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In vivo cytokine gene transfer by gene gun reduces tumor growth in mice

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ABSTRACT **Implantation of tumor cells modified by *in vitro* cytokine gene transfer has been shown by many investigators to result in potent *in vivo* antitumor activities in mice. Here we describe an approach to tumor immunotherapy utilizing direct transfection of cytokine genes into tumor-bearing animals by particle-mediated gene transfer. *In vivo* transfection of the human interleukin 6 gene into the tumor site reduced methylcholanthrene-induced fibrosarcoma growth, and a combination of murine tumor necrosis factor α and interferon γ genes inhibited growth of a renal carcinoma tumor model (Renca). In addition, treatment with murine interleukin 2 and interferon γ genes prolonged the survival of Renca tumor-bearing mice and resulted in tumor eradication in 25% of the test animals. Transgene expression was demonstrated in treated tissues by ELISA and immunohistochemical analysis. Significant serum levels of interleukin 6 and interferon γ were detected, demonstrating effective secretion of transgenic proteins from treated skin into the bloodstream. This *in vivo* cytokine gene therapy approach provides a system for evaluating the antitumor properties of various cytokines in different tumor models and has potential utility for human cancer gene therapy.**

In situ secretion of certain cytokines by genetically modified tumor or immune system cells can induce inflammatory and/or immune responses which inhibit the growth of certain transplantable tumors. Transgenic cytokines including interleukin 2 (IL-2), IL-4, IL-6, tumor necrosis factor α (TNFα), interferon γ (IFNγ), and granulocyte/macrophage-colony-stimulating factor have been effective in mediating either T-cell-dependent or inflammatory responses leading to tumor regression or rejection (1–6). Mice treated with cytokine-gene-modified tumor cells often rejected subsequent challenges with unmodified tumor cells (4–6). Several clinical trials have been initiated involving use of cytokine-gene-modified autologous tumor cells (7, 8) or tumor-infiltrating lymphocytes (9, 10) for treatment of patients with advanced cancers.

Here we describe an *in vivo* cancer gene therapy approach in which cytokine genes are introduced into tumor-bearing animals by Accell (Agracetus) particle-mediated gene transfer (11–13). This technology utilizes an adjustable electric discharge to generate a shock wave which accelerates DNA-coated gold particles into target cells or tissues, resulting in gene transfer. When compared with other gene delivery methods such as lipofection, calcium phosphate precipitation, or electroporation, the particle-mediated, or "gene gun," technique can achieve up to 100-fold higher transgene expression levels *ex vivo* or *in vitro* (11, 12). This method has previously allowed highly efficient gene transfer to skin and liver tissues of live animals (13). In this study, we have developed an *in vivo* gene therapy system by delivering cytokine genes directly into tumor-bearing animals. This *in vivo* and *in situ* gene transfer protocol is designed to bypass the *ex vivo* gene transfer approach commonly employed in previous cancer gene therapy studies.

With this strategy, we investigated several cytokines previously reported to have antitumor effects in defined animal models, including IL-2, IL-6, TNFα and IFNγ. Among a wide array of functions, IL-6 probably serves most importantly as a mediator of inflammation (14). Antitumor activity against several murine tumor cell lines has been described (15). Also, IL-6 gene-transfected tumor cells have been shown to have slower growth or reduced tumorigenicity (16, 17). IFNγ plays a key role in the host response to virus or antigen stimulation and enhances monocyte and natural killer cell function (18). It can exert direct antitumor activity and modulate immune responses by mediating delayed-type hypersensitivity, increasing expression of major histocompatibility complex-encoded antigens on tumor cells, and enhancing tumor cell susceptibility to lysis (18, 19). In addition, IFNγ gene-transfected tumor cells have been shown to exhibit reduced tumorigenicity (20, 21). The antitumor activities of TNFα have also been well documented. Produced by tumor-infiltrating lymphocytes and activated macrophages, it activates cytotoxic T cells and natural killer cells (4). TNFα gene-modified tumor cells have also been demonstrated to have reduced tumorigenicity (22, 23). Although it has potent antitumor activity, systemic administration of TNFα causes severe toxicity (24). As a result, TNFα gene-transfected tumor-infiltrating lymphocytes or tumor cells have been injected to deliver a high local concentration of the cytokine for induction of immune responses (24). IL-2 is known to exert antitumor activity through specific cytotoxic T lymphocytes (25). IL-2 gene-transduced tumors have been demonstrated to attract infiltrating T lymphocytes (26) and can elicit a systemic immune response (27). It is believed that $CD8^+$ cells are the major IL-2-activated effector cells (25), although recent studies have shown that natural killer cells also play a critical role in rejecting IL-2-producing tumors (28).

We report here that efficient *in vivo* transgenic cytokine expression and positive antitumor effects were systematically obtained from human IL-6 (hIL-6), TNFα, murine IFNγ (mIFNγ), and mIL-2 gene treatments. This gene transfer system allows evaluation of the antitumor effects of various cytokines against different tumor types *in vivo* and measurement of the pharmacological kinetics of transgenic cytokine expression. Potential application of this system to gene therapy of human cancers is discussed.

MATERIALS AND METHODS

Cytokine Genes and Expression Plasmids. BCMGIL-6 and BCMGIL-2, containing a hIL-6 or mIL-2 cDNA cloned into a

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Abbreviations: IL, interleukin; IFNγ, interferon γ; TNFα, tumor necrosis factor α; prefix m, murine; prefix h, human.

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BCMG-neo expression vector (17), were kindly provided by Drew Pardoll, Johns Hopkins University, Baltimore, MD. The β-galactosidase gene in pCMVβ (Clontech) was replaced with cDNA encoding hTNFα, mTNFα (R & D Systems, Minneapolis), or mIFNγ (kindly provided by G. Opdenakker, Rega Institute, Leuven, Belgium) to create pCMVhTNF, pCMVmTNF, and pCMVmIFN, respectively. BCMGneo or pCMVluc (13) was used as the control vector as appropriate.

Tumor Cell Lines and Subcutaneous Tumors. The SP1 methylcholanthrene-induced fibrosarcoma (29) and Renca renal carcinoma cell lines (kindly provided by Robert Wiltrout, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD) were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 1% minimal Eagle's medium nonessential amino acids, and gentamicin at 50 μg/ml. Animal use was performed under protocols approved by the Institutional Animal Care and Use Committee. For induction of tumors, 6- to 8-week-old female C57BL/6 or BALB/c mice (Harlan–Sprague–Dawley) were injected subcutaneously on the abdomen with 1×10^5 SP1 or Renca cells, either 1 or 5 days prior to the first DNA treatment.

***In Vivo* Gene Transfer.** Plasmid DNA was purified on Qiagen columns (Qiagen, Chatsworth, CA), precipitated onto gold particles (1–3 μm) at a density of 2.5 μg of DNA per mg of particles, and evenly distributed onto a Mylar sheet (1.8×1.8 cm^2; 0.1 mg of particles per cm^2) as described (30). For cotransfection experiments, two separate cytokine gene plasmid preparations were mixed and coprecipitated onto the gold particles at a dosage of 1.25 μg of each plasmid per mg of gold particles. Mice were placed on the Accell device (Agracetus) with the target tissues in the path of the particles. Particles were then accelerated by a 20-kV electric discharge, resulting in delivery of the DNA-laden particles to the target tissue.

Cytokine Tumor Gene Therapy Protocol. Established murine tumor cell lines were inoculated subcutaneously into the appropriate mouse host strain on the indicated days prior to the onset of treatment. After the mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (20 mg/kg), the surgically exposed tumor site and/or the epidermis overlaying the transplanted tumors was treated *in situ* with test cytokine genes or control DNA. Each treatment with DNA-coated gold particles delivered 0.8 μg of plasmid DNA per site. In experiments where the tumor was directly exposed to the accelerated particles, a 2-cm incision was made near the tumor site, the subcutaneous tissue was exposed, and test or control plasmid DNA was delivered into the dermis layer at the site of tumor cell injection. The incision was closed with stainless steel clips, and the epidermal tissue overlaying the injected tumor cells was bombarded with the DNA-coated particles. Four to five subsequent epidermal transfections were performed on alternate days in all experiments. In experiments testing the effects of cytokine gene transfer to the epidermis directly over the subcutaneous tumor, particle-mediated gene delivery was performed four or five times at 1- to 2-day intervals as indicated for specific experiments. Antitumor effects were measured 2 weeks (Renca) or 3 weeks (SP1) after the first treatment. The mice were euthanized, the tumors present were excised and weighed, and the average tumor weight in the test and control groups was determined. For survival analysis, mice were sacrificed when tumors reached a predetermined size (≥100 mm^2), or earlier if the tumor had erupted through the skin.

ELISAs and Immunoperoxidase Staining for Cytokines. Transfected tissues were homogenized and centrifuged, and the supernatants were analyzed by an ELISA specific for hIL-6 (R & D Systems), human TNFα (Biosource, Camarillo, CA), mTNFα (Endogen, Cambridge, MA), or mIFNγ (Genzyme, Boston, MA). Twenty-four hours following gene transfer, tissues at the target sites (1–2 cm^2) were excised and fixed for 1 hr with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tissues were then dehydrated in a graded series of ethanol and embedded in paraffin. Thin (5-μm) tissue sections were deparaffinized and incubated for 30 min with polyclonal rabbit anti-recombinant hIL-6 or anti-recombinant hTNFα antibodies (Genzyme). To eliminate crossreactivity with mIL-6 or mTNFα, primary antibodies were first incubated overnight in supernatant medium from lipopolysaccharide-stimulated mouse bone marrow cells. Sections were washed with 0.05 M Tris, pH 7.5/10% ethanol/0.25% Tween 20 and incubated with biotinylated goat anti-rabbit IgG for 30 min. After washing, ABC-peroxidase reagent was applied (Vector Laboratories) and avidin–biotin immunocomplexes were visualized with metal-enhanced diaminobenzidine and hydrogen peroxide (Pierce).

RESULTS AND DISCUSSION

Antitumor effects and transgene expression levels of three sets of cytokine genes were evaluated in the present gene therapy study, utilizing a particle-mediated technique for *in vivo* gene transfer. These cytokine genes, including IL-6, IFNγ, TNFα, and IL-2, were initially selected for their reported antitumor effects in specific mouse tumor models and were later found in preliminary screening (data not shown) to confer an apparent antitumor activity. Systematic experiments using these cytokines were then executed, with the results described below.

IL-6. Data from several independent experiments showed that subcutaneous methylcholanthrene-induced fibrosarcoma (SP1) tumors exhibited a reduced growth rate when tumor-bearing mice were treated with hIL-6 DNA, compared with cohorts that received a control DNA vector (Fig. 1*A*). In initial experiments, both epidermal and dermal tissues surrounding the inoculated tumor were treated. Later experiments showed that epidermal transfections alone resulted in a similar reduction in tumor growth (Fig. 1*B*), and that the majority of hIL-6 expression resulted from epidermal gene delivery (Fig. 1*D*). At the discharge voltages used in this study, DNA-coated gold particles effectively transfect epidermal tissues; however, particle penetration to the subcutaneous tumor cells is limited. Thus, these findings suggest that localized expression of hIL-6 by nearby normal tissues plays a key role in the antitumor response. When the epidermal DNA treatments were initiated 5 days after tumor cell inoculation, a similar level of tumor growth reduction was observed (50%), suggesting that *in vivo* transfection with the hIL-6 gene is effective against established tumors.

Histology of the bombarded tissues revealed that the gold particles penetrated three to five cell layers and were primarily located in the skin tissue peripheral to the tumor mass. Immunohistochemistry with an antibody specific for hIL-6 distinctly showed transgenic hIL-6 expression by squamous cells in the epidermal layer (Fig. 2 *Upper*). To measure the level of transgenic IL-6 production, serum samples, tumors and nearby tissues were collected from *in vivo* transfected mice following euthanization. Two days after transfection, IL-6 levels reached 40 pg/ml in serum (Fig. 1*C*) and remained at detectable levels (1–5 pg/ml) for up to 7 days as measured by ELISA. The tumor and associated skin tissue extracts contained much higher levels of IL-6, ranging from 200 to 600 pg/ml (Fig. 1*D*). Thus, high levels of hIL-6 were expressed locally and in serum, compared with the mean physiological concentration of IL-6 in human serum, 1.6 pg/ml (31). The high-level, localized transgene expression apparently creates a cytokine protein gradient from which the transgenic hIL-6 can dissipate into the blood stream via diffusion. Although in the present experiments the mechanism by which IL-6 suppresses tumor growth is not quite clear, both cytotoxic T-cell and inflammatory responses have been previously demonstrated (32).

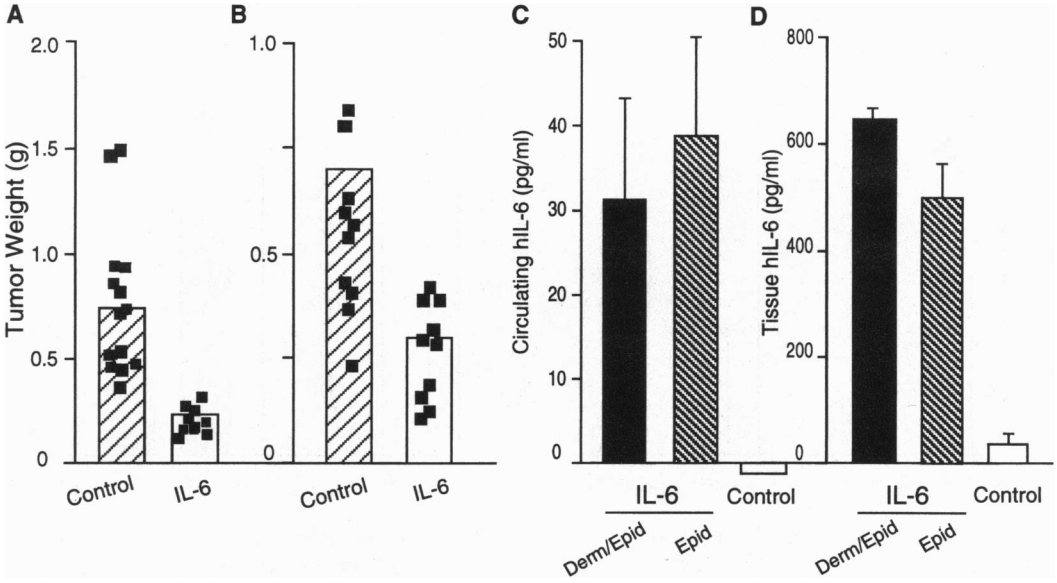

FIG. 1. *In vivo* expression and antitumor effects of a hIL-6 transgene in the mouse fibrosarcoma tumor (SP1) model. (*A*) Particle-mediated gene transfer of hIL-6 DNA to dermal and epidermal tissues overlaying a subcutaneous tumor results in reduction of tumor growth. The tumor reduction effect from one representative experiment is depicted; two other independent experiments showed similar results. Bars, average tumor weight; filled squares, individual tumor weights. (*B*) Bombardment of epidermal tissue alone results in similar antitumor effects. (*C*) Expression of transgenic hIL-6 protein in bombarded mouse skin and tumor tissues determined upon euthanization. Derm, dermis; Epid, epidermis. (*D*) Detection of transgenic hIL-6 protein in the circulation of test animals. Serum samples were obtained prior to and 1 week after the hIL-6 gene treatment regime was completed.

IFNγ plus TNFα. Cotransfection of IFNγ and TNFα genes *in vivo* by particle bombardment resulted in a drastic reduction of subcutaneous Renca tumor growth (Fig. 3 *A* and *B*). Three experiments, each with 11 or 12 animals per treatment set, showed highly reproducible effects. Compared with controls, average tumor weights excised from mIFNγ/hTNFα-treated animals were reduced by 42%, 48%, and 69% (one-tailed aggregate P value < 0.002). As in the IL-6 experiments, we found that epidermal gene deliveries alone resulted in a reduction of tumor growth (Fig. 2*B*). Results of three independent mIFNγ/mTNFα experiments showed average tumor weight reductions of 58%, 69%, and 87% (one-tailed aggregate P value ≤ 0.001). Also, mIFNγ/mTNFα gene treatments initiated 5 days rather than 1 day after tumor cell inoculation also resulted in a 35% reduction in average tumor weight (one-tailed P value $= 0.06, n = 10$). Tissue and serum collected for cytokine measurements 24 hr after transfection contained high levels of transgenic IFNγ and TNFα (Fig. 3 *C* and *D*). Circulating IFNγ levels in the test animals were 186 pg/ml compared with <1 pg/ml in the controls, which is significantly higher than the normal physiological concentration of IFNγ in human serum, <10 pg/ml (33).

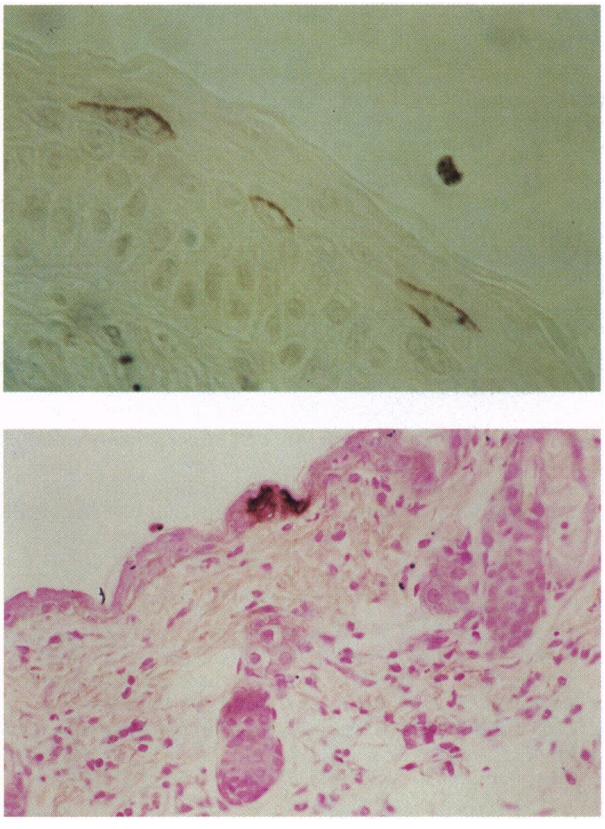

FIG. 2. Immunoperoxidase staining of transgenic hIL-6 (*Upper*) and hTNFα (*Lower*) proteins expressed in bombarded skin tissues 24 hr after *in vivo* transfection with the corresponding cDNAs.

Immunohistochemical analysis revealed that transgenic hTNFα was produced by the epidermal squamous cells (Fig. 2 *Lower*). Since the three cytokines studied here showed high expression levels and similar immunohistochemical staining patterns, these studies confirm that particle-mediated gene transfer is a highly efficient technology for expression of transgenic cytokines *in vivo*.

IL-2 plus IFNγ. The effects of mIL-2/IFNγ cytokine gene therapy on the survival of tumor-bearing mice were examined. Renca tumor-bearing mice treated with a combination of murine IL-2 and IFNγ genes had significantly increased survival compared with mice that received a control vector (Fig. 4). In the two experiments shown, 25% of the treated animals remained tumor-free (>60 days), demonstrating that strong immune and/or inflammatory responses capable of eradicating the tumor were induced.

In this study we evaluated an alternative approach for expression of cytokine transgenes both locally and systemically in tumor-bearing animals. Using two distinct tumor systems, we demonstrated that IL-6 was effective in reducing SP1 fibrosarcoma growth and that both TNFα/IFNγ and IL-2/

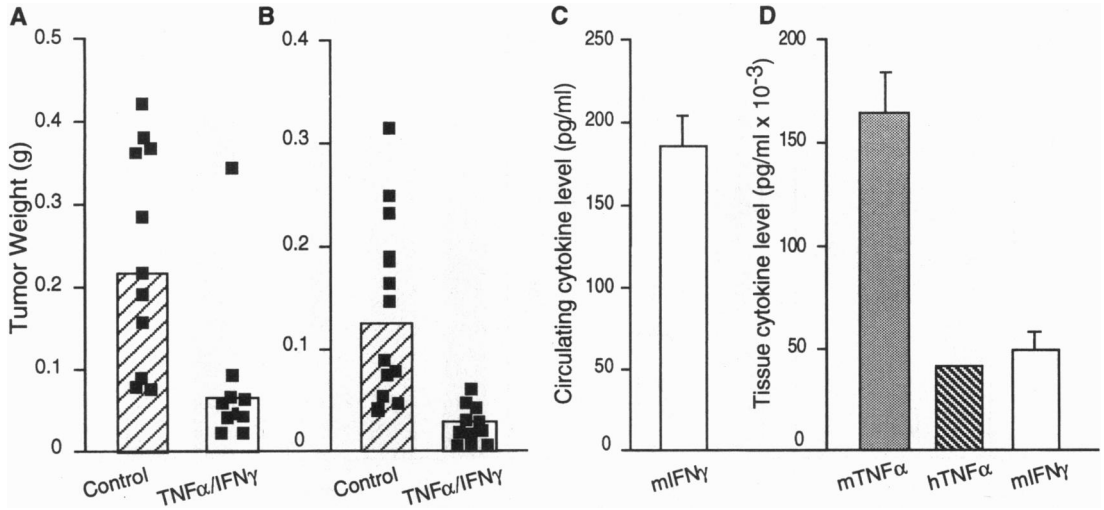

FIG. 3. Antitumor effects mediated by *in vivo* cotransfection of IFNγ and TNFα genes in a mouse Renca tumor model. Average and individual tumor weights measured 14 days after the first gene delivery are depicted. Bars, average tumor weight; filled squares, individual tumor weights. Each set consisted of 12 mice. (*A*) Tumor reduction effect measured after one dermal and five epidermal treatments with hTNFα and mIFNγ expression plasmids or with control DNA (pCMVluc). Two other independent experiments showed similar results. (*B*) Increased effectiveness of the murine TNFα gene. In these experiments, mice were treated with five epidermal cotransfections of mTNFα and mIFNγ genes. (*C* and *D*) Transgenic cytokine expression in serum and transgenic skin tissues, respectively, collected 24 hr after a single epidermal transfection. ELISAs were performed with commercial kits.

IFNγ gene combinations inhibited Renca tumor growth. In addition, IFNγ/IL-2 gene cotransfection resulted in significantly extended survival of Renca tumor-bearing mice. However, when tested with the current treatment regimen, a murine IL-4 gene construct did not affect the growth of Renca tumors (data not shown). This indicates that the current *in vivo* gene transfer approach, which results in cytokine secretion in a paracrine fashion, may be effective for certain cytokines but not for others. The efficient *in vivo* production of cytokines that can be achieved by the procedures defined in this study will allow a systematic evaluation of the antitumor activities of additional or newly identified cytokine genes.

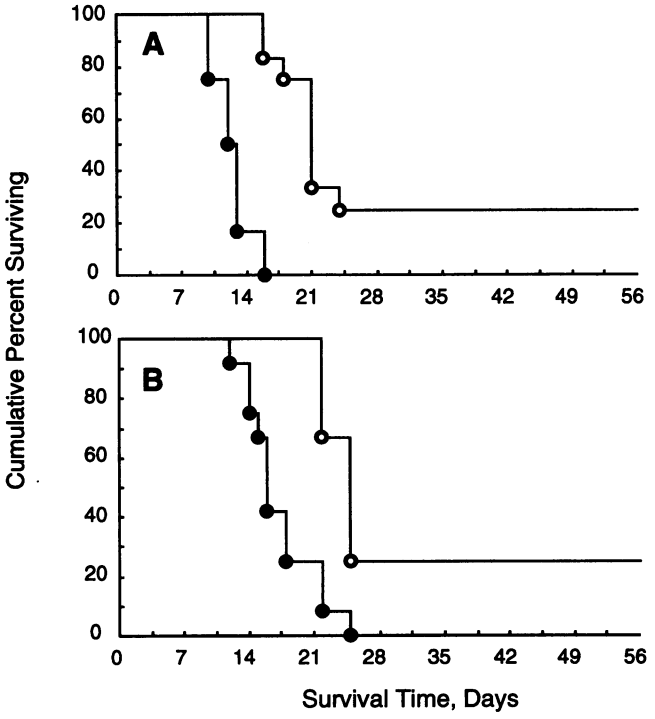

FIG. 4. Kaplan and Meier plots of the cumulative percent survival of Renca tumor-bearing mice bombarded with mIFNγ and mIL-2 genes (○) or pCMVluc as a control (●). Mice were euthanized when the tumor exceeded 1 cm in diameter or erupted through the skin; the interval between tumor cell injection and euthanization is used as the individual survival time. Epidermal bombardments with mIFNγ and mIL-2 genes were as described in Figs. 1 and 3. Two independent experiments (*A* and *B*) measuring survival times showed statistically significant differences ($n = 12$, $P \leq 0.005$, Wilcoxon rank-sum test) between treated and control animals.

In conclusion, this experimental tumor gene therapy system allows cytokine genes to be delivered to specific tissues *in vivo*, creating high peritumoral cytokine concentrations without apparent systemic toxicity. Particle-mediated gene transfer methods are minimally or not dependent on the specific characteristics of the target cell or tissue, since they deliver DNA via a physical force. We therefore suggest that there is potential utility of particle-mediated gene transfer for human cancer gene therapy. Although the underlying mechanisms involved remain to be determined, it is plausible that expression of certain cytokines in the tumor microenvironment mediates a localized or regional immune response which ultimately inhibits tumor growth.

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