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# Immunological Response in the Mouse Brain: II. Experimental Immunotherapy Model Against Transplanted Mouse Lymphoma in the Brain

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## Abstrct

An experimental model of immunotherapy for intracranially transplanted mouse lymphoma is described. In Winn-type neutralization assays in the brain, slight efficiency of immune lymphocytes was observed, on the other hand, the nude mice with intracranially transplanted mouse lymphoma were successfully treated by a single intravenous transfer of immune lymphocytes. This effect was totally eliminated by the treatment of lymphocytes with anti Thy-1 antibody and complement before transfer, whereas the passage through a nylon-wool column had no effect on tumor rejection. By the treatment with anti Lyt antibodies and complement, Lyt-$2^+$ cytotoxic T lymphocytes appeared to be responsible for the effector T-cell subpopulations in a systemic transfer study. The effectiveness of tumor-specific cytotoxic T-cell lines, which were established and maintained without added IL-2 and mainly consisted of Lyt-$2^+$ T-cells, was demonstrated not only in the Winn neutralization test in the brain, but also in the adoptive transfer study. On the other hand, the noncytotoxic cell line that entirely consisted of Lyt-$1^+$ T-cells had no such effect.

In conclusion, the rejection of mouse lymphoma in athymic nude mice was considered to be mediated by Lyt-$2^+$ cytotoxic T lymphocytes.

## Introduction

In several syngeneic animal tumor models, the adoptive transfer of tumor sensitized lymphocytes can cause total regression of established tumors and permanently cure the animals of disease[2,45]. The immunity to allografts or syngeneic tumor cells has been shown to be mediated mainly by T cells, referred to as cytotoxic T lymphocyte (CTL)*[6]. Passive transfer of immunity

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索引語：脳腫瘍，実験腫瘍，免疫，脳内，免疫療法

* The abbreviations used are: CM, complete medium; Con A, concanavalin A; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; IC, intracranial; ID, intradermal; IL-2, interleukin 2; MLTC, mixed lymphocyte-tumor cell culture; TCGF, T-cell growth factor.

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for the treatment of cancer in animals and man has been attempted[45]. Functional subclasses of T lymphocytes on Lyt antigens have been extensively studied and CTLs have been found to express the Lyt-$2^+$ antigen[4,5,37]. CTL had been considered to be the main effector cell population in both allograft and syngeneic tumor rejection, because of its capacity to kill tumor cells directly in vitro, and the generation of these cells during rejection[6]. Therefore, the activation and expansion of the CTL population has been attempted[15,27] and numerous CTL clones have been successfully established using interleukin 2(IL-2)[11,12,16,22,40,50,52]. However, contrary to our expectations, there have been few reports indicatives that IL-2-dependent CTL clones are effective in protecting against the allo- or syngeneic tumor growth in in vivo systemic transfer studies[11,52]. On the other hand, recent observations have demonstrated that allografts[34,35], or syngeneic tumors[19,21,23] are rejected by systemic transfer of Lyt-$1^+$ cells, but not by Lyt-$2^+$ CTL populations. Therefore, the determination of an effector T lymphocyte subpopulation is essential to promote adoptive immunotherapy against malignancies in humans.

Although several experimental models have been reported dealing with adoptive immunotherapy to intraperitoneally[7,8,17,18,23] or subcutaneously[16,19,21,29,39,41,46] transplanted tumors, few studies have dealt with the intracranial tumor model[43,44,48,52] probably because of the immunological peculiality of the central nervous system (CNS)[1].

Recently, the authors demonstrated a slightly low but detectable immunological response in the brain using transplanted mouse lymphoma in the comparative experiments with extracranial immunological responses[30]. In the present study experimental immunotherapy to intracranially transplanted mouse lymphoma is described, and the importance of CTL as the effector cells in tumor rejection is also demonstrated.

## Materials and Methods

**Mice:** Breeding pairs of nude mice and their heterozygous littermates with BALB/c and C57BL/6 backgrounds respectively, were originally purchased from Clea Japan Inc., Tokyo, Japan and nude or their heterozygous (BALB/c$\times$C57BL/6)$F_1$ mice, denoted CB6$F_1$ or CB6$F_1$-nu/nu, were raised and maintained under specific pathogen free conditions in the Facilities of Experimental Animals, Faculty of Medicine, Kyoto University. Male and female mice were used between 6 and 12 weeks of age.

**Tumors:** RL♂1, together with RL♂6 and RL♀9 (kindly provided by Dr. Nakayama, The Center of Adult Disease, Osaka, Japan), is a radiation-induced leukemic cell line of BALB/c origin which has been maintained in BALB/c mouse in its ascitic form. Other tumors used in the present study were P815 (methylcholanthrene-induced mastocytoma of DBA/2 origin), and EL-4 (dimethylbenzanthrecene-induced leukemia of C57BL/6 origin). P815 and EL-4 cells were maintained in the corresponding syngeneic strain of mice in their ascitic forms.

**In Vivo Immunization:** The procedures with RL♂1 have been previously described[33]. In brief, 6 to 8-wk-old male and female CB6$F_1$-nu/+ mice received intradermal (ID) injections on their backs with $5\times10^5$ RL♂1 cells in a volume of 0.1 ml Hanks' balanced salt solution

(HBSS). The tumor grew to 5–10 mm in diameter in about 2 weeks and then regressed spontaneously in the following 4 weeks. This is in accordance with the previous observation[38]. After complete regression of the tumor, the mice were boosted three times with increasing numbers of viable RL♂1 cells up to $5\times10^7$ cells at an interval of two weeks. Three weeks after the last immunization, spleen cells were used as donors of responding cells in mixed lymphocyte-tumor cell culture (MLTC), or as effector immune spleen cells for the study either of Winn-type neutralization assay or for adoptive transfer studies.

**Intracranial Transplantation of Tumors:** RL♂1 cells ($10^4$) suspended in a volume of 0.01 ml of HBSS were injected under sterile conditions through the right frontal cranial bone to a depth of 2 mm with a 0.05 ml glass microsyringe (Hamilton Co., Reno, NA) and a 27-gauge Yaoi needle[28].

**Preparation of Effector Cells:** Spleens of $CB6F_1$-nu/+mice that had been immunized against RL♂1 in vivo were aseptically removed, pooled and crushed gently with a loose-fitting glass homogenizer in HBSS. After removing large cell clumps and debris with cotton wool, the spleen cells obtained were washed three times and resuspended in HBSS.

Details for generation and maintenance of cytotoxic and noncytotoxic cell lines, CTLL-D4 and D4f, have been previously described[33]. Briefly, MLTC was performed between the RL♂1-immune $CB6F_1$-nu/+ spleen cells as responders and 10,000 R-irradiated RL♂1 cells as stimulators in 2 ml of complete medium (CM) in 24-well multidish culture plates (Corning Glass Works, Science Products Division, Corning, NY). CM used in this experiment was composed of RPMI-1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% FCS (Flow Laboratories, Inc., Rockvile, MD), $5\times10^{-5}$ M 2-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cultures were incubated for 5 days at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After incubation, the cells were harvested, washed and then resuspended in RPMI-1640 supplemented with 10% FCS and assayed for cytotoxic activity by chromium release assay[37]. An aliquot of MLTC cells was used further for the secondary in vitro micro-MLTC on the same day and this was prepared in CM to which 5% (vol/vol) IL-2 was added. Thus, each culture contained $5\times10^2$ MLTC cells as responders, $5\times10^5$ irradiated (2,000 R) $CB6F_1$-nu/+ spleen cells as feeders and $5\times10^4$ irradiated (10,000 R) RL♂1 cells as stimulators in a final volume of 0.2 ml per well of a flat-bottomed 96-well microculture plate (Corning Glass Works). Cytotoxic cell lines against RL♂1 derived from the secondary micro-MLTC were selected on day 14 after the initiation of culture, and transferred to and maintained in 2 ml cultures that contained $5\times10^6$ feeder cells and $5\times10^4$–$1\times10^5$ stimulator cells in CM. After transferring the proliferating cells from microculture, no IL-2 was added to the culture. After the initial expansion, CTL lines were passed after 7 to 14 days by transferring $1\times10^5$ cells to the well of a 24-well culture plate together with both feeder and stimulator cells. Among the CTL lines able to proliferate in culture, CTLL-D4 was chosen for its high proliferative response and specific cytotoxic activity against RL♂1. A noncytotoxic cell line, D4f, was obtained by culturing CTLL-D4 only with the splenic feeder cells. D4f cells thus prepared completely lost its cytotoxic activity against RL♂1. Cytotoxic activity of CTLL-D4 and D4f

cells against RL♂1 measured by a 5-hr chromium release assay was about 50–60% and 0%, respectively, at the effector to target cell ratio of 1 : 1. CTLL-D4 cells consisted of mixed populations with regard to Lyt phenotype expression; 15–25% Lyt-$1^{+}23^{-}$, 10–15% Lyt-$1^{-}23^{+}$ and 60–75% Lyt-$1^{+}23^{+}$ cells. D4f cells consisted entirely of Lyt-$1^{+}23^{-}$ cells.

**Treatment of Lymphocytes:** An anti Thy-1.2 monoclonal antibody (HO-13.4), originally supplied from the Cell Distribution Center, Salk Institute of Health, San Diego, CA, was used for depletion of Thy-$1^{+}$ cells at a 1/100 dilution. Anti Lyt-1.2 and anti Lyt-2.2 monoclonal antibodies supplied from Meiji Institute of Health Science, Tokyo, Japan, were used at a 1/50 dilution. Immune spleen cells were prepared as described above and adjusted to a concentration of $2 \times 10^7$ cells/ml in Medium-199 (GIBCO) containing the desired concentration of the antibody. Cells were incubated for 60 min at 4°C with intermittent agitation, and they were then centrifuged and resuspended with preselected nontoxic rabbit serum as complement (C) source for 30 min at 37°C with occasional shaking. After treatment, the cells were washed three times and resuspended at a desired concentration in HBSS.

**IL-2:** SD rat spleen cells ($2.5 \times 10^6$) were incubated for 24 hrs at 37°C in 5% $CO_2$ in the presence of 5 $\mu$g/ml Concanavalin A (Con A). The resulting culture supernatants were harvested, centrifuged and passed through 0.45 $\mu$m filters (Millipore Corp., Bedford, MA). One lot of the rat Con A supernatants was selected and used for TCGF.

**Winn-type Neutralization Assay:** The Winn-type neutralization assay[51)] was carried out using $CB6F_1$-nu/nu mice as recipients. The effector cells, prepared as described above and adjusted to the desired concentration, were mixed with an equal volume of tumor cells ($2 \times 10^6$/ml). The mixture was injected intracranially in a volume of 0.01 ml as described above. The mice wihch survived for more than 60 days after inoculation were considered as cured. The control mice, intracranially injected with 0.01 ml HBSS or effector cells only, also survived for more than 60 days after injection.

**In Vivo Assays of Adoptive Immunotherapy:** $CB6F_1$-nu/nu mice were also used as the recipients in this assay. Various numbers of effector cells in 0.5 ml of sterile HBSS were intravenously transferred into the tail vein of $CB6F_1$-nu/nu mice, which had been inoculated with $1 \times 10^4$ tumor cells intracranially 3 days earlier. The mice which survived for more than 60 days after tumor inoculation were considered cured of tumor. The control mice, intravenously injected with 0.5 ml HBSS or effector cells only, also survived for more than 60 days after injection.

## Results

**IC Neutralization Assay:** Injection of a mixture of either $5 \times 10^4$ (E/T=5 : 1) or $2 \times 10^5$ (E/T=20 : 1) normal spleen cells with $1 \times 10^4$ RL♂1 cells into the IC site of $CB6F_1$-nu/nu mice resulted in no effect on survival time compared with the injection of tumor cells alone (Fig. 1). Mixed-inoculation of immune spleen cells and tumor cells had a significant effect on survival time (E/T=5 : 1 $p<0.05$, 20 : 1 $p<0.02$), however, no mouse was cured permanently. In this sense, the neutralizing effect of immune spleen cells is limited. As shown in Fig. 2, the cytotoxic

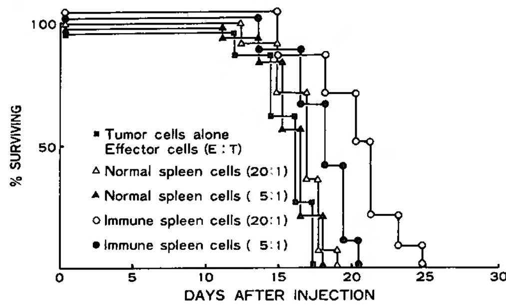


**Fig. 1.** Intracranial neutralization assay using spleen cells. $CB6F_1$-nu/nu mice were injected with either $1\times10^4$ RL♂1 cells (■), or admixtures of RL♂1 and effector cells in a volume of 0.01 ml, respectively. Effector cells are: normal $CB6F_1$-nu/+ spleen cells E/T=20 : 1 (△); 5 : 1 (▲); RL♂1-immune $CB6F_1$-nu/+spleen cells E/T=20 : 1 (○); 5 : 1 (●). Each group consisted of eight to ten mice.

T-cell line, CTLL-D4, had a neutralizing effect. The mice that received an admixture of CTLL-D4 and RL♂1 cells, at an E/T ratio of 20 : 1, were cured except for one mouse. This effect of CTLL-D4 is reduced in proportion to the decrease in the E/T ratio. On the other hand, the noncytotoxic cell line, D4f, had no effect on survival time in the Winn-type neutralization assay. The cytotoxic activities of immune and normal spleen cells used in this study were 30–40% and 10–20%, respectively, at an E/T ratio of 20 : 1 in the 5-hr $^{51}$Cr-release assay, whereas the cytotoxic activity of CTLL-D4 cells was approximately 50–60% at an E/T ratio of 2 : 1. In this experiment, the cytotoxic activity in vitro correlates well with an in vivo effect on survival. However, the neutralizing effect of CTLL-D4 cells is not complete enough to cure the mice permanently, whereas the admixture of CTLL-D4 and RL♂1 injected intradermally could cure the mice permanently[29]. Specific effects on the neutralization of the tumor were also studied (Table 1). Various tumor cells, RL♂6, RL♀9 and EL-4, were inoculated together with CTLL-D4 cells at an E/T ratio of 20 : 1. The effect on survival was only seen when CTLL-D4 cells were inoculated together with RL♂1 cells. The present results are in agreement with the specific

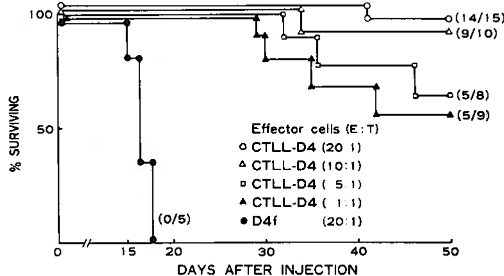


**Fig. 2.** Intracranial neutralization assay using cultured cell lines. $CB6F_1$-nu/nu mice were injected with admixture of RL♂1 and effector cells at various E/T ratios. Effector cells are CTLL-D4, E/T=20 : 1 (○), 10 : 1 (△), 5 : 1 (□), 1 : 1 (▲), and D4f, E/T=20 : 1 (●). Each group consisted of five to fifteen mice.

**Table 1.** Specific effect of CTL line on winn-type neutralization assay.

| Tumor[a] | MST[b] ± SD | Number of mice rejected/tested |
|---|---|---|
| RL♂1 | >60 | 7/7 |
| RL♂6 | 14.3±3.1 | 0/8 |
| RL♂9 | 15.2±2.3 | 0/7 |
| EL-4 | 13.2±2.9 | 0/6 |

[a] CTLL-D4 cells ($2\times10^5$) were admixed with various tumor cells ($1\times10^4$), and then injected intracranially with a volume of 0.01 ml.
[b] Median survival time in days.

cytotoxic effect of CTLL-D4 cells in vitro[33] and that found in an in vivo subcutaneous neutralization assay[29].

**Adoptive Transfer of Effector Cells:** In order to investigate the effector mechanisms in IC tumor rejection, various numbers of effector cells were adoptively transferred into $CB6F_1$-nu/nu mice which had been inoculated with $1\times10^4$ tumor cells intracranially 3 days earlier.

To examine the dose of spleen cells that cured the mice (Table 2), the mice transplanted intracranially with $1\times10^4$ RL♂1 cells received different numbers of spleen cells. All the mice that had recieved more than $1\times10^8$ immune spleen cells were cured, whereas the mice that had received either normal spleen cells or less than $1\times10^8$ immune spleen cells were not cured completely. The mice that had received more than $1\times10^8$ immune spleen cells were resistant to an additional IC challenge of RL♂1 cells, and were cured permanently (data not shown).

The specific effect of immune spleen cells on IC tumor rejection after intravenous transfer was also investigated (Table 3). The mice, which were transplanted intracranially with the tumors, RL♂1, RL♂6, RL♀9 or EL-4, were treated with a single intravenous injection of $1\times10^8$ RL♂1-immune spleen cells. The results showed that only the mice inoculated with RL♂1

**Table 2.** Dose response of spleen cells necessary to reject IC injected tumors in nude mice after IV transfer.

| Number of cells transferred[a] | MST[b] ± SD | Number of mice rejected/tested |
|---|---|---|
| No transfer | 15.3±1.2 | 0/10 |
| (Immune spleen) | | |
| $0.2\times10^8$ | 17.6±2.4 | 0/9 |
| $0.5\times10^8$ | 19.8±3.5 | 7/11 |
| $1.0\times10^8$ | >60 | 12/12 |
| $1.5\times10^8$ | >60 | 10/10 |
| (Normal spleen) | | |
| $0.5\times10^8$ | 15.8±2.4 | 0/8 |
| $1.0\times10^8$ | 16.1±2.1 | 0/9 |

[a] Various doses of $CB6F_1$-nu/+ spleen cells immunized against RL♂1 in vivo were intravenously transferred into $CB6F_1$-nu/nu mice bearing 3-day RL♂1 ($1\times10^4$) tumors.
[b] Median survival time in day.

**Table 3.** Specific effect of immune spleen cells on tumor rejection after IV transfer in nude mice[a].

| Tumor[b] | MST[c]±SD | Number of mice rejected/tested |
|---|---|---|
| RL♂1 | >60 | 8/8 |
| RL♂6 | 13.4±1.3 | 0/8 |
| RL♀9 | 14.8±1.7 | 0/9 |
| EL-4 | 12.6±2.1 | 0/8 |

[a] Spleen cells of $CB6F_1$-nu/+mice immunized against RL♂1 were used for effector cells at a dose of $1\times10^8$.
[b] Various kinds of tumor cells ($1\times10^4$) were intracranially injected in $CB6F_1$-nu/nu mice 3 days before IV transfer of immune spleen cells.
[c] Median survival time in days.

were cured. The adoptive transfer of RL♂1-immune spleen cells had no effect on the growth of other tumor cells.

**Analysis of Effector Populations in Immune Spleen Cells:** In order to clarify the effector population necessary to reject the tumor in an adoptive transfer study, immune spleen cells were treated before transfer into $CB6F_1$-nu/nu mice (Table 4). The complement dependent cytotoxity test was carried out to eliminate desired effector subsets. The effect of immune spleen cells to cure the mice were totally eliminated by the treatment with anti Thy-1.2 Ab and C. The results indicated that Thy-1.2 positive cells, grossly identical with T cells, were necessary to reject the tumors. In the reciprocal experiment, the immune spleen cells were passed through a nylon-wool column to enrich the non-B cells and non-adherent cells. This treatment had no effect on the capacity of immune spleen cells to cure the mice after adoptive transfer. The mice that received the spleen cells treated with anti Lyt-2.2 Ab and C were not cured, whereas two out of six mice that received spleen cells treated with anti Lyt-1.2 Ab and C were cured. Therefore, we can conclude that the Thy-1.2 positive T cells were necessary, and that especially Lyt-2 positive cells play a major role in curing the mice with intracranially transplanted lymphoma.

The T-cell mediated tumor rejection was further examined in vivo. Either the cytotoxic

**Table 4.** Effect of treatment of immune spleen cells on tumor rejection.

| Treatment[a] | MST[b]±SD | Number of mice rejected/tested |
|---|---|---|
| none | >60 | 12/12 |
| anti Thy-1.2+C | 15.8±1.5 | 0/10 |
| anti Lyt-1.2+C | 24.6±4.8 | 2/6 |
| anti Lyt-2.2+C | 16.1±2.1 | 0/6 |
| NWP[c] | >60 | 8/8 |

[a] Treatment of immune spleen cells was performed before IV transfer ($1\times10^8$) in nude mice bearing 3-day tumors.
[b] Median survival time in days.
[c] Nylon wool passage ($5\times10^7$).

**Table 5.** Effect of cell lines on tumor rejection after IV transfer.

| Effector cells[a] | Cell number | MST[b]±SD | Number of mice rejected/tested |
|---|---|---|---|
| No cell transfer | | 15.3±1.2 | 0/10 |
| D4f (noncytotoxic) | $5\times10^7$ | 14.3±2.5 | 0/8 |
| | $1\times10^7$ | 15.1±3.1 | 0/7 |
| CTLL-D4 (cytotoxic) | $2\times10^7$ | 24.4±5.6 | 2/7 |
| | $1\times10^7$ | 19.5±4.7 | 0/6 |

[a] Effector cells were intravenously transferred into CB6$F_1$-nude mice bearing 3-day RL♂1 tumors.
[b] Median survival time in days.

cell line CTLL-D4 or noncytotoxic cell line D4f were transferred into the CB6$F_1$-nu/nu mice that had been inoculated with RL♂1 intracranially 3 days earlier (Table 5). The CTLL-D4 cells mainly consisted of a Lyt-$2^+$ cytotoxic population, whereas the D4f cells consisted entirely of Lyt-$1^+2^-$ cells. Some mice were cured by the transfer of CTLL-D4 cells, but none were cured by transfer of nocytotoxic cells, D4f. These results indicate that Lyt-$2^+$ cytotoxic T-cells play a major role in this type of tumor rejection.

## Discussion

In the present study, two types of successful immunotherapy models, both local tumor neutralization and systemic transfer of effector cells, were demonstrated in IC tumor transplanted mice, and Lyt-$2^+$ CTL populations were strongly suggested to play a major role in this type of tumor rejection.

Numerous reports indicate that it is possible to obtain regression of established immunogenic murine or rats tumors by the passive transfer of T cells from tumor-immune donors[2,6]. In most of these studies, however, immunotherapy had to be accompanied by chemotherapy[7,23,39] or by irradiation of the host[19] in order to be successful, and the tumors used were invariably immunogenic to the host[42,43,44]. These tumor systems, therefore, are only partially representative of the situation occurring in humans, since human tumors are only weakly or not immunogenic[1,3].

The advancement in immunology has enabled us to use monoclonal antibodies[32] and IL-2[22] to investigate the details of an immune system, and to promote the clinical application of immunotherapy to malignancies[3,27]. Since the discovery of IL-2, the elucidation of the in vivo function of CTL and the establishment of an efficient immunotherapeutic method were expected to follow because many CTL clones with different functions could be easily established and maintained[11,12,40,50,52]. However, few studies have shown such IL-2-dependent clones to be effective on protecting against the allo- or syngeneic tumor growth in in vivo systemic transfer assays[11,52]. Several explanation for such in vivo inefficiency of exogenous IL-2-dependent CTL clones or lines are possible: 1) CTL is not actually an effector subset[19,21,34,35], 2) the life span of such CTL clones are altered in vivo due to the extremely low level of TCGF[13], or the presence of an inhibitor to IL-2[26], 3) the loss of normal migration pattern caused by long-term

culture in IL-2[10], and 4) loss of specificity in the CTL clone[49]. CTLL-D4 cells belong to an oligoclonal cell line, and consisted of a mixed population of Lyt-$1^+23^-$, Lyt-$1^-23^+$ and Lyt-$1^+23^+$, producing IL-2 in the culture supernatant stimulated with irradiated tumor cells in the presence of splenic feeder cells[33]. Although long-term culture of bulk MLTC cells without addition of TCGF has been considered to be impossible, we obtained CTLL-D4 cells by limiting dilution methods under 500 cells/well condition. This oligoclonal CTL line had in vivo efficiency because the addition of exogenous IL-2 is not necessary, and cell-cell interaction between helper T cells and cytotoxic T cells played a major role in in vivo tumor rejection[29].

Recently, the importance of administration of IL-2 together with the CTL clone to enhance and maintain the cytotoxic activity has been stressed[9,14,41]. However, experimental studies on immunotherapy have been carried out using subcutaneously or intraperitoneally transplanted tumor models, probably because of the peculiar immunological response in the central nervous systems (CNS)[1]. The brain has been thought to be an "immunologically privileged site" because of its incapability of inducing allograft or even xenograft reactions[36]. Recent observations, however, suggest that an immunological response can be built to intracranial tumors[20,31,42,47]. We have already demonstrated the occurrence of immunological responses in the mouse brain, and concluded that the resistance in the brain against transplanted lymphomas is not essentially different from that in the periphery[30].

CTL generated in vitro has been shown to be effective against murine tumors in a neutralization assay in the brain. Romani et al.[43] reported that the intracerebral growth of a highly immunogenic murine lymphoma in an immunologically depressed host can be impaired by means of local adoptive immunotherapy. Systemic adoptive immunotherapy showed that CTL obtained from a secondary in vitro culture is as effective as that generated in a primary in vitro sensitization culture, whereas cultured normal lymphocytes or freshly harvested spleen cells have no therapeutic activity, the crucial points being the number of CTL injected and time of CTL admin istration[44].

Shibuya et al.[48] reported on the systemic adoptive transfer of immunity by using gliosarcoma T9 cells in rat. Rejection of IC T9 challenge was obtained after transfer, in recipients of "hyperimmune" spleen cells, but was less (60% maximum) than that noted after ID challenge (100%). Their explanations for this difference were that: 1) IC tumor growth is more rapid than growth in ID site and is less rapidly inhibited, 2) immune cells, such as CTL, cannot reach the IC site in sufficient numbers as easily as they reach ID growing tumors, and 3) the brain is relatively deficient of antigen presenting cells.

Yamasaki et al.[52] succeeded in adoptive immunotherapy using tumor specific CTL clone for mouse malignant gliomas, and stressed the importance of dependency on dose and time of intravenous administration of CTL clone. They speculated that the increased intracranial pressure due to the tumor growth could be a cause of the incomplete efficiency of adoptive immunotherapy. It is conceivable that the mice may not be cured because of the increased intracranial pressure due to the rapid growth of the tumor. Nevertheless an immune reaction strong enough to regress the tumor can be produced in the CNS.

In our model using a radiation-induced leukemia RL♂1, the intracranially-transplanted tumors killed the mice even when the mice were injected with only one thousand tumor cells (data not shown). In the Winn-type neutralization assay, as shown in Fig. 1 and Fig. 2, a few mice were not cured by the admixture of RL♂1 cells with highly cytotoxic cells, CTLL-D4, although most of the mice survived for more than 60 days after mixed-inoculation, which is in contrast to the previous report that all the mice were cured by intradermal mixed-inoculation of CTLL-D4 cells and RL♂1, even at the E/T ratio of 1 : 1[29]. This discrepancy might be due to: 1) spread of injected cells to remote sites via the subarachnoid space, since the brain parenchyma is easily damaged by mechanical injection, and 2) increased intracranial pressure due to the growth of tumor cells.

Thus, although many problems remain to be solved, the present study opens a new perspective for therapeutic application of immunotherapy against brain tumors.

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## References

1) Apuzzo MLJ: Immunological aspects of intrinsic glial tumors. J Neurosurg **55**: 1–18, 1981.
2) Borberg H, Oettgen HF, Choudry K, et al: Inhibition of established transplants of chemically induced sarcoma in syngeneic mice by lymphocytes from immunized donors. Int J Cancer **10**: 539–547, 1972.
3) Bullard DE, Bigner DD: Applications of monoclonal antibodies in the diagnosis and treatment of primary brain tumors. J Neurosurg **64**: 2–16, 1985.
4) Cantor H, Boyse EA: Functional subclasses of T lymphocytes bearing different Ly antigens I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. J Exp Med **141**: 1376–1389, 1975.
5) Cantor H, Boyse EA: Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of $Ly^+$ cells in the generation of killer activity. J Exp Med **141**: 1390–1399, 1975.
6) Cerottini JC, Brunner KT: Cell-mediated cytotoxity, allograft rejection and tumor immunity. Adv Immunol **18**: 67–132, 1974.
7) Cheever MA, Greenberg PD, Fefer A: Specific adoptive therapy of established leukemia with syngeneic lymphocytes sequentially immunized in vivo and in vitro and nonspecifically expanded by culture with interleukin 2. J Immunol **126**: 1318–1322, 1981.
8) Cheever MA, Greenberg PD, Fefer A, et al: Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by in vivo administration of purified interleukin 2. J Exp Med **155**: 968–980, 1982.
9) Cheever MA, Greenberg PD, Irle C, et al: Interleukin 2 administered in vivo induces the growth of cultured T cells in vivo. J Immunol **132**: 2259–2265, 1984.
10) Dailey MO, Fathman CG, Butcher EC, et al: Abnormal migration of T lymphocyte clones. J Immunol **128**: 2134–2136, 1982.
11) Dailey MO, Pillemer E, Weissman IL: Protection against syngeneic lymphoma by a long-lived cytotoxic T cell clone. Proc Natl Acad Sci USA **79**: 5384–5387, 1982.
12) DeVries JE, Spits H: Cloned human cytotoxic T lymphocyte (CTL) lin s reactive with autologous melanoma cells. I. In vitro generation, isolation, and analysis to phenotype and specificity. J Immunol **132**: 510–519, 1984.

13) Donohue JH, Rosenberg SA: The fate of interleukin-2 after in vivo administration. J Immunol **130**: 2203-2208, 1983.
14) Donohue JH, Rosenstein M, Chang AE, et al: The systemic administration of purified interleukin 2 enhances the ability of sensitized murine lymphocytes to cure a disseminated syngeneic lymphoma. J Immunol **132**: 2123-2128, 1984.
15) Eberlein TJ, Rosenstein M, Spiess PJ, et al: Generation of long-term T-lymphoid cell lines with specific cytotoxic reactivity for a syngeneic murine lymphoma. J Natl Cancer Inst **69**: 109-116, 1982.
16) Eberlein TJ, Rosenstein M, Rosenberg SA: Regression of a disseminated syngeneic solid tumor by systemic transfer of lymphoid cells expanded in interleukin 2. J Exp Med **156**: 385-397, 1982.
17) Engers HD, Glasebrook AL, Sorenson GD: Allogeneic tumor rejection induced by the intravenous injection of Lyt-2$^{+}$ cytolytic T lymphocyte clones. J Exp Med **156**: 1280-1285, 1982.
18) Engers HD, Lahaye T, Sorenson GD, et al: Functional activity in vitro effector T cell populations. II. Antitumor activity exhibited by syngeneic anti-MoMULV-specific cytolytic T cell clones. J Immunol **133**: 1664-1670, 1984.
19) Fernandez-Cruz E, Woda BA, Feldman JD: Elimination of syngeneic sarcoma in rats by a subset of T lymphocytes. J Exp Med **152**: 823-841, 1980.
20) Fioretti MC, Circolo A, Bianchi R, et al: Immunochemotherapy studies with murine lymphoma cells growing in mouse brain. I. studies with tumor-associated histocompatibility antigens. Cancer Immunol Immunother **9**: 145-152, 1980.
21) Fujiwara H, Fukuzawa M, et al: The role of tumor-specific Lyt-1$^{+}$2$^{-}$ T cells in eradicating tumor cells in vivo. I. Lyt-1$^{+}$2$^{-}$ T cells do not necessarily require recruitment of host's cytotoxic T cell precursors for implementation of in vivo immunity. J Immunol **133**: 1671-1676, 1984.
22) Gillis S, Smith KA: Long-term culture of tumor-specific cytotoxic T cells. Nature (Lond.) **268**: 154-155, 1977.
23) Greenberg PD, Cheever MA, Fefer A: Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1$^{+}$2$^{-}$ lymphocytes. J Exp Med **154**: 952-963, 1981.
24) Greenberg PD, Cheever MA: Treatment of disseminated leukemia with cyclophosphamide and immune cells: tumor immunity reflects long-term persistence of tumor-specific donor T cells. J Immunol **133**: 3401-3407, 1984.
25) Grimm EA, Mazumder A, Zhang HZ, et al: Lymphokine-activated killer cell phenomenan. lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med **155**: 1823-1841, 1982.
26) Hardt C, Rollinghoff M, Pfizenmaier K et al: Lyt-23$^{+}$ cyclophosphamide-sensitive T-cells regulate the activity of an interleukin 2 inhibitor in vivo. J Exp Med **154**: 262-274, 1981.
27) Kedar E, Weiss DW: The in vitro generation of effector lymphocytes and their employment in tumor immunotherapy. Adv Cancer Res **38**: 171-287, 1983.
28) Keyaki A, Handa H, Yamashita J, et al: Growth-inhibitory effect of prostaglandin $D_2$ on mouse glioma cells. J Neurosurg **61**: 912-917, 1984.
29) Keyaki A, Kuribayashi K, Sakaguchi S, et al: Effector mechanisms of syngeneic anti-tumour responses in mice. I. Cytotoxic T lymphocytes mediate neutralization and rejection of radiation-induced leukemia RL♂1 in the nude mouse system. Immunol **56**: 141-151, 1986.
30) Keyaki A, Handa H, Yamashita J, et al: Immunological response in the mouse brain: I. Transplantation immunity to mouse lymphoma graft. Arch Jpn Chir **57**: 113-123, 1988.
31) Kida Y, Cravioto H, Hochwald GM, et al: Immunity of transplantable nitrosourea-induced neurogenic tumors. II. Immunoprophylaxis of tumors of the brain. J Neuropathol Exp Neurol **42**: 122-135, 1983.
32) Köhler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (Lond.) **256**: 495-497, 1975.
33) Kuribayashi K, Keyaki A, Sakaguchi S, et al: Effector mechanisms of syngeneic anti-tumour responses in mice. I. Establishment and characterization of an exogenous IL-2-independent cytotoxic T- lymphocyte line specific for radiation-induced leukemia RL♂1. Immunol **56**: 127-140, 1986.
34) Loveland BE, Hogarth PM, Cereding RH, et al: Cells mediating graft rejection in the mouse. I. Lyt-1 cells mediate skin graft rejection. J Exp Med **153**: 1044-1057, 1981.
35) Loveland BE, McKenzie IFC: Which T cells cause graft rejection? Transplantation **33**: 217-221, 1982.

36) Medawar PB: Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Brit J Exp Path **29**: 58–69, 1948.
37) Nakayama E, Shiku H, Stockert E, et al: Cytotoxic T cells: Lyt phenotype and blocking of killing activity by Lyt antisera. Proc Natl Acad Sci USA **76**: 1977–1981, 1979.
38) Nakayama E, Shiku H, Takahashi T, et al: Definition of a unique cell surface antigen of mouse leukemia RL♂1 by cell-mediated cytotoxity. Proc Natl Acad Sci USA **76**: 3486–3490, 1979.
39) North RJ: Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J Exp Med **55**: 1063–1074, 1982.
40) Palladino MA, Obata Y, Stockert E, et al: Characterization of interleukin 2-dependent cytotoxic T-cell clones: specificity, cell surface phenotype, and susceptibility to blocking by Lyt antisera. Cancer Res **43**: 572–576, 1983.
41) Palladino MA, Welte K, Carroll AM, et al: Characterization of interleukin 2 (IL-2)-dependent cytotoxic T-cell clones. V. Transfer of resistance to allografts and tumor grafts requires exogenous IL-2. Cell Immunol **86**: 299–307, 1984.
42) Puccetti P, Campanile GF, Romani L, et al: Antilymphoma graft responses in the mouse brain: A study of T-dependent functions. Int J Cancer **31**: 769–774, 1983.
43) Romani L, Fioretti MC, Bianchi R, et al: Intracerebral adoptive immunotherapy of a murine lymphoma antigenically altered by drug treatment in vivo. J Natl Cancer Inst **68**: 817–822, 1982.
44) Romani L, Bianchi R, Puccetti P, et al: Systemic adoptive immunotherapy of a highly immunogenic murine lymphoma growing in the brain. Int J Cancer **31**: 477–482, 1983.
45) Rosenberg SA, Terry WD: Passive immunotherapy of cancer in animals and man. Adv Cancer Res **25**: 323–388, 1977.
46) Rosenstein M, Eberlein TJ, Rosenberg SA: Adoptive immunotherapy of established syngeneic solid tumors: Role of T lymphoid subpopulations. J Immunol **132**: 2117–2122, 1984.
47) Scheinberg LC, Edelman FL, Levy WA: Is the brain "an immunologically privileged site"? I. Studies based on intracerebral tumor homotransplantation and isotransplantation to sensitized hosts. Arch Neurol **11**: 248–264, 1964.
48) Shibuya N, Hochgeschwender U, Kida Y, et al: Immunity to transplantable nitrosourea-induced neurogenic tumors. III. Systemic adoptive transfer of immunity. J Neurupathol Exp Neurol **43**: 426–438, 1984.
49) Shortman K, Wilson A, Scollay R: Loss of specificity in cytolytic T lymphocyte clones obtained by limit dilution culture of Lyt-2$^+$ T cells. J Immunol **132**: 584–593, 1984.
50) Tyler JD, Galli S, Snider ME, et al: Cloned Lyt-2$^+$ cytolytic T lymphocytes destroy allogeneic tissue in vivo. J Exp Med **159**: 234–243, 1984.
51) Winn HJ: Immune mechanisms in homotransplantation. II. Quantitative assay of the immunologic activity of lymphoid cells stimulated by tumor homografts. J Immunol **86**: 228–239, 1961.
52) Yamasaki T, Handa H, Yamashita J, et al: Specific adoptive immunotherapy with tumor-specific cytotoxic T-lymphocyte clone for murine malignant gliomas. Cancer Res **44**: 1776–1783, 1984.

## 和文抄録

# マウス脳内における免疫応答
# 2. 脳内移植腫瘍モデルにおける実験的免疫療法

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BALB/C マウスに放射線で誘発された白血病細胞 RL♂1 を用い，(BALB/cXC57BL6)$F_1$(CB6$F_1$) マウスを免疫して得たリンパ球を抗 Thy-1, 抗 Lyt 抗体と補体により処理し，CB6$F_1$-nu/nu マウスを recipient として，腫瘍中和および移入試験をおこなった．その結果，Lyt-$2^+$ T細胞が移植同系腫瘍の拒絶に必要なエフェクター細胞であることが判明した．また，RL♂1 に特異的な細胞障害性を有し，かつT細胞増殖因子(IL-2)の添加なしで長期継代培養可能な Lyt-$2^+$ $2^+$ より成るT細胞株を作製した．そして，あらかじめ RL♂1 を脳内に移植した CB6$F_1$-nu/nu マウスに経静脈的に細胞株を移入したところ，抗腫瘍効果が確認された．