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Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors

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ABSTRACT **Particle-mediated (gene gun) *in vivo* delivery of the murine interleukin 12 (IL-12) gene in an expression plasmid was evaluated for antitumor activity. Transfer of IL-12 cDNA into epidermal cells overlying an implanted intradermal tumor resulted in detectable levels (266.0 ± 27.8 pg) of the transgenic protein at the skin tissue treatment site. Despite these low levels of transgenic IL-12, complete regression of established tumors (0.4–0.8 cm in diameter) was achieved in mice bearing Renca, MethA, SA-1, or L5178Y syngeneic tumors. Only one to four treatments with IL-12 cDNA-coated particles, starting on day 7 after tumor cell implantation, were required to achieve complete tumor regression. This antitumor effect was CD8+ T cell-dependent and led to the generation of tumor-specific immunological memory. By using a metastatic P815 tumor model, we further showed that a delivery of IL-12 cDNA into the skin overlying an advanced intradermal tumor, followed by tumor excision and three additional IL-12 gene transfections, could significantly inhibit systemic metastases, resulting in extended survival of test mice. These results suggest that gene gun-mediated *in vivo* delivery of IL-12 cDNA should be further developed for potential clinical testing as an approach for human cancer gene therapy.**

Interleukin 12 (IL-12), a bimolecular glycoprotein consisting of a 35- and a 40-kDa subunit, was originally identified as a factor that stimulates natural killer cells (1, 2) and promotes maturation of cytotoxic T lymphocytes (CTL) (3, 4). It has recently been demonstrated that local or systemic treatment with recombinant (r) IL-12 protein mediates profound antitumor effects *in vivo*, causing regression of established subcutaneous tumors and tumor metastases (5, 6). However, systemic administration of rIL-12 caused dose-dependent toxicity in mice (7) and in human trials (8). Thus, a delivery mechanism that can provide relatively low levels of IL-12 at the target tissue might be advantageous in that it could generate an antitumor effect without causing systemic toxicity. Indeed, as cancer gene therapy has evolved, recent studies have produced encouraging results, showing that murine fibroblasts (9) or tumor cells (10) transduced *in vitro* with the IL-12 gene using a retroviral vector were able to induce antitumor immune responses. These data suggest that peritumoral IL-12 delivery may be as efficacious as systemic administration and avoid many undesirable side effects.

The particle-mediated method for gene delivery by gene gun utilizes a shock wave to accelerate DNA-coated gold particles into target cells or tissues. At submicrogram quantities of DNA per dose for *in vitro* or *in vivo* gene transfer, the gene gun can deliver thousands of DNA copies intracellularly into test tissues, resulting in high level transgene expression (11). As this method is cell surface receptor-independent, it can successfully deliver genes into a wide spectrum of mammalian cell types (12, 13). We have recently demonstrated that a particle-mediated, *in vivo* cytokine gene therapy reduces tumor growth in mice (14). Treatments with interferon-γ and tumor necrosis factor α shortly after the implantation of tumor cells inhibited tumor growth and prolonged the survival of tumor-bearing mice. To more closely approximate clinical situations, and to take advantage of the findings that IL-12 more effectively stimulates activated than naive T cells (15, 16), we evaluated the effect of IL-12 gene therapy on the growth of established tumors. In this study, we utilized the gene gun technology for *in vivo* IL-12 cDNA delivery into the skin overlying the implanted, established tumor tissues.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, DBA/2, and A/J female mice between 8 and 12 weeks of age were obtained from Harlan–Sparague–Dawley or Taconic Farms. All animal experiments were conducted in accordance with principles stated in ref. 17.

Murine Tumor Models. Six established mouse tumor cell lines were employed in this study, namely Renca carcinoma, MethA sarcoma (both syngeneic in BALB/c mice), L5178Y lymphoma, P815 mastocytoma (both syngeneic in DBA/2 mice), SA-1 sarcoma, and B16 melanoma (syngeneic in A/J and C57BL/6 mice, respectively). Tumor cell cultures were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and gentamicin at 50 μg/ml. MethA sarcoma, SA-1 sarcoma, P815 mastocytoma, and L5178Y lymphoma were grown as ascites in syngeneic mice for 1 week before injecting intradermally (i.d.). Mice were shaved in the abdominal area and injected i.d. with 1×10^6 (or 1×10^5, in the case of B16 tumor) tumor cells in 50 μl phosphate-buffered saline (PBS). Tumor growth was monitored two to three times a week by measuring two perpendicular tumor diameters using calipers.

IL-12 Gene Expression Vector. We constructed a plasmid (pWRG3169) containing coding sequences for the p35 and p40 subunits of murine (m) IL-12, linked tandomly in the same direction and each driven by its own cytomegalovirus (CMV) i/e promoter/enhancer, a simian virus 40 (SV40) sd/sa intron sequence, and a bovine growth hormone polyadenylylation sequence. The murine 35- and 40-kDa IL-12 subunit cDNA clones were isolated from mouse lymphocyte cDNA libraries by PCR cloning. The PUC19 plasmid backbone was derived from a bluescript SK(+) vector with an ampicillin-resistance gene (see Fig. 1*A*). A control vector containing a luciferase

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Abbreviations: IL-12, interleukin 12; CTL, cytotoxic T lymphocyte; mAb, monoclonal antibody; r, recombinant; m, murine; CMV, cytomegalovirus; SV40, simian virus 40; Luc, luciferase.

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(Luc) cDNA expression plasmid containing the CMV promoter was constructed as described by Cheng *et al.* (12).

***In Vivo* and *in Vitro* Gene Transfer.** The experiments utilized a helium-pulse Accell (gene gun) device that was designed by D. McCabe (Agracetus, Inc). Plasmid DNA was precipitated onto 2 μm gold particles. Particles were suspended in a solution of 0.1 mg of polyvinyl pyrrolidone per ml in absolute ethanol. This DNA/gold/particle preparation was coated onto the inner surface of a Tefzel tubing by using a tube loader (Agracetus), and the tubing was cut into 0.5-inch segments to result in delivery of 0.5 mg gold and 1.25 μg plasmid DNA per transfection. For tumor therapy, mouse skin overlying and surrounding the target tumor was transfected *in vivo* with IL-12 or Luc cDNA expression vectors starting from day 7 after i.d. implantation of 1×10^6 of five different types of tumor cells, except for B16 tumor, which was implanted at 10^5 cells. Each treatment consisted of four transfections (5 μg plasmid DNA/treatment) with a 300 psi helium gas pulse. One transfection was directly over the tumor site, and three additional treatments were evenly spaced around the circumference of the tumor in a triangle pattern. The *in vitro* particle bombardment gene transfer was performed as described (18).

IL-12 Bioassay. For determining transgenic IL-12 expression following *in vivo* gene transfer, blood was obtained by cardiac puncture, and skin tissue samples containing four transfection sites were collected in 0.5 ml of general extraction buffer, thoroughly minced with scissors, and sonicated before collecting the supernatant. The level of transgenic IL-12 protein was determined by a cell proliferation bioassay by using murine Con A-activated splenocytes as described (19). Briefly, spleen cells (5×10^6/ml) from naive BALB/c mice were stimulated with Con A (5 μg/ml) for 4 days at 37°C. Serial dilutions of the test samples (cell culture supernatants, serum, or skin tissue homogenates) were incubated with the activated spleen cells (2×10^4 cells/well) for 48 h, and the level of cell proliferation was measured by [^{3}H]thymidine incorporation. Serially diluted recombinant murine IL-12 (R & D Biosystems) was used as a standard. Anti-mIL-12 monoclonal antibody (mAb) (kindly provided by M. Gately, Hoffman–La Roche) was used to ensure that the bioactivity of the samples was due to IL-12. The sensitivity of this assay was about 10 pg/ml for rIL-12 standard protein and cell culture samples, and about 100 pg/ml for serum and skin tissue extracts.

Immunohistochemistry. The *in vivo* transfected skin tissues were sectioned in a cryostat (8 mm), placed on silanated slides and allowed to air dry. Test tissues were then fixed with acetone at 4°C for 10 min, air dried, washed in PBS for 10 min, and incubated with the anti-IL12 mAb (10 mg/ml) for 60 min at room temperature. Reacted tissues were rinsed two times in PBS and incubated with a biotinylated secondary antibody (rabbit anti-rat IgG; Vector Laboratories) for 60 min at room temperature. After rinsing with PBS, localization of the antibody binding was visualized with peroxidase staining and developed with metal enhanced 3,3-diaminobenzidine-H_2O_2. Sections were rinsed in PBS and counterstained with hematoxylin, dehydrated in ethanol, treated with xylene, and mounted with permount.

IL-12 Gene Therapy of Spontaneous Metastasis. A spontaneous metastasis model using weakly immunogenic P815 tumor has been described (20). DBA/2 mice were injected i.d. with 1×10^6 P815 cells. The skin overlying and surrounding the tumor was transfected with IL-12 cDNA or Luc cDNA on days 12 and 14 of tumor growth. Surgical excision of the tumor was performed on day 15 of tumor growth, and additional transfections of the skin on both sides of abdomen were performed on days 16, 18, and 20. Survival of the mice was followed.

Generation of Cytotoxic T Lymphocytes and Cytotoxic Assay. Tumor-specific CTL were generated *in vitro* as described (21). Briefly, spleen cells (5×10^6), derived from BALB/c mice that had rejected Renca tumors due to IL-12 gene therapy and had remained tumor-free for 2 months, or from age-matched naive mice, were cocultured with 5×10^4 mytomicin C-treated Renca cells in 24-well culture plates in complete RPMI 1640 media. After culturing for 5 days *in vitro*,

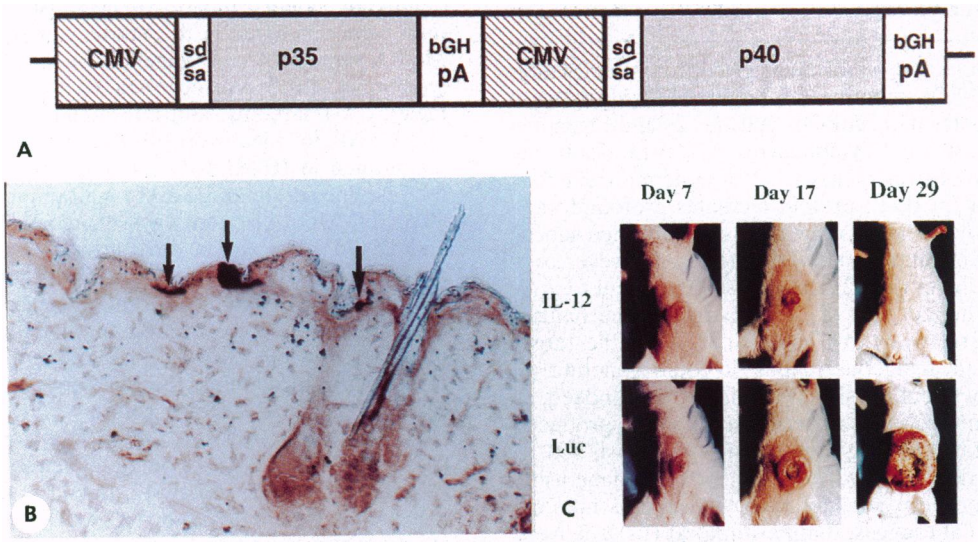

FIG. 1. *In vivo* transfer of IL-12 cDNA expression plasmid into mouse skin leads to regression of intradermally implanted, established tumors. (*A*) IL-12 cDNA gene construct engineered in the pWRG 3169 expression plasmid. CMV, cytomegalovirus i/e promoter; sd/sa, the SV40 splicing donor/splicing acceptor site; bGH PA, bovine growth hormone polyadenylylation signal sequence. (*B*) Detection of transgenic IL-12 protein in gene gun-treated skin tissues at 24 h after IL-12 cDNA delivery. Plasmid DNA was precipitated onto 2 μm gold particles. Mice were shaved in the abdominal area, and the epidermis was transfected with a 300 psi helium gas pulse by using the helium-pulse Accell device (gene gun). Immunoperoxidase assay demonstrates the presence of IL-12 protein (arrows) in the epidermal cell layers of the test mice. (*C*) Antitumor effect in MethA sarcoma model. Mouse skin overlying and surrounding the target tumor was transfected *in vivo* with IL-12 or Luc cDNA expression vectors on days 7 and 10 after i.d. implantation of 1×10^6 MethA cells. At each treatment, mice received four transfections (5 μg plasmid DNA/treatment). One transfection was directly over the tumor site, and three additional treatments were evenly spaced around the circumference of the tumor in a triangle pattern. Photographs of test mice were taken on days 7, 17, and 29 after tumor cell implantation. Whereas all mice treated with the control (Luc) plasmid DNA developed large tumors, three of eight mice treated with IL-12 gene exhibited complete tumor regression as shown here; the other five mice had reduced tumor growth.

graded numbers of viable effector cells and ^{51}Cr-labeled Renca cells (10^4) were placed into the round-bottomed wells of 96-well plates. After incubating for 4 hr at 37°C, radioactivity in supernatants was determined.

RESULTS AND DISCUSSION

Transgenic Expression of IL-12 *in Vitro* and *in Vivo*. We constructed a plasmid (pWRG3169) containing coding sequences for the p35 and p40 subunits of mIL-12, linked tandomly in the same direction and each driven by its own CMV i/e promoter/enhancer, a SV40 sd/sa intron sequence, and a bovine growth hormone polyadenylylation sequence (Fig. 1*A*). This version of a mIL-12 vector was found to be 3- to 8-fold more efficient in expressing IL-12 protein in B16 tumor cells transfected *in vitro* or in murine skin transfected *in vivo* than the same cDNA clones constructed in a single operon with an internal ribosome entry site linkage. This vector was also more efficient than using gold beads coated with a mixture of two different expression plasmids, one for each IL-12 subunit (data not shown). *In vitro* and *in vivo* expression of IL-12 was performed with this tandom IL-12 gene construct and compared with expression by a control vector containing a Luc cDNA expression plasmid. The level of transgenic IL-12 was determined by a cell proliferation bioassay. Upon *in vitro* gene gun-mediated delivery of 1.25 μg pWRG3169 DNA into 1×10^6 B16 (murine melanoma) cells, 49.8 ± 10.2 ng of functionally active IL-12 were detected at 24 h posttransfection. At 24 h after *in vivo* gene transfer into skin tissue, 266.0 ± 27.8 pg of mIL-12 were detected per 0.172 ± 0.026 g of fresh weight tissue within a standard 1.5×1.5 cm^2 full thickness skin biopsy that contained four gene gun-treated sites. Because of the limited sensitivity of the current IL-12 bioassay for serum and skin tissue extracts (≥100 pg/ml), we were unable to detect the low levels of IL-12 that might have been released into serum of test mice. We have previously shown (14) that very low levels of other cytokines, such as interferon-γ, interleukin 6, or granulocyte/macrophage colony-stimulating factor, can be detected in serum of mice undergoing gene therapy on the skin. It is important to note that the amount of IL-12 detected in the *in vivo* skin transfection sites was 1/400 to 1/40,000 of the dosage (0.1–10 μg) of the systemically injected rIL-12 protein which resulted in both antitumor effects and toxicity in mice (5–7).

Skin tissue overlying a 7-day i.d. Renca tumor was treated with IL-12 expression plasmid by gene gun delivery and biopsied 24 h later. Histologic examination revealed that the gold particles primarily penetrated to the epidermal cell layers of the mouse skin tissue but not into the underlying tumor cells. Accordingly, immunohistochemical staining of the skin tissue 24 h following gene gun delivery with pWRG3169 revealed that transgenic IL-12 was expressed only in the epidermal cell layers (Fig. 1B).

Tumor Regression and Suppression of Tumor Growth Following IL-12 Gene Therapy. It is known that certain murine immunogenic tumors can induce a T cell-mediated immune response that is best detected on days 7–9 of tumor growth in defined tumor models (22–24). Therefore we started the IL-12 cDNA treatments at 7 days postimplantation of tumor cells with the hope of enhancing the already activated endogenous antitumor immune response. Using this experimental strategy, the *in vivo* delivery of the chimeric IL-12 genes into skin tissues overlying established 7-day tumors resulted in complete tumor regression or suppression of tumor growth in four tumor models (Figs. 1*C* and 2). In mice bearing Renca, L5178Y, MethA, or SA-1 tumors, complete tumor regression was achieved in 87.5% (7/8), 87.5% (7/8), 57% (4/7), and 37.5% (3/8) of the tested mice, respectively (Fig. 2). Nearly identical results were achieved with Renca tumors after a single IL-12 cDNA treatment on day 7 (data not shown). In contrast, tumors grew progressively in most of the untreated mice (data not shown) or mice treated with Luc gene (Fig. 2). Furthermore, in mice bearing P815 mastocytoma or B16 melanoma, a significant suppression of tumor growth was achieved (Fig. 2). For example, on day 13 post-P815 tumor cell implantation, the mean tumor diameter in mice treated with IL-12 gene was 8.89 ± 0.27 mm versus 12.28 ± 0.46 mm in mice treated with Luc control gene in the same expression plasmid ($P < 0.001$). Likewise, on day 15 post-B16 tumor cell implantation, tumor diameter in mice treated with IL-12 gene was 6.30 ± 0.45 mm versus 11.8 ± 0.31 mm in mice treated with Luc gene ($P < 0.001$). However, the observed suppression of tumor growth was transient and all mice eventually died from progressing tumors. Whether or not a modification in gene transfer schedules could improve the result of therapy of these two weakly immunogenic tumors is unclear and warrants further evaluations, especially since most human tumors are believed to be weakly or not immunogenic.

It is important to note that for all tested mouse tumor models, the tumors were already well established at the beginning of the therapy, and had reached 5–8 mm in diameter. To our knowledge, this is the first evidence that an IL-12 gene therapy protocol can cause a complete regression of

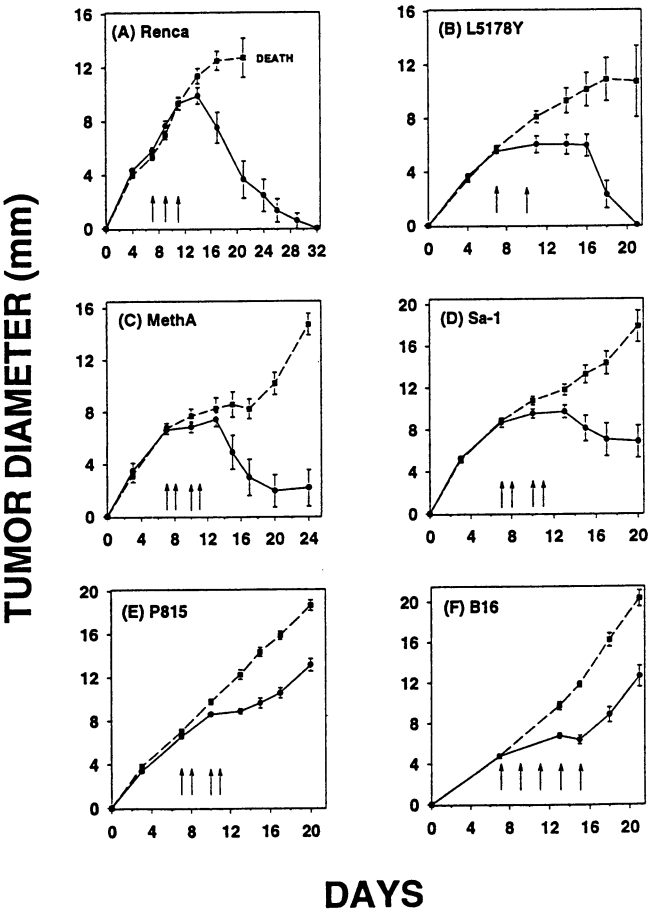

FIG. 2. Kinetics of regression of established murine tumors following *in vivo* IL-12 gene therapy. The gene therapy procedure was started at 7 days after i.d. injection of 1×10^6 (or 1×10^5, in the case of B16 tumor) indicated tumor cells. At each treatment, mice received four transfections with IL-12 DNA (circles) or with control DNA, pCMVLuc (squares). The arrows on each graph indicate the days following tumor injection on which gene transfer treatments were carried out. Mean tumor diameters ± SEM are shown for 7–8 mice per group except for B16 tumor model (12 mice per group). The IL-12 gene therapy experiments were repeated five times with the Renca tumor system, two times with MethA and P815 tumors, and once with L5178Y and B16 tumor models, and similar results were obtained.

established, relatively large tumors. Previous studies have shown that IL-12 gene therapy using retroviral vectors resulted in prevention of tumor development (9), or regression of small, 3-day-old MCA207 sarcomas in 33% of treated mice (10). It is also noteworthy that only 1–4 days of therapy (using four gene gun treatments per tumor site on each day of therapy) resulted in tumor regression or growth suppression in virtually all of our experiments. In previous studies using recombinant protein therapy, tumor regression required daily injections of IL-12 at doses from 0.1 to 10 μg for 1 week (6), or 5 days a week for 3–4 weeks (5) .

It is also important to note that in our IL-12 gene therapy protocol, the normal skin tissue overlying an established tumor is intentionally transfected topically to incite the existing antitumor immune response. The results presented in Figs. 1 and 2, in conjunction with our previous findings using other cytokine genes (14), indicate that transgenic IL-12 production by normal epidermal cells in the vicinity of the tumor is responsible for the antitumor effect of IL-12 gene therapy. Therefore, the *in vivo*, particle-mediated IL-12 gene transfer protocol is drastically different from other currently employed procedures of cancer gene therapy, where a therapeutic vector is either introduced into the tumor or other cells *in vitro*, or injected directly into the tumor mass *in vivo*.

Involvement of CD8$^+$ T Cells in Tumor Regression. To determine the role of CD4$^+$ and CD8$^+$ T cells in the observed tumor regression, we injected Renca tumor-bearing mice with anti-CD4 or anti-CD8 mAb on the next day after the beginning of IL-12 gene therapy and then 4 days later. This protocol was based on our previous findings showing that the same mAbs caused depletion of more than 90% of relevant T cell subsets in mice for 4–5 days following a single injection (25, 26). Fig. 3 provides direct evidence that the IL-12 gene therapy-induced tumor regression required CD8$^+$ cells, in that *in vivo* depletion of CD8$^+$ T cells, but not the depletion of CD4$^+$ T cells, abrogated the effect of IL-12 gene therapy. These data are in agreement with the findings of Brunda *et al.* (5) that tumor regression caused by rIL-12 is mediated by CD8$^+$ T cells, and not by CD4$^+$ T cells. In fact, depletion of CD4$^+$ T cells with anti-CD4 mAb appeared to result in slightly accelerated tumor regression (Fig. 3), implying that CD4$^+$ T cells may have suppressed the anti-tumor effect of IL-12 in this tumor model. Indeed, it has been shown that established tumors induce Th2-like CD4$^+$ T suppressor cells, which can inhibit CD8$^+$ T cell-mediated immune responses (24, 27, 28). The beneficial effect of anti-CD4 mAb treatment for tumor immunotherapy with rIL-2 protein (25) or IL-12 gene (29) has been previously reported. Supporting data show that IL-12 protein can activate tumor-specific CD8$^+$ T cells *in vitro* (30) and mediate an anti-suppressive effect on Th2 CD4$^+$ T cells *in vivo* (31, 32).

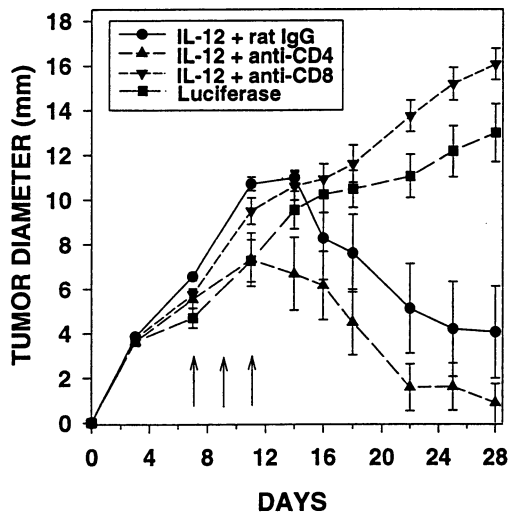

FIG. 3. Tumor regression caused by IL-12 gene requires CD8$^+$ T cells. BALB/c mice were injected i.d. with 1×10^6 Renca cells. Skin was transfected with IL-12 or Luc cDNA expression vectors on days 7, 9, and 11 posttumor implantation (arrows). Anti-CD4 mAb (clone GK1.5) or anti-CD8 mAb (clone 2.43), both obtained from the Trudeau Institute (Saranac Lake, NY), were administered intraperitoneally on days 8 (300 μg/mouse) and 12 (150 μg/mouse) after tumor implantation. Control groups included mice that were treated with the IL-12 gene and received rat IgG (Sigma) at the same doses and schedule as the anti CD8-and CD4 mAb, or mice treated with the Luc gene instead of the IL-12 gene. Mean tumor diameters ± SEM are shown for eight mice per group.

Anti-Metastatic Effect of Local IL-12 Gene Therapy. The results showing that tumor regression caused by local IL-12 gene therapy requires CD8$^+$ T cells suggest that local IL-12 gene delivery might result in a systemic antitumor effect. To test this hypothesis, we used the P815 tumor cells that metastasize into the visceral organs several days after the i.d. implantation, thereby causing the death of the mice even when the primary tumor has been surgically removed (20). In this system, an excision of the primary P815 tumor on day 12 (data not shown) or day 15 (Fig. 4) posttumor implantation was followed by death of all treated mice by day 37 or 29, respectively. However, when the skin overlying the i.d. P815 tumors were transfected with IL-12 cDNA on days 12 and 14 posttumor cell implantation, followed by tumor excision on day 15, and three additional IL-12 cDNA skin transfections at the abdominal sites adjacent to the excised primary tumor, a significant prolongation of the survival was observed (Fig. 4). These results suggest that local delivery of IL-12 gene into the skin tissue overlying and surrounding the primary tumor can

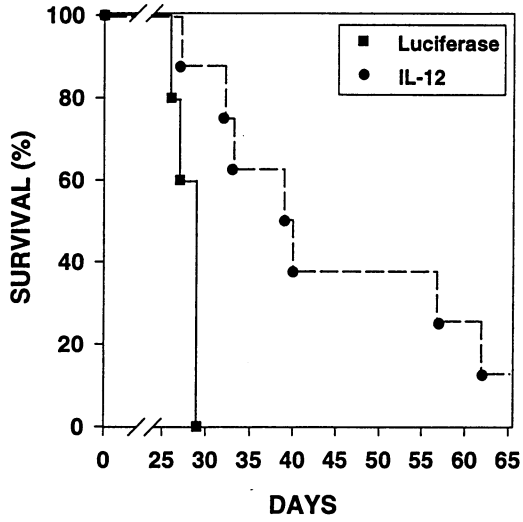

FIG. 4. Anti-metastatic effect of IL-12 gene therapy on P815 tumors. DBA/2 mice were injected i.d. with 1×10^6 P815 cells. Skin tissues overlying and surrounding the target tumor were treated with IL-12 cDNA delivered by gene gun (8 mice/group) or Luc cDNA (5 mice/group) on days 12 and 14 after tumor cell implantation. Surgical excision of the tumor was performed as described (20) on day 15, when tumor size reached about 13 mm in diameter. Additional transfections of skin on both sides of the abdomen were performed on days 16, 18, and 20 after implantation of tumor cells. All mice treated with the control Luc cDNA died in 28.0 ± 0.6 days after tumor cell implantation. Death was caused by spontaneous metastases of tumor cells into the internal organs, primarily the liver, as was evidenced by macroscopic examination (data not shown). IL-12 gene therapy effectively prolonged the survival of mice (survival time 41.4 ± 4.9 days, $P < 0.05$), and one of eight mice was "cured." This experiment was repeated without additional transfections posttumor excision, and we observed that all of the Luc cDNA treated mice ($n = 11$) died in 43.9 ± 7.1 days, whereas 5 of 12 (41.6%) IL-12 gene therapy-treated mice survived for at least 180 days and thus were considered cured.

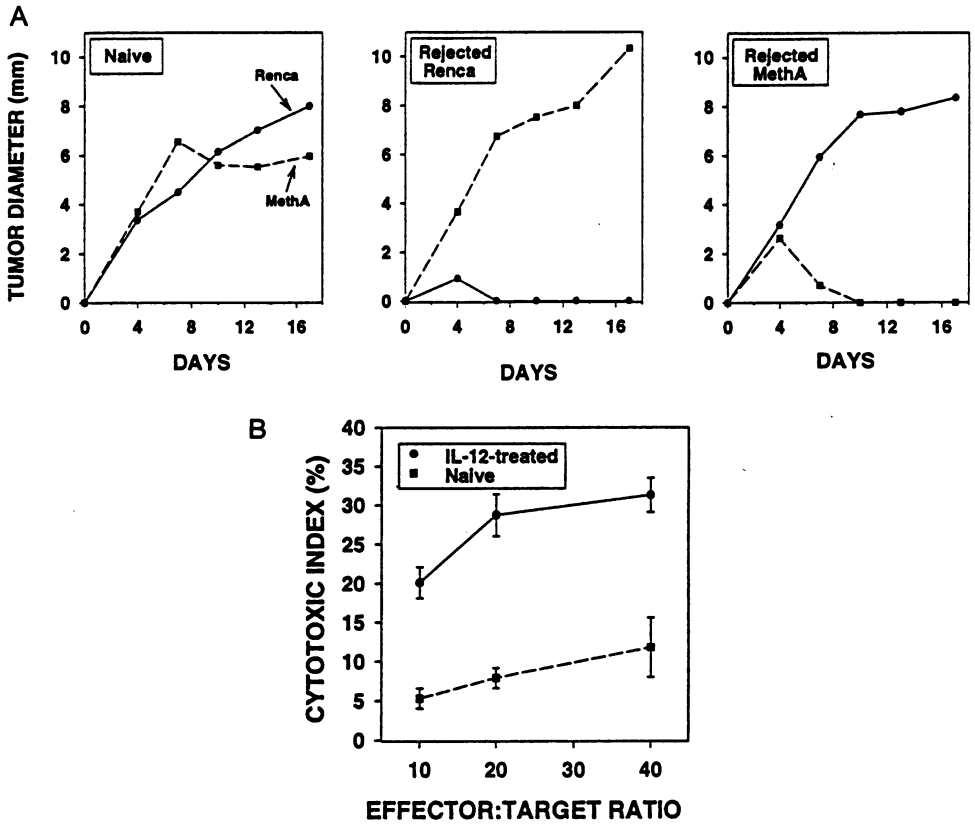

FIG. 5. IL-12 gene therapy in tumor-bearing mice results in development of tumor-specific immunological memory. (*A*) Rejection of secondary tumor challenge following IL-12 gene therapy. BALB/c mice that rejected Renca or MethA tumors following IL-12 gene therapy were injected one month later with 1×10^6 of both Renca cells (circles) and MethA cells (squares) on the right and the left side of abdomen, respectively. As a control, the tumor cells were injected into age-matched naive BALB/c mice. Data are presented as the means of five to eight mice per group. The experiment was repeated using mice that rejected L5178Y tumors and were secondarily challenged with L5178Y or P815 tumor cells, and similar results were obtained. (*B*) Induction of CTL activity in mice that rejected tumors following IL-12 gene therapy. Tumor-specific CTL were generated *in vitro* as described. Mean ± SEM of four mice per group. Spleen cells from IL-12 gene-treated mice generated 3- to 4-fold higher levels of CTL activity than spleen cells from naive mice ($P < 0.005$).

augment systemic antitumor immune response even against a weakly immunogenic tumor, and this can lead to eradication of established spontaneous metastases in mice. Therefore, such human metastatic cancers as subcutaneous T-cell lymphoma or melanoma may provide excellent models for future clinical application of the current IL-12 gene therapy approach.

Immunological Memory in Mice Following IL-12 Gene Therapy. It has been recently shown that tumor regression caused by rIL-12 protein therapy results in development of a memory immune response against the tumor (33). We evaluated if the mice that rejected tumors following the *in vivo* IL-12 gene therapy developed tumor-specific immunity. Fig. 5*A* shows that the mice which rejected Renca tumors and were tumor-free for 1 month resisted a second challenge with Renca cells but developed tumors when challenged with MethA tumor cells. Inversily, the mice that rejected MethA tumors following IL-12 gene therapy resisted the second challenge with MethA cells, but developed tumors when challenged with Renca cells. These results demonstrate that mice which rejected their tumors following skin transfection with IL-12 gene develop tumor-specific immunological memory against a secondary tumor challenge. Furthermore, spleen cells from the mice that rejected Renca tumors, in contrast to spleen cells from naive mice, exhibited CTL activity upon stimulation with Renca cells *in vitro* (Fig. 5*B*). In a similar study, using mice that rejected L5178Y tumors, we found that the generated CTL were tumor-specific, in that they lysed the L5178Y target tumor cells but not the syngeneic P815 target cells (data not shown).

Our study shows that gene gun-mediated *in vivo* delivery of an IL-12 expression plasmid into skin tissue overlying tumor sites is an effective approach for tumor immunotherapy in various murine tumor models, leading to eradication or suppression of established intradermal tumors and their spontaneous metastases. Remarkably, the local amount of detectable IL-12 at the treatment tissue site is 1/400 to 1/40,000 of the dosage of rIL-12 protein employed for efficacy studies in the same or similar mouse tumor models (5, 6, 33). In view of the apparent dose dependent toxicity of human rIL-12 protein in clinical trials (8), this dosage difference between the recombinant protein delivered systemically and transgenic protein produced via *in vivo* DNA delivery may make the present IL-12 gene therapy approach more favorable for clinical considerations. Given the efficacy, simplicity, and potential cost-effectiveness of the gene gun-mediated IL-12 gene therapy approach, further preclinical development of this approach is warranted in order to consider initiation of human cancer clinical trials.

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