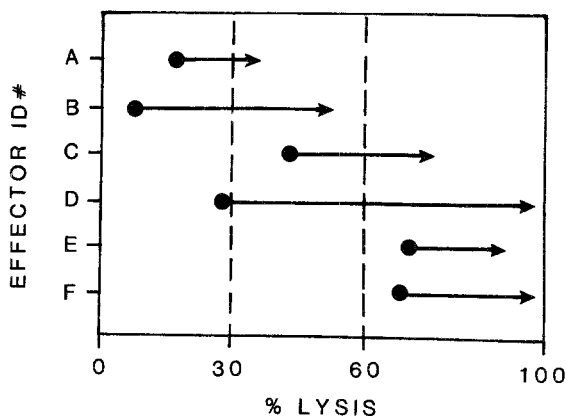

Fig. 4. Effect of rIL-2 and PHA on LMAK effector function assayed on U373 glioma target cells. Lytic activity of each LMAK preparation in the absence of any added materials is shown with a solid dot, the magnitude of the change in killing with added IL-2 or PHA is shown by the length of the bar and the direction of the change is indicated by the arrowhead and the position of the bar relative to the dot. (A) IL-2 (200 u/ml) stimulation of LMAK effector function. (B) PHA (1 μg/ml) stimulation of LMAK effector function. Both IL-2 and PHA stimulation of effector function were run simultaneously with identical effector cells.

The mean survival after surgery and immunotherapy was 31 weeks and median survival was 30 weeks. At present, with only one third of the patients enrolled in the study, the 30 weeks survival is not significantly different from the historical controls. Young *et al.* [28] reported that patients that received surgery alone after recurrence of the high
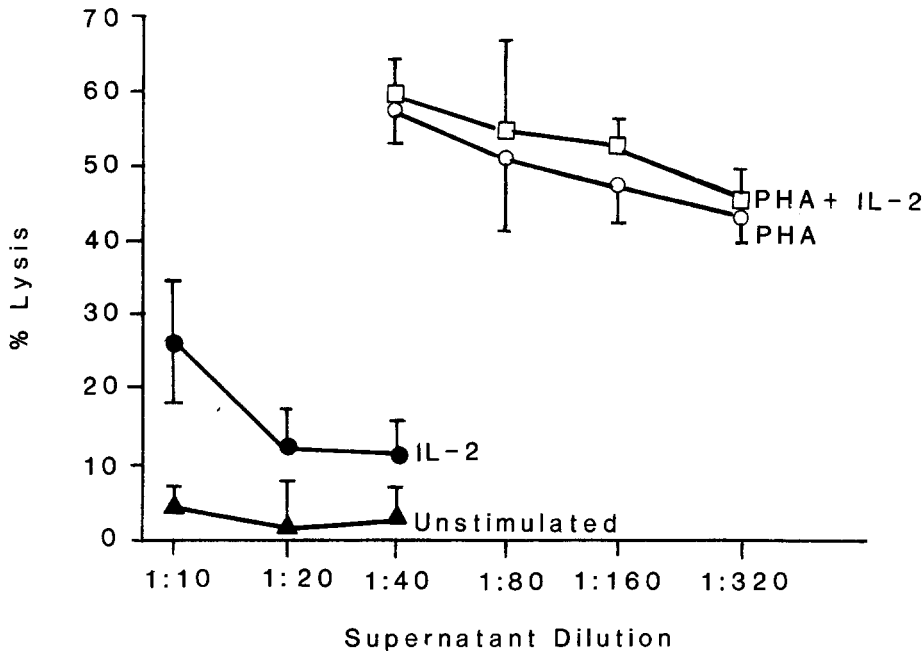
Fig. 5. PHA and IL-2 induced production of Tumor Necrosis Factor (TNF) and Lymphotoxin (LT) from LMAK cells. LMAK incubated in media (--▲--)), rIl-2 (200 u/ml) (--●--), PHA (1 μg/ml) (--○--), and rIL-2 + PHA (--□--) for 24 hours and cell free supernatants collected. Dilutions of the supernatants were assayed for TNF/LT activity in a standard L-929 bioassay. (See Materials and methods).

grade glioma survived 27 weeks. None of the various arms of the protocol have enough patients to draw reliable conclusions. When PHA is included as part of the therapy, no adverse reactions were observed.

Radiographic evaluation of response to therapy

Radiographic evaluation of the patients before and after therapy was performed. A total of 11 patients had the same scan pre-operatively and 40–70 days post-operatively using either contrast-enhanced Computed Tomography (CECT) or Gadolinium-DPTA enhanced Magnetic Resonance Imaging (Gd-MRI). Measurements of the primary tumor were taken in three perpendicular axes. Tumor growth was defined as an incremental increase in tumor size in two of the measured axes, increasing mass effect, or 'new' tumor growth (enhancing lesions). A decrease in size of the enhancing lesion was defined by a decrease in 2 of the three perpendicular axes of > .5 cm compared to the preoper-

ative scan. Three of 11 evaluable cases (27%) demonstrated a decrease in the size of the enhancing lesion after the first cycle of surgery and MAK + IL-2 therapy. Only one (patient # 6) of 3 evaluable patients demonstrated a decrease in size of the enhancing lesion after the second cycle of surgery and immunotherapy. Patient # 6 did not demonstrate any decrease in enhancing lesion after the first surgery and immunotherapy with MAK cells and IL-2, while demonstrating a smaller enhancing lesion after the second surgery and immunotherapy with MAK cells, IL-2, and PHA.

The significance of the decreased size of the enhancing lesion remains unclear. It is possible that the surgery could have lead to a decrease in size of the lesion, which was reflected in the scan, or the implanted lymphocytes, IL-2, and PHA could have triggered the destruction of the tumor as demonstrated by a decrease in the size of the enhancing lesion. A vascular leak could have been induced by the surgery, lymphocytes, or IL-2, resulting in the enhancing lesions being studied.

Clinical response observed after therapy

The clinical response to therapy for the 19 patients after their first surgery is reported in Table 4. The clinical status of these patients was evaluated after surgery, MAK cell and IL-2 therapy and includes changes from the postoperative period and the first few months followup. Seven patients (37%) clinically improved after therapy, eight (42%) showed no change in clinical status, and four (21%) were worse after therapy. Mean survival in those that improved clinically was 35 weeks, in those that did not change clinically was 39 weeks and was 7 weeks in those that were worse after therapy. There was no correlation between the clinical response and the observed radiographic response.

Several patients showed dramatic objective improvements. Patient #2 has resolution of right sided hemiparesis, after therapy, that lasted 7–8 months. Patient #3 had slowed mentation and partial aphasia which resolved after therapy and the improvement lasted 5 months. The mental confusion in patient #4 resolved for 4 months. Although these patients improved clinically, it is difficult to critically evaluate the improved clinical status in this study and determine if the observed change could be accounted by surgery alone, or if the immunotherapy contributed to this change.

Complications occurring in this study

Few complications were observed after surgical debulking of the glioma and adoptive immunotherapy. Three patients had complications related to their glioma or surgery prior to entry into this

Table 3. Response to therapy

Patient Number	Surgery* (+/-PHA)		Karnofsky status (pre/post op)		Clinical[+] response (after surgery I)	Radiographic response[++]		Survival (weeks)[+++]
	I	II	I	II		I	II	
1	0	0	70/70	70/70	0	0	0	41
2	0	0	70/70	70/70	I	S	0	65
3	0	+	70/70	70/70	I	0	NA	35
4	0	+	70/70	70/70	0	0	NA	56
5	0	+	70/70	70/70	I	0	NA	22
6	0	+	80/90	90/90	0	S	NA	53
7	0	+	80/90	90/90	0	0	S	53
8	0	ND	70/70	ND	W	NA	NA	12
9	+	+	70/70	50/50	I	NA	NA	22
10	+	ND	70/70	ND	W	NA	NA	6
11	+	+	70/80	80/50	I	0	NA	40
12	+	ND	70/40	ND	W	NA	NA	5
13	+	ND	70/70	ND	0	0	NA	41
14	+	+	70/70	70/70	I	0	NA	41
15	+	+	70/80	80/80	0	NA	NA	20
16	+	+	80/80	80/80	0	S	NA	30
17	+	+	70/70	70/0	0	NA	NA	18
18	+	ND	70/70	ND	W	NA	NA	5
19	+	+	80/80	80/80	I	NA	NA	17

* = This data indicated whether PHA was employed with surgery I or II; + = PHA employed, 0 = no PHA employed, ND = not done.

[+] = Clinical response; I = improved, 0 = no change, w = worse.

[++] = The radiographic response was scored as a change form baseline preoperative CT or MRI scan with contrast to a scan 40–70 days after surgery and immunotherapy: 0 = no change, L = larger, S = smaller, NA = scans not available.

[+++] = Survival – is measured form the date when the patient underwent reoperation for recurrent glioma (ie. surgery I).

study. Patient # 1 had thrombophlebitis, patient # 13 had the middle cerebral artery compromised during surgery, and patient # 15 had a scalp wound infection prior to referral. Four patients had a reduction in their Karnofsky score immediately after surgery (Table 3). Two patients (# 11, 12) had new onset of hemiparesis after resection of highly vascular gliomas. Fever > 100° F was seen in 26% of the patients in the first 48 hours and 26% had one measurement > 100° F greater than 48 hrs after surgery. None of these elevations in temperature were considered related to the immunotherapy, and probably reflected postoperative pulmonary atelectasis. Other complications that occurred included: pulmonary emboli (# 1), thrombophlebitis (# 5), pneumonia (# 10), cerebrovascular accident (# 9). None of these were thought to represent effects of the immunotherapy.

Patient # 1 died of pulmonary emboli. Patient # 1 had prior problems with postoperative thrombophlebitis prior to entry into this study, did not have thrombophlebitis after undergoing surgery and immunotherapy twice. At the time he died of as a result of the PE, he was semicomatose and had massive regrowth of the glioma. On these grounds we feel that this PE was not directly related to the surgery and immunotherapy administered during this study. Furthermore, this 5% (1/19) incidence of death due to pulmonary emboli in this is similar to the 3.4% death rate due to PE in patients undergoing intracranial surgery [29–31].

In general, the patients tolerated the surgical procedures well, with minimal morbidity, and problems with increased intracranial pressure were not noted after MAK + IL-2, or MAK + IL-2 + PHA therapy.

Discussion

Recent clinical trials employing adoptive immunotherapy employ autologous lymphokine-activated lymphocytes with broad antitumor reactivity to treat a number of neoplasms. Studies have demonstrated that culturing lymphocytes with IL-2 generates cytotoxic antitumor killer cells that have broad specificity, such that most neoplastic cells can be killed *in vitro* [2, 32]. Two types of IL-2 stimulated effector lymphocytes have been generated *in vitro* [3, 5, 33]. One category of effector cell is the non-T large granular lymphocyte, commonly termed Lymphokine Activated Killer (LAK) cell, and the second type of effector is the non-MHC restricted T cell.

Table 4. Therapy of high grade gliomas: survival in glioma patient groups

Patient group	Therapy (+/− PHA) surgery		Patients surviving		Duration of survival (weeks)	
	Surgery		Alive/total	%	DX to death*	MAK I to death[+]
	I	II				
1	−	−	0/2	0	78 (78–85)	53 (41–65)
2	−	ND	0/1	0	81	12
3	−	+	2/5	40	60 (43–74)	44 (22–56)
4	+	+	3/7	43	66 (29–134)	27 (17–41)
5	+	ND	1/4	25	69 (20–185)	14 (5–41)
TOTALS			6/19	32%	71 (N = 19)	30 N = 19

* Average survival from diagnosis (DX) to death in weeks, and range of survival given in parentheses.
[+] Duration from the first MAK cell therapy (first surgery) to death.

LAK cells are generated *in vitro* by stimulating peripheral blood lymphocytes with rIL-2 alone for 3–5 days and generate activated large granular lymphocytes that have the CD16 surface marker and do not have surface CD3 [32]. These effector lymphocytes have proven useful in the control of metastatic tumor in animal models [8–11]. LAK cells are currently being tested for anti-tumor activity in human cancer patients and responses have been seen mainly in patients with melanoma and renal cell carcinoma [12, 14].

Activated T cells with a broad range of lytic activity against neoplasms can also be generated if T cells are stimulated with PHA or anti-CD3, in addition to rIL-2, *in vitro*. These activated T cells have been given various names: 1) PHA Activated Killer (PAK) Cells [34, 35], 2) Mitogen Activated IL-2 Stimulated Killer (MAK) Cells, 3) Autologous Stimulated Lymphocytes (ASL) [33], and CD3 or T-LAK [4, 5].

Several studies have examined the effect of treating recurrent high grade gliomas with surgery, activated lymphocytes and rIL-2. Jacobs *et al.* [20], Merchant *et al.* [21], Yoshida *et al.* [22], and Barba *et al.* [36] employed PBL stimulated *in vitro* with only IL-2 for 3–5 days to generate LAK cells. Ingram *et al.* [19, 37] employed PBL stimulated with PHA and IL-2 for 10 days *in vitro* to generate the effectors (mainly T cells). Two of these studies reported improvement more than would be expected from surgery alone [19, 22, 37].

In these protocols employing local implants of activated lymphocytes, IL-2 was administered with activated lymphocytes (LAK or T cells) to attempt to maintain their biologic activity. Previous studies in animal models of metastatic disease found that LAK cells alone were not effective in preventing the growth of metastasis; however, when both IL-2 and LAK cells were combined, significant anti-tumor activity was demonstrated [10, 11]. *In vitro* IL-2 can enhance the anti tumor lytic activity of effector cells. We were however interested if other mitogens might more effectively trigger anti-tumor lytic activity and cytokine release.

PHA is well known to not only to trigger lymphocytes *in vitro* to become effector cells capable of killing tumor targets, but also to trigger the release of large amounts of cytokines [26, 27, 34, 38, 39]. Current studies have found that predominantly T cell MAK effector cells become much more cytotoxic *in vitro* on U373 glioma target cells and produce high levels of TNF or LT in the presence of PHA. IL-2 can enhance effector function but it is not as predictable as that stimulated by PHA. Low levels of cytokine production is stimulated by IL-2, while PHA stimulates much more TNF/LT activity. Thus, lymphocytes might be more effective killers and more effective cytokine producers *in vivo* when placed in the presence of PHA rather than IL-2.

We have begun a Phase I/II study to examine the use of PHA *in vivo* to enhance the biologic activity of MAK cells. Baseline studies of unstimulated PBL killing of K562 and U373 tumor target cells, demonstrated 7–18% of the patients had high levels of spontaneous LAK and natural killer activity *in vitro*. This suggests that the glioma patients immune system was stimulated by having the tumor to produce high levels of effector cell activity. With IL-2 activation *in vitro* the percentage of patients lymphocytes demonstrating high levels of killing activity increased to 47–76%.

Overall, 19 patients have been treated in this phase I/II trial. Three of eleven patients had a decrease in size of the enhancing lesion on CT and/or MRI. Seven patients improved clinically. Median survival after therapy of all patients treated with surgery and immunotherapy was 30 weeks. Lastly, we have found the use of PHA *in vivo* to be safe and not associated with toxicity.

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