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An Experimental Approach to Specific Adoptive Immunotherapy for Malignant Brain Tumors

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Abstract

With the aid of interleukin 2 (IL-2), two phenotypically different cytotoxic T lymphocyte (CTL) clones were established with target specificity against syngeneic murine malignant brain tumor (a methylcholanthrene-induced ependymoblastoma of C57BL/6 mouse origin, 203-glioma). Furthermore, the cloned CTL lines were characterized in vitro, and their in vivo effectiveness was investigated by intracerebral (i.c.) tumor neutralization assay and adoptive immunotherapy with the clones for i.c. tumor-bearing mice. Each CTL clone retained an IL-2 dependency with a defined functional activity. G-CTLL 1 with a phenotype of Lyt-1$^-$.2.3$^+$ exhibited a target cytotoxicity against 2 kinds of murine glioma cells, syngeneic 203-glioma and allogeneic RSV-M glioma (Schmitt-Ruppin rous sarcoma virus-induced malignant astrocytoma). It is noted that G-CTLL 1 cells produced gamma interferon (IFN) by stimulation with glioma antigens. The spontaneous release of gamma IFN paralleled the amounts of exogeneous IL-2 added into the cultures, but IL-2 had no synergistic effects on IFN release in the presence of tumor antigens. Furthermore, by adding anti-mouse gamma IFN antibody, the IFN production of G-CTLL 1 cells was inhibited but their lytic potential was hardly reduced in vitro. In contrast, G-CTLL 2 cells expressed a cell surface phenotype of Lyt-1$^+$.2.3$^+$ with more restricted target specificity against only syngeneic 203-glioma cells, although they showed a weaker cytotoxicity than G-CTLL 1 cells and no release of gamma IFN. The in vivo therapeutic efficacy using G-CTLL 1 cells was confirmed in both adoptive immunotherapy and tumor neutralization assays. It was thus suggested that the killing mechanism may be associated with the direct lytic activity of CTL cloned cells or the tumor-specific production of gamma IFN by the clones.

Introduction

Recently advanced immunological techniques, particularly the use of interleukin 2 (IL-2), have permitted the controlled progressive growth and the long-term culture of immunized T

Key word: Brain tumor, Cytotoxic T lymphocyte, Gamma interferon, Immunotherapy, Interleukin 2, Malignant glioma.

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lymphocytes with defined immunological functions[1,3,8]. With the aid of IL-2, a large number of homogeneous populations such as cytotoxic T lymphocytes (CTL) have been provided by cloning technology[4,10]. On the other hand, it is well known that interferon (IFN) has a variety of immunobiological properties including antitumor activity[5,7,11].

The purpose of the present study was to determine the usefulness of adoptive immunotherapy with tumor-specific CTL clones for malignant brain tumors. Thus, three kinds of experiments were carried out in a system of mouse malignant glioma (methylcholanthrene-induced ependymoblastoma, 203-glioma): (1) The tumor-specific CTLs were further cultured for a long-term period in the presence of IL-2 in order to obtain cloned CTL lines. (2) The cloned cells established by IL-2 were investigated for their in vitro characterization, and (3) the in vivo efficacy was examined in mice inoculated intracerebrally (i.c.) with the glioma cells. Finally, the in vivo tumor killing mechanism of CTL clones was discussed.

Materials and Methods

Animals and Tumor Cells:

Male C57BL/6 mice, 6 to 8 weeks old, were used for all experiments. A methylcholanthrene-induced ependymoblastoma of C57BL/6 mouse origin, 203-glioma, was used as target cells. For examination of target specificity, effector cells were assessed for lytic activity against other 7 kinds of syngeneic and allogeneic tumor cell lines (Table 1).

In vivo Immunization and Lymphocyte Preparation:

The tumor cell suspensions of 203-glioma cells were prepared by trypsinization. Five$\times 10^5$ cells/ml were injected i.c. All of the mice died with a 3.2 weeks of median survival time[13]. For s.c. inoculation, the tumor cells were injected into the left flank. Immunized T lymphocytes from spleens and regional lymph nodes from i.c. and s.c. tumor-bearing mice, respectively, were prepared after passing through a nylon wool column[13].

Preparation of IL-2:

Partially purified IL-2 was used; briefly, C57BL/6 mouse spleen cells, or Wistar rat spleen or mesenteric lymph node cells (5×10^6/ml) were cultured with 2 μg/ml concanavalin A (Con A) for 18 hours (hr). The crude supernatants (Con A-sup) were decanted twice every 18 hr, centrifuged and resterilized by milipore filtration. For partial purification, the con A-sup was concentrated by ammonium sulphate precipitation, and subjected to gel filtration with Sephadex G-100 column and subsequent ion-exchange chromatography with diethylaminoethyl cellulose. For bioassay, IL-2 activity was determined by the capacity to support the exponential growth of a murine IL-2 dependent CTL clone (G-CTLL 1)[14]. The IL-2 titer in the test sample was calculated by a reciprocal titer of the test samples, which gave 50% of maximal counts of standard.

Antibody:

Anti-Thy-1 monoclonal antibody (mAb) (at 10 μg/ml) and Lyt-1 and Lyt-2 mAb (at 20 μg/ml) against T-cell differentiation antigens from Becton Dickinson, Sunnyvale, Calif were used[13]. Rabbit anti-asialo-GM1 antibodies (at a 1/100 dilution) against surface antigens of mouse NK

cells from Wako Pure Industries, LTD were used[13].

Assay for CTL:

The assay of T-cell mediated cytotoxicity was estimated in triplicate[13]. Briefly, after treatment with mitomycin C (50 μg/ml for 45 min), 3×10^4 target cells in 0.1 ml were incubated for 24 hr prior to the addition of effector cells. The mixtures at various effector to target (E/T) ratios were incubated in a final volume of 0.2 ml. After 18 hr of incubation, target cell suspensions were prepared and viable tumor cell numbers were calculated.

$$\text{CTL activity (\%)} = \left(1 - \frac{\text{surviving target cells after incubation with effector cells}}{\text{surviving target cells after incubation with medium alone}}\right) \times 100$$

Tumor-Specific CTL Clone:

Immunized regional lymph node T cells (10^6/0.5 ml) were cultured with antigen-presenting cells (5×10^4/0.5 ml) from spleens of s.c. tumor-bearing mice in the presence of IL-2. Cell clusters cytotoxic against 203-glioma cells were distributed at limiting dilution (0.3 cells/well).

Winn-Type Intracranial Tumor Neutralization Assay:

A mixture of cloned CTL and 5×10^5 203-glioma cells at E/T ratios of 2/1 and 10/1 was injected i.c. in a total volume of 10 μl of saline. The survival and mortality were recorded by daily observation of mice after i.c. injection.

Adoptive Immunotherapy:

On Day 7 after i.c. inoculation with 5×10^5 203-glioma cells, cloned CTL at various doses (1×10^6, 5×10^6, 1×10^7, 2×10^7, and 5×10^7 cells) were injected intravenously (i.v.) in a total volume of 1 ml of saline. In additional experiments, 1×10^7 cloned CTL were injected i.v. various days after i.c. inoculation with 5×10^5 203-glioma cells.

Results

Establishment of Cloned CTL Line by IL-2

Two tumor-specific CTL clones (G-CTLL 1 and 2) were established by IL-2, and it was found that these two clones retained in an IL-2 dependent exponential proliferation (Fig. 1) and continued to maintain the definite cytotoxicity for syngeneic 203-glioma cells (Table 1). G-CTLL 1 clone exhibited a lytic activity against 2 kinds of murine glioma cells, syngeneic 203-glioma and allogeneic RSV-M glioma. It was suggested that G-CTLL 1 cells mediated a mouse glioma-associated antigen-specific cytotoxicity. The cell surface phenotypes of G-CTLL 1 cells were Thy-1$^+$, Lyt-1$^-$.2.3$^+$, and asialo-GM1$^-$ (Table 2). The cytotoxicity was blocked by adding only Lyt-2 mAb, indicating that G-CTLL 1 cells originated from CTL[6,9]. It was of note that the gamma IFN production of G-CTLL 1 cells was enhanced by tumor antigen or mitogen stimulation (Table 3). Contrasted with G-CTLL 1 cells, G-CTLL 2 cells showed a more restricted target specificity but a weaker cytotoxicity against only syngeneic 203-glioma cells (Table 1). The cell surface phenotypes of G-CTLL 2 cells were Thy-1$^+$, Lyt-1$^+$.2.3$^+$ and asialo-GM1$^-$ (Table 2).

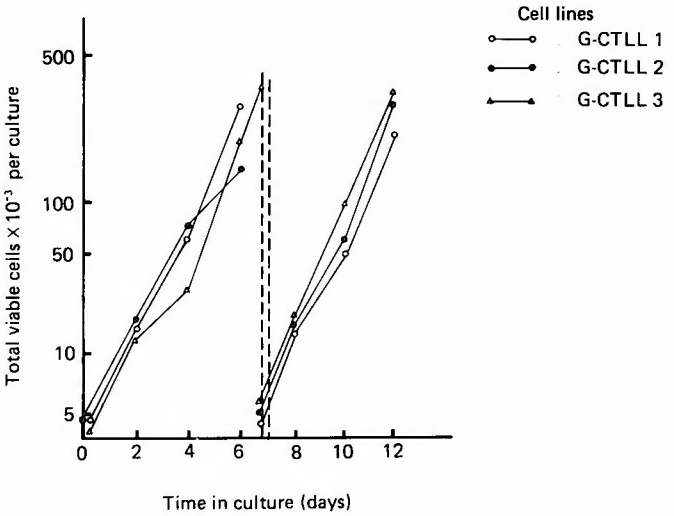

Figure 1. Growth curves of G-CTLL 1 and G-CTLL 2 clones.

When seeded at a concentration of 5×10^3 cells per ml, the CTL clones reached a saturation density of approximately 3×10^5 cells per ml after 6 to 7 days in culture, representing a doubling time of approximately 24 hours. The proliferative growth was totally dependent upon the presence of interleukin 2. G-CTLL 3 is another established clone without showing any target specificity.

In Vivo Anti-Tumor Activity of G-CTLL **1** *Cells*

(A). I.C Tumor Neutralization Assay: At E/T ratio of 10/1, all of 6 mice remained alive for over 15 weeks in a case of injection with cloned CTL. Even at the E/T ratio of 2/1, median survival time was prolonged to 5.6 weeks in a group with administration of CTL clones (Table 4).

(B). Adoptive Immunotherapy: It was observed that the therapeutic efficacy of G-CTLL 1 cells was dependent upon both dose and timing of i.v. infusion of the cloned cells (Table 5 and 6).

Table 1. Target specificity ef cloned CTL lines.

Tumor cell line	Mouse origin	Cytotoxic activity (% lysis)							
		Day 35		Day 180		Day 330		Day 400	
		G-CTLL 1	G-CTLL 2	G-CTLL 1	G-CTLL 2	G-CTLL 1	G-CTLL 2	G-CTLL 1	G-CTLL 2
203-glioma	C57BL/6	80.8**	62.4**	78.6**	60.2**	85.2**	68.0**	84.2**	66.4**
B-16 melanoma	C57BL/6	5.2	4.0	3.6	2.8	4.8	3.4	2.9	4.2
YM-12 fibrosarcoma	C57BL/6	4.6	5.4	4.4	ND	4.0	ND	3.3	3.6
EL 4 thymoma	C57BL/6	6.0	8.6	5.8	ND	3.8	ND	3.2	4.0
RSV-M glioma	C_3H/He	40.2*	3.8	31.8*	4.2	30.6*	4.0	34.0*	4.4
C-1300 neuroblastoma	A/J Ax	3.8	4.2	3.8	3.6	3.8	4.8	3.6	3.0
P 815 mastocytoma	DBA/2J	4.0	4.8	4.2	ND	4.0	ND	5.6	3.4
YAC-1 T-cell lymphoma	A/Sn	3.6	3.4	3.8	4.0	5.0	3.6	4.0	3.8

After starting continuous culture, both G-CTLL 1 and 2 cells were chronologically assessed for their CTL activity against a panel of 8 tumor cell lines. Effector : target cell ratio of 10 : 1. Values are significant (*$p<0.01$, **$p<0.005$).

ND=not done.

Table 2. Surface markers of G-CTLL 1 and G-CTLL 2 clones.

Treatment with antibody	CTL activity (%) G-CTLL 1	G-CTLL 2
Thy-1	7.5 ↓	6.9 ↓
Lyt-1	64.7	15.2 ↓
Lyt-2	7.0 ↓	14.9 ↓
Lyt-1+Lyt-2	3.8 ↓	2.8 ↓
asialo GM1	58.0	49.2
no treatment	67.9	46.4

Surface phenotypes of the two CTL clones were evaluated by a complement-mediated antibody-dependent cytotoxicity assay, using Thy-1, Lyt-1, Lyt-2 and asialo-GM1 antibodies (see Materials and Methods). Values of representative percentages are significantly eliminated compared to those of untreated groups by the selection method (↓ $p<0.01$).

It was suggested, however, that the effectiveness was inhibited by increased i.c. pressure due to tumor progressive growth (Table 6).

Discussion

To better understand the significant role of T-cell dependent immunosurveillance in the brain, the authors previously investigated the time course of syngeneic Ts and CTL activities

Table 3. Augmentign tumor-tpecific production of gamma interferon by G-CTLL 1 clone.

Tumor Cell Lines	IFN activity(U/ml) E/T=2/1	CTL activity (%) E/T=2/1	E/T=10/1
203-glioma (H-2^b)	140**	62.4**	80.8**
B-16 (H-2^b)	33	ND	4.6
YM-12 (H-2^b)	10	ND	3.6
EL 4 (H-2^b)	< 2	ND	5.8
RSV-M (H-2^a)	156**	18.8*	28.6*
C-1300 (H-2^k)	4	ND	3.3
P815 (H-2^d)	< 2	ND	2.2
YAC-1 (H-2^a)	32	ND	1.8

G-CTLL 1 cells were cultured with various kinds of tumor cells for 14 hours at the effector to target cell (E/T) ratio of 2 : 1 or 10 : 1. Both interferon (IFN) activity in the supernatants and cytotoxic T-lymphocyte (CTL) acyivity of the clone were assayed. The type of IFN was determined by elimination of IFN activity after treatment with either ph 2.0 or anti-gamma IFN antibody.
ND=not done.

Table 4. Winn-type neutralization assay.

G-CTLL 1 : Tumor Ratio (i.c. : 5×10^5)	Survival & Mortality		
	MST	TDR	D/T
10 : 1	15<	0	0/6
2 : 1	5.6*	100	5/5
0 : 1	3.2	100	12/12

A mixture of G-CTLL 1 with 5×10^5 203-glioma cells at various effector to target (E/T) ratios was injected intracerebrally (i.c.).

* Value is significantly lower than that of group injected without G-CTLL 1 cells ($p<0.05$).

MST=median survival time (week). TDR=tumor death rate at 8 weeks in tumor-bearing mice (%). D/T=number of mice died after tumor cell inoculation/total numcer of mice inoculated intracerebrally.

Table 5. Dose depedency of G-CTLL 1 cells in adoptive immunotherapeutic effectiveness.

Dose of Adoptive Transfer (i.v. on day 7 after i.c.)	Survival & Mortality		
	MST	TDR	D/T
G-CTLL 1 : 5×10^7	15<	0	1/14#
2×10^7	15<	0	0/10
1×10^7	6.1*	50	4/8
5×10^6	5.9*	50	5/10
1×10^6	3.8	100	8/8
Ts : 5×10^7	2.0**	100	5/5
Tc : 5×10^7	3.6	100	4/4
PBS only	3.3	100	4/4

G-CTLL 1 cells, immune T cells (Tc or Ts), or only PBS (1 ml) were injecyed intravenously (i.v.) on Day 7 after intracerebrally (i.c.) inoculation of 5×10^5 cells of 203-glioma. In the transfer of more than 5×10^6 G-CTLL 1 cells, a significant effectiveness was observed compared to that injected with PBS alone (*$p<0.05$).

All mice except for one (this mouse died at 10 weeks) survived over 15 weeks.

** Value is significantly lower than that injected with PBS alone ($p<0.05$).

Tc=regional lymph node T lymphocytes on Day 14. Ts=splenic T lymphocytes on Day 3. MST=median survival time (week). TDR=tumor death rate at 8 weeks in tumor-bearing mice (%). D/T=number of mice died after tumor cell inoculation/total number of mice inoculated intracerebrally. PBS=phosphate buffered saline.

Table 6. Time dependency of G-CTLL 1 cells in adoptive immunotherapeutic effectiveness.

i.v. of G-CTLL 1 (i.c. : 5×10^5)		Survival & Mortality					
		day 0	day 3	day 5	day 7	day 10	day 16
1×10^7	MST	10<	6.6*	6.0*	6.8*	2.6**	2.4**
	TDR	0	40	60	40	100	100
	D/T	0/5	2/5	3/5	2/5	5/5	5/5
PBS	MST	ND	ND	ND	3.3	ND	2.3**
	TDR	ND	ND	ND	100	ND	100
	D/T	ND	ND	ND	4/4	ND	3/3

One $\times10^7$ G-CTLL 1 cells were injected i.v. various days after intracerebral (i.c.) inoculation of 5×10^5 203-glioma cells. Values are significantly higher and lower than those of mice injected with PBS alone, respectively (MST=3.3, TDR=100 and D/T=4/4 in Table 7) (*$p<0.05$, **$p<0.05$).

MST=median turviva. time (week). TDR=tumor death rate at 8 weeks in tumor-bearing mice (%). D/T=number of mice died after tumor cell inoculation/total number of mice inoculated intracerebrally PBS=phosphate buffered saline. ND=not done.

after tumor cell inoculation[12]. It was found that the induction of Ts activity preceeded that of the CTL activity at the initial stage of tumor-bearing hosts, exhibiting a faster turnover of life. It has been postulated that in the brain, which is considered as incompletely immunologically privileged organ, vascular changes permit tumor antigens to leave the brain as the tumor grows[2,13]; in response to the new antigenic stimulus, lymphocytes proliferate and, most importantly, Ts are generated predominantly. However, the reason and mechanism for the Ts generation have remained unclarified.

Furthermore, it was suggested from another independent previous experiments that the tumor-specific CTL were activated with target specificity by both IFN and IL-2 in a different way[15]. It was observed that the CTL activation by IL-2 remained for a longer time, although a longer lag time (more than 5 days) after initial culture was required. Furthermore, it was found that IL-2 influenced Lyt-1$^+$.2.3$^+$ CTL to proliferate and develop the lytic potential. In contrast, even a 3-hour incubation with IFN could enhance the cytotoxicity, but the augmenting effects were observed no longer 5 days later[15]. Additionally, it was found that IFN activated Lyt-1$^-$.2.3$^+$ CTL and increased their proportion of the total cell population with a simultaneous decrease of Lyt-1$^+$.2.3$^+$ CTL. Therefore, it seems likely that IL-2 may provide a growth of CTL populations and that IFN can accelerate recruitment of new effectors, causing activation of the lytic process.

Accounting for these above previous results, we have found out the best culture conditions for establishment of glioma-specific CTL clones. By using immune T-cells on day 14, showing a peak lytic potential after s.c. 203-glioma cell inoculation[13], we successfully obtained two phenotypically different kinds of CTL clones with the aid of IL-2. It should be stressed that G-CTLL 1 cells produced a large amount of gamma IFN by stimulation with tumor-specific antigen. Since gamma IFN plays a pivotal role in antitumor effectiveness[5,7,11], the in vivo effector mechanism may involve gamma IFN release by the cloned CTL. Further examinations on adoptive immunotherapy with CTL clones will be worth undertaking before testing alternative antitumor mechanisms in the experimental approach to human malignant brain tumors.

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和文抄録

悪性脳腫瘍に対する特異的養子免疫療法の実駅的研究

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インターロイキン2 (IL2) を用い，実験的悪性グリオーマ株 (C57BL/6 マウス由来のメチルコラントレン誘発上衣芽腫，203-glioma) に対する特異的キラーT細胞クロン株 (G-CTLL 1及び G-CTLL 2) を樹立したので，その in vitro characterization 並びに in vivo における抗腫瘍効果を脳腫瘍モデルを用いた Winn's neutralization assay と養子免疫療法により検討した．両クロン株ともに 203-glioma に対し高いキラー活性を有し，IL2 依存性の増殖を保持した．G-CTLL 1の表面抗原は Lyt-1(−)，Lyt-2(+)，asialo-GM1 (−) であり，2種類のマウスグリオーマ株 (同系203-glioma 及び Schmitt-ruppin rous sarcoma virus誘発異系悪性グリオーマ株，RSV-M glioma) に対しキラー活性を示した．更に，G-CTLL 1はグリオーマ抗原あるいはマイトゲン刺激によりガンマ型インターフェロン (IFN) を産生する事が判明した．G-CTLL 1の spontaneous IFN production は IL2 濃度依存性に増量したが，腫瘍抗原刺激下では IL2 の IFN 産生に対する相乗効果はみられなかった．抗ガンマ型 INF 抗体により G-CTLL 1の IFN 産生は抑制されたが，203-glioma に対するキラー活性は低下しなかった．一方，G-CTLL 2 の表面抗原は Lyt-1 (+)，Lyt-2(+)，Thy-1(+)，asialo-GMI(−) であり，G-TLL 1と比較して G-CTLL 2 は IFN 産生能を有さず，203-glioma に対するキラー活性は低いものの腫瘍特異性は高く 203-glioma のみに細胞障害性を示した．in vivo における抗腫瘍効果はよりキラー活性を示した G-CTLL 1 で検索した．203-glioma の脳腫瘍モデルにおいて，Winn's neutralization assay のみならず養子免疫療法でその抗腫瘍性が発揮されている事が確認された．この抗腫瘍効果には G-CTLL 1 の高いキラー活性あるいは G-CTLL 1 が産生したガンマ型 IFN の宿主免疫促進効果に起因することが示唆された．