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Vaccination with irradiated tumor cells engineered to secrete murine granulocyte–macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity

(tumor immunology/gene transfer)

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ABSTRACT **To compare the ability of different cytokines and other molecules to enhance the immunogenicity of tumor cells, we generated 10 retroviruses encoding potential immunomodulators and studied the vaccination properties of murine tumor cells transduced by the viruses. Using a B16 melanoma model, in which irradiated tumor cells alone do not stimulate significant anti-tumor immunity, we found that irradiated tumor cells expressing murine granulocyte–macrophage colony-stimulating factor (GM-CSF) stimulated potent, long-lasting, and specific anti-tumor immunity, requiring both CD4$^+$ and CD8$^+$ cells. Irradiated cells expressing interleukins 4 and 6 also stimulated detectable, but weaker, activity. In contrast to the B16 system, we found that in a number of other tumor models, the levels of anti-tumor immunity reported previously in cytokine gene transfer studies involving live, transduced cells could be achieved through the use of irradiated cells alone. Nevertheless, manipulation of the vaccine or challenge doses made it possible to demonstrate the activity of murine GM-CSF in those systems as well. Overall, our results have important implications for the clinical use of genetically modified tumor cells as therapeutic cancer vaccines.**

The use of autologous cancer cells as vaccines to augment anti-tumor immunity has been explored throughout this century (1). Although a few patients have appeared to benefit from this approach, the responses observed generally have been only partial and short-lived. Strategies to improve the efficacy of such vaccinations, including the use of nonspecific immunostimulants such as bacille Calmette–Guérin and *Corynebacterium parvum*, have resulted in little improvement. Recent studies involving the use of genetically modified tumor cells as vaccines have nonetheless generated renewed enthusiasm for the concept of cancer vaccines. Such studies have shown that the transduction of murine tumor cells with genes for interleukin 4 (IL-4) (2–4), IL-2 (5, 6), interferon γ (γ-IFN) (7, 8), tumor necrosis factor type α (TNF-α) (9–11), granulocyte colony-stimulating factor (G-CSF) (12), JE (13), IL-7 (14, 15), and IL-6 (16) leads to rejection of the genetically modified cells by syngeneic hosts. Moreover, several studies indicate that cells expressing γ-IFN, IL-2, TNF-α, IL-4, IL-6, or IL-7 increase systemic immunity as well, since mice vaccinated with transduced cells reject a subsequent challenge of nontransduced cells and, in some cases, a preexisting tumor (4, 16).

While these latter studies provide a firm basis for believing that gene transfer will prove to be a powerful tool for altering the immunogenicity of tumors, they also suggest that it will be critically important to identify, by means of comparative studies, which gene products or combinations thereof are best able to stimulate anti-tumor immunity in a wide variety of tumor models, and what characteristics of specific tumor models, if any, influence the ability to detect the immunostimulatory activity of specific gene products. Toward these ends, we have generated a variety of recombinant retroviruses encoding different potential immunomodulators and compared the vaccination properties of both live and irradiated tumor cells transduced by the viruses in several different tumor models. We show below that in a B16 melanoma model (17), in which nontransduced irradiated cells possess little ability to stimulate systemic anti-tumor immunity, a previously unidentified molecule, murine granulocyte–macrophage CSF (GM-CSF), is the most potent stimulator of systemic anti-tumor immunity of the 10 molecules tested. In addition to reporting on the characteristics of the immune response induced by GM-CSF-expressing B16 cells, we have examined the activity of GM-CSF in a number of tumor models used previously by others to identify cytokines with anti-tumor activity. We demonstrate that analysis of the effects of cytokine expression in these models is problematic, since at the vaccine and challenge doses used previously in studies with live transduced cells, vaccination with irradiated cells alone generates systemic anti-tumor immunity at levels comparable to those induced by live transduced cells.

MATERIALS AND METHODS

Tumor Models. B16-F10 melanoma cells (17), kindly provided by Michael Wick (Dana–Farber Cancer Institute), CT-26 colon carcinoma cells (5) and Lewis lung carcinoma cells (16) obtained from ATCC, RENCA renal carcinoma cells (4), and CMS-5 fibrosarcoma cells (6), kindly provided by Eli Gilboa, were maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum and penicillin/streptomycin. WP-4 fibrosarcoma cells (9), kindly provided by Steve Rosenberg, were grown in RPMI medium containing 10% (vol/vol) fetal calf serum, 2 mM glutamine, and penicillin/streptomycin. Animals used were 6- to 12-week-old C57BL/6 females (The Jackson Laboratory) for B16, Lewis lung, and WP-4 experiments, and 6- to 12-week-old BALB/c females (The Jackson Laboratory) for CT-26, RENCA, and CMS-5 studies.

Recombinant Retroviruses. Tim Springer (Harvard Medical School) kindly provided the cDNAs for murine ICAM-1 and CD2. Other cDNAs were generously provided by Frank Lee

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Abbreviations: IL, interleukin; γ-IFN, interferon γ; TNF-α, type α tumor necrosis factor; GM-CSF, granulocyte–macrophage colony-stimulating factor.

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and Ken-ichi Arai (DNAX). The precise cDNA sequences subcloned into MFG by standard procedures (18) were as follows: murine IL-2 (19), bp 49–564; murine IL-4 (20), bp 56–479; murine IL-5 (21), bp 44–462; murine IL-6 (22), bp 49–684; murine GM-CSF (23), bp 174–619; murine γ-IFN (24), bp 70–561; murine ICAM-1 (25), bp 30–1657; murine CD2 (26), bp 48–1079; murine IL-1 receptor antagonist (H.H. and R.C.M., unpublished data), bp 16–563; human TNF-α (27), bp 86–788.

CRIP packaging cell lines producing the different viruses were generated as described (28, 29). Cytokines secreted by the infected, unselected B16 populations were assayed 48 hr after plating 1×10^6 cells in 10-cm dishes containing 10 ml of medium. IL-1 receptor antagonist secretion was measured from infected, unselected 3T3 cells 24 hr after plating 5×10^6 cells in a 10-cm dish containing 10 ml of medium. The cytokine bioassays for murine IL-2, IL-6, γ-IFN, and GM-CSF were kindly performed by Kathy Sill, Jonathan Keller, and Howard Young (National Cancer Institute). Briggs Morrison (Dana–Farber Cancer Institute) kindly performed the IL-4 bioassay. Cytokines were assayed as follows: murine IL-2, using CTLL cells (30) and ELISA (Collaborative Biomedical, Bedford, MA); murine IL-4, using CT4S cells (30) and ELISA (Endogen, Cambridge, MA); murine IL-5, using an ELISA (Endogen); murine IL-6, using T1165 cells (30) and ELISA (Endogen); murine GM-CSF using FDCP-1 cells (30) and ELISA (Endogen); murine γ-IFN, using vesicular stomatitis viral inhibition (30) and ELISA (Genzyme); human TNF-α using L929 cells (30) and ELISA (R & D Systems, Minneapolis); murine IL-1 receptor antagonist, using ^{125}I-labeled IL-1β binding inhibition (31). Expression of murine ICAM-1 and CD2 in B16 target cells was determined by standard procedures (30) on an EPICS-C FACS analyzer (Coulter) using antibodies YN1/1.47 (39) and RM2/5, respectively (provided by Tim Springer).

Vaccinations. Tumor cells were treated with trypsin, washed once in medium containing serum, and washed twice in Hanks' balanced saline solution (HBSS) (GIBCO) before injection. Trypan blue-resistant cells were suspended to the appropriate concentrations and injected in 0.5 ml of HBSS. Indicated tumor cells (after suspension in HBSS) received 3500 rads (1 rad = 0.01 Gy) from a ^{137}Cs source discharging 124 rads/min. Irradiation of tumor cells did not abrogate secretion of cytokine *in vitro* over the course of 7 days.

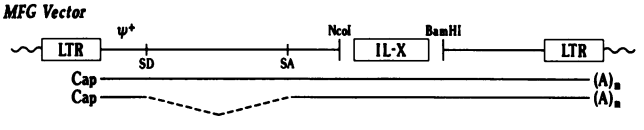
Histology. Tissues for histologic examination were fixed in 10% neutral buffered formalin, processed to paraffin embedment, and stained with hematoxylin and eosin.

Cell Depletions. Mice were depleted of lymphocyte subsets by standard procedures (30) using monoclonal antibody (mAb) GK1.5 (32) for CD4$^+$ cells, mAb 2.43 (33) for CD8$^+$ cells, and PK136 (34) for natural killer (NK) cells. Examination of splenocytes and lymph node cells by fluorescence-activated cell sorting revealed that the depleted subset represented <0.5% of the total lymphocytes, with normal levels of other subsets.

Cytotoxic T-Cell Assay. CD8 blockable lytic activity against γ-IFN-treated B16 targets was determined by standard procedures (30) using splenocytes obtained 2 weeks after vaccination.

RESULTS

To facilitate the rapid screening of a variety of gene products for their influence on the immunogenicity of tumor cells, and to make possible the simultaneous transfer of multiple genes into cells, we used retroviral-mediated gene transfer (35) for our studies. DNA sequences encoding the cytokine and adhesion molecules shown in Fig. 1 were inserted into the retroviral vector MFG (L. Spain, P. Robbins, and R.C.M., unpublished data) and the resulting constructs were introduced into CRIP cells (28) to generate recombinant virus with



cDNA	Titer (copy number)	Level of Expression
MFG mulL-2	1.0	5000 U/ml
MFG mulL-4	0.25	15 ng/ml
MFG mulL-5	2.0	250 ng/ml
MFG mulL-6	0.5	400 ng/ml
MFG muGM-CSF	2.0	300 ng/ml
MFG muγ-IFN	0.1	20 ng/ml
MFG mulL-1RA	1.0	30 ng/ml
MFG mulCAM	0.5	+ FACS
MFG muCD2	0.5	+ FACS
MFG human TNF-α	0.5	400 ng/ml

FIG. 1. MFG recombinant retroviruses encoding cytokines and adhesion molecules. Moloney murine leukemia virus (Mo-MuLV) long terminal repeat (LTR) sequences are used to generate both a full-length viral RNA (for encapsidation into virus particles) and a subgenomic mRNA (analogous to the Mo-MuLV env mRNA), which is responsible for expression of inserted sequences. The vector retains both sequences in the viral gag region shown to improve the encapsidation of viral RNA and the normal 5′ and 3′ splice sites necessary for generation of the env mRNA. Protein coding sequences are inserted between the *Nco* I and *Bam*HI sites in such a way that the initiation codon of the inserted sequence is placed precisely at the position of the viral env initiation codon, and a minimal 3′ nontranslated sequence is included in the insert. No selectable marker exists in the vector.

amphotropic host range. B16 melanoma cells were exposed to viral supernatants and transduced cells were characterized for the efficiency of infection and secretion of gene product (Fig. 1). The expression of each gene product in B16 cells was comparable or greater than the levels reported in previous cytokine gene transfer studies.

Experiments Involving Live Cells. To assess directly the effect of the cytokine and adhesion molecules upon the tumorigenicity of B16 cells, transduced cells were inoculated subcutaneously into C57BL/6 mice, their syngeneic host, and the mice were examined every few days for tumor formation. While modest delays in tumor formation were associated with synthesis of IL-4, IL-6, γ-IFN, and TNF-α, only cells secreting IL-2 were completely rejected. Several cytokines produced distinctive systemic syndromes, presumably as a consequence of a progressively increasing number of cells expressing the cytokines *in vivo*. GM-CSF-transduced cells induced a fatal toxicity manifested by profound leukocytosis (polymorphonuclear leukocytes, monocytes, and eosinophils), hepatosplenomegaly, and pulmonary hemorrhage. IL-5-expressing cells showed a striking peripheral eosinophilia and splenomegaly. IL-6-expressing cells caused hepatosplenomegaly and death. TNF-α-expressing cells induced wasting, shivering, and death.

Rejection of IL-2-transduced cells made it possible to examine their potential to generate systemic immunity. Mice were first inoculated with IL-2-expressing B16 cells and, subsequently, over the course of 1 month, challenged with nontransduced B16 cells. All animals succumbed to this challenge, regardless of the time of challenge, with only an occasional delay in tumor formation (data not shown). IL-2-secreting cells were then superinfected with a second retrovirus, and the doubly transduced cells were evaluated in similar protection experiments. Only the combination of GM-CSF and IL-2 generated potent systemic protection, with a majority of the mice surviving tumor challenge long term (Fig. 2*A*).

Experiments Involving Irradiated Cells. The facts that cells expressing both IL-2 and GM-CSF, but not IL-2 alone, conferred systemic protection on vaccinated hosts and that cells secreting GM-CSF alone grew progressively led us to

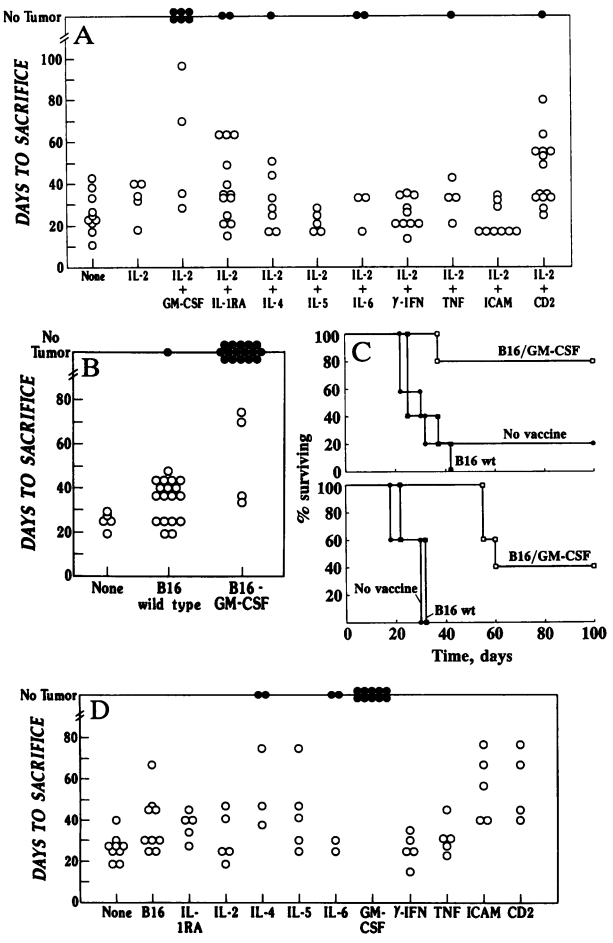


FIG. 2. B16 melanoma cell vaccinations. (*A*) Syngeneic C57BL/6 mice were vaccinated subcutaneously in the abdomen with 5×10^5 live transduced B16 cells as indicated. Animals were challenged 7–14 days later with 5×10^5 live nontransduced B16 cells subcutaneously in the back. All vaccinating inocula were rejected. Mice were sacrificed when challenge tumors reached 2–3 cm (longest diameter) or severe ulceration or bleeding developed. (*B*) Mice were vaccinated with 5×10^5 irradiated (3500 rads) GM-CSF-transduced or nontransduced B16 cells. Animals were challenged 7 days later with 5×10^5 live nontransduced B16 cells. (*C*) Mice were first inoculated with live nontransduced B16 cells (*Upper*, 5×10^4 B16; *Lower*, 1×10^5 B16). Three days later, animals received 4×10^6 irradiated GM-CSF-transduced or nontransduced B16 cells. wt, Wild type. (*D*) Mice were vaccinated with 5×10^5 irradiated (3500 rads) transduced cells as indicated. Animals were challenged 7 days later with 1×10^6 live nontransduced B16 cells. ○, Animal succumbed to tumor challenge; ●, animal protected from tumor challenge.

consider whether IL-2 might be functioning primarily in a local fashion to mediate rejection of the vaccinating cells. Therefore, we next examined whether GM-CSF cells that were inactivated by irradiation would be capable of inducing systemic anti-tumor immunity. As shown in Fig. 2*B*, vaccination of mice with irradiated B16 cells expressing GM-CSF alone did indeed lead to potent anti-tumor immunity, with most of the mice surviving their tumor challenge. The systemic immunity was long lasting in that the majority of mice vaccinated with irradiated cells that express GM-CSF and that were subsequently challenged with nontransduced cells several months after vaccination remained tumor free. The systemic immunity was also specific in that GM-CSF-expressing cells did not protect mice from a challenge of Lewis lung carcinoma cells (16), another tumor of C57BL/6 origin, and GM-CSF-expressing Lewis lung carcinoma cells did not protect mice from a challenge of nontransduced B16 cells (data not shown). An important finding was that nontransduced irradiated B16 cells elicited only minimal effects upon the growth of challenge cells (Fig. 2*B*), thus indicating that inactivation of the vaccinating cells *per se*, by either irradiation or expression of IL-2, does not necessarily lead to generation of anti-tumor immunity. In addition to conferring potent protection against challenge with nontransduced cells, irradiated B16 cells expressing GM-CSF were also capable of mediating the rejection of a preestablished tumor, while irradiated nontransduced cells were not, at least not at the doses tested (Fig. 2*C*). Similar results were also obtained in studies in which established metastases were generated through the intravenous injection of nontransduced cells (data not shown).

To determine whether irradiated cells expressing other gene products were capable of inducing similar levels of anti-tumor immunity, cell populations expressing different gene products were also tested for vaccination activity after their irradiation (Fig. 2*D*). GM-CSF-expressing cells were the most potent, with IL-4- and IL-6-expressing cells showing reduced activity.

Characterization of the Immune Response Stimulated by GM-CSF-Expressing B16 Cells. Examination of the site of vaccination with irradiated GM-CSF-expressing cells revealed an extensive local influx of immature, dividing monocytes, granulocytes (predominantly eosinophils), and activated lymphocytes and paracortical hyperplasia in the draining lymph node. In contrast, in mice vaccinated with nontransduced irradiated cells, only a mild influx of inflammatory cells was seen, which consisted primarily of lymphocytes, and a comparatively smaller enlargement of the draining lymph node was observed. The challenge site of mice vaccinated with irradiated GM-CSF-expressing cells demonstrated a large number of eosinophils, monocytes, and lymphocytes, while only patches of lymphocytes were seen at the challenge site in mice vaccinated with irradiated cells. Virtually no responding cells were observed in naive animals challenged with live B16 cells.

To determine which cells were critical for systemic immunity, a series of mice were depleted of $CD4^+$, $CD8^+$, or NK cells by administration of antibodies *in vivo*; they were subsequently vaccinated with irradiated GM-CSF-expressing cells. Both $CD4^+$ and $CD8^+$ T cells were required for effective vaccination, since depletion of either T-cell subset before vaccination abrogated the development of systemic immunity, whereas depletion of NK cells had little or no effect (Fig. 3*A*). Depletion of $CD4^+$ or $CD8^+$ cells after vaccination also abrogated anti-tumor immunity (data not shown). While mice vaccinated with nontransduced cells possessed little detectable CD8-blockable tumor-specific cytotoxicity, this level was significantly enhanced in mice vaccinated with GM-CSF-expressing cells (Fig. 3*B*).

Activity of GM-CSF in Other Tumor Models. To establish the generality of the host response to vaccination with irradiated GM-CSF-expressing cells, we first examined the ability of irradiated, nontransduced cells representing a variety of murine tumors to elicit systemic immunity, since the earlier studies of Prehn and Main (36) and Klein and coworkers (37) had indicated that some murine tumors are inherently immunogenic when inactivated by irradiation or other means. For these experiments, we focused on several tumor models that had been used previously to identify cytokines possessing activity in tumor rejection or tumor challenge assays. These tumors included (*i*) CT-26, a colon carcinoma-derived cell line, used in studies that identified the activity of IL-2 (5); (*ii*) CMS-5, a fibrosarcoma-derived cell line, used to identify the activity of IL-2 and γ-IFN (6, 7); (*iii*) RENCA, a renal cell carcinoma-derived cell line, used to identify the activity of IL-4 (4); and (*iv*) WP-4, a fibrosarcoma-derived cell line, used to identify the activity of TNF-α

(9). As shown in Fig. 4*A*, we found that in each tumor model examined, irradiated cells possessed potent vaccination activity, comparable to that reported previously with live cells expressing the various cytokines tested at similar vaccine and challenge doses. In spite of the very significant vaccination activity of nontransduced cells, manipulation of the vaccine and challenge doses did make it possible to demonstrate that in these models, as well as in the Lewis lung carcinoma system (data not shown), GM-CSF-expressing cells were more efficacious than irradiated cells alone in eliciting systemic immunity (Fig. 4*B*).

DISCUSSION

The experiments reported here were motivated by a large number of recent studies that had convincingly shown that the host response to tumor challenge can be dramatically influenced by inoculation of tumor cells genetically engineered to express particular cytokines. Our current studies extend those findings in several important ways. First, the generation of highly transmissible retroviral vectors encoding a variety of different potential immunomodulators made it possible to assess rapidly the relative potency of not only many of the molecules that had been studied by others but also a number of gene products never before examined. No previous study had compared the relative activity of different gene products in a single tumor model or examined the activity of a single gene product in multiple models. Such comparative studies are critical both for establishing the generality of an immunostimulatory effect of a gene product and for ultimately determining the gene product or mixture of gene products best able to stimulate anti-tumor immunity in a wide variety of tumor models.

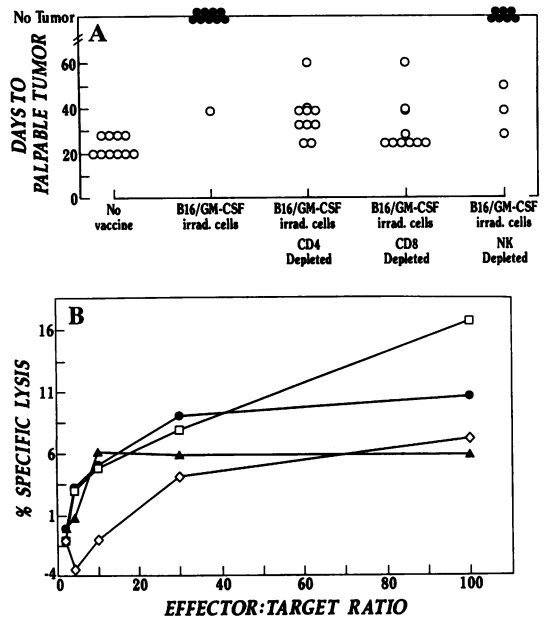


FIG. 3. GM-CSF vaccinations require CD4$^+$ and CD8$^+$ lymphocytes. (*A*) Mice were depleted of CD4$^+$, CD8$^+$, or NK1.1$^+$ cells by administration of antibodies beginning 1 week before vaccination. Fourteen days after receiving 1×10^6 irradiated GM-CSF-transduced B16 cells, animals were challenged with 1×10^5 live nontransduced B16 cells. ○, Animal succumbed to tumor challenge; ●, animal protected from tumor challenge. (*B*) Splenocytes were harvested 14 days after vaccination and stimulated *in vitro* for 5 days with γ-IFN-treated B16 cells. CD8 blockable cytotoxic T-lymphocyte activity was determined in a 4-hr ^{51}Cr release assay on γ-IFN-treated B16 targets at various effector/target cell ratios. Splenocytes from naive C57BL/6 and BALB/c mice served as controls. □, GM-CSF; ●, Allo; ◇, naive; ▲, B16 irradiation.

The most important finding from our studies was that GM-CSF, a cytokine most often associated with the growth and differentiation of hematopoietic progenitors, proved to be the most powerful immunostimulant of the 10 molecules tested. This result is particularly intriguing in light of several recent reports suggesting that GM-CSF may play an important role in the maturation and/or function of specialized antigen presenting cells (38). The possibility that localized expression of GM-CSF by vaccinating cells might specifically enhance tumor-antigen presentation by host antigen presenting cells is compatible with our finding that both CD4$^+$ and CD8$^+$ cells were required for the anti-tumor response, since the B16 cells used in our study do not express detectable amounts of class II major histocompatibility complex molecules, even after γ-IFN treatment, and therefore are unlikely to be able to prime antigen-specific CD4$^+$ cells. Studies to specifically characterize the role of epidermal Langerhans cells, dendritic cells, and other potential antigen presenting cells in vaccination studies involving GM-CSF-expressing cells as well as the normal role of GM-CSF in hematopoiesis and immunity through the generation of mouse strains carrying disruptions of the GM-CSF gene remain to be done.

In addition to identifying the activity of GM-CSF, our studies also indicated that two gene products previously shown to stimulate anti-tumor immunity, IL-4 and IL-6, are

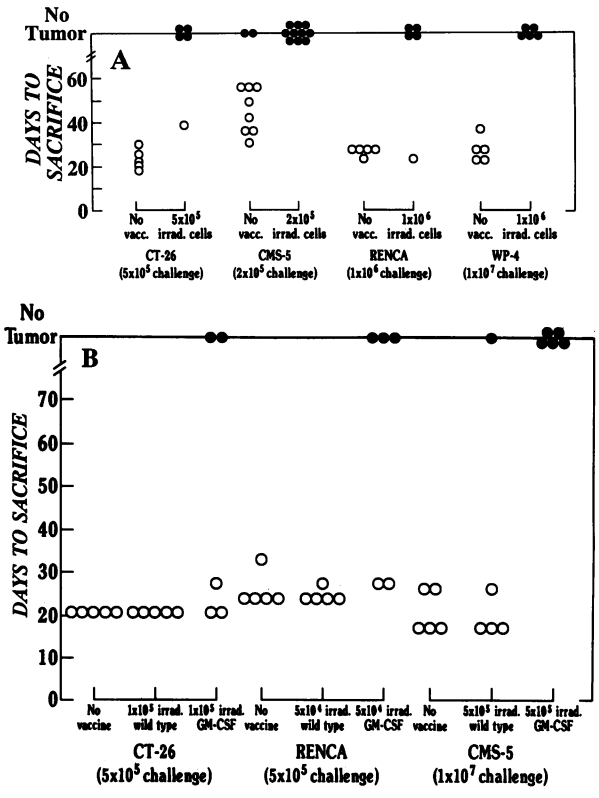


FIG. 4. Irradiated GM-CSF-transduced tumor cells stimulate increased systemic immunity relative to nontransduced irradiated tumor cells. (*A*) Irradiated nontransduced murine tumor cell lines used in previous cytokine transfection studies are immunogenic. Irradiated (3500 rads) nontransduced tumor cells were administered subcutaneously in the abdomens of syngeneic mice. Animals were challenged 1–3 weeks later with live, nontransduced cells subcutaneously on the back. ○, Animal succumbed to tumor challenge; ●, animal protected from tumor challenge. (*B*) Irradiated GM-CSF-transduced tumor cells stimulate increased systemic immunity relative to nontransduced irradiated tumor cells. Mice were vaccinated with irradiated GM-CSF-transduced cells or nontransduced cells. Animals were challenged 1–3 weeks later with live, nontransduced tumor cells. ○, Animal succumbed to tumor challenge; ●, animal protected from tumor challenge.

active in the B16 model as well. In contrast, we were unable to detect the activity of three other gene products (γ-IFN, TNF-α, IL-2) previously identified as immunostimulators. Our inability to detect the activity of these gene products could be due to a variety of factors related to levels of cytokine expression, specific characteristics of the different tumor models, and potential differences in the properties of live and irradiated cells as vaccines. While further studies will be necessary to resolve these issues, the experiments reported here nevertheless highlight a limitation of previous cytokine gene transfer studies involving live transduced cells that may be relevant. No previous study involving live transduced tumor cells had directly compared the vaccination activity of transduced cells to that of the nontransduced cells, due to the progressive growth of the nontransduced cells. Accordingly, the studies provided no information regarding the inherent immunogenicity of the tumor model used. Since both Prehn and Main (36) and Klein and coworkers (37) have demonstrated that certain murine tumors are inherently immunogenic and can sometimes elicit potent systemic anti-tumor immunity, it is important to assess such properties of tumors, so that the contribution of cytokine expression to the immunity observed can be better evaluated. In this regard, our findings show that at the vaccine and challenge doses commonly used in previous studies with the RENCA, CMS-5, CT-26, and WP-4 cell lines, irradiated cells alone conferred immunity comparable to that reported previously with live transduced cells. These results raise the possibility that the immunostimulatory activity of some gene products previously identified may largely be attributable to their ability to promote the local destruction of vaccinating cells. If so, both the use of irradiated cells and the establishment of conditions of vaccine and challenge cell doses in which nontransduced irradiated cells possess little detectable vaccination activity, as we have done in the current studies, would appear to be very important for comparing the immunostimulatory activity of different gene products.

Finally, several features of the experimental system described here have important implications for the clinical use of genetically engineered tumor cells as therapeutic vaccines. First, the combination of high titer and high gene expression afforded by the MFG vector system would obviate the need for selection of transduced cells among a bulk tumor cell population, thereby minimizing the time required for culturing primary tumor cells prior to vaccination and maximizing the antigenic heterogeneity represented in the vaccinating inoculum. Second, to the extent that either the *in vitro* manipulation of tumor cells or retroviral integration might pose the risk of conferring a more malignant phenotype upon the transduced cells, the use of irradiated rather than live cells as cancer vaccines would appear to be extremely important. Moreover, since primary tumor explants likely contain nonneoplastic elements as well, irradiation of the tumor samples before vaccination will also prevent the possibility of the autonomous growth of nonneoplastic cells induced by autocrine synthesis of their own growth factors.

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