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Induction of Specific Cytotoxic T Lymphocytes against Autologous Brain Tumor by Crossreactive Allo-tumor Cell Stimulation

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Cytotoxic T lymphocytes (CTL) against autologous malignant brain tumor were generated in peripheral blood lymphoid cells (PBL) prepared from a patient with a malignant brain tumor by stimulation of the cultured PBL for 7 days with attenuated crossreactive malignant melanoma (MM2) cells pretreated with mitomycin C. The crossreactive MM2 cells were effective for antigen stimulation for CTL induction in place of autologous glioblastoma cells, which are difficult to expand in culture. The optimal ratio between nylon wool-passed T lymphocytes and nylon wool-adherent accessory cells to induce CTL in the patient's PBL was found to be 25 to 1. *In vitro*-activated CTLs induced by MM2 were cytotoxic not only to MM2, but also to the autologous tumor cells in an HLA class I-restricted manner, and their surface phenotype was found to be $CD3^+$ and $CD8^+$. CTL therapy using crossreactive allogeneic tumor cells as the stimulator could be clinically valuable to treat malignant brain tumors.

Key words: Autologous tumor — CTL — Crossreactive allogeneic tumor — Malignant brain tumor

Cytotoxic T lymphocytes (CTL) specific for syngeneic tumors were clarified to be one of the effector cell populations for both establishing and maintaining antitumor immunity.[1-3] In the postoperative therapy of malignant brain tumor, CTL therapy as a specific immunotherapy is expected to be useful, as well as chemotherapy or radiation therapy, even though the mechanisms involved in regulating cellular immunity, especially against autologous tumors in the human, are not yet fully understood.[1,2,4,5] In order to establish a passive specific immunotherapy against malignant brain tumors using activated CTL, we have developed a method to induce CTL directed to the tumor by stimulating the patient's peripheral blood lymphoid cells (PBL). However, a major problem is that an autologous malignant brain tumor cell line often can not be established and expanded in culture sufficiently to allow its use for stimulation. Here we describe the induction of autologous glioblastoma-specific CTL by stimulation with crossreactive allogeneic tumor cells and the immunological analysis of these activated CTL.

MATERIALS AND METHODS

Establishment of tumor cell lines In our laboratory, a human glioma mass was minced, suspended in Ca, Mg-free phosphate-buffered (0.01 *M*, pH 7.2) saline (PBS) and digested for 4 h in the presence of 0.2% (w/v) collagenase (Seikagaku Kogyo Co., Ltd., Tokyo) and 0.1% (w/v) deoxyribonuclease (Sigma Chemicals Co., Ltd., St. Louis, MO) at room temperature. A single cell suspension was prepared by passing the digest through nylon mesh and washing the cells twice with PBS. The tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% pooled human cord serum (HCS) and 100 μg/ml of kanamaycin (Meiji Seika Co., Ltd., Tokyo). The cultured cells were maintained in 25 cm^2 plastic flasks (Nunc, Roskilde, Denmark) at 37°C in a humidified automatic CO_2 incubator (Asthec Co., Ltd., Fukuoka) with 7% CO_2 and 93% air. The tumor specimen was taken from a 72-year-old male glioblastoma patient; HLA class I A (2, 24), B (51, 60), C (—, —). This autologous glioblastoma cell line (GM 051989) was propagated after one month's culture. In the same way, we also established other human tumor cell lines, i.e., one glioblastoma (GM 011191), two malignant melanomas (MM 040689, MM1, MM 011890, MM2), one gastric cancer (GC 121288) and one squamous cell carcinoma (SCC 062089) in our laboratory. HLA typing of the tumor donor patients was performed using Japanese standard HLA serotyping trays established in National Sakura Hospital, Central Kidney Transplantation Center (Sakura, Chiba).

Preparation of PBL PBL were purified by Ficoll-Conray density gradient (1.078) centrifugation at 2100 rpm for 30 min from the brain tumor patient's peripheral blood leukocyte population obtained by automatic leukapheresis (Haemonetics V50, Braintree, MA) at 1450 rpm. The purified PBL were washed three times with PBS and resuspended in serum-free culture medium (ASF 104, Ajinomoto Co., Ltd., Tokyo) supplemented with 4% HCS and 100 μg/ml of kanamycin. The viable cells were counted by a usual dye exclusion method.

Induction of human glioblastoma-specific CTL The tumor-bearing patient's PBL were cultured with attenuated crossreactive allogeneic tumor cells pretreated with 100 μg/ml mitomycin C for the purpose of tumor antigen stimulation, instead of autologous brain tumor cells, at the ratio of 3×10^7 PBL to $1\text{–}3\times10^5$ tumor cells in 10 ml of ASF104 supplemented with 4% HCS, 100 μg/ml kanamycin, 50 μM 2-mercaptoethanol (2ME) and 20 mM HEPES for 7 days in an automatic CO_2 incubator (Asthec Co., Ltd.) with 7% CO_2 and 93% air. The allogeneic tumor cell line was selected from allogeneic tumor lines which shared a part of the HLA class I antigens of the original patient in the human cell bank of our laboratories. Since there is an optimal ratio between T lymphocytes and accessory cells to induce CTL directed to autologous tumor cells *in vitro*, the accessory cell population was readjusted by adding back various numbers of nylon wool column-adherent cells to the purified nylon wool-passed lymphocyte population.[6] After 7-day stimulation culture, the activated PBLs were tested for cytotoxic activity by 10-h ^{51}Cr-release assay. A dose of 1–2 U/ml of recombinant interleukin (rIL)-2 (100–200 IU/ml; Takeda Pharmaceutical Ind., Osaka) could be added on day 3 of the 7-day culture for therapeutic CTL induction. In this experiment, CTL was induced without rIL-2.

Treatment of cytotoxic cells with various monoclonal antibodies The surface phenotype of cytotoxic cells was defined by the use of monoclonal anti-CD3 (OKT3) or anti-CD4 (OKT4) or anti-CD8 (OKT8) antibody (Ortho Diagnostics Systems Inc., Raritan, NJ) and rabbit complement. These antibodies were used at the concentration of 50 μg/ml. The low-toxicity rabbit complement for human lymphocytes was prepared in our laboratory.

Cytotoxicity test Target cells were adjusted to the concentration of 1×10^7/ml with Hanks' solution (pH 7.2) containing 10% fetal calf serum(FCS) and 0.5 ml of the tumor target cell suspension was incubated with 50 μCi of ^{51}Cr in the form of sodium chromate for 45 min at 37°C. The cells were washed twice with Hanks' solution and once with DMEM, then resuspended in DMEM. An appropriate number of activated PBL and 1.5×10^4 ^{51}Cr-labeled target cells in DMEM supplemented with 5% FCS, 20 mM HEPES, 5×10^{-5} M 2ME and 100 μg/ml of kanamycin, were mixed in each well of round-bottomed 96-well microtiter plates in quadruplicate. The total volume of each sample was adjusted to 0.2 ml, and the plates were centrifuged for 1 min at 600g and then incubated for 10 h at 37°C to obtain optimal specific ^{51}Cr release by the activated lymphocyte-mediated cytotoxicity. If target cells are relatively resistant to the lysis, the incubation time could be set at 16 h. After the incubation, the plates were centrifuged at 1000 rpm for 5 min, then a 0.1 ml aliquot of the supernatant was taken from each well and its radioactivity was measured in a well-type gamma-scintillation counter (Packard, Auto-Gamma 5000, Meriden, CT). The percentage of target cell lysis was expressed in the figures as specific ^{51}Cr release calculated by means of the following formula.[1]

$$\text{Percent specific } ^{51}\text{Cr release} = \{(\text{CPM experimental} - \text{CPM spontaneous})/(\text{CPM maximum} - \text{CPM spontaneous})\} \times 100$$

RESULTS

Specific target cell lysis by activated cytotoxic effector cells in PBL of a glioblastoma patient induced by *in vitro* crossreactive allogeneic tumor cell stimulation In the case of our 72-year-old male glioblastoma patient, we could not prepare enough autologous glioma cells for CTL induction. Hence, we tried to activate peripheral lymphoid cells of the patient by stimulation with mitomycin C-treated allogeneic tumor cells from our tumor cell bank, and a MM2 having similar HLA class I antigens to the original tumor was selected.

The activated effector cells stimulated by MM2 clearly showed crossreactive killing activity against not only the stimulator tumor cells (MM2), but also MM1 and GM011191, while they did not show effective cytotoxicity against allogeneic tumor cells such as GC121288 and SCC062089, as shown in Fig. 1. The tumors MM1 and GM011191 expressed partially shared HLA class I A and B loci with the autologous glioblastoma, whereas GC121288 and SCC062089 showed little commonality of HLA class I molecules with the autologous glioblastoma. The killing activity mediated by the activated effector cells seemed to involve a shared HLA class I molecule(s), in particular A24, of MM2, MM1, and GM011191 as a self epitope(s).

Cold target inhibition of killing activity mediated by activated effector cells This HLA-restricted specific killing activity by the activated effector cells was confirmed by cold target inhibition assay. Cold target cells were added to the cytotoxicity assay mixture in 10-fold excess over ^{51}Cr-labeled target cells. The cytotoxic activity to MM2 of the effector cells induced by stimulation with MM2 was clearly inhibited not only by MM2, but also by autologous glioblastoma cells, as well as by some crossreactive tumor cells, such as MM1 or GM011191 having partially shared HLA class I molecules, whereas non-crossreactive allogeneic cold target tumor cells such as GC121288 or SCC062089 did not inhibit their cytotoxic activity, as shown in Fig. 2.

Analysis of optimal ratio of T cells and accessory cells for cytotoxic cell induction in the patient's PBL We investigated the optimal ratio of T lymphocytes and adherent accessory cells to induce maximum CTL activity against crossreactive MM2 as the target. The T lym-

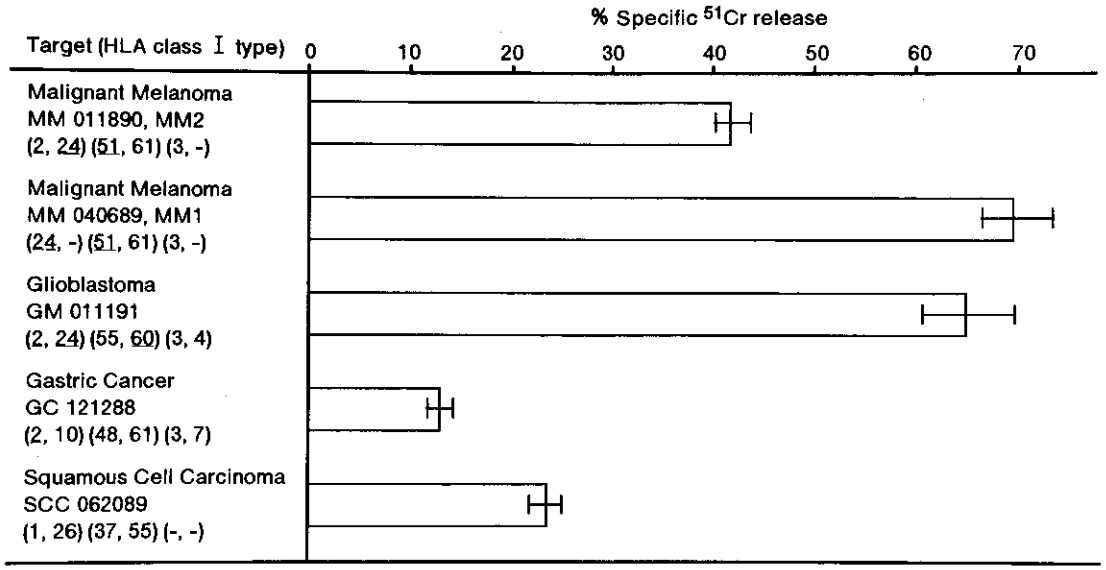

Fig. 1. Specificity of activated effector cells induced by crossreactive allogeneic tumor stimulation. Killer T cells induced by stimulation with MM2 were found to have HLA class I-restricted killer activity against target cells. Thus, MM2 can substitute for the autologous glioma cells as the stimulator for specific CTL induction. Responder HLA class I type, (2, 24) (51, 60) (—, —); stimulator MM 011890 HLA class I type, (2, 24) (51, 61) (3, —); effector/target ratio=40. MM, malignant melanoma; CTL, cytotoxic T lymphocytes.

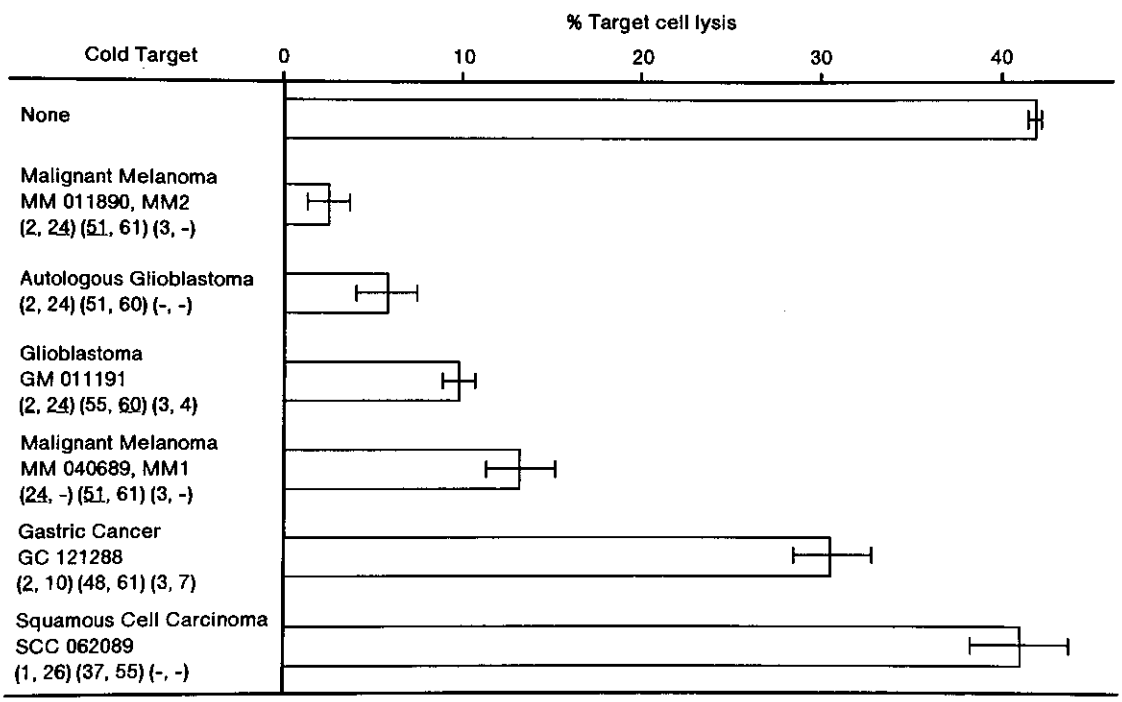

Fig. 2. Cold target inhibition of activated cytotoxic T lymphocytes (CTL). Cytolytic activity of induced CTL against the hot targets was inhibited by unlabeled autologous tumor cells or other crossreactive allogeneic tumor cells. Responder HLA class I type, (2, 24) (51, 60) (—, —); stimulator HLA class I type, (2, 24) (51, 61) (3, —); effector/target/cold target ratio=20/1/10.

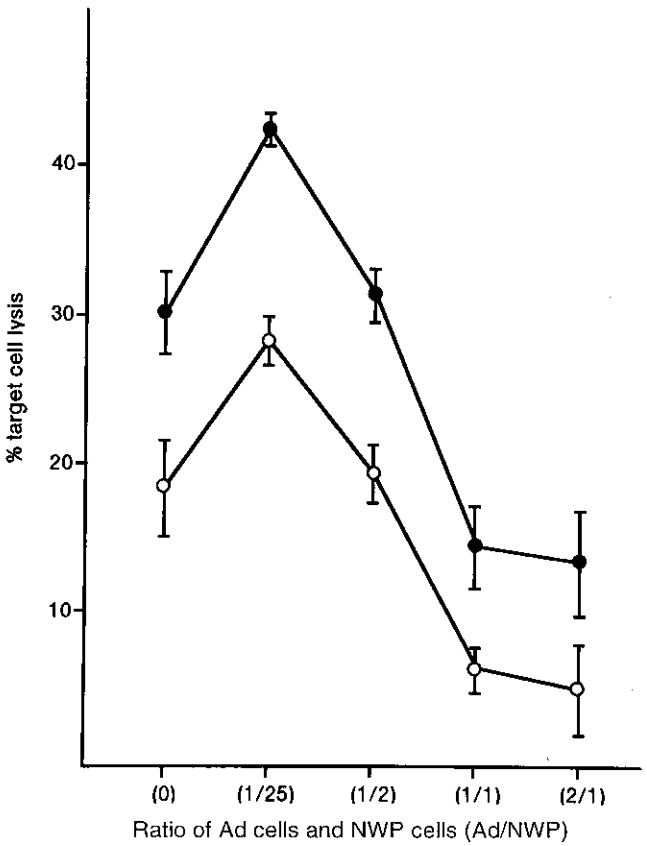

Fig. 3. Target cell lysis of cytotoxic T cell with various ratios of adherent (Ad) cells and nylon wool-passed (NWP) T cells (Ad/NWP). After 7-day culture at various ratios of NWP T lymphocytes and accessory cells with allogeneic stimulation, the induced killer cells were assayed for killer activity against MM2 (the stimulator). The optimal ratio of NWP T lymphocytes and accessory cells was 25 : 1. ○, Effector/target ratio=20; ●, effector/target ratio=40.

phocytes were purified from the brain tumor patient's PBL by passage through a nylon wool column, and accessory cells were collected from the nylon wool-adherent cells. The cell mixtures of various ratios of T lymphocytes and accessory cells were separately cultured for 7 days with stimulation by MM2. After the 7-day culture, the killing activity of each sample was assayed. As shown in Fig. 3, the optimal ratio of T lymphocytes to accessory cells was 25 to 1. Cytotoxic cells could not be induced in the presence of a large number of accessory cells. Furthermore, Fig. 4 shows that the killing activity induced in nylon wool column-passed (NWP) T lymphocytes of PBL was very much higher than that induced in crude PBL.

Surface phenotype of activated killer cells The surface phenotype of the cells possessing killing activity induced in this glioblastoma patient's PBL was analyzed by the use of monoclonal anti-CD3 (OKT3), anti-CD4 (OKT4) or anti-CD8 (OKT8) antibody and rabbit complement (C). As shown in Fig. 5, the killing activity was diminished by treatment with anti-CD3 or anti-CD8 antibody and complement. On the other hand, treatment with anti-CD4 and complement did not reduce the killing activity, thus clearly indicating that this killing activity was mediated by $CD8^+$ T cells.

Killer activity against autologous glioblastoma cells It is important for specific immunotherapy that PBL activated by *in vitro* stimulation with allogeneic tumor (MM2) cells should be tested for cytotoxic activity against autologous tumor cells. As shown in Fig. 6, this patient's stimulated NWP T cells were confirmed to show stronger killing activity against autologous glioblastoma cells than unstimulated NWP T cells. This was supported by the result of a cold target inhibition assay against MM2 with autologous glioblastoma (Fig. 2).

These data, taken together, indicate that the CTL induced by stimulation with crossreactive allogeneic MM2 also showed specific killing activity against autologous glioblastoma cells.

DISCUSSION

Autologous tumor-specific CTLs form a population of killer cells which can be useful for passive immunotherapy. The existence of tumor-specific antigens which stimulate the host's immune systems has been studied in various tumor systems,[7] and mutant oncogene products such as Akt,[8] Ras[9] and P-53[10] mutant gene peptides and tumor rejection antigen peptides such as MAGE antigens[11, 12] have been identified as tumor-specific antigens which are expressed on HLA class I molecules and are recognized by specific CTL in various tumor systems.

Our killer lymphocytes induced *in vitro* by stimulation with crossreactive allogeneic tumor cells were specific CTL as judged from specific killing activity, cold target inhibition assay, and surface phenotype analysis (Figs. 2, 3 and 6). These data suggest that crossreactive allogeneic tumor cells with partially shared HLA class I antigens could substitute for the autologous tumor as stimulators for the purpose of generating glioma-specific CTLs. The ideal CTL therapy against glioma would require the use of autologous glioma-specific CTLs. In the clinical situation, however, a sufficient quantity of autologous malignant brain tumor cells is not always available for CTL induction by restimulation of the patient's lymphocytes. If crossreactive allogeneic tumor cell lines can be used as stimulators for the CTL induction against autologous glioma, specific therapy might be feasible for more patients.[13]

The clinical therapeutic potential of CTL against autologous tumor has been much discussed during the last

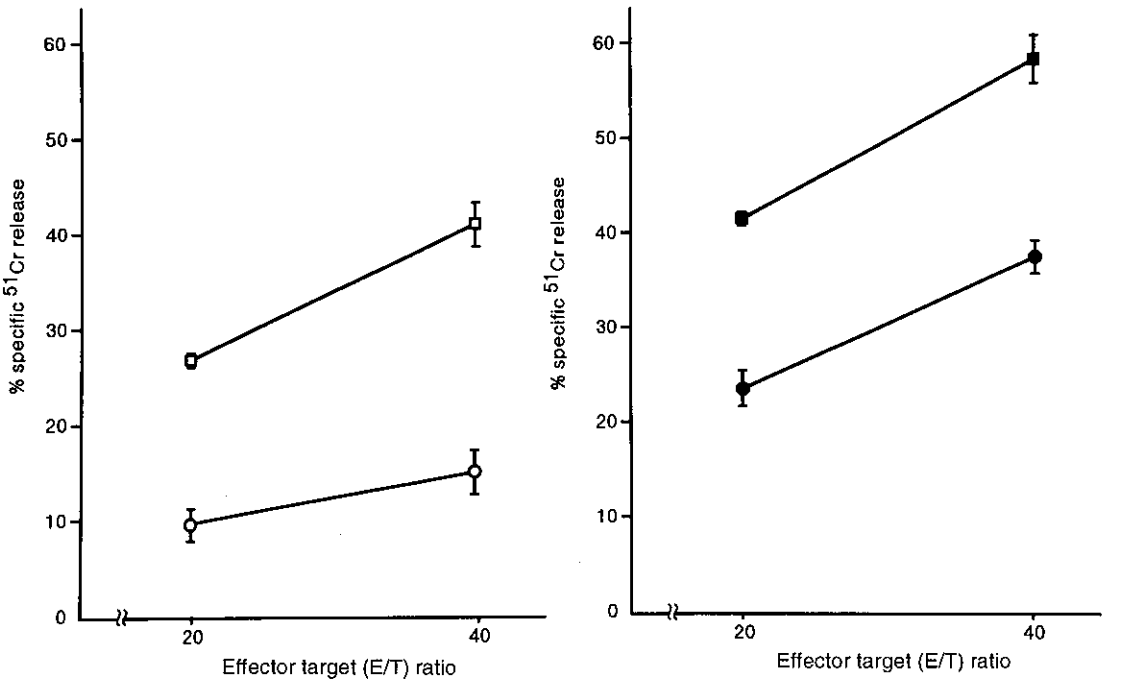

Fig. 4. Activity of cytotoxic T cells induced from the patient's crude PBL and the NWP T lymphocytes adjusted to the optimal ratio with accessory cells. Killer activity against MM2 as the stimulator from NWP T lymphocytes was very much higher than that from crude PBLs. Effector HLA class I, (2, 24) (51, 60) (—, —); stimulator HLA class I, (2, 24) (51, 61) (3, —); ○, unstimulated PBL; □, stimulated PBL; ●, unstimulated NWP T lymphocytes; ■, stimulated NWP T lymphocytes. NWP, nylon wool column-passed; MM, malignant melanoma; PBLs, peripheral blood lymphoid cells.

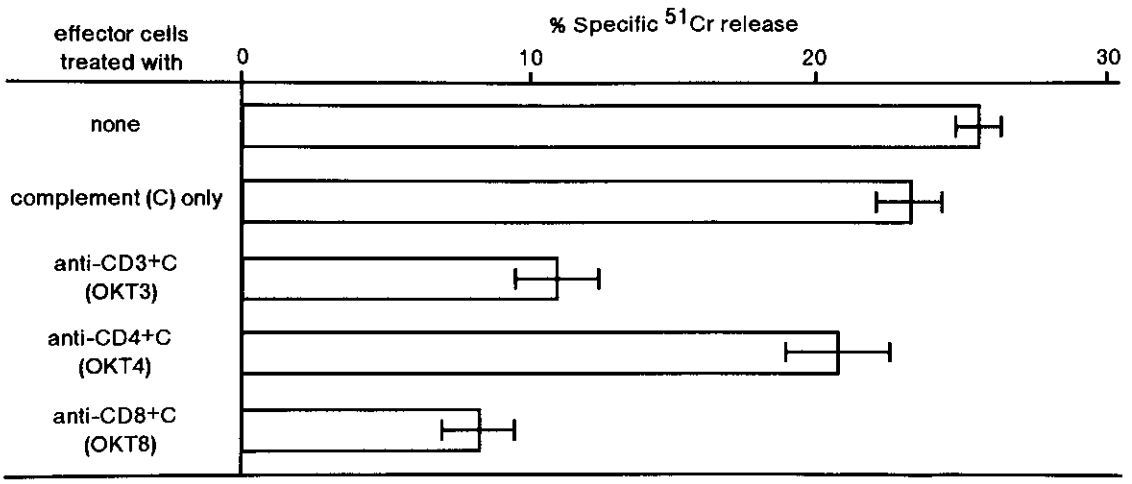

Fig. 5. Surface phenotype of activated killer T cells. The killing activity against crossreactive allogeneic melanoma MM2 as the stimulator was largely removed by treatment with anti-CD3 or CD8 antibody and complement. Anti-CD4 and complement did not reduce the activity. Effector/target ratio = 10. MM, malignant melanoma.

decade. The lymphokine-activated killer cells (LAK) developed by Rosenberg's group were given much attention.[14, 15] However, theoretically, CTL therapy should be more effective than LAK because CTL has specific killing activity against the tumor. In contrast to LAK therapy, CTL therapy requires several critical steps for the induc-

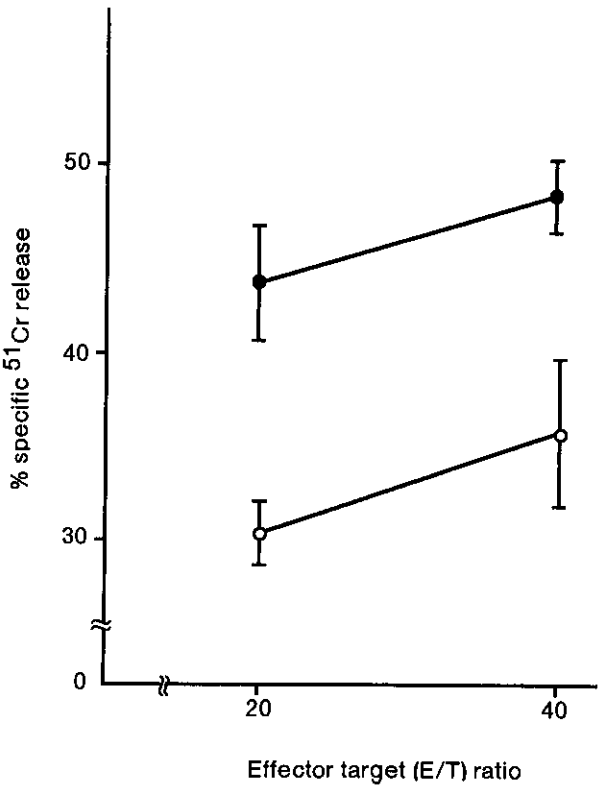

Fig. 6. Specific killer activity of CTL against autologous glioblastoma cells. Even without recombinant IL-2, CTL induced by crossreactive allogeneic melanoma MM2 showed significantly higher killing activity against autologous glioma cells, compared to the unstimulated T cells. ○, unstimulated NWP T lymphocytes; ●, stimulated NWP T lymphocytes. CTL, cytotoxic T lymphocytes; IL-2, interleukin 2; MM, malignant melanoma; NWP, nylon wool column-passed.

tion of CTL. First, since the specific CTL clone size for the autologous tumor in patients' PBL is quite limited, the CTL should be activated by autologous tumor cells in order to magnify the tumor-specific cytolytic activity, and a large number of tumor cells from each individual patient is usually required for the CTL induction. Our technique of applying established crossreactive allogeneic tumor cell lines as the stimulator should increase the number of glioma cases treatable by CTL therapy. Secondly, consistent and effective *in vitro* methods for inducing potent CTL are needed. Thirdly, *in vitro* expansion of autologous tumor-specific CTL is necessary, although this step could be circumvented by addition of growth factors, such as recombinant IL-2 and some other cytokines such as IL-4, IL-6, and IL-7. Among these processes, the second step is crucial. Malignant brain tumor patients are often in an immuno-suppressed state in which tumor-derived factors, such as transforming growth factor-β, play a role,[16] and PBLs of the brain tumor-bearing host may show an abnormal constitution of white blood cells, including lower levels of $CD3^+$ T lymphocytes and relative monocyte predominancy. In our protocol, T cells purified from crude PBLs on a nylon wool column were adjusted to give the optimal ratio (25 : 1) between T lymphocytes and accessory cells for stimulation of CTL.

Fujimoto *et al.* demonstrated significance of an activating factor for CTL, so-called killer T lymphocyte activating factor (KAF) derived from macrophages at the optimal ratio.[17] The KAF could replace monocyte function. It acted at the CTL induction phase, as well as the effector phase. Further, the KAF-augmented CTL possessed specificity against autologous tumor and other crossreactive tumor cell lines.

Many problems remain in applying CTL therapy to clinical cases.[18–22] CTL therapy of human brain tumors might be practical by local injection, i.e., via the Ommaya reservoir, into the post-operative tumor cavity.[23, 24] We have treated our glioblastoma patient by CTL therapy alone for one year after left frontal tumor removal. So far, no evidence of tumor recurrence on CT and no neurological findings have been seen, and there have been no serious side effects (unpublished results). These results seem very promising for the development of effective CTL therapy.

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