Basic Studies on Local Adoptive Immunotherapy with Lymphokine-activated Killer (LAK) Cells and It's Clinical Application to Malignant Gliomas

Lymphokine-activated Killer (LAK) 細胞を用いた悪性神経膠腫に対する局所養子免疫療法の基礎研究と臨床応用

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Basic Studies on Local Adoptive Immunotherapy with Lymphokine-activated

Summary

Basic studies of local adoptive immunotherapy with lymphokine-activated killer (LAK) cells and recombinant interleukin-2 (rIL-2) were carried out along with its clinical application using peripheral blood lymphocytes (PBLs) obtained from 22 patients with malignant glioma including 19 recurrent patients. The LAK cells obtained from the patients were active enough to kill both allogeneic and autologous glioma cells, though the cytotoxicities of patients' PBLs and rIL-2-induced LAK cells were less than those of healthy control subjects.

The cytolytic activity of LAK cells to Daudi cells (LAK activity) was correlated positively with that to glioma-derived cells, enabling us to substitute the LAK activities of rIL-2-induced LAK cells for the cytotoxicities of LAK cells to glioma cells. On light microscopic observation of a co-culture of LAK cells and glioma-derived cells, the target cells attacked by the LAK cells shrank, became round, and lysed within 60 minutes.

Phenotype analysis of patients' LAK cells showed a marked increase in HLA-DR and IL-2 receptor (IL-2 R, CD25) positive cells, that was observed also in those of the control subjects, although the percentage of Leu-3 (CD4) positive cells was less than that in the healthy controls. Anticonvulsants and the cerebrospinal fluid of glioma patients did not affect the generation of LAK cells and the cytotoxic activities of rIL-2 induced LAK cells. Dexamethasone (glucocorticoid) inhibited the generation of LAK activity by rIL-2, but did not have any inhibitory effect on LAK cells already induced by rIL-2.

In clinical trials, we administered both LAK cells ($1-2 \times 10^9$) and rIL-2 ($5-30 \times 10^4$ JRU) into the tumor cavity through a previously implanted Ommaya reservoir and catheter system onde a week several times. Of 15 patients receiving this immunotherapy in whom residual tumors were detected on CT scan, three had complete response. Malignant gliomas in the other patients, however, grew again from under the granulation tissue formed by the injected LAK cells and rIL-2, resulting in a fatal outcome. The mean survival time of 16 patients with recurrent malignant gliomas was 16 months. Side effects associated with this immunotherapy were dependent upon the amount of injected rIL-2 and would be controlled by reducing the amount of rIL-2.

Although this new immunotherapeutic approach has resulted in marked tumor regression in some patients for whom no other effective therapy is available at present, it requires further development and improvement to deliver the injected LAK cells to deep-seated tumor cells before its clinical use is generally accepted.

Introduction

Gliomas (tumors of neuroepithelial tissue) account for 36% of all primary brain tumors in Japan (1), with most gliomas being malignant and recurring inevitably in spite of surgical treatment followed by radiation and chemotherapy. The mean survival time (MST) of patients with malignant gliomas treated with current therapies is approximately 15 months from the time of surgery in Japan (1) and 12 months in the United States (2).

In the hope of improving patient survival, many neurosurgeons have conducted numerous clinical trials in search of better treatment modalities. Immunotherapy has for many years been an attractive candidate, because such treatment does not harm normal cells and the survival of glioma patients seems to be correlated with the degree of lymphocytic infiltration (3, 4). Takakura et al. (5, 6) have already reported increased survival times in patients with malignant brain tumors treated with transfusions of allogeneic bone marrow cells or intratumoral infusions of autologous and allogeneic activated lymphocytes. Other investigators have also reported improvement or stabilization of the clinical course of patients with brain tumors treated with intratumoral injections of autologous lymphocytes (7, 8).

Recent advances in molecular biology and cellular immunology have renewed interest in a new class of lymphoid killer cell that is capable of killing tumor cells in vitro and/or in vivo (9, 10, 11). These lymphokine-activated killer (LAK) cells are produced by the incubation of peripheral blood mononuclear cells (PBMCs)

with interleukin-2 (IL-2) alone for 2 days or more (9), and express potent cytotoxicity against a variety of tumor cells. Although many questions remain to be resolved concerning LAK cells, the efficacy of this form of adoptive immunotherapy to malignant gliomas should be clarified. Gliomas are located only in the central nervous system and rarely metastasize to the extra-neural organs. We therefore tried local adoptive immunotherapy consisting of intralesional LAK cell injection with IL-2 through a previously implanted Ommaya reservoir and catheter system.

The present study was designed in order to clarify the following questions on local adoptive immunotherapy of malignant gliomas with LAK cells.

1. Are the cytotoxic activities of LAK cells generated from the PBMCs of glioma patients comparable to those of LAK cells from healthy subjects?
2. Do glioma cell-derived suppressive factors in cerebrospinal fluid (CSF) of the patients inhibit the cytotoxic ability of administered LAK cells?
3. Do anticonvulsants and steroids that are often administered to glioma patients inhibit the functions of injected LAK cells or IL-2?
4. Can injected LAK cells kill malignant glioma cells in vivo as well as in vitro?
5. Are adverse effects of this immunotherapy too severe to apply to patients with malignant gliomas?
6. Is the outlook for the future of local adoptive

immunotherapy to malignant gliomas favorable?

The results presented in this paper suggest that this type of local adoptive immunotherapy is useful as an adjuvant to the treatment on malignant gliomas, even though several problems remain to be elucidated.

Materials and Methods

Materials

Monoclonal antibodies including anti-Leu 2a (CD8), 3a (CD4), 4 (CD3), 7 (CD57), 11 (CD16), HLA-DR, and IL-2 receptor (IL-2 R, CD25) were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA, U.S.A.). Recombinant interleukin-2 (rIL-2, 10^7 Japan Reference Units = JRU/mg) was supplied by Shionogi Pharmaceutical Inc. (Osaka). Anticonvulsants were provided from the companies supplying each drug: diphenylhydantoin from Dainihon Pharmaceutical Inc. (Osaka), phenobarbital from Fujinaga Pharmaceutical Inc. (Tokyo), and Valproic acid by Kyowa Hakko Inc. (Tokyo). Culture media and reagents for use on cultures of LAK cells were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). Ficoll-Paque was obtained from Pharmacia Inc. (Piscataway, NJ, U.S.A.), and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.).

Patient Profile

This study involved 22 patients (17 males and 5 females) with malignant gliomas. The age ranged from 14 to 60 years old. Eight patients suffered from glioblastoma multiforme, seven with astrocytoma grade 3, four with oligodendroglioma grade 3, one with gemistocytic astrocytoma, and two with malignant ependymoma (Table 1). The tumors of twenty patients were located in the supratentorial region, one in the brainstem (Case 18), and one in the upper cervical spinal cord (Case 9). Nineteen patients had tumors that recurred (six had second recurrences) and three patients (Case 12, 13, 17) had no recurrent tumor on computed tomographic scan (CT scan). Previous treatment by surgery and radiotherapy was carried out in all cases from 3 to 135 months, and chemotherapy was conducted in 14 cases from 4 to 59 months before this treatment. All patients had more than a 60 % Karnofsky score as performance status.

Production of Lymphokine-activated Killer Cells

For in vivo application of adoptive immunotherapy, their PBMCs were separated by processing 4 to 6 liters of whole blood for 2-3 hours, using a continuous-flow cell separator (Model COBE 2997, IBM Corp., Cobe Laboratories, Lakewood, CO, U.S.A.). Acid-citrate-dextrose was used as the anticoagulant. The erythrocyte-containing PBMCs suspension was carefully layered on Ficoll-Paque and centrifuged at 400 x g for 20 minutes. The separated PBMCs were transferred and washed three times with 0.9% sodium

chloride. The cells were then suspended again at a concentration of 1-2 x 10^6/ml in complete medium (CM) consisting of RPMI 1640 or AIM-V, containing 2% heat-inactivated human AB serum (HTLV-III negative), 2 mM glutamine, 50 μg/ml of gentamycin sulfate, and 1000 JRU/ml of rIL-2. The cells were then cultured in 75 cm^2 polystyrene flasks (Miles Scientific Inc., Naperville, IL, U.S.A.) in 30-40 ml of CM at 37°C with 5% CO_2 for 3-4 days.

For in vitro experiments, the separated PBMCs or LAK cells obtained from 22 patients and 10 healthy control subjects were incubated in 30 ml of CM at a concentration of 1-2 x 10^6/ml in 75 cm^2 polystyrene flasks. Anticonvulsants or dexamethasone (DX) were added to CM at determined concentrations. Diphenylhydantoin (DPH), phenobarbital (PB), and valproic acid (VP) were selected out of anticonvulsants because of frequent prescription.

For the experiments using CSF, CSF obtained from three glioma patients (one of three was Case 1) at the terminal stage was added to the same amount of CM. In the experiments on CSF, PBMCs obtained from the same patient as CSF were used.

Preparation of Tissue Culture Glioma Target Cells

The human glioma-derived U251 cells (12) and glioma cells obtained at the time of surgery were used in this study. Freshly resected tumor samples were minced into 1 mm^3 pieces and transferred into 75 cm^3 polystyrene flasks in Eagle's minimum essential medium (MEM) containing 20% fetal calf serum (FCS). After tumor outgrowth began, the medium was changed regularly and

the cells were subcultured when needed using 0.25% trypsin to release the tumor cells from the flask. The malignant and glial properties of these lines have been established by ultrastructural (13), biochemical (14), and biophysical techniques (15).

For measurement of cytotoxic activities of LAK cells to killing of target cells, cultured glioma cells were released from the flasks using phosphate buffer saline (PBS) without Ca^{2+} or Mg^{2+} containing 0.02% EDTA for 30 minutes. A single-cell suspension was prepared by repeated pipetting of PBS with glioma cells, and adding the same amount of RPMI 1640 containing 20% heat-inactivated human AB serum.

Measurement of Cytotoxicities

The cytotoxic activities of lymphocytes were measured using a standard 4-hour chromium-51 (^{51}Cr) release assay (9). The target cells were NK (natural killer)-sensitive K562 cells for NK activity and NK-resistant Daudi cells for LAK activity. U251 and three cultured glioma-derived cells obtained at the time of operation that were histologically diagnosed as glioblastoma were used for the allogeneic glioma target cells. For the cytolysis to autologous glioma cells, glioma-derived cells obtained from four patients (Case 1,2,3,8) were selected. The target cells (10^6) were incubated and labeled with 1.85 MBq sodium chromate-51 ($Na_2{}^{51}CrO_4$) for 60 minutes at 37°C in 0.2 ml culture medium consisting of RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum and 50 μg/ml gentamycin sulfate. The

^{51}Cr-labeled cells were then washed three times in culture medium and passed through a nylon mesh to eliminate aggregates. Labeled target cells (10^4) in 100 ul medium were added to varying numbers of effector cells in round-bottomed 96-well microtiter plates (Lynbrook Chemical, Hampton, CT, U.S.A.).

The plates were centrifuged at 45 x g for 3 minutes, then incubated for four hours at 37 C in 5% CO_2. After incubation, the plates were centrifuged at 175 x g for 5 minutes. The supernatants were harvested with the Skarton supernatant harvesting system (Skarton, Sterling, VA) and counted in a gamma counter. The effector/target ratio (E/T ratio) was 20/1 for NK activity, 10/1 for LAK activity, and 200/1, 100/1 and 50/1 for LAK killing of target glioma cells. The percentage of specific lysis was calculated by the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100\%$$

based on the mean count per minute of triplicate wells for the experimental release, and on the mean count per minute of 12 wells for the spontaneous and maximal release.

For measuring inhibitory effects by CSF obtained from the patients, 100 μl of CSF instead of CM was added to the target cells.

Microscopic Observation of LAK Cell Killing to Glioma Cells

To remove extracellular IL-2, LAK cells were transferred

from the CM to MEM by centrifugation at 700 x g for 10 minutes at 20 °C and resuspended in MEM. LAK cells in MEM were added to the petri dishes containing the glioma-derived cells at an E/T ratio of 25/1. The co-cultures of LAK and glioma cells in petri dishes were incubated at 37 °C in 5% CO_2 and the change with the passage of time was observed under Olympus light microscope.

Surface Markers of Mononuclear Cells

Fresh or cultured mononuclear cells were adjusted to a concentration of 10^6 cells/ml in PBS containing 0.1% sodium azide buffer and stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies specific for different surface markers on human mononuclear cells. The cells were incubated with monoclonal antibodies pretitered for 30 minutes at 4 °C to give optimal staining. The monoclonal antibodies included anti-Leu 2a, 3a, 4, 7, 11, HLA-DR, and IL-2 R. The stained cells were washed three times with the PBS-sodium azide buffer, and resuspended in 1 ml of the same buffer containing 2% FCS for flow cytometric analysis by Spectrum IV analyzer (Ortho Diagnostic Systems K.K. Tokyo).

Therapy Schedule

Patients with malignant gliomas were treated with craniotomy for extensive removal of the tumor followed by placement of a catheter into the postoperative tumor cavity. The catheter was connected to an Ommaya reservoir placed beneath the galea. Two or

three weeks after surgery, PBMCs were harvested from the patients with leukopheresis and incubated in CM as described above. Before administration of LAK cells, samples of culture medium were tested by Gram staining to check for the presence of microorganisms, with infusions from the Ommaya reservoir being carried out only if the Gram stain was negative. In addition, aliquots of the medium were cultured for fungi and for aerobic bacteria during 14 days. In no case was there evidence of microorganism growth in the cultures.

On immunotherapy treatment days, the suspended and loosely adherent LAK cells ($1-2 \times 10^9$) were harvested, washed twice in 0.9 % sodium chloride (NaCl), and resuspended in a 5-ml infusion medium consisting of 0.9 % NaCl with $5-30 \times 10^4$ JRU of rIL-2. Then, under sterile conditions, the cell suspension was injected through a No. 23 needle into the reservoir at a rate of 1 ml per minute. Repeated leukopheresis was performed once a week and the goal of the total number of LAK cells for intracranial administration was 5×10^9. Glucocorticoid therapy was limited during immunotherapy and used only in patients who exhibited long-standing side effects due to infusions of LAK cells and rIL-2.

Before and after immunotherapy, patients received CT scans with and without contrast enhancement. The CT scans were studied to assess changes in the appearance of the brain and tumor in response to treatment. Tumor size was determined by contrast-enhanced CT scanning. In the case of residual tumors existing in CT before treatment, response to the therapy was scored as either

complete response (CR: complete resolution of tumor), partial response (PR: >50% reduction of tumor volume), minor response (MR: 25%-50% reduction of tumor mass), no change (NC: <25% reduction of tumor mass or <25% increase in tumor volume), or progressive disease (PD: >25% increase in tumor volume within 4 weeks). The response to treatment of seven patients was also evaluated by magnetic resonance imaging (MRI).

Statistical Analysis

The cytotoxicities and phenotypic data of the different groups were compared by means of paired-T test.

Results

Cytotoxicity and Phenotype of LAK cells

We studied spontaneous and IL-2-induced cytotoxic activities on PBMCs obtained from 22 glioma patients and 10 healthy control subjects. The results are shown in Fig. 1 Each patient's immunological profile is also shown in Table 2. The spontaneous cytotoxicity to K562 cells (NK activity) was 19.8% $\pm$ 7.7% in the patients and 28.7% $\pm$ 6.4% in the control group at an E/T ratio of 20/1, and the cytotoxicity to Daudi cells (LAK activity) was 2.9% $\pm$ 2.8% in the patients and 7.8% $\pm$ 2.8% in the control group at an E/T ratio of 10/1. Incubation with 1000 JRU/ml of rIL-2 for three days markedly induced the NK and LAK activities to a comparable degree both in the patients and in the control group.

Interleukin-2-induced NK activity was 62.1% ± 16.8% in the patients and 78.4% ± 9.5% in the control group, and IL-2-induced LAK activity was 41.5% ± 16.7% in the patients and 62.3% ± 10.8% in the control group. Spontaneous and IL-2-induced NK and LAK activities in the patients were reduced compared to the healthy control group ($p<0.01$).

We also studied the cytotoxicities of LAK cells to four cultured allogeneic glioma cell types in six patients (Cases 1, 2, 3, 8, 11 and 12) and cultured autologous glioma-derived cells of four patients (Cases 1, 2, 3 and 8). No considerable difference in the vulnerability to LAK cells was found among the four different allogeneic cultured glioma-derived cells (Table 3a). The cytotoxicity to autologous glioma cells was similar to that to allogeneic U251 glioma cells (Table 3b). The ability to kill U251 glioma cells was proportional to the LAK activity, and definite correlations between the cytotoxicities to the U251 glioma cells and Daudi cells were demonstrated in Fig. 2 ($r = 0.969$).

Under microscopic observation of co-cultures with glioma-derived cells and LAK cells, the glioma-derived cells were morphologically large (about 100 μm long), flat, and adhered tightly to the surface of the petri dishes. In contrast, the LAK cells were much smaller (about 10 μm diameter). The large difference in size and shape between the cell types made it easy to identify them in the co-cultured cells. In the co-cultured cells, the LAK cells were also seen to move and bind to the target cells within 30 minutes. The glioma-derived cells

attacked by LAK effector cells, on the other hand, shrank, became round, and detached themselves from the surface of the flask within 60 minutes (Fig. 3).

With regard to phenotypic subsets of LAK cells, the responsiveness of peripheral blood lymphocytes to rIL-2 was almost the same in both healthy control subjects and the patients (Table 4). The ratio of Leu HLA-DR and CD 25 (IL-2 R) positive cells markedly increased by rIL-2 stimulation for three days ($p<0.001$). The ratio of CD57 (Leu-7)-positive cells slightly increased ($p<0.05$). The ratio of CD4 (Leu-3a + 3b)-positive cells in the patients' PBLs was lower than that of the healthy controls. Therefore, the CD4/CD8 ratio of the patients was much lower than that of the healthy control subjects. Six patients whose ages were over 50 years old (Cases 2, 8, 13, 17, 19 and 22) were excluded in the statistics of Table 4 because the ratio of CD8-positive cells was rather low for aging and the ratio of CD4/CD8 was unexpectedly high (Table 2).

Effect of CSF, Glucocorticoid, and Anticonvulsants on Cytotoxicities of LAK Cells

We investigated the effect of CSF on the generation of LAK cells and the cytotoxicity of IL-2-induced LAK cells. The CSF in three glioma patients at the terminal stage did not change any cytotoxicities of the LAK cells (Table 5). We also examined the effect of glucocorticoid (steroid) and anticonvulsants on the generation of LAK cells and cytotoxicities of LAK cells, using the PBMCs in seven patients (Cases 4, 5, 7, 9, 11, 12 and 13).

DX inhibited the generation of LAK activities but had no effect on the NK activity or cytotoxicity of IL-2-induced LAK cells (Figs. 4 and 5). Anticonvulsants did not affect the induction of LAK cells or cytotoxicities of IL-2-induced LAK cells at all. The analysis of phenotyping subsets revealed that DX inhibited the expression of Leu HLA-DR and IL-2 R in LAK cells (Table 6). The anticonvulsants also did not affect the ratio of the phenotyping subsets.

Response to Treatment

Of the 15 patients with residual tumors treated by local adoptive immunotherapy with LAK cells and IL-2, three had CR (Figs. 6 and 7) and three had MR on CT scan (Table 2). The incidence of objective responses including CR and PR was three of 15 (20%). Response to the treatment was not related directly to the cytotoxicity of the patients' LAK cells and appeared to vary with the histologic type of glioma. Concerning patients with oligodendroglioma grade 3, two of four had CR. Most of the patients who were not successful in this immunotherapy did not have recurrent tumor inside the LAK cell-injected cavities. Those tumors recurred from under the surface of the cavity (Figs. 8 and 9), and rarely occupied the cavity (Fig. 9) The MST of 19 patients with recurrent malignant gliomas was 16 months (Fig. 10).

On CT scan examined soon after injection of LAK cells and rIL-2, a lesion appeared on contrast enhancement along the tumor

cavities was found in 11 of 22 patients (Figs. 11 and 12). This type of contrast enhancement could be differentiated from tumor recurrence because the enhanced lesions usually disappeared within one month (Fig. 12). The new or enlarged low-density area surrounding the enhanced lesion was demonstrated in three patients (Cases 3, 13 and 14) and was consistent with increases in cerebral edema (Fig. 12b). MRI obtained from seven patients exhibited the same findings as the CT scan, though MRI was superior to CT scan in demonstrating the existence of the tumor and surrounding edema (Fig. 6).

Pathological Changes after Immunotherapy

We have had six reoperated cases after this immunotherapy due to recurrent or residual tumors. Three cases (Cases 1, 9 and 10) were operated on within two months after the last injection of LAK cells and rIL-2. The histology of removed glioma tissues showed dead LAK cells piling up inside the tumor cavities (Fig. 13a) and the formation of granulation tissue on the surface of the cavity. The granulation tissue like a capsule accompanied marked infiltration of eosinophilic leukocytes mingling with tumor cells (Fig. 13b). Malignant glioma cells were located below the capsules on the surface of the tumor cavity and appeared to be protected from attacks by LAK cells (Fig. 13c). In the tumor tissues of the other three patients obtained more than four months after the last immunotherapy, we could no longer find any special histological reactions related to the treatment.

Complications

Complications occurred in 12 of 22 cases undergoing adoptive immunotherapy with LAK cells and rIL-2. Fever and headache were common systemic side effects and were often associated with nausea and/or vomiting. These toxicities occurred one or two hours after LAK cells and rIL-2 administration and subsided within a few days. In principle, the severity of these complications was proportional to the amount of administered rIL-2, although there was a considerable difference depending on individuals. Human serum albumin (5%) was included in injected LAK cell suspension in Cases 15 and 16 for retaining activities of LAK cells in a saline solution. However, The toxicity with albumin was, however, so severe that the use of albumin has not been continued. Generally, administration of rIL-2 less than 5×10^4 JRU per injection did not incur severe side effects. Glucocorticoid therapy was used in four patients (Cases 2, 7, 13 and 14) who exhibited long-standing (more than three days) severe side effects.

Convulsion occurring in one patient (Case 7), followed by hemiparesis, might be caused by temporary decreasing cerebral blood flow due to the large amount of injected rIL-2. Meningitis also occurred in one case (Case 15). The infection appeared to be a result of administration of albumin and postoperative wound infection. The patient required removal of the Ommaya reservoir and cleaning of the tumor cavity.

Discussion

Adoptive immunotherapy using intravenous infusion of a large quantity of LAK cells and high-dose rIL-2 has proved successful in mediating the regression of some metastatic solid tumors in humans (10, 11). This immunotherapy (LAK therapy) caused severe systemic side effects, but can result in marked tumor regression in some patients for whom no other effective therapy is available at present (11). This evidence aroused interest in the use of LAK cells and rIL-2 on malignant gliomas because definite advantages can be expected in the treatment of primary brain tumors. Intracranial gliomas rarely metastasize to extraneural organs and the volume of CSF is small (about 150 ml). Therefore, local adoptive immunotherapy can be potent and can conserve the amount of LAK cells and IL-2 for the treatment.

For these distinctive features of brain tumors, we undertook local adoptive immunotherapy consisting of intracranial administration of LAK cells with rIL-2 through a previously implanted Ommaya reservoir and catheter system. We have investigated several basic problems regarding this immunotherapy as well as the clinical application. The initial in vitro study was carried out to determine whether LAK cells with strong cytotoxicity can be obtained from the glioma patients.

Although a few reports have appeared demonstrating that LAK cells derived from glioma patients showed similar levels of cytotoxicity to those derived from healthy individuals (16, 17), the cytotoxicities of LAK cells obtained from the patients in our study were less than those of healthy control subjects as were

the spontaneous cytotoxicities of PBMCs. This is rather an expecting result, considering the compromising status of cellular immunity in glioma patients. They have impaired delayed cutaneous hypersensitivity (18), reduced the numbers of circulating lymphocytes (18), depressed NK activity (19), and decreased in vitro proliferative responses to mitogens (20). Most of the patients in this study, moreover, had recurrent tumors, and they had received chemotherapy or steroids (prednisolone 15-30 mg daily) before this treatment.

The analysis of their phenotyping subsets revealed a selective depletion of CD4-positive cells and a lower CD4/CD8 ratio as indicated before (21). The ratio of Leu-HLA-DR and IL-2 R-positive cells in LAK cells markedly increased in both healthy control subjects and the patients. The ratio of CD57-positive cells implying NK cells slightly increased. Since the ratio of CD4-positive cells in the patients' PBLs was lower than that of the healthy controls, the CD4/CD8 ratio of the patients was much lower than that of the healthy control subjects. It is obvious that LAK cells obtained from patients in this study, however, at least demonstrated sufficient activity to kill a variety of tumor cells including glioma cells because IL-2-activated cytotoxicity is a remarkably robust immunological function (17).

For measuring the cytotoxicities of LAK cells, we mainly carried out standard ^{51}Cr release assay using K562 (NK activity) and Daudi (LAK activity) cells. The measurement methods of those activities are established, simple, and highly reproducible. There was no large difference in the vulnerability to LAK cells

among the four different allogeneic cultured glioma cells, and the cytotoxicity to autologous glioma cells was almost the same as that to allogeneic U251 glioma cells. The LAK activities were correlated with the cytotoxicities to allogeneic glioma cells (U251), so that they could represent their killing activities to autologous glioma cells. Based on these experimental results, the cytotoxicity to autologous glioma cells were not measured routinely.

Microscopic observation of co-cultures with LAK cells and autologous glioma-derived cells demonstrated that the large glioma-derived cells reacted to small LAK effector cells by shrinking, becoming round, and rapidly detaching themselves from the surface of the flask. It appears that twenty times as many LAK cells in measuring LAK activity (E/T ratio = 10/1) needed for cytotoxic assay to glioma-derived cells (E/T ratio = 200/1) results from the great difference in size between the target cells. Glioma-derived cells are at least ten times longer in size than Daudi cells for LAK activity. The findings of injured glioma-derived cells are consistent with an apoptotic rather than an osmotic lysis mechanism of cell death that supports a common cytotoxic mechanism for cytotoxic T lymphocytes (CTLs), NK cells, and LAK cells (22).

The next series of experiments examined whether the CSF in glioma patients inhibits the generation of LAK cells and the cytotoxicities of LAK cells. The culture medium containing patients' CSF instead of conditioned medium was presumably more analogous to in vivo environments. Human glioblastoma cells secrete an immunosuppressive factor which is capable of

suppressing both IL-2 dependent T-cell proliferation and the generation of cytotoxic T-cells in allogeneic mixed leukocyte cultures (23). Purification of this substance shows that the factor is identical to transforming growth factor beta$_2$ (TGF-β_2) (24). The cyst fluid of brain tumors also contains immunosuppressive factors that inhibit the activation of lymphocytes by mitogens (25). These findings indicated that the CSF in glioma patients including suppressive factors might protect glioma cells from being attacked by LAK cells.

Using the CSF obtained at the terminal stage from three patients that were assumed to contain a large quantity of suppressive factors, their inhibitory effects on the potency of LAK cells and rIL-2 were investigated. The results demonstrated that even such CSF expressed no inhibitory effect on the generation of LAK cells and IL-2-induced cytotoxic activities against allogeneic tumor cells. This seems to be partly because the concentration of suppressive factors in the CSF is lower than that needed to act on the inhibition of the LAK activity cells, and because the mediation of such factors is totally inhibited by higher concentrations of IL-2. In the previous studies, the suppressive effect by TGF-β_2 on the induction of LAK cell activity can also be reduced by higher concentrations of rIL-2 (26). The glioma cyst fluid also did not inhibit the killing action of LAK cells in vitro (27).

In clinical application of LAK therapy on malignant gliomas, the effect of glucocorticoids and anticonvulsants should be elucidated. Glucocorticoids are used to reduce brain edema and

anticonvulsants are essential to suppress seizures in most patients with glial tumors. Glucocorticoids are also quite effective in relieving side effects associated with LAK therapy (28). Those drugs, however, may reduce the clinical effectiveness of immunotherapy. Glucocorticoids inhibit the generation of LAK cell activity at pharmacological doses (29, 30), and anticonvulsants also exert inhibitory effects on cellular immunity (31).

We examined the inhibitory action caused by these indispensable drugs on LAK cell generation and IL-2-induced LAK cell killing of tumor cells. In the present study, DX did inhibit generation of LAK activity from PBMCs by rIL-2, while it had no inhibitory effect on the generation of NK activity or on the cytotoxicities of IL-2-induced LAK cells. Anticonvulsants could not affect IL-2 action on PBMCs or LAK cells.

These results obtained from in vitro studies seem to be favorable for LAK therapy. The administration of anticonvulsants can continue during the adoptive immunotherapy. In addition, glucocorticoids can be used to immediately relieve side effects caused by the immunological treatment in patients because they can no longer inhibit the cytotoxicities of infused LAK cells. The administration of glucocorticoids steroids should be avoided or minimized only during the harvesting of PBMCs, even if the previous study demonstrated that the ability to generate LAK cells is not influenced by the administration of glucocorticoids (16).

In the clinical trial of our therapeutic schedule, 22 patients with malignant gliomas were treated. The MST of the 19

patients with recurrent malignant gliomas was 16 months, for whom no other effective therapy was available. Although the incidence of objective responses to this new immunotherapy was 20% on CT scan, our results seem to be even better than conventional therapy of recurrent malignant gliomas, considering the MST of recent reports on the treatment of malignant gliomas by chemotherapy following surgery was almost 9 months (32, 33). Thus far, only a few clinical reports have appeared on this type of immunotherapy on malignant gliomas except for two preliminary phase I studies (34, 35).

Barba et al. (36) reported that one of nine patients with recurrent malignant gliomas had a PR on CT scan. They also noticed that severe neurological side effects occurred in all patients undergoing treatment and were related to increases in cerebral edema that appeared to be mediated by the immunotherapy. They continued injections of rIL-2 for five days. Initially, rIL-2 was given at 7×10^3 JRU/kg and was increased in a graded fashion in subsequent patients up to 4.2×10^4 JRU/kg. The large amount of rIL-2 in their therapeutic schedule seemed to cause brain edema by a presumed increase in capillary permeability as well as a decrease in systemic vascular resistance by intravenous administration of rIL-2 which leads to loss of intravascular fluid into the soft tissues (37).

Although severe neurological side effects are major problems of local LAK therapy, only one patient that suffered from hemiparesis following epileptic seizure has been experienced. Long-standing severe systemic side effects that were limited to

high fever, headache and vomiting were also observed in a few patients. Since these side effects were assumed to be due to the infusion of rIL-2, we were able to relieve the toxicities of the therapy by reducing the amount of rIL-2 per injection from 3×10^5 JRU to 5×10^4 JRU. Kimoto et al. (38) confirmed that a high concentration of rIL-2 was not necessary when the LAK activity was potent, with the minimum requirement being 35 JRU/ml. In fact, Yoshida et al. (39) reported that definite tumor regression was observed in six of 23 patients with recurrent malignant gliomas by a smaller amount of rIL-2 injections than that in our therapeutic schedule.

The tumor responses noted in six (CR: 3, MR: 3) of 15 patients not only demonstrated that LAK cells can kill tumor cells in vivo, but also that regional intratumoral adoptive immunotherapy using currently available techniques has a definite limitation. This limitation is due to both the characteristics of the tumor and the treatment. In patients with recurrent malignant glial tumors, tumor cells usually extend beyond areas that are radiographically abnormal, as indicated on CT scans by either contrast enhancement or peritumoral low density (40). The value of LAK cells is limited because they must make cell-to-cell contact to kill tumor cells (22) and they appear incapable of seeking out distant tumor cells.

Our study on regional pathological changes soon after LAK therapy also supported the same limitation of this immunotherapy. On the surface of the residual tumors, the formation of granulation tissue was observed, associated with marked eosinophilic infiltration and gliosis. The glial tumor cells

were located under the granulation tissue like a capsule that protected against LAK cell killing. The formation of this granulation tissue is estimated to be completed within a few weeks after the initial injection of LAK cells and rIL-2.

These results indicate that injections of LAK cells and IL-2 after capsule formation are ineffective because LAK cells cannot reach the tumor cells by the barrier of the granulation tissue. In fact, the tumors recurred from below the capsules and invaded deeply in the opposite direction against the cavities. Successful LAK therapy of malignant gliomas, therefore, remains dependent upon the ability to deliver the LAK cells to distant occult tumor cells. In the near future, these problems might be overcome by treating patients before tumor cells infiltrate deep into the surrounding brain. This can be done by improving the traffic of lymphocytes to brain tumors, or by exploiting lymphocytes already existing in the brain like tumor-infiltrating lymphocytes (TILs).

We have little experience in this type of immunotherapy on malignant gliomas. The present study, however, suggest that this therapy might be effective for patients with recurrent malignant gliomas, especially the malignant cystic type of oligodendrogliomas, for whom no effective therapy is available or in whom standard therapy has failed. In the same way that surgery, radiotherapy, and chemotherapy have needed time and experience, local adoptive immunotherapy for malignant gliomas requires further investigation before its clinical use is generally accepted. The ultimate role of this therapy, if any,

in the treatment of malignant gliomas remains to be elucidated.

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Legends

Fig. 1 NK and LAK activity in patients with malignant gliomas (n = 22) and the healthy control group (n = 10) before and after /incubation with 1000 JRU/ml of rIL-2 for 3 days. (E/T ratio of 20:1 for NK activity and 10:1 for LAK activity)

Fig. 2 Correlation between cytotoxicities of LAK cells against Daudi cells (target cells for LAK activity) and those against U251 glioma cells.

Fig. 3 Photomicrograph of LAK cells killing an autologous glioma cell; change with the passage of time. Autologous LAK cells (Case 2) move and bind to the autologous cell within 30 minutes. The target cell attacked by LAK cells becomes round and shrinks within 60 minutes. Full picture width = 130 μm.

Fig. 4 The effects of anticonvulsants and DX on the generation of cytotoxicities by rIL-2. Spontaneous NK and LAK activities were measured using the PBMCs of the patients. Patients' PBMCs were incubated in CM containing 1000 JRU/ml of rIL-2 for three days with anticonvulsants and DX (10^{-6}M). The concentrations of anticonvulsants are 25 μg/ml of DPH, 30 μg/ml of phenobarbital PB and 100 μg/ml of VP.

Fig. 5 The effects of anticonvulsants and dexamethasone (DX) on the cytotoxicities of LAK cells. LAK cells generated by 5 days' stimulation of rIL-2 were incubated again in complete medium (CM)

containing rIL-2 (1000 JRU/ml) for two more days with anticonvulsants and DX (10^{-6}M). The concentrations of anticonvulsants were as same as those in Fig. 4.

Fig. 6 MRI (SE 500/17) in Case 2. a: Pretreatment MRI demonstrating residual recurrent cystic oligodendroglioma (grade 3) in the right parietal region. b: MRI obtained two weeks after treatment showing that the tumor and mass effect totally disappeared.

Fig. 7 CT scan with contrast enhancement in Case 5. a: Pretreatment scan showing a left frontal residual glioma (gemistocytic astrocytoma). b: Posttreatment scan showing disappearance of enhanced lesion.

Fig. 8 CT scan with contrast enhancement in Case 1. a: Posttreatment CT scan showing catheter, and residual-enhanced tumor on the surface of the cavity in the frontal lobe. b: CT scan 1 month after LAK therapy. The tumor became larger in the opposite direction of the cavity. c: CT scan 5 months after LAK therapy. The tumor covered the entire lateral ventricle.

Fig. 9 CT scan with contrast enhancement in Case 9. a: Pretreatment scan showing left frontal tumor cavity and residual glioma (astrocytoma grade 3). b: Posttreatment scan demonstrating the distinct ring-enhanced lesion in the circumference of the cavity. c: CT scans two months after the

last injection of LAK cells showing recurrence of the tumor. The tumor occupied most of the cavity.

Fig. 10 Survival after local LAK therapy in 19 patients with recurrent malignant glioma. The Kaplan-Meier curve shows probability of survival in months. Eight of 19 patients were alive at the time of analysis.

Fig. 11 CT scan with contrast enhancement in Case 2.
a,b: Pretreatment scan showing residual tumor with surrounding brain edema. c,d: CT scan after the first injection of 10^9 LAK cells and 2×10^5 rIL-2. Ring enhancement in the circumference of the cavity appeared after treatment. The catheter inside the cavity is distinctly observed in b and d.

Fig. 12 CT scan with contrast enhancement in Case 13. a: Pretreatment scan showing no residual tumor. b: Posttreatment scan demonstrating the appearance of the ring-enhanced lesion accompanied by the surrounding low-density area assuming brain edema. c: CT scan one month after steroid treatment. Ring enhancement and brain edema disappeared.

Fig. 13 Histology of removed glioma tissues after local adoptive immunotherapy (Case 1, x 80). This patient received the first injection of LAK cells and rIL-2 eight weeks before and the last injection three weeks before. a: Dead LAK cells piling up inside the tumor cavity. Nuclei of some LAK cells were stained with hematoxylin. b: Formation of granulation tissue on the surface of

the cavity. Eosinophilic leukocytes markedly infiltrate and mingle with gliosis. c: Malignant glioma cells below the granulation tissue. The tumor cells are found in the edematous tissue below the capsule. Full picture width = 0.45 mm

Table 1. Patients' profiles for adoptive immunotherapy

Case	Age, Sex	Pathology	Previous Treatment (1. Operation, 2. Radiotherapy, 3. Chemotherapy)	Time after Previous (first, second) Treatment (months)
1.	34, M	Astrocytoma grade 3	1. SR 2. 58 Gy	40
2.	50, M	Oligodendroglioma grade 3	1. SR 2. 50 Gy	135
3.	22, F	Ependymoma grade 3	first: 1. SR 2. 20 Gy second: 3. ACNU (80 mg), VCR (1 mg)	82, 5
4.	32, M	Oligodendroglioma grade 3	1. SR 2. 50 Gy	28
5.	40, M	Gemistocytic astrocytoma	first: 1. PR 2. 70 Gy 3. ACNU (80 mg) second: 1. PR 2. 20 Gy	35, 13
6.	14, M	Ependymoma grade 3	1. TR 2. 40 Gy 3. ACNU (50 mg)	59
7.	42, M	Astrocytoma grade 3	1. SR 2. 50 Gy	38
8.	56, F	Glioblastoma	1. SR 2. 50 Gy 3. ACNU (100 mg x 5)	22
9.	37, M	Glioblastoma	first: 1. PR 2. 50 Gy 3. ACNU (100 mg x 2), VCR (1 mg x 4) second: 1. PR 3. ACNU 60 mg	23, 4
10.	20, M	Astrocytoma grade 3	1. PR 2. 38 Gy	6
11.	37, F	Glioblastoma	1. PR 2. 60 Gy 3. ACNU (100 mg), VM-26 (150 mg)	73
12.	30, M	Oligodendroglioma grade 3	1. SR 2. 50 Gy	6
13.	51, M	Astrocytoma grade 3	1. SR 2. 60 Gy 3. ACNU (100 mg x 2), VCR (1 mg x 4)	8
14.	46, M	Glioblastoma	1. TR 2. 60 Gy 3. ACNU (140 mg), VCR (1.75 mg x 2)	5
15.	33, M	Astrocytoma grade 3	1. PR 2. 60 Gy 3. ACNU (100 mg), VCR (1 mg x 2)	36
16.	46, M	Glioblastoma	1. SR 2. 72 Gy 3. ACNU (100 mg x 4), VCR (1 mg x 6)	7
17.	52, F	Glioblastoma	1. TR 2. 72 Gy 3. ACNU (100 mg x 2), VCR (1 mg x 6)	5
18.	45, M	Astrocytoma grade 3 (pons)	first: 1. PR 2. 50 Gy second: 1. PR 2. 20 Gy	19, 3
19.	52, F	Oligodendroglioma grade 3	first: 1. PR 2. 50 Gy second: 1. SR	67, 14
20.	47, M	Glioblastoma	1. SR 2. 72Gy 3. ACNU (100 mg x 2), VCR (1 mg x 6)	8
21.	35, M	Astrocytoma grade 3	1. TR 2. 72 Gy 3. ACNU (100 mg x 2), VCR (1 mg x 6)	6
22.	60, M	Glioblastoma	first: 1. TR 3. ACNU (100 mg x 4) second: 1. SR 2. 72 3. ACNU (100 mg x 2), VCR (1 mg x 6)	17, 11

TR, Total Removal; SR, Subtotal Removal; PR, Partial Removal; VCR, Vincristine

Table 2. Patients' immunological profile and clinical responses

Case	CD4/CD8	NK, LAK activity rIL-2(-)		(+)		Total of Administered LAK cells and rIL-2		Survival Time (months)	Tumor Response
		NK%	LAK	NK	LAK	x 10^9	x 10^5JRU	* dead	
1.	0.85	23	5	74	43	4.0	5.6	16*	MR
2.	1.94	30	6	56	35	3.2	5.6	21*	CR
3.	1.13	30	8	67	66	6.0	4.2	18*	MR
4.	1.87	23	0	52	32	5.0	4.2	13*	NC
5.	0.92	23	9	61	75	3.6	3.5	35	CR
6.	1.07	19	1	36	31	2.8	3.5	36	(-)
7.	0.91	30	4	71	71	4.6	7.0	13*	MR
8.	0.88	21	5	62	43	3.5	6.3	11*	NC
9.	0.99	11	1	60	28	4.1	6.3	9*	NC
10.	0.41	28	1	69	34	3.2	4.9	4*	PD
11.	1.60	19	0	62	38	3.8	4.2	18*	(-)
12.	0.99	21	5	86	59	3.3	3.5	32	(-)
13.	1.92	19	3	93	47	3.5	4.2	31	(-)
14.	0.52	28	5	77	59	3.5	4.9	24	(-)
15.	1.03	16	4	72	28	4.0	4.2	14	NC
16.	0.88	8	0	52	31	1.2	3.5	5*	NC
17.	1.50	10	0	58	29	5.0	2.8	12	(-)
18.	1.72	8	0	80	50	4.5	4.2	5*	NC
19.	2.09	8	0	31	13	5.0	2.8	10	CR
20.	1.46	25	3	62	44	5.0	2.1	10	NC
21.	0.89	20	0	23	14	4.0	2.1	10	(-)
22.	1.46	25	4	62	44	5.0	4.2	6	NC

Tumor Response: CR, complete response; PR, partial response; MR, minor response; NC, no change; PD, progressive disease;

(-): no detected tumor on CT scan before treatment

Table 3 Cytotoxicities of LAK cells to cultured glioma cells

a. Cytotoxic assay to allogeneic cultured glioma cells (n = 6)

	Target cells			
	U251	Y.D.	K.T.	N.N.
E/T ratio	%			
200 : 1	45.2 ± 4.1	33.4 ± 5.1	42.6 ± 6.3	48.2 ± 7.5
100 : 1	38.5 ± 3.8	26.7 ± 3.2	34.5 ± 4.9	39.6 ± 6.3
50 : 1	27.8 ± 3.4	21.0 ± 4.5	26.2 ± 3.1	32.2 ± 4.4

b. Comparison between cytotoxicities to autologous and allogeneic U251 cultured glioma cells

	Target cells (E/T ratio = 200 : 1)	
	Autologous	U251
	%	
Case 1	42.5	40.6
Case 2	50.8	38.3
Case 3	49.7	49.5
Case 8	48.4	44.2

Table 4. Phenotyping subsets in PBLs and LAK cells obtained from the healthy control group and the patients.

Phenotype	Controls (n = 10)		Patients (n = 16)	
	PBL	LAK	PBL	LAK
CD4 (Leu 2a)	34.8± 7.2	33.3± 8.8	32.1±13.2	33.5±11.5
CD3 (Leu 4)	71.0±10.5	70.2± 9.3	68.6± 6.8	67.5± 7.1
CD57 (Leu 7)	16.6± 6.2	20.2± 5.9*	14.5± 7.9	19.3±10.4*
Leu HLA-DR	14.5± 6.2	41.0± 8.5**	16.1± 9.6	42.0±15.7**
CD16 (Leu 11)	14.1± 5.9	12.2± 4.6	9.6± 7.1	7.7± 4.0
CD4 (Leu 3a+3b)	44.6±10.7	46.2± 8.3	34.5±13.5	35.1±13.8
CD25 (IL-2 R)	1.3± 0.7	7.2± 4.1**	1.1± 0.8	5.9± 4.6**
CD4/CD8	1.52±0.20	1.60±0.28	1.07±0.39	1.10±0.30

* $p<0.05$ ** $p<0.001$

Table 5. Effect on cytotoxicities of LAK cells by CSF obtained from malignant glioma patients.

Case	Generation of LAK cells		IL-2 Induced LAK cells		^{51}Cr release assay* in patient's CSF	
	IL-2	IL-2 + CSF	IL-2	IL-2 + CSF		
	a.		b.		a.*	b.*
Patient A	22.6±2.5	20.8±2.7	35.9±3.1	34.1±1.9	23.5±1.8	36.5±3.3
B	29.7±2.1	27.9±3.2	40.4±2.2	38.4±2.5	28.2±2.5	39.6±2.3
C	24.6±1.5	25.3±2.0	29.9±2.2	28.4±1.7	23.7±2.0	29.2±4.1

IL-2-induced LAK cells were incubated with or without CSF for two more days.

* : CSF exists only during ^{51}Cr release assay. a*, b* : The LAK cells used in the experiment were the same as those in experiment a. or b. respectedly.

Table 6. The effects of anticonvulsants (DPH, PB, VPA) and DX on phenotyping expression of LAK cells.

Phenotype	Control	IL-2	IL-2 + DPH	IL-2 + PB	IL-2 + VPA	IL-2 + DX
CD57 (Leu 7)	15.3± 7.7	20.8±10.0	20.5± 8.3	22.1± 5.2	21.5± 6.1	19.8±4.3
CD16 (Leu 11)	9.6± 7.1	7.7± 4.2	8.7± 3.8	7.5± 3.2	8.0± 2.7	8.1±3.9
Leu HLA-DR	16.2± 9.9	42.0±15.7	40.2± 9.7	43.1±11.5	39.7±10.8	16.5±5.7*
CD25 (IL-2 R)	1.1± 0.8	6.7± 3.6	6.3± 2.9	6.8± 3.2	7.0± 3.1	3.0±1.4*

Control: phenotyping subsets of peripheral blood lymphocytes (PBLs) separated by Ficoll-Paque. The PBLs were incubated with IL-2 (1000 JRU/ml) and the concentration of each drug was determined for three days. The concentrations of the drugs in the medium were: DPH, 20 μg/ml; PB, 50 μg/ml; VPA, 100 μg/ml; DX, 10^{-6}M n = 7 *$p<0.01$

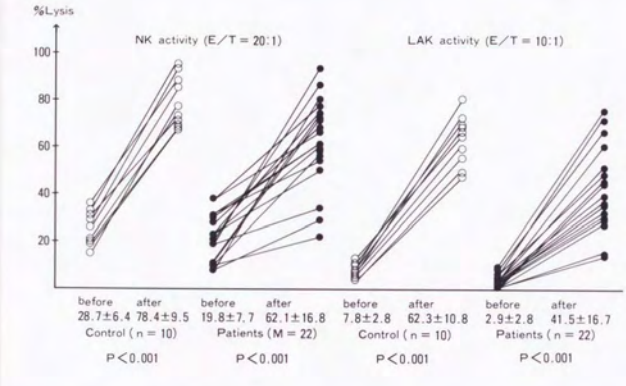

%Lysis
NK activity (E/T = 20:1)
LAK activity (E/T = 10:1)
100
80
60
40
20
before after
28.7±6.4 78.4±9.5
Control (n = 10)
P<0.001
before after
19.8±7.7 62.1±16.8
Patients (M = 22)
P<0.001
before after
7.8±2.8 62.3±10.8
Control (n = 10)
P<0.001
before after
2.9±2.8 41.5±16.7
Patients (n = 22)
P<0.001

Fig. 1

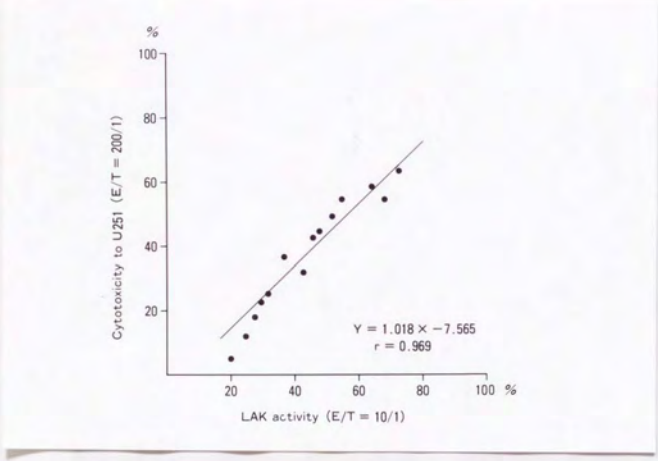

%
100
80
60
40
20
Cytotoxicity to U251 (E/T = 200/1)
Y = 1.018 × −7.565
r = 0.969
20
40
60
80
100 %
LAK activity (E/T = 10/1)

Fig. 2

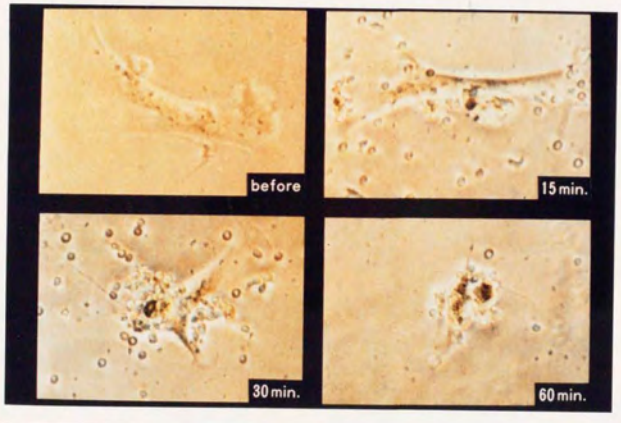
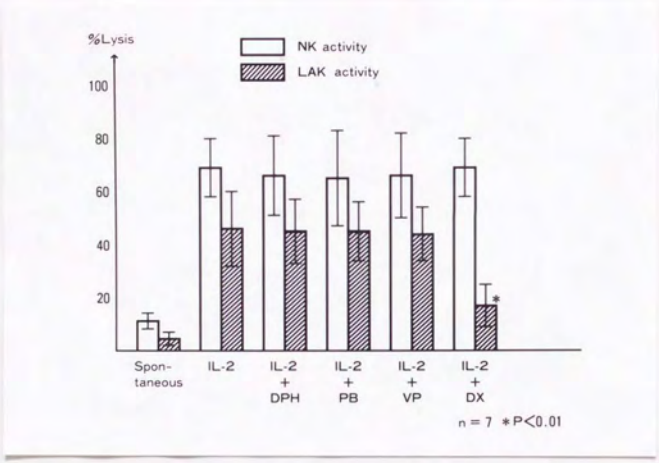


Fig. 3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NK activity
LAK activity
100
80
60
40
20
Spon-
taneous
IL-2
IL-2
+
DPH
IL-2
+
PB
IL-2
+
VP
IL-2
+
DX
*
n = 7 *P<0.01

Fig. 4

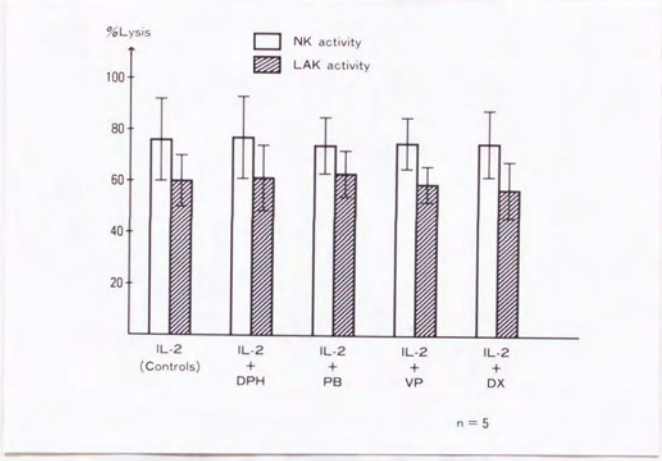
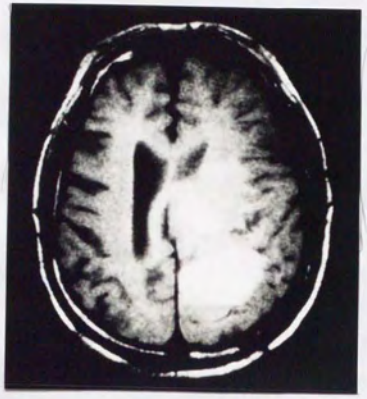

%Lysis
NK activity
LAK activity
100
80
60
40
20
IL-2 (Controls)
IL-2 + DPH
IL-2 + PB
IL-2 + VP
IL-2 + DX
n = 5

Fig. 5



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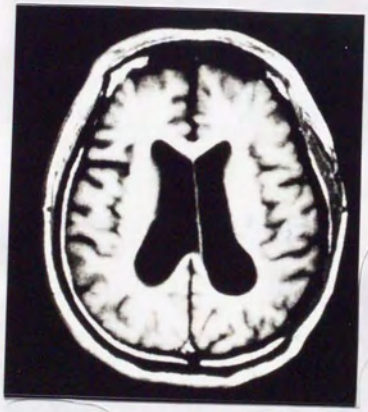

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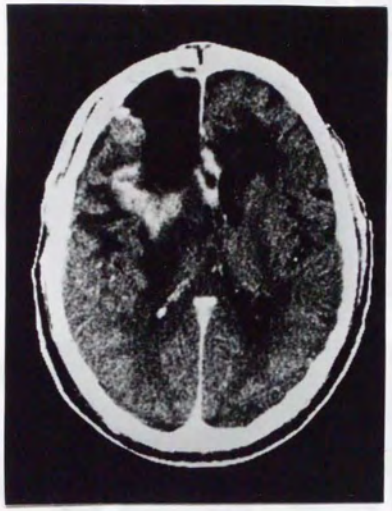
Fig. 6



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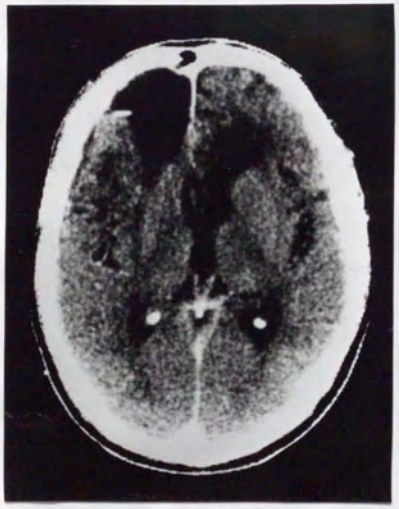

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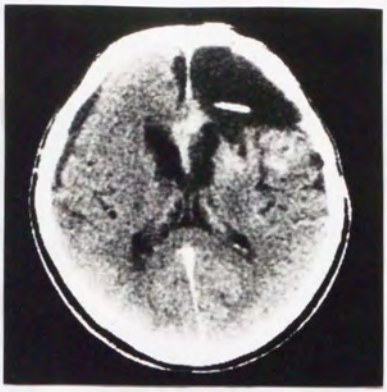
Fig. 7



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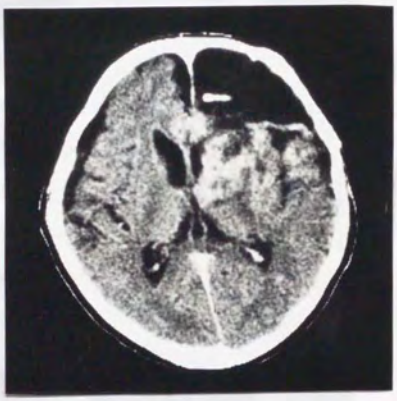

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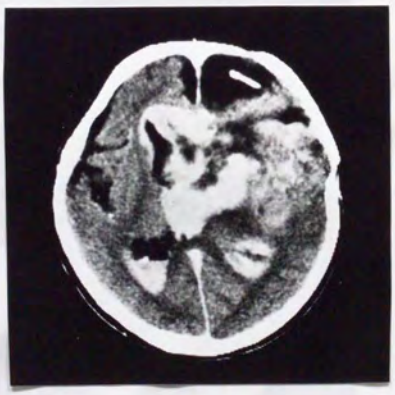
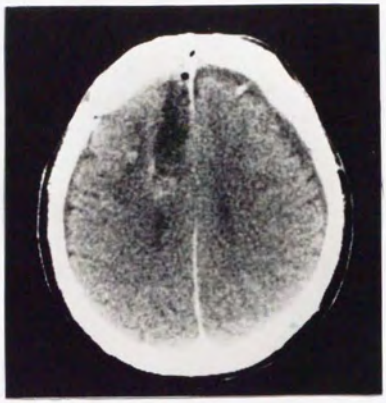


c

Fig. 8

a



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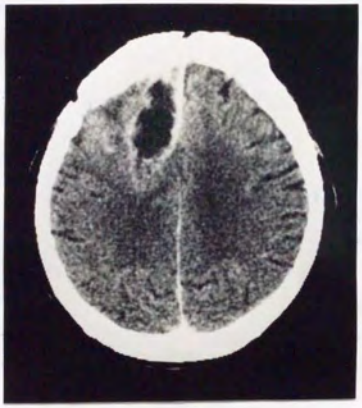

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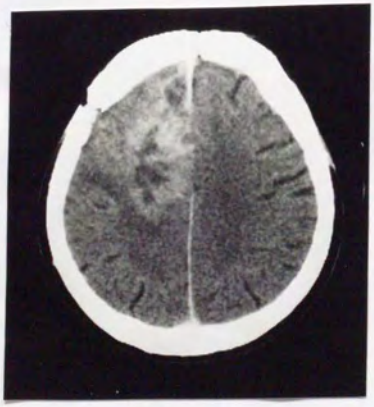


Fig. 9

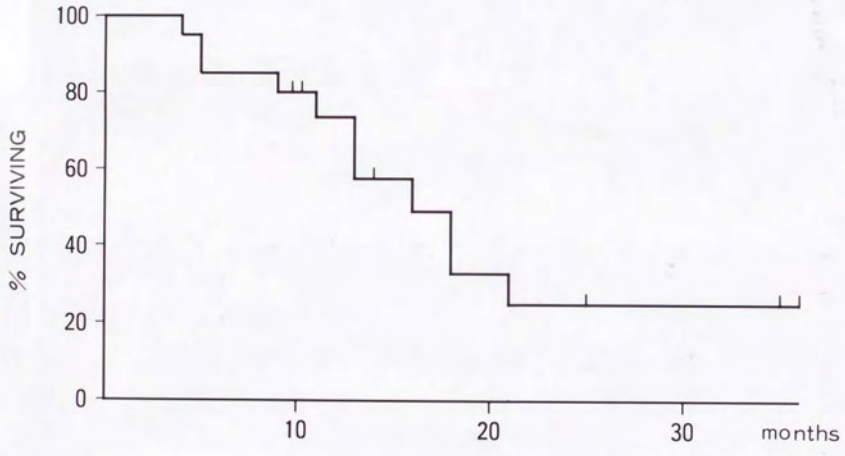
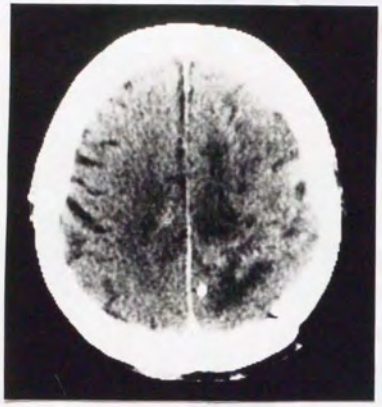


Fig. 10



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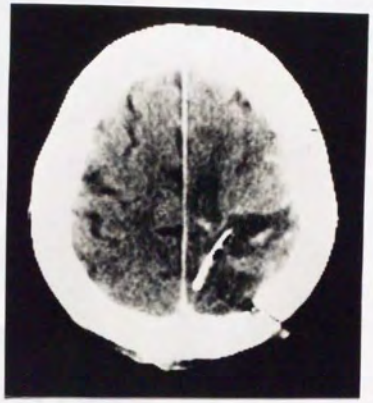

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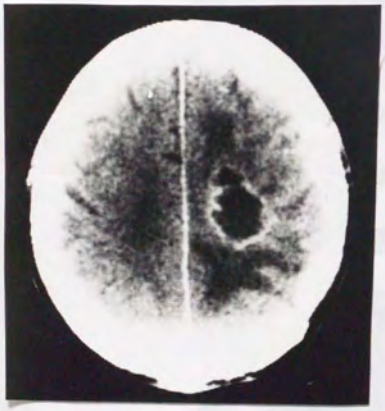

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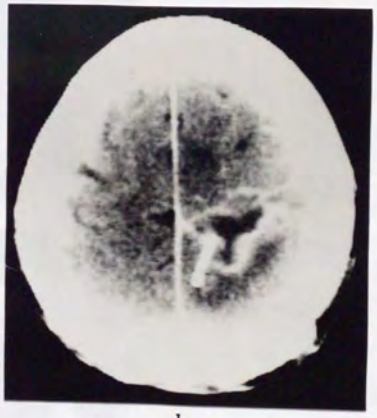

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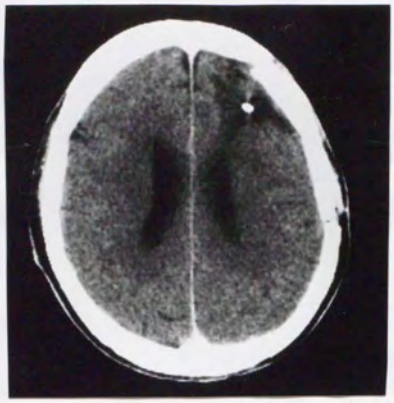
Fig. 11



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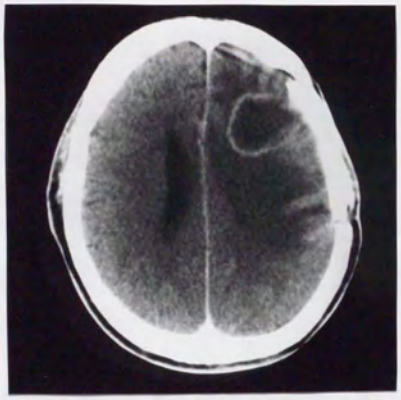

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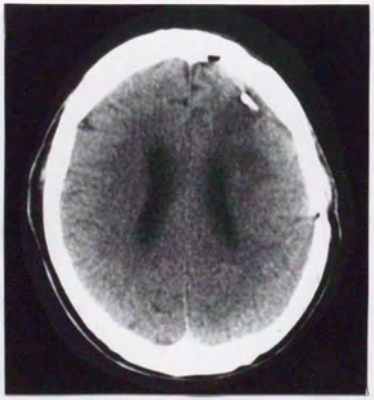
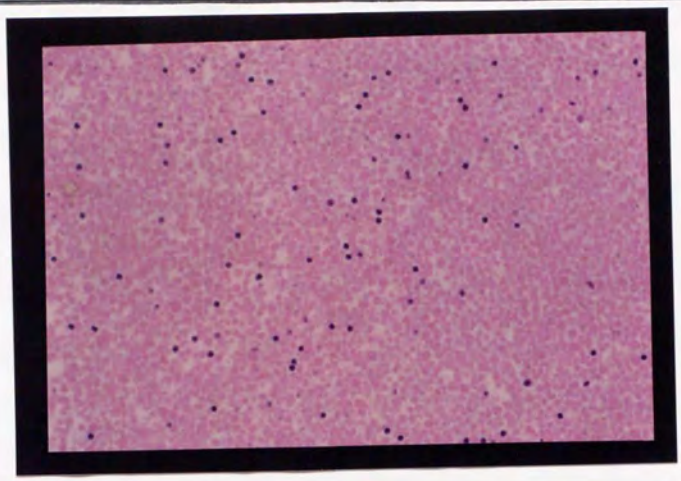


c

Fig. 12

a



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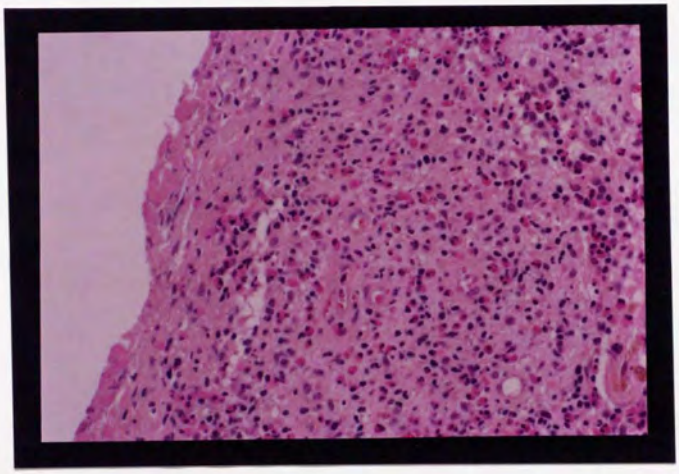

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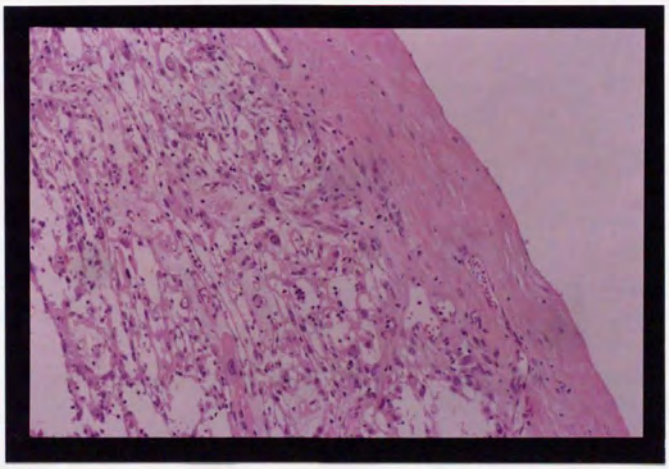
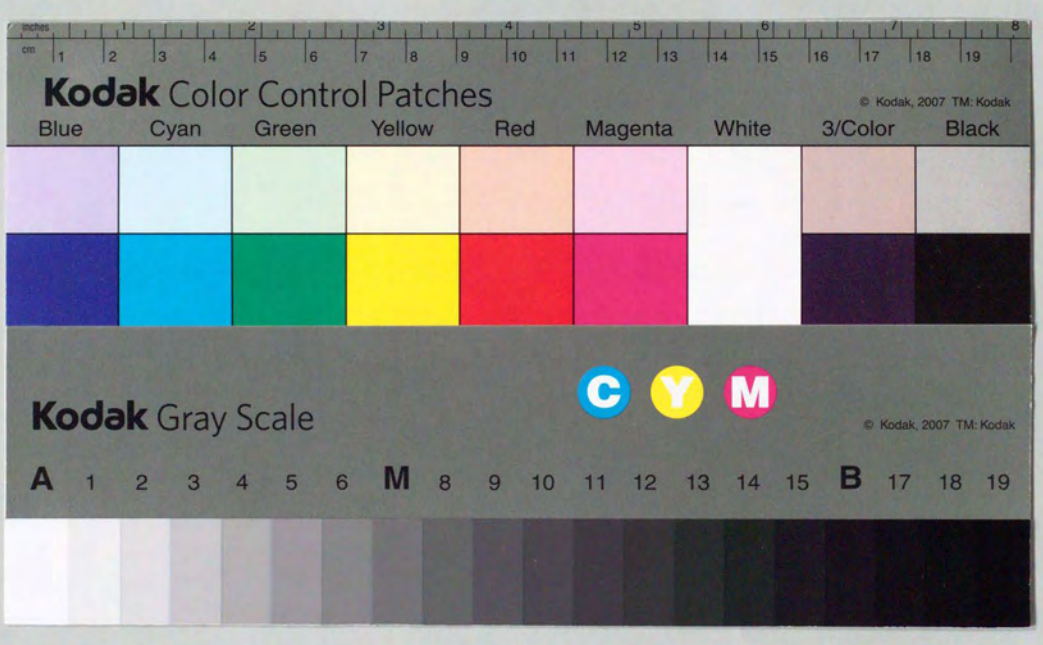


Fig. 13

膀胱に対する局所遺伝子免疫療法の基礎研究と臨床応用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cm
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© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19