Effects of Local Injection of \textit{ex Vivo} Expanded Autologous Tumor-specific \textit{T} Lymphocytes in Cases with Recurrent Malignant Gliomas$^1$

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ABSTRACT

\textbf{Purpose:} The aim of this report was to indicate both the advantages and disadvantages of local cell transfer therapy using \textit{ex vivo} expanded autologous tumor-specific \textit{T} lymphocytes (ATTLs) for recurrent cases of malignant gliomas.

\textbf{Experimental Design:} Subjects are 10 cases of malignant gliomas consisting of 7 cases of glioblastoma multiforme, 2 cases of anaplastic astrocytoma, and 1 case of anaplastic oligoastrocytoma. All cases were recurrences. ATTLs were induced by coculturing peripheral blood mononuclear cells with autologous tumor cells in medium containing interleukin-1, -2, -4, and -6 and administered into the local tumor site in total numbers of 3–247 $\times 10^7$ cells. As end points, tumor response and survival time were analyzed observing clinical state.

\textbf{Results:} Five cases responded to this therapy (namely, one case showed complete remission, and four cases had a partial response). There were three cases of no change, and two cases of progressive disease. The overall tumor response rate was 50%. No complications were noticed, except for two cases of minor local hemorrhage and eight cases of temporary fever. Nine cases died of further tumor progression, and one case died of aspiration pneumonia, and the cause-specific survival analysis indicates that the median survival time was 5 months from the initial ATTL injection.

\textbf{Conclusions:} The results suggest that local administration of ATTLs is effective in recurrent malignant gliomas in that it demonstrated a high benefit:risk ratio with minor side effects. Although its antitumor effect may be temporary in some advanced cases, it is highly possible that the tumor could be stabilized when conditions are optimal.

INTRODUCTION

Tumor-specific immunotherapy for malignant gliomas has risen in importance because of its high benefit:risk potential. Among various immunological effector cells, CTLs have been considered to play a crucial role in tumor rejection \textit{in vivo}. CTLs can be defined as CD8$^+$ \textit{T} lymphocytes that kill tumor cells specifically in the MCH class I-restricted manner; however, recent analyses indicate that tumor-specific CD4$^+$ \textit{T} lymphocytes are not only capable of killing tumor cells but are also capable of playing an important role in CTL-mediated tumor cell killing (1, 2). Despite the presence of these highly efficient effector cells \textit{in vivo}, antitumor immune mechanisms are suppressed in tumor-bearing hosts by factors such as a structural change in T-cell receptor (3) and secretion of transforming growth factor $\beta$ (4), IL-$6$ (5), or prostaglandin E$_2$ (6) from tumor cells. Therefore, our global scheme of adoptive immunotherapy using ATTLs, consisting of CD8$^+$ and CD4$^+$, is straightforward, featuring \textit{ex vivo} expansion of ATTLs under optimized conditions followed by administration to the host for tumor eradication. Although there are many animal studies showing that immunological cell therapy against gliomas by CTLs is very effective (7–9), the clinical applications of this therapy have been limited (10–13). Because the induction of autologous CTLs or ATTLs against spontaneous brain tumor in rodents is practically impossible, most of these animal models use alloreactive \textit{T} cells against a homogeneous experimental tumor cell population. As a result, it is difficult to translate these animal data into human patients bearing spontaneous malignant gliomas with heterogeneous cell populations.

We have demonstrated a method of inducing autologous CTLs (14, 15) or ATTLs (16) from human PBMCs using tumor cells irradiated by X-rays or fixed with formalin as target antigen. Based on these studies, we expanded ATTLs using the same method and experimentally administered them to end-stage cases of recurrent malignant gliomas to test toxicity, feasibility, and potential effectiveness of ATTLs (17). Using these data, we report the results and characteristics of 10 consecutive cases treated with local administration of \textit{ex vivo} expanded ATTLs.

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$^2$ The abbreviations used are: IL, interleukin; ATTL, autologous tumor-specific \textit{T} lymphocytes; PBMC, peripheral blood mononuclear cell; Gd, gadolinium; MRI, magnetic resonance image; KPS, Karnofsky performance status; CR, complete remission; PR, partial response; PD, progressive disease; ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; CSF, cerebrospinal fluid.
therefore excluded from this study: (a) patient age < 70 years; (b) pathologically proven WHO grade III and IV glioma at the last surgery; (c) recurrence after surgery, radiotherapy, and chemotherapy; and (d) presence of evaluable tumor volume represented by Gd-enhanced area on T1-weighted MRIs. Exclusion criteria were as follows: (a) prior chemotherapy within 4 weeks; and (b) serious concomitant disease precluding completion of the protocol.

Pathological specimens were evaluated by two pathologists at the University Hospital of Tsukuba, and recurrence was determined through enlargement of Gd-enhanced area and mass effect on follow-up magnetic resonance imaging associated with clinical evaluation. Local administration of ATTLs was performed in 13 cases of malignant gliomas during this period, and clinical evaluation. Local administration of ATTLs was performed within 4 weeks; and (b) serious concomitant disease precluding completion of the protocol.

Table 1 Background of 10 cases treated with ATTLs

<table>
<thead>
<tr>
<th>No.</th>
<th>Age/sex</th>
<th>KPS</th>
<th>Pathology</th>
<th>Surgery</th>
<th>Radiation (Gy)</th>
<th>Adjuvant therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24/F</td>
<td>20%</td>
<td>GBM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (partial)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.8 (brain)</td>
<td>PAV, MTX, IFN-β, IA ACNU, IA CDDP</td>
</tr>
<tr>
<td>2</td>
<td>62/M</td>
<td>30%</td>
<td>GBM</td>
<td>1 (partial)</td>
<td>64.8 (brain)</td>
<td>IA ACNU, IFN-β</td>
</tr>
<tr>
<td>3</td>
<td>64/M</td>
<td>20%</td>
<td>GBM</td>
<td>1 (partial)</td>
<td>63.6 (brain)</td>
<td>PAV × 2, IFN-β</td>
</tr>
<tr>
<td>4</td>
<td>53/M</td>
<td>20%</td>
<td>AA</td>
<td>2 (partial)</td>
<td>64.8 (brain)</td>
<td>PAV, CDDP, VP-16, IFN-β</td>
</tr>
<tr>
<td>5</td>
<td>27/F</td>
<td>20%</td>
<td>AA</td>
<td>2 (partial)</td>
<td>44.5 (brain)</td>
<td>IFN-β</td>
</tr>
<tr>
<td>6</td>
<td>23/F</td>
<td>40%</td>
<td>AOA</td>
<td>5 (partial)</td>
<td>55.8 (brain)</td>
<td>PAV × 4, IFN-β</td>
</tr>
<tr>
<td>7</td>
<td>62/F</td>
<td>20%</td>
<td>GBM</td>
<td>2 (partial)</td>
<td>64.8 (brain)</td>
<td>PAV, IFN-β</td>
</tr>
<tr>
<td>8</td>
<td>61/F</td>
<td>30%</td>
<td>GBM</td>
<td>2 (partial)</td>
<td>55.8 (brain)</td>
<td>PAV, IFN-β</td>
</tr>
<tr>
<td>9</td>
<td>70/M</td>
<td>50%</td>
<td>GBM</td>
<td>2 (partial)</td>
<td>64.8 (brain)</td>
<td>PAV, IFN-β</td>
</tr>
<tr>
<td>10</td>
<td>58/M</td>
<td>20%</td>
<td>GBM</td>
<td>2 (partial)</td>
<td>68.4 (brain)</td>
<td>PAV × 3</td>
</tr>
</tbody>
</table>

Average 50.4 62.2 (brain)

<sup>a</sup> GBM, glioblastoma multiforme; AA, anaplastic astrocytoma; AOA, anaplastic oligoastrocytoma.

<sup>b</sup> Partial, partial removal.

<sup>c</sup> MTX, methotrexate.

<sup>d</sup> IA, intraarterial.

<sup>e</sup> CDDP, cisplatin.

MATERIALS AND METHODS

Patient Population. All patients were assessed and supervised by the Department of Neurosurgery at the University Hospital of Tsukuba during the period of December 1996 to September 2001. Eligible criteria were as follows: (a) patient age < 70 years; (b) pathologically proven WHO grade III and IV glioma at the last surgery; (c) recurrence after surgery, radiotherapy, and chemotherapy; and (d) presence of evaluable tumor volume represented by Gd-enhanced area on T1-weighted MRIs. Exclusion criteria were as follows: (a) prior chemotherapy within 4 weeks; and (b) serious concomitant disease precluding completion of the protocol.

Pathological specimens were evaluated by two pathologists at the University Hospital of Tsukuba, and recurrence was determined through enlargement of Gd-enhanced area and mass effect on follow-up magnetic resonance imaging associated with clinical evaluation. Local administration of ATTLs was performed in 13 cases of malignant gliomas during this period, and the following 3 cases were not evaluable for efficacy and were therefore excluded from this study: (a) an 8-year-old girl with anaplastic gangliogloma of the thalamus who was treated with an insufficient number of ATTLs induced from paraffin-embedded tissue without killing assay; (b) a 40-year-old woman with right frontal anaplastic astrocytoma who received 42 Gy of high-LET radiation 1 month after the final administration of ATTLs; and (c) a 60-year-old woman with left frontal glioblastoma who was treated with one local injection with ATTLs and two local injections with natural killer cells that were induced because of insufficient amount of target tumor cells for ATTL induction. These 10 cases are summarized in Table 1. They consist of seven cases of glioblastoma multiforme, two cases of anaplastic astrocytoma and one case of anaplastic oligoastrocytoma (Table 1). There were five males and five females, with an average age of 50.4 years. All cases were diagnosed as recurrence after multiple surgical procedures, irradiation of 50–65 Gy of γ-rays, and chemotherapy using procarbazine, ACNU, and vincristine, combined with IFN-β. In these cases, the initial ATTLs were administered at least 5 weeks after previous treatment. After the response to ATTL treatment was determined following the protocol, dexamethasone was administered as clinically indicated at the end stage on recurrence in six cases (cases 2, 3, 5, 6, 8, and 9). In case 10, 4–16 mg of dexamethasone were used daily during the course of ATTL administration because of rapid tumor progression. No steroid was used in cases 1, 4, and 7. The KPS of these patients at the time of treatment ranged from 20% to 50%. Preliminary results of cases 1–4 have been reported previously with a main focus on the toxicity, feasibility and early response of this treatment (17).

Procedures. Our procedures to induce ATTLs have been described previously (14). Fresh operative tumor specimen obtained at surgery was immediately minced and gently rinsed with Dulbecco’s PBS followed by culture in two or three 60-mm plates containing 5 ml of DMEM with 10% FCS. Two days before initiation of ATTL induction, the cultured tumor cells were trypsinized and plated at a concentration of 1 × 10⁵ tumor cells/well in 2 ml of the same culture media in 10–20 wells of a 24-well culture plate, depending on the number of the tumor cells harvested. When the tumor cells adhered to the bottom of the plates the next day, they were irradiated with 50 Gy of X-rays (cases 3–10) or fixed for 3 h with freshly prepared 10% formalin in PBS (cases 1 and 2) followed by thorough rinse with PBS and incubation overnight in 2 ml of induction medium RHAM-α (18) containing 5% autologous plasma. Patients’ PBMCs were prepared from approximately 30 ml of heparinized peripheral blood with a conventional preparation kit (Lymphoprep; Nycomed Pharma A.S.). Separated PBMCs were plated at a concentration of 1 × 10⁶ PBMCs/well on these tumor cells, which had been irradiated or fixed beforehand. They were cocultured in 2 ml of RHAM-α with 5% autologous plasma containing 167 units/ml IL-1β (Ohtsuka Pharmaceuticals Co.), 67 units/ml IL-2 (Shionogi & Co., Ltd.), 67 units/ml IL-4 (Genzyme Co., Cambridge, MA), and 134 units/ml IL-6 (Ajinomoto Inc.). The plates were periodically shaken to ensure that the PBMCs were in contact with the tumor cells, and the half of
the culture media was changed every 2 days to maintain and facilitate the growth of ATTLs. After 10–14 days, when the target tumor cells disappeared almost completely, lymphocytes were transferred into 6-well culture plates at a concentration of \(2 \times 10^6\) lymphocytes/well, in which \(2 \times 10^5\) tumor cells were previously plated and irradiated or fixed as mentioned. They were cocultured once more in 5 ml of the same induction media for restimulation to expand ATTLs for approximately 7 days.

The \textit{in vitro} cytotoxicity of the resulting ATTLs was determined by crystal violet staining assay as described previously (14, 15, 19). Briefly, ATTLs were added to the precultured target cells as effectors at the indicated E:T ratio. After 24 h of coculture, the resulting adherent target cells were fixed with 10% (v/v) formalin and stained with crystal violet solution (0.4% in water). Then, the fixed cells in plates were washed gently with running water and dried at room temperature. Dye in the fixed cells of each well was eluted by methanol, and the \(A_{570}\) nm was determined. The final surviving fraction was calculated from the \(A_{570}\) nm of each sample and that of control wells obtained from only the target cells precultured in a separate plate. The sensitivity of this method has been reported to be equivalent to \(^{51}\text{Cr} \text{release assay at E:T ratios } < 10\) (19).

The phenotypes of the ATTLs were characterized by staining with FITC-labeled monoclonal antibodies (Becton Dickinson, Mountain View, CA) against CD3, CD4, and CD8 surface antigens. Flow cytometry was performed by FACScan with LYSIS II software (Becton Dickinson) following the manufacturer’s instructions. Mycoplasma assay, endotoxin assay, and DNA fingerprinting were performed before each administration, as described previously (17).

The chronological scheme of the therapeutic procedure is shown in Fig. 1. Target tumor tissues taken at the first operation were submitted for routine primary culture. It takes approximately 3 weeks from separation of PBMCs to induction of a sufficient number of ATTLs by the method mentioned above. The resulting ATTLs were administered once weekly as a bolus in a 2-ml suspension, via Ommaya reservoir, into the cavity created by the initial surgery in all cases except for case 6, in which intrathecal injections were performed. Ommaya reservoir implant with irrigation of the cavity was performed 1–3 days before the first administration in all cases except for cases 3 and 6. This procedure, tumor volume reduction was not attempted. In case 3, ATTLs were injected more than 3 weeks after Ommaya reservoir implant. In case 6, ATTLs were administered into the lateral ventricle and the lumbar subarachnoid space targeting the disseminated tumor cells. Pretreatment MRIs were taken 2–5 days before the first ATTL administration in all cases. Early change was monitored by MRIs taken 1 and 4 weeks after the last injections, and then MRIs were followed-up at 3-months intervals or sooner, as clinically indicated. Evaluation was performed following the guidelines reported by Therasse et al. (20), except for the measurement of tumor size. The tumor regression rate was calculated based on the three-dimensional volume of the Gd-enhanced area on T1-weighted MRI as mentioned in the previous report (17).

Ethics. The ethical committee of the University of Tsukuba (The Special Committee for Medical Ethics) gave approval to administer this therapy to these patients, and written informed consent was obtained from all patients before initiating any procedure related to this treatment.

RESULTS

Numbers and characterizations of ATTLs induced from these patients are summarized in Table 2. The number of ATTLs per single injection ranged from \(0.7 \times 10^7\) to \(144.0 \times 10^7\) (average number, \(26.5 \times 10^7\)), and the total number ranged from \(3.0 \times 10^7\) to \(247.0 \times 10^7\) (average number, \(74.1 \times 10^7\)). Final numbers of injected ATTLs varied depending on the culture condition, including the initial number of PBMCs and the amount of target tumor cells for initial induction and restimulation. Phenotype analyses show that the proportion of CD4+ T cells was greater than that of CD8+ T cells in more than two inductions in six cases. The percentage of surviving tumor cells ranged from 3% to 58% after exposure to ATTLs at E:T ratios indicated.
Clinical results were evaluated by two end points: tumor response and survival (Table 3). Five cases responded to this therapy, namely, one case of CR and four cases of PR on Gd-enhanced T1-weighted MRI. In these responders, the regression rates of tumor volume ranged from 54% to 100% on MRI. Three cases demonstrated tumor regression ranging from 6% to 23%, and they were determined as no change. Case 10 showed tumor growth by 24% during the treatment and was assessed as PD.

### Table 2
Number and characterization of ATTLs administered to each patient

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>No. of ATTLs/injection ((\times 10^7))</th>
<th>Total no. of ATTLs ((\times 10^7))</th>
<th>Phenotype (% CD4:CD8)</th>
<th>Surviving tumor cells ((E:T) ratio)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24/F</td>
<td>2.0</td>
<td>7.0</td>
<td>95.2:4.8</td>
<td>10.2 (10)</td>
</tr>
<tr>
<td>2</td>
<td>62/M</td>
<td>0.7</td>
<td>3.0</td>
<td>46.5:53</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>64/M</td>
<td>11.0</td>
<td>21.3</td>
<td>33.8:66</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>53/M</td>
<td>2.0</td>
<td>3.0</td>
<td>20.7:4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>27/F</td>
<td>2.0</td>
<td>2.0</td>
<td>16.5:77</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>23/M</td>
<td>19.1</td>
<td>98.5</td>
<td>70.4:29</td>
<td>53 (4)</td>
</tr>
<tr>
<td>7</td>
<td>62/F</td>
<td>38.0</td>
<td>247.0</td>
<td>96.5:3.5</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>61/F</td>
<td>17.5</td>
<td>48.3</td>
<td>60.7:39</td>
<td>58</td>
</tr>
<tr>
<td>9</td>
<td>70/M</td>
<td>11.2</td>
<td>90.15</td>
<td>35.2:64</td>
<td>56</td>
</tr>
<tr>
<td>10</td>
<td>58/M</td>
<td>78.0</td>
<td>232.8</td>
<td>51.2:48</td>
<td>55</td>
</tr>
</tbody>
</table>

\^a E:T ratio is 8 if it is not specified in parentheses. Absent values are mainly due to lack of target tumor cells.

### Table 3
Clinical results

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Tumor volume ((cc))</th>
<th>Regression rate (%)</th>
<th>Response</th>
<th>From initial CTL injection to outcome (months)</th>
<th>Time to recurrence (months)</th>
<th>Outcome (KPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>130</td>
<td>70</td>
<td>PR</td>
<td>3</td>
<td>1</td>
<td>Dead</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>65</td>
<td>NC^a</td>
<td>2</td>
<td>1</td>
<td>Dead</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>8</td>
<td>NC</td>
<td>4</td>
<td>2</td>
<td>Dead</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>65</td>
<td>PR</td>
<td>23</td>
<td>16</td>
<td>Dead</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>100</td>
<td>CR</td>
<td>33</td>
<td>22</td>
<td>Dead</td>
</tr>
<tr>
<td>6^b</td>
<td>IC:72</td>
<td>+17</td>
<td>PD</td>
<td>5</td>
<td>—</td>
<td>Dead</td>
</tr>
<tr>
<td>7^c</td>
<td>LS:11</td>
<td>82</td>
<td></td>
<td>—</td>
<td>—</td>
<td>Dead</td>
</tr>
<tr>
<td>8</td>
<td>88</td>
<td>54</td>
<td>PR</td>
<td>1</td>
<td>—</td>
<td>Dead</td>
</tr>
<tr>
<td>9</td>
<td>27</td>
<td>23</td>
<td>NC</td>
<td>10</td>
<td>7</td>
<td>Dead</td>
</tr>
<tr>
<td>10</td>
<td>72</td>
<td>+24</td>
<td>PD</td>
<td>5</td>
<td>—</td>
<td>Dead</td>
</tr>
</tbody>
</table>

\^a NC, no change.

\^b In this case, disseminated tumor in the lumbar subarachnoid (LS) space regressed remarkably without recurrence; however, there was 17% growth in the intracranial (IC) primary site.

\^c This patient died of aspiration pneumonia and renal failure before recurrence was noticed clearly.

Clinical results were evaluated by two end points: tumor response and survival (Table 3). Five cases responded to this therapy, namely, one case of CR and four cases of PR on Gd-enhanced T1-weighted MRI. In these responders, the regression rates of tumor volume ranged from 54% to 100% on MRI.
the lumbar subarachnoid space significantly on MRI. However, the primary intracranial lesion enlarged, and the patient died 5 months after treatment. Thus, the overall response of this case is PD, although ATTLs were effective to the dissemination (17). The overall tumor response rate of our series was 50% (CR and PR). No change in KPS between pre- and posttreatment was seen in cases 1, 2, 3, 7, 8, and 10, although transient improvements in consciousness level and vital signs were observed in cases 1 and 3. Case 4 was discharged from the hospital and stayed home for more than 15 months, and the highest KPS for this case was 40%. Case 5 left the hospital walking with slight right hemiparesis and motor aphasia. She stayed home for more than 20 months, and her highest KPS was 60%. In case 6, intrathecal ATTL injection relieved her lumbar pain markedly with an improvement in KPS from 40% to 60% for approximately 3 months. Also, case 9 left hospital with a KPS of 70% and remained in the same state for more than 6 months. With regard to complications, there were four cases of local hemorrhage detected only by MRI (cases 1, 4, and 8) or observed at autopsy (case 7); however, none of these cases demonstrated hematoma-related clinical symptoms. Although temporary low-grade fever was noticed in all cases except for cases 2 and 9, treatment-related infections or acute allergic reactions were not observed. A theoretical concern is the occurrence of leukoencephalopathic changes caused by local administration of ATTLs. ATTL-related white matter change might occur in case 5 because serial magnetic resonance imaging demonstrated atrophic process of the brain as the tumor regressed (Fig. 2). However, it is difficult to determine whether this process was caused by ATTL injection or because the patient had received radiotherapy previously and there was no pathological evidence. Also, there was no histological evidence of leukoencephalopathy in the vicinity of the tumor cavity in the autopsy specimen of case 7.

All patients died during follow-up. Because case 7 died suddenly of massive aspiration pneumonia and severe lactic acidosis 3 weeks after ATTL treatment, nine cases in this series died of tumor progression and were available for cause-specific survival analysis by the Kaplan-Meier method (Fig. 3). The average and median survival times of these 9 cases were 9.7 and 5 months, respectively.

Case 5 was a 27-year-old woman with a left temporo-parieto-frontal anaplastic astrocytoma. Because her neurological status deteriorated rapidly due to tumor growth during radiotherapy, surgical decompression and subsequent ATTL protocol were carried out. After local injection of ATTLs with a total number of $38 \times 10^7$ cells, the residual tumor in the medial fronto-temporal region was reduced in size and disappeared on MRI 3 months later (Fig. 3). She was discharged with a KPS of 60% due to slight right hemiparesis and motor aphasia.
and her neurological status had been stable for 21 months. However, recurrence was noticed at the left cerebral peduncle of the midbrain, deeper than the initial tumor location. She suffered rapidly progressive right hemiparesis, left ophthalmoplegia, and consciousness disturbance, and she died 33 months after the final administration of ATTLs.

Case 7 was a 62-year-old woman with a right frontal glioblastoma multiforme. Tumor recurrence was noted on the MRI, and ATTL injection resulted in 54% regression of the enhanced area and was associated with minor hemorrhage in the injected cavity (Fig. 4). However, the patient suffered massive aspiration pneumonia followed by renal failure and severe lactic acidosis 15 days after the last ATTL administration. Autopsy gave no evidence to indicate a direct relationship between ATTL administration and her aspiration pneumonia. Marked lymphocyte infiltration was shown in the tumor corresponding to the enhanced area on MRI (Fig. 5a). Histopathology of the specimen obtained at the initial surgery did not show
marked lymphocyte infiltration as compared with the autopsy specimen (Fig. 5b). Immunohistochemical analysis indicated that these infiltrating cells were positive for leukocyte common antigen, and most of these cells were positive for UCHL-1 staining. Furthermore, most of these cells were CD8+ and CD4−. These pathological findings indicate that there was a marked infiltration of CD8+ T lymphocytes at the tumor close to the cavity.

**DISCUSSION**

To enhance the therapeutic response of malignant gliomas, various immunotherapies have been developed and attempted (21). Among them, adoptive cell transfer therapy using ex vivo activated autologous lymphocytes has been considered as one of the promising approaches. Autologous LAK cells have been administered to glioma patients in some clinical studies (22–25) with certain positive results. However, LAK cell therapy alone is not considered very efficient for clinical use at present because of its low killing activity, nonspecificity, and the side effects associated with simultaneous IL-2 administration. Clinical trials using more specific and efficient effectors have been reported in limited number. Kuruse et al. (10) reported five patients with malignant gliomas treated by intracavitary alloreactive CTLs and IL-2, and they were able to stabilize tumor growth in three grade III gliomas. Also, Plautz et al. (11) reported 10 cases of malignant gliomas treated with T cells expanded from inguinal lymph nodes that drain the tumor vaccine site; however, the results were 8 cases of PD and 2 cases of PR. They also treated 12 cases of newly diagnosed gliomas using the same method with improved results (12). Furthermore, Quattrocchi et al. (13) reported a pilot study of local injection of autologous tumor-infiltrating lymphocytes combined with adjuvant chemotherapy on six cases of recurrent malignant gliomas with overall responses of one CR, four PRs, and one case of PD. A combination of active immunization and specific adoptive cellular immunotherapy was also tried on 15 patients with recurrent astrocytoma by Holladay et al. (26), with a focus on the feasibility of this treatment. We reported previously (17) on toxicity and feasibility when the early responses of cases 1–4 of the present study illustrated a temporary reduction in tumor volume to various degrees without significant toxicity. Case 4 in this series was considered minor response (regression of 29%) in that previous report (17); however, further observation demonstrated remarkable tumor reduction on magnetic resonance imaging after that paper was published, and the final response was PR, as indicated in Table 3. The present study reports one of the highest response rates reported to date for adoptive cell transfer therapy alone in cases of recurrent malignant gliomas.

The first technical difficulty faced by our procedure is in the primary culture of tumor tissue because it is sometimes very difficult to harvest a sufficient number of target cells required for killing assay or restimulation for ATTl expansion as shown in Table 2. The second barrier is that it is occasionally difficult to deliver them to the tumor site to achieve direct contact with tumor cells in vivo. To reproduce in vitro response after local administration in vivo, tumor cells should be exposed to the cavity space. Membranous tissue is occasionally formed along the wall of the cavity, which usually contains high-protein fluid and tumor debris. Thus, we injected ATTls into the tumor cavity immediately after Ommaya reservoir implant with tumor

![Fig. 5 Photomicrographs of the autopsy specimen taken from case 7. Histopathological appearance indicates marked infiltration of mononuclear cells in the tumor tissue (×100; H&E staining; a) as compared with that taken at the time of initial surgery (×100; H&E staining; b). Immunohistochemical staining with UCHL-1, which identifies T lymphocyte, showed that most of these mononuclear cells are T cells, and these were also CD8+.](image-url)
cavity clean up in eight cases. This procedure is also beneficial because the Ommaya reservoir is usually not patent for a long period when it is implanted in closed tumor cavities containing protein-rich fluid with tumor debris. In case 6, the condition of the disseminated tumor in the CSF was very similar to in vitro culture conditions, and it was possible for ATTLs to contact tumor cells directly in the CSF as they settled together in the subarachnoid space by gravity, demonstrating a good response against these disseminated tumors. This may indicate that CSF dissemination could be a good indication of intrathecal administration of ATTLs, as mentioned recently by Clemons-Miller et al. (27).

We previously reported the results of an inhibition assay using anti-CD3, anti-CD4, and anti-CD8 antibodies and anti-MHC class I and II antibodies on the killing activity of ATTLs induced from case 6 (17, 28). It was shown that their killing activity was inhibited by anti-CD3, anti-CD8 and anti-MHC class I antibodies, whereas anti-CD4 and anti-MHC class II antibodies did not show any inhibition, indicating that tumor cell killing of the induced ATTLs was MHC restricted. However, we found that CD4+ T cells grew fast and predominated the culture population in the course of ATTL induction in cases 1, 5, 6, 7, and 9 and that these CD4+ -rich lymphocytes did not exhibit high killing activity against autologous tumor cells in vitro. The tumor volume reduction on magnetic resonance imaging would be much smaller if in vitro killing activity at a defined E:T ratio were directly transferred to the in vivo state. Nevertheless, as is shown in Table 2, the population of CD4+ T cells was greater than that of CD8+ T cells in some good responders, indicating a possibility of further immunological reactions in vivo after direct local administrations of ATTLs in the presence of CD4+ cells. This does not contradict the findings on the autopsy specimen in case 7, in which a great number of CD8+ T cells were infiltrating the tumor tissue, despite the fact that most ATTLs administered were CD4+ T cells (Table 2). These observations support the hypothesis that in vivo autoactivation of CD8+ CTLs and subsequent antitumor immunological reactions both occur as a result of the presence of CD4+ T cells. Therefore, it is speculated that ex vivo expanded CD8+ CTLs alone may fail to kill heterogeneous tumors in vivo, resulting in recurrence from escaped clones, and that the presence of CD4+ T cells should be required for optimal induction of CTLs via MHC class II-mediated pathway as mentioned recently (1, 2).

Although further recurrence was noticed in all cases, we consider that cases 4, 5, and 9 clearly benefited from this treatment. Quality of life improved for a certain period in these cases, especially in cases 4 and 5, where the patients recovered from a bed-ridden state. However, the survival time curve might shift slightly to the right without a higher plateau, and the benefits for survival time might be small in most glioblastoma cases, despite a temporary reduction in tumor size on magnetic resonance imaging. This may largely be due to the fact that most patients in this series were advanced or end-stage recurrences with large tumor volumes. In particular, unavoidable steroid administration in case 10 might be another factor responsible for treatment failure despite the administration of a large number of ATTLs. Therefore, to improve the response and survival rate with this immunotherapy, patients should be treated in an earlier stage with smaller tumor volumes, and those patients dependent on steroid administration should be excluded. With regard to eradinating clones escaped from CD8+ T cells, a higher population of CD4+ T cells or a combination of natural killer cells might be useful. In addition, intra-arterial administration of ATTLs may be beneficial for tumor cells that have infiltrated the surrounding area, although it may require a great number of ATTLs to achieve sufficient local accumulation.

In conclusion, it was shown that local administration of ATTLs is effective in recurrent malignant gliomas as compared with former adoptive cell transfer therapy and that this treatment demonstrated a high benefit:risk ratio with minor side effects. Although the antitumor effect of this treatment may be temporary in some advanced cases, it is highly possible that local administration of ATTLs consisting of CD4+ and CD8+ T cells could stabilize the tumor for a significant period, if the conditions mentioned above are optimal.

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