Jpn. J. Cancer Res. 84, 689–696, June 1993

# Interferon-γ-producing Tumor Induces Host Tumor-specific T Cell Responses

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**We investigated the mechanism of host immune responses against two interferon-γ (IFN-γ) gene-transduced tumors, plasmacytoma MOPC104E(Muγ) and mammary cancer SC115(Kγ), which originally had weak immunogenicity. Both IFN-γ-producing tumor cells had reduced tumorigenicity and were rejected by syngeneic mice. The rejection was completely blocked by *in vivo* treatment with anti-CD8 or anti-IFN-γ monoclonal antibodies. While anti-CD4 monoclonal antibody also blocked the rejection of SC115(Kγ), it enhanced the initial tumor growth of MOPC104E(Muγ). Specific protection against subsequent challenge with the respective parental tumor cells was demonstrated in mice which rejected the IFN-γ-producing tumor cells. Cultured lymphocytes derived from immunized mouse spleens had cytotoxic T cell activity against parental tumor cells, as well as against cells that produced IFN-γ. These findings indicate that the antitumor effects are mediated by cytotoxic T cells and, partly, by helper T cells, and that locally secreted IFN-γ plays an important role in generating these effector cells.**

Key words: Interferon-γ — Gene transfer — Tumorigenicity — Anti-tumor immunity

Tumor cell-targeted cytokine gene therapy, a new approach developed for treating malignant tumors,[1] involves the use of tumor cells that have been genetically engineered to produce a particular cytokine; these cells then produce locally increased cytokine concentrations when injected into mice. Cytokines that are effective in this respect include interferon-γ (IFN-γ[5]),[2-6] interleukin (IL)-2,[7,8] IL-4,[9,10] IL-7,[11] tumor necrosis factor-α[12-14] and granulocyte colony-stimulating factor.[15]

IFN-γ plays an important role in inducing cytotoxic T lymphocytes[16] and in tumor regression mediated by tumor-infiltrating lymphocytes.[17] We previously reported that expression of IFN-γ cDNA in transplantable mouse neuroblastoma C1300 cells enhanced major histocompatibility complex (MHC) class I expression and augmented specific anti-tumor immunity in the recipient.[3] Although other investigators have also reported reduced tumor cell tumorigenicity after INF-γ gene transfer, the immune responses induced against various tumors were distinct in some respects, in that one showed non-specific[6] and the other showed tumor-specific immune responses, similar to those of C1300.[5]

In the study described here, we performed tumor cell-targeted IFN-γ gene transfer to two other weakly immunogenic murine tumors, and we extended our previous observations; our findings suggest the presence of a specific T cell-mediated anti-tumor immune response. We found not only that tumor-specific cytotoxic T lymphocytes (CTL) reacted to both parental and gene-transduced tumor cells, but also that $CD4^+$ T cells were involved in this system, depending upon the tumor used.

## MATERIALS AND METHODS

**Mice** Inbred 6- to 12-week-old female BALB/c mice and male DS mice, a subline from dd mice, were obtained from Shizuoka Laboratory Animal Center and Aburabi Laboratories, Shionogi & Co. Ltd., Shiga, respectively. BALB/c nu/nu mice (8 to 12 weeks old) were obtained from the Facility of Experimental Animals, Faculty of Medicine, Kyoto University.

**Tumors** Plasmacytoma MOPC104E has been described elsewhere.[18] Shionogi Carcinoma 115 (SC115),[19] established from spontaneous mammary carcinoma in the DS mouse, was obtained from K. Yamaguchi (Shionogi Research Laboratories, Shionogi & Co. Ltd., Osaka). Tumor cell lines were maintained in RPMI 1640 medium (for MOPC104E) or Dulbecco's modified minimal essential medium (for SC115), supplemented with 10% fetal bovine serum. In the animal studies, tumor cells were suspended in 0.2 ml of Hanks' balanced salt solution and were inoculated s.c. into the right flank of syngeneic mice. Tumor size, assessed in terms of the diameter, was measured twice a week. YAC1 and RL♂1[20] were used as natural killer (NK)-sensitive targets in a cytotoxicity assay.

**Retroviral IFN-γ gene transduction into tumor cells** Tumor cells were transduced with cDNA, using a retro-

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[5] Abbreviations: IFN-γ, interferon-γ; IL, interleukin; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocytes; NK, natural killer; mAb, monoclonal antibody; MLTC, mixed lymphocyte tumor cell culture.

virus vector, as previously described.[2,3] Briefly, MOPC-104E and SC115 cells were infected with the supernatant of $\phi$2(Mu$\gamma$)8 or $\phi$2(K$\gamma$)1 packaging cells in the presence of 8 $\mu$g/ml of Polybrene (Sigma, St. Lous, MO). Both $\phi$2(Mu$\gamma$)8 and $\phi$2(K$\gamma$)1 produce mouse IFN-$\gamma$-expressing retroviruses, pSVX(Mu$\gamma\Delta$As) and WB-(KMu$\gamma$), respectively. Clonal derivates, MOPC104E-(Mu$\gamma$) from MOPC104E and SC115(K$\gamma$) from SC115, were isolated by G418 (Geneticin; Sigma) selection, expanded, and assessed for IFN-$\gamma$ production. G418-resistant lines, MOPC104E(neo) and SC115(neo), transduced with pZIP.NEO.SV(X)1, were obtained as controls for IFN-$\gamma$ gene-transduced cells.

**Mouse IFN assay** The culture supernatant from $5\times10^4$ cells in 2 ml of medium in a 24-well culture plate was collected after 24 h and assayed for IFN. The assay was based on the antiviral activity of the supernatants, as measured by the reduction in the cytopathic effect of vesicular stomatitis virus on L cells, as described previously.[21] The titer was expressed in reference units (U) as calibrated against the NIH reference mouse IFN-$\alpha/\beta$ (G002-904-511).

**Antibodies** The monoclonal antibodies (mAb) used in an ascitic form in this study were: anti-CD8 [anti-Lyt2.2 for BALB/c mice[22]; anti-Lyt2.1, American Type Culture Collection HB129, for DS mice], anti-CD4 (GK 1.5),[23] rat mAb against mouse IFN-$\gamma$ (R4-6A2),[24] and monomorphic anti-MHC class I mAb (M1/42).[25] A rabbit anti-asialo GM1 antibody[26,27] was purchased from Wako Pure Chemical, Osaka.

**Flow cytometry** Tumor cell surface phenotypes were analyzed by indirect fluorescence using a FACScan (Becton Dickinson, Mountain View, CA). Single-cell suspensions were incubated with the mAb. Bound antibodies were detected by incubation with the appropriate FITC-coupled rabbit anti-mouse or -rat IgG.

**Administration of mAb *in vivo*** Mice were injected i.v. with anti-asialo GM1 (20 $\mu$l/mouse, days 0, 7, and 14), anti-CD8 (100 $\mu$l/mouse, day 0), anti-CD4 (50 $\mu$l/mouse, days 0, 4, 7), or anti-IFN-$\gamma$ mAb (50 $\mu$l/mouse, days 0, 7) after tumor inoculation.

**Mixed lymphocyte tumor cell culture (MLTC)** After tumor regression, mice were boosted twice with i.p. injections of $3\times10^6$ of the corresponding tumor cells at 2-week intervals. Spleen cells from these mice were cocultured as responding cells in the presence of 100-Gy-irradiated tumor cells as stimulators, as described previously.[28] After a 5-day incubation, cells were harvested and assayed for cell-mediated cytolysis.

**Assay of cytolytic activity** The cytolytic activity of spleen cells and MLTC cells was determined using a $^{51}$Cr-release assay as described previously.[28] When the cold target inhibition assay was performed, varying numbers of unlabeled competitor cells were added to the $1\times10^4$ labeled target cells per well. The effector to $^{51}$Cr-target cell ratio was always 3:1 and the cold to hot cell ratio varied from 0.6:1 to 10:1. Cold target cells were omitted from control wells to determine the uninhibited cytolysis of effector cells.

## RESULTS

**IFN-$\gamma$ gene transduction into tumor cells** Two different types of tumor cell lines, MOPC104E and SC115, were transduced with IFN-$\gamma$ cDNA using a retroviral vector. Transduced cells were selected by G418, and the cloned lines, obtained by limiting dilution, MOPC104E(Mu$\gamma$) and SC115(K$\gamma$), were used in the following experiments. The cloned lines continuously produced about 300 and 50 U/ml of IFN-$\gamma$, respectively, throughout the study. The *in vitro* cell growth of MOPC104E(Mu$\gamma$) and SC115(K$\gamma$) was comparable to that of their respective parental tumor cells (data not shown).

**Inability of IFN-$\gamma$-producing tumor to grow *in vivo*** Parental MOPC104E and SC115 are highly tumorigenic,

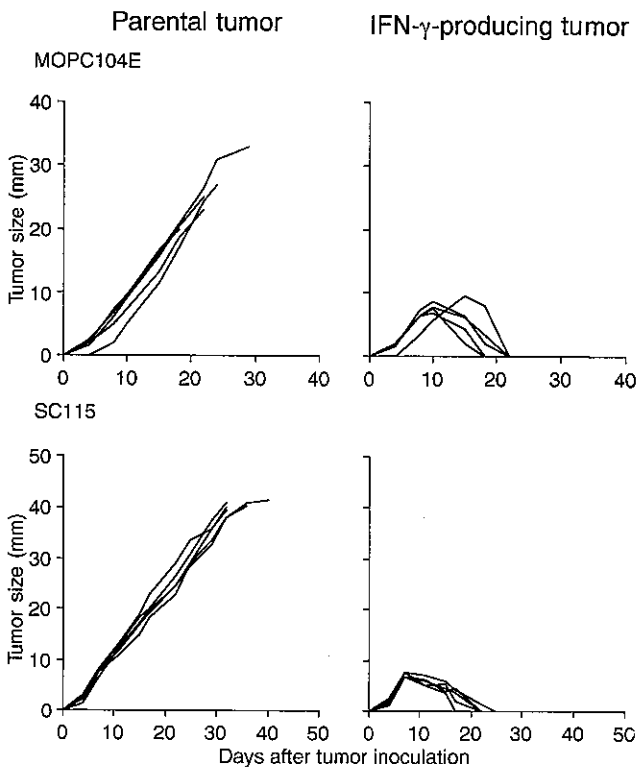


Fig. 1. Loss of tumorigenicity in IFN-$\gamma$-producing tumor cells. Groups of five mice were inoculated s.c. with $1\times10^6$ of the indicated parental or IFN-$\gamma$-producing tumor cells. Tumor growth, as mean diameter, was monitored twice a week. One representative experiment of three is shown.

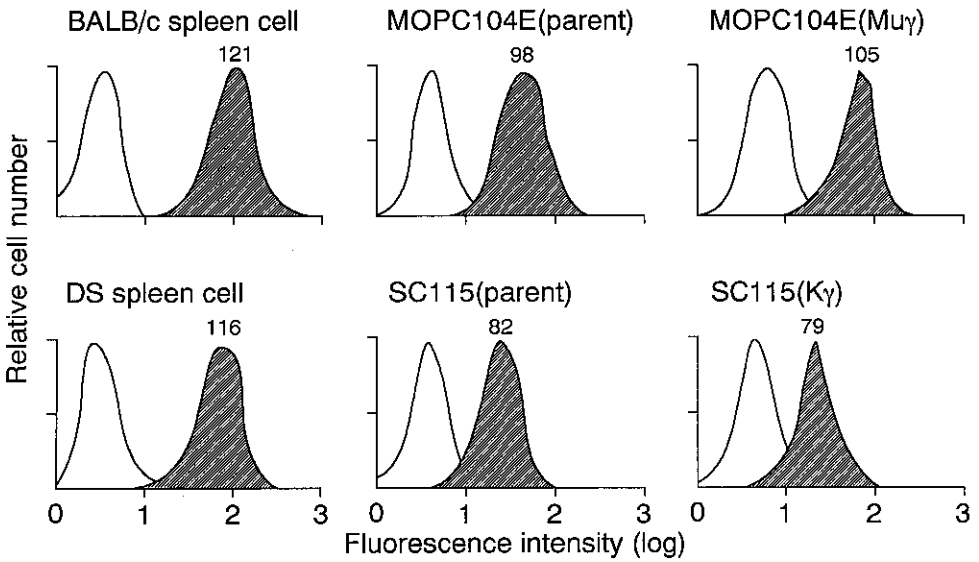


Fig. 2. MHC class I expression of IFN-γ-producing tumor cells. Cells were labeled with a monomorphic anti-MHC class I mAb (M1/42), followed by FITC-conjugated goat anti-rat IgG. As a control, the first layer mAb was omitted. The number on top of each shaded area represents the mean fluorescence intensity of the peak.

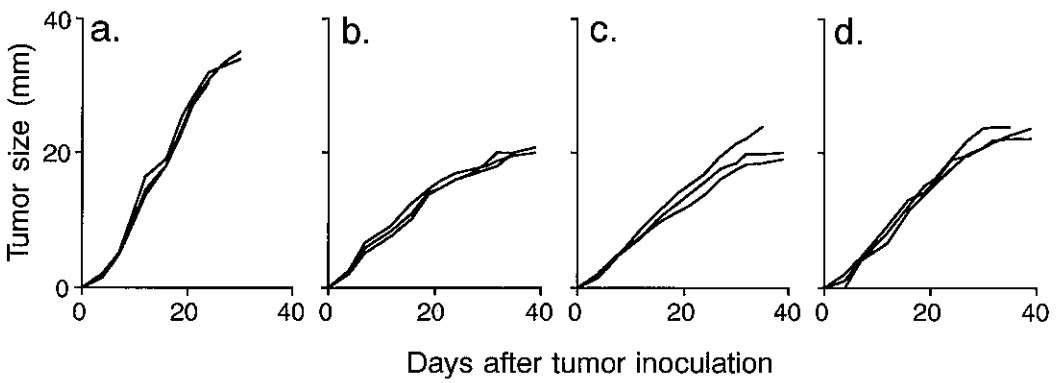


Fig. 3. MOPC104E(Muγ) tumor growth in nude mice. BALB/c nu/nu mice were given s.c. injections of $1\times10^6$ MOPC104E-(parent) (a) or MOPC104E(Muγ) (b, c, d), followed by *in vivo* administration of anti-asialo GM1 (c) or anti-IFN-γ (d) as described in "Materials and Methods."

and injection of as few as $5\times10^4$ cells produces tumors in syngeneic mice. In mice inoculated with $1\times10^6$ MOPC-104E(Muγ) or SC115(Kγ) cells, tumors appeared transiently but regressed, whereas parental tumor cells grew progressively, as shown in Fig. 1. Even at cell concentrations up to $1\times10^7$, mice rejected the IFN-γ-producing tumors. Neo-gene-transduced tumor cells grew in a manner similar to that of the parental cells (data not shown).

**MHC class I antigen expression of IFN-γ-producing tumor cells** In this study, both tumors expressed MHC class I antigen before gene transduction, at a level comparable to that of syngeneic spleen cells. Transduction of the IFN-γ genes did not augment the expression of MHC class I antigen in either of the tumors (Fig. 2). No MHC class II antigen was expressed before or after transduction with the IFN-γ gene (data not shown).

**Enhancement of host NK activity by IFN-γ producing tumors** Since NK cells might be involved in tumor rejection and since IFN-γ is a potent activator of NK cells, we assessed the influence of tumor-secreted IFN-γ on host NK activity using athymic nude mice. When $1\times10^6$ MOPC104E(Muγ) cells were inoculated s.c. into syngeneic BALB/c nu/nu mice, no tumor regression was ob-

served (Fig. 3). However, the tumor growth was slower than that of parental MOPC104E, despite a similar growth rate *in vitro*. Mice bearing MOPC104E(Muγ) or parental MOPC104E were killed 7, 14, or 21 days after s.c. tumor inoculation and the NK activity of their spleen cells was measured by determining *in vitro* cytotoxicity against the NK-sensitive cell lines, YAC1 or RL♀1. NK activity significantly increased with the tumor growth (Fig. 4). Furthermore, to test whether an elevated NK response was involved in the retarded growth of MOPC104E(Muγ), we examined the effect of *in vivo* administration of mAb against NK cells and IFN-γ, using nude mice. As shown in Fig. 3, tumor growth of MOPC104E(Muγ) was not enhanced by anti-asialo GM1 or by anti-IFN-γ, at a concentration that was sufficient to eliminate either NK or IFN-γ activity *in vivo*.

**Effects of various mAbs on the growth of IFN-γ-producing tumor** BALB/c and DS mice were injected s.c. with either MOPC104E(Muγ) or SC115(Kγ) cells ($1\times10^6$/mouse), respectively, and were treated *in vivo*

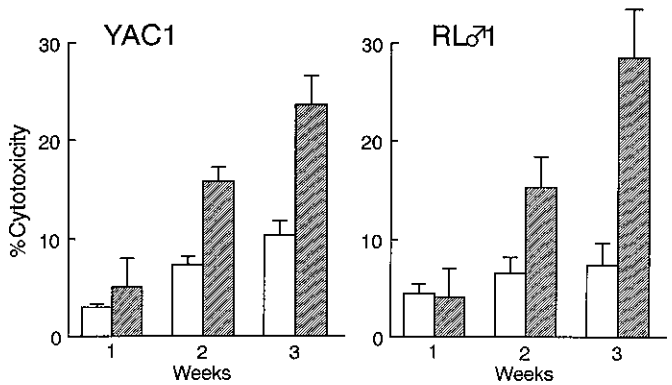


Fig. 4. Enhancement of NK activity by IFN-γ-producing tumor. BALB/c nu/nu mice were inoculated s.c. with $1\times10^6$ tumor cells. Spleen cells, isolated at various time points, were used in an *in vitro* cytotoxicity assay. Spleen cells from mice bearing MOPC104E(parent) (open columns); spleen cells from mice bearing MOPC104E(Muγ) (hatched columns). Effector to target cell ratio was always 100:1. Data represent mean±SE (n=3). Lytic activity of spleen cells from normal mice against YAC1 and RL♀1 was 5.6±2.2 (mean±SE) and 7.3±2.7, respectively, and that of spleen cells from mice treated with polyI:C was 23.7±4.6 and 23.4±4.6, respectively.

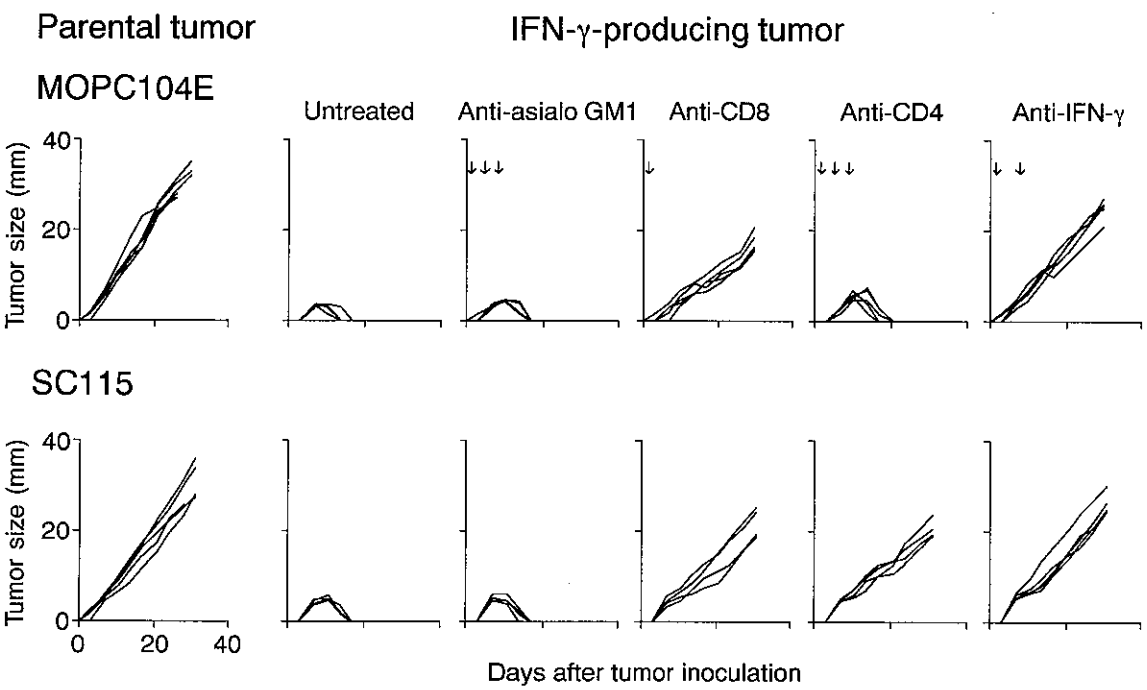


Fig. 5. Effects of *in vivo* administration of various antibodies on the growth of IFN-γ-producing tumors. Mice were inoculated s.c. with $1\times10^6$ IFN-γ-producing tumor cells, followed by *in vivo* administration of the various antibodies shown; tumor growth was observed. As controls, parental tumor cells were inoculated. Each group consisted of 4 to 5 mice. One representative experiment of three is shown.

with anti-CD8, anti-CD4, or anti-IFN-γ mAb. Tumor rejection of MOPC104E(Muγ) and SC115(Kγ) was blocked by anti-CD8 or anti-IFN-γ mAb (Fig. 5). In contrast to the slight prologation of the rejection time for MOPC104E(Muγ), anti-CD4 mAb completely blocked the rejection of SC115(Kγ). Administration of anti-asialo GM1 antibody was not effective for either tumor, as was seen in nude mice.

**CTL induction by IFN-γ-producing tumors** To determine whether $CD8^+$ CTL were induced *in vivo*, MLTC was performed. The respective spleen cells of mice that rejected MOPC104E(Muγ) or SC115(Kγ) were cultured, and their cytolytic activity was assessed. MLTC cells were cytotoxic against IFN-γ-gene-transduced tumor cells, as well as against parental tumor cells, in both models (Fig. 6). This cytolytic activity was blocked by anti-CD8 mAb (data not shown). Anti-MOPC104E-(Muγ) MLTC cells had consistently greater cytolytic activity against IFN-γ non-secreting parental tumor cells, whereas SC115(Kγ) MLTC cells had greater cytolytic activity against IFN-γ-secreting tumor cells than against the parental cells. In both cases, unrelated syngeneic tumor cells were not lysed.

The cold target inhibition assay demonstrated that cold MOPC104E(Muγ) and parental MOPC104E inhibited lysis against each of the labeled targets to the same degree (Fig. 7). However, cold parental SC115 cells had no inhibitory effect on labeled SC115(Kγ) cell lysis, although parental SC115 cell lysis was inhibited equally by both cold SC115(Kγ) and parental SC115.

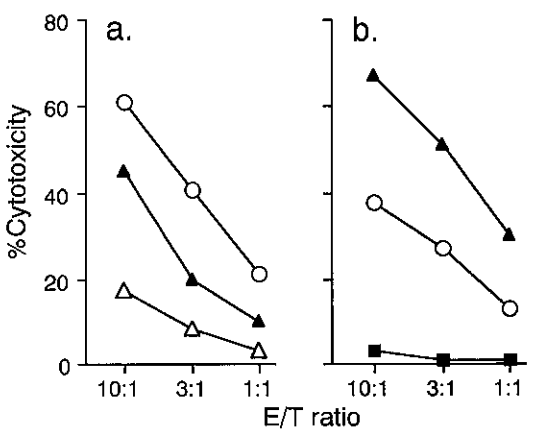


Fig. 6. CTL induction by IFN-γ-producing tumors. Spleen cells from mice that rejected MOPC104E(Muγ) (a) or SC115-(Kγ) (b) were stimulated *in vitro* with the corresponding irradiated tumor cells, and were used in a $^{51}Cr$ release assay against the following targets; parental tumor (○), IFN-γ-producing tumor (▲), RL♀8(△), and SC42 (■). E/T, effector to target.

**Tumor-specific memory is generated by IFN-γ-producing tumors** Mice that rejected IFN-γ-gene-transduced tumors were challenged 5 to 12 weeks later in the opposite flank with $1 \times 10^6$ parental tumor cells. No tumors appeared in the mice challenged with either MOPC104E or SC115. On the other hand, RL♀8 and SC42, tumors of the same genetic background as MOPC104E and SC115, respectively, were not rejected in most of the mice examined (Table I).

## DISCUSSION

We previously reported that the tumorigenicity of a murine malignant neuroblastoma,C1300, was reduced *in vivo* after IFN-γ-gene transduction.[3] In the present study, we confirmed the effect of IFN-γ-gene transduction in two other types of murine tumor cell lines, MOPC-

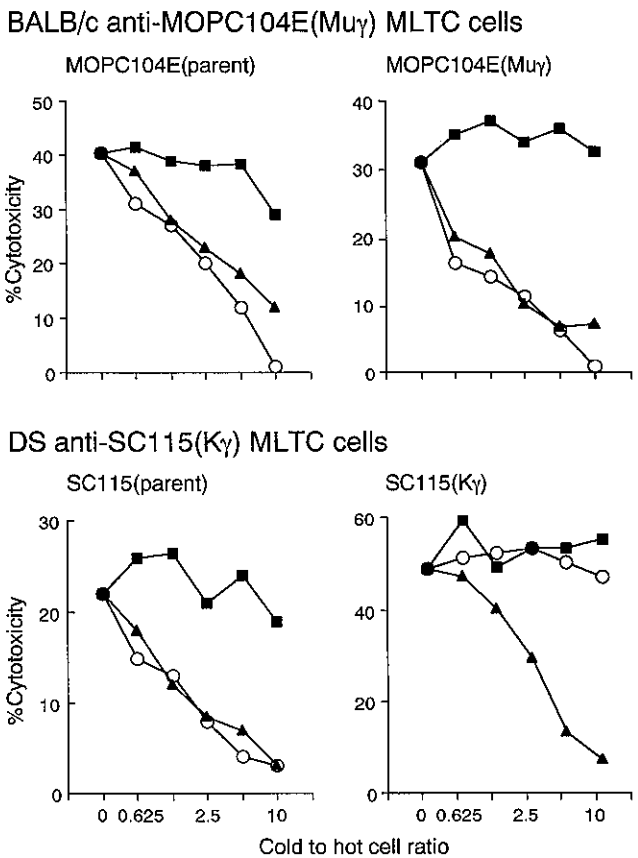


Fig. 7. Cold target inhibition assay. The cytolytic activity of MLTC cells against parental or IFN-γ-producing tumors, shown in each panel, was determined by adding different numbers of cold competitor target cells. Cold targets were parental tumor (○), IFN-γ-producing tumor (▲), and YAC1 (■). Effector to hot target cell ratio was always 3:1.

Table I. Specific Immunity of Mice that Rejected IFN-γ-producing Tumors

| Rejected by mice | Challenged with[a] | No. of mice with tumor/No. of mice challenged |
|---|---|---|
| MOPC104E(Muγ) | MOPC104E(parent) | 0/10 (10/10)[b] |
| | RL♀8 | 9/10 (10/10)[b] |
| SC115(Kγ) | SC115(parent) | 1/10 (5/5)[b] |
| | SC42 | 8/10 (5/5)[b] |

*a*) Mice were injected s.c. with $1\times10^6$ tumor cells, and tumor growth was observed.
*b*) Unimmunized syngeneic mice were injected s.c.; all mice had tumors.

104E and SC115, and examined in more detail the cellular mechanisms leading to the rejection of these tumors.

Although the amounts of IFN-γ produced by MOPC-104E(Muγ) and SC115(Kγ) differed by about a factor of 6 (300 U vs. 50 U), both tumor cells were rejected in syngeneic mice. With regard to the C1300 neuroblastoma in our previous study, syngeneic mice rejected the high producer transfectant [C1300(Muγ)12; 50 U/ml], but not the low producer [C1300(Muγ)3; 5 U/ml].[3] This suggests that a certain amount of IFN-γ must be secreted for tumor rejection to occur, depending on the tumor's potential antigenicity, growth rate, and susceptibility to effector cells. Furthermore, transplantable tumors with no or weak antigenicity may not necessarily be rejected in syngeneic mice after tumor cell targeted-cytokine gene transfer. Therefore, discriminating between tumors is important for the application of this potential therapeutic system.

IFN-γ augments the expression of MHC class I antigen,[29,30] and MHC class I antigens can dramatically enhance the immunogenicity of tumor cells.[31] The effect of IFN-γ might be related, in part, to the increased expression on the tumor cell surface.[3,5] However, this was not the case in the present study. MHC class I molecules seemed to be expressed on these tumor cell surfaces before IFN-γ gene transduction. Thus, there may be no relation between the increases in MHC class I antigens and the reduction of tumorigenicity. Recently, it has been reported that the products of two genes, proteasome and peptide transporter, act to modify MHC class I-binding antigen presentation upstream within the pathway.[32-34] These two genes are located in the MHC region and are transcriptionally up-regulated by IFN-γ. Therefore, it is possible that IFN-γ secreted above some level enhances the activities of these molecules and increases the tumor-specific antigenic peptides bound to MHC class I on the cell surfaces, resulting in the efficcient induction of effectors, as will be described below.

Our *in vivo* and *in vitro* findings confirmed that, even if the targeted tumor cells for IFN-γ gene transduction were of a different origin, the generated effector cells are of a specific T cell nature. Administration of anti-CD8 mAb *in vivo* abolished the regression of both tumors, suggesting an important role of $CD8^+$ T cells in tumor elimination. While the spontaneous regression of MOPC104E-(Muγ) was not affected significantly, injection of anti-CD4 mAb *in vivo* enhanced SC115(Kγ) tumor growth even though the tumor cells did not express MHC class II antigen on their surface. However, injection of anti-CD4 mAb at later time points (days 7, 11, and 14 after tumor inoculation) did not block the tumor regression of SC115-(Kγ), and eight of ten examined mice rejected SC115-(Kγ) (unpublished data). These results suggested that, at least in SC115(Kγ), $CD4^+$ T cells played a pivotal role as a helper T cell population in tumor-specific CTL induction, especially during the early stage of tumor growth. Such heterogeneity of T cells participating in anti-tumor responses against different tumors might not reflect the direct effect of IFN-γ itself, as has been suggested in the case of IL-7,[11] but might reflect qualitative differences in tumor antigens, such as binding capacity to MHC class I or II molecules.

We found that the inhibition of IFN-γ-producing tumor growth was reversed by neutralizing anti-IFN-γ mAb. Moreover, the growth of parental MOPC104E and SC115 was impaired when they were admixed with corresponding IFN-γ-producing cells in the mixed tumor transplantation assay (data not shown). These results suggests that the presence of this cytokine in the tumor milieu is essential for the induction of anti-tumor T cell effector populations. On the other hand, the role of NK cells appears to be negligible, since MOPC104E(Muγ) implanted in nude mice exhibited similar growth patterns in the presence and absence of anti-asialo GM1, which abrogates the NK activity *in vivo*. The retarded growth of MOPC104E(Muγ) in nude mice could be ascribed either to macrophages activated by IFN-γ secreted by the tumor cells or to a growth disadvantage of engineered tumor cells *in vivo*.

In accordance with the results obtained from the *in vivo* studies, MLTC of immunized spleen cells generated $CD8^+$ CTL directed not only against the IFN-γ-producing tumor cells, but also against the parental tumor cells. Thus, the CTL population, expanded *in vitro*, recognized the cross-reactive antigen(s) shared by the parent and its IFN-γ gene transduced partner in both tumor systems.

Although the reason is unclear, the cytotoxic activity of MLTC cells against parental MOPC104E was consistently more potent than that against MOPC104E-(Muγ). On the other hand, anti-SC115 MLTC cells were consistently more cytotoxic against SC115(Kγ) than the

parental cells. However, based on cold target inhibition assay, CTL in SC115(Kγ) MLTC cells seemed to consist of at least two populations, one being specific for SC115-(Kγ), and the other being crose-reactive with the parent and its gene-transduced partner SC115(Kγ). This was confirmed in clones established from the MLTC cells, and SC115(Kγ)-specific CTL did not lyse parental SC115 (unpublished data). The antigen recognized by the SC115(Kγ)-specific CTL may be different from that recognized by the cross-reactive CTL.

Our results indicated that the reduced tumorigenicity of IFN-γ gene-transduced tumors was mediated by the induction of tumor-specific effector T cells that recognized antigens cross-reactive between parental and gene-transduced tumor cells.

*(Received January 25, 1993/Accepted March 19, 1993)*

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