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# Immunotherapy of Bladder Cancer with Cytokine Gene-modified Tumor Vaccines[1]

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## ABSTRACT

**This study explored the use of cytokine gene-modified tumor cells as cellular vaccines for the treatment of bladder cancer. The mouse MBT-2 tumor is an excellent model for human bladder cancer. This carcinogen-induced tumor of bladder origin resembles human bladder cancer in its etiology and histology and responds to treatment in a manner similar to that of its human counterpart. In a previous study we have shown that interleukin 2 (IL-2)-secreting, irradiated, MBT-2 cell preparations were capable of curing animals from orthotopically established tumors and engendered protective immunological memory in the cured animals. In this study we have compared the effectiveness of several cytokines and found that while IL-1α, IL-1β, and γ-interferon were only weakly effective in the therapeutic vaccination protocol, granulocyte-macrophage colony-stimulating factor was almost as effective as but not superior to IL-2, as reported previously for another tumor model system. Induction of cytotoxic T-lymphocyte correlated only poorly with the therapeutic benefit of the cytokine gene-modified tumor cell preparations, questioning its prognostic value for the development of improved genetically modified tumor vaccines.**

## INTRODUCTION

Bladder cancer, especially presenting as superficial disease, is responsive to immunotherapeutic agents such as *Bacillus Calmette-Guérin* (1) and may represent a good candidate for immunological intervention. A growing number of studies have shown that genetically engineered tumor cells expressing cytokines such as IL-2,[3] IFN-γ, IL-4, IL-6, IL-7, or GM-CSF could immunize mice against a subsequent challenge with parental tumor cells (2). Moreover, in several instances, growth-inactivated tumor cell preparations engineered to secrete cytokines were also active in tumor-bearing animals, reducing tumor progression and even curing some animals from a preestablished tumor (3–7). The murine MBT-2 cell line, derived from a carcinogen-induced bladder tumor in a C3H mouse, is an excellent model to evaluate new approaches for the treatment of bladder cancer (8). This highly malignant tumor retains the histological appearance of a poorly differentiated transitional cell carcinoma; treatments that have shown promise in this murine model have been similarly effective in human bladder cancer (9, 10). Toward the development of effective tumor vaccines for the treatment of bladder cancer, we have shown in a recent study that irradiated IL-2, but not IFN-γ, gene-modified MBT-2 cells were capable of curing mice from a preexisting orthotopically induced tumor and engendering immunological memory in the cured animals (6). In this study, we have explored the therapeutic benefit of additional cytokines, including IL-1α, IL-1β, and GM-CSF. IL-1 was shown to be cytostatic or cytotoxic to tumor cells in culture and had a limited antitumor effect *in vivo* (11). The large number of biological properties ascribed to IL-1 clearly illustrate the pleotropic nature of this cytokine and its role in T-cell-dependent immune responses as well as in inflammatory reactions, although not all of its properties represent its true function *in vivo*. GM-CSF is well known for its effects on the growth and differentiation of hemopoietic progenitors and more recently was implicated in the maturation and function of bone marrow-derived dendritic cells (12). In a comparative study, Dranoff *et al.* (7) have shown that GM-CSF-secreting tumor cell preparations provided the best protection to mice implanted with B16.F10 melanoma tumors. We, therefore, wished to see whether the use of GM-CSF-secreting tumor cells will improve the therapeutic outcome in mice carrying an orthotopically induced MBT-2 bladder tumor beyond what was seen by using IL-2-secreting tumor cell preparations (6). Our observations show that in the MBT-2 bladder tumor model, the therapeutic benefit of using IL-2-secreting tumor cell preparations is equal to or better than that of GM-CSF-secreting tumor cells.

Survival of tumor bearing animals is a reliable measure for assessing the effectiveness of tumor vaccine preparations. However, survival studies require large numbers of animals and take a long time for completion. We, therefore, tested whether induction of CTL in vaccinated animals could be used as a surrogate end point for prescreening purposes. Our observations indicate that induction of tumor-specific CTL correlates poorly with the therapeutic benefit of cytokine-secreting vaccine preparations and question its prognostic value in the development of cytokine gene-modified tumor vaccines.

## MATERIALS AND METHODS

**Animals and Tumor Cells.** Female C3H/He mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Swiss *nu/nu* nude mice were obtained from a colony bred at Memorial Sloan-Kettering Cancer Center (New York, NY). MBT-2 cells were supplied by Dr. T. Ratliff (Washington University, St. Louis, MO). Cells were grown in tissue culture in RPMI 1640 supplemented with 10% fetal bovine serum, 200 mM L-glutamine, and 10 mM nonessential amino acids.

**Orthotopic Implantation of MBT-2 Cells into the Bladder of C3H Mice.** This procedure was performed as described previously (6). Briefly, animals were anesthetized by i.p. injection with pentabarbitol. Under magnification, a 0.8-cm incision was made transversely in the abdomen just above the pubis; the anterior abdominal wall muscles were incised and the bladder was delivered into the surgical field. Using a 1.0-ml tuberculin syringe, MBT-2 cells in 50 μl of PBS were injected into the bladder wall. The incision was closed in one layer using a 5.0 Prolene suture. The procedure was well tolerated and postoperative mortality was less than 5%.

**Retroviral Vector Design and Derivation of Cytokine-secreting MBT-2 Cell Lines.** All retroviral vectors used in this study were derived from the Moloney murine leukemia virus and are based on the high titer N2 retroviral vector, which also contains the bacterial neoselectable gene (13). cDNAs corresponding to cytokines were fused to various promoters present in the retroviral vector as shown in Fig. 1. N2/IL-2 was described previously (14). Mouse GM-CSF and IL-1β cDNAs were obtained from Genentech (San Francisco, CA) and mouse IL-1α cDNA was obtained from Hoffman La Roche (Nutley, NJ). The GM-CSF and IL-1β cDNAs were cloned into the pBC140 vector (15). The IL-1α cDNA was cloned into the DC/TK vector (6), and the IFN-γ cDNA was cloned into the DC/SV/R vector (16). Vector DNA was

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[3] The abbreviations used are: IL, interleukin; IFN, interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor; CTL, cytotoxic T-lymphocyte; PBS, phosphate-buffered saline; cDNA, complementary DNA; BSA, bovine serum albumin; NK, natural killer; i.d., intradermal.



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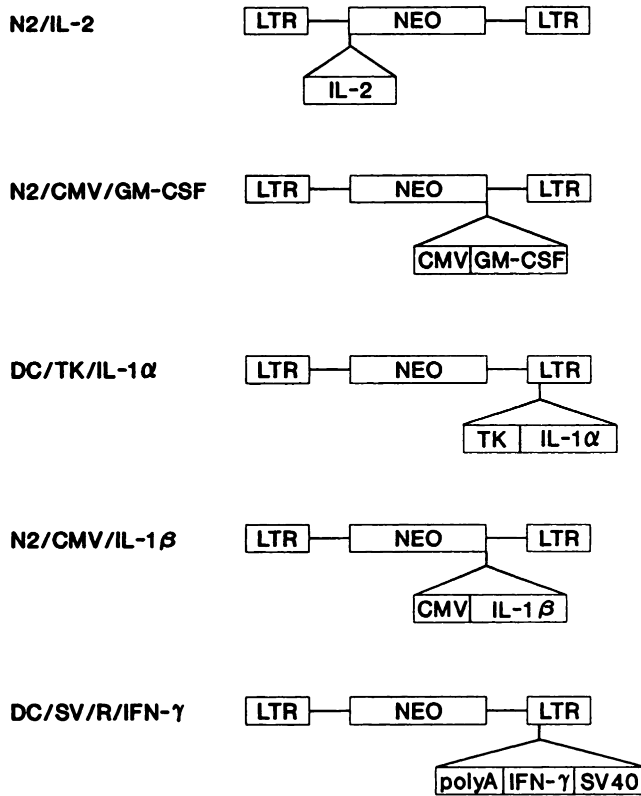


Fig. 1. Structure of retroviral vectors containing cytokine cDNA expression cassettes (for additional details see "Materials and Methods"). *LTR*, long terminal repeat; *CMV*, cytomegalovirus; *polyA*, polyadenylate; *SV*, simian virus; *TK*, thymidine kinase.

transfected into a packaging cell line (GP+envAM12), and virus-containing cell-free supernatant was used to infect MBT-2 cells. Clones stably transduced with vector DNA were isolated and the expression of each cytokine was determined by measuring secretion of the cytokine into the cell supernatant using a bioassay or enzyme-linked immunosorbent assay. Clones secreting the highest level of cytokine were chosen for further studies (measurements are given in units or ng/ml/$10^6$ cells/24 h): MBT-2/IL-2, 72 units; MBT-2/GM, 14 ng; MBT-2/IFN, 732 units; MBT-2/IL-1α, 0.28 unit; MBT-2/IL-1β, 0.32 unit. As a control cell line, we used MBT-2 cells transduced with the parental vector, N2A (17). Parental MBT-2 cells or cells transduced with the N2A vector (MBT-2/N2A) did not secrete detectable amounts of cytokines used in this study.

**Cytotoxicity and Europium Release Assay.** Two mice were immunized three times by i.p. injections of $5 \times 10^6$ irradiated (7000 rads) cell preparations. Fourteen days later, pooled splenocytes were isolated as described previously (14), except that a 5-day stimulation with irradiated tumor cells was substituted with a 3-day incubation in the presence of 10 units/ml of human recombinant IL-2. The nonadherent cells were collected and incubated with MBT-2 cells at various effectors to target ratios and cytotoxicity was determined in a 5-h europium diethylenetriamine pentaacetate release assay as described in detail by Volgmann *et al.* (18). Europium diethylenetriamine pentaacetate release was measured by time-resolved fluorescence (Delta fluorometer; Wallach, Inc., Gaithersburg, MD).

**Immunohistochemistry.** For immunohistological staining, slides of frozen tissue sections were fixed in cold acetone for 10 min, washed with PBS, then placed in a 0.1% hydrogen peroxide solution to remove background peroxidase activity in the tissue, washed again in PBS, and then incubated with biotin-conjugated primary monoclonal antibody diluted in 2% BSA/PBS. To stain for NK cells, rat anti-LGL-1 monoclonal antibody, a kind gift from Dr. Llewellyn Mason, was used at a 1:100 dilution, followed by peroxidase-conjugated mouse anti-rat IgG (Boehringer Mannheim). For staining with anti-macrophage (MØ), -CD4, and -CD8 antibody, slides were air dried for 1 h and fixed in 1% freshly prepared paraformaldehyde solution in PBS for 5 min. Slides were washed in PBS and then blocked for 15 min in avidin, washed, blocked for 15 min in biotin (Avidin/Biotin blocking kit; Vector Laboratories, Inc.), washed and blocked in 10% normal goat serum (Vector Laboratories, Inc.), diluted in PBS for 15 min, and washed, and then primary antibody was added. Purified rat IgG2b anti-mouse MØ (Mac I; Pharmingen) was used at 1:100 dilution in 2% BSA/PBS; purified rat IgG2a anti-mouse CD4 (clone RM-4-5; Pharmingen) was used at 1:50 dilution in 2% BSA/PBS; and purified rat IgG2a anti-mouse CD8α (clone 53-6.7; Pharmingen) and purified rat IgG1 anti-mouse CD8β (clone 53-5.8; Pharmingen) were mixed, each at a 1:50 dilution in 2% BSA/PBS. Tissue sections were incubated with antibody for 1 h at room temperature in a humid chamber. They were then washed in PBS and secondary antibody was applied for 30 min at room temperature. Biotinylated goat anti-rat IgG (Southern Biotec) at 1:200 dilution in PBS was used against the CD4 and CD8 antibodies. The sections were then washed in PBS, and streptavidin-horseradish peroxidase (Boehringer Mannheim) was applied at a 1:500 dilution in PBS for 1 hour at room temperature in a humid chamber. The tissues were then washed in PBS, developed in dimethylaminoazobenzene substrate for 10 min, washed, counterstained in hematoxylin for 1 minute, washed, dipped in acid-alcohol solution, washed, dipped in lithium solution, and washed, mounted, and coverslipped using Aquamount (Baxter Products). Antibody specificity was confirmed on sections of normal lymph node, spleen, and the RAW 264.7 macrophage cell line.

## RESULTS

MBT-2 cells were infected with retroviral vectors carrying the IL-2, IFN-γ, IL-1α, Il-1β, or GM-CSF expression cassettes; cytokine-secreting cell lines were isolated as described in "Materials and Methods" and Fig. 1. Tumors grew progressively in all animals given i.d. injections of $1 \times 10^5$ or more parental MBT-2 cells. MBT-2 cells secreting IFN-γ, IL-1α, IL-1β, or GM-CSF (MBT/IFN, MBT/IL-1α, MBT-2/IL-1β, or MBT-2/GM, respectively) grew as well, albeit more slowly, whereas IL-2-secreting cells (MBT-2/IL-2) were rejected. MBT-2/IL-2 cells also failed to grow in T-cell-deficient Swiss *nu/nu* mice, whereas the growth of MBT-2 cells secreting IFN-γ, IL-1α, IL-1β, or GM-CSF was unaffected (data not shown). Histochemical analysis has revealed that CD4+ as well as CD8+ T-cells were present at the site of injection of all cytokine-secreting tumor cells, whereas no T-cells could be seen at the site of injection of unmodified MBT-2 or MBT-2/N2A cells (Table 1). NK cells were found at the injection site of MBT-2/IL-2 cells, and macrophages were present at the site of injection of MBT-2/IFN and MBT-2/GM cells, as well as MBT-2 and MBT-2/N2A cells. It therefore appears that IL-2, but not the other cytokines tested, induced a cellular infiltrate which ultimately led to the rejection of the tumor cells. However, T-cells, which were present at the site of injection of IL-2-secreting MBT-2 cells, did not contribute to their rejection. It is therefore possible that NK cells, the presence of which was indicated by immunohistochemical analysis, may be responsible for the rejection of IL-2-secreting tumor cells.

**Treatment of Tumor-bearing Animals with Cytokine-secreting MBT-2 Cells.** Since induction of a systemic antitumor immune re-

Table 1 *Lymphocyte infiltration at the site of injection with cytokine secreting cells*[a]

| Cells | Lymphocytes | | | |
|---|---|---|---|---|
| | CD4+ | CD8+ | Mϕ | NK |
| Control | –[b] | – | + | – |
| N2A | – | – | + | – |
| IL-2 | + | + | – | + |
| GM-CSF | + | + | + | – |
| IFN-γ | + | + | + | – |
| IL-1α | + | + | – | – |
| IL-1β | + | + | – | – |

[a] Two weeks following the intradermal injection of $1 \times 10^6$ cytokine-secreting MBT-2 cells and control cells, the site of injection was resected and examined by hematoxylin and eosin or immunohistochemical staining.

[b] –, no cells of this type observed; +, infiltration of the particular type of lymphocytes was observed in the tumor specimen.



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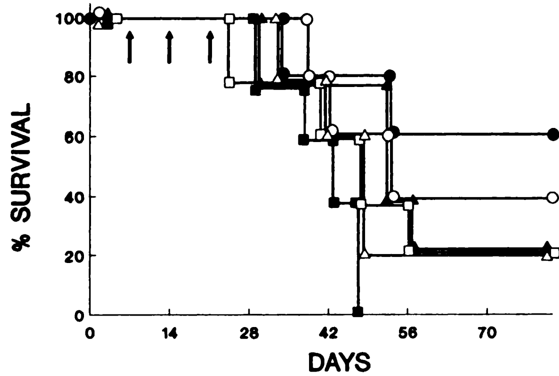


Fig. 2. Treatment of tumor-bearing mice with cytokine gene-modified MBT-2 cells. Tumors were established in the bladder of C3H mice by orthotopic implantation of $2 \times 10^4$ MBT-2 cells into the bladder wall of the animal. Vaccinations consisting of i.p. injections of $5 \times 10^6$ irradiated (7000 rads) MBT-2 cells were performed 7, 14, and 21 days post-tumor implantation (*arrows*). Mice were evaluated on a daily basis and were sacrificed when moribund. Each treatment group consisted of 5 mice. ■, MBT-2/N2A; ●, MBT-2/IL-2; ○, MBT-2/GM; □, MBT-2/IFN; △, MBT-2/IL-1α; ▲, MBT-2/IL-1β.

sponse in the mouse may or may not correlate with rejection of the cytokine-secreting tumor cells, the ability of tumor cell preparations to induce therapeutically beneficial immune responses in tumor-bearing animals was investigated by using tumor cells that were growth inactivated by X-irradiation. In the experiment shown in Fig. 2, tumors were established in the bladder by injecting $2 \times 10^4$ MBT-2 cells into the bladder wall as described previously (6). Tumor mass became palpable in all animals by day 14 and lung metastases were apparent on day 21. Left untreated, tumor-bearing mice would live no longer than 7 weeks. Histological examination in selected animals 7 days postimplantation has confirmed the presence of a vascularized tumor composed of cells resembling in appearance the inoculated MBT-2 cells (data not shown). Seven days post-tumor implantation, $5 \times 10^6$ irradiated, unmodified, and cytokine-secreting MBT-2 cells were injected i.p. into the tumor-bearing animals (5 animals/group). Injections of irradiated cells were repeated two additional times at weekly intervals (Fig. 2, *arrows*). Treatment of tumor-bearing mice with MBT-2 cells harboring the parental vector N2A had no measurable therapeutic benefit, since all animals died within 6–7 weeks posttumor implantation. On the other hand, as reported previously, IL-2-secreting tumor cells were highly effective in reducing tumor growth and led to complete tumor regression in 3 of 5 animals. In the other two mice, the original tumor continued to grow, albeit more slowly, and the mice eventually died. GM-CSF-secreting tumor cell preparations were also effective but less so than IL-2-secreting cells, inducing tumor regression in 2 of 5 animals (see below). The therapeutic benefit of IFN-γ-, IL-1α-, or IL-1β-secreting MBT-2 cells was more limited, leading to tumor regression in only 1 of 5 animals.

The limited therapeutic benefit of GM-CSF-secreting cells, as compared to IL-2-secreting cells, was somewhat unexpected in view of the findings of Dranoff *et al.* (7). However, the small number of mice used in each treatment group (5 animals/group) precluded a firm conclusion on this issue. To further evaluate the potential therapeutic benefit of IL-2- and GM-CSF-secreting tumor cell preparations, the experiment shown in Fig. 2 was repeated, except that each treatment group consisted of 20 mice. As shown in Fig. 3, whereas treatment of the tumor-bearing mice with MBT-2/IL-2 cells succeeded in "curing" 10 of 20 mice (50%), treatment with MBT-2/GM cells induced tumor regression in 7 of 20 mice (35%). This difference was not found to be significant ($P < 0.3$).

In a previous study, we have shown that animals cured of a preestablished MBT-2 tumor by treatment with IL-2-secreting tumor cells were highly resistant to a subsequent challenge with MBT-2 cells, indicating that immunological memory persisted in the cured animals. To confirm and extend these observations, the mice cured by treatment with IL-2- or GM-CSF-secreting MBT-2 cells as shown in Fig. 3 were implanted intravesically with a second dose of parental MBT-2 cells. As shown in Table 2, whereas 5 of 5 mice in the control group of age-matched C3H mice developed a tumor and eventually died, no tumors formed in animals cured by treatment with either IL-2-or GM-CSF-secreting MBT-2 cells.

**Induction of Tumor-specific CTL in Mice Vaccinated with Cytokine-secreting MBT-2 Cells.** To see whether the therapeutic benefit of cytokine-secreting MBT-2 cells correlates with the induction of tumor-specific CTL, mice were given i.p. injections three times at weekly intervals with parental or cytokine-secreting irradiated MBT-2 cells; 2 weeks after the last injection spleens were isolated and the presence of tumor-specific CTL was determined using a standard cytotoxicity assay. As shown in Fig. 4, IL-2- and GM-CSF-vaccinated animals induce high levels of tumor-specific CTL (65 and 73%, respectively; effector:target cell ratio, 200:1); IL-1α- and IL-1β-vaccinated animals induce moderate levels of CTL (37 and 41%, respectively; effector:target cell ratio, 200:1), and IFN-γ-vaccinated animals induce a low level of CTL (27%; effector:target cell ratio, 200:1), barely above the background of nonspecific lysis (13–14%). We have noted previously that high levels of cytotoxicity are generated in mice given injections of IFN-γ-secreting CMS-5 tumor cells. This difference can be attributed to the difference in the intrinsic tumorigenicity of the tumors, the poorly immunogenic MBT-2 tumor, and the highly immunogenic CMS-5 tumor. Specificity of lysis was indicated by the fact that neither animals given injections of vector DNA-harboring cells (N2A) nor untreated animals (control) exhibited appreciable cytotoxicity against MBT-2 cells; animals given injections of various cytokine-secreting MBT-2 cells did not exhibit cytotoxicity against another histoidentical tumor, 38C13 (data not shown).

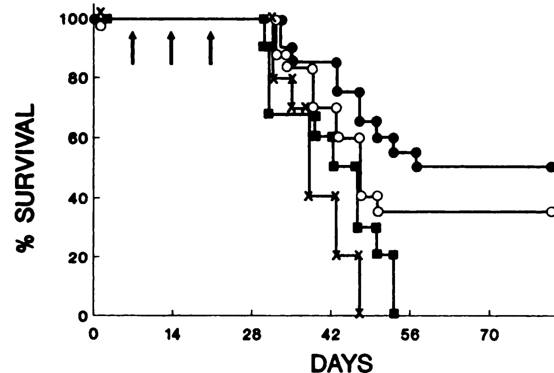


Fig. 3. Treatment of tumor-bearing mice with cytokine gene-modified tumor cells. Comparison between IL-2 and GM-CSF. Experimental design was as shown in Fig. 2, except that each treatment group with cytokine-secreting tumor cells consisted of 20 mice. ×, control mice; ■, MBT-2/N2A; ●, MBT-2/IL-2; ○, MBT-2/GM.

Table 2 *Challenge of C3H mice cured from their original tumor with parental MBT-2 cells*[a]

| Treatment | No. of mice with tumors/ No. of mice injected |
|---|---|
| IL-2 | 0/10 |
| GM-CSF | 0/7 |
| Age-matched controls | 5/5 |

[a] Mice cured by treatment with IL-2- or GM-CSF-secreting MBT-2 cells (Fig. 3) and 5 age-matched untreated mice were challenged with $2 \times 10^4$ MBT-2 cells orthotopically and followed for over 60 days.



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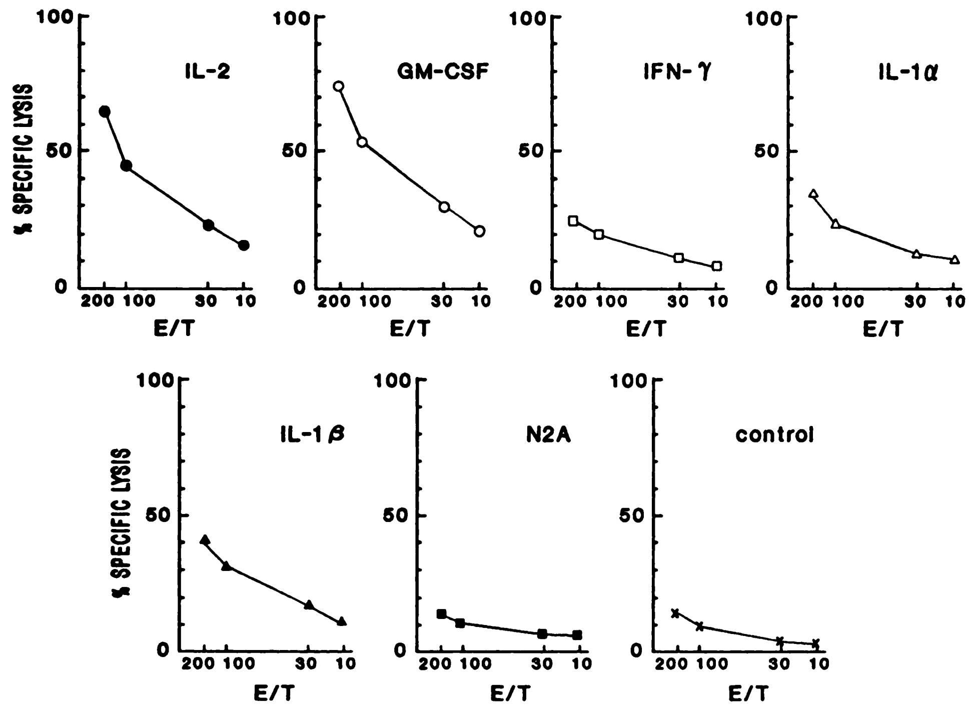


Fig. 4. Induction of tumor-specific cytotoxic cells in animals vaccinated with cytokine-secreting MBT-2 cells. C3H mice were inoculated i.p. with unmodified or vector-transduced, irradiated MBT-2 cells. Fourteen days later, pooled splenocytes from 2 animals were aseptically removed and cytotoxicity was determined in a 5-h europium diethylenetriamine pentaacetate release assay as described in "Materials and Methods." Unmodified MBT-2 cells were used as targets. *E/T*, effector:target cell ratio.

## DISCUSSION

Systemic administration of cytokines to cancer patients has had limited therapeutic benefit, in part due to unacceptable levels of toxicity resulting from the administration of high doses of cytokine to the patient. Use of cytokine-secreting tumor cells was designed to circumvent this limitation by providing the desired cytokine in a concentrated form at the vicinity of the tumor cells which encode the putative tumor antigens against which an immune response is induced. The utility of cytokine gene-modified tumor cells as vaccines was first indicated in studies showing that s.c. injected cytokine-secreting tumor cells were rejected and induced a state of immunity that protected the animal from a subsequent challenge with unmodified tumor cells (2). Nevertheless, the use of live tumor cells as vaccines and vaccination of healthy rather than tumor-bearing animals has limited relevance to cancer patients. More recent reports that cytokine gene-modified tumor cells used in an irradiated form had considerable therapeutic benefit in tumor-bearing animals are therefore encouraging (3–7). We have previously shown that treatment of mice with IL-2-secreting irradiated MBT-2 cells is capable of curing mice bearing an orthotopically implanted tumor and that immunological establishment of immune memory persisted in the cured animals (6).

In a comparative study, Dranoff *et al.* (7) have shown that GM-CSF-secreting tumor vaccines provided the best protection to mice implanted s.c. with B16.F10 melanoma tumor cells, whereas other cytokines had little if any therapeutic benefit (7). In this study using the MBT-2 bladder tumor model, we found that whereas GM-CSF had considerable therapeutic benefit, IL-2-secreting vaccines provided equal or better protection in tumor-bearing animals. In yet another study, Vieweg *et al.* have observed that IL-2-secreting vaccines were superior to GM-CSF-secreting vaccines in a rat model for prostate cancer (19). The differences between our observations and the observations of Dranoff *et al.* (7) may reflect differences in the susceptibility and response of the B16.F10 melanoma and MBT-2 bladder tumors to various cytokines secreted from the irradiated tumor cells. It is possible, although unlikely, that the observed differences were of a technical nature, since the MBT-2 cells did secrete GM-CSF in a range reported to be effective in the B16.F10 model and since mice vaccinated with the GM-CSF-secreting MBT-2 cells induced a potent CTL response (Fig. 4). Our observations, taken together with the study of Dranoff *et al.* (7), highlight the possible variability among tumors in their response to cytokines secreted from the genetically engineered cells and caution against premature conclusions as to which cytokine is most effective in the context of a tumor vaccination protocol.

Survival of tumor-bearing animals treated with various tumor vaccine preparations constitutes a reliable measure for their therapeutic benefit. Nevertheless, the length of time required for the completion of each experiment and the need to use large cohorts of animals hamper speedy progress in the development of improved vaccination strategies. Clearly, a simpler end point that could be used for prescreening purposes would be highly desirable. Since it is well documented that CTLs constitute an important effector arm in the antitumor immune response, a possibly useful end point would be to measure the induction of CTL responses in animals treated with various tumor vaccine preparations. In this study, we have measured the presence of tumor-specific CTL in mice vaccinated with cytokine-secreting tumor vaccines and found only a partial correlation between CTL induction and therapeutic benefit. IFN-γ and IL-1α or IL-1β induced a low to intermediate level of CTL and exhibited a low index of therapeutic benefit, whereas IL-2- or GM-CSF-secreting tumor cells exhibited a significant therapeutic benefit and induced high levels of CTL. Nevertheless, GM-CSF-secreting vaccines reproducibly induced higher levels of CTL than IL-2-secreting vaccines, which was not predictive of the fact that their therapeutic benefit was inferior to that of IL-2-secreting vaccines. Therefore, our studies cannot recommend the use of CTL induction as an alternative end point to assess the therapeutic benefit of cytokine gene-modified tumor vaccines. This conclusion is consistent with and reinforces previous observations that question the reliability of *in vitro* cytotoxicity assays as predictors of therapeutic efficacy *in vivo* (20, 21).



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