

Reduction of End-stage Malignant Glioma by Injection with Autologous Cytotoxic T Lymphocytes

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Autologous cytotoxic T lymphocytes (CTL) against primary-cultured malignant gliomas were generated from peripheral blood mononuclear cells *in vitro* in 4 patients. Activities of the CTL were highly specific to the corresponding autologous glioma and were inhibited, in one patient, with antibodies against CD3, CD8 and MHC-class I molecules. When the CTL were injected 3 times into the primary-tumor-resected cavity via an Ommaya tube, reduction of the recurrent tumors with magnetic resonance imaging (MRI)-measured volumes exceeding 45 cm³ was observed in 3 patients. In a patient with glioblastoma multiforme (GBM), the tumor volume (estimated, 130 cm³) was rapidly reduced to 1/3, although re-recurrence of the tumor followed 40 days later. A slight but distinct rapid reduction of the tumor volume was observed in another GBM patient and in an anaplastic astrocytoma patient; essentially no change was observed in a further GBM patient. These results suggest that adoptive immunotherapy with autologous CTL will be clinically effective against end-stage malignant gliomas.

Key words: Autologous cytotoxic T lymphocytes — Malignant glioma — Brain tumor — Adoptive immunotherapy

Cytotoxic T lymphocytes (CTL) have shown higher cytotoxicity than lymphokine-activated killer (LAK) cells against tumors.^{1–10} Unfortunately, there have been practical problems in clinical application of CTL, i.e., (1) difficulty of obtaining a sufficient number of live target tumor cells for repeated stimulation of CTL growth^{5,9–12} for prolonged culture periods and, consequently, (2) difficulty of obtaining a sufficient number of CTL for treatment of a patient. Expansion of primary cultured tumor cells and establishment of tumor cell lines are not practical for every patient since tumor cells cannot always be successfully subcultured.¹³ However, novel methods have been developed to overcome the first problem in our laboratory. The operative fresh specimens of tumors are useful for the induction of autologous CTL from the peripheral blood mononuclear cells (PBMC) when supplied as minced tissue.¹⁴ Formalin-fixed paraffin-embedded tumor sections are applicable as the source of tumor antigens^{8,15} and fixed cultured tumor cells are also stimulative for the expansion of autologous CTL.¹⁶

The combination of these methods provided us with an increased potential to generate autologous CTL for the treatment of end-stage malignant glioma patients. Here we report initial clinical treatments of 4 patients to whom autologous CTL were applied directly into the tumor-resected cavities via Ommaya tubes.

MATERIALS AND METHODS

Cases From 4 patients with malignant gliomas, autologous CTL were generated. The gliomas of 3 patients were pathologically diagnosed as glioblastoma multiforme (GBM), while the other patient had anaplastic astrocytoma (AAS).

Patient No. 1 (Y. S.) was a 23-year-old woman with a right frontal tumor. Subtotal removal of the tumor was carried out on June 8, 1994. Pathological diagnosis was AAS at this stage. Then the patient was given 65 Gy of radiation. Chemotherapy was performed by intratumoral injection with the antitumor drug mixture (PAV-MTX, 2 mg/injection, twice a week, 4 weeks) mixture at the total dose of 16 mg. Magnetic resonance imaging (MRI) on October 5, 1995, revealed regrowth of the tumor, and then interferon α (3×10^6 units) was administered i.v. twice a week, with no effect. Surgical mass reduction of the recurrent tumor that was pathologically identified as GBM was performed on October 25. However, due to continued regrowth of the tumor, her consciousness level gradually deteriorated to stupor and respiratory status to Cheyne-Stokes type before the administration of CTL. The second operative specimens were used for primary culture of the tumor cells and the resulting cultured tumor cells were employed for the generation of autologous CTL.

Patient No. 2 (A. N.) was a 53-year-old man with a right parietal tumor. Subtotal removal was carried out on June 10, 1997, and radiation therapy with 65 Gy fol-

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lowed. Pathological diagnosis was AAS, grade III. The operative specimens were used for primary culture of the tumor cells. Regrowth of the tumor was observed in September, 1997, at which stage it was suspected to have transformed to GBM. Before the administration of CTL, rapid regrowth of the tumor was observed upon MRI.

Patient No. 3 (S. M.) was a 63-year-old man with a left parietal tumor. Subtotal removal was performed on December 24, 1996, and radiation therapy with 64 Gy followed. Pathological diagnosis was GBM. Regrowth of the tumor was observed in March, 1997. His consciousness level gradually deteriorated to stupor before the administration of CTL.

Patient No. 4 (K. K.) was a 62-year-old man with a right parietal tumor. Subtotal removal was performed on December 12, 1996, and radiation therapy with 64 Gy followed. Pathological diagnosis was GBM. Regrowth of the tumor was observed in April, 1997. Before the administration of CTL, his consciousness level had gradually deteriorated to stupor because of continued regrowth of the tumor.

Tumor cells and blood lymphocytes After obtaining written informed consent from each patient or their family and research ethical approval as a variation of LAK therapy in the Department of Neurosurgery, Institute of Clinical Medicine, University of Tsukuba, we started primary culture of the tumor cells from fresh operative specimens in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). In patients No. 2–No. 4, sufficient amounts of tumor cells grew *in vitro* for generation of CTL. These tumor cells were harvested by trypsinization and reseeded into 24-well culture plates. After the cells had adhered on the culture plate surface, they were washed with Dulbecco's phosphate-buffered saline (PBS). The tumor cells were cultured in DMEM containing 5% autoplasm for 3 days, then irradiated with 50 Gy of X-rays and used as antigens to induce CTL. In patient No. 1, 4 months was required to obtain approximately 10^5 tumor cells, which were reseeded in 1 well of a 24-well culture plate. They were fixed for 3 h with freshly prepared 10% formalin in PBS, then washed thoroughly with PBS and incubated in culture medium overnight; they then served as antigens. Target tumor cells used in the killing assay were the autologous tumor cells and allogeneic human malignant renal carcinoma cell lines TUHR3TKB, TUHR4TKB and OS-RC-2, and the gastric adenocarcinoma cell line GT3TKB. These cell lines were obtained from routine cultures in the RIKEN Cell Bank. A conventional Ficoll-Paque method (Lymphosepal, Tokyo) was used to separate the PBMC from 40 ml of heparin-containing peripheral blood. The PBMC were washed successively with PBS and with culture medium containing 5% autologous plasma by centrifugation at 2,000 rpm (700g) for 5 min at room temperature.

Culture medium and cytokines RHAM α medium, originally developed for human lymphocyte culture,¹⁷⁾ was used for induction of autologous CTL after supplementation with 5% autologous plasma and recombinant human interleukin (IL)-1 β (Otsuka Pharmaceutical Co., Ltd., Osaka, 167 U/ml), IL-2 (Takeda Chemical Industries, Co., Ltd., Osaka, 67 IU/ml), IL-4 (Ono Pharmaceutical Co., Ltd., Osaka, 67 U/ml) and IL-6 (Ajinomoto Co., Inc., Tokyo, 134 U/ml).

Generation of CTL PBMC were suspended at a concentration of 2×10^6 cells/ml in the culture medium and placed on primary-cultured tumor cells previously irradiated or on the fixed tumor cells in the wells of a 24-well culture plate. The plate was shaken periodically to ensure that the PBMC came into contact with the tumor cells. Half the culture medium was changed every 2 days until the lymphocytes began to grow. Then, the CTL preparation was maintained at a suitable dilution with the remaining tumor cells in the culture medium. After approximately 10 days in culture, the CTL were transferred to 6-well culture plates and restimulated with irradiated tumor cells (patients No. 2–No. 4). For patient No. 1, the CTL were restimulated with mouse anti-human CD3 monoclonal antibody (OKT-3, Nichirei Co., Tokyo) which was precoated on the bottom of a culture plate at a concentration of 0.05 μ g/ml and 0.2 ml/cm².

In the present study, the time from the operation to the first administration of CTL was 146, 140, 130, and 184 days in patients No. 1, No. 2, No. 3, and No. 4, respectively. In patient No. 1, CTL were induced and cultured continuously for 20 days for the first administration, for a further 21 days for the second, for a further 14 days for the third, and for a further 22 days for the fourth. In patient No. 2, CTL induction was started 3 times independently from autologous PBMC and cultures were continued for 21, 20, and 16 days for the first, second, and third administrations. In patient No. 3, CTL induction was started 3 times independently from autologous PBMC and cultured for 24, 23, and 23 days for the first, second, and third administrations. In patient No.4, CTL induction was started 3 times independently from autologous PBMC and cultured for 20, 27, and 20 days for the first, second, and third administrations.

Before the injection into the patients, the CTL were pelleted and washed 3 times with saline (10 ml) containing 1% human serum albumin (HSA) by centrifugation at 1,400 rpm (250g) for 5 min, then resuspended in 2 ml of saline containing 4% HSA and 670 U of IL-2 (Shionogi & Co., Ltd., Osaka).

Inspection of CTL Before every injection of the CTL preparation, the polymerase chain reaction (PCR) detection method described by Harasawa *et al.*¹⁸⁾ was used to ensure there had been no mycoplasma contamination. The level of endotoxin was determined by monitoring the

LAL gel-formation rate with the Toxinometer ET-208 according to the manufacturer's protocol (Wako Junyaku, Co., Tokyo).

It was confirmed that the generated CTL and the corresponding target tumor cells were derived from the same patient by DNA fingerprinting after PCR to detect single locus variable number of tandem repeats (VNTR) in genomic DNA.¹⁹⁻²¹⁾ The PCR reaction mixture in a final volume of 20 μ l consisted of 200 ng of DNA, PCR-buffer [60 mM Tris-HCl, pH 9.0; 3.5 mM MgCl₂; 15 mM (NH₄)₂SO₄], 2% dimethyl sulfoxide, 200 μ M each of dAPT, dCTP, dGTP and dTTP, 6.0 pmol of each PCR primer and 0.5 U *Taq* DNA polymerase (Amplitaq, TaKaRa Shuzo Co., Kyoto). The following 3 sets of microsatellite-specific PCR primers were applied: D1S80,¹⁹⁾ D17S30²⁰⁾ and DXS52.²¹⁾ The PCR was performed for 32 cycles: each cycle was carried out at 95°C for 1 min, 60°C for 2 min, and 72°C for 10 min with D1S80 and D17S30 primers. With the DXS52 primer, each cycle was 95°C for 1 min, 58°C for 10 min, and 72°C for 10 min. The PCR products were stained with 0.02 % ethidium bromide for 20 min under gentle shaking after electrophoresis in a 2% agarose gel at 100 V for 30 min.

Cytotoxic activity The target tumor cells were seeded in 96-well culture plates at a density of 5×10³ cells/well in 100 μ l of RHAM α medium containing 5% autoplasm and were precultured overnight. Appropriate numbers of CTL, which had been adjusted to the desired effector-to-target (E/T) ratio, were added to the wells with 100 μ l of RHAM α medium containing 5% autoplasm. The plates were incubated for 24 h at 37°C in a humidified CO₂ incubator and then the wells were washed once gently with an appropriate amount of PBS. Adherent target cells were fixed for 1 h with 10% formalin (200 μ l/well). The number of adherent target cells was measured as the number of surviving cells by the crystal violet staining (CV) assay as described previously.¹⁵⁾ This CV assay used in the present study is as sensitive and accurate for assessment of the killing activity of CTL against adherent target cells as the standard ⁵¹Cr-release cytotoxicity assay for E/T ratios of 10 or lower (see the comparison of standard curves, Fig. 1 in ref. 15).

The numbers of adherent cells were expressed as absorbance at 450 nm. The killing activities were expressed as follows:

$$\text{Killing activities (\%)} = [1 - (A - AL) / (AT - B)] \times 100$$

where *A* is the absorbance in each well; *AL* is the mean of absorbances in the wells containing the lymphocytes without target cells; *AT* is the mean of absorbances in the wells containing target cells without lymphocytes; *B* is the mean of absorbances of blank wells. All measurements were done in triplicate for each observation point. Note that the tumor cell lines grew rapidly during the 24-h

incubation and this resulted in negative values of killing activity if the tumor cells were not vigorously killed by the CTL.

The inhibition assay of cytotoxicity of CTL could be performed at an E/T ratio of 4 in patient No. 3. Target tumor cells were seeded in 96-well culture plates at a density of 5×10³ cells/well with 100 μ l of RHAM α medium containing 5% autoplasm and were precultured overnight. The target cells were then pretreated with monoclonal antibodies against MHC-class I and -class II (Dako, W6132 clone, 16.5 μ g/ml and Dako, CR3143 clone, 20.3

Table I. Phenotype of the Autologous CTL at the First Administration (%)

Patient No.	CD3 ⁺	CD4 ⁺	CD8 ⁺
1	97.7	95.4	2.3
2	100	19.8	73.5
3	100	33.2	83.5
4	98.1	45.8	53.6

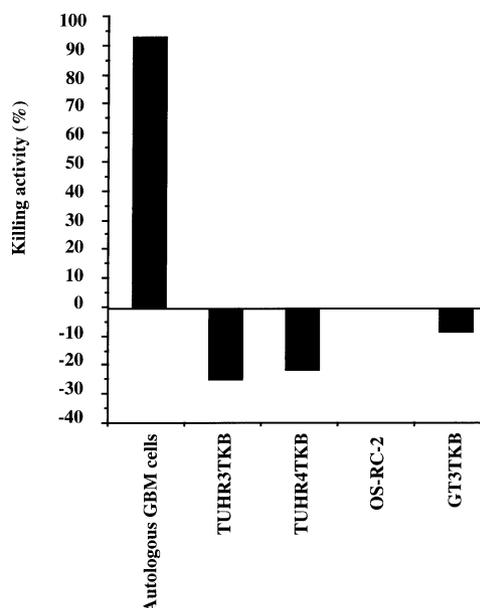


Fig. 1. Specific cytotoxicities of the CTL in patient No. 1. Cytotoxic activities were determined by co-culturing CTL on the target tumor cells and measuring surviving adherent tumor cells after crystal violet staining (see "Materials and Methods"). Assay was performed at an E/T ratio of 10 for 24 h. The CTL killed most of the autologous tumor cells but did not kill any allogeneic renal carcinoma cells (TUHR3TKB, TUHR4TKB, and OS-RC-2) or gastric adenocarcinoma cells (GT3TKB). The negative values of killing activity resulted when tumor cell lines proliferated during the 24-h incubation.

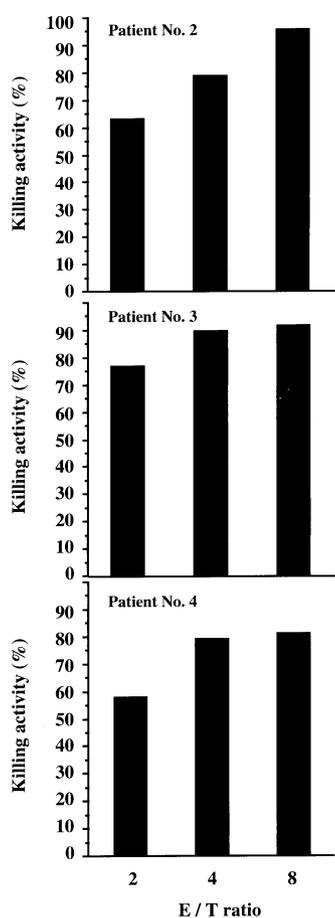


Fig. 2. Specific cytotoxicities of the autologous CTL from patients No. 2–No. 4. Cytotoxic activities were determined by co-culturing with corresponding autologous target tumor cells. Assays were performed at E/T ratios of 2, 4 and 8 for 24 h.

$\mu\text{g/ml}$, respectively) at 37°C for 4 h. As effector cells, CD8^+ cells were selected by means of magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), and were pretreated with antibodies against CD3, CD4 and CD8 (Nichirei Co., Nu-T3 clone, $5 \mu\text{g/ml}$, Nu-Th/i clone, $5 \mu\text{g/ml}$, Nu-Ts/c clone, $5 \mu\text{g/ml}$) at 4°C for 30 min.

Cell-surface CD antigens CTL were characterized by staining with fluorescein isothiocyanate-labeled monoclonal antibodies (Becton-Dickinson, Mountain View, CA) against CD3, CD4, and CD8 surface antigens. Flow cytometry was performed with a FACScan (Becton-Dickinson).

Tumor volume calculation on MRI Tumor volume estimation was based on the gadolinium-enhanced areas of MRI using a computerized light table and an image analysis program, Bild/2D (Computer Bild Co., Tokyo).

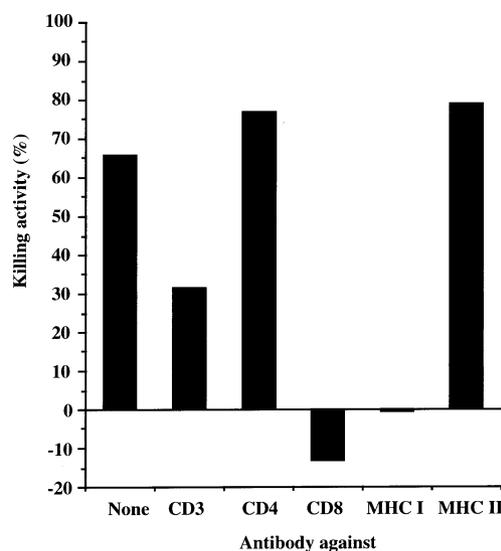


Fig. 3. Inhibition of cytotoxicity of CTL by monoclonal antibodies against surface proteins in patient No. 3. The effector cells were pretreated with monoclonal antibodies against CD3, CD4 and CD8 at 4°C for 30 min. The target cells were pretreated with antibodies against MHC-class I and -class II at 37°C for 4 h and incubated with the effector cells at an E/T ratio of 4 at 37°C for 4 h. None, no treatment; CD3, CD4, CD8, MHC I, MHC II, pretreated with anti-CD3, anti-CD4, anti-CD8, anti-MHC I, anti-MHC II antibody, respectively.

RESULTS

Characteristics of CTL Flow cytometry of the phenotype of administered lymphocytes revealed that in patient No. 1 $\text{CD3}^+ \text{CD4}^+ \text{CD8}^-$ cells comprised 95.4% of the CTL (Table I). However, in patients No. 2, No. 3, and No. 4, the major population in the CTL was shared by $\text{CD3}^+ \text{CD4}^- \text{CD8}^+$ cells, viz., 73.5, 83.5, and 53.6%, respectively.

The CTL from patient No. 1 killed over 90% of autologous tumor cells at an E/T ratio of 10 for 24 h *in vitro* (Fig. 1). Due to the very slow growth of the GBM tumor cells and resulting shortage of the target cells, the killing assay with the autologous tumor cells of patient No. 1 could not be performed at any other E/T ratio. The CTL did not kill allogeneic malignant cell lines such as human renal carcinoma OS-RC-2, TUHR3TKB, and TUHR4-TKB, or gastric adenocarcinoma GT3TKB (Fig. 1). Negative killing activities displayed in Fig. 1 indicate that the target allogeneic carcinoma cells grew during the 24-h incubation period of the assay.

As shown in Fig. 2, a dose-response relation between killing activity of autologous CTL and E/T ratio was observed in patients No. 2, No. 3, and No. 4. For every patient, more than 50% of the brain tumor cells were killed by the corresponding autologous CTL at the low E/

T ratio of 2 for 24 h *in vitro*. At an E/T ratio of 8, nearly complete killing of the tumor cells was observed in patients No. 2 and No. 3, and 80% killing in patient No. 4.

The killing activity of the CTL from patient No. 3, as a representative of the 4 patients, was inhibited by pre-treatment with monoclonal antibodies against CD3, CD8 and MHC-class I, but not by antibodies against CD4 and MHC-II, at an E/T ratio of 4 (Fig. 3).

Table II. Numbers of the Injected CTL and Their Culture Period for Each Treatment of the End-stage Patients

Patient No.	Diagnosis	Injections				Total
		1st	2nd	3rd	4th	
1	GBM ^{a)}	2.0 ^{b)}	3.0	1.4	2.8	9.2
2	AAS	2.2	0.8	2.0	—	5.0
3	GBM	11	3.0	7.3	—	21.3
4	GBM	0.7	1.3	1.0	—	3.0

a) Abbreviations: GBM, glioblastoma multiforme; AAS, anaplastic astrocytoma.

b) Unit=10⁷ cells.

Clinical treatment with CTL and observation on MRI

In all cases, no other treatment for gliomas was given except the least conventional osmotherapy for 4 weeks before the CTL administration. Prior to the CTL injection, possible contamination of mycoplasma in the preparation was checked and excluded. Endotoxin content was also confirmed to be less than 0.5 ng/ml. The individual identity of target tumor cells and the CTL was confirmed by DNA fingerprinting after amplifying single locus VNTR in genomic DNA by PCR with three pairs of DNA primers (see "Materials and Methods"). All bands in each of the three PCR products showed identical patterns in each patient (data not shown).

In patient No. 1, who was at the end-stage of GBM (see the section on Cases), the first injection of autologous CTL suspended in 2 ml of saline containing 4% HSA and 670 U of IL-2 (Table II) was applied to the tumor-resected cavity via an Ommaya tube. Two days later, a drastic decrease of the gadolinium-enhanced areas on MRI was observed (axial sections A, C and coronal sections B, D in Fig. 4). Since the previous three sequential observations on MRI enabled us to estimate the linear

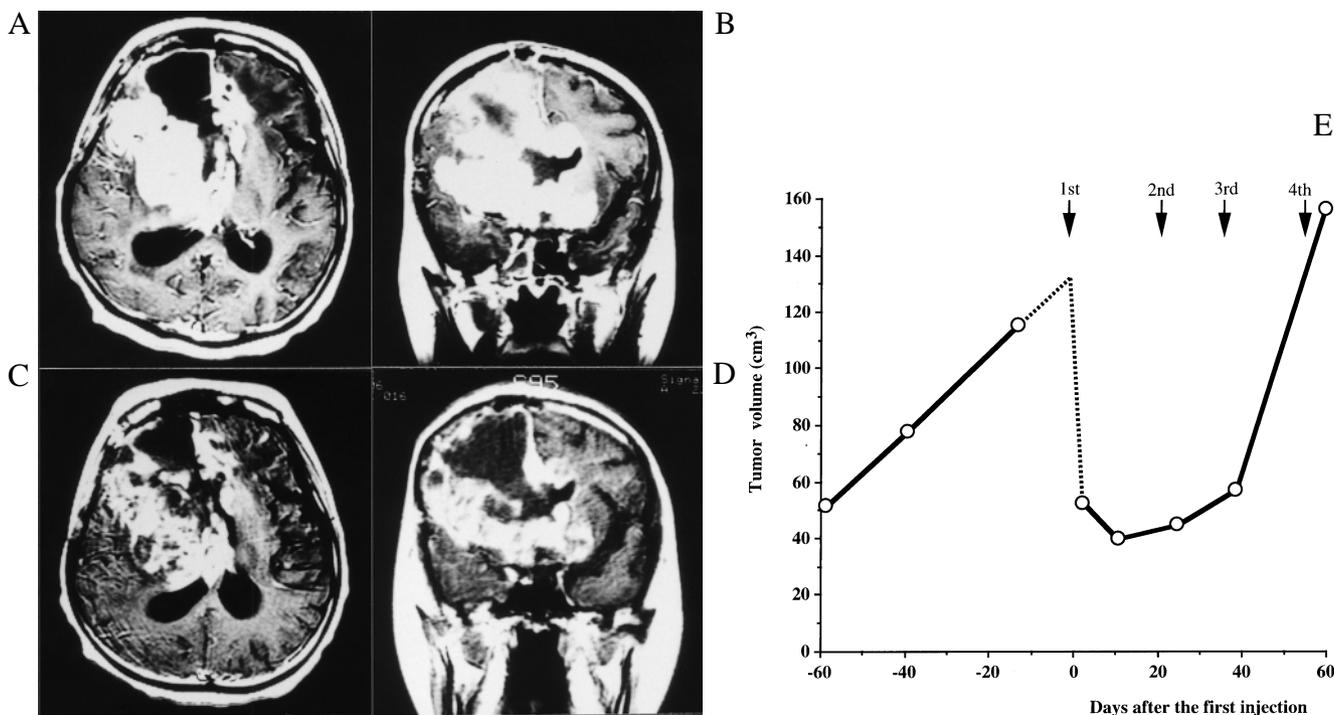


Fig. 4. Reduction of volume of the tumor treated with the autologous CTL in patient No. 1. Axial (A, C) and coronal (B, D) slice images of MRI before (A, B) and after (C, D) the first injection of CTL in patient No. 1. These are T1-weighted images enhanced with gadolinium. The tumor appears as the large high-intensity area in the right frontal lobe. E: changes of the tumor volume in patient No. 1. Arrows indicate injections of the autologous CTL. The first injection was defined as day 0; second injection was day 20; third, day 34; fourth, day 56. The dotted line was extrapolated from the MRI-observed points at days -60, -41, and -14. By day 2, a drastic reduction of the tumor volume was apparent.

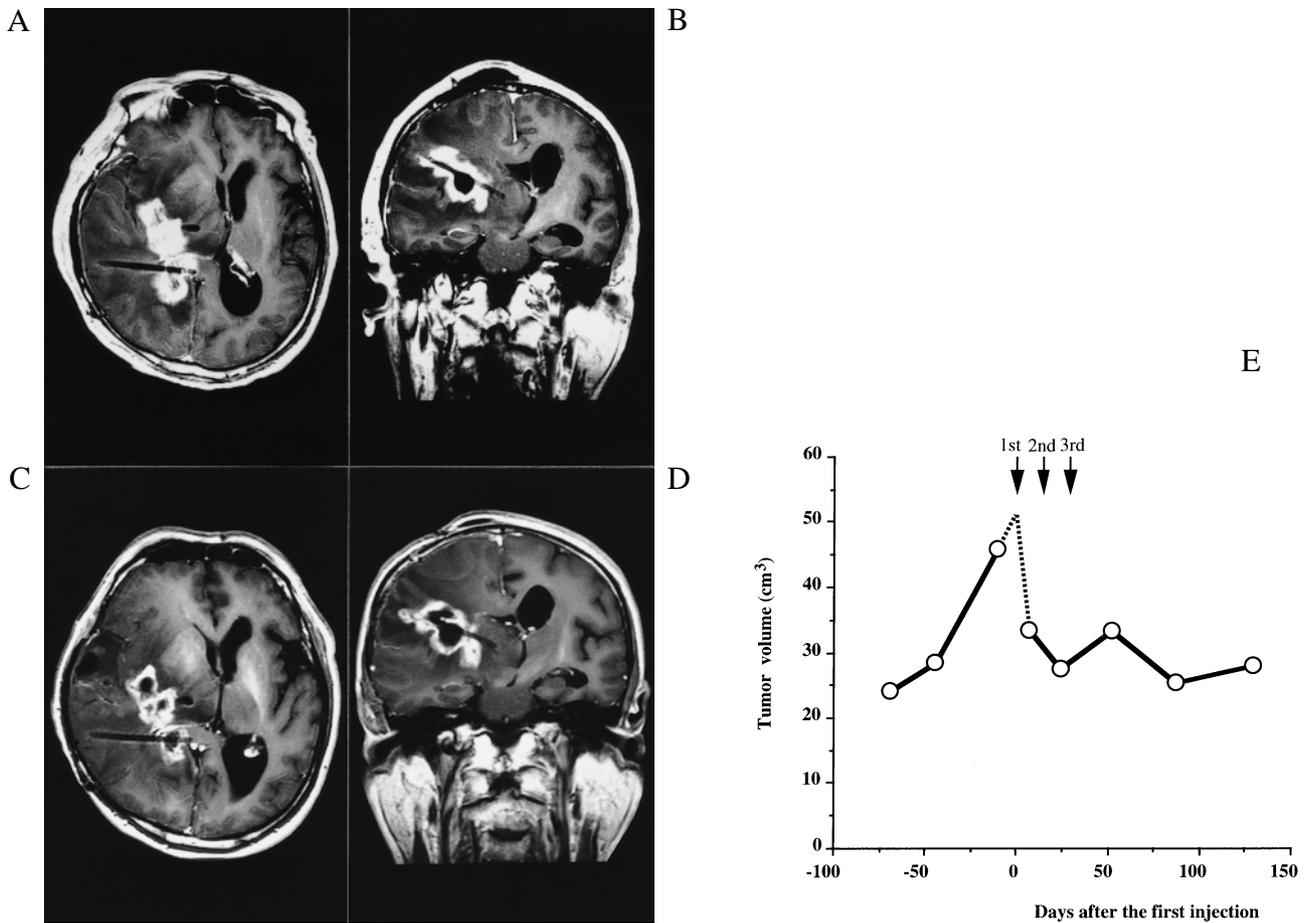


Fig. 5. Reduction of volume of the tumor treated with the autologous CTL in patient No. 2. Axial (A, C) and coronal (B, D) slice images of MRI before (A, B) and after (C, D) the first injection of CTL in patient No. 2. Images of inserted Ommaya tubes are seen in the mid-left of A and C and in the upper-left of B and D. E: changes of the tumor volume in patient No. 2. Arrows indicate injections of the autologous CTL. The first injection was defined as day 0; second injection was day 14; third, day 24. The dotted line was extrapolated from the MRI observed points at days -69, -45, and -11. By day 7, a reduction of the tumor volume was apparent.

increase of the recurrent tumor volume (Fig. 4E), the tumor was estimated to have decreased in size to be 1/3 of the volume just before the CTL injection.

Twenty days later, the second injection of CTL was performed through two routes simultaneously; one via the Ommaya tube and the other via a lumbar tap. Each injection contained 1.5×10^7 cells in 2 ml of saline containing 4% HSA and 335 U of IL-2. A further 14 days later, the third injection of CTL was given via an Ommaya tube with an accompanying injection of CTL via a lumbar tap (a total of 1.4×10^7 cells). However, as MRI taken 3 days after the third injection of CTL showed minor bleeding and regrowth of the tumor, the fourth injection of CTL (1.3×10^7 cells with 670 U of IL-2) was carried out intra-arterially by inserting a microcatheter into the left anterior cerebral artery. The following day, a further two CTL

injections were given; one into the cavity via the Ommaya tube and the other via a lumbar tap (each injection contained 1.5×10^7 cells and 335 U of IL-2). After these treatments, since the patient's status of respiration deteriorated due to right pleural effusion caused by light pneumonia, the adoptive immunotherapy with autologous CTL was stopped. The growth rate of the recurrent tumor was more rapid than before as calculated on MRI (Fig. 4E). The patient died at 111 days from the start of CTL therapy due to brain herniation by the regrowth of the tumor; the family did not consent to autopsy.

In the other 3 patients, all injections were administered to the tumor-removed cavity via an Ommaya tube. For each injection, the CTL number shown in Table II was suspended in 2 ml of saline containing 4% HSA and 670 U of IL-2.

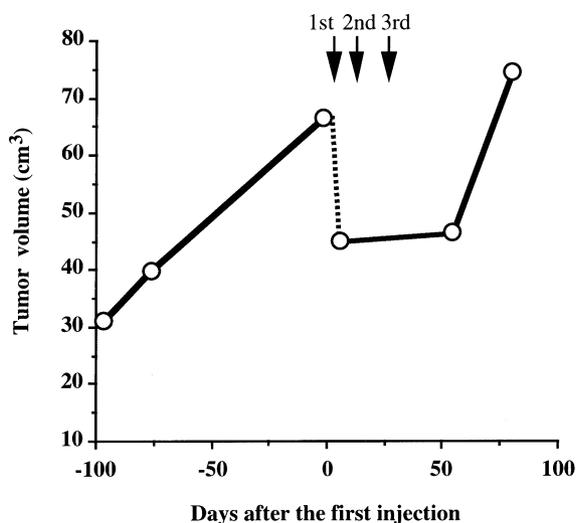


Fig. 6. Transient volume reduction of the tumor treated with autologous CTL in patient No. 3. Arrows indicate injections of CTL. The first injection of CTL was defined as day 0; second injection was day 7; third, day 14.

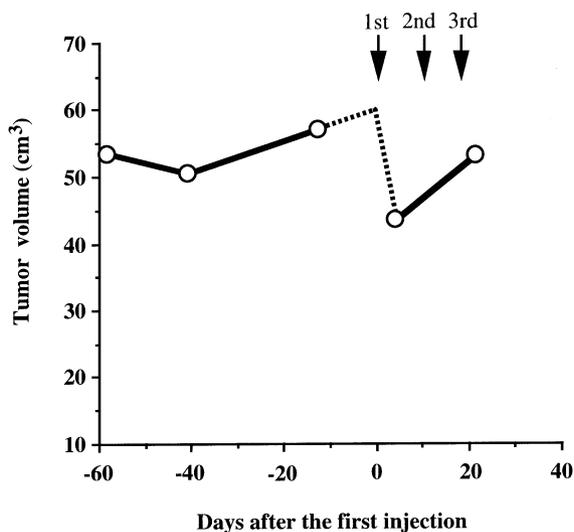


Fig. 7. Changes in volume of the tumor treated with autologous CTL in patient No. 4. Arrows indicate injections of CTL. The first injection of CTL was defined as day 0; second injection was day 11; third, day 18.

In patient No. 2, intervals of the first-second and first-third injections were 14 days and 24 days, respectively. On MRI, the tumor volume was measured at 3 points before the injections and at 5 points after the commencement of treatment. In this patient, 2 Ommaya tubes were inserted into the tumor cavity to permit the repeated

administration of CTL suspension. The image width of the Ommaya tubes provided a standard measure width on MRI which assisted in determining tumor volume before and after the CTL administration (Fig. 5, A-D). Apparent shrinkage of the gadolinium-enhanced areas on MRI can be seen when Fig. 5A (axial section) is compared with Fig. 5C and when Fig. 5B (coronal section) is compared with Fig. 5D. The estimated tumor volume revealed reduction from more than 47 cm³ to less than 34 cm³ (Fig. 5E). At the time of writing, the tumor seems static and no regrowth has been observed for more than 1 year.

In patient No. 3, intervals of the first-second and first-third injections were 7 days and 14 days, respectively. On MRI, the tumor volume was measured at 3 points before and 3 points after the CTL injections. The estimated tumor volume revealed reduction from 67 to 45 cm³ after the first injection (Fig. 6). However, apparent regrowth of the tumor was observed 2 months later. The patient died 4 months later due to the recurrent tumor; consent was not given for autopsy.

In patient No. 4, intervals of the first-second and first-third injections were 11 days and 18 days, respectively. The tumor volume was measured at 3 points before and 2 points after the commencement of CTL injections. In this patient, although the tumor volume seemed to change from 56 to 45 cm³, we considered that this did not represent a significant reduction (Fig. 7). The tumor continued to grow slowly after the CTL therapy. The patient died from pneumonia 2 months after the first CTL injection; consent was not given for autopsy.

Aside from some minor bleeding in the tumor cavity of patient No. 1 there was no deterioration of the patients' general condition during the clinical course of CTL therapy in all cases.

DISCUSSION

Although many reports have described the establishment and characterization of tumor-specific murine CTL lines and their possible therapeutic effects,²²⁻²⁶ the induction of human CTL against glioma has not been easy, since autologous CTL are exclusively required for clinical treatment of the tumor. One of the key obstacles, we assume, is the preparation of sufficient live target tumor cells for repeated stimulation of CTL growth^{5,9-12} over a prolonged culture period. However, expansion of primary cultured tumor cells and establishment of tumor cell lines are not practical for every patient since tumor cells cannot always be successfully subcultured.¹³ In this context, stable sources of the tumor-specific antigen are desirable for the induction of autologous CTL.

In patient No. 1, we were unable to obtain a sufficient amount of the target cells due to the extremely slow growth rate of primary cultured tumor cells. Therefore,

we fixed the tumor cells to stimulate PBMC continuously, since we have successfully induced and restimulated CTL on fixed tumor sections²⁷⁾ and/or fixed cultured tumor cells.¹⁶⁾ Furthermore, after initial activation of the lymphocytes, the murine anti-human CD3 monoclonal antibody was used as the stimulator to maintain growth of the CTL. This may have contributed to the preferential growth of the CD4⁺ cell population. CD4⁺ T cells, which have not necessarily been considered "typical killer cells" as CD8⁺ cells are,^{28,29)} but rather "helper cells,"³⁰⁾ in the present T cell population showed high killing activity and specificity against autologous tumor cells (Fig. 1).

We had thought initially that more than 10⁹ autologous CTL cells should be applied to the tumor-removed cavity of the patient, even if the CTL were shown to be able to kill target tumor cells at an E/T ratio of 1 to 10 *in vitro*. However, surprisingly, the tumor mass decreased dramatically after injection of CTL. The response (Fig. 4E) could not be explained in terms of the injected number of CTL and their demonstrated killing activity *in vitro*. The general immune system may have been involved in this response in patient No. 1. Although at present we have no evidence showing that this CD4⁺ CTL population also contained helper T cells cooperatively acting against the autologous GBM cells, this possibility should be taken into consideration to explain how the small number of administered lymphocytes (2×10⁷ cells for the first injection) caused the drastic decrease of the recurrent tumor volume within 2 days (Fig. 4E). If helper T cell were present in the CD4⁺ CTL population, they might activate killer T cell precursors that had infiltrated *in vivo* into the highly vascularized glioma tissue.³¹⁾

In the other 3 cases, live tumor cells obtained by primary cultures were used to induce CTL, and to re-stimulate the induced lymphocytes. In all cases, the induced CTL had high killing activities against autologous tumor cells (Fig. 2). Restriction of the killing of CTL by antibodies against CD3, CD8 and MHC-class I was confirmed in patient No. 3, whose HLA-serotype was A2, A24, B52, B62, (B75), -/- (M. Hayashida, RIKEN Cell Bank, personal communication) (Fig. 3). However, HLA-subtypes were not known in other patients.

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In patients No. 2 and No. 3, we consider that the slight but obvious reduction of tumor volumes observed on MRI was caused by the injected CTL and their high killing activity. In patient No. 4, the reduction of tumor volume was not considered significant. This patient received the smallest number of CTL (Table II). Therefore, we consider that the use of greater number of injected CTL in future studies may allow more effective treatment of malignant gliomas.

Regrowth of the tumor in patients No. 1 and No. 3 may have been due to inadequate delivery of CTL to the tumor site distant from the tip of the Ommaya tube. However, as it is widely known that GBM contains morphologically different types of tumor cells, the regrowth may also have been due to the development of a tumor cell population with different antigenicity, i.e., non-responders to the present CTL.

In patient No. 1, bleeding was observed in the tumor-removed cavity 3 days after the third injection of CTL. This bleeding was probably caused by exposure of the abnormal capillary in the tumor³¹⁾ to the free space produced after the drastic reduction of the tumor mass. However, the bleeding did not influence the neurological condition of the patient and no other adverse effect was observed. In future clinical trials involving CTL, a pilot application will be necessary to avoid possible severe bleeding.

The present results suggest that, although there are many practical problems which must be addressed, such as induction of autologous CTL, stable supply of unknown tumor antigens for repeated stimulation of CTL expansion, and CTL delivery to unknown location of tumors, adoptive immunotherapy with autologous CTL is effective in the treatment of malignant gliomas.

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