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Cytological Characteristics of Human Glioma-infiltrating Lymphocytes Stimulated with Recombinant Interleukin 2 and an Anti-CD3 Antibody

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Tumor-infiltrating lymphocytes (TIL) were generated from 10 glioma specimens by using recombinant interleukin-2 and an anti-CD3 antibody (CD3+TILs). We obtained more than 1×10^9 cells in 5 cases, more than 5×10^8 cells in 2 cases, and about 1×10^8 cells in 3 cases during three weeks of incubation from small specimens ranging in weight from 0.5 to 2.0 g. In 4 cases, TILs were expanded following stimulation with only rIL-2 (CD3-TILs). The growth rate of CD3-TILs was less than that of CD3+TILs. Cytotoxicity of CD3+TILs was lower than that of lymphokine-activated killer (LAK) cells in a standard 4h ^{51}Cr release assay. Cold target inhibition was undertaken in three cases and specific cytotoxicity could be shown in only one case. CD3+TILs mainly consisted of CD3-positive cells, ranging from 63.2 to 99.9%. The ratio of CD4-positive cells to CD8-positive cells was not constant. The expression of Leu 7 and CD16 was low. The present study did not confirm previous findings that TILs were more tumor-selective and potent than LAK cells. Furthermore, the results on *in vitro* antitumor activity of those cells were not necessarily consistent with the results on their clinical activity. Further careful work is necessary on the preparation of immunocytes and the subsequent adoptive immunotherapy.

Key words: Tumor-infiltrating lymphocyte — Human glioma — Anti-CD3 antibody — Interleukin-2 — Lymphokine-activated killer cell

Adoptive transfer of lymphokine-activated killer (LAK) cells,[1] which can be produced by culture of peripheral blood lymphocytes (PBL) with interleukin 2 (IL-2), has recently been used against malignant tumors.[2–7] In addition, Rosenberg *et al.* reported[8] that the cytolytic activity of IL-2-stimulated tumor-infiltrating lymphocytes (TIL) is greater than that of LAK cells. However, few clinical trials of adoptive immunotherapy (AIT) with TILs have been published,[3, 9, 10] primarily because this therapy has certain shortcomings, such as difficulty in obtaining enough TILs.

CD3 is a lymphocyte surface antigen and an anti-CD3 antibody is clinically used to prevent acute rejection of transplanted kidneys. This antibody also elicits the generation of killer lymphocytes.[11–14] We previously[15] succeeded in obtaining enough LAK cells from PBL of patients with malignant brain tumors by using an anti-CD3 antibody. Furthermore, we also tried to use anti-CD3 antibody for generating TILs from gliomas.

We report here that we could readily obtain enough TILs for AIT from small amounts of glioma tissues by using an anti-CD3 antibody with rIL-2. We have also investigated the difference in cytolytic activity and cell surface phenotypes between TILs and LAK cells.

The abbreviations used are: LAK cells, lymphokine-activated killer cells; rIL-2, recombinant interleukin-2; TIL, tumor-infiltrating lymphocytes; AIT, adoptive immunotherapy; CM, complete medium; FBS, fetal bovine serum.

MATERIALS AND METHODS

Origin of cells Ten patients were studied ranging in age between 3 and 80 years (Table I). Specimens resected surgically from these patients were between 0.5 and 2.0 g in weight. Histopathologically two of them were low-grade astrocytomas, seven were high-grade astrocytomas, and one was ganglioglioma grade 3. No patient had received prior chemotherapy or irradiation except case 2, who had received chemotherapy (ACNU) and radiotherapy (60 Gy).

Culture medium Complete medium (CM) consisted of TIL medium 1 (IBL Industries) supplemented with 2% human AB serum. In certain cases, serum of the patients was used instead of AB serum.

Preparation of TILs and tumor cells Single cell suspensions of TILs and tumor cells were obtained by enzymatic digestion. Resected tumor was collected from surgery and transported in a sterile fashion. Necrotic tissue, fatty tissue, clotted blood, and apparently normal tissue were removed and the remaining specimen was minced into small pieces with surgical blades. The chopped tissue was washed at least three times with RPMI 1640 medium to remove peripheral blood. This tissue was then dissociated by mechanical stirring for 1 h at room temperature in a flask containing hyaluronidase (175 U/g), collagenase (1400 U/g), and deoxyribo-

nuclease (1350 U/g). The resulting mixture was filtered, and the cells were washed twice with RPMI 1640, and resuspended at a final concentration of 1.0×10^6 cells/ml in CM with 500 U/ml of rIL-2 (supplied by Shionogi Chemical Industries). The flasks were incubated overnight at 37°C in 5% CO_2, then the nonadherent cells were harvested and placed on Ficoll-Paque for single density gradient separation at 400*g* for 20 min. The cells at the interface were collected . The adherent cells were cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO_2 and used later as target cells.

Growth of TILs The cell suspensions were washed twice with RPMI 1640 and resuspended at a concentration of 2.5×10^5 cells/ml in CM with 500 U/ml of rIL-2. The suspension was placed in 6- or 24-well culture plates coated previously with an anti-CD3 antibody (Muromonab OKT3, Ortho Diagnostics) at 10 or 15 μg/ml in PBS and the plates were then incubated at 37°C in 5% CO_2 for approximately 7 days. The medium was partially replaced after 3 to 4 days. After activation, the cells were harvested, resuspended at a final concentration of 1.0×10^6 cells/ml in CM with 500 U/ml of rIL-2, and incubated at 37°C in 5% CO_2. The cell concentration was maintained at approximately 2×10^6/ml.

Growth and generation of LAK cells Heparinized blood samples (10–40 ml) were obtained from the same patients during surgery. PBL were isolated by Ficoll gradient centrifugation (400*g*, 25 min), washed twice with PBS, and finally suspended in CM with 500 U/ml of rIL-2 at a concentration of 1×10^6 cells/ml. Half of the cells were placed in 6- or 24-well culture plates coated previously with an anti-CD3 antibody and these cells were designated CD3+LAK cells. The remainder were placed in usual 6- or 24-well culture plates and these cells were designated CD3-LAK cells. Cytolytic activity and surface phenotypes of TILs and LAK cells were measured on the same day.

Cultured tumor targets and autologous glioma cell targets K562, the NK cell-sensitive erythroleukemia cell line, Daudi, the NK cell-resistant Burkitt's lymphoma cell line, U251MG, a glioblastoma cell line, and autologous glioma cells obtained by the above-mentioned method were cultured and used as targets.

Assay of cytolytic activity The cytolytic activity of TILs and LAK cells was tested *in vitro* in a standard ^{51}Cr release assay. Target cells and effector lymphocytes were cocultured for 4 or 20 h. The effector:target ratio was 20:1 except in the case of cold target inhibition. All determinations were made in triplicate and the percentage lysis was calculated by means of the formula:

$$\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100\%.$$

In addition, specific cytotoxicities of TILs and LAK cells against autologous glioma cells were assessed by cold target inhibition. Autologous glioma cells (5×10^5) were labeled with 50 μCi of Na_2CrO_4 (Amersham, Japan) for 120 min at 37°C. Unlabeled autologous glioma cells of U251MG as cold targets were added in advance at a concentration of 5×10^3/well in triplicate to 4×10^5 effector lymphocytes in 96-well round-bottomed plates. Labeled target cells were then washed and added at the same concentration to the plates. The E:T ratio (40:1) and the ratio of cold and hot targets (1:1) were fixed. After 20 h at 37°C in a humidified CO_2 incubator, 100 μl aliquots of supernatants were harvested and counted in a gamma counter. The percentage of lysis was calculated by the above formula. If the percentage of lysis against labeled autologous glioma cells with unlabeled U251MG cells was higher than that against labeled autologous glioma cells with unlabeled autologous glioma cells, those effector lymphocytes were considered to have specific cytotoxicity.

Cell surface analysis Surface phenotypes of TILs and LAK cells were measured by FACS. The staining reagents included anti-Leu 2, anti-Leu 3, anti-Leu 4, anti-Leu 7, anti-Leu 11, anti-HLA-DR, and anti-Tac antibodies.

RESULTS

Growth and expansion of TILs After about two weeks of incubation, we could readily obtain more than 1×10^8 TILs from each of the surgical specimens, which ranged between 0.5 and 2.0 g in weight (Table I). At least 5×10^8 TILs are needed for AIT. We obtained more than 1×10^9 cells in 5 cases, more than 5×10^8 cells in 2 cases, and about 10^8 cells in 3 cases, after three weeks of incubation. If we had not used TILs for cytolytic assay, we could have obtained more TILs. There was no relationship between age and growth rate. We also tried to expand TILs stimulated only with rIL-2 in 7 cases and we obtained more than 10^7 cells in cases 2, 5, 7, and 8, but not more than 10^8 cells after three weeks of incubation except in case 7. The growth rate of TILs stimulated with rIL-2 alone was lower than that of TILs stimulated with rIL-2 and an anti-CD3 antibody. The characteristic patterns of growth and expansion were that, within the first week in culture, many viable glioma cells and a few lymphocytes were evident and then, by the end of the second week, the number of TILs gradually increased, while the number of glioma cells decreased. Usually viable glioma cells disappeared by the end of the third week, but, in case 1, it took more than 3 weeks to activate TILs. We discontinued incubation of TILs at about 50 days in culture, because cytotoxicity of TILs at that time was almost undetectable.

Table I. Patients' Profiles, Weight of Specimens, and Cell Numbers of TILs

Patient	Age/Sex	Pathological diagnosis		Weight of specimen (g)	Original cell number ($\times 10^6$)	Total cell number ($\times 10$)[a]	
						CD3+TILs[b]	CD3−TILs[c]
1. H.S.	33/M	Astrocytoma	grade 4	2.0	1.1	0.9	—[d]
2. T.M.	40/M	Astrocytoma	grade 2	1.7	0.8	10.0	0.9
3. Y.S.	27/M	Astrocytoma	grade 3	2.0	ND[e]	2.3	—
4. M.K.	35/M	Astrocytoma	grade 3	1.5	1.8	13.0	ND
5. H.K.	74/M	Astrocytoma	grade 4	2.0	ND	9.7	0.9
6. T.S.	57/M	Ganglioglioma	grade 3	2.0	2.1	11.5	ND
7. T.T.	41/M	Astrocytoma	grade 4	0.7	0.7	14.6	4.6
8. S.T.	58/F	Astrocytoma	grade 4	1.0	ND	8.8	0.7
9. M.S.	80/F	Astrocytoma	grade 4	0.7	1.0	1.6	—
10. W.N.	3/F	Astrocytoma	grade 2	0.5	1.0	10.0	ND

a) Total cell number in three weeks of incubation.
b) TILs stimulated with rIL-2 and an anti-CD3 antibody.
c) TILs stimulated with rIL-2 alone.
d) More than 10^7 cells could not be obtained.
e) Not done.

Growth and expansion of LAK cells The results for LAK cells were described previously.[15] Briefly, in all cases, more than 5×10^8 CD3+LAK cells and more than 1×10^8 CD3-LAK cells were obtained after 2 weeks of incubation (data not shown). The growth rate of CD3+ LAK cells was greater than that of CD3-LAK cells and TILs. In elderly patients, it was slightly more difficult to expand CD3+LAK cells, but it was not difficult to obtain enough CD3+LAK cells from their PBLs.

Cytolytic activity of TILs and LAK cells TILs and LAK cells were assayed for activity against K562, Daudi, allogeneic glioma, and autologous glioma cells by using a standard ^{51}Cr release assay (Table II). Labeled target cells and effector lymphocytes were cocultured for 4 or 20 h. A 20 h assay could be used only against allogeneic glioma and autologous glioma cells, because spontaneous release of chromium from K562 and Daudi cells after 20 h in culture was too high (spontaneous cpm/maximum cpm >0.3). Generally, as compared with LAK cells, cytolytic activity of TILs against all kinds of tumor cells was low, especially against autologous and allogeneic glioma cells as measured by a standard 4 h assay (Fig. 1). The percentage lysis was not increased even after 20 h in culture. Cytotoxicity of TILs against autologous glioma cells was analyzed in all cases, but specific cytotoxicity was not detectable by this assay. In addition, cold target inhibition was used in cases 7, 8, and 10 for investigating the specific cytotoxicity of TILs and LAK cells against autologous glioma cells. The E:T ratio and the ratio of hot targets and cold targets were fixed (E:T=40:1, hot targets:cold targets=1:1). In case 7 alone, the percentage of lysis against hot autologous glioma cells with cold U251MG cells was very much higher than that of lysis against hot autologous glioma cells with cold autologous glioma cells. Those results indicated that the TILs in case 7 had specific cytotoxicity against autologous glioma cells. In other cases, the percentages were almost the same. Even in case 7, the percentage lysis against autologous glioma cells was low using a standard 4 h ^{51}Cr assay, so cold target inhibition had to be used if we intended to ascertain specific cytotoxicity against autologous glioma cells. We also used this method to investigate the specificity of LAK cells against autologous glioma cells, but we could not find specific cytotoxicity against these cells in any case (data omitted).

Surface phenotypes of TILs Phenotypic analysis of TILs was performed in all cases between days 14 and 48 in culture and was retested twice in three cases. Table III lists the surface phenotypes. TILs mainly consisted of CD3-positive cells, ranging from 63.2 to 99.9% (mean $93.1\pm24.0\%$). The ratio of CD4-positive cells and CD8-positive cells was not constant. The expression of Leu 7 was detected on $25.3\pm39.0\%$ of TILs, but CD16 was expressed only on $2.6\pm12.2\%$ of TILs. HLA-DR was expressed highly, ranging from 38.5 to 99.0% (mean $81.6\pm34.8\%$). We also studied the difference in surface phenotypes between TILs and LAK cells (Table IV). No significant difference between TILs and CD3+LAK cells was noted, but there were obvious differences between TILs and CD3-LAK cells. In the case of CD3-LAK cells, CD16 and Leu 7 were highly expressed, while there was little expression of CD3. In three cases, surface phenotypes of TILs stimulated with rIL-2 alone were analyzed, but no differences were noted. These findings indicated that the TILs consisted mainly of T-cell lymphocytes.

Table II. Cytotoxic Activity of CD3+TILs

Patient	Days in culture	% Killing (E:T=20:1–40:1)[a]							
		K562	Daudi	Autoglioma[b]		Alloglioma[c]		Cold inhibition	
				4 h[d]	20 h[e]	4 h	20 h	auto+auto[f]	auto+U251[g]
1	35	0	0	0	—[h]	—	—	—	—
2	21	77.8	100	17.4	—	31.4	33.1	—	—
	28	56.3	100	0	—	9.9	—	—	—
	48	0	8.5	—	—	—	—	—	—
3	27	31.1	20.2	0.3	—	6.8	—	—	—
	40	2.6	6.7	5.0	—	—	—	—	—
	48	0.7	2.1	—	—	—	—	—	—
4	41	3.7	—	—	—	—	—	—	—
	55	3.0	0.4	0	—	—	—	—	—
5	13	8.8	13.0	7.4	14.3	—	—	—	—
	27	2.9	—	—	—	—	—	—	—
	43	10.2	12.6	—	—	3.8	—	—	—
6	15	1.5	3.5	18.9	—	—	—	—	—
	22	6.5	4.3	1.4	—	—	—	—	—
	34	2.8	8.8	—	—	—	—	—	—
7	15	23.8	9.1	—	—	0	6.9	—	—
	21	9.9	9.1	3.5	21.7	1.0	—	—	—
	35	38.0	0	1.7	0	0.9	—	11.7	73.4
	42	7.3	13.5	—	—	2.6	4.7	—	—
8	14	26.4	11.7	3.9	3.0	4.1	18.6	11.9	17.2
	21	15.9	32.3	—	60.8	8.3	37.3	61.8	43.6
	35	43.2	7.8	—	69.2	19.5	8.3	75.7	73.4
9	35	33.1	28.0	0	11.6	—	—	—	—
10	20	35.4	20.1	3.6	26.6	4.5	27.1	41.0	41.0
	28	13.4	27.3	0.6	36.4	5.5	31.2	51.1	42.1
	34	0	23.2	3.7	14.9	0	2.6	38.0	38.9

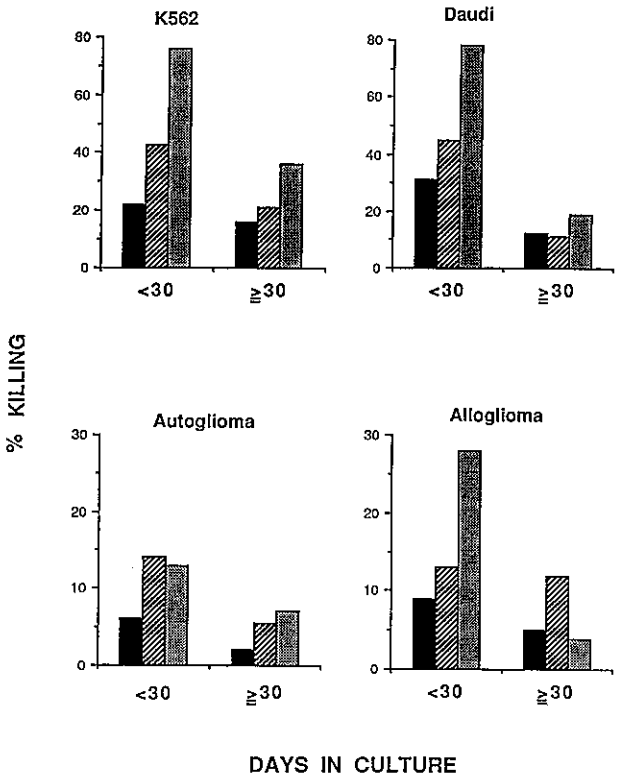
a) The effector: target ratio was 20:1 except in the case of cold target inhibition.
b) Short-term cultured autologous glioma cells were used.
c) Short-term cultured allogeneic glioma cells were used in cases 2, 3, and 5. U251MG cells were used in cases 7, 8, 9, and 10.
d) Target cells and effector cells were cocultured for 4 h.
e) Target cells and effector cells were cocultured for 20 h.
f) Labeled autologous glioma cells and unlabeled autologous glioma cells were used as target cells.
g) Labeled autologous glioma cells and unlabeled U251MG cells were used as target cells.
h) Not done.

DISCUSSION

Recent experiments have demonstrated[16] that the adoptive transfer of LAK cells plus IL-2 can mediate tumor regression in a variety of human tumors, including brain tumors.[2–7] In addition, Rosenberg *et al.* have reported[8] that the cytolytic activity of TILs stimulated with rIL-2 is greater than that of LAK cells.

There have been few reports of clinical trials of AIT with TILs, especially against brain tumors,[3, 9, 10] because it is not easy to obtain enough TILs. Availability of TILs is one of the major shortcomings of this therapy, but, as described above, we could obtain enough TILs by using an anti-CD3 antibody. An antibody against CD3 of T-cell receptor complex elicits the generation of killer lymphocytes[11–14] and we have reported[3, 15] that more than 10^8 CD3+LAK cells could be readily obtained from 10–20 ml of heparinized blood. The anti-CD3 antibody which we used is available for human use and we have been able to undertake AIT with CD3+LAK cells without major side effects. Therefore an anti-CD3 antibody can be safely used as a stimulator of lymphocytes.

A 4 h ^{51}Cr release assay is usually used to analyze cytolytic activity of lymphocytes. While this is a standard method, there are certain shortcomings: 1) incubating



labeled target cells with effector lymphocytes for 4 h is insufficient, 2) only cytotoxicity against floating cells or monolayer cells can be analyzed, and 3) it is difficult to show specific cytotoxicity against autologous tumor cells. We also analyzed cytolytic activity of TILs and LAK cells using this method. Sawamura *et al.*[17] and Belldegrun *et al.* [18] have reported that there is no difference in cytotoxicity between TILs and LAK cells, but our results indicate that the cytotoxicity of TILs is lower than that of LAK cells against all kinds of tumor cells. However, these results are different from the data which Rosenberg *et al.* obtained in mice.[8] Thus, a question arises as to whether these results really show that TILs are less suitable for AIT as compared to LAK cells.

Fig. 1. Cytolytic activity of TILs and LAK cells against K562, Daudi, autologous glioma cells, and allogeneic glioma cells *in vitro* versus time in culture. Cytotoxicity against all targets was higher in the first 30 days in culture, followed by a gradual decrease in the cytotoxicity of the cells. Activity of LAK cells, especially aCD3-LAK cells, was higher than that of TILs against each of the targets. TIL: Tumor-infiltrating lymphocytes (■). LAK+: LAK cells stimulated with rIL-2 and an anti-CD3 antibody (▨). LAK−: LAK cells stimulated with rIL-2 alone (▩).

Table III. Surface Phenotypes of TILs

Patient	Days in culture	aCD3[a]	% of positive cells						
			CD3	CD4	CD8	CD16	CD25	Leu 7	HLA-DR
1	40	+[b]	96.7	61.4	44.8	0.3	3.8	7.2	95.6
2	21	+	63.2	32.3	42.6	23.5	10.7	36.8	69.2
	48	+	97.7	61.9	43.6	0.4	3.9	1.5	87.3
		−[c]	99.4	26.4	69.4	1.9	58.9	19.4	88.8
3	27	+	94.6	23.3	80.4	0.2	2.9	22.0	97.6
4	41	+	99.9	2.5	81.2	0.4	1.3	ND[d]	98.9
5	14	+	99.0	72.5	41.5	0.8	69.1	8.5	99.0
	42	+	98.6	20.9	74.5	0.2	0.7	45.7	ND
		−	99.2	1.6	92.4	2.0	46.3	ND	84.5
6	15	+	98.6	83.4	49.4	3.6	89.0	12.6	38.5
7	15	+	98.2	57.3	41.7	1.3	3.6	42.9	94.3
		−	98.9	57.8	42.2	1.2	7.2	66.1	97.5
	35	+	67.0	43.2	66.9	1.0	0.6	50.3	81.2
		−	99.8	34.0	79.0	0.7	1.8	62.3	84.6
8	14	+	98.7	30.7	70.2	0.5	45.2	14.8	82.4
9	35	+	98.9	82.0	47.8	0.4	37.4	3.0	70.2
10	19	+	99.4	63.5	64.1	1.0	4.0	58.7	65.5

a) Anti-CD3 antibody.
b) TILs stimulated with rIL-2 and anti-CD3 antibody.
c) TILs stimulated with rIL-2 alone.
d) Not done.

Table IV. Differences in Surface Phenotypes between TILs and LAK Cells

Effector	% of positive cells (mean±SD)						
	CD3	CD4	CD8	CD16	CD25	Leu 7	HLA-DR
CD3+TIL[a)]	93.1±12.0	48.8±24.3	57.6±15.0	2.6±6.0	20.9±28.6	25.3±18.5	81.6±17.4
CD3+LAK[b)]	94.5±6.3	43.9±26.2	56.5±25.1	2.5±2.5	21.0±21.6	21.8±16.0	67.6±15.0
CD3−LAK[c)]	74.7±17.5	30.0±22.0	56.3±21.0	18.9±11.0	18.6±22.9	35.6±25.6	72.4±26.1

a) TILs stimulated with rIL-2 and anti-CD3 antibody.
b) LAK cells stimulated with rIL-2 and anti-CD3 antibody.
c) LAK cells stimulated with rIL-2 alone.

There is insufficient evidence to address this issue, but we think the results of the standard ^{51}Cr release assay do not necessarily reflect *in vivo* cytotoxicity because of the above shortcomings. Therefore we extended the duration of culture of labeled target cells with effector lymphocytes to 20 h, but even by this method, the superiority of TILs could not be shown *in vitro*.

Specific cold target inhibition has been used for analyzing specific cytotoxicity against autologous tumor cells. We have analyzed specific cytotoxicity using this method in three cases and could show specificity in one case. The optimum strategy is to change the E:T ratio and the ratio of labeled and unlabeled tumor cells and to repeat this assay. Because it was difficult to obtain enough autologous glioma cells from small specimens and also because, even when it was possible, we intended to utilize the TILs to treat the patients, we could not repeat this assay often. The most important purpose of analyzing cytotoxicity *in vitro* is to anticipate the effectiveness of TILs or LAK cells *in vivo*. However, even if TILs have specific cytotoxicity against autologous glioma cells, those lymphocytes are not necessarily effective at AIT. Therefore it is very difficult to know in advance in which cases AIT is likely to be effective.

Cell surface marker analysis of TILs has been reported by various groups.[15, 18–23] The characteristic pattern of our results was that the expression of CD3 was detected on 93.1% of TILs, and this is high compared with other reports. There was no difference between surface phenotypes of TILs and those of CD3+LAK cells. The high expression of CD3 is perhaps due to the use of the anti-CD3 antibody.

We repeated phenotyping during the culture period in three cases, and observed decreasing Leu 7 and CD16 expression. Belldegrun *et al.*[18] have shown increasing CD3- and CD4-positive cells with decreasing CD8- and HLA-DR-positive cells, but our results did not show consistent changes in the expression of CD3, CD4, CD8, CD25, and HLA-DR.

We have to resolve many problems in order to enhance the effectiveness of AIT. 1) Do TILs really recognize tumor-associated antigens? 2) What kind of lymphocytes is most effective, CD4-positive cells, CD8-positive cells or other kinds of cells? 3) Are TILs stimulated with anti-CD3 antibody really effective as effector cells of AIT, and are they really more effective than LAK cells? 4) Is there any relationship between cytotoxicity and expression of MHC on tumor cells? Some clinical trials of AIT with TILs have been reported and we also have undertaken AIT with TILs and LAK cells in some patients with malignant gliomas, but no-one has yet been able to obtain satisfactory results.[3, 9, 10] If we can resolve the problems, we should be able to obtain more effective cells, and AIT could become a useful therapeutic approach against malignant gliomas.

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