

Autologous Natural Killer Cell Therapy for Human Recurrent Malignant Glioma

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Abstract. Background: Natural killer (NK) cells are highly efficient in the cellular immune response against malignant tumors without restriction of major histocompatibility complex. However, clinical studies using autologous NK cells have been reported in only a very limited number of cases, due to the fact that selective NK expansion is difficult to achieve in this patient population. Here, we report the results of adoptive immunotherapy in patients with recurrent malignant gliomas using autologous NK cells that were expanded *ex vivo* by a novel method. Patients and Methods: Peripheral blood mononuclear cells (PBMCs) were prepared from patients with malignant gliomas, and were co-cultured with an irradiated human feeder cell line (HFWT) in RHAM- α medium supplemented with 5% autologous plasma and interleukin-2. The resulting NK cell-rich effector cells were injected into 9 patients (16 courses) with recurrent malignant glioma (6 cases of WHO grade-3 glioma and 3 cases of grade-4 glioma). Results: The mean frequency of NK cells among lymphocytes was $82.2 \pm 10.5\%$. A combination of focal and intravenous injections was performed in 10 courses. Intravenous injection alone was performed in 6 courses. Further, intravenous injection of low-dose interferon beta (6×10^6 IU/week) was performed as an adjuvant therapy in all courses to achieve maximum benefit for enrolled patients. Clinical evaluation demonstrated 3 PR, 2 MR, 4 NC and 7 PD in a total of 16 courses of treatment. Severe neurological toxicity was not observed in any of the patients. Conclusion: It was demonstrated that NK cell-rich effector cells were expanded *ex vivo* from PBMCs in all nine cases of recurrent malignant glioma and that

NK cell therapy was safe and partially effective in patients with recurrent malignant gliomas.

Natural killer (NK) cells are one of the most essential cell types, and they rank with cytotoxic T lymphocytes (CTLs) as regards strong cytotoxic activity against malignant tumor cells in the cellular immune response. NK cells express several types of NK receptors. One class of NK receptors, the killer inhibitory receptors (KIRs), are inhibitory and the remaining receptors facilitate the activation of NK cells (1). Major histocompatibility complex (MHC) -class I molecules on malignant tumor cells bind to KIRs and inactivate NK cells (2). Therefore, it is recognized that the function of NK cells is partially compensatory for the cytotoxic function of CTLs against tumor cells *via* MHC-class I molecules. Recent studies have revealed active interactions between NK cells and dendritic cells (DCs), which are the most important antigen-presenting cells inducing MHC-class I-restricted immune response (3,4). Thus, NK cells play an important role in cellular immune responses in cooperation with DCs, CTLs and other cell types. Therefore, the administration of these cells, pre-activated *in vitro*, the so-called adoptive immunotherapy, has a potential to contribute to treatment of malignant tumors. The efficacy of adoptive immunotherapy in patients with malignant tumors has previously been reported by our laboratory and other laboratories using lymphokine-activated killer (LAK) cells, activated T lymphocytes, or CTLs (5-13). In particular, direct injection of autologous CTLs into the post-operative cavities of brain tumor patients resulted in a high response rate in cases involving recurrent gliomas (12, 13). However, this type of therapy requires several complex and tedious steps, including the primary culture of autologous tumor cells, long-term incubation of peripheral blood mononuclear cells (PBMCs) on irradiated tumor cells, a cocktail of four cytokines, an enriched culture medium for CTL induction, *etc.* Indeed, this type of therapy can only be performed in a limited number of patients.

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Table I. Characteristics of patients who received NK therapy.

Case No.	Age/ Sex	Region of tumor	Diagnosis	Treatment history			Pretreatment	
				Surgical operation	Rad. (Gy)	Others	Vol. (cc)	K.P.S. (%)
1	44/M	Lt. frontal	AO rec.	Partial	64	Chem	50	50
2	48/F	Lt. frontal	AOA rec.	Partial	65	Chem	23	50
3	27/F	Lt.front-temporal	AA rec.	Partial	45	CTL	5	50
4	52/F	Pontine	GBM rec.	Biopsy	65	Chem	34	20
5	23/F	Tectal	AA rec.	Partial	60	Chem	23	50
6	56/M	Lt. temporal	GBM rec.	Partial	>60	Chem	45	20
7	70/M	Rt. frontal	GBM rec.	Partial	60	-	24	80
8	53/M	Rt. thalamic	AA rec.	Biopsy	74	Chem	21	40
9	39/M	Rt. medulla	AA rec.	Partial	50	Chem	5	80

AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; rec., recurrence; Rad., radiotherapy; Chem., chemotherapy; Vol., tumor volume.

On the other hand, NK cells can be activated in the relatively short term without the use of target autologous tumor cells (14-18). However, clinical applications of NK cells have been reported in only a very limited number of cases, due to the difficulty of the large-scale expansion of NK cells (14,19). In our previous paper, we reported the selective expansion of human NK cells from PBMCs by using a specific feeder cell line (19, 20). Using this method, 58- to 401-fold expansion of lymphocytes containing more than 70% NK cells has been successfully achieved (19).

Here, we report an autologous type of NK cell therapy for use in patients with recurrent malignant glioma, which is among the most rarely cured tumor types despite intensive available treatments such as surgical removal, radiotherapy and chemotherapy. The two-year survival rates of patients in Japan with anaplastic glioma and glioblastoma are 41.4% and 21.3%, respectively (21); similar results have been observed in various countries (22).

Patients and Methods

Patients. Eligible criteria: (a) A histologically proven malignant glioma after surgical tumor resection; (b) appearance of re-growing residual tumor by radiographical imaging; (c) absence of previous treatment such as chemotherapy, radiotherapy, or immunotherapy within 4 weeks before the present therapy; (d) absence of any other difficult-to-treat disease such as autoimmune disease or hemopoietic disease; (e) primary culture of the resected tumor cells and associated trials of autologous CTL induction cultures resulted in failure. Although not necessarily a prerequisite for the present clinical trial, we suggested in our previous report that CTL therapy should be the primary adoptive immunotherapy choice for the treatment of recurrent malignant glioma (12,13). Enrolled patients and their characteristics are listed in Table I.

Expansion of NK cells. PBMCs were prepared from heparinized peripheral blood obtained from each patient using a conventional

preparation kit (Lymphoprep, Nycomed Pharma, Oslo, Norway). The cells were washed once with Dulbecco's phosphate-buffered saline (PBS), then once with culture medium and centrifuged at 1,400-rpm (300 g) for 10 min. Before addition of the PBMCs to the NK cell expansion culture a human feeder cell line, HFWT cells (1×10^5), were plated in each well of 24-well culture plates and were irradiated with X-rays (50 Gy) after overnight incubation. The PBMCs (1×10^6 cells/ml, 1 ml/well) were then cultured on the irradiated HFWT cells. RHAM α medium supplemented with 5% autologous plasma and interleukin-2 (IL-2, 200U/ml) was used for the culture. The NK expansion culture was continued with appropriate changes of the medium including IL-2 until the adherent feeder cells disappeared. Six or seven days after the culture, the cells were collected and washed. The cell suspension was diluted again with culture medium to 5×10^5 /ml and was transferred into 75-cm² culture flasks. The culture was continued for 14 days after the preparation of PBMCs.

Flow cytometry. To evaluate the subpopulation of cultured lymphocytes, the cells were stained with monoclonal antibodies (mAbs), *i.e.*, FITC-labeled anti-CD3 (UCHT1, IgG1), -CD16 (3G8, IgG1), PE-labeled anti-CD56 (VKN75, IgG1), -CD158a (VI NK12, IgM), and -CD158b (VI NK8, IgG2b) purchased from BD PharMingen (SanDiego, CA, USA). Isotype-matched control mAbs were used as negative controls. Cells were stained with these mAbs for 30 min at 4°C. After washing the cells, they were immediately analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Cytotoxicity assay and quantitative measurement of cytokine protein level. The non-radioisotopic crystal violet (CV) staining assay was adopted for ecological reasons in our laboratory and has been used for the determination of the actual cytotoxicity of cytotoxic T lymphocytes (CTL) to anchorage-dependent target tumor cells; this previously described assay is compatible with the standard ⁵¹Cr-release assay at an effector / target (E/T) ratio of 10 or lower (15). Since HFWT cells are anchorage-dependent tumor cells, we also used this assay to determine the cytotoxicity of NK cells.

A microparticle-based flow cytometry assay using the BD human TH1/Th2 cytokine CBA kit-II purchased from BD PharMingen was

adopted for the quantitative measurement of protein levels of several cytokines (interleukin-2: IL-2, interleukin-4: IL-4, interleukin-6: IL-6, interleukin-10: IL-10, tumor necrosis factor- α : TNF α and interferon- γ : IFN- γ). Briefly, mixed microparticles providing capture surfaces for specific proteins were cultured for 3 h with the medium used for the cytotoxicity assay. Then, the microparticles were analyzed by a FACScan flow cytometer. The sensitivity for each cytokine using this assay is 7.1 μ g/ml for interferon- γ and 2.6-3.0 for other cytokines.

Injection of expanded lymphocytes into patients. All patients were scheduled to receive one or more courses of treatment. One course was defined as three injections once a week. Exceptions have been indicated in the text. After the evaluation of cell proportion with flow cytometry, whole expanded lymphocytes, mainly consisting of NK cells, were injected into each patient into the tumor cavity and intravenously, where possible. As material for intracavitary injection, we used the Ommaya reservoir system commonly used for the therapeutic treatment of brain tumors. The Ommaya reservoir was made newly or revised before the injection, if necessary. A minimal dose of IL-2 (less than 100 IU/kg (weight) / one injection) was added with the lymphocytes in order to maintain their activity by the time of injection. For the clinical study design, intravenous injection of low-dose interferon beta (IFN- β , 6x10⁶ IU/week) was performed as an adjuvant therapy in all patients to achieve maximum benefit for enrolled patients. Glucocorticoid was used for only one patient (patient No. 6).

Evaluation of response and adverse effects to the treatment. Responses and adverse effects to the treatment were evaluated at 4 or 5 weeks after the beginning of each treatment course. A response was determined by comparing clinical signs as well as tumor size measured before and after treatment, following the slightly modified guidelines reported by Therasse *et al.* (23). In the present study, the modification of the guidelines was undertaken for the measurement of tumor size, which was calculated based on the volumetry observed by magnetic resonance (MR) imaging using contrast-enhancing material in all cases. The three-dimensional volumetry was reported for the brain tumors (6, 12, 13). The response was considered to be a complete response (CR) if all measurable tumor tissue had disappeared. A partial response (PR) was defined as a 50% decrease in the total tumor volume of all lesions persisting for four weeks, without increase in the size of any tumor and without the appearance of any new tumor. A minor response (MR) was defined as a 25% decrease in the total tumor volume of all lesions persisting less than four weeks, without an increase in the size of any tumor and without the appearance of any new tumor. A progressive disease (PD) was defined as an increase of over 25% in the total tumor volume of all lesions, or by the appearance of any new tumor. A case of no-change (NC) was defined when the response was excluded from CR, PR, MR and PD.

We represented adverse effects in each course by a neurological toxicity grading scale modified by Hayes (6). Grades 0-IV were defined in the following manner: Grade 0, normal or no change in neurological status compared with the baseline examination; Grade I, mild lethargy and/or irritability or visual, motor, or sensory symptoms, without change in neurological examination; Grade II, moderate lethargy, disorientation, or psychosis lasting less than 48 hours or mild increase in preexisting deficits; Grade III and Grade IV followed Hayes' report (6). Severe neurological toxicity was defined as Grade III or more.

Statistical analyses. Student's *t*-test was used for examination of significance between two groups for *in vitro* studies.

Ethics. The ethical committees of the University of Tsukuba and RIKEN, Japan, approved this therapy. Written informed consent was obtained from each patient before the initiation of any procedure related to this treatment.

Results

Selective expansion of NK cells from PBMCs of patients with malignant glioma. The HFWT cell line is the most essential material required for the selective expansion of NK cells. This type of tumor has no surface antigen responding to anti-HLA A, B, C or anti-HLA DR, DP, DQ. In our previous report, we demonstrated that the HFWT cell line is very sensitive to NK cells and it can act as an NK cell inducer (19). In preliminary tests, we confirmed the selective expansion of NK cells from PBMCs of patients with recurrent malignant glioma after culturing the cells in a medium containing IL-2, with or without irradiated HFWT cells. The latter condition is used for what is known as a LAK cell culture. Figure 1 illustrates the most pronounced example of cell contrast; the cells in this experiment were derived from patient No. 5, and were expanded from whole PBMCs and cultured in a medium containing IL-2 with or without irradiated HFWT cells. The difference between cells was quite pronounced; in fact the proportion of CD3⁺CD16⁺CD56⁺ NK cells reached 96.0% in the condition with HFWT cells, and only 6.8% in the condition without HFWT cells. The characteristics of the cells expanded with or without the irradiated HFWT cells in seven patients (patients No. 1-7) are summarized in Table II. (We could not study the analysis in patients No. 8 and 9 due to lack of PBMCs.) The mean increase in expansion of the cells in the former condition and in the latter condition was 112.9-fold and 13.3-fold, respectively. As regards the CD16⁺CD56⁺ NK cells, obvious differences were observed between the cells subjected to the two types of expansion methods, *i.e.*, 85.9% in the former condition and 23.4% in the latter condition. The lymphocytes expanded with irradiated HFWT cells revealed stronger cytotoxicity against two types of target cell lines (HFWT as an MHC class I-negative cell line and a glioblastoma cell line, TKB-34RGB, as an MHC class I-positive cell line) than those expanded without irradiated HFWT cells (Figure 2 A).

The expanded lymphocytes from patient No. 3 showed strong cytotoxicity against not only non-irradiated HFWT cells, but also against autologous tumor cells and various allogeneic tumor cell lines. The expanded lymphocytes from patient No. 4 also showed strong cytotoxicity against HFWT cells and against two types of allogeneic tumor cells (Figure 2-B). More than 50% of all examined tumor cells were killed by the lymphocytes at an E/T ratio of 4.

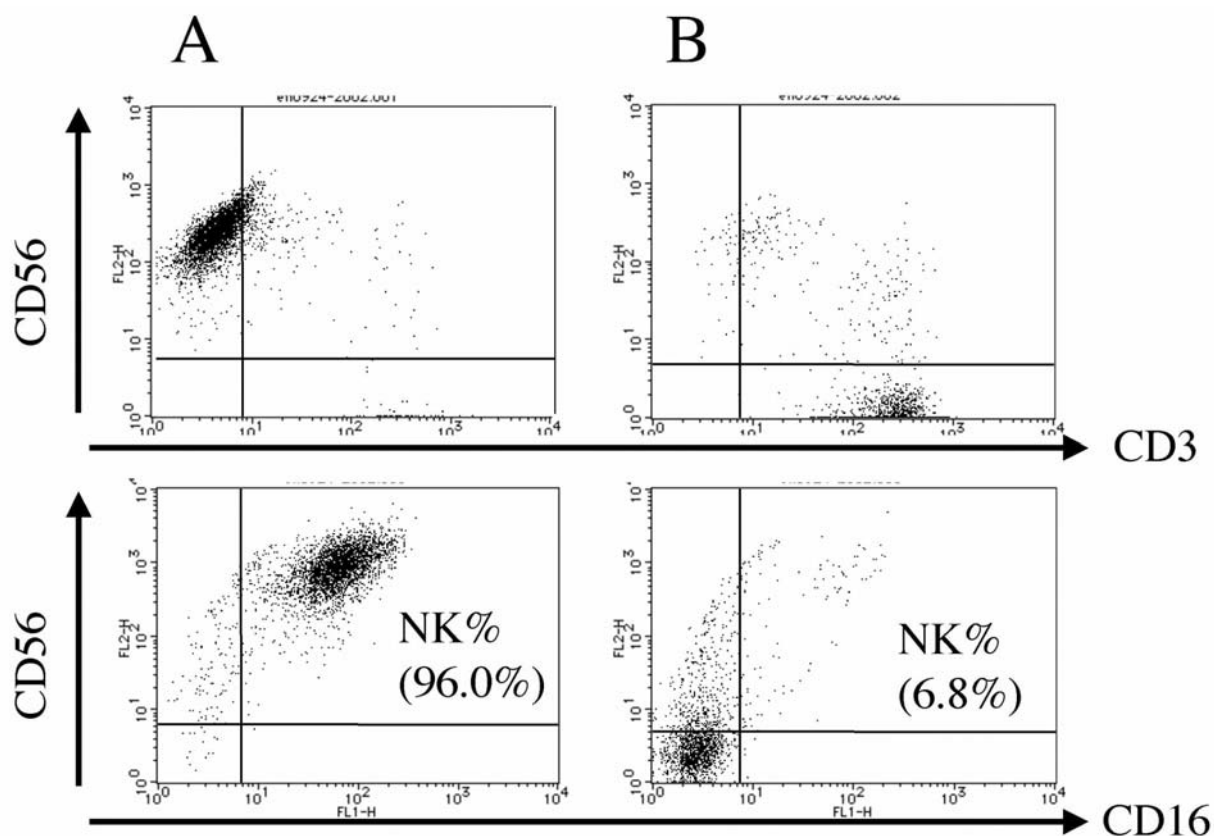


Figure 1. Expanded cells cultured with HFWTs and IL-2 from the PBMCs of patient No. 5 (A); this group was compared with an IL-2-only culture of otherwise identical cells (B). The cultured lymphocytes were stained with anti-CD3, -CD16, -CD8, -CD56 and -CD4. Isotype-matched control mAbs were used as negative controls.

Table II. Characteristics of cells expanded with irradiated HFWT and IL-2 (A) or with IL-2 (B) in seven patients.

	A	B	Student's <i>t</i> -test
Cell expansion (-fold / 2 weeks)	112.9±106.9	13.3±13.2	<i>p</i> =0.033
CD16+CD56+ cell (%)	85.9±9.6	23.4±15.3	<i>p</i> =0.001
Cytotoxicity (%), target; HFWT *	94.7±5.6	42.0±18.9	<i>p</i> =0.007
Cytotoxicity (%), target; TKB-34RGB *	62.6±35.4	28.6±21.4	<i>p</i> =0.026

* cytotoxicity (%)=100 - surviving target cells (%) (E/T ratio=1)

In addition, protein levels of several cytokines (IL-2, IL-4, IL-6, IL-10, TNF α and IFN- γ) in each well used for the cytotoxicity assay were analyzed. IFN- γ was secreted by NK cells that were not in the presence of target cells. Furthermore, a much higher concentration of IFN- γ was detected in the medium in which NK cells killed the target cells (Figure 3 and Table III). TNF- α showed similar changes

in concentration, although there was no statistical significance. No remarkable change was observed between the cytokine concentrations of IL-2, IL-4, IL-6 and IL-10 in the medium with NK cells only or in the medium with both NK cells and target cells. A high concentration of IL-2 was considered to be an artifact due to culturing effector cells with high-dose IL-2 and the high concentration of IL-6 in Figure 3-B was considered to be production from the target cells.

Clinical responses to the treatment. Table I shows the characteristics of 9 patients who received the NK therapies used in this study. The nine patients in the series ranged in age from 23 to 70 years (5 men and 4 women). Histological diagnoses were anaplastic gliomas, 6 cases (anaplastic astrocytoma, 4 cases; anaplastic oligodendroglioma, 1 case; anaplastic oligoastrocytoma, 1 case) and glioblastoma, 3 cases. All patients received radiation therapy and 7 patients received several types of chemotherapy more than 4 weeks before the NK therapies. The Karnofsky performance scales of these patients at the time of treatment ranged from 20% to 80%. The total dose of injected cells and

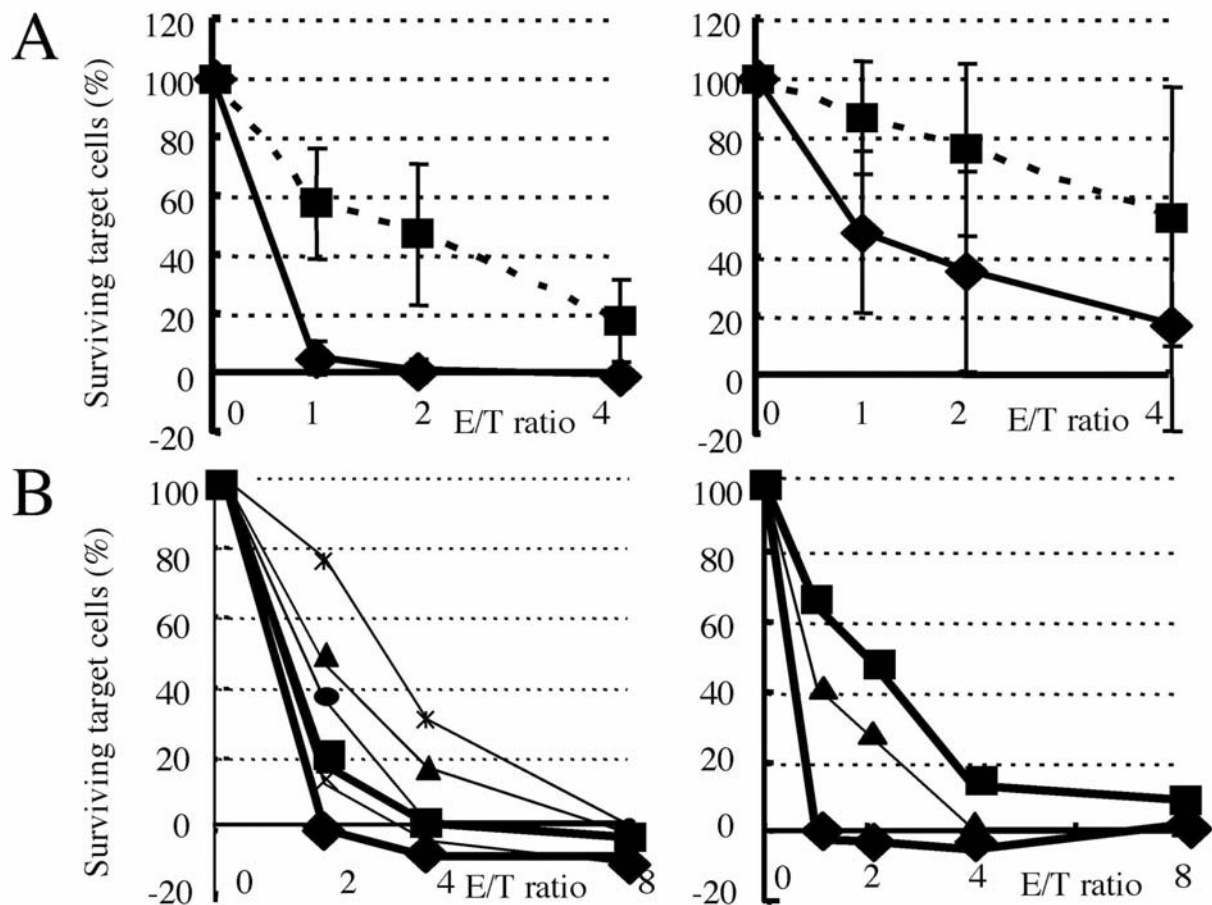


Figure 2. *A-left & right.* The cytotoxicity of the lymphocytes expanded by using HFWTs and IL-2 from seven patients was examined against the HFWT cell line (left figure) or a glioblastoma cell line (TKB-34RGB: right figure) used as target tumor cells; a non-radioisotopic crystal violet (CV) staining assay (24 hours) at an E/T ratio of 0, 1, 2 and 4 (continuous line) was applied for the analysis. Additionally, lymphocytes expanded with IL-2 only were also examined (dashed line).

B-left. The CV staining assay was used for the determination of the cytotoxicity of expanded lymphocytes from patient No. 3 against HFWT (◆), autologous brain tumor cell line (■: TKB-19RGB) and various target brain tumor cell lines (*: TKB-7RGB, ▲: TKB-9RGB, ●: TKB-30RGB, ■: TKB-19RGB and ×: TKB-17RGB). The lymphocytes were examined at an E/T ratio of 0, 2, 4 and 8 for 24 hours. *B-right.* The CV staining assay was used for the determination of the cytotoxicity of expanded lymphocytes from patient No. 4 against three target tumor cell lines (◆: HFWT, ■: TKB-34RGB and ▲: TKB-17RGB).

injected sites in these ten cases treated with autologous NK cell therapy are shown in Table IV. Considering all cases together, from 11.3- to 90.1-fold expansion of lymphocytes from PBMCs was observed. The absolute number of the expanded NK cells depended on the frequency of initial NK cells among the PBMCs ($R^2=0.736$, $p=0.0135$, data not shown). The mean frequency of NK cells (defined as CD16+CD56+ cells) among the cultured lymphocytes was $82.2 \pm 10.5\%$. More than 25% of the lymphocytes had KIRs responding to CD158a or CD158b.

The expanded cells were injected into tumor cavities (0.4 - to 2.3×10^9 cells per one course) combined with intravenous injection (0.2 - to 3.7×10^9 cells per one course) for ten courses (total dose, 0.6 - to 5.1×10^9 cells per one course). In 6 courses,

intravenous injection alone was performed (0.6 - to 6.5×10^9 cells per one course), because of unexpected closing of the Ommaya reservoir tubing or inoperative condition of the patients for revision of the reservoirs. Figure 4 demonstrates the progression of the tumor volume as an enhanced lesion on T1WI or as a high-intensity lesion on T2WI in MR imaging measured in each case. Apparent tumor regression was observed in cases No. 3, 4, 5 and 8. The results of treatment for each patient are also shown in Table IV. There were 3 PR, 2 MR, 4 NC and 7 PD in a total of 16 courses of treatment in 9 patients. Neurological toxicity grading scales were Grade 0 or Grade I in all courses, although all patients exhibited transient fever. Since case No. 6 underwent other therapy within one month after completion of a treatment course, the

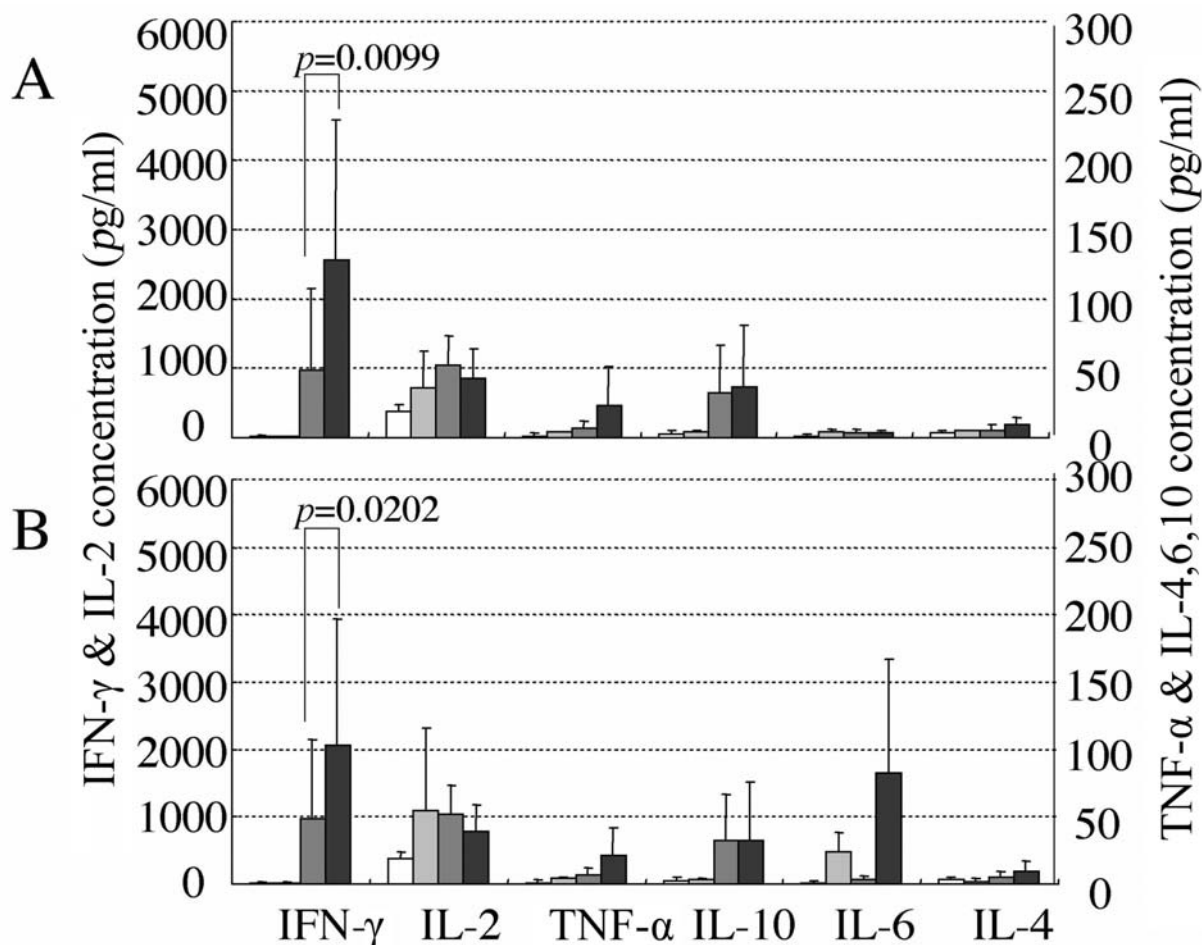


Figure 3. A. The concentrations of various cytokines were evaluated in each of the wells after the cytotoxicity assay against HFWTs (shown in Figure 2). Left bar, the mean concentrations in medium only; middle-left bar, the concentrations in the medium with HFWT cells (1×10^4 cells / $200 \mu\text{l}$); middle-right bar, the concentrations in the medium with NK cells (8×10^4 cells / $200 \mu\text{l}$); and right bar, the concentrations in the medium with NK cells and HFWT cells. B. The concentrations were evaluated in each of the wells after the cytotoxicity assay against the glioblastoma cell line (TKB-34RGB).

clinical outcome was evaluated in the other 8 patients. Four patients are still alive (KPS=80%, 70%, 40%, 10%) and 4 patients died as a result of malignant glioma.

Autologous NK cell therapy – presentation of an illustrative case. Patient No.5 was a 23-year-old woman with a tectal tumor, who developed headaches beginning in April, 2001. Partial removal of the tumor was carried out on June 19, 2001. The pathological diagnosis was anaplastic astrocytoma. The patient was given 60Gy of radiation. Chemotherapy was performed by intravenous injection with the antitumor drug ACNU. Despite these intensive treatments, intratumoral bleeding with tumor regrowth occurred on August 23, 2001. The patient was admitted to our hospital for additional treatment consisting of immunotherapy. Surgical mass reduction of the recurrent tumor revealed the same pathological diagnosis. Two courses of NK therapy were

carried out from December 20, 2001 to June 6, 2002. An expansion curve and phenotypes of cultured cells in case 5 are shown in Figure 5-A and -B. Adverse effects were mild fever, headache and nausea. Post-treatment MR imaging revealed tumor regression when compared with images taken before the treatment (Figure 6). Afterwards, the patient underwent additional NK therapy. However, tumor remission did not occur after the third course.

Discussion

To the best of our knowledge, with the exception of LAK cell therapy, this is the first report of autologous NK cell therapy for use in patients with brain tumors. We ascertained that this type of therapy induced a decrease in tumor volume in some cases, without any severe neurogenic toxicity. We concluded that the acceleration of

Table III. Cytokine releasing and cytotoxicity of the expanded cells against two target cell-lines (HFWT & TKB-34RGB).

Case No.	NK cell (%)	IFN- γ	Cytokine releasing from cultured cells (pg/ml), target: HFWT				Cytotoxicity (%)	IFN- γ	Cytokine releasing from cultured cells (pg/ml), target: TKB-34RGB				Cytotoxicity (%)
			TNF- α	IL-10	IL-6	IL-4			TNF- α	IL-10	IL-6	IL-4	
1	76.4	6200	6.0	2.9	2.1	11.9	97	90	5.0	5.9	187.5	22.4	-2
2	71.2	5000	80.0	41.8	6.8	5.6	92	4460	61.0	34.8	31.6	5.0	58
3	91.4	2520	8.4	33.0	3.5	6.2	87	1590	14.1	19.2	38.3	8.9	101
4	96.0	1900	18.8	12.8	1.8	8.4	102	1710	26.3	13.8	194.4	7.2	36
5	96.0	410	11.6	5.9	3.9	4.5	89	290	6.6	5.7	25.1	0.0	83
6	86.9	n.p	n.p	n.p	n.p	n.p	100	n.p	n.p	n.p	n.p	n.p	76
7	83.1	4930	14.4	122.4	3.3	18.7	96	4200	12.9	117.4	16.9	12.8	86

n.p., not performed.

Table IV. Responses and neurological toxicities of NK therapy.

Case No.	Course of therapy	Total (x10 ⁹)	dose (-fold)	Injected route (i.c. %)	Response to the therapy	Neurological toxicity	Posttreatment K.P.S. (from initial NK cell injection to outcome)	
1	1	3.1	43.7	<i>i.v.</i>	(0)	NC	Grade 0	Dead(18 months)
2	1	3.3	43.6	<i>i.v. +i.c.</i>	(50)	PD	Grade 0	Dead (6 months)
3	1	0.6	11.3 #	<i>i.v.</i>	(0)	PR	Grade 0	50%
	2	3.5	52.5	<i>i.v.</i>	(0)	PD	Grade 0	40%
	3	6.5	90.1	<i>i.v.</i>	(0)	PD	Grade 0	Dead(11 months)
4	1	0.7	12.1 #	<i>i.v. +i.c.</i>	(51)	NC	Grade 0	10%
	2	2.5	42.6	<i>i.v.</i>	(0)	MR	Grade 0	10% (15 months)
5	1	5.1	52.7	<i>i.v. +i.c.</i>	(43)	PR	Grade I*	70%
	2	4.6	64.1	<i>i.v. +i.c.</i>	(50)	PR	Grade I*	70%
	3	4.0	54.9	<i>i.v. +i.c.</i>	(50)	PD	Grade 0	70% (12 months)
6	1	0.6	13.9	<i>i.v. +i.c.</i>	(65)	PD	Grade 0	Dead**
7	1	4.3	59.6	<i>i.v. +i.c.</i>	(13)	NC	Grade 0	Dead (4 months)
8	1	1.8	63.6	<i>i.v. +i.c.</i>	(50)	MR	Grade 0	40%
	2	1.7	23.8 #	<i>i.v. +i.c.</i>	(50)	PD	Grade 0	40% (5 months)
9	1	0.8	11.5	<i>i.v.</i>	(0)	NC	Grade 0	80%
	2	3.2	44.7	<i>i.v. +i.c.</i>	(50)	PD	Grade 0	80% (5 months)

Data regarding cell expansion that proceeded with difficulty are included in the calculation.

* Headache, nausea and irritability.

** Another therapy was performed one month after the period of NK therapy.

i.v.: intravenous; *i.c.*: intracranial.

tumor growth might have been depressed in 9 courses out of all 16 courses (56%). Furthermore, in 2 out of the 9 cases (22%), or in 3 out of the 16 courses (19%), the reduction of tumor volume to less than half of the pretreatment volume persisted for over 4 weeks. This response rate was not necessarily higher than the response rates of 11-28% in cases with advanced cancers including malignant gliomas carried out with the combination of high-dose IL-2 and LAK cells (5, 6, 8-10). However, high-dose IL-2 and LAK therapy often causes severe neurological toxicity (21-33%) (5, 6). The response and the toxicity may depend strongly on the dose of IL-2. We believe that an elevation in the response rate to autologous

NK cell therapy can be achieved, if this treatment is combined with a feasible dose of IL-2 or radiation therapy. Furthermore, combined therapies consisting of NK cell therapy and other immunotherapies with an MHC-class-I restriction, such as CTL therapy, might reasonably be expected to provide a breakthrough in the treatment of malignant tumors. In the present study, we used low dose IFN- β (6x10⁶ IU/week) to obtain maximum clinical benefit for enrolled patients, as it has been reported that IFN- β promotes NK cell-mediated cytotoxicity *in vitro* and *in vivo* (24). Although high-dose IFN- β therapy with/without other adjuvant treatments has been tried resulting in partial benefit in treatment for recurrent malignant gliomas,

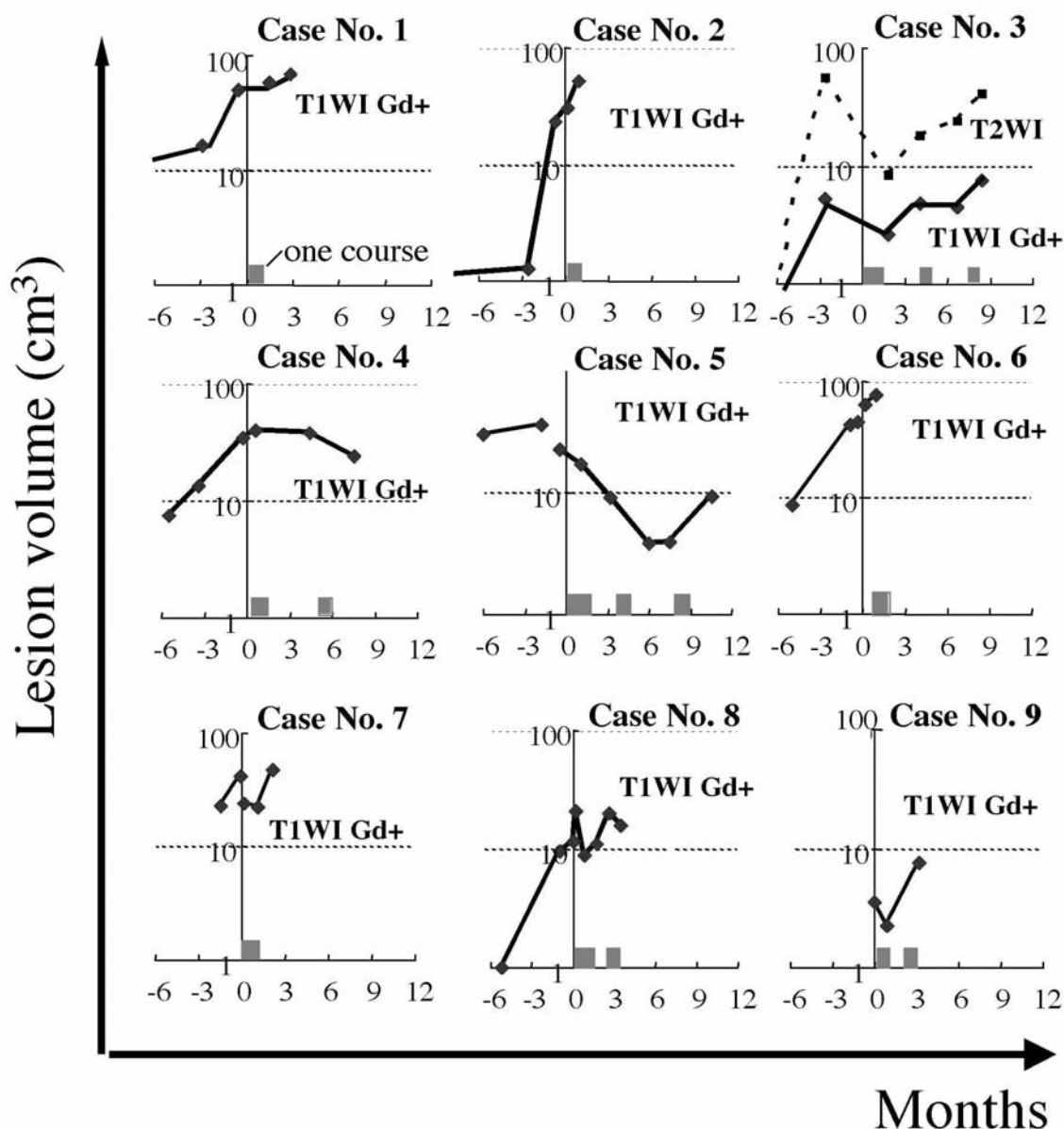


Figure 4. The progression of the tumor volume, measured as an enhanced lesion (T1WI) or as a high-intensity lesion (T2WI) on MR imaging in each case is demonstrated. The tumor size was calculated based on the three-dimensional volumetry of the lesion.

recent studies used IFN- β as a drug- or radio- sensitizer (25-27). We were unable to find any report indicating that low-dose IFN- β alone can induce tumor regression in recurrent malignant gliomas.

Several authors have noted that NK cells, which play an important role in the cellular immune response, possess strong cytotoxicity against malignant tumors (1, 14, 16-19, 28-30). However, clinical trials of autologous NK therapy have been reported in very limited numbers (14). One explanation for the lack of trials is the difficulty of

selective and highly efficient expansion of autologous NK cells from the PBMCs of patients. Ordinary NK cell expansion systems using antibodies or several feeder cells including K562 cells require several complex steps and/or a very large number of PBMCs from a patient (14, 16-18). In contrast, our novel method of NK cell expansion on HFWT feeder cells led to selective and highly efficient expansion of autologous NK cells. According to our data, 11.3- to 90.1-fold expansion of lymphocytes containing more than 70% activated NK cells was obtained within 2

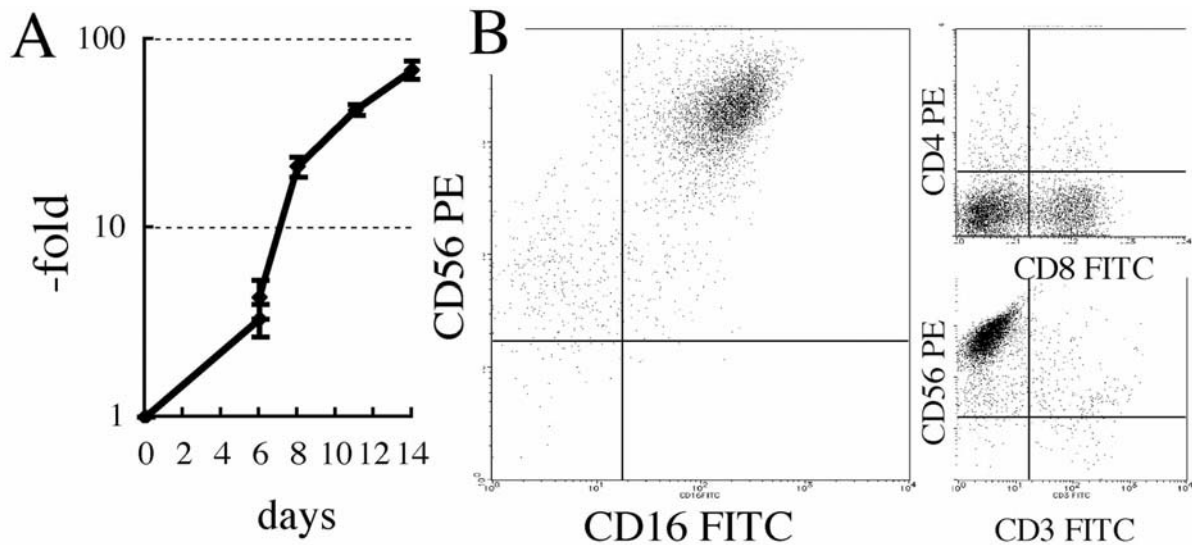


Figure 5. The expansion curve and the phenotypes of cultured cells in case No. 5 are shown. A. PBMCs (1×10^6 cells/well) were cultured on 1×10^5 irradiated HFWT cells with IL-2 (200 IU/ml). Six days after the culture, the collected cells were transferred into 75-cm² culture flasks. The culture was continued for 14 days after the preparation of PBMCs. B. The cultured lymphocytes were stained with several antibodies and analyzed by a flow cytometer. NK cells defined as CD16⁺CD56⁺ cells by flow cytometry comprised 91.1 % of the total lymphocytes.

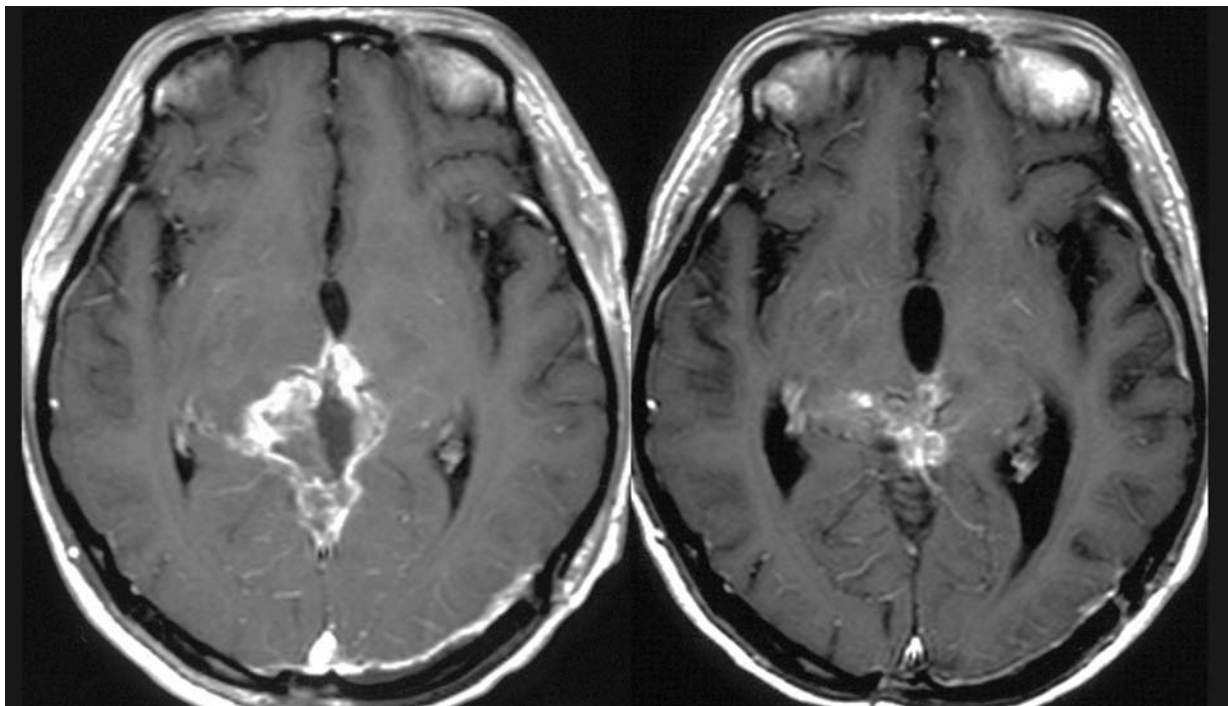


Figure 6. MR images before and after two courses of NK therapy in patient No. 5, with a tectal recurrent glioblastoma, are shown.

weeks in all ten patients with recurrent malignant glioma, without any additional purification steps. Even in the patients with a low KPS score, the expanded lymphocytes revealed strong cytotoxicity against various types of tumor cell as shown in Figure 2.

In the present cases, we attempted to administer adoptive immunotherapy using NK cells by combined injections *via* an intravenous route and a direct route into the tumor cavity for 10 out of 16 courses. When administering adoptive immunotherapy, several cell injection routes (*e.g.*,

intravenous injection, focal injection *via* the feeding vessels of the tumor, and direct intratumoral injection *via* the tumor cavity) can be selected. Among these routes, intravenous injection is preferred for cell delivery in several types of adoptive immunotherapies, *e.g.*, LAK cell therapy and CTL therapy. Previous histological reports have indicated the migration of LAK/NK cells injected intravenously into tumorous tissue. However, several authors have claimed that only a low concentration of LAK/NK cells in such tumors would be expected, even if a large number of cells were to be injected *via* non-feeding vessels (14, 16, 31, 32). Furthermore, as regards brain tumor models, Holladay and colleagues claimed that antitumor activity against established intracerebral gliomas was not exhibited by intravenous injection of LAK/NK cells (33). As regards focal injection *via* the feeding vessels of the tumor, several reports have demonstrated an enormous distribution of NK cells into the tumor site, as shown by positron emission tomography, or by video microscopy of the feeding vessels (18, 31). Moreover, intracavitary LAK and CTL therapies have been reported and have successfully achieved tumor regression in some cases (5-7,12,13). Such focal injection *via* the feeding vessel or direct injection into a tumor cavity would be expected to lead to direct cytotoxicity. However, the procedure using these injection routes presents a complexity and an uncertainty. Indeed, in 6 out of the 16 courses, direct injection into the tumor cavity was not performed because of unexpected troubles of the Ommaya reservoir system. Moreover, interestingly, intravenous injection alone achieved a remarkable tumor regression in 2 courses (No.3-course 1 and No.4-course 2). The best injection route of NK cells should be proved by further study of the NK cell therapy.

The results of the present study (Table IV) support the feasibility of continued use of autologous NK cell therapy. This type of therapy has several advantages when compared with other adoptive immunotherapies performed in our hospital using LAK cells or CTLs: a) tumor specimens of each case are not necessary for autologous NK cell expansion, although it is essential for CTL expansion; b) NK cells can be expanded in a relatively short time (2 weeks) compared with CTLs (3 weeks or more); c) NK cells possess strong cytotoxicity compared with LAK cells *in vitro* (Table II). On the other hand, there are also considerable disadvantages when using autologous NK cell therapy as well as other adoptive immunotherapies, *e.g.*, limitations of the reaction span and the presence of resistant tumor cells against injected cells. For instance, although the NK cell therapy in cases No. 3, 5 and 8 was effective in the first course, tumor remission did not occur during or after the following courses. This phenomenon might suggest that some tumor cells, resistant to NK cells, survived and grew again. This phenomenon should be examined carefully in further clinical trials.

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