

# Radiation Improves Intratumoral Gene Therapy for Induction of Cancer Vaccine in Murine Prostate Carcinoma

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## ABSTRACT

Our goal was to convert murine RM-9 prostate carcinoma cells *in vivo* into antigen-presenting cells capable of presenting endogenous tumor antigens and triggering a potent T-helper cell-mediated immune response essential for the generation of a specific antitumor response. We showed that generating the major histocompatibility complex (MHC) class I+/class II+/Ii– phenotype, within an established subcutaneous RM-9 tumor nodule, led to an effective immune response limiting tumor growth. This phenotype was created by intratumoral injection of plasmid cDNAs coding for interferon gamma, MHC class II transactivator, and an antisense reverse gene construct (RGC) for a segment of the gene for Ii protein (–92,97). While this protocol led to significant suppression of tumor growth, there were no disease-free survivors. Nevertheless, irradiation of the tumor nodule on the day preceding initiation of gene therapy yielded 7 of 16 mice that were disease-free in a long-term follow up of 57 days compared to 1 of 7 mice receiving radiotherapy alone. Mice receiving radiotherapy and gene therapy rejected challenge with parental RM-9 cells and demonstrated specific cytotoxic T-cell activity in their splenocytes but not the mouse cured by radiation alone. These data were reproduced in additional experiments and confirmed that tumor irradiation prior to gene therapy resulted in complete tumor regression and specific tumor immunity in more than 50% of the mice. Increasing the number of plasmid injections after tumor irradiation induced tumor regression in 70% of the mice. Administering radiation before this novel gene therapy approach, that creates an *in situ* tumor vaccine, holds promise for the treatment of human prostate carcinoma.

## OVERVIEW SUMMARY

We investigated sequential tumor irradiation followed by intratumoral gene therapy for *in situ* induction of a cancer vaccine in murine RM-9 prostate carcinoma. DNA plasmid vectors encoding for interferon- $\gamma$  major histocompatibility complex (MHC) class II transactivator, and an antisense reverse gene construct (RGC) for a segment of Ii protein gene were designed and injected into RM-9 tumors. This approach causes genetic modification of tumor cells *in situ* leading to the expression of MHC class I+/class II+/Ii– phenotype and converts these cells into antigen-presenting cells capable of triggering a robust antitumor immune response. We showed that intratumoral gene therapy caused

a significant but transient suppression of tumor growth. Irradiation of the tumor nodule, 1 day prior to gene therapy, enhanced gene therapy efficacy, and led to approximately 50% tumor-free mice. These mice rejected RM-9 tumor challenge and showed specific cytotoxic T-cell activity. Radiation improved the therapeutic effect of gene therapy and induction of cancer vaccine.

## INTRODUCTION

CARCINOMA OF THE PROSTATE is the most common malignant tumor in men, with more than 180,400 newly diagnosed cases annually, resulting in greater than 31,900 deaths

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each year (Jemal *et al.*, 2002). Localized prostate carcinoma is sensitive to conventional radiotherapy using megavoltage photons (x rays), however, residual disease often causes clinical relapse (Powell *et al.*, 1997; Gray *et al.*, 2001). Once the regional lymph nodes become involved, 85% of patients develop distant metastases in 5 years (Haas and Pontes, 1995). Locally advanced prostate cancer and metastatic prostate cancer present a difficult therapeutic problem for clinicians. Locally advanced prostate cancer is treated by radiation and androgen blockade but this therapy fails in a significant number of patients resulting in tumor recurrence and disease progression. Metastatic prostate cancer is first treated by androgen blockade, however, within 18 months it usually becomes hormone refractory and patients have an expected survival of less than a year (Hillman *et al.*, 1999). Given the limitations of current therapies, immunotherapy and gene therapy are being explored to eradicate residual primary and metastatic disease (Hillman *et al.*, 1999; Steiner and Gingrich, 2000; Harrington *et al.*, 2001). These approaches include autologous dendritic cells pulsed with PSMA peptides (Tjoa *et al.*, 1998) or transfected with prostate-specific antigen (PSA) mRNA (Heiser *et al.*, 2002), autologous granulocyte-macrophage colony-stimulating factor (GM-CSF)-transduced tumor cells (Simons *et al.*, 1999), immunization with vaccinia vector encoding PSA (rV-PSA) (Eder *et al.*, 2000) and intratumoral cytokine interleukin-2 (IL-2) DNA gene therapy (Belldegrun *et al.*, 2001). The goal of active immunotherapy is to induce a potent immune response to break tolerance and eradicate micrometastatic disease. Used in conjunction with definitive local therapies (i.e., radiation and radical prostatectomy), and effective systemic treatments (e.g., hormone ablation), active immunotherapy has the potential to extend disease-free survival.

We previously investigated the combined use of local tumor irradiation with systemic immunotherapy for prostate cancer in a model of human PC-3 prostate carcinoma cells implanted in the prostate of nude mice (Hillman *et al.*, 2001). The therapeutic effect of tumor radiation was enhanced by systemic IL-2 therapy. However, all mice died of recurrent tumor. In the current study, we have tested the combination of radiation with a novel *in situ* tumor cell vaccine, turning tumor cells into antigen-presenting cells (APC) to present endogenous tumor antigens to activate CD4<sup>+</sup> helper T cells. To elicit a specific anti-tumor response, tumor-associated antigens (TAA) on tumor cells must be presented to helper T cells and cytotoxic T cells in the context of major histocompatibility complex (MHC) molecules via APC. Activation of tumor-specific helper T cells has been found to be essential for optimal activation of cytotoxic T cells and for the generation of long-term immunological memory (Ostrand-Rosenberg *et al.*, 1990; Armstrong *et al.*, 1997; Pardoll and Topalian, 1998; Xu *et al.*, 2000; Wang *et al.*, 2001). Helper T cells recognize antigens in the context of MHC class II molecules but the invariant chain restricts endogenous peptide presentation by MHC class II molecules. The invariant chain Ii is a type-II membrane glycoprotein that acts as a chaperone molecule inhibiting binding of endogenous antigens to newly synthesized MHC class II molecules in the endoplasmic reticulum (ER) (Koch *et al.*, 1982; Stockinger *et al.*, 1989; Guagliardi *et al.*, 1990). This mechanism allows only exogenous peptide binding to MHC class II molecules and limits the repertoire of peptides presented by MHC class II molecules. It

has been hypothesized that tumor peptide presentation by MHC class II molecules is blocked in the presence of Ii and that this can explain, in part, the failure of the immune system to eradicate tumor. Ostrand-Rosenberg and colleagues demonstrated that transfection of MHC class I+, MHC class II- murine tumors with genes for MHC class II  $\alpha$  and  $\beta$  chains leads to MHC class II-expressing tumor cells which prime mice against challenge with the parental tumor (Ostrand-Rosenberg *et al.*, 1990; Armstrong *et al.*, 1997, 1998a,b; Qi *et al.*, 2000). Supratreansfecting such cells with the gene for the Ii protein abrogated the immunopotentiating effect (Clements *et al.*, 1992; Armstrong *et al.*, 1997). If Ii is suppressed, nascent MHC class II molecules in the ER pick up peptides from the pool transported there for binding to MHC class I molecules. A potent CD4<sup>+</sup> T-helper cell response to these novel MHC class II-presented epitopes leads to enhanced priming of dendritic cells, a robust cytotoxic T lymphocyte (CTL) response to MHC class I-presented determinants, antibodies, and memory. Because of allele heterogeneity, it is not practical in humans to induce the MHC class II+/Ii- phenotype by transfecting with individual MHC class II genes. However, one can induce endogenous MHC class II molecules by transfecting a gene for the MHC class II transactivator (CIITA), which induces both MHC class II molecules and the immunoregulatory Ii protein (Chang and Flavell, 1995; Reith and Mach, 2001). The Ii protein is then suppressed by an antisense method (Qiu *et al.*, 1999). This method is clinically feasible because of the monomorphic nature of Ii gene.

We have developed a novel prostate cancer vaccine by genetic modification of tumor cells with genes causing upregulation of MHC class I and class II molecules and suppression of Ii protein. These preclinical studies were performed in the RM prostate cancer model syngeneic in normal C57BL/6 mice (Thompson *et al.*, 1989; Nasu *et al.*, 1999). DNA plasmid vectors carrying the genes for the CIITA gene or interferon (IFN)- $\gamma$  gene or a reverse gene construct to Ii were constructed. We found that RM-9 cells can be induced to express the MHC class I+/class II+/Ii- phenotype *in vitro* by transfection with these vectors. Intratumoral gene therapy using these vectors to genetically modify the RM-9 cells in established tumors *in situ* resulted in inhibition of tumor growth. The efficacy of *in situ* induction of the cancer vaccine was enhanced by tumor irradiation administered prior to intratumoral gene therapy.

## MATERIALS AND METHODS

### *Tumor model*

RM-9, a murine prostate cancer cell line, was derived from independent primary prostate tumors induced in the Zipras/myc-9-infected mouse prostate reconstitution (MPR) model system using C57BL/6 mice as previously described (Thompson *et al.*, 1989). Cells were maintained *in vivo* by serial subcutaneous passages and also cultured *in vitro* in culture medium (CM) consisting of Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin, and 10 mM HEPES buffer (Gibco BRL, Life Technologies, Grand Island, NY) (Hall *et al.*, 1997; Nasu *et al.*, 1999). Cells were passaged, *in vitro*, by trypsinization using

0.25% trypsin. For *in vivo* implantation, RM-9 cells were washed in Hanks' balanced salt solution (HBSS) and injected subcutaneously at  $2 \times 10^5$  cells in 0.1 ml HBSS, in 4–6-week-old C57BL/6 mice (Harlan Sprague Dawley Inc., Indianapolis, IN). To fit the radiation apparatus, cells were injected in the middle of the back, 1.5 cm from the tail. Mice were shaved prior to injection for accurate location of the injection site and for monitoring tumor growth. Mice were housed and handled in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. The Wayne State University Animal Investigation Committee approved the animal protocol.

#### Gene expression vectors

The plasmids pEF/Bsd/CIITA, (pCIITA) and pcDNA (3)/IFN- $\gamma$  (pIFN- $\gamma$ ) were constructed with cytomegalovirus (CMV) promoters based on constructs from Invitrogen (Carlsbad, CA) by standard molecular biology techniques. The plasmid Ii-RGC (pIi-RGC) was constructed by cloning an Ii gene fragment of base pairs from -92 to 97 (where A in the AUG start codon is position 1) into RSV.5 vector in a reverse orientation, being driven by a RSV promoter to avoid promoter competition when large amounts of Ii-RGC were used. This construct was selected for our studies because it was more effective than the same construct driven by a CMV promoter. Ii-RGC (-92 to 97) was further cloned into E1/E3-deleted adenoviral vectors (Ad-vectors) (Quantum, Montreal, Canada) according to the manufacturer's instructions to form recombinant adenovirus containing Ii-RGC (Ad-Ii-RGC). The IL-2 containing plasmid (pIL-2), pNGVL-hIL-2 plasmid (CMV promoter/enhancer/intron A), was obtained from National Gene Vector Laboratory (University of Michigan, Ann Arbor, MI). Plasmids were expanded by culturing transformed *Escherichia coli* DH5 $\alpha$  competent cells in LB Broth with an appropriate selective antibiotic: ampicillin for pCIITA, pIFN $\gamma$ , and pIi-RGC and kanamycin for pIL-2 (Life Technologies). Plasmids were purified with Giga Kits (Qiagen, Valencia, CA) and purity was assayed by electrophoresis in a 1% agarose gel.

#### Transduction of RM-9 cells with Ad-vectors in vitro

RM-9 cells were plated at  $2 \times 10^5$  cells per well in 6-well plates. After 24-hr incubation at 37°C in 5% CO<sub>2</sub> incubator, cells were washed twice with phosphate-buffered saline (PBS) and Ad-vectors were added in 0.5 ml PBS at different multiplicity of infection (MOI). Cells were infected for 1 hr at 37°C and 5.5 ml CM were added. Cells were further incubated for 96 hr.

#### Transfection of RM-9 cells with plasmid vectors in vitro

RM-9 cells were plated at  $2 \times 10^5$  cells per well in 6-well plates. After 24-hr incubation at 37°C in 5% CO<sub>2</sub> incubator, cells were washed twice with PBS. DNA plasmid vectors (3 or 6  $\mu$ g DNA) were mixed with PolyFect transfection reagent (Qiagen) then added in duplicate wells in 1.5 ml CM. After 2 days incubation, cells were removed and replated in 24-well plates at  $2 \times 10^4$  with selective antibiotics: blasticidin-S-HCL for pCIITA and hygromycin B for pIFN- $\gamma$  (Life Technologies). An-

tibiotic resistant colonies were selected and expanded further in cultures with selective antibiotics. Transfected clones were identified by expression of MHC molecules.

#### Expression of MHC class I and class II molecules and Ii protein

Cells transfected with plasmids and transduced with Ad-vectors, were washed with HBSS, removed by trypsinization and washed twice with HBSS. To assay for cell surface expression of MHC class I and II molecules, cells were labeled with fluorescein isothiocyanate conjugated (FITC) antibodies monoclonal antibodies (mAb). Anti-H-2K<sup>b</sup> mAb directed against MHC class I alloantigen or anti I-A/I-E mAb specific to MHC class II alloantigens (Pharmingen, San Diego, CA) were used in a direct immunofluorescence assay (Younes *et al.*, 1995). Cells were labeled for 45 min on ice, washed twice in HBSS, and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Gates were set for nonspecific binding using cells labeled with FITC mouse IgG<sub>2a</sub> to control for anti-MHC class I mAbs or FITC rat IgG<sub>2a</sub> to control for anti-MHC class II mAb. To test for intracellular Ii expression, cells were fixed with 2% formalin, washed once with 1 M glycine and twice with HBSS containing 0.2% saponin. Cells were labeled with rat anti-mouse Ii mAb In.1 (Koch *et al.*, 1982) for 1 hr on ice, washed twice in saponin buffer and labeled with FITC-goat F(ab')<sub>2</sub> anti rat IgG (ICN Pharmaceuticals, Aurora, Ohio) for 30 min on ice (Qiu *et al.*, 1999). Cells were analyzed on a FACScan flow cytometer setting gates for nonspecific binding using cells labeled with FITC-goat F(ab')<sub>2</sub> anti-rat IgG. Murine A20 lymphoma cells were used as a control for positive MHC class II molecules and Ii staining.

**Radiation.** An apparatus developed for radiotherapy of mouse prostate tumors (Hillman *et al.*, 2001) was adapted for the radiation of subcutaneous tumors located in the middle of the back, at 1.5 cm from the tail. Acrylic jigs were designed to place anesthetized mice in the supine position with their forelimbs and hindlimbs restrained by posts for reproducible and accurate positioning of the subcutaneous tumor on the back as described previously (Hillman *et al.*, 2001). Three jigs were positioned on an aluminum frame mounted on the x-ray machine to irradiate three mice at a time. Lead shields of 6.4 mm thickness were designed with three cutouts for the three mice to expose the area of the tumor to photon irradiation while shielding the rest of the mouse body (Hillman *et al.*, 2001). The radiation dose to the tumor and the scattered dose to areas of the mouse outside of the radiation field were carefully monitored. Photon irradiation was performed with a Siemens Stabilipan X ray set (Siemens Medical Systems, Inc) operated at 250 kV, 15 mA with 1 mm copper filtration at a distance of 47.5 cm from the target.

#### Intratumoral treatment with DNA plasmid vectors

Mice were injected subcutaneously with RM-9 cells at  $2 \times 10^5$  cells in 0.1 ml HBSS. On day 7 post-cell injection, established RM-9 tumors were treated with intratumoral injections of mixtures of DNA plasmid vectors. CIITA, IFN- $\gamma$ , and IL-2 DNA plasmids were injected at a dose of 3  $\mu$ g per injection per day while Ii-RGC DNA plasmid was injected at 31  $\mu$ g per in-

jection per day. We used approximately 10 times more Ii-RGC than CIITA in order to insure that each cell transfected with a CIITA gene was also transfected with an Ii-RGC, and to ensure that there would be sufficient suppression of the Ii protein in light of Ii induction caused by CIITA. A total of 40  $\mu\text{g}$  plasmid was injected per mouse, and the total amount of plasmid DNA was adjusted when needed using empty plasmid DNA to result in the same total DNA amount in all groups. Plasmids vectors were mixed with a liposome formulation of cationic lipid DMRIE [1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide/cholesterol] (DMRIE-C, Gibco, Life Technologies) 2–4 min prior to injection at a ratio of 1:5 w/w, DMRIE/DNA. Four injections of plasmids were given once a day, on days 7, 8, 9, and 10 after cell injection. Experimental groups consisted of 7–8 mice/group. Group 1, the control group received PBS. Group 2 received injections of a mixture of pCIITA plus pIFN- $\gamma$  plus empty plasmid. Group 3 received injections of a mixture of pCIITA plus pIFN- $\gamma$  plus pIL-2 plus empty plasmid. Group 4 received injections of a mixture of pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC. Mice were monitored for tumor growth and survival. Tumors were measured in three dimensions, three times per week, with a caliper. Tumor volume was calculated using the equation:  $0.5236 \times \text{length} \times \text{width} \times \text{height}$ . In all experiments, when tumors reached 1.5 cm in greatest diameter or 1 cm with ulceration, mice were moribund and sacrificed in accordance with animal facilities regulations.

#### *Combination of radiation and intratumoral gene therapy*

Mice were injected subcutaneously with RM-9 cells at  $2 \times 10^5$  cells in 0.1 ml of HBSS. Mice with established tumors were treated on day 6 with selective tumor irradiation administered at a single dose of 8 Gy photons. A day later, on day 7, intratumoral injections of plasmid mixtures were initiated and continued on day 8, 9, and 10 as described above. Experimental groups of 7–8 mice per group were treated either with PBS, or tumor irradiation and PBS intratumorally, or tumor irradiation and a mixture of pCIITA plus pIFN- $\gamma$  plus pIL-2 plus empty plasmid, or tumor irradiation and injections of a mixture of pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC. The doses of plasmids and conditions of injection were identical to those given in groups 3 and 4, above. Mice were monitored for tumor growth and survival. Tumors were measured in three dimensions, three times per week, with a caliper. Tumor volume was calculated using the equation:  $0.5236 \times \text{length} \times \text{width} \times \text{height}$ . Mice with no evidence of tumor at day 57 underwent challenge with  $1 \times 10^5$  parental RM-9 tumor cells injected subcutaneously in the opposite flank; as a control, three naïve mice also underwent challenge in this fashion.

#### *Cytolysis assay*

Splenocytes were isolated from the spleens of mice challenged with RM-9 cells and from two naïve mice that were not challenged. Splenocytes at  $5 \times 10^6$  per milliliter were stimulated with  $10^5$  per milliliter of mitomycin-C-treated RM-9 cells or RM-9/IFN- $\gamma$  clone cells in 24-well plates in the presence of 60 international units of recombinant hIL-2 (Chiron Corporation, Emeryville, CA) for 4 days. These stimulated splenocytes were used as effector cells in a standard 4 hr  $^{51}\text{Cr}$  release as-

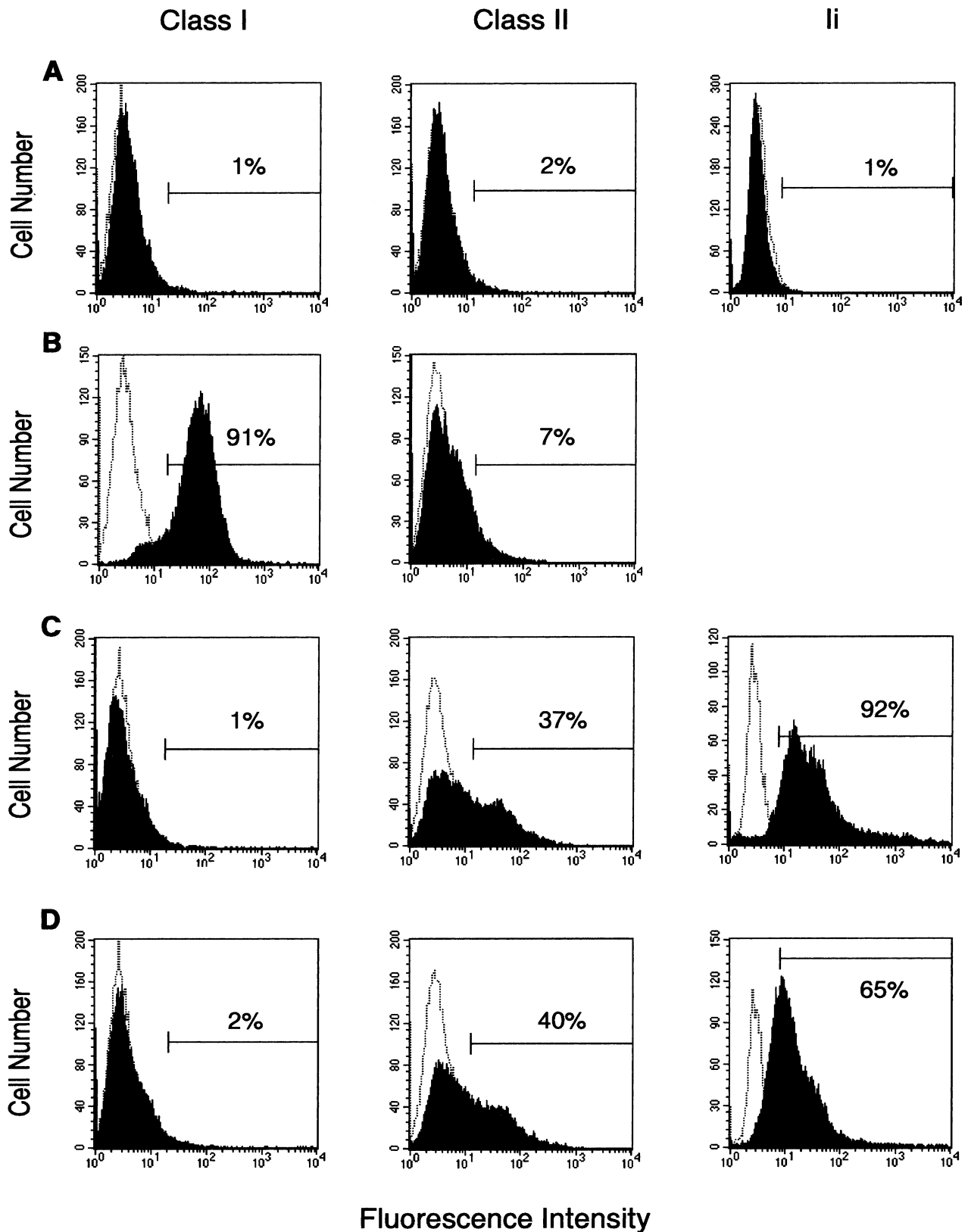
say to measure the cytolysis of specific targets RM-9, RM-9/IFN- $\gamma$  and the unrelated syngeneic target EL-4 murine thymoma. Briefly, effector cells were plated in 96-well plates and serially diluted in triplicate wells to result in effector to target ratio of 100:1, 50:1, 25:1, and 12.5:1. Targets cells were labeled with 250  $\mu\text{Ci}$   $^{51}\text{Cr}$  and used at 5000 cells per well. After 4 hr of incubation, release of  $^{51}\text{Cr}$  was measured in the cell supernatant using a gamma counter (Packard, Downers Grove, IL). The percent cytotoxicity was calculated using the mean of the triplicate wells taking into account  $^{51}\text{Cr}$  spontaneous release obtained from target cells incubated alone in medium and  $^{51}\text{Cr}$  maximal release obtained by detergent lysis of target cells.

*Statistical analysis.* The BMDP Release 7 statistical software package program was used for data analyses. Wald's tests of significance were used to establish statistically significant differences in subcutaneous tumor sizes among the various treatment groups. Subsequent analyses of the data consisted of pair-wise comparison of treatment groups that resulted in the production of unadjusted  $p$  values. In order not to inflate the overall significance level of 0.05, the Holm simultaneous testing procedure was applied to provide adjusted  $p$  values and to identify treatment group comparisons that were statistically significant. Survival curves were constructed by the Kaplan-Meier product limit method. The Mantel-Haenszel  $\chi^2$  test was used to compare the survival curves. On the establishment of statistical significance at the 0.05 level, the Holm simultaneous testing procedure was utilized to conduct a multiple pair-wise comparisons of survival curves and to identify the significant ones while maintaining the global statistical significance level of 0.05.

## RESULTS

#### *Induction of MHC class I and class II molecules and suppression of Ii protein in RM-9 cells by genetic modification in vitro*

RM-9 cells were negative for expression of MHC class I and class II molecules and for intracellular Ii protein (Fig. 1A). To upregulate these molecules, RM-9 cells were transfected, *in vitro*, with DNA plasmids containing genes for either IFN- $\gamma$  or the CIITA transactivator of MHC class II gene expression. Cell clones were selected and assayed for expression of MHC molecules and intracellular Ii. Most RM-9/IFN- $\gamma$  clones expressed MHC class I molecules but not MHC class II molecules (Fig. 1B). Of 16 clones transfected with pIFN- $\gamma$ , only 1 upregulated MHC class II, but at a low density (mean fluorescence intensity [MFI] = 17). All clones expressed MHC class I molecules on 85–90% of the cells at relatively high density (MFI = 80). RM-9/CIITA clones showed MHC class II molecule upregulation on approximately 40% of the cells but were negative for MHC class I molecules (Fig. 1C). MHC class II upregulation was accompanied by upregulation of intracellular Ii protein (Fig. 1C). These data show the need for expression of both genes (CIITA and IFN- $\gamma$ ) to induce the MHC class I+/class II+/Ii+ phenotype in RM-9 cells. Interestingly, RM-9/CIITA and RM-9/IFN- $\gamma$  clones were tumorigenic when implanted subcutaneously in mice. Expression of MHC class I or MHC class II molecules was not sufficient to abrogate RM-9 tumorigenicity in mice.

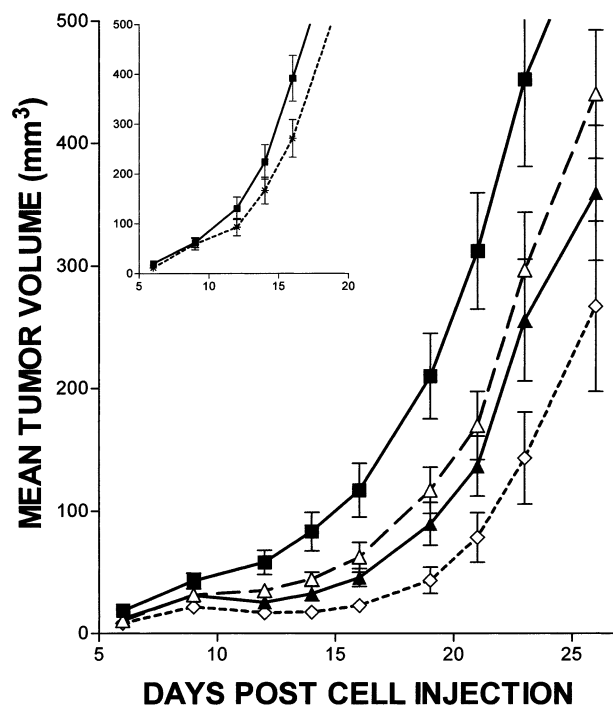


**FIG. 1.** Phenotype of RM-9 (A), RM-9/IFN- $\gamma$  (B), RM-9/CIITA clones (C) and Ii suppression of RM-9/CIITA clone with Ad-Ii-RGC (D). RM-9 cells were transfected with the DNA plasmids pCIITA or pIFN- $\gamma$  and selected for 4 weeks in the presence of blasticidin or hygromycin B, respectively. Stable clones RM-9/CIITA (B) and RM-9/IFN- $\gamma$  (C) were labeled with monoclonal antibodies (mAbs) specific to major histocompatibility complex (MHC) class I and class II antigens and to Ii. RM-9/CIITA clone was transduced with Ad-Ii-RGC at 10 multiplicity of infection (MOI) for 96 hr, then cells were assayed for MHC class II antigens and Ii expression (D). Cells were analyzed by flow cytometry and gates were set to exclude nonspecific binding using isotype Ab controls (dotted lines).

To downregulate Ii protein expression induced with MHC class II molecules, an Ad-vector carrying the reverse gene construct (-92,97) to Ii (Ad-Ii RGC) was used to transduce RM-9/CIITA cell clone. Transduction of RM-9/CIITA cells with Ad-Ii RGC induced a decrease in Ii expression (from 92% to 65% cells) and a marked shift to lower levels of Ii expression in all cells (decrease in MFI from 112 to 36) without affecting the expression of MHC class II molecules (Fig. 1D). The plasmid used for generating Ad-Ii RGC was used for therapy of established *in vivo* tumors.

#### RM-9 tumor gene therapy with DNA plasmid vectors

Because both IFN- $\gamma$  and CIITA DNA plasmids were required to induce MHC class I and class II molecules, respectively, a combination of both plasmids together was tested *in vivo* for the treatment of established RM-9 subcutaneously tumors (Fig. 2). In addition, a suboptimally therapeutic dose of an IL-2 plasmid was added as a cytokine adjuvant to enhance and/or sustain the antitumor immune response. Finally, the requirement for concurrent suppression of Ii protein was tested with an Ii-RGC DNA plasmid. Established subcutaneous RM-9 tumors



**FIG. 2.** Growth of RM-9 tumors treated with intratumoral injections of DNA plasmid vectors. Established subcutaneous RM-9 tumors were treated on day 7 with a combination of DNA plasmid vectors given once a day for 4 consecutive days as detailed in Materials and Methods. Control group 1 received phosphate-buffered saline (PBS; closed squares). Group 2 received pCIITA plus pIFN- $\gamma$  (open triangles). Group 3 received pCIITA plus pIFN- $\gamma$  plus pIL-2 (closed triangles). Group 4 received pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC (open diamonds). The mean tumor volume of 7–8 mice per group and standard error (SE) bars are shown. Inset shows the mean tumor volume of 5 mice per group with SE bars, after PBS (closed squares) or empty plasmid treatment (asterisks).

of 0.3–0.4 cm diameter were treated with four intratumoral injections of plasmids given once per day for 4 consecutive days as detailed in Materials and Methods. Treatment with pCIITA and pIFN- $\gamma$  delayed tumor growth relative to the control mice ( $p = 0.07$ ). Addition of pIL-2 to pCIITA and pIFN- $\gamma$  further delayed tumor growth significantly compared to control ( $p = 0.006$ ). Combining pIi-RGC with the plasmids pCIITA, pIFN- $\gamma$ , and pIL-2 completely inhibited tumor growth until day 18 ( $p = 0.0001$  compared to control). Tumor growth inhibition could not be ascribed to a nonspecific effect (e.g., CpG effect) because treatment of tumors with intratumoral empty plasmid for 4 days did not affect tumor growth (inset in Fig. 2). Furthermore, the response was lower in mice that did not receive pIi-RGC (groups 2 and 3; Fig. 2) and instead received 30  $\mu$ g empty plasmid. In subsequent weeks, tumor regrowth was observed in all treatment groups but reflected the initial differences in tumor growth rates among the three plasmid treatment groups (Fig. 2). These treatments did not increase ultimate survival of the mice. All mice died or were terminated by day 29 because of large tumors in control and treatment groups. Twenty percent of the mice receiving pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC survived up to 38 days and died with large tumors (data not shown). These data were reproduced in separate experiments.

#### RM-9 tumor irradiation followed by intratumoral plasmid gene therapy

Tumors were selectively irradiated and treated a day later with intratumoral plasmid gene therapy. Established RM-9 subcutaneous tumors of 0.3–0.4 cm were irradiated with 8 Gy photons on day 6 after cell injection. From day 7, tumors were injected with PBS, pCIITA plus pIFN- $\gamma$  plus pIL-2, or with pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC for 4 consecutive days. A single high dose of photon radiation significantly delayed growth of RM-9 tumors compared to tumor growth in control mice (Fig. 3A and 3B;  $p = 0.0002$ ). This delay was observed until day 19, but tumor re-growth was observed by day 21 in 6 of 7 mice (Fig. 3B). One of the mice in that group was tumor-free at day 21 and remained so at day 57 (Fig. 3B). Comparing gene therapy with all the plasmids (Fig. 2) to radiation alone (Fig. 3) showed that tumor growth inhibition was observed up to days 18–20 in both groups followed by tumor regrowth in the majority of the mice with no statistically significant differences between the two groups. Combining radiation and pCIITA, pIFN- $\gamma$ , and pIL-2 treatment further inhibited tumor growth compared to control mice ( $p < 0.0001$ ). In this treatment group, 2 of 8 mice had tumor regression beginning on day 19 and complete disappearance by day 26; both mice remained tumor free by day 57 (Fig. 3C). In an unusual pattern of response, 1 mouse showed tumor reduction on days 19–28, regrowth on days 28–54, but then complete regression by day 57 (Fig. 3C). Addition of Ii-RGC plasmid to the combined therapy induced tumor regression by day 19 and complete disappearance of tumor by day 21 in 5 of 8 mice (62%) (Fig. 3D). Surprisingly, one of these mice showed tumor regrowth on day 42, had a 1.1-cm ulcerated tumor by day 57, and was sacrificed. The other 4 mice remained tumor-free at day 57 (Fig. 3D). Tumor regression induced by radiation and pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC was not only highly significant com-

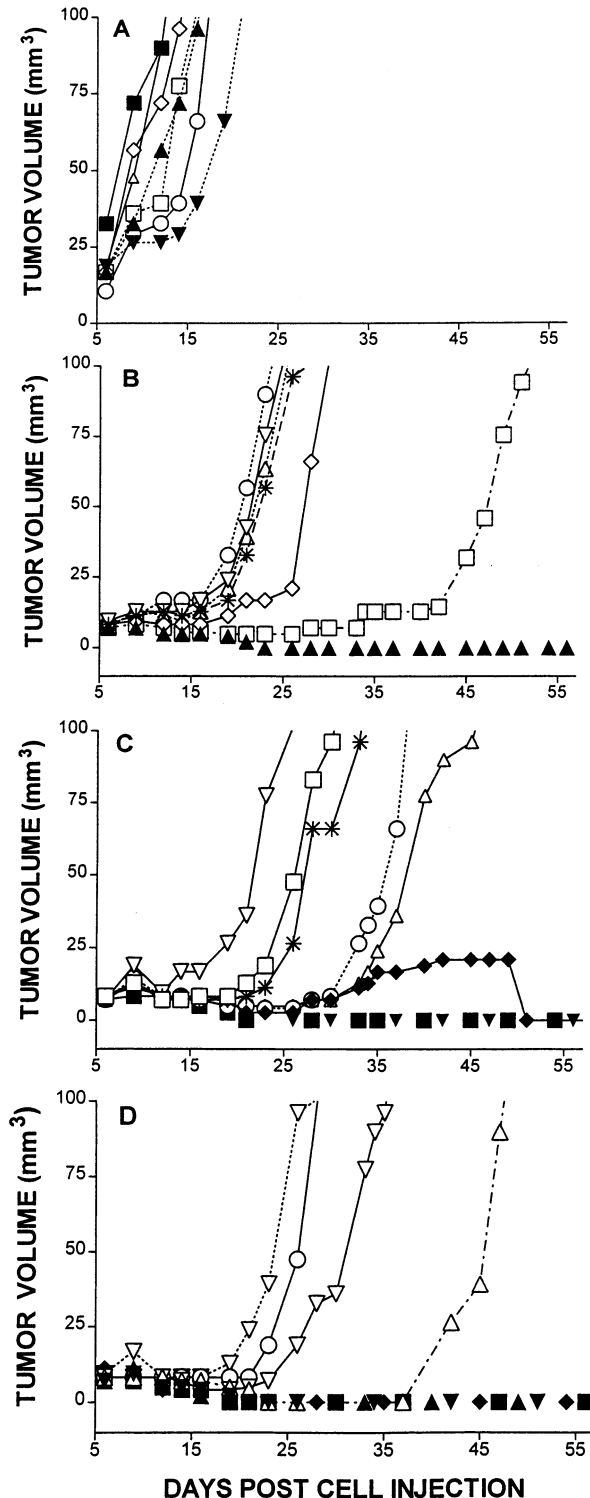
pared to control mice ( $p < 0.0001$ ), but was also significant compared to mice treated by radiation combined with pCIITA plus pIFN- $\gamma$  plus pIL-2 without pIi-RGC ( $p < 0.05$ ). Comparisons between pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC gene therapy alone (Fig. 2) and radiation plus pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC gene therapy (Fig. 3) showed that initially, tumor growth was inhibited in both groups and

tumor sizes were small up to day 19. However, the main difference between these two groups is that by day 21 after radiation plus gene therapy, the tumor disappeared in 5 of 8 mice in contrast to treatment by gene therapy only in which all tumors in 8 of 8 mice grew rapidly to large sizes.

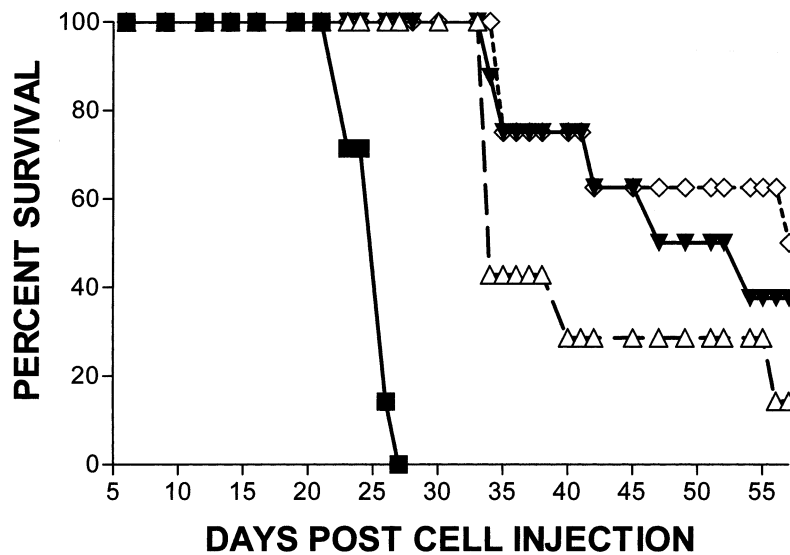
Survival follow-up showed that by day 27, all mice in the control group were dead (Fig. 4). Overall statistical differences among the four Kaplan-Meier curves were significant ( $p = 0.0003$ ). Radiation alone or combined with pCIITA plus pIFN- $\gamma$  plus pIL-2 caused a significant survival increase compared to control mice ( $p < 0.01$ ) (Fig. 4). Addition of pIi-RGC resulted in a greater increase in survival compared to control mice ( $p < 0.008$ ; Fig. 4). The differences between survival curves of radiation-treated mice and radiation plus gene therapy treated mice were not statistically significant. The main difference among these groups is that after radiation and gene therapy, a higher percentage of surviving mice were tumor-free. By the end of the observation period (day 57), 14% of mice in the radiation alone group, 37.5% in the group receiving radiation plus pCIITA plus pIFN- $\gamma$  plus pIL-2, and 50% in the group receiving radiation plus pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC were tumor-free (Table 1). Mice that were tumor-free at day 57 were rechallenged with parental tumor (see below).

#### Response to challenge tumor and antitumor cytotoxic activity in mice cured by radiation and plasmid therapy

All eight tumor-free mice after radiation or radiation and plasmid therapy were challenged on day 57 with  $1 \times 10^5$  RM-9 cells subcutaneously as were three naïve untreated mice. The three naïve mice developed tumors by day 7–10 (Table 1). The one tumor-free mouse after radiation developed a tumor by day 13 after challenge, while the seven tumor-free mice after combined radiation/plasmid therapies did not develop tumors by day 21 (Table 1). On day 21 after tumor challenge, all mice tumor-free or bearing challenge tumor were sacrificed. Their splenocytes were isolated and stimulated for 4 days with mitomycin C-treated RM-9 cells or RM-9/IFN- $\gamma$  cells. Cytotoxicity of these stimulated cells was then assayed against RM-9, RM-9/IFN- $\gamma$ -specific targets or EL-4 nonspecific target. Only when splenocytes were stimulated with RM-9/IFN- $\gamma$  cells and tested against  $^{51}\text{Cr}$ -labeled RM-9/IFN- $\gamma$  target, was cytotoxic activity detected at a level greater than 10% (Fig. 5A). Low levels of killing were obtained with splenocytes from the mouse rendered



**FIG. 3.** Growth of RM-9 tumors treated with irradiation followed by intratumoral injections of DNA plasmid vectors. Established subcutaneous RM-9 tumors were irradiated with 8 Gy photons on day 6. On day 7, intratumoral plasmid therapy with pCIITA plus pIFN- $\gamma$  plus pIL-2  $\pm$  pIi-RGC was initiated for 4 consecutive days as detailed in Materials and Methods. Control group 1 received phosphate-buffered saline (PBS; A). Group 2 received radiation and PBS (B). Group 3 received radiation combined with pCIITA plus pIFN- $\gamma$  plus pIL-2 (C). Group 4 received radiation combined with pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC (D). In each panel, the tumor volume of 7–8 individual mice is represented by a different symbol. By day 57, tumor-free mice were observed in 1 of 7 mice in group 2 (B), 3 of 8 in group 3 (C), and 4 of 8 in group 4 (D).



**FIG. 4.** Survival of mice treated with tumor irradiation followed by intratumoral injections of DNA plasmid vectors. The survival of mice from experiment shown in Figure 3 is reported until day 57 of tumor rechallenge. The surviving mice on day 57 in groups 2–4 were free of tumor. Group 1: control (closed squares); group 2: radiation (open triangles); group 3: radiation plus pCIITA plus pIFN- $\gamma$  plus pIL-2 (closed triangles); group 4: radiation plus pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pli-RGC (diamonds).

tumor-free by radiation but that did not reject the tumor challenge on day 57. Tumor-free mice from combined radiation/plasmid therapies and resistant to tumor challenge showed higher level of cytotoxicity against RM-9/IFN- $\gamma$  target reaching up to 28–38% killing at 100:1 E/T ratio when all plasmids including pli-RGC were used in plasmid therapy (Fig. 5A). When splenocytes were stimulated with RM-9 cells, the level of cytotoxicity against RM-9/IFN- $\gamma$  target was lower (range, 10–20%) at 100:1 E/T (Fig. 5B) and no killing was observed against RM-9 target. Splenocytes stimulated with RM-9/IFN- $\gamma$  cells and tested against RM-9 target showed 0–10% killing (data not shown). RM-9/IFN- $\gamma$  cells expressed MHC class I molecules and a low level of MHC class II molecules whereas RM-9 cells were negative for both. Splenocytes from all mice had either no killing of EL-4 cells or 3–7% killing (data not shown). Splenocytes from naïve mice or normal mice challenged with

RM-9 cells that developed tumors had no significant toxicity against the three targets (data not shown).

#### *Reproducibility of antitumor immune response induced by tumor irradiation followed by intratumoral plasmid gene therapy*

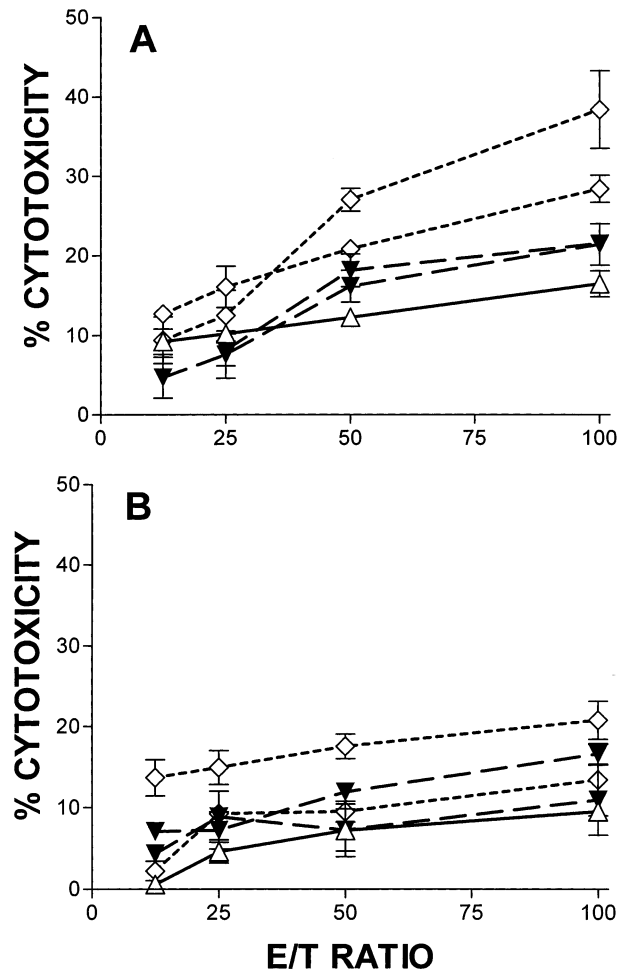
Additional experiments were conducted to confirm the findings presented in Figures 2–5 and Table 1. Established RM-9 subcutaneous tumors of 0.3–0.4 cm were treated with either intratumoral injections of the pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pli-RGC plasmid mixture without radiation or received 8-Gy photon radiation followed 1 day later by intratumoral plasmid therapy using the same doses and conditions as those described in Materials and Methods. Mice were followed up to 64 days and the final outcome from each treatment group is summa-

TABLE 1. ANTITUMOR RESPONSE OF MICE TREATED WITH TUMOR IRRADIATION FOLLOWED BY INTRATUMORAL GENE THERAPY

	<i>Tumor-free mice</i>	
	<i>Posttreatment</i>	<i>Postchallenge tumor</i>
Control	0/7	NA
Radiation	1/7	0/1
Rad + pCIITA + pIFN- $\gamma$ + pIL-2	3/8	3/3
Rad + pCIITA + pIFN- $\gamma$ + pIL-2 + pli-RGC	4/8	4/4
Naïve mice	NA	0/3

The proportion of tumor-free mice at the end of the observation period, day 57 after radiation and plasmid therapy, is presented for the experiment described in Figures 3 and 4. Tumor-free mice and naïve mice were challenged with RM-9 cells on day 57, the proportion of challenge-tumor free mice by day 21 is reported.





**FIG. 5.** RM-9-specific cytotoxic activity of splenocytes from mice cured by radiation alone and radiation plus plasmid therapy. Splenocytes were isolated from mice cured by radiation alone and by combined radiation/plasmid therapy, and challenged with RM-9 cells. Splenocytes were stimulated for 4 days *in vitro* with RM-9/IFN- $\gamma$  cells (A) or RM-9 cells (B) and assayed against  $^{51}\text{Cr}$ -labeled target RM-9/IFN- $\gamma$  in a cytotoxicity assay as described in Materials and Methods. Each curve represents data from an individual mouse treated either with radiation (open triangles), radiation plus pCIITA plus pIFN- $\gamma$  plus pIL-2 (closed triangles), or radiation plus pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC (diamonds). Each point on the curves is the mean percent cytotoxicity of triplicate wells with standard error (SE) bars at each E/T ratio. Because of the complexity of these assays, two mice in each radiation/plasmid combination were tested and reported.

rized in Table 2. Treatment with the combination of all the plasmids without prior tumor irradiation caused tumor growth delay but no complete regression, confirming our previous findings presented in Figure 2. Radiation alone or combined with empty plasmid caused transient tumor growth delay but no complete tumor regression, showing that radiation with or without empty plasmid is not sufficient to induce a significant antitumor response.

Treatment of a large number of mice with radiation followed by the pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC plasmid

mixture resulted in complete tumor regression in 7 of 13 mice, confirming our initial findings shown in Figure 3 and Table 1. We have also tested whether the seven tumor-free responding mice treated with radiation and all the plasmids, pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC were immune to rechallenge with parental RM-9 tumor cells. These mice were challenged on day 64 with  $1 \times 10^5$  RM-9 cells given subcutaneously in the flank. After a long follow-up of 30 days after tumor challenge, none of these seven mice showed any sign of challenge tumor growth on the flank or tumor regrowth at the initial site of RM-9 subcutaneous cell injection (Table 2). Thus, these mice were completely free of tumor at 3 months after treatment and were immune to tumor rechallenge. These data confirm our previous findings (Table 1) that all mice rendered free of tumor by this regimen are resistant to specific tumor rechallenge with RM-9 cells. As a control, in the same challenge experiment, five naïve mice injected in the flank with  $1 \times 10^5$  RM-9 cells subcutaneously developed RM-9 tumors by day 7–10 (Table 2). In a separate experiment, three mice rendered tumor-free by radiation and plasmid combination were challenged on day 60 with unrelated EL-4 tumor cells given subcutaneously at  $1 \times 10^5$  cells in the flank. These three mice developed EL-4 tumors by 10–12 days similarly to five naïve mice injected with  $1 \times 10^5$  EL-4 cells. Splenocytes isolated from tumor-free mice, at 30 days after RM-9 cells rechallenge and stimulated *in vitro* with mitomycin-C-treated RM-9/IFN- $\gamma$  cells showed approximately 18% cytotoxic activity against RM-9/IFN- $\gamma$  (80:1 E/T ratio), whereas the activity against EL-4 was 2–3% (data not shown).

#### *Treatment of RM-9 tumors with radiation and five injections of DNA plasmid vectors*

To improve the outcome of radiation and plasmid gene therapy, we tested the effect of an additional administration of plasmids 2 days after the end of plasmid treatment. Mice were treated with radiation on day 6, then a mixture of pCIITA plus pIFN- $\gamma$  plus pIL-2 plus pIi-RGC was intratumorally administered on days 7–10. An additional injection of plasmids was given 2 days later. Radiation caused a transient inhibition of tumor growth compared to untreated mice as observed in Figure 3 (data not shown). Treatment with radiation and five administrations of plasmid therapy induced tumor regression in 5 of 7 mice (data not shown).

## DISCUSSION

In the process of malignant transformation, prostate epithelial cells show a loss of MHC molecules, which may contribute to the failure of the immune system to control cancer progression. MHC class I determinants were detected in 100% of benign prostatic hyperplasia (BPH), 59% of primary tumors, and 34% of metastatic tumors, while MHC class II determinants were expressed in 100% of BPH, 19% of primary tumors, and 5% of lymph node metastases (Sharpe *et al.*, 1994). Its expression was found to be positively correlated with the malignancy of colon adenocarcinoma (Jiang *et al.*, 1999). Our strategy was to genetically modify murine prostate carcinoma tumors that are negative for MHC class I and class II molecules, to induce upregulation of these molecules on the cell sur-

TABLE 2. ANTITUMOR RESPONSE OF RM-9-BEARING-TUMOR MICE TREATED WITH RADIATION AND PLASMID GENE THERAPY AND RESPONSE TO RECHALLENGE

Treatment Group	Tumor-free mice		
	Posttreatment	Postchallenge tumor	
		RM-9	EL-4
Control	0/10	—	—
pCIITA + pIFN- $\gamma$ + pIL-2 + pIi-RGC	0/7	—	—
Radiation	0/11	—	—
Radiation + empty plasmid	0/5	—	—
Radiation + pCIITA + pIFN- $\gamma$ + pIL-2 + pIi-RGC	7/13	7/7	—
Radiation + pCIITA + pIFN- $\gamma$ + pIL-2 + pIi-RGC	3/6	—	0/3
Naïve mice	NA	0/5	0/5

The proportion of tumor-free mice at the end of the observation period, day 64 after radiation and plasmid therapy, is presented for additional repeated experiments. Tumor-free mice and naïve mice were challenged with RM-9 cells or EL-4 cells on day 64, the proportion of challenge-tumor free mice by day 30 is reported.

face and increase tumor immunogenicity. To improve the presentation of endogenous tumor peptides by MHC class II molecules, novel gene expressing vectors were designed to decrease the synthesis of the immunoregulatory Ii protein. Our purpose was to create the MHC class I+/II+/Ii- phenotype to let tumor cells simultaneously present both MHC class I- and class II-restricted epitopes. Induction of MHC class II molecules and Ii by CIITA and suppression of Ii by Ii-RGC antisense, is a clinically practical method because CIITA and Ii genes are monomorphic. Transfecting each patient with genes for his or her own MHC class II alleles is not clinically practical.

We showed that *in vitro*, RM-9 murine prostate carcinoma cells that are negative for MHC antigens can be induced to express MHC class II molecules by transfection with CIITA DNA plasmid. Furthermore, MHC class II molecules were not induced with IFN- $\gamma$  DNA plasmid but this plasmid did effect class I molecule induction. RM-9 cells may be defective in the CIITA gene and IFN- $\gamma$  gene cannot induce class II antigens (Steimle *et al.*, 1994). CIITA is considered to be a master regulatory factor required for class II molecules and Ii protein (Steimle *et al.*, 1994; Chang *et al.*, 1995; Reith *et al.*, 2001). Sequences targeted by CIITA are located upstream to the structural genes for MHC class II molecules and Ii protein. Ii protein is always coincided with the induction of MHC class II molecules (Chang *et al.*, 1995). This regulation of Ii expression presumably prevents an immunologic response to endogenous antigens. Such a response in transformed tumor cells is therapeutic.

Given the *in vitro* demonstration that combined plasmids pCIITA, pIFN- $\gamma$ , and Ad-Ii-RGC could generate the MHC class I+/class II+/Ii- phenotype, we proceeded to test the biologic effect of generating this phenotype *in vivo* in RM-9 subcutaneous tumor nodules. Tumor growth was suppressed for several days after intratumoral lipid delivery of plasmids coding for CIITA, IFN- $\gamma$ , IL-2, and Ii-RGC genes. Omission of the Ii-RGC from this mixture reduced this effect. Addition of IL-2 plasmid enhanced the response probably by acting as an adjuvant cytokine, helping to strengthen and sustain the activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. IL-2 plasmid was used at a 3- $\mu$ g subtherapeutic dose compared to the 50- $\mu$ g tumoricidal dose used in other studies (Saffran *et al.*, 1998). Similarly when

used at an adjuvant level, IL-2 plasmid enhances PSA DNA vaccines (Kim *et al.*, 2001). We recently demonstrated the biological activity of the CIITA, IFN- $\gamma$ , IL-2, and Ii-RGC genes inserted in adenoviral vectors in the murine MC-38 colon adenocarcinoma model *in vitro* and in the Renca model *in vitro* and *in vivo* (Hillman *et al.*, 2003). We showed that intratumoral injection of these vectors in Renca tumors induced a potent antitumor immune response. We found that this gene therapy approach using adenoviral vectors failed to induce a potent antitumor response in the RM-9 model (unpublished observations). It should be noted that the Renca model is weakly immunogenic and more responsive to immunotherapy approaches than the RM-9 model.

In this study, we tested whether tumor irradiation administered 1 day prior to intratumoral gene therapy enhanced the antitumor response. In previous studies, we found that radiation significantly decreased tumor burden and also induced an inflammatory response (Younes *et al.*, 1995; Dezso *et al.*, 1996; Hillman *et al.*, 2001). Thus, we selected the sequence of tumor irradiation first followed by gene therapy to trigger an immune response. This sequence takes advantage of (1) the presence of inflammatory cells in the vicinity of the tumor mobilized by radiation-induced tissue damage participating in the immune response triggered by the gene therapy (Younes *et al.*, 1995; Dezso *et al.*, 1996; Hillman *et al.*, 2001); (2) the pool of tumor proteins and peptides generated from radiation-induced tumor apoptosis (Bellone *et al.*, 1997); (3) MHC class I+/class II+/Ii- tumor cells have sufficient time to actively present TAA before undergoing apoptosis; and (4) radiation also increases gene transduction efficiency and duration of expression after plasmid delivery (Stevens *et al.*, 1996). RM-9 tumor irradiation alone caused a transient but significant degree of tumor growth inhibition and increased host survival as shown in other tumor models (Younes *et al.*, 1995; Hillman *et al.*, 2001). However, tumor irradiation, given prior to gene therapy, clearly enhanced the therapeutic effect of intratumoral gene therapy, with 43.8% mice tumor-free (7/16), at day 57 in contrast to plasmid therapy alone in which no mice showed complete tumor regression. The proportion of tumor-free surviving mice after radiation and CIITA, IFN- $\gamma$ , IL-2, and Ii-RGC gene therapy was 50% compared to 37.5% with all plasmids except Ii-RGC, and

14% with radiation alone. Repeated experiments with a larger number of mice confirmed that tumor irradiation prior to CIITA, IFN- $\gamma$ , IL-2, and Ii-RGC gene therapy resulted in complete tumor regression in more than 50% of the mice while radiation alone or radiation combined with empty plasmid caused only transient tumor growth delay. The 1 mouse of 7 cured by radiation in the previous experiment (Table 1) seems to be incidental compared to 0 of 11 in repeated experiments (Table 2). Addition of an intratumoral administration of CIITA, IFN- $\gamma$ , IL-2, and Ii-RGC plasmids 2 days after the fourth injection of the same plasmid mixture improved the outcome of radiation and gene therapy causing tumor regression in 70% of the mice. These data show reproducibility in our findings and confirm that radiation is needed to increase the efficacy of subsequent genetic modification of RM-9 cells *in situ* into a cancer vaccine in order to induce complete tumor regression as a result of upregulation of MHC molecules and decreased expression of Ii protein on the tumor cells by plasmid transfection. These findings lend support for further studies to optimize the dose and schedule of radiation and plasmid therapy. Furthermore, these data, coupled with the pattern of early tumor regression after intratumoral therapy both with and without radiation therapy, suggest that suppression of Ii protein synthesis in MHC class II-expressing tumor cells augments vaccine activity, and are consistent with studies of Ii suppressed cell-based tumor vaccines (Qiu *et al.*, 1999).

Tumor challenge experiments strengthen our findings demonstrating that prior tumor irradiation enhanced the efficacy of cancer vaccine induction by *in situ* genetic modification of RM-9 cells. All mice rendered disease-free by radiation combined with plasmid therapy rejected RM-9 tumor challenges in contrast to one mouse cured by radiation alone in which the challenge tumor grew. Rejection of RM-9 tumor challenge, in mice rendered tumor-free by radiation combined with CIITA, IFN- $\gamma$ , IL-2, and Ii-RGC gene therapy, was reproduced in additional experiments (Table 2). Moreover, mice with complete tumor regression induced by radiation and gene therapy did not reject unrelated EL-4 tumor cells. These data indicate that plasmid therapy combined with radiation generated a systemic and specific immune response against tumor challenge while radiation alone did not. Specific immunity is confirmed by the significant specific antitumor cytotoxicity measured in the splenocytes of mice rejecting the challenge tumor compared to a lower level in the radiation treated mouse not immune to challenge. This cytotoxic activity was observed only when mouse splenocytes were stimulated with class I+ RM-9 cells (RM-9/IFN- $\gamma$  clone) and tested against this cell line as a target suggesting the generation of specific CD8<sup>+</sup> T-cell cytotoxic activity. The significance of these observations is emphasized by the fact that the RM series of prostatic cancer cell lines are poorly immunogenic, grow aggressively, and invade locally when implanted into prostates of syngeneic mice. In previous studies, only transient tumor regression followed intratumoral administration of Ad-IL-12, without systemic immunity or T-cell activity (Nasu *et al.*, 1999). RM-1 cells transduced with canarypox ALVAC viruses carrying cytokine genes induced antitumor activity that was primarily mediated by natural killer (NK) cells (Griffith *et al.*, 2001). These studies also showed induction of tumor-specific CD4<sup>+</sup> regulatory T cells that inhibited tumor-specific CD8<sup>+</sup> cytotoxic T cells and specific tumor

immunity (Griffith *et al.*, 2001). In our study, intratumoral plasmid therapy did not effect complete regression of established tumors, unless the tumors were irradiated prior to plasmid therapy. Radiation might have destroyed or limited suppressive immunoregulatory T cells. In addition, radiation could have contributed by debulking the tumor and increasing gene transduction of surviving cells thus improving efficiency of *in situ* genetic modification leading to an immune response that eradicated remaining tumor cells. Stevens *et al.* (1996) have demonstrated that radiation improves transfection efficiency of plasmid DNA in normal and in malignant cells *in vitro* resulting from radiation-induced DNA breaks and DNA repair mechanisms. They showed that radiation followed by plasmid transfection caused enhanced integration of the transgene.

Clinically, we have developed a regimen of fractionated radiation with neutrons combined with photons that resulted in a significant decrease in tumor recurrence (Forman *et al.*, 1998, 1999). At 3 years, no evidence of recurrence was found in 91% of patients with stage T1 disease, in 86% of stage T2 disease, and 61% of stage T3/T4 disease (Forman *et al.*, 1999). The local control of stage T3/T4 (61%) was improved over photon radiation (35–40%) but such treatment did not prevent disease progression in a significant number of patients. Progression and metastases are attributed to micrometastases at presentation and small volumes of residual disease following local therapy. Combining radiation and intratumoral genetic induction of cancer vaccine promises to extend disease-free and progression-free survival for patients with localized prostate cancer. Transrectal ultrasound guidance for gene injections into the prostate has been established as a clinically feasible procedure (Beldegrun *et al.*, 2001).

Further studies are ongoing to clarify the mechanisms by which radiation improves the efficacy of gene therapy, to optimize the conditions of radiation/plasmid combination to increase therapeutic efficacy, and to test this novel approach in orthotopic transplants both for local tumor eradication and control of spontaneous metastases.

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