Regression of Bladder Tumors in Mice Treated with Interleukin 2 Gene–Modified Tumor Cells

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Summary

This study explored the use of interleukin 2 (IL-2) and interferon γ (IFN-γ) gene–modified tumor cells as cellular vaccines for the treatment of bladder cancer. The mouse MBT-2 tumor used 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 its human counterpart. Using retroviral vectors, the human IL-2 and mouse IFN-γ genes were introduced and expressed in MBT-2 cells. The tumor-forming capacity of the cytokine gene–modified MBT-2 cells was significantly impaired, since no tumors formed in mice injected intradermally with either IL-2- or IFN-γ-secreting cells, using cell doses far exceeding the minimal tumorigenic dose of parental MBT-2 cells. Furthermore, mice that rejected the IL-2- or IFN-γ-secreting tumor cells became highly resistant to a subsequent challenge with parental MBT-2 cells, but not to 38C13 cells, a B cell lymphoma of the same genetic background. To approximate the conditions as closely as possible to the conditions prevailing in the cancer patient, inactivated cytokine-secreting cells were used to treat animals bearing tumors established by orthotopic implantation of MBT-2 cells into the bladder wall of the animal. Treatment of mice carrying a significant tumor burden with IL-2-secreting MBT-2 cells had a significant inhibitory effect on tumor progression with extended survival. Moreover, in 60% of the mice the tumor regressed completely and the animals remained alive and free of detectable tumor for the duration of the observation period. Treatment of tumor-bearing animals with IL-2-secreting MBT-2 cells was superior to the use of cisplatin, a chemotherapeutic agent used in the treatment of bladder cancer. The therapeutic effect of IFN-γ-secreting cells was minimal and treatment with unmodified MBT-2 cells had no effect on tumor growth or survival, showing that the parental MBT-2 cells were nonimmunogenic in this experimental setting. Most importantly, mice that exhibited complete tumor regression after treatment with IL-2-secreting MBT-2 cells became resistant to a subsequent challenge with a highly tumorigenic dose of parental MBT-2 cells, indicating that long-term immunological memory was established in the "cured" mice.

Metastasis of malignant neoplasms is responsible for most therapeutic failures in clinical oncology (1). Active immunization protocols directed against the patient's own cancer cells may constitute an effective and minimally invasive treatment modality for cancer patients whose primary tumor has been successfully treated. Clinical studies using tumor vaccines consisting of irradiated tumor cells or fractions obtained from tumor cells have on occasion shown clinical responses or prolongation of disease-free interval and survival, but in general have shown little therapeutic effect and have failed to provide a solid foundation for future progress (2).

The identification of soluble factors as modulators of immune responses has led to their incorporation in experimental protocols designed to augment antitumor immune responses. For example, systemic administration of IL-2, alone or in combination with other treatments, had profound inhibitory effects on tumor progression in the experimental animal but exhibited only limited therapeutic benefit when administered to cancer patients (3, 4). The limited efficacy of IL-2 in cancer immunotherapy is explained at least in part by the toxicity resulting from the necessity to administer high doses of the cytokine to the patient (5).

Since most immunomodulators act as local hormones whose accumulation in the serum is prevented by virtue of their short half-life, it was argued that local delivery of cytokines would have a pronounced therapeutic benefit (6, 7). Several studies have attempted to induce the regression of established tumors or to induce immunological memory by repeated injections of cytokines at the site of the tumor (6–10). Overall, these studies have failed to demonstrate a distinct advantage for

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this mode of cytokine delivery, perhaps because it did not approximate closely enough the physiological rate of cytokine secretion required to elicit an optimal immune response. Another approach that would result in the highly localized secretion of cytokines at the site of the tumor would consist of inserting cytokine genes into tumor cells (11, 12). Recent studies have shown that genetically engineered tumor cells expressing cytokines such as IL-2 (13–15), IFN-γ (16–18), IL-4 (19), IL-6 (20), IL-7 (21), or TNF (22) could immunize mice against a subsequent challenge with parental tumor cells.

Many of the experimental systems used to evaluate the effectiveness of cytokine gene–modified tumor cells as cancer vaccines suffer from drawbacks that limit their relevance to human cancer: (*a*) use of animal tumor models that bear little, if any, similarity to human cancer; (*b*) use of live rather than inactivated cells in immunization protocols, a fact also precluding the direct determination of the intrinsic immunogenicity of the tumor cells used in the study; (*c*) immunization of healthy animals against a subsequent challenge with parental tumors, rather than treatment of tumor-bearing animals with the cytokine-secreting cells and demonstration of persistence of immunological memory in the cured animals; (*d*) heterotopic, rather than orthotopic, implantation of tumor cells; (*e*) measuring the growth of the primary tumor rather than measuring suppression of metastasis derived from the implanted tumor; (*f*) lack of controlled comparison to an established vaccination or treatment protocol.

Using an increasingly relevant animal model, Golumbek et al. (19) have shown that IL-4-expressing RENCA cells derived from a spontaneously arising renal cell carcinoma were capable of curing mice with a preestablished, albeit small, tumor burden, and that a fraction of the cured mice were also resistant to a subsequent challenge with parental tumor cells. More recently, Porgador et al. (18, 20) have shown that treatment of mice with IL-6 or IFN-γ gene–modified, irradiated, tumor cell preparations derived from a Lewis lung carcinoma clone (D122) was capable of suppressing the metastatic spread of a preestablished tumor, leading to the complete cure of a significant fraction of treated animals.

In this study we used a mouse tumor model to develop a cytokine gene–modified tumor vaccination strategy for the treatment of bladder cancer. Epidemiological studies support the notion that transitional cell carcinoma, the most common form of bladder cancer, may result from exposure to external carcinogens (23). Although transurethral resection followed by intravesical Bacillus Calmette-Guerin (BCG)[1] treatment will result in prolonged remission, a significant fraction of patients will eventually relapse locally and/or progress to metastatic disease (24). Some patients with metastatic disease respond to cytotoxic chemotherapy such as methotrexate, inblastin, adriamycin, and cisplatin, or cisplatin plus radiotherapy (reviewed in reference 23). However, most complete responders will eventually relapse and die. Because of the serious limitations of available therapy of bladder cancer, new approaches are clearly needed. Bladder cancer, especially presenting as superficial disease, is responsive to immunotherapeutic agents such as BCG (24), and may represent a good candidate for immunological intervention using tumor vaccines.

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 to the treatment of bladder cancer. The MBT-2 tumor was induced by the oral administration of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide (FANFT), a potent carcinogen inducing bladder neoplasms in 80–90% of mice, and is highly specific for the urothelium (25). This highly malignant tumor retains the histological appearance of a poorly differentiated transitional cell carcinoma and resembles, both grossly and histologically, its human counterpart. The MBT-2 is considered a useful model, as treatments that have shown promise in this murine model have been similarly effective in human bladder cancer (23, 26).

Looking toward the development of effective tumor vaccines for the treatment of bladder cancer, we assessed the ability of IL-2 or IFN-γ gene–modified MBT-2 cells to induce a state of immunity in the mouse against parental unmodified tumor cells. In this study we have shown that irradiated IL-2, but not IFN-γ, gene–modified MBT-2 cells were capable of curing mice from a significant burden of parental tumor implanted orthotopically into the bladder wall. Moreover, mice cured from their existing tumors were resistant to a later challenge with a highly tumorigenic dose of parental unmodified MBT-2 cells.

Materials and Methods

A 528-bp-long DNA fragment encoding the human IL-2 cDNA was obtained from the plasmid pBCll/RSV/ΔT (27) by digestion with restriction enzymes BamHI and HindIII. A 620-bp-long DNA fragment encoding the mouse IFN-γ cDNA was obtained from the plasmid PBRMuIFNg by digestion with restriction enzymes Sau3AI and SspI (28). A 852-bp-long DNA fragment encoding the herpes simplex virus (HSV) thymidine kinase (TK) promoter was obtained from plasmid PHSV106 (29) by digestion with restriction enzymes BamHI and BglII. A 794-bp-long DNA fragment encoding the major immediate early human CMV promoter was obtained from plasmid pRR23 (30) by digestion with restriction enzymes BalI and SmaI. N2 is a retroviral vector derived from the genome of Moloney murine leukemia virus (MoMLV), containing the bacterial neomycin resistance (*neo*) gene, which is used as a selectable marker (31). A schematic diagram presenting the various vector constructs used in these studies is shown in Fig. 1. Vector N2/IL-2 was constructed by cloning the IL-2 cDNA into a unique BclI site present upstream from the initiation codon of the *neo* gene, generating a bicistronic transcriptional unit. The TK promoter–encoding DNA fragment was fused to the IL-2 cDNA and cloned into a SnaBI site present in the 3′ LTR of a modified N2 vector (32) to generate vector construct DC/TK/IL-2. The prefix DC is short for double copy, which describes this vector design, where the foreign gene is inserted into the 3′ LTR of the retroviral vector. For more details on DC vectors see Hantzopoulos et al. (32). The CMV promoter–containing DNA fragment was fused to the IFN-γ cDNA fragment and cloned into a unique XhoI site present downstream from the *neo* gene coding sequences to

[1] *Abbreviations used in this paper:* BCG, Bacillus Calmette-Guerin; DC, double copy; TK, thymidine kinase.

generate vector N2/CMV/IFNg, and the TK promoter–IFN-γ cDNA fusion product was cloned into the SnaBI site present in the 3′ LTR of a modified N2 vector to generate vector DC/TK/IFNg. DCA is a previously described vector in which the human ADA minigene was cloned into the 3′ LTR of the N2 vector (32). Retroviral vector constructs were converted into corresponding virus by transfection into the helper-free amphotropic packaging cell line GP+envAM12 (33). G418-resistant colonies were pooled, and virus-containing cell-free supernatant was used to infect MBT-2 cells in the presence of 8 μg/ml polybrene. Clonal derivatives of MBT-2 cells were isolated by G418 selection, expanded to cell lines, and secretion of IL-2 or IFN-γ into the cell supernatant was measured. Absence of replication-competent virus in the cytokine-producing MBT-2 cells was shown by the inability to transfer G418 resistance to NIH 3T3 cells.

Cytokine Assays. Supernatant from 2 × 10^6 semiconfluent cells in a 6-cm plate were collected after 48 h and assayed for the presence of human IL-2 or mouse IFN-γ. IL-2 activity was determined using IL-2-dependent human primary lymphoblasts in a proliferation assay as previously described (34). IFN-γ activity was measured using a bioassay based on its antiviral activity as determined by the reduction of the cytopathic effects of vesicular stomatitis virus on L cells (35).

Tumor Cell Lines and Animal Studies. The transplantable FANFT-induced MBT-2 tumor (25) was obtained from Dr. T. Ratliff (Washington University, St. Louis, MO). MBT-2 cells were grown in vitro in RPMI supplemented with 10% FCS (Hyclone Labs, Logan, UT) and 2 mM L-glutamine. 6–8-wk-old C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Tumor cell injections were done using freshly prepared tumor cells removed from culture plates by trypsinization, washed twice in PBS, and resuspended in PBS at a concentration of 10^7 cells/ml. When indicated, cells were inactivated by irradiation (7,000 rad delivered at a rate of 86 rad/min, using a ^{137}Cs source animal irradiator [Gamma Cell-40; Nordion International, Kanato, Ontario, Canada]).

Orthotopic Implantation of MBT-2 Cells into the Bladder Wall of C3H Mice. A detailed description of the procedure will by provided elsewhere (Connor, J., W. Heston, E. Gilboa, and W. Fair, manuscript in preparation). Briefly, animals were anesthetized by intraperitoneal injection of 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 delivered into the surgical field. Using a 1.0-cc 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 <5%. Tumors grew at the site of inoculation in all animals injected intravesically with 10^4 or more MBT-2 cells, and metastases in the lung became apparent 3 wk posttumor inoculations.

Results

Generation of MBT-2 Cell Lines Expressing IL-2 and IFN-γ. Fig. 1 shows the retroviral vector constructs used to introduce and express the human IL-2 and mouse IFN-γ genes in MBT-2 cells. The retroviral vectors used in this study were derived from MoMLV and are based on the high-titer N2 retroviral vector (31), which also contains the bacterial *neo*-selectable gene. Vector DNA was transfected into a packaging cell line (GP+envAM12), and virus-containing cell-free supernatant

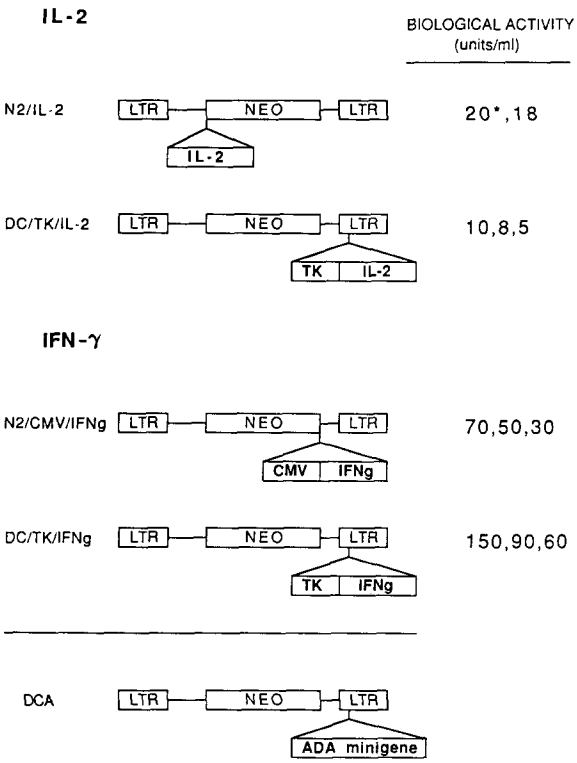

Figure 1. Structure of retroviral vectors containing the human IL-2 or the mouse IFN-γ cDNAs, and cytokine production in MBT-2 cells. For details on retroviral vector design, see Materials and Methods. Amount of IL-2 or IFN-γ secreted by representative clones derived from MBT-2 cells transduced with the cytokine gene–containing vector is shown (see Materials and Methods). Parental MBT-2 cells or MBT-2 clones transduced with the DCA vector did not secrete detectable levels of either cytokine. *MBT-2 clones secreting IL-2 or IFN-γ used in subsequent studies, referred to in the text as MBT/IL-2 or MBT/IFN-γ, respectively.

was used to infect MBT-2 cells. Clones stably transduced with vector DNA were isolated, and expression of the IL-2 or IFN-γ genes was determined by measuring the secretion of the cytokines into the cell supernatant using a bioassay for each cytokine (for additional details, see Materials and Methods and references 14, 17, and 32). The amount of biologically active IL-2 or IFN-γ secreted from representative clones for each retroviral vector is listed in Fig. 1. Clones secreting the highest level of each cytokine, denoted by an asterisk in Fig. 1 and referred to in the text as MBT/IL-2 and MBT/IFN-γ, were chosen for further studies. Secretion of IL-2 or IFN-γ had no discernable effects on cell morphology or on the growth rate of the MBT-2 cells in culture when compared to parental MBT-2 cells or MBT-2 cells transduced with another retroviral vector encoding the human ADA minigene (DCA). Parental MBT-2 cells or clones transduced with the control DCA vector did not secrete detectable levels of either IL-2 or IFN-γ.

Tumorigenic Potential of IL-2- or IFN-γ-secreting MBT-2 Cells. Tumors grew progressively in all animals injected intradermally with 10^4 or more parental MBT-2 cells, or with MBT-2 cells transduced with the control DCA retroviral vector. Conversely, injection of up to 10^5 MBT/IL-2 or 2.5 × 10^5 MBT/IFN-γ cells failed to grow in the animal. Thus,

Table 1. *Intravesical Installation of MBT-2 Cells in Mice Immunized with IL-2 or IFN-γ Gene-modified Cells*

Exp.	Injection*	No. of parental MBT-2 cells instilled intravesically‡	No. of mice with tumors per number of mice injected
1	MBT/IL-2	2×10^4	0/5
	MBT/IL-2	4×10^4	0/5
	MBT/IL-2	8×10^4	0/5
	MBT/IL-2	1.6×10^5	0/5
	MBT/IL-2	3.2×10^5	0/5
	Medium	2×10^4	5/5
2	MBT/IFN-γ	4×10^4	0/5
	MBT/IFN-γ	8×10^4	0/5
	MBT/IFN-γ	2×10^5	0/5
	MBT/IFN-γ	4×10^5	0/5
	Medium	2×10^4	5/5

* 10^5 MBT/IL-2 or 1.5×10^5 MBT/IFN-γ viable cells were injected intradermally.
‡ 3 wk post injection (intradermal) of cytokine-secreting cells.

local secretion of IL-2 or IFN-γ from the genetically modified cells abrogated their tumorigenicity in vivo. Spleen cells derived from mice that rejected the IL-2- or IFN-γ-secreting tumor cells exhibited cytotoxicity in vitro against parental MBT-2 cells but not against unrelated target (data not shown), consistent with previous studies showing that a CTL response was induced by the cytokine gene-modified tumor cells (13–15, 17–20).

IL-2-secreting MBT-2 Cells Protect Mice from a Subsequent Challenge with Parental, Unmodified Tumor Cells. In general, cytokine-secreting tumor cells failed to grow in the syngeneic host, albeit only in a number of cases were they also capable of immunizing mice against the parental tumor (13–22). We therefore examined the ability of IL-2- or IFN-γ-secreting MBT-2 cells to induce a protective immune response against parental, unmodified MBT-2 cells. In the experiment summarized in Table 1, mice were injected intradermally with either medium alone, with 10^5 live MBT/IL-2, or with 1.5×10^5 live MBT/IFN-γ cells. 3 wk later mice were challenged with increasing doses of parental MBT-2 cells, and the appearance of a tumor at the site of injection was monitored. In previous studies assessing the immunogenicity of cytokine gene-modified tumor cells, the tumor challenge was implanted at ectopic sites, usually under the skin or intradermally. It is, however, well documented that tumor cells outside their natural milieu behave quite differently than in their organ of origin (36). To reproduce the environment that exists in human metastatic bladder cancer, we developed a method to implant MBT-2 cells into the bladder wall of the mouse (intravesical instillation). This technique, which consists of a simple surgical procedure, is both easy and reproducible and will be described in detail elsewhere (Connor et al., manuscript in preparation). In the animals that underwent intravesical instillation of MBT-2 cells, tumors formed significantly faster, metastasized earlier, and were fatal much more quickly than in animals that had intradermal implantation of cells.

As shown in Table 1, tumors grew in all control animals inoculated through intravesical instillation with 2×10^4 MBT-2 cells. On the other hand, if mice were first injected intradermally with IL-2- or IFN-γ-secreting cells, no tumors formed even at very high challenge doses. The specificity of the protection was indicated by the fact that the growth of an unrelated tumor (38C13, a B cell lymphoma of the same genetic background) was not affected (data not shown).

IL-2- and IFN-γ-secreting MBT-2 Cells Affect the Course of Disease in Tumor-bearing Animals. To test the effectiveness of cytokine gene-modified tumor vaccines under conditions that would approximate the conditions prevailing in the cancer patient, we sought to determine whether IL-2- or IFN-γ-expressing MBT-2 cells are capable of affecting the course of disease in tumor-bearing animals. Since the use of live tumor cells as vaccines is undesirable, the cytokine-secreting cells were inactivated by X-irradiation. Immunization with inactivated cell preparations also enabled us to determine the intrinsic immunogenicity of the unmodified MBT-2 cells.

In the experiment shown in Fig. 2, tumors were established in the bladder of mice by intravesical instillation of 2×10^4 MBT-2 cells. 7 d postimplantation of tumor cells, 5×10^6 irradiated unmodified MBT-2 cells, or irradiated cytokine-secreting cells, were injected intraperitoneally in the tumor-bearing animals. Existence of tumors in all animals could be established at that time by palpation, and histological examination in selected animals has confirmed the presence of a vascularized tumor composed of cells resembling in appearance the inoculated MBT-2 cells. Injections of irradiated cells were repeated three additional times at weekly intervals (Fig. 2 *A*, *arrows*). Treatment of mice with irradiated parental MBT-2 cells had no effect on average tumor size (Fig. 2 *A*), and animals in this group and the untreated group died within 5–6 wk post-intravesical instillation of MBT-2 cells (Fig. 2 *B*). On the other hand, treatment of the tumor-bearing mice with irradiated IL-2-secreting MBT-2 cells had a significant inhibitory effect on tumor progression. Moreover, in three of five mice the tumor regressed completely, and the animals remained free of detectable tumor >8 wk, at which time they were rechallenged with MBT-2 cells (see below). In the other two mice the original tumors continued to grow, albeit more slowly, and the mice eventually died. Treatment of mice with IFN-γ-secreting cells had a less pronounced effect both on tumor growth and survival. Combined treatment with both IL-2- and IFN-γ-secreting cells was not additive.

Cisplatin is the most active single chemotherapeutic agent used in the treatment of bladder cancer (23). Cisplatin exhibited a modest antitumor effect in the MBT-2 model (25, 26). It was therefore of interest to compare side by side the effectiveness of treatment with IL-2-secreting MBT-2 cells and cisplatin in mice carrying an established tumor. In the experiment shown in Fig. 3, mice were treated with cisplatin once, 7 d post-intravesical instillation of MBT-2 cells. Treat-

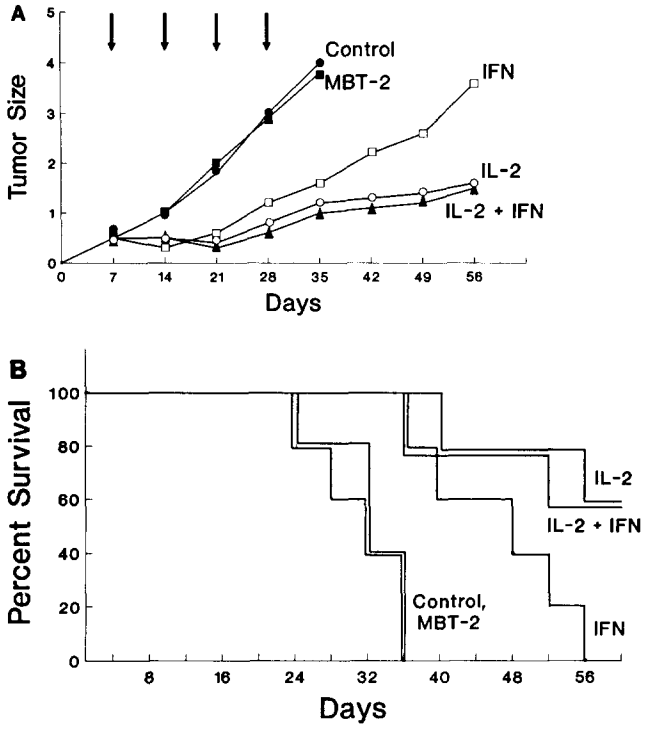

Figure 2. Treatment of tumor-bearing mice with cytokine gene–modified, X-irradiated MBT-2 cells. Tumors were established in the bladder of C3H mice by intravesical instillation of 2 × 10^4 MBT-2 cells. 7 d later, animals (five mice per group) were injected intraperitoneally with 5 × 10^6 X-irradiated cells that consisted of either unmodified MBT-2 cells, MBT/IL-2 cells, MBT/IFN-γ cells, or a combination of both MBT/IL-2 and MBT/IFN-γ cells. Injections were repeated weekly for a total of four times, as indicated by the arrows. (*Control*) Mice injected with PBS. (*A*) The origin and irregular shape of the bladder-induced tumors prevented an accurate measure of tumor size. Tumors were therefore graded on a relative scale of 0–5: 0, no tumor; 0.5, palpable but not visible tumor; 1.0, small visible tumor; 2–4, increasingly larger tumors; and 5, abdomen full of tumor with erosion through the skin. Mice were killed when moribund. Average tumor size was calculated for each group of five or remaining mice. (*B*) Survival of mice >60 d correlated with complete tumor regression.

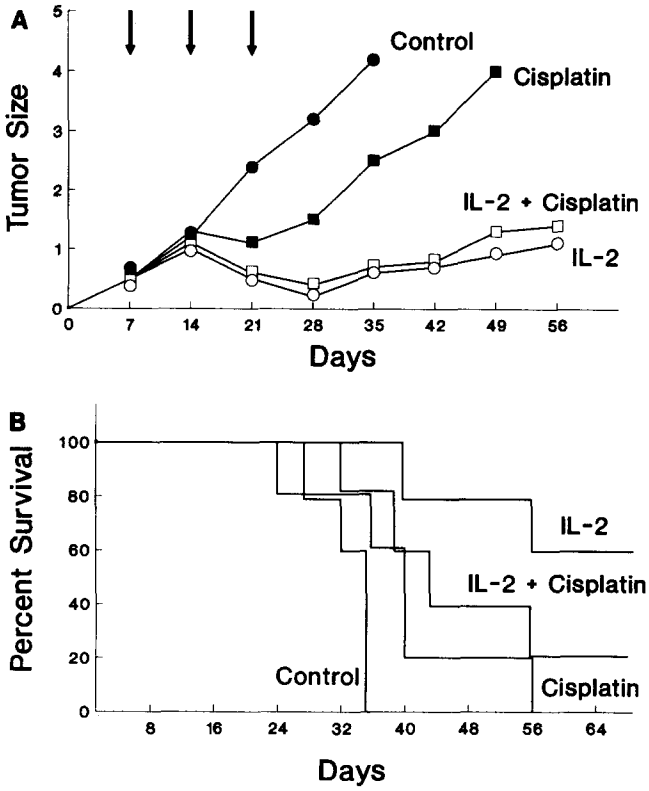

Figure 3. Treatment of tumor-bearing mice with cisplatin and X-irradiated IL-2 gene–modified MBT-2 cells. Tumors were established in the bladders of C3H mice by intravesical instillation of 2 × 10^4 MBT-2 cells. Treatment with cisplatin was performed once, at day 7. Cisplatin (*cis*-diamine dichloroplatinum; Bristol Laboratories, Evansville, IN) was dissolved in sterile H_2O and injected intraperitoneally using a dose of 6 mg/kg body weight in a total volume of 0.1 cm^2, as previously described (26). Intraperitoneal injections of X-irradiated MBT/IL-2 cells were initiated at day 14 and injections were repeated at weekly intervals twice more, as indicated by the arrows. Complete tumor regression occurred in mice that survived >60 d (*B*). Five mice were used in each treatment group.

ment with irradiated IL-2-secreting cells was initiated 14 d after establishment of the tumor, and repeated twice more at weekly intervals, as indicated. At the commencement of treatment with IL-2-secreting cells (day 14), the primary tumors were clearly visible to the naked eye, and since macrometastases can be detected in the lung 1 wk later, i.e., 3 wk post–intravesical instillation inoculation of tumor cells, it is reasonable to assume that micrometastases were already present in the lung at the time when treatment with IL-2-secreting cells was initiated. As previously noted, while cisplatin had a modest effect on tumor growth and survival (25, 26), treatment with IL-2-secreting tumor cells had a dramatic effect on tumor growth, leading to complete regression of the 14-d-old visible tumors in three of five animals, which remained free of tumor >9 wk (at which time they were again inoculated with MBT-2 cells, see below). The decreased effectiveness of combined treatment with cisplatin (given on day 7) and IL-2-secreting cells (initiated on day 14) could be attributed to toxic effects of cisplatin.

Since a significant proportion of tumor-bearing mice were apparently cured by treatment with IL-2-secreting MBT-2 cells, it was of interest to test whether such mice would be protected from a second challenge with a highly tumorigenic dose of parental MBT-2 cells. When mice exhibiting long-term tumor regression were rechallenged via intravesical instillation with parental MBT-2 cells, no tumor growth was observed in nine of nine mice tested. By contrast, five of five age-matched control mice inoculated with fivefold less MTB-2 cells developed a tumor at the site of inoculation (Table 2). This result indicates that immunological memory was established in the tumor-bearing mice treated with inactivated IL-2-secreting cells.

Discussion

Although recent studies have shown that cytokine gene–modified tumor cells are capable of immunizing mice against the parental tumor, the choice of the animal model and/or the experimental design used have often limited their

relevance to human cancer. The emphasis in the current study was to test the effectiveness of cytokine gene–modified tumor cells as cellular vaccines using an animal model and an experimental design that approximates as closely as possible the conditions prevailing in the cancer patient. The MBT-2 mouse tumor model used in this work is an excellent model for human bladder cancer, not only because this carcinogen-induced tumor of bladder origin resembles in its etiology and histology bladder cancer in humans, but also because the MBT-2 tumor responds to treatment in a manner similar to its human counterpart (23, 26).

In this study we have shown that intradermal injection of IL-2 gene–modified MBT-2 cells into tumor-bearing animals is capable of curing mice with a considerable tumor burden (Figs. 2 and 3). Most importantly, the mice cured of their tumor became resistant to a subsequent challenge with a very high dose of parental MBT-2 cells, indicating that immunological memory persisted in the cured mice (Table 2). Since recurrence of metastatic, and in some cases local, disease is the major cause of death in cancer patients, this observation suggests that cytokine gene–modified tumor vaccines could provide protection to the cancer patient (in remission) against minimal residual disease.

The effectiveness of cytokine gene–modified tumor vaccines in tumor-bearing animals was demonstrated in recent studies. Golumbek et al. (19) have shown that live IL-4-expressing RENCA cells (a renal cell carcinoma cell line) were capable of curing mice from a small burden of tumor, and that 50% of the cured mice were resistant to a subsequent challenge with parental tumor cells. Porgador et al. (18, 20) have shown that treatment of mice with inactivated IL-6 or IFN-γ-expressing D122 cells (a Lewis lung carcinoma–derived cell line), but not unmodified tumor cells, had a significant inhibitory effect on preestablished lung micrometastases, and led to the complete cure of a significant proportion of mice.

Additional features of this experimental system increase its relevance to human cancer. In this study cytokine-secreting cells inactivated by irradiation were used to treat tumor-bearing animals. Previous studies, with the notable exception of those by Porgador et al. (18, 20), have used live cytokine-secreting cells as immunogens. Ths use of live tumor cells as cellular vaccines in cancer patients is of course undesirable. In animal models, live cells could be used to immunize because cytokine-secreting cells lose their in vivo growth potential, i.e., tumorigenicity. In studies in which live cytokine-secreting cells were used as immunogens, the intrinsic immunogenicity of the tumor cells could not be determined, and therefore it was unclear whether the cytokine contributed to the induction of antitumor immunity or whether its only function was to inhibit tumor growth in the mouse. In view of the fact that spontaneously arising tumors are apparently nonimmunogenic (although bladder tumors may be considered weakly immunogenic since they respond to treatment with BCG [24]), the use of even weakly immunogenic tumor cells in animal models is undesirable (37). Using irradiated tumor cells, we could show that unmodified MBT-2 cells were nonimmunogenic in the experimental setting used and that the protective effect seen in tumor-bearing animals could be attributed to the action of IL-2 (Fig. 2). It is interesting to note in this regard that IFN-γ was more effective than IL-2 in reducing the tumorigenicity of MBT-2 cells (up to 2.5×10^5 IFN-γ-secreting cells injected intradermally did not form a tumor compared with only 1.0×10^5 IL-2-secreting cells). Nevertheless, irradiated IL-2-secreting MBT-2 cells were superior to IFN-γ-secreting cells in the treatment of tumor-bearing mice (Fig. 2). This is a clear indication that different cellular mechanisms are responsible for the observed antitumor effect and the immunogenic potential of the cytokine-expressing tumor cell.

Another important feature of this experimental system was that the parental MBT-2 tumor cells were implanted orthotopically into the mouse bladder. It is well recognized that the site of implantation of the tumor can greatly influence its properties and that orthotopic implantation represents a more accurate model to follow in vivo behavior (36). Indeed, MBT-2 cells injected intradermally or subcutaneously grow slowly and metastasize poorly. In contrast, when injected into the bladder wall of the mouse, they grow faster and metastasize extensively to the lung, mimicking more closely the behavior of metastatic human bladder cancer.

To assess the effectiveness of a new treatment strategy, comparison with an established treatment protocol is highly informative. We therefore compared side by side the effectiveness of IL-2 gene–modified MBT-2 cells with that of cisplatin, a commonly used chemotherapeutic agent used in the treatment of cancer, including bladder cancer (23). The superior effect elicited by IL-2 gene–modified MBT-2 cells compared with that of cisplatin is evident (Fig. 3), further recommending for consideration this form of immunotherapy for the treatment of bladder cancer.

Although the use of tumor-bearing mice as subjects of immunological intervention enhances the relevance of this animal model to human cancer, initiation of treatment with IL-2-secreting MBT-2 cells shortly after establishment of the parental tumor in the bladder of the experimental mouse fails to take into account the fact that cancer patients generally become candidates for immunotherapy after a long history of disease, including treatments designed to reduce the tumor burden present at the time of diagnosis. The concern has been raised that such individuals may be in a state of generalized or tumor-specific immunosuppression (38, 39), and therefore the question, not addressed in our experimental design, is whether treatment with cytokine-secreting cells would also be able to reverse a possible state of immune unresponsiveness. However, patients with recurrent superficial bladder cancer refractory to currently available intravesical therapies are not in a state of generalized immunosuppression and may represent a patient population suitable for such treatment. This approach has the added advantage that, if successful, it would enable bladder preservation and avoid the significant psychologic sequelae of radical cystectomy and urinary diversion.

In summary, the murine bladder model described in this study represents an improved model to evaluate the effective-

ness of cytokine gene-modified tumor vaccine. It would be of particular interest to evaluate in this experimental system the use of additional promising cytokines such as IL-6 (20), as well as combinations of cytokines. Such studies are in progress.

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References

1. Fidler, I.J., and C.M. Balch. 1987. The biology of cancer metastasis and implications for therapy. *Curr. Progr. Surg.* 24:137.
2. Livingston, P.O. 1991. Active specific immunotherapy in the treatment of patients with cancer. *In* Immunology and Allergy Clinics of North America: Human Cancer Immunology II. H.F. Oettgen, editor. W.B. Saunders Company Ltd., London. 401–423.
3. Cheever, M.A., J.A. Thompson, D.J. Peace, and P.D. Greenberg. 1986. Potential uses of interleukin 2 in cancer therapy. *Immunobiology.* 172:365.
4. Rosenberg, S.A. 1988. Immunotherapy of cancer using Interleukin 2: current status and future prospects. *Immunol. Today.* 9:58.
5. Lee, R.E., M.T. Lotze, J.M. Skibber, E. Tucker, R.O. Bonow, F.P. Ognibene, J.A. Carrasquillo, J.H. Shelhamer, J.E. Parrillo, and S.A. Rosenberg. 1989. Cardiorespiratory effects of immunotherapy with interleukin-2. *J. Clin. Oncol.* 7:7.
6. Bubenik, J., P. Perlmann, M. Indrova, J. Simova, T. Jandlova, and J. Neuwirt. 1983. Growth inhibition of an MC-induced mouse sarcoma by TCGF (IL-2)-containing preparations. *Cancer Immunol. Immunother.* 14:205.
7. Forni, G., M. Giovarelli, A. Santoni, A. Modesti, and M. Forni. 1986. Tumour inhibition by interleukin-2 at the tumour/host interface. *Biochim. Biophys. Acta.* 865:307.
8. Pizza, G., G. Severini, D. Menniti, C. De Vinci, and F. Corrado. 1984. Tumour regression after intralesional injection of interleukin 2 (IL-2) in bladder cancer. Preliminary report. *Int. J. Cancer.* 34:359.
9. Forni, G., M. Giovarelli, and A. Santoni. 1985. Lymphokine-activated tumor inhibition *in vivo*. *J. Immunol.* 134:1305.
10. Forni, G., T. Musso, C. Jemma, D. Boraschi, A. Tagliabue, and M. Giovarelli. 1989. Lymphokine-activated tumor inhibition in mice. *J. Immunol.* 142:712.
11. Bubenik, J., N.N. Voitenok, J. Kieler, V.S. Prassolov, P.M. Chumakov, D. Bubenikova, J. Simova, and T. Jandlova. 1988. Local administration of cells containing an inserted IL-2 gene and producing IL-2 inhibits growth of human tumours in nu/nu mice. *Immunol. Lett.* 19:279.
12. Bubenik, J., J. Simova, and T. Jandlova. 1989. Immunotherapy of cancer using local administration of lymphoid cells transformed by IL-2 cDNA and constitutively producing IL-2. *Immunol. Lett.* 23:287.
13. Fearon, E.R., D.M. Pardoll, T. Itaya, P. Golumbek, H.I. Levitsky, J.W. Simons, H. Karasuyama, B. Vogelstein, and P. Frost. 1990. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell.* 60:397.
14. Gansbacher, B., K. Zier, B. Daniels, K. Cronin, R. Bannerji, and E. Gilboa. 1990. Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J. Exp. Med.* 172:1217.
15. Ley, V., P. Langlade-Demoyen, P. Kourilsky, and E.-L. Larsson-Sciard. 1991. Interleukin 2-dependent activation of tumor-specific cytotoxic T lymphocytes *in vivo*. *Eur. J. Immunol* 21:851.
16. Watanabe, Y., K. Kuribayashi, S. Miyatake, K. Nishihara, E. Nakayama, T. Taniyama, and T. Sakata. 1989. Exogenous expression of mouse interferon gamma cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity. *Proc. Natl. Acad. Sci. USA.* 86:9456.
17. Gansbacher, B., R. Bannerji, B. Daniels, K. Zier, K. Cronin, and E. Gilboa. 1990. Retroviral vector-mediated gamma-interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. *Cancer Res.* 50:7820.
18. Porgador, A., R. Bannerji, Y. Watanabe, M. Feldman, E. Gilboa, and L. Eisenbach. 1993. Anti-metastatic vaccination of tumor-bearing mice with two types of gamma-interferon gene inserted tumor cells. *J. Immunol.* 150:1458.
19. Golumbek, P.T., A.J. Lazenby, H.I. Levitsky, L.M. Jaffee, H. Karasuyama, M. Baker, and D.M. Pardoll. 1991. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science (Wash. DC).* 254:713.
20. Porgador, A., E. Tzehoval, A. Katz, E. Vadai, M. Revel, M. Feldman, and L. Eisenbach. 1992. Interleukin 6 gene transfection into Lewis lung carcinoma tumor cells suppresses the malignant phenotype and confers immunotherapeutic competence against parental metastatic cells. *Cancer Res.* 52:3679.
21. Aoki, T., K. Tashiro, S. Miyatake, T. Kinashi, T. Nakano, Y.

Oda, H. Kikuchi, and T. Honjo. 1992. Expression of murine interleukin 7 in a murine glioma cell line results in reduced tumorigenicity *in vivo*. *Proc. Natl. Acad. Sci. USA.* 89:3850.
22. Asher, A.L., J.J. Mulé, A. Kasid, N.P. Restifo, J.C. Salo, C.M. Richert, G. Jaffe, B. Fendly, M. Kriegler, and S.A. Rosenberg. 1991. Murine tumor cells transduced with the gene for tumor necrosis factor-alpha. *J. Immunol.* 146:3227.
23. Raghavan, D., W.U. Shipley, M.B. Garnick, P.J. Russell, and J.P. Ritchie. 1990. Biology and management of bladder cancer. *N. Engl. J. Med.* 322:1129.
24. Herr, H.W., R.A. Badalament, D.A. Amato, V.P. Laudone, W.R. Fair, W.F. Whitmore, Jr. 1989. Superficial bladder cancer treated with bacillus Calmette-Guerin: a multivariate analysis of factors affecting tumor progression. *J. Urol.* 141:22.
25. DeKernion, J.B., M.S. Soloway, and L. Persky. 1974. Chemotherapy of experimental transitional-cell carcinoma. *Urology.* 4:63.
26. Soloway, M.S., and W.M. Murphy. 1979. Experimental chemotherapy of bladder cancer—systemic and intravesical. *Semin. Oncol.* 6:166.
27. Cullen, B.R. 1988. Expression of a cloned human interleukin-2 cDNA is enhanced by the substitution of a heterologous mRNA leader region. *DNA (NY).* 7:645.
28. Morris, A.G., and G. Ward. 1987. Production of recombinant interferon by expression in heterologous mammalian cells. *In* Lymphokines and Interferons: A Practical Approach. M.J. Clemens, A.G. Morris, and A.J.H. Gearing, editors. IRL Press, Inc., Washington, DC. 61–72.
29. McKnight, S.L. 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. *Nucleic Acids Res.* 8:5949.
30. Wano, Y., B.R. Cullen, P.A. Svetlik, N.J. Pfeffer, and W.C. Greene. 1987. Reconstitution of high affinity IL-2 receptor expression is a human T-cell line using a retroviral cDNA expression vector. *Mol. Biol. Med.* 4:95.
31. Armentano, D., S.-F. Wu, P.W. Kantoff, T. von Ruden, W.F. Anderson, and E. Gilboa. 1987. Effect of internal virus sequences on the utility of retroviral vectors. *J. Virol.* 61:1647.
32. Hantzopoulos, P., B. A. Sullenger, G. Ungers, and E. Gilboa. 1989. Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. *Proc. Natl. Acad. Sci. USA.* 86:3519.
33. Markowitz, D., S. Goff, and A. Bank. 1988. Construction and use of a safe and efficient amphotropic packaging cell line. *Virology.* 167:400.
34. Zier, K. 1982. Functional and antigenic properties of cultured T-cells in the cell mediated lympholysis (CML) assay. *Hum. Immunol.* 4:147.
35. Lewis, J.A. 1987. Biological assays for interferons. *In* Lymphokines and Interferons: A Practical Approach. M.J. Clemens, A.G. Morris, and A.J.H. Gearing, editors. IRL Press, Inc., Washington, DC. 73–87.
36. Fidler, I.J. 1990. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes Memorial Award Lecture. *Cancer Res.* 50:6130.
37. Hewitt, H.B., E.R. Blake, and A.S. Walder. 1975. A critique of the evidence for active host difference against cancer, based on personal studies of 27 murine tumours of spontaneous origin. *Br. J. Cancer.* 33:241.
38. Kamo, I., and H. Friedman. 1977. Immunosuppression and the role of suppressive factors in cancer. *Adv. Cancer Res.* 25:271.
39. North, R.J. 1985. Down-regulation of the antitumor immune response. *Adv. Cancer Res.* 45:1.