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Immunology

Expression of murine interleukin 7 in a murine glioma cell line results in reduced tumorigenicity *in vivo*

(gene therapy/tumor regression)

TOMOKAZU AOKI*, KEI TASHIRO†, SHIN-ICHI MIYATAKE*, TATSUO KINASHI†, TORU NAKANO†, YOSHIFUMI ODA*, HARUHIKO KIKUCHI*, AND TASUKU HONJO†‡

Departments of *Neurosurgery and †Medical Chemistry, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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ABSTRACT **We have examined the immunoregulatory effect of local and continuous secretion of interleukin 7 (IL-7) from murine glioma cells (203-glioma) engineered by murine IL-7 gene transfection. Secretion of IL-7 from glioma cells did not result in morphology or growth rate changes but did reduce tumorigenicity *in vivo* in proportion to the amount of IL-7 produced. This reduction in tumorigenicity could be reversed in a dose-dependent fashion by injection of anti-IL-7 neutralizing monoclonal antibody at the tumor site. Mice immunized with IL-7-producing glioma cells showed a specific immune response to 203-glioma but not to two other syngeneic cell lines (B-16, a melanoma, and YM-12, a fibrosarcoma). IL-7-producing glioma cells were not rejected in mice depleted of $CD8^+$ cells but were rejected in mice depleted of $CD4^+$ or $NK1.1^+$ cells. These results suggest that $CD8^+$ T cells may play an important role in tumor rejection.**

Various investigators have transfected the major histocompatibility complex class 1 (1), interleukin 4 (IL-4) (2), interferon γ (3), IL-2 (4, 5), granulocyte-colony-stimulating factor (6), and tumor necrosis factor α (7, 8) genes directly into tumor cells. Expression of such genes in tumor cells has resulted in reduction of the tumorigenicity *in vivo*, probably because it prevents the decrease in expression of immunogenic determinants on tumor cells and the resultant insufficient recognition of tumor cells by the immune system.

IL-7 was originally defined as a bone marrow stromal cell-derived soluble growth factor inducing proliferation of pre-B cells (9). cDNAs encoding murine and human IL-7 have been cloned (10, 11). IL-7 has been shown to function as a growth factor for T cells in an IL-2-independent fashion (12, 13) and to enhance the generation of cytotoxic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells (14, 15). More recently, IL-7 has been found to induce cytokine secretions and tumoricidal activity by human peripheral blood monocytes (16).

We therefore focused our attention on the possible antitumor activity of IL-7 *in vivo* mediated by CTLs, LAK cells, and monocytes. To construct cell lines that constitutively produce IL-7, we transfected murine ependymoblastoma cells (203-glioma) with an expression vector containing murine IL-7 cDNA. These IL-7-producing glioma cells were injected into syngeneic mice to determine the effect on tumorigenicity *in vivo*.

MATERIALS AND METHODS

Tumor Cell Lines. A methylcholanthrene-induced malignant glioma cell line (ependymoblastoma, 203-glioma; refs. 17 and 18), a methylcholanthrene-induced fibrosarcoma (YM-12; ref. 17), and a spontaneously occurring malignant melanoma (B-16; ref. 19) were used. These three tumor cell lines originated from C57BL/6 mice and were cultured in Dulbecco's modified minimal essential medium (Nissui, Tokyo) with 10% heat-inactivated fetal bovine serum (Grand Island Biological, Grand Island, NY) and antibiotics (penicillin G, 200 units/ml; streptomycin sulfate, 50 μg/ml).

Plasmid Construction and Transfection. Murine IL-7 cDNA was isolated from pSRα-mIL-7 (ref. 20; kindly provided by S. Nishikawa) by *Pst* I/*Xmn* I digestion and ligated to the *Xho* I site of BMGNeo plasmid (ref. 21; kindly provided by H. Karasuyama) by standard methods (22). The 203-glioma cells were transfected by electroporation (23). Cells (10^6) were washed and suspended in 40 μl of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) in the presence of 10 μg of BMGNeo or BMGNeo-mIL-7 plasmids. Cells were subjected to three 50-μsec pulses (400 V, 1.0 kV/cm) with an electroporation machine (Shimadzu, Kyoto). After incubated for 48 hr in complete medium, cells were selected for 2 weeks in medium with G418 (200 μg/ml). Clones were isolated and expanded to cell lines.

IL-7 Assay. Culture supernatant from 2×10^6 transfected cells in a 6-cm plate was collected after 48 hr and assayed for IL-7 activity by [^{3}H]thymidine uptake of the IL-7-dependent pre-B-cell line DW34 (ref. 20; kindly provided by S. Nishikawa). Serial dilutions of IL-7 were incubated with 10^4 DW34 cells in a volume of 200 μl for 48 hr; this was followed by a 6-hr incubation with 1 μCi (37 kBq) of [*methyl*, 1′,2′-^{3}H]- or [*methyl*-^{3}H]thymidine (Amersham). One unit of activity was defined as the amount of IL-7 inducing 50% of maximal proliferation in a 200-μl cell culture.

Antibodies. Rat anti-human/murine IL-7 monoclonal antibody (mAb) was purchased from Genzyme. In the presence of murine IL-7 at 5 ng/ml, an antibody concentration of 5 μg/ml was required to achieve a 90% neutralization of bioactivity *in vitro* (according to manufacturer's specifications). mAbs against Lyt-2 (CD8) or L3T4 (CD4) were purchased from Becton Dickinson. mAb against NK1.1 [specific antigen on natural killer (NK) cells; ref. 24] obtained from the American Type Culture Collection was prepared from ascites. Control mAb (mouse myeloma IgG1) was purchased from Zymed Laboratories.

Animal Study. Male C57BL/6 mice, 6–8 weeks old, were purchased from the Shizuoka Animal Center (Shizuoka, Japan). Cells were injected subcutaneously into the right flank of the animal in 100 μl of PBS via a 26-gauge needle on a 1-ml syringe. Tumor size was measured in millimeters by using a caliper and was recorded as mean diameter [longest surface length (a), width (b), and thickness (c), $(a + b + c)/3$]. Anti-IL-7 mAb (1 or 10 μg) or control mouse IgG was

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Abbreviations: CTL, cytotoxic T lymphocyte; IL, interleukin; LAK, lymphokine-activated killer; mAb, monoclonal antibody; NK, natural killer.

‡To whom reprint requests should be addressed.

Table 1. IL-7 activity in supernatants from transfected cells

Cell line	Plasmid	Activity, units/ml
203-glioma	—	<0.1
203-glioma-Neo	BMGNeo	<0.1
7G1	BMGNeo-mIL-7	42.3
7G2	BMGNeo-mIL-7	17.5
7G3	BMGNeo-mIL-7	9.8
7G4	BMGNeo-mIL-7	52.4
7G5	BMGNeo-mIL-7	45.2
7G6	BMGNeo-mIL-7	21.7
7G7	BMGNeo-mIL-7	12.4

Each transformed cell type was seeded at 10^5 cells and, after 2 days, the culture supernatants were tested for IL-7 activity by [^{3}H]thymidine incorporation of the IL-7-dependent cell line DW34. One unit of activity was defined as the amount of IL-7 that induced 50% of the maximal proliferation of DW34 cells.

coinjected with 10^6 cells, and injections of the mAb (1 or 10 μg) around the site of tumor growth were repeated every other day for 3 weeks. Mice were injected intraperitoneally with 250 μg of anti-L3T4, anti-Lyt-2, or anti-NK1.1 in 0.5 ml of PBS just prior to subcutaneous tumor cell inoculation and were given 200 μg of the mAb every 7 days to maintain a suppression of helper T cell, CTL, or NK cell activity, respectively (3, 24, 25).

DNA Extractions and Polymerase Chain Reaction (PCR) Assay. Genomic DNA was isolated from tumor cell lines or tumor biopsy specimens obtained from mice. The cells were incubated at 37°C for 1 hr with proteinase K (200 μg/ml) and 1% SDS in 10 mM Tris·HCl (pH 8.0), and DNA was isolated by phenol extraction and ethanol precipitation (26). PCR using DNA was carried out as described (27). Primers were 5′-ATGATACAAAGGAAGCTGCT-3′ (positions 759–778; sense strand) and 5′-TTATATACTGCCCTTCAAAA-3′ (positions 1015–996; antisense strand).

RESULTS

Establishment of Glioma Cell Lines That Constitutively Secrete Murine IL-7. A murine IL-7 expression plasmid (BMGNeo-mIL-7) was constructed by using a bovine papilloma virus-based expression vector containing a neomycin-resistance gene (BMGNeo). Cells of the 203-glioma line (a methylcholanthrene-induced ependymoblastoma) were transfected with BMGNeo or BMGNeo-mIL-7 by electroporation. Twelve clones with BMGNeo-mIL-7 and 5 clones with BMGNeo were isolated by G418 selection. By PCR we confirmed that BMGNeo-mIL-7 plasmids were present in all 12 isolated clones (data not shown). Culture supernatants from transfected cells were assayed for IL-7 activity by using the IL-7-dependent pre-B-cell line DW34 (Table 1). Two clones (the highest IL-7 producer, 7G4, and the lowest IL-7 producer, 7G3) were selected for use in the following experiments. In each clone, IL-7 activity was neutralized by the anti-IL-7 mAb (data not shown). No detectable IL-7 activity was found in supernatants from either parental 203-glioma cells or 203-glioma cells transfected with BMGNeo alone (Table 1). Moreover, we could not find obvious differences in cell morphology and cell proliferation among parental 203-glioma cells, 203-glioma cells with BMGNeo alone, and 203-glioma cells with BMGNeo-mIL-7 (data not shown).

***In Vivo* Tumorigenicity of IL-7-Producing 203-Glioma Cells.** To determine whether or not the constitutive and local IL-7 production from the genetically engineered cells might influence their tumorigenicity *in vivo*, parental 203-glioma cells, BMGNeo-transfected 203-glioma cells, BMGNeo-mIL-7-transfected 203-glioma cells (high producer, 7G4; low producer, 7G3) were injected subcutaneously into the flank of syngeneic C57BL/6 mice. Three weeks after tumor cell injection, we evaluated tumor sizes. When the number of tumor cells injected was between 7.5×10^4 and 2.5×10^5, parental and control BMGNeo-transfected 203-glioma cells showed tumor formation, whereas IL-7-producing cells did not (Table 2). When the injected cell number was over $>10^6$, even IL-7 producing cells showed tumor formation 3 weeks later after injection (Table 2). However, within 3 months tumor growth slowed down and tumor regression could be observed in the case of 10^6 cells (data not shown). When injected cell numbers were between 10^5 and 7.5×10^5, the high producer (7G4) showed a lower rate of tumor formation than the low producer (7G3) (Table 2).

Anti-IL-7 mAb Inhibits the Reduction of Tumorigenicity *in Vivo*. To determine the relationship between IL-7 secretion from genetically engineered glioma cells and their reduced tumorigenicity *in vivo*, 10^6 IL-7-producing cells (7G4) were simultaneously injected with 1 or 10 μg of anti-IL-7 mAb. One microgram of anti-IL-7 mAb completely blocked IL-7 activity in 1 ml of supernatant from 10^6 7G4 cells in a 48-hr culture (data not shown). Additionally, anti-IL-7 mAb (1 or 10 μg) was injected every other day for 3 weeks around the tumor site. We used an isotype-matched mAb as control. The results indicate that anti-IL-7 mAb inhibited the reduction of tumorigenicity in IL-7-producing cells, whereas the control antibody did not (Fig. 1). The blocking activity correlated with the dose of anti-IL-7 mAb injected. After removal of anti-IL-7 mAb treatment, the growth of 7G4 cells slowed down again.

Gene Deletion and Loss of IL-7 Production in IL-7-Transfected 203-Glioma Cells Restores Tumorigenicity *in Vivo*. When the number of tumor cells injected was $<10^6$ and the observation period was >3 months, tumor formation, although unexpected, occurred in five mice. DNA was extracted from these five tumors whose *in vivo* tumorigenicity had been restored, and we tested for the presence of transfected IL-7 genes by PCR. Interestingly, the murine IL-7 gene had been lost from one of the five tumors (Fig. 2). Next, culture supernatants from the five tumor cell lines obtained by biopsy were assayed for IL-7 activity on the IL-7-dependent pre-B-cell line DW34. We found markedly reduced IL-7 activity compared with that of parental IL-7-transfected 203-glioma cell lines (7G3 and 7G4) (Table 3). We therefore suspect that IL-7 production plays an essential role in the observed reduction of tumorigenicity *in vivo*.

Mice Rejecting IL-7-Producing 203-Glioma Cells Had Specific Antitumor Activity. Mice were first injected subcutaneously with 10^6 IL-7-producing cells (7G4). Eight to ten weeks

Table 2. *In vivo* tumorigenicity of IL-7-transfected 203-glioma cell lines

Cell line	No. of mice with tumor/no. of injected mice								
	5×10^4	7.5×10^4	10^5	2.5×10^5	5×10^5	7.5×10^5	10^6	5×10^6	10^7
203-glioma	1/3	4/5	10/10	10/10	10/10	9/10	10/10	ND	5/5
203-glioma-Neo	1/5	4/5	9/10	10/10	10/10	10/10	10/10	5/5	5/5
7G3	0/3	0/3	1/10	3/10	8/10	10/10	10/10	5/5	5/5
7G4	0/3	0/5	0/10	0/10	1/10	5/10	9/10	5/5	5/5

Tumor formation was assessed 3 weeks after subcutaneous injection of 5×10^4 to 1×10^7 tumor cells in syngeneic C57BL/6 mice. Mice with tumors >2 mm in diameter were scored positive.

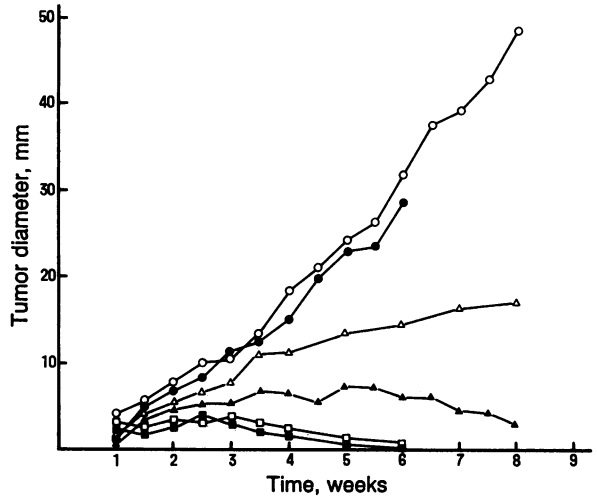

FIG. 1. Tumor inhibition is reversed by anti-IL-7 neutralizing mAb. Anti-IL-7 mAb was coinjected with 10^6 tumor cells, and injection of the mAb (1 or 10 μg) about the site of tumor growth in C57BL/6 mice was repeated every other day for 21 days. Mean tumor sizes of five mice (SD < 15%) were plotted. ■, IL-7-producing 203-glioma (7G4) plus control IgG; □, 7G4 plus no treatment; ▲, 7G4 plus anti-IL-7 mAb (1 μg); △, 7G4 plus anti-IL-7 mAb (10 μg); ●, 203-glioma-Neo plus control IgG; ○, 203-glioma-Neo plus no treatment.

later, the mice rejecting the IL-7-producing cells were reinjected subcutaneously with 7.5×10^4, 10^5, or 5×10^5 203-glioma, YM-12 (fibrosarcoma), or B-16 (melanoma) cells. The 203-glioma and YM-12 cells had almost the same growth rate *in vitro*, whereas B-16 grew more rapidly than 203-glioma (data not shown). Mice reinjected with 203-glioma did not show any tumor formation 3 weeks after tumor reinjection, but mice with YM-12 or B-16 did (Table 4). These three tumor cell lines were derived from C57BL/6 mice. The acquired antitumor immunity in these mice was therefore specific for 203-glioma.

Mice Depleted of $CD8^+$ Cells Are Incapable of Rejecting IL-7-Producing 203-Glioma Cells. The above results, and those of a preliminary histological analysis that revealed a massive lymphocyte infiltration of the IL-7-producing 203-glioma, but not of the parental 203-glioma, suggest that the cellular immune system is important for tumor rejection. To elucidate which type(s) of effector cell plays a major role in tumor rejection, mice were injected with IL-7-producing cells

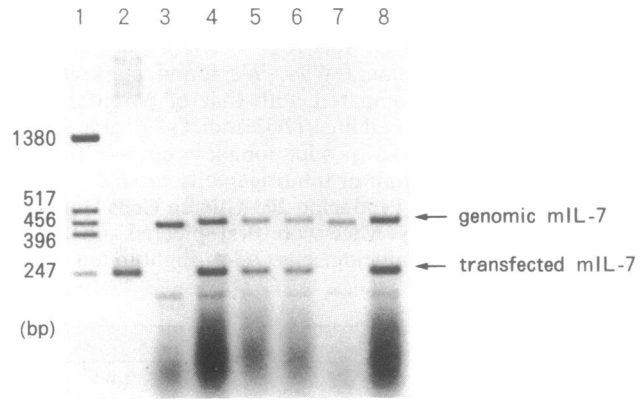

FIG. 2. Gene deletion and loss of IL-7 production by IL-7-transfected 203-glioma cells. Lane 1, Bluescribe M13(+) DNA digested with *Hin*fI as a size marker [sizes in base pairs (bp) at left]; lane 2, BMGNeo-mIL-7 plasmid; lane 3, 203-glioma-Neo; lanes 4–8, DNA samples from five tumors with restored tumorigenicity. All samples were examined by PCR. mIL-7, murine IL-7.

Table 3. IL-7 activity in supernatants from five tumors whose tumorigenicity was restored *in vivo*

Cell line	Activity, unit(s)/ml
203-glioma-Neo	<0.1
7G3	8.1
7G4	47.4
Sample 1	0.7
Sample 2	0.1
Sample 3	0.2
Sample 4	<0.1
Sample 5	0.5

Supernatants from five tumor cell lines obtained by biopsy were assayed for IL-7 activity. Samples 1–3 originated from 7G3; samples 4 and 5 were from 7G4.

(10^6 7G4 cells per mouse) together with anti-CD4 (L3T4), anti-CD8 (Lyt-2), or anti-NK1.1 mAb and with an isotype-matched mAb, as a control. Depletion of T-cell subsets was assessed by flow cytometric analysis of lymph node cells. A greater than 95% depletion was achieved (3, 25). Splenocytes from mice depleted of $NK1.1^+$ cells showed little killer activity against YAC-1 (NK-sensitive cells) (data not shown) (24). Fig. 3 shows that the reduced tumorigenicity observed in normal mice was not observed in mice depleted of $CD8^+$ cells, but was in mice depleted of $CD4^+$ or $NK1.1^+$ cells.

DISCUSSION

Murine glioma cells transfected with the murine IL-7 gene had reduced tumorigenicity *in vivo*. Whether or not systemic administration of therapeutic (high) doses of IL-7 can induce antitumor activity *in vivo* and yet produce only slight side effects remains to be determined. In the case of low-dose systemic administration, IL-7 did not result in apparent side effects (28). However, systemic administration of high doses of cytokines other than IL-7 to obtain therapeutic antitumor activity has often resulted in severe side effects (29, 30).

Attempting to avoid these potential difficulties, Nishimura *et al.* (31) studied the therapeutic efficacy of adoptive tumor immunotherapy by *in vivo* administration of IL-2 slowly released from a miniosmotic pump. We have modified this initial idea by using tumor cells transfected with a cytokine gene to maintain a therapeutic local cytokine concentration at the site of the growing tumor. If the cytokine gene and cell line are mismatched, cells transfected with the cytokine gene may grow autonomously and, instead of reduced tumorigenicity, manifest tumorigenicity *in vivo* may occasionally result (32–35).

A methylcholanthrene-induced 203-glioma that originated from C57BL/6 mice was selected for our study, because our laboratory had been investigating the characteristic immunological responses to this glioma cell (17, 18). We chose the IL-7 gene because IL-7 enhances the generation of CTLs,

Table 4. Tumor incidence of 203-glioma, YM-12, and B-16 cells in mice rejecting the IL-7-producing 203-glioma 7G4

	No. of mice with tumor/no. of mice injected		
	Immunized mice		Nonimmunized mice,
Cell line	10^5 cells	5×10^5 cells	10^5 cells
203-glioma	0/5	0/5	5/5
YM-12	4/5	5/5	5/5
B-16	5/5	5/5	5/5

C57BL/6 mice were injected subcutaneously with the indicated number of cells from the indicated syngeneic tumor cell lines, after rejection of 10^6 IL-7-producing 203-glioma cells. Mice with tumors >2 mm in diameter were scored positive.

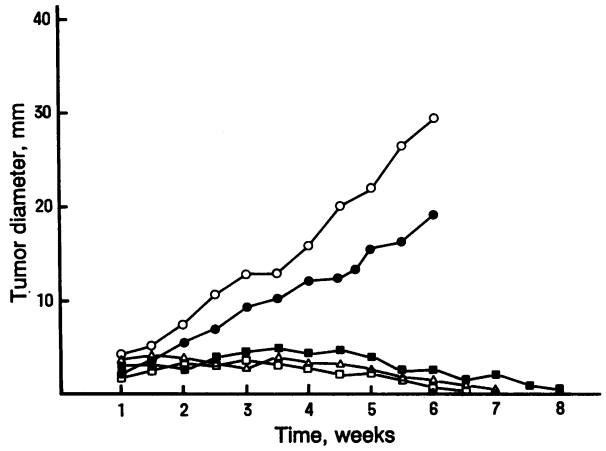

FIG. 3. Growth of IL-7-producing glioma cells in mice depleted of $CD8^+$, $CD4^+$, or $NK1.1^+$ cells. C57BL/6 mice were injected with IL-7-producing cells (10^6 7G4 cells per mouse) together with anti-$CD4^+$, anti-$CD8^+$, or anti-$NK1.1^+$ mAb and, as control, with an isotype-matched mAb. Mean tumor sizes of five mice (SD < 15%) were plotted. ○, Control mice (no treatment) injected with 203-glioma-Neo; ●, mice depleted of $CD8^+$ cells injected with IL-7-producing glioma (7G4); ■, mice depleted of $NK1.1^+$ cells with 7G4; △, mice depleted of $CD4^+$ cells with 7G4; □, control mice (no treatment) with 7G4.

LAK cells, and monocytes (14–16). We further confirmed that this 203-glioma did not change its morphology or growth rate even when cultured with high concentrations of IL-7 *in vitro* (data not shown).

Retrovirus-mediated gene transfer is a popular, stable, and efficient method but, due to integration into host chromosomes, may have harmful effects, including inactivation of important genes, activation of silent protooncogenes, and others. On the other hand, since the BMGNeo vector replicates as a multicopy episome and has little harmful effect on host chromosomes, cells transfected with cytokine genes in this vector constitutively secrete large quantities of cytokines (21). We therefore made use of the bovine papilloma virus-based expression vector BMGNeo.

IL-7-transfected 203-glioma cells showed reduction in tumorigenicity in proportion to their ability to produce IL-7 (Table 2). This reduction of tumorigenicity of IL-7-producing glioma cells was inhibited by anti-IL-7 neutralizing mAb in a dose-dependent fashion (Fig. 1). We extracted DNA from IL-7-transfected glioma cells whose tumorigenicity had been restored *in vivo*, and we assayed for IL-7 activity in culture supernatants from these glioma cells. In one case, loss of the IL-7 gene had occurred (Fig. 2), and a marked decline in the ability to produce IL-7 was noted in all cases examined (Table 3). Further, we suspect that those glioma cell lines that still had the IL-7 gene might have become unable to produce IL-7 due to various point mutations or deletions within the promoter region or mIL-7 cDNA of the plasmids. The exact mechanisms by which IL-7-transfected glioma cells lost the IL-7 gene or became unable to produce IL-7 efficiently are, however, unknown. These results demonstrate that IL-7 secretion plays an essential role in the observed reduction of tumorigenicity *in vivo*.

IL-7-producing tumor cells were found to have no differences in growth rate or morphology *in vitro* (data not shown). However, IL-7-producing glioma cells resulted in reduced tumorigenicity *in vivo*. Mice rejecting IL-7-producing glioma cells showed specific antitumor activity (Table 4). These results suggest that host immunity plays an important role in the reduction of tumorigenicity *in vivo*. IL-7-producing glioma cells were not rejected by mice depleted of $CD8^+$ cells but were rejected by mice depleted of $CD4^+$ or $NK1.1^+$ cells (Fig. 3). Mice immunized with IL-7-producing glioma cells had a specific immune response to 203-glioma cells, but not to two other syngeneic tumor cells (YM-12, B-16) (Table 4). Preliminary data revealed a reduced tumorigenicity in mice depleted of Mac-1^+ (36) cells (data not shown). Reduced tumorigenicity such as that observed in normal mice was also observed in beige mice [C57BL/6(bg^J/bg^J); NK/LAK activity-impaired mice; ref. 37] but not in nude (athymic) mice [C57BL/6(*nu/nu*); T-cell deficiency; ref. 38] (data not shown). $CD8^+$ cells therefore seem to play an important role in the reduction of tumorigenicity of IL-7-producing 203-glioma cells *in vivo*.

Although most CTL responses require T helper cells (4, 39), T help is not required for a CTL response *in vivo* (40, 41). The results of this study support the idea that $CD8^+$ T cells have the ability to induce an antitumor cytotoxic response in the absence of $CD4^+$ helper T cells *in vivo*, on the condition that there is local and continuous IL-7 secretion around the tumor.

It is widely accepted that enhanced expression of major histocompatibility complex (MHC) class 1 antigens on tumor cells results in reduced tumorigenicity *in vivo* (42). We did not, however, observe enhanced MHC class 1 expression on IL-7-producing glioma cells (data not shown). We cannot explain the finding of reduced tumorigenicity *in vivo* described here by assuming a potentiation of immune recognition of glioma cells by MHC-restricted killer T cells, since we do not know whether any tumor-specific antigens on these cells would be enhanced. The nature of putative tumor-specific antigens on our glioma cells remains to be elucidated.

Glioma cells have been shown to release various cytokines, such as an IL-1-like factor (43), an IL-3-like factor (44), an IL-6-like factor (45), an interferon β-like factor (46), and a transforming growth factor β_2-like factor (47). We found that culture supernatants from 203-glioma cells contained no IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, interferon γ, or tumor necrosis factor α (data not shown). However, we cannot rule out that other factors from 203-glioma cells contribute to the modulation of reduction of tumorigenicity *in vivo*.

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