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Treatment of bladder carcinomas using recombinant BCG DNA vaccines and electroporative gene immunotherapy

Chi-Feng Lee[1,3], Sun-Yran Chang[2], Dar-Shih Hsieh[3], and Dah-Shyong Yu[3]

[1]Graduate Institute of Life Sciences, National Defence Medical Centre, National Defence University, Taipei, Taiwan, ROC; [2]National Defence Medical Bureau, Ministry of National Defence, Taipei, Taiwan, ROC; and [3]Laboratory of Uro-oncology, Division of Urology, Department of Surgery, Tri-Service General Hospital, Taipei, Taiwan, ROC.

Intravesical immunotherapy with live *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) is the treatment of choice for superficial bladder cancers. Nevertheless, a significant proportion of patients do not respond to this therapy, and adverse effects are common. Here, we report the cloning of recombinant mycobacterial DNA vaccines and demonstrate the ability of multicomponent and multisubunit DNA vaccines to enhance Th1-polarized cytokine-mediated responses as well as effector cell responses. Splenocytes from immunized groups of mice were restimulated *in vitro* and examined for cytotoxicity against murine bladder tumur (MBT-2) cells. We used four combined recombinant BCG DNA vaccines (*poly-rBCG*) for electroporative gene immunotherapy (EPGIT) *in vivo*, and found that tumor growth was significantly inhibited and mouse survival was prolonged. Increased immune cell infiltration and induction of apoptosis were noted after treatment with *poly-rBCG* alone, with the murine interleukin-12 (*mIL-12*) vaccine alone, and—most significantly—with the *poly-rBCG+ mIL-12* vaccine combination. Electroporation of *poly-rBCG+ mIL-12* resulted in complete tumor eradication in seven of eight mice ($P<.01$) within 28 days. Thus, EPGIT using multicomponent multisubunit BCG is highly effective in the treatment of bladder cancer. This approach presents new possibilities for the treatment of bladder cancer using recombinant BCG DNA vaccines.

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Intravesical immunotherapy with live *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) is successful in reducing the recurrence and progression of superficial bladder cancer after transurethral resection of the tumor.[1] Routine complete response rates of 60–70% have been reported.[2,3] Nevertheless, a significant proportion of patients do not respond to BCG therapy, and adverse effects are common.[4] These findings limit its use in clinical practice. Thus, BCG immunotherapy could be improved, and more effective alternative BCG treatment strategies are likely to evolve from such therapy.

Even though BCG is known to be very effective, its antitumor mechanism remains unclear. It is generally accepted that it activates multiple cellular components such as $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and macrophages, which are the major factors for successful cancer immunotherapy. The release of cytokines by these activated immune cells and infected bladder epithelial cells may augment the immune response, resulting in the destruction of local cancers. This mechanism involves attachment of the bacterium to the urothelium via fibronectin, and antigen presentation by the urothelial cells leading to granulocyte and monocyte attraction. A subsequent Th1 cytokine polarized response is characterized by the production of elevated levels of interleukin-2 (IL-2), interleukin-12 (IL-12), interferon-gamma (IFN-γ), and possibly a major histocompatibility complex (MHC) class I presentation of tumor rejection antigens on the urothelial cell surface to specific $CD8^+$ T lymphocytes.[5]

Although there have been some reports of successful attempts to reduce adverse effects and increase efficacy using nonviable heat-inactivated BCG and BCG subfractions,[6,7] others have found this to be less effective than treatment with viable organisms.[8–10] Treatment failure was blamed on the lack of urothelial retention of these BCG products and inadequate delivery of the BCG components.

Since cytokines appeared to be important for efficient BCG therapy, the next treatment approach combined lower BCG doses with cytokines such as interferon-α-2b[11,12] or IL-12,[13] among others. This approach shows promise. Combining BCG with intravesical IFN-γ instillations[14] may further improve the effectiveness of treatment without increasing its adverse effects, possibly because of greater enhancement of Th1-mediated suppression of Th2 cytokine responses and an enhanced

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Address correspondence and reprint requests to: Prof D-S Yu, Department of Surgery, Tri-service General Hospital, No 325, Sec 2, Cheng-Kung Rd., Neihu 114, Taipei, Taiwan, ROC.
E-mail: leendmc@ndmctsgh.edu.tw

local capacity to destroy bladder tumor cells.[15–17] Indeed, to be effective, cancer immunotherapy must stimulate the focal synthesis of cytokines, as well as activate, increase, and deploy antitumor effector cells.

Modified tumor cell vaccines have been proposed to activate antitumor effector cells, but initial attempts at producing them failed.[18] However, the use of immunologically active mycobacterial subcomponents instead of live BCG for preventing recurrences of superficial bladder cancer is an attractive prospect. Although the mechanism has been poorly investigated, BCG subcomponents have been found to stimulate Th1 cell differentiation and enhance non-MHC-restricted cytotoxicity against bladder tumor cells.[17] This understanding of how BCG subcomponents can stimulate immune activity underlies antitumor DNA vaccine strategies. Thus, these vaccines can be instilled in the bladder where, as with live BCG, they can activate local antitumor immunity by increasing the concentrations of cytokines within the proximity of the tumor, leading to MHC class I and cytotoxic T-cell expression, and a Th1-polarized response.[19–21]

Here, we investigated the effects of a combination of four BCG DNA vaccines (*poly-rBCG*) and one murine IL-12 (*mIL-12*) DNA vaccine on tumor cell killing and tumor-specific immune responses, and evaluated the efficacy of this combination in treating mice with bladder tumors. We showed that *Ag85A*, *Ag85B*, *Mpt64*, *PstS3*, and *ESAT6* have the potential to contribute to the treatment of bladder carcinomas, by enhancing the cellular immune response.

Materials and methods

Mice and animal care

Pathogen-free 4-week-old female C_3H/HeN mice (Animal Centre of National Defence Medical Centre) were used for these experiments. Animal care and experimental procedures were in accord with the institutional guidelines.

Mycobacterial strains

M. bovis BCG Connaught strain (Connaught Laboratories Limited; Ontario, Canada) was obtained as a freeze-dried vaccine and was rehydrated with phosphate-buffered saline (PBS). A single strain of *M. tuberculosis* (from a patient in the Tri-Service General Hospital, Taipei, Taiwan) was grown for approximately 1 month at 37°C in a modified Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose.

Molecular cloning and DNA sequencing

Mycobacterial genomic DNA samples (*M. tuberculosis* or *M. bovis* BCG) were used as polymerase chain reaction (PCR) templates to produce copies of the *Ag85A*, *Ag85B*, *Mpt64*, *PstS3*, and *ESAT6* genes. These genes were submitted to GenBank (*Ag85A*, AY207395; *Ag85B*, AY207396; *Mpt64*, AY208674; *PstS3*, AY207397; and *ESAT6*, AY207398). The PCR was performed with gene-specific primers (Table 1) for 30 cycles, each cycle consisting of 15 s at 94°C, 1 minutes at 56°C, and 2 minutes at 72°C. These DNA fragments were purified and ligated into a *pCRII-TOPO* vector (Invitrogen Life Technologies, Carlsbad, CA), and then subcloned into the eucaryotic expression vector *pCMV-V5-His6* (a generous gift of Dr Y-L Lin, Academia Sinica, Taipei, Taiwan). The *ESAT6* gene was ligated to the 3′ ends of each of the other four genes (i.e., *Ag85A*, *Ag85B*, *Mpt64*, and *PstS3*). All genes encoding antigens were fused to a mouse *immunoglobulin kappa* (*Igκ*) chain leader sequence and amplified with a 5′ primer containing a *Sal*I restriction site and a 3′ primer designed with an *Nhe*I restriction site.

The *mIL-12* 35-kDa light chain (*p35*) and 40-kDa heavy chain (*p40*) were amplified from a cDNA library of C_3H/HeN mouse spleen cells. The positive clones were inserted into the *pIRES* vector (Clontech Laboratories, Palo Alto, CA), which is a mammalian expression vector that allows expression of two genes of interest at high levels by cloning *IL-12 p40* and *IL-12 p35* into two cloning sites, A and B. This permits both the genes of interest to be translated from a single bicistronic mRNA.

To construct the *mIL-12* vaccine fusion with various (red, yellow and green) fluorescent proteins (FP), the genes for *p35*, *p40* (p35 and p40 are peptide subunits of mIL-12), and *p35p40* (p35p40 is a single-chain peptide of mIL-12) were ligated to the plasmid vectors *pEGFP-N1*, *pERFP-N1*, and *pEYFP-N1* (Clontech Laboratories), respectively. All constructs were used in expression studies *in vivo*. Figure 1 schematically illustrates the design of three expression systems: the eucaryotic *poly-rBCG* system, and the heterodimeric *mIL-12* system with or without FP on the C terminal end.

In vivo *direct DNA vaccine transfer to tumor and muscle tissues*

Four *rBCG* DNA vaccines (each of the four plasmids coding for one mycobacterial antigen in combination with the ESAT6 and mouse Igκ signal sequence) and heterodimeric *mIL-12* DNA vaccine (*PIRES-p40p35*) were used. Briefly, 4–6-week-old female C_3H/HeN mice were anaesthetized in groups of eight with an intraperitoneal injection of ketamine HCl/xylazine (100 μg/15 μg per mouse) before plasmid injection and electroporation. For the transfer of *poly-rBCG* intratumoral genes, MBT-2 cells (approximately 5×10^6 cells) were subcutaneously inoculated into the backs of 5-week-old female C_3H/HeN mice, and tumors were allowed to develop for about 2 weeks. The tumors were then excised and chopped into small pieces, and equal amounts of tumor were subcutaneously inoculated into the right flank of 6-week-old mice. At 1–2 weeks after inoculation, when the tumors were about 5–7 mm in diameter, 30 μg of each naked mycobacterial DNA vaccine in 120 μl of normal saline solution was injected into them using a syringe (Becton Dickinson, Franklin Lakes, NJ). For

Table 1 Sequences of the gene-specific primers

Gene and orientation	Sequence of primer
Ag85A	
Sense	5′-GTC GAC TTT TCC CGG CCG GGC TTG-3′
Antisense	5′-GCT AGC GTC TGT TCG GAG CGA GGC G-3′
Ag85B	
Sense	5′-GTC GAC TTC TCC CGG CCG GGG CTG-3′
Antisense	5′-GCT AGC GCC GGC GCC TAA CGA ACT C-3′
Mpt64	
Sense	5′-CGCG GTC GAC GCG CCC AAG ACC TAC TGC-3′
Antisense	5′-CCGG GCT AGC GGC CAG CAT CGA GTC GAT C-3′
PstS3	
Sense	5′-CCGT CTC GAG TGT GGT AAC GAC GAC AAT GTG-3′
Antisense	5′-GCGG GCT AGC GGC GAT CGC GTT GAC CGC-3′
ESAT6	
Sense	5′-CGGC GCT AGC ATG ACA GAG CAG CAG TGG-3′
Antisense	5′-CC ATC GAT TGC GAA CAT CCC AGT GAC G-3′
Igκ	
Sense	5′-CAC ACC ATG GAT TTT CAA GTG CAG AT-3′
Antisense	5′-GCT AGC CTA GTG CCA TGG TGT CGA CTC CTC TGG ACA TTA TGA CTG-3′
mIL12-p35	
Sense	5′-TCTAGA GCT AGC ATG TGT CAA TCA CGC TAC-3′
Antisense	5′-GTCGAC GGC GGA GCT CAG ATA GCC-3′
mIL12-p40	
Sense	5′- GCTAGC GCT AGC ATG TGT CCT CAG AAG CTA-3′
Antisense	5′-CTCGAG GGA TCG GAC CCT GCA GGG-3′

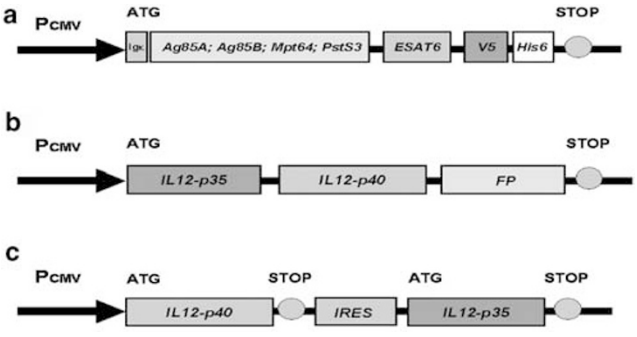


Figure 1 Schematic drawing showing the expression systems of *poly-rBCG* used in the four vaccines and the *mIL-12* used in the fifth vaccine as adjuvant. (**a**) Constructs of *poly-rBCG* DNA vaccines (*pCMV-AE*, *pCMV-BE*, *pCMV-ME*, and *pCMV-PE*). (**b**) Single chain *mIL-12* DNA fused with FP genes (*pEYFP-p35p40*). (**c**) Bicistronic *mIL-12* DNA vaccine (*pIRES-p40p35*) contained within the internal ribosome entry site (*IRES*).

the intramuscular transfer of *mIL-12* genes, the quadriceps femoris muscles of 6-week-old female C_3H/HeN mice were injected with 100 μg of *mIL-12* vaccine. Negative control mice were injected with the empty vector only.

Electroporative transfection

To improve the DNA transfer efficiency, electroporation was used[22] at the site of each injection for electroporative gene immunotherapy (EPGIT). Briefly, two-needle array electrodes (BTX, San Diego, CA) were inserted into the tumor masses or muscle fibers immediately after DNA injection. The distance between the electrodes was 5 mm, and the array was inserted longitudinally relative to the tumor masses or fibers. Electric pulses were generated using a square-wave electroporator (Model 830; BTX). *In vivo* electroporation parameters were as follows: distance between the electrodes, 100 V/cm; pulse duration, 50 ms; 10 pulses with reversal of polarity. These were selected based on a previous report[22] and our preliminary experiments.

Detection of poly-rBCG *DNA vaccines and* mIL-12 *fusion with FP*

Poly-rBCG DNA vaccines containing the V5-His6 tag were detected in paraffin-embedded 5-μm-thick sections of bladder tumors by staining with a fluorescein isothiocyanate (FITC)-labelled monoclonal antibody to V5 (Invitrogen Life Technologies) according to the manufacturer's directions. Briefly, about 7 days after EPGIT with either the empty control vector alone or with

the four combined recombinant mycobacterial DNA vaccines fused with a V5-His6 tag or coexpressed *mIL-12*, mice ($n=6$) were killed. Their tumors were harvested, fixed in 10% paraformaldehyde, immunostained, and viewed under a fluorescence microscope (Xenon arc lamp and FITC filter on a Zeiss Axioskop). Images were acquired with color charge-coupled-device camera and frame-grabbing equipment. To determine expression rates, the expression efficiency of the electroporation-mediated *poly-rBCG* gene was measured using flow cytometric analysis on a FACSCalibur instrument (BD Biosciences, San Diego, CA) to detect tumor cells expressing mycobacterial antigens fused with the V5-His tag. Staining with various FITC-labelled anti-V5 and biotin-labelled anti-His monoclonal antibodies (Invitrogen Life Technologies), cells positive for *poly-rBCG* were gated into different groups of MBT-2 cell populations. To demonstrate the expression of mIL-12 in the muscle cells, five mice per treatment group were killed about 7 days after electroporation of recombinant *mIL-12* DNA fused with either the EGFP, ERFP, or EYFP plasmids. Their quadriceps femoris muscles were sectioned fresh (without freezing or fixation) into 5-μm-thick sections and mounted in PBS for immediate confocal microscopy analysis. This was performed on a Zeiss LSM510 system (Carl Zeiss, Jena, Germany). Images were directly imported into the system's software.

Reverse transcription (RT)-PCR procedure

To determine individual expressions of the five mycobacterial genes, the RNA levels were examined by RT-PCR amplification. From lysates of bladder tumor cells transfected with *poly-rBCG* + *mIL-12*, total RNA was extracted with TRIzol (Gibco BRL, Grand Island, NY), using the manufacturer's directions. RT-PCR was performed using the method described by Wang et al[23] to amplify *Ag85A*, *Ag85B*, *Mpt64*, *PstS3*, *ESAT6*, and α-*actin* (internal control group) mRNAs. An initial denaturation of 1 minutes at 95°C was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Primers are listed in Table 1. All the five *rBCG*s were amplified by using undiluted cDNA, and α-*actin* was amplified using a 1:50 dilution. These cDNA quantities were selected empirically so that PCR amplification did not reach a plateau in 25 cycles. In total, 10 μl of each PCR product was electrophoresed on 2% agarose gel. The intensity of each fluorescent band was normalized against the corresponding control α-*actin* cDNA fluorescent band and compared with those of DNA standards using the digital ChemiGenius image analysis system (Syngene, Cambridge, UK). Analysis was carried out using automated GeneTools software (Syngene).

Preparation of cytotoxic T-lymphocyte (CTL) effector cells and cytotoxic assay

Effector cells were derived from splenocytes as precursor of CTLs. The responding splenocytes were obtained from sequentially immunized mice 14 days after electroporative immunogene therapy of *poly-rBCG*, resuspended in complete RPMI medium (Invitrogen Life Technologies) and cultured at a concentration of 5×10^6 splenocytes per culture well together with 10 μg of MBT-2 extracts in a total volume of 2 ml. The effector cells were harvested after 5 days of culture. Two cell lines, both originating from C3H mice, were transfected with *poly-rBCG* and used as target cells. MBT-2 is a transitional cell carcinoma, but Sq-1979 is a squamous cell carcinoma. Target cells were labelled with 100 μCi $Na_2(^{51}Cr)O_4$ for 1 h at 37°C in RPMI-1640 medium containing 10% fetal calf serum (FCS) and subjected to cytotoxicity assays. Labelled target cells (10^4/well) were incubated in triplicate with effector cells in a total volume of 200 μl RPMI in 96-well microtiter plates at different effector:target cell ratios. After 12 h of coincubation, specific lysis was determined as: specific lysis (%) = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release varied from 5 to 10%. All experiments were performed in triplicate, and each group consisted of five immunized mice.

Cytometric bead array (CBA) assay

The CBA (BD Biosciences) uses flow cytometry to detect mouse cytokines from small-volume serum samples. To distinguish between Th1 and Th2 reactions, the BD mouse Th1/Th2 cytokine CBA kit was used to measure protein levels of IL-2, IL-4, IL-5, IFN-γ, and tumor necrosis factor (TNF)-α in a single serum sample at the same time. Briefly, mouse serum from four groups (empty vector control, *poly-rBCG* alone, *mIL-12* alone, or *poly-rBCG* + *mIL-12*; $n=8$ each) was collected on days 0, 7, 14, and 21 after vaccine treatment. In all, 50 μl of serum and 10 μl of mixed CBA beads were added and incubated at room temperature for 2 h, washed, and then incubated with a second phycoerythrin-labelled anti-cytokine antibody per test. Flow cytometry was performed using a dual-laser FACSCalibur instrument (BD Biosciences). Data were displayed by using CellQuest software and were analyzed with CBA analysis software (BD Biosciences). The expected sensitivity was in the picogram-per-milliliter range. The data are the means ± SEM of the serum cytokine levels from the indicated groups.

Immunohistochemistry of CD4, CD8a, CD3e, CD19, and F4/80

To distinguish between cellular and humoral immune responses, simultaneous immunohistochemistry was used to detect the effects of treatment on immune cell responses. Samples of the tumor tissue were collected from the experimental groups of mice 7 days after vaccination. Detection of CD4, CD8a, CD3e, CD19, and F4/80 was assessed in paraffin-embedded sections using the avidin–biotin–peroxidase complex method.[24] Sections (4 μm) were deparaffinized and then treated with 3.0% hydrogen peroxide (Sigma, St Louis, MO) to block the endogenous peroxidase activity. Antigens were retrieved by immersing the slides in 1 × ChemMate buffer

for antigen retrieval (S2031; Dako, Kyoto, Japan) and then microwaving them at 700 W for 10 min. The protocol for the LSAB2 Kit peroxidase (Dako) was followed for each sample. The sections were blocked with 5% fetal bovine serum/PBS for 30 minutes and then incubated at room temperature with the CD4, CD8a, CD3e, CD19, and F4/80 primary antibodies diluted 1:25 (BD PharMingen, San Diego, CA), respectively, followed by the secondary antibody diluted 1:50 (BD PharMingen) for 90 min. The samples were then rinsed and incubated with streptavidin–biotin–peroxidase complex and diaminobenzidine tetrachloride (AEC Ready-To-Use Kit, Dako) as chromogens, and counterstained with Mayer's hematoxylin (Sigma). Sections of spleen and MBT-2 transferred with empty vector were used as positive and negative controls, respectively.

Histopathological analysis and terminal dUTP nick-end labelling (TUNEL) assay

At 14 days after EPGIT, five further paraffin-embedded sections of the tumor tissue samples from the experimental groups of mice were resectioned and cut in the middle at the site of the original tumor inoculation. The tissue was then fixed in 10% buffered formalin and routinely evaluated on 4-μm-thick sections stained with hematoxylin and eosin (H&E) for histopathological analysis. The proportion of viable bladder tumor cells in each tumor at five randomly selected areas was calculated using an image analyser (MAC Scop, Nagano, Japan), and the overall effect of *poly-rBCG* EPGIT was evaluated. TUNEL staining was performed using an ApopTag Peroxidase *In situ* Apoptosis Detection kit (Intergen Company, Purchase, NY). The manufacturer's instructions were followed with some modifications.[25] An apoptosis index (percentage) was calculated for each of the five samples at the five areas as the proportion of TUNEL-positive MBT-2 cells with respect to the total number of MBT-2 cells evaluated in a $\times 200$ or $\times 400$ magnification counting field. Only non-necrotic MBT-2 cells were counted to determine the apoptotic index. Approximately 2500 such cells were evaluated for each section of the indicated groups.

Western blot analysis of Bcl-2 protein

To monitor the changes in expressions of Bcl-2 and Bax proteins, samples of tumor tissues were collected from the experimental groups of mice every 7 days for 28 days after EPGIT. Tumor lysate (i.e., tissue lysed with 1% Triton-X 100, 150 mM NaCl, 10 mM Tris-HCl pH 7.0, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride for 30 minutes on ice) was centrifuged for 10 minutes at 15,000 *g*, and the supernatant was diluted in PBS. Samples were electrophoresed on a 15% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto an Immobilon-P transfer membrane (Millipore, Bedford, MA). Blots were blocked with 5% skim milk in PBS plus 0.5% Tween 20 for 1 hour and incubated with anti-Bcl-2 antibody (BD PharMingen) at a dilution of 1:1500 for 1 hour, or with anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000 for 1 hour. The blots were then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:1000; Amersham Biosciences, Piscataway, NJ) for 1 hour and then developed using the Super Signal West Pico Chemiluminescent kit (Pierce Chemical Co, Rockford, IL) according to the manufacture's directions. The chemiluminescent signal was captured in real time using a digital Chemi-Genius image analysis system (Syngene). The dynamic range of the camera, coupled with automated GeneTools software analysis (Syngene), ensured accurate quantification of protein expression on chemiluminescent Western blot images.

Monitoring of tumor volumes and mouse survival rates

Tumor growth was monitored every 3 days for a period of 33 days after gene therapy by means of calliper measurements. Tumor volume was calculated according to the following formula: volume $V = 0.52 \times L \times W \times H$, where L is the length or largest diameter, W is the width or smallest diameter, and H is the height (all in millimeters). The means $\pm$ SEMs of the tumor volumes were calculated for eight independent mice in the treatment groups. The cumulative survival rates of treated MBT-2-bearing mice were periodically determined over 90 days of observation.

Statistical analysis

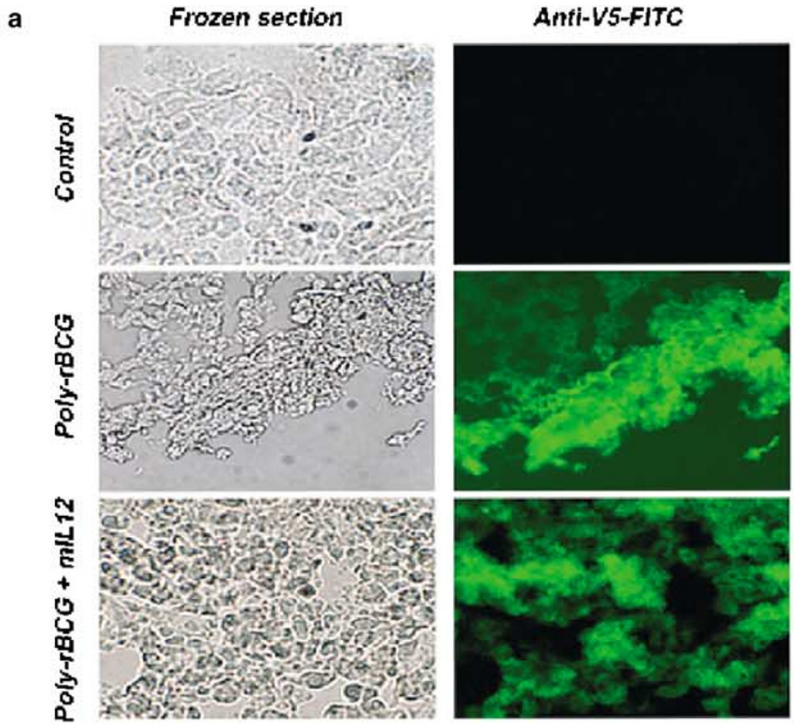
The mean density $\pm$ SD was calculated for each rBCG antigen mRNA expressed, and analyzed with Tukey's Honestly Significantly different test. Differences were considered to be statistically significant at $P < .05$. The mean tumor volumes $\pm$ SEMs were calculated for the inhibition of tumor growth and analyzed with the generalized estimating equation (GEE) test. Differences were considered to be statistically significant at $P < .01$. For cumulative survival rates, Kaplan–Meier estimates were calculated for multiple comparisons methods, and differences were considered to be statistically significant at $P < .05$.

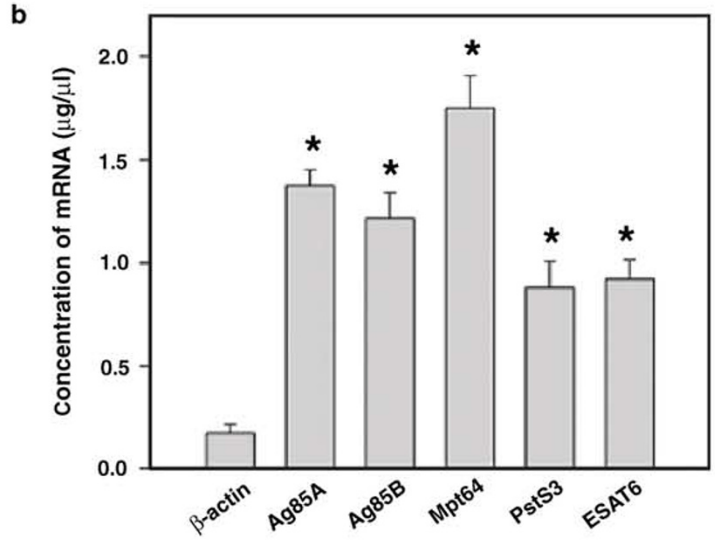
Results

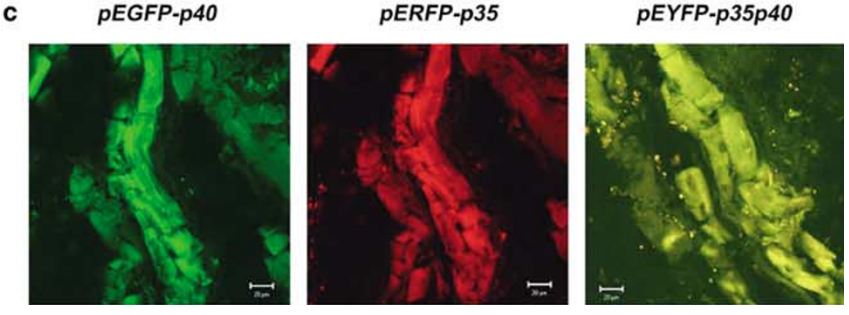
Expression of mycobacterial antigens and mIL-12

The expression of four recombinant DNA vaccines via EPGIT were examined in the MBT-2 implants by immunofluorescence microscopy. Immunohistochemistry of the bladder tumor also indicated the level of expression and uniform distribution of these four proteins (Fig 2a).

Figure 2 Expressions of *poly-rBCG* in the bladder tumor and *mIL-12* in the quadriceps femoris muscle using EPGIT *in vivo*. (**a**) Protein expression of poly-rBCG (green) was assessed in the tumor by means of immunofluorescence microscopy ($\times 60$). (**b**) mRNA expression of *Ag85A*, *Ag85B*, *Mpt64*, *PstS3*, and *ESAT6* genes in the tumor mass was determined by RT-PCR. *Significant difference from the α-*actin* control ($P < .05$). (**c**) Protein expression and colocalization of murine *IL-12 pEGFP-p40* (green), *pERFP-p35* (red), and *pEYFP-p35p40* (yellow) in the quadriceps femoris muscle was examined using confocal microscopy (bar = 20 μm).


a
Frozen section
Anti-V5-FITC
Control
Poly-rBCG
Poly-rBCG + mIL12


b
Concentration of mRNA (μg/μl)
2.0
1.5
1.0
0.5
0.0
β-actin
Ag85A
Ag85B
Mpt64
PstS3
ESAT6


c
pEGFP-p40
pERFP-p35
pEYFP-p35p40

Compared with the negative controls, the expression rate in mice vaccinated with *poly-rBCG* measured by flow cytometry and divided by the total counting cells that were fluorescently labelled was above 72% on day 7 and about 48% at day 14 after EPGIT (data not shown), indicating a high efficiency of *poly-rBCG* expression.

To monitor individual expression of the five mycobacterial genes, mRNA levels were demonstrated by RT-PCR amplification using specific primers for each antigen. In mice bearing tumors ($n=5$) transfected with *poly-rBCG + mIL-12* via EPGIT, the expression of individual antigen was measured with densitometry and compared with α-actin internal control after RT-PCR amplification and gel electrophoresis. The expression levels of *Ag85A*, *Ag85B*, *Mpt-64*, *PstS3*, and *ESAT6* were 1.39 ± 0.08, 1.22 ± 0.13, 1.75 ± 0.16, 0.88 ± 0.13, and 0.92 ± 0.09 g mRNA/ml lysate, respectively (Fig 2b). These results confirmed active transcription of all five genes and expression of all antigens encoded by them. The expression levels of mRNA between intratumorally transferred five-component *poly-rBCG* via EPGIT were significantly higher than those of internal controls ($n=5$, $P<.05$).

To demonstrate the expression of *mIL-12* in the muscle cells via electroporation, the quadriceps femoris muscle of C_3H/HeN mice was electroporatively transfected with plasmids containing *IL-12* subunits *p40*, *p35*, and *p35p40*, and reporter genes specifying EGFP, ERFP, and EYFP, respectively. Confocal microscopy indicated that *IL-12* subunits were coexpressed and uniformly presented (Fig 2c).

Tumor eradication

The growth of MBT-2 implants was compared over 33 days after single transfers using EPGIT with either the empty transfection vector (*pCMV-V5-His6*), *poly-rBCG*, the recombinant *mIL-12* vaccine, or *poly-rBCG + mIL-12*. Note that the *mIL-12* vaccine was deposited in the quadriceps muscle, whereas the other vectors were delivered to the tumor locally. The growth of MBT-2 implants transferred with *poly-rBCG*, *mIL-12*, and *poly-rBCG + mIL-12* by means of EPGIT *in vivo* was significantly inhibited, as compared with MBT-2 implants transferred with control *pCMV-V5-His6* ($n=8$, $P<.01$, GEE test; Fig 3a). On the day when the gene was transferred, the mean tumor volume of MBT-2 implants was $536.60\pm78.83\,mm^3$. After 16 days, the mean tumor volume of MBT-2 implants transferred with control *pCMV-V5-His6* rapidly increased to $1788.5\pm291.71\,mm^3$ (Fig 3a). However, the mean tumor volumes of MBT-2 implants transferred by *poly-rBCG* alone and *mIL-12* alone increased to $939.75.5\pm150.24$ and $739.38\pm112.07\,mm^3$, respectively (Fig 3a). The tumor volumes in mice treated with *poly-rBCG* alone or with *mIL-12* vaccine alone were thus already significantly reduced by day 16 after the start of treatment. On day 16, the mean tumor volume of MBT-2 treated with *poly-rBCG + mIL-12* was reduced significantly to $134.00\pm29.81\,mm^3$ (Fig 3a). The tumors continued to shrink and were eventually eradicated by 28 days (Fig 3a). Moreover, suppressed growth rate was also observed in the untreated distant right flank MBT-2 tumor that was inoculated and established at the same time, when compared with that of the equivalent control tumor in the control group ($n=8$, $P<.01$, GEE test). On the other hand, our results demonstrate that suppressed growth rate cannot be achieved with fewer than four combined *rBCG* DNA vaccines. It seems that the use of four different rBCG antigens is necessary.

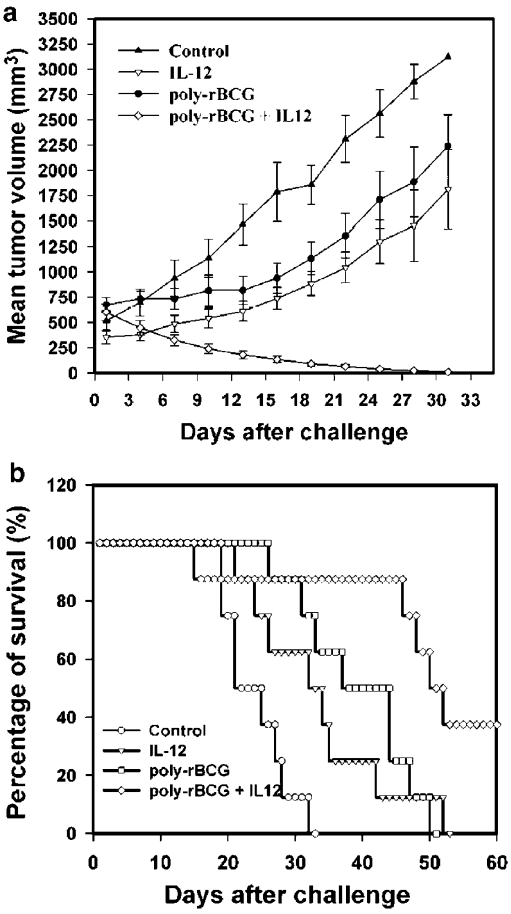


Figure 3 Effects of *poly-rBCG* priming on tumor growth and survival. Intratumoral *poly-rBCG* (30 μg each, an equal mixture of the four *rBCG* DNA vaccines) with or without intramuscular *mIL-12* (100 μg, *pIRES-p40p35* DNA vaccine) was delivered via EPGIT *in vivo*. Measurements were made every 3 days for 33 days after treatment ($n=8$). (**a**) The time course of tumor mass progression. Both *poly-rBCG* and *mIL-12* were able to suppress bladder tumor growth ($P<.01$). *Poly-BCG + mIL-12* resulted in significant tumor inhibition and regression ($P<.01$). (**b**) The cumulative survival curve of EPGIT mice. The mice were observed for 90 days and the cumulative survival rate was calculated. Coinjection of *mIL-12* significantly increased the survival rate elicited by *poly-rBCG* ($P<.05$). Data for all groups and treatments are shown as means ± SEM.

Effect of treatment on animal survival

All three treatments (with *mIL-12*, *poly-rBCG*, and *poly-rBCG + mIL-12*) increased the survival of mice ($n=8$,

$P<.05$; Fig 3b). However, survival of mice treated with *poly-rBCG* + *mIL-12* was substantially increased within the 90-day observation period, when compared with the survival of the other three groups. At a time when no mice survived in the *mIL-12* group and *poly-rBCG* group (i.e., day 50), 40% of mice in the *poly-rBCG* + *mIL-12* group were still alive (Fig 3b). The cumulative survival of mice treated with *poly-rBCG* + *mIL-12* was dramatically higher than for the other three groups around day 45 after treatment. All mice treated with vector alone, *mIL-12* alone, or *poly-rBCG* alone died before the end of the experiment (by day 90). On the other hand, only one mouse died on day 20, four died before day 50, and the rest survived in the *poly-rBCG* + *mIL-12* group.

Antitumor responses of CTLs enhanced by MBT-2 implants expressing poly-rBCG

Splenocytes from *poly-rBCG*-vaccinated mice were stimulated *in vitro* with MBT-2 extracts, and the lytic activities of the cells against MBT-2 cells were examined as CTL responses. As shown in Fig 4a, effector cell activities that were generated by spleen cells from mice with intratumoral *poly-rBCG*-transferred MBT-2 resulted in potent *poly-rBCG*-specific CTL responses. Spleen cells from C_3H/HeN mice immunized with *poly-rBCG* developed greater *poly-rBCG*-specific lytic activities than did effector cells from the other experimental groups (Student's *t*-test; $P<.05$). Moreover, *in vitro* sensitization and cytotoxicity assays with MBT-2 extracts also showed that such augmented cytotoxic responses are tumor-specific ($P<.05$). More interestingly, splenocytes prepared from mice vaccinated with *poly-rBCG* showed significant cytotoxic activity against Sq-1979 cells cotransfected with *poly-rBCG*, but not against syngeneic Sq-1979 cells at a high effector/target ratio (60:1). Thus, immunotherapy for bladder cancer using *poly-rBCG* strongly elicited tumor-specific CTL responses.

Th1-polarized serum systemic IFN-γ response to electroporated vaccines

To distinguish the Th1 and Th2 pathways that are directed by the electroporated vaccines, serum levels of IL-2, IL-4, IL-5, IFN-γ, and TNF-α were analyzed using a mouse Th1/Th2 cytokine CBA, and monitored at the same time. Serum IFN-γ level was below 3 pg/ml in control mice without treatment. Mice of study groups ($n=8$ each) treated with the *mIL-12* vaccine with or without *poly-rBCG* were found to have substantially and significantly higher Th1-polarized serum IFN-γ induction than the untreated controls. The serum IFN-γ level increased gradually after the transfer of *mIL-12* alone (means ± SEM; 1500 ± 78) and *mIL-12* + *poly-rBCG* (1354 ± 105 pg/ml) into the quadriceps femoris muscle and MBT-2 implants, respectively, and peaked on day 7 after EPGIT. Significantly, the Th1-polarized serum IFN-γ levels from the experimental mice stayed higher over the first 14 days following EPGIT, with an average of 1466 ± 111 pg/ml. This concentration still stayed significantly higher up to day 21 (583 ± 58 pg/ml) in mice receiving *poly-rBCG* + *mIL-12*. In the group of mice receiving *poly-rBCG* alone, the serum IFN-γ level stayed around 113 ± 9 pg/ml up to day 21. Thus, the combined treatment succeeded in prolonging the *poly-rBCG*-induced elevation in Th1-polarized interferon levels (Fig 4b). On the other hand, in the MBT-2-bearing C_3H/HeN mice transferred with *poly-rBCG* vaccine with or without *mIL-12*, no elevations of IL-2, IL-4, IL-5, or TNF-α levels were found.

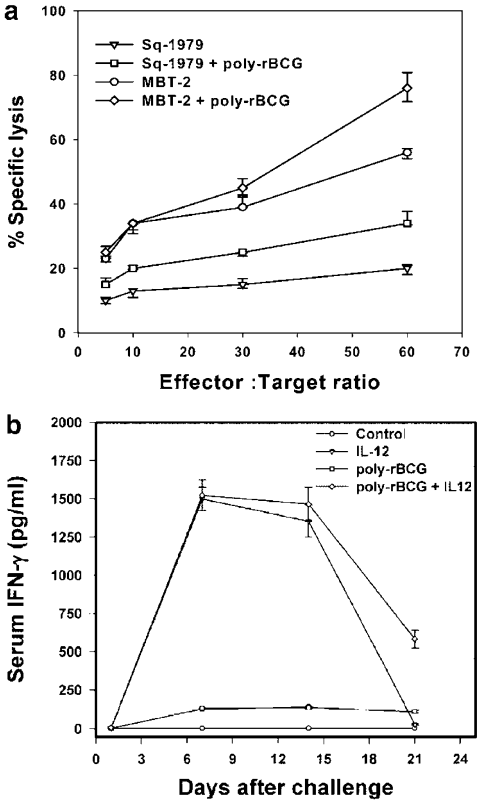


Figure 4 Antitumor CTL response and Th1-polarized immune response elicited by electroporated *poly-rBCG* DNA vaccines. (**a**) Splenocytes from MBT-2 mice immunized with *poly-rBCG* developed high MBT-2-specific lytic activity. Splenocytes from *poly-rBCG*-vaccinated mice were stimulated with MBT-2 extracts and assessed for their lytic activities against bladder tumors. Each bar represents the means ± SD of specific lysis values obtained from five mice per group. (**b**) Induction of IFN-γ levels by the electroporated vaccines. When the serum IL-2, IL-4, IL-5, IFN-γ, and TNF-α levels of treated MBT-2-implanted mice were compared using the same test, four groups of mice ($n=8$ each) treated with the *mIL-12* vaccine with or without *poly-rBCG* were found to have substantially and significantly higher serum IFN-γ levels over at least the first 14 days following EPGIT. These results suggest that the combined treatment succeeded in prolonging the poly-rBCG-induced elevation in systemic Th1 interferon, but not in Th2 cytokine levels.

Effect of treatment on immune cell response

To validate the cellular immune responses that are elicited by the treatment with *poly-rBCG* and *mIL-12*, we assessed the induction of T cells, B cells, and macrophages in

samples of MBT-2 tissue. Using standard immunohistochemical procedures, markers for T cells (CD3e, CD_4, and CD8a), B cells (CD19), and macrophages (F4/80) were compared in MBT-2 implants from untreated mice and from mice treated for 1 and 2 weeks with empty vector, *poly-rBCG* alone, *mIL-12* alone, or *poly-rBCG* + *mIL-12*. In tumors treated with *poly-rBCG*, *mIL-12*, or *poly-rBCG* + *mIL-12*, the concentrations of all markers except for the B-cell marker were increased at 1 week (Fig 5), and at 2 weeks the macrophage marker concentration was still above control levels (data not shown). Thus, all treatments appeared to elicit a cellular (Th1)-type response, rather than a humoral (Th2) response within MBT-2 implants. For any given marker, concentrations in tumor sections from mice treated with *poly-rBCG* + *mIL-12*, *poly-rBCG* alone, or *mIL-12* alone were highest, intermediate, and lowest, respectively. The results of the combined (*poly-rBCG* + *mIL-12*) treatment appeared to be due to the sum of a *poly-rBCG* effect and an *IL-12* effect.

Histopathological analysis

At 14 days after treatment, histopathological analysis of intratumoral MBT-2 sections stained with H&E revealed that the distribution of viable MBT-2 cells was reduced in comparison with that of the control group ($98.7 \pm 1.5\%$; $P < .01$), not only in MBT-2 implants transferred with *poly-rBCG* ($38.3 \pm 3.7\%$) and *mIL-12* ($49.8 \pm 4.8\%$) but also in those receiving *poly-rBCG* + *mIL-12* ($18.3 \pm 2.7\%$). Reduction in cell numbers, cell membrane fragmentation, and nuclear degradation in MBT-2 tumors transferred

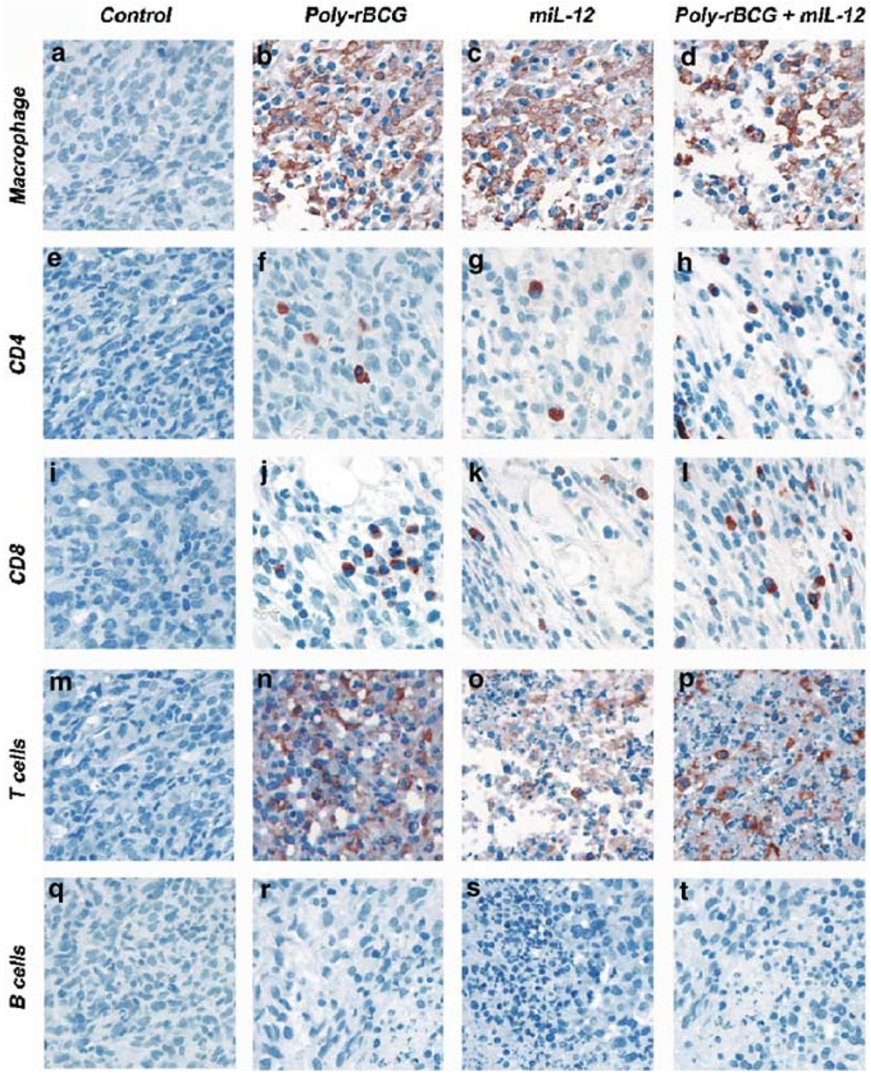


Figure 5 Immunohistochemical analyses of cellular immune responses in MBT-2 implants treated with empty vector, *poly-rBCG*, *mIL-12*, or *poly-rBCG* + *mIL-12*. Tests were performed 1 week after EPGIT. Sections were stained immunohistochemically using specific antibodies to a macrophage marker (rat anti-mouse F4/80, **a**–**d**), CD4 lymphocyte marker (rat anti-mouse CD4, **e**–**h**), CD8 lymphocyte marker (rat anti-mouse CD8a, **i**–**l**), T-cell marker (hamster anti-mouse CD3e, **m**–**p**) or B-cell marker (rat anti-mouse CD19, **q**–**t**). In tumors treated with *poly-rBCG*, *mIL-12*, or *poly-rBCG* + *mIL-12*, the staining of all markers increased except for B cells ($\times 60$).

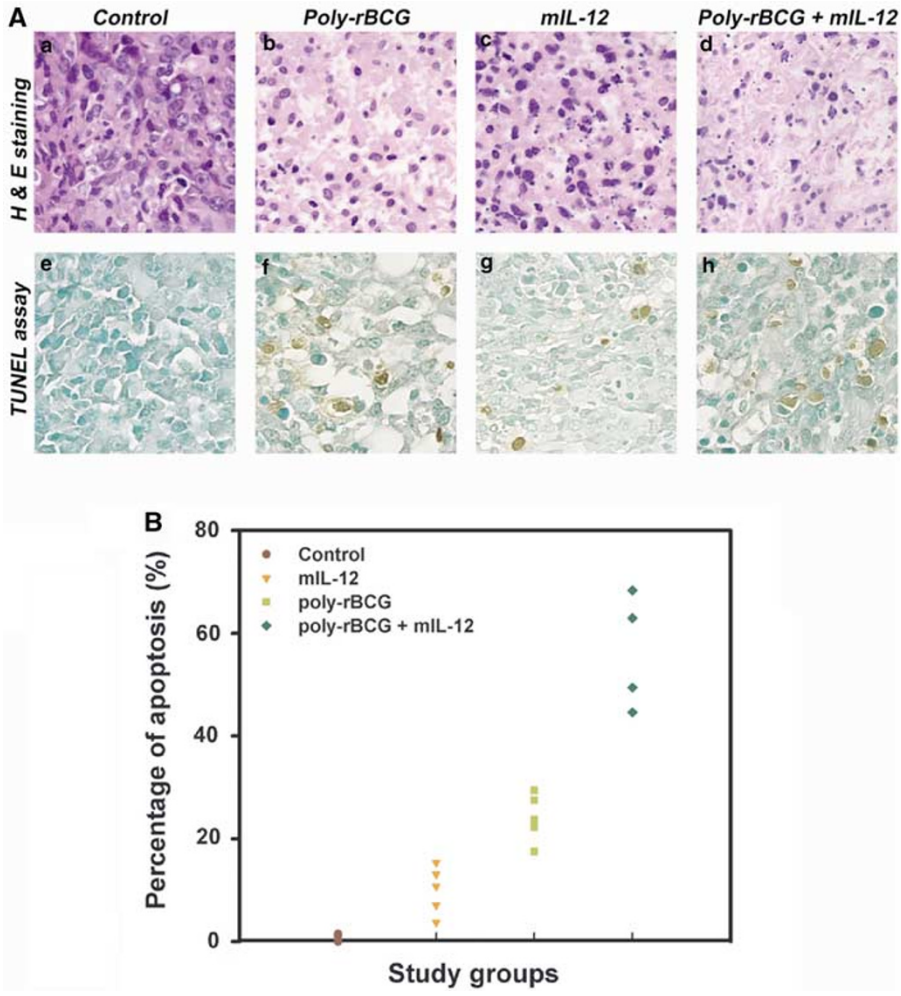


Figure 6 Histology of H&E staining and apoptosis TUNEL assay in the sections of intratumoral MBT-2 cells after EPGIT. (**a**) H&E staining (**a**–**d**) of the sections of MBT-2 cells revealed that reductions in cell numbers, increased cell membrane fragmentation, and nuclear degradation in MBT-2 cells receiving *poly-rBCG* alone or *poly-rBCG* + *mIL-12* was more definite than in those receiving MBT-2 in the *pCMV-V5-His6* control group. Furthermore, the MBT-2 sections (**e**–**h**) revealed that more TUNEL-positive cells were found in the *poly-rBCG* + *mIL-12*-transferred tumors and in those receiving *poly-rBCG* alone than were found in the control group (× 60). Tissues were taken 14 days after EPGIT. (**b**) The average percentage of apoptotic cells is shown for each mouse ($n = 5$). TUNEL analysis indicated that the combined *poly-rBCG* + *mIL-12* treatment was associated with more apoptosis (44.6–68.6%) than the *poly-rBCG* alone (17.5–29.4%) and *mIL-12* alone (3.8–13.1%) treatments. Controls (no treatment) showed a low background level of apoptosis (0–1.5%).

with *poly-rBCG* alone and *poly-rBCG* + *mIL-12* were more obvious than in the *pCMV-V5-His6* control group (Fig 6a). Grossly, the nuclear staining of MBT-2 implants treated with *poly-rBCG* alone and *poly-rBCG* + *mIL-12* was less basophilic than that of tumors receiving *pCMV-V5-His6* empty vector controls.

Effect of treatment on apoptosis

TUNEL assay was used to assess the apoptotic change in the MBT-2 implants induced by our DNA vaccines. Immunohistochemical analysis indicated that all three types of treatment led to apoptosis. The combined *poly-rBCG* + *mIL-12* treatment was associated with the greatest extent of apoptosis, ranging from 44.6–68.6%. Separate treatment with *poly-rBCG* and *mIL-12* led to less apoptosis, ranging from 17.5–29.4% for *poly-rBCG* alone, and 3.8–3.1% for *mIL-12* alone. Finally, treatment controls resulted in background apoptosis levels ranging from 0 to 1.5%. These results suggest a synergistic effect between *poly-rBCG* and *mIL-12* vaccines individually (Fig 6b). Macroscopically, the TUNEL staining of MBT-2 implants treated with *poly-rBCG* alone and *poly-rBCG* + *mIL-12* was more intense than that of transferred *pCMV-V5-His6* empty vector controls. Apoptosis levels were also confirmed by typical morphological changes, including cell shrinkage, condensed chromatin, and fragmented nuclei, and generation of the typical 196-bp DNA ladder upon gel electrophoresis (data not shown).

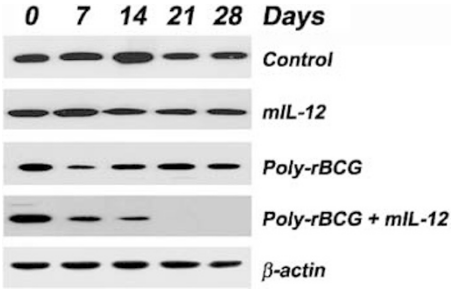


Figure 7 Western analysis of Bcl-2 protein expression in MBT-2 implants after EPGIT with *poly-rBCG* and *mIL-12*. After EPGIT with *poly-rBCG in vivo*, Bcl-2 protein expression was downregulated on day 7, but returned to control levels after day 14. After EPGIT with *poly-rBCG* + *mIL-12 in vivo*, Bcl-2 protein expression was downregulated between days 7 and 14, and was undetectable after day 14. In all other mice (*mIL-12* and empty vector control), no changes in Bcl-2 protein expression were observed.

Post-treatment Bcl-2 protein expression in tumors

Several genes, especially those of the *Bcl-2* gene family, regulate apoptosis. Since the TUNEL assay results indicated increased apoptosis after treatment, the possibility that Bcl-2 and Bax could modulate this increase was investigated. In animals treated with *poly-rBCG* + *mIL-12*, intratumoral Bcl-2 protein expression was downregulated at day 7 and further downregulated by day 14. By day 21, Western blots indicated undetectable levels of Bcl-2 in these tumors (Fig 7). In mice treated with *poly-rBCG* only, Bcl-2 protein expression was also downregulated by day 7, but had partially rebounded by day 14 and thereafter (Fig 7). No changes in Bcl-2 protein expression were observed in any other mice (treated or untreated). At the same time, Bax protein expressions were unchanged in all treatment groups of mice, or were minimally upregulated intratumorally in mice treated with *poly-rBCG* (data not shown).

Discussion

Intravesicular instillation of BCG is considered to be the most effective prophylaxis for recurrent superficial bladder cancer and treatment for carcinoma *in situ*. Although the virulence of *M. bovis* has been remarkably attenuated, the instillation of live BCG occasionally causes serious side effects, such as high fever, granulomatous prostatitis, pneumonitis, hepatitis, and BCG sepsis.[4] These disadvantages of BCG immunotherapy prompted us to evaluate other recombinant BCG DNA vaccines with potent antitumor activity but without severe side effects. Although the mechanism of intravesicular BCG action in preventing or suppressing bladder cancer growth is unknown, the efficacy of BCG is believed to be caused by antigen-mediated local production of cytokines and stimulated accumulation of inflammatory cells.[20] The alternative to BCG considered in this study is a mixture of DNA vaccines that specify multiple BCG antigens, that cause these antigens to be synthesized and secreted in bladder tumors, and that ultimately induce recruitment of immunocompetent cells and the associated accumulation of cytokines.

Of the five BCG antigen-specific genes incorporated in our four vaccine constructs (Fig 1), all have been gene inserts in previous DNA vaccines that have elicited antigen-specific responses. Other genes inserted into our DNA vaccines include the gene for *Igκ*, which signals cells to secret more protein.[26,27] All these antigens were equally expressed in the tissue (Fig 2a). This uniform pattern of localization is in contrast to the heterogeneous pattern (uniform and speckled) reported by Velaz-Faircloth et al,[28] and may indicate that the efficient secretion of the antigen components from the cells prevented intracellular concentrations from reaching the levels that cause antigen aggregation and speckle formation. The demonstration that all five gene-specific antigens in our vaccines were fully expressed also suggests effective delivery of *poly-rBCG* by electroporation to the bladder tumor and to the quadriceps femoris muscle. This result agrees with previous demonstrations that functioning DNA vaccines could be efficiently delivered to muscle[22,29] as well as tumors.[30,31]

We attempted to reproduce the efficacy of live BCG organisms in immunotherapy for superficial transitional cell bladder carcinoma while avoiding the adverse effects associated with this treatment. For this, we adopted a multisubunit multicomponent approach. This approach caused the growth of MBT-2 implants transferred with *poly-rBCG*, *mIL-12*, and *poly-rBCG* + *mIL-12* by means of EPGIT *in vivo* to be significantly inhibited, as compared with MBT-2 implants transferred with control *pCMV-V5-His6*. Combined gene-transfection tumor vaccines have been found to be highly effective, and perhaps more effective than live BCG in eliciting antitumor responses. However, those vaccines have generally consisted of only two component genes: *IL-7* and *B7.1*,[32] macrophage colony stimulating factor and *IL-4*,[33] or *B7* and *IL-12*.[34] Our use of four combined mycobacterial vectors led to broader and more enhanced responses than those attainable using only a two-component vaccine. Thus, the use of five different mycobacterial antigens is necessary. One, or the combination of two of the four mycobacterial vectors is not sufficient to achieve the observed effects in the subcutaneous bladder tumor model. In addition, the hallmark of successful treatment of bladder cancers using attenuated BCG is apparently the induction of an *in situ* (bladder) Th1 immune response.[20] This is marked by an increase in Th1-type cytokines (IFN-γ, IL-12, and IL-2) and effector cells (cytotoxic CD4, NK, and CD8).[17,35] Here, we demonstrated that *poly-rBCG* might facilitate more induction of IFN-γ than either negative controls, and this induction was significantly enhanced by the addition of *mIL-12*. Similarly, *poly-rBCG* was also shown to increase the concentration of macrophages and T cells, in particular $CD4^+$ and $CD8^+$ cells, within treated tumors. Evidence that these are antitumor effector cells is shown by our findings of retarded growth of tumor implants in response

to *poly-rBCG*; complete remission of these implants in response to *poly-rBCG + mIL-12*; substantially increased survival of mice treated with *poly-rBCG + mIL-12*; and increased apoptosis, especially in response to the combined treatment of *poly-rBCG + mIL-12*.

Although the mechanism underlying multicomponent immunization has not been fully elucidated in the present study, the focusing of rBCG antigens within the confined area of transfection might facilitate cognate presentation of CTL epitopes and Th1 epitopes.[36,37] The effects were associated with an enhancement of the cellular immune response. Cognate presentation of these epitopes on antigen-presenting cells is an effective stimulus of CTLs.[38,39] Transfection of *poly-rBCG* into MBT-2 is thought to add a Th epitope or CTL epitope to the bladder tumor cells. Immunization with plasmid DNA encoding recombinant BCG subunits showed much higher CTL responses than did immunization with the control empty vector. We have shown that induction of effective CTLs occurs significantly following combination therapy with *poly-rBCG*. In addition, *poly-rBCG* and *poly-rBCG + mIL-12* vaccines mediated the enhancement of both $CD8^+$ and $CD4^+$ T-cell responses, indicating that these constructs target the MHC class I and class II presentation pathways, whereas *mIL-12* alone appears to mediate the enhancement of the $CD4^+$ response only (Fig 5). Thus, *mIL-12* was recognized as a treatment adjuvant that stimulates the elevation of the IFN-γ level and $CD4^+$ T-cell responses.

Apoptosis is important for the resolution of pathophysiological as well as normal processes,[40,41] and influences tumor sensitivity to radioimmunotherapy.[42] For tuberculosis, leprosy, and sarcoidosis, the high turnover of epithelioid cells in granulomata is attributed to apoptosis and an influx of mononuclear phagocytes.[43] Similarly, BCG antigens may act as apoptosis inducers.[44] In the present study, the elevated numbers of apoptotic cells in bladder tumors responding to treatment with *poly-rBCG* and *poly-rBCG + mIL-12* can explain reduced tumor growth and tumor eradication (Fig 6a). Further, a Th1-polarized antitumor response (IFN-γ release, T-lymphocyte and macrophage influx) leading to significant apoptotic regression of tumor cells was observed. Thus, Bcl-2, an inhibitor of apoptosis, was found to be downregulated, consistent with the post-treatment timing of apoptotic cell increases (Fig 7). These data suggest that apoptosis contributes to the mechanism of the treatment-induced tumor regression, and provide a rationale for further functional characterization of rBCG immunotherapy.

Although the study using *poly-rBCG + mIL-12* showed that tumor cells were eradicated by day 28 (Fig 3a and b), some mice still died from cachexia. Further autopsy and histopathology of the study group using *poly-rBCG + mIL-12* also showed that tumor cells were eradicated, but enhanced systemic toxicity such as pulmonary oedema and splenomegaly was found in some mice (data not shown). To understand whether the systemic toxicity was caused by the expression of *poly-rBCG* or by the enhancement of IFN-γ stimulated by *mIL-12*, individual or combined *poly-rBCG* DNA vaccine constructs were analyzed in pathogen-free 4-week-old C_3H/HeN female mice (data not shown). Long-term monitoring after six treatments with EPGIT every 2 weeks for 3 months revealed no splenomegaly by autopsy. In addition, histopathological analysis of heart, liver, spleen, lung, and kidney revealed no difference between the study groups and the healthy mice controls. Serum was also collected every week after EPGIT for biochemical analysis and we found no differences with mice treated with empty vectors or healthy mice controls ($P < .0001$). This further evidence strongly supports the advantage of *poly-rBCG* and its potential usage on the intravesicular instillation of *poly-rBCG*.

Systemic elevation of IL-12 was shown to be more important than local IL-12 elevation in the cure of established tumors and spontaneous metastases.[45] However, it is generally believed that when injected intramuscularly—a systemic route of administration—the expression rate as well as toxicity increase. On the other hand, other studies have shown that IL-12 is more effective and less toxic when it is injected into the tumor locally, but the expression rate is lower.[46] In the present study, we found that electroporation-mediated administration of *mIL-12* cDNA to muscle significantly inhibited the growth of not only poly-rBCG-transferred bladder tumor but also untreated distant tumors. Several approaches could be studied to improve efficacy in this regard. An additional interesting point is that the only place to establish an orthotopic bladder-cancer model is in the bladder itself, and not in other sites such as the flank of the mouse.

Methods of gene delivery for either therapeutic or experimental purposes rely largely on viral vectors. However, serious concerns have been voiced about the use of such vectors, especially when clinical trials are involved. Gene transfer by electroporation *in vivo* was found to be effective for introducing DNA into murine hepatocellular carcinomas and melanomas in a mouse model.[46,47] Like other nonviral methods, electroporation has a variety of advantages, because all of the tissues and cells could become targets. To further improve antitumor efficacy of *poly-rBCG* and the clinical applicability of gene therapy for bladder carcinoma, we have used an *in vivo* electroporation method of gene transfer. These strategies facilitate the delivery efficiency of DNA vaccine and increase the screening efficiency of *poly-rBCG* in this model. Our study shows that EPGIT using *poly-rBCG* exerts strong antitumor activity with the adjuvant of *IL-12* vaccination in a subcutaneous bladder tumor model. We have thus provided evidence of the potential use of rBCG vaccines for the treatment of bladder carcinomas and, possibly, other compartmentalized neoplasms.

Abbreviations

BCG, bacillus Calmette–Guérin; CBA, cytometric bead array; CTL, cytotoxic T lymphocyte; EPGIT, electroporative gene immunotherapy; FITC, fluorescein isothio-

cyanate; H&E, haematoxylin and eosin; Ig*k*, immunoglobulin kappa; MBT-2, murine bladder tumor; poly-rBCG, four combined recombinant BCG DNA vaccines; TUNEL, terminal dUTP nick-end labeling.

Acknowledgments

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