

Original Research Report

Immune responses to repetitive adenovirus-mediated gene transfer and restoration of gene expression by cyclophosphamide or etoposide

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Abstract

Background. One major concern about adenoviral vectors for repetitive gene delivery is the induction of an immune response to the vector, thus impeding effective gene transduction.

Methods. To assess the immune response to the adenoviral vector, repetitive gene dosing was performed into rhesus monkey cervix and C3H mouse skin using the adenoviral vector carrying the *lacZ* gene. Three repetitive intracervical injections of adenovirus-*lacZ* were done in the rhesus monkey at the intervals of 4 weeks. Gene expression on the second and third injection was completely suppressed.

Results. Anti-adenovirus IgG levels and neutralizing antibody titers in the rhesus monkey significantly increased after the first injection of adenovirus. In the C3H mouse, neutralizing antibody titers significantly increased after the first injection of adenovirus-*lacZ* at more than 10⁸ plaque-forming unit (PFU). The repetitive expression of *lacZ* gene in the mouse skin markedly decreased when the second injection is done more than 2 weeks after the first injection. Chronic low-dose treatment with cyclophosphamide or etoposide markedly suppressed neutralizing antibody titers in the mouse serum and restored the gene expression in the mouse skin on the second and third injection.

Conclusions. It is suggested that repetitive gene expression by adenovirus-mediated transfer may be reduced by circulating neutralizing antibodies and could be restored by chronic low-dose treatment with cyclophosphamide or etoposide.

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Introduction

Human adenoviruses have proven to be effective vectors for the delivery and expression of foreign genes. A disadvantage of using adenovirus as a vector is that adenoviruses do not integrate their genome into the chromosomes of the host cells. Thus, gene expression is

transient and repetitive administration of the virus may be required for effective expression of the therapeutic gene. Especially, repetitive gene delivery is required to induce tumor reduction of cancer. Although employment of adenoviral-based vector delivery systems has a number of advantages for cancer gene therapy as above, repetitive administration of adenovirus vectors will stimulate an immune response in vivo to the vector, resulting in possible impedance of gene delivery. Adenovirus-mediated repetitive gene transfer has been studied in several animal models: airway of the mouse [1,2], cotton rat, and rhesus monkey [3,4]; liver of the dog [5] and rabbit [6]; and intestine of rat [7]. In these studies using adenovirus, it was shown that

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repetitive gene transfer could be achieved at a reduced efficiency. Serum anti-adenovirus neutralizing antibody titers differ depending upon administration routes of adenovirus because topical application of adenovirus exhibits the lower neutralizing activity compared with systemic administration [7,8].

Cervical cancer is a good model of human papilloma-virus-induced carcinogenesis and seems an ideal one for genetic intervention. Previous studies have shown that adenovirus-*p53* can suppress the growth of human cervical cancer cells in vitro and in vivo [9,10], and that the β -galactosidase gene is highly expressed in the rhesus monkey cervix following adenovirus-mediated gene transfer [11]. There are no reports of systemic immune responses to adenoviral administration into the cervix. We addressed here the biosafety and the antibody-mediated immune response to the adenoviral vector for 5 months after repetitive adenoviral injections into the rhesus monkey cervix.

The immunosuppressant FK506 decreases the humoral response and prolongs expression of adenovirus-mediated transgene [12,13]. The other immunosuppressant cyclosporin A prolongs expression of the adenovirus-mediated transgene but a second administration of adenovirus had only a minimal effect on transgene expression [5]. Although repeated deliveries would be required for cancer patients, repetitive adenovirus-mediated gene expression has yet to be established. To address the reversal of inhibition of repetitive gene expression by adenovirus-mediated immune response, an adenoviral vector (Ad5CMV-*lacZ*) carrying the *Escherichia coli lacZ* gene was also used to transduce mouse skin. Repetitive subcutaneous injections were introduced to detect the reversal of adenovirus-mediated immune responses by anti-cancer agents.

Materials and methods

Animals

After review and approval by both the Animal Care and Use Committee and the Biosafety Committee at the University of Texas M. D. Anderson Cancer Center, 15 reproduction-competent rhesus monkeys (aged 5–16 years) were selected (Table 1) and female C3H mice aged 5–6 weeks were purchased from Harlan Co. (Houston, TX). To assess the health status of each monkey, and to establish baseline values prior to inoculating the animals with adenovirus, hematology and serum biochemistry tests were performed. The monkeys were carefully monitored for side effects of virus infection. No infections, stenosis, vaginal thermal injuries, fistulae, or other abnormalities were noted. To detect virus, sera were cultured on 293 cells. All monkeys had negative cultures on day 3. HeLa cells

Table 1

Characteristics of monkeys and adenoviral entries into the monkey cervixes

Monkey number	Age	Weight progeny (kg)	<i>n</i>	Adenoviral entries (PFU)
Single abrasion with Ad5CMV- <i>lacZ</i> ^a				
J9	10	8.6	2	2×10^8
L163	9	6.6	0	2×10^9
L879	10	5.5	1	2×10^9
J47	14	5.5	1	2×10^{10}
L745	17	8.6	10	2×10^{10}
Three injections with Ad5CMV- <i>lacZ</i> ^b				
L571	5	5.2	2	2×10^8
L741	16	6.9	9	2×10^9
L577	7	7.0	2	2×10^9
L345	5	7.5	1	2×10^{10}
L87	8	5.8	2	2×10^{10}
Three injections with Ad5CMV- <i>lacZ</i> + Ad5CMV- <i>p53</i> ^c				
L515	11	5.6	3	2×10^{10}
J23	6	5.3	1	2×10^{10}
L559	10	6.5	4	2×10^{10}
L57	10	7.9	5	2×10^{10}
J3	6	7.3	2	2×10^{10}

^a Adenoviruses at each dose (300 μ l) were placed on gelfoam against the abraded monkey cervix.

^b Adenoviruses at half of each dose (150 μ l) were injected into the anterior and posterior cervix of the monkey, respectively.

^c Ad5CMV-*lacZ* at half of each dose (150 μ l) and Ad5CMV-*p53* at half dose (150 μ l) were injected into the anterior and posterior cervix of the monkey, respectively.

were used to detect replication competent adenovirus-*lacZ* and adenovirus-*p53* according to the previous report [14]. Briefly, HeLa cells were incubated with samples of cervical discharge and cell lysates from cervical biopsies for 5 days and harvested. The HeLa cell lysate was incubated with a new batch of HeLa cells. This process was repeated again to obtain the third set of cell extracts for PCR analysis. PCR assay detected the E1 DNA in a reconstitution of one plaque-forming unit (PFU) of wild-type virus in 10^9 PFU of recombinant viruses. Adenoviral replication was not detected in the samples of cervical discharge and cervical biopsies on day 3. None of the monkeys developed any clinical signs of viral infection or inflammation. Repetitive exposure to the adenoviral vector also did not increase the inflammatory response in the injected cervix, which was estimated by H&E staining. Physical examination revealed no fever, lymphadenopathy, conjunctivitis, tachypnea, or tachycardia at any of the time points. No abnormalities were found in complete blood counts or sedimentation rate, nor were abnormalities observed in serum electrolytes, transaminases, or blood urea nitrogen and creatinine (data not shown). No monkeys died during this experiment.

Virus administration

To detect the gene expression and the anti-adenovirus antibody produced after topical application of Ad5CMV-*lacZ*, five monkeys underwent abrasion of the cervix with

sandpaper until punctuate bleeding points appeared. Ad5CMV-*lacZ* was then placed on gelfoam and applied to the abraded cervix. This treatment method is considered to be very useful for clinical application because of its safety and non-invasiveness, whereas this topical application was examined to estimate the local adenoviral transduction on the cervix. Three different doses were used: 1 monkey received 2×10^8 PFU/300 μ l; 2 monkeys received 2×10^9 PFU/300 μ l; and 2 monkeys received 2×10^{10} PFU/300 μ l. Three days later, all five monkeys underwent cone biopsy via loop electrosurgical excision procedure (LEEP) of the cervix. Blood samples were drawn on days 0, 3, 28, 31, 59, and 150.

To detect the gene expression and the anti-adenovirus antibody produced after injection of Ad5CMV-*lacZ* at different doses, five monkeys each received injections of Ad5CMV-*lacZ* in two divided doses in the anterior and posterior cervix through an insulin syringe (gauge 28 1/2). Three different doses were used: 1 monkey received 2×10^8 PFU/200 μ l; 2 monkeys received 2×10^9 PFU/200 μ l; and 2 monkeys received 2×10^{10} PFU/200 μ l. Three days later, all five monkeys underwent cone biopsy via LEEP of the cervix. The same doses of Ad5CMV-*lacZ* as used before were injected into the anterior and posterior cervix on days 28 and 56. All five monkeys underwent punch biopsy of the cervix 3 days after the second and third injection. Blood samples were drawn on days 0, 3, 28, 31, 56, 59, 90, 120, and 150.

To detect the gene expression and the anti-adenovirus antibody produced after injection of Ad5CMV-*lacZ* and Ad5CMV-*p53*, five monkeys received injections of Ad5CMV-*lacZ* in the anterior cervix and injections of Ad5CMV-*p53* in the posterior cervix through an insulin syringe. Single doses of Ad5CMV-*lacZ* (1×10^{10} PFU/100 μ l) into the anterior cervix and Ad5CMV-*p53* (1×10^{10} PFU/100 μ l) into the posterior cervix were given to each monkey on days 0, 28, and 56. Three days after each injection, all five monkeys underwent punch biopsy of the cervix. Blood samples were drawn on days 0, 3, 28, 31, 56, 59, 90, and 150.

To study the dose-dependency of neutralizing antibody titers after injection of Ad5CMV-*lacZ* at different doses, eighteen C3H mice each received single injection of Ad5CMV-*lacZ* in the right posterior flank through an insulin syringe. Three mice were used for each treatment. Six different doses were used: each group received 1×10^{10} PFU/100 μ l; 1×10^9 PFU/100 μ l; 1×10^8 PFU/100 μ l; 1×10^7 PFU/100 μ l; 1×10^6 PFU/100 μ l; and 1×10^5 PFU/100 μ l. Blood samples were drawn on day 28.

To detect the gene expression after repetitive injection of 1×10^{10} PFU Ad5CMV-*lacZ*, 24 C3H mice each received repetitive injection of Ad5CMV-*lacZ* in the right posterior flank through an insulin syringe. Three mice were used for each treatment. Ad5CMV-*lacZ* was injected into the mouse skin at each time point as shown in Fig. 4.

To detect the gene expression and neutralizing antibody titers after injection of Ad5CMV-*lacZ* in C3H mice treated with each drug preparation, 50 μ l of viral suspension (containing 5×10^9 PFU) was injected into the intradermal tissue of right posterior flank of each mouse through an insulin syringe after shaving hairs. Each drug preparation in 100 μ l solution was injected every day from 3 days before the first injection of Ad5CMV-*lacZ* until sacrifice.

Drugs

Pamindronate (Aredia, Ciba-Geigy Ltd., Basel, Switzerland), methylprednisolone (Solu-Medrol, Abbott Lab., North Chicago, IL), FK506 (Fujisawa Pharmaceutical Co., Osaka, Japan), cisplatin (Bristol-Myers Squibb Co., Princeton, NJ), paclitaxel (Taxol, Bristol-Myers Squibb), mitomycin C (Bristol-Myers Squibb), cyclophosphamide (Bristol-Myers Squibb), etoposide (VP-16, Bristol-Myers Squibb), 5-FU (SoloPak Lab. Inc., Elk Grove Village, IL), and doxorubicin (Chiron Therapeutics, Emeryville, CA) were injected into the mice to inhibit immune response to adenoviral vector. Dose of each drug was determined according to the dose usually used in human clinical cancer treatment. Pamindronate, methylprednisolone, and FK506 were injected intramuscularly. Cisplatin, paclitaxel, mitomycin C, and doxorubicin were injected intraperitoneally.

X-Gal staining

The injected rhesus monkey cervix and mouse skin samples were obtained and embedded with tissue-freezing compound (Miles Inc., Elkhart, IN). Samples with 4- μ m thickness were fixed with 100% acetone at -20°C . Samples transduced by Ad5CMV-*lacZ* were visualized by staining with X-gal (β -galactosidase substrate, 5-bromo-4-chloro-3-indolyl- μ -D-galactopyranoside; Sigma Chemical Co., St. Louis, MO). Nuclear fast red (1 mg/ml, Sigma) was used as counterstaining.

Detection of anti-adenovirus IgG and IgA

A standard enzyme-linked immunosorbent assay (ELISA) procedure was followed to detect anti-adenovirus IgG in rhesus monkey serum using a cell-free extract prepared by sonication and centrifugation of the cell pellet obtained from 6×10^7 293 cells infected with Ad5CMV-*lacZ*. The control antigen was prepared by processing 6×10^7 uninfected cells. Peroxidase-conjugated goat anti-human IgG (Sigma) and *o*-phenylenediamine dihydrochloride (OPD, Sigma) were used as a secondary antibody and a substrate, respectively.

Vaginal secretions were collected for ELISA to detect anti-adenovirus IgA antibody levels. Peroxidase-conjugated goat anti-human IgA (Sigma) was used as a secondary antibody. Local IgA was not detected before

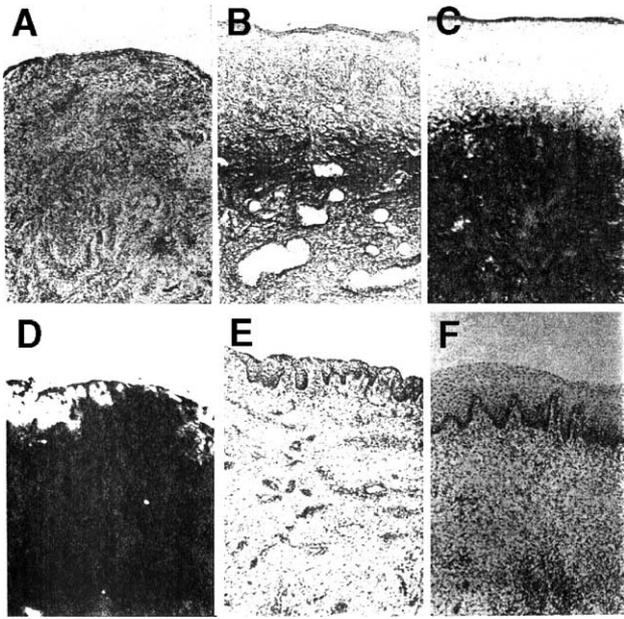


Fig. 1. Transgene expression in the rhesus monkey cervix transduced by Ad5CMV-lacZ. The cervix was abraded and Ad5CMV-lacZ at 2×10^{10} PFU was then placed against the abraded cervix and stained by X-gal (A). Ad5CMV-lacZ doses of 1×10^8 PFU (B), 1×10^9 PFU (C), and 1×10^{10} PFU (D) were injected into the anterior and posterior cervix and stained by X-gal. Each cervix after the second (E) and third injection (F) with Ad5CMV-lacZ at 1×10^{10} PFU was stained by X-gal.

and after the adenoviral injection in any of the 15 monkey cervixes.

Assays for anti-adenovirus neutralizing antibodies

Neutralizing antibodies measure the ability of the monkey serum to prevent β -gal expression of human cervical cancer SiHa cells by Ad5CMV-lacZ. Each serum dilution (50 μ l) was mixed with Ad5CMV-lacZ (3×10^5 PFU in 50 μ l), incubated for 1 h at 37°C, and applied to SiHa cells in 96-well plates (1×10^4 cells/well). After 2 days incubation at 37°C, cells were fixed with 0.5% glutaraldehyde solution and stained for β -galactosidase expression. Detection of anti-adenovirus reactivity was based on readings at an optical density of 630 nm (OD₆₃₀). The titer of neutralizing antibody for each sample was reported as the dilution with which 50% of cells of staining in the well was inhibited compared with non-immunized serum.

Image analysis of β -gal expression

The degree of β -gal expression in the mouse skin was quantitated on digitized images of the X-gal-stained slides using a Magiscan Images Analysis System (Joyce-Loebl, Ltd., Dukesway, England) and a Nikon light microscope

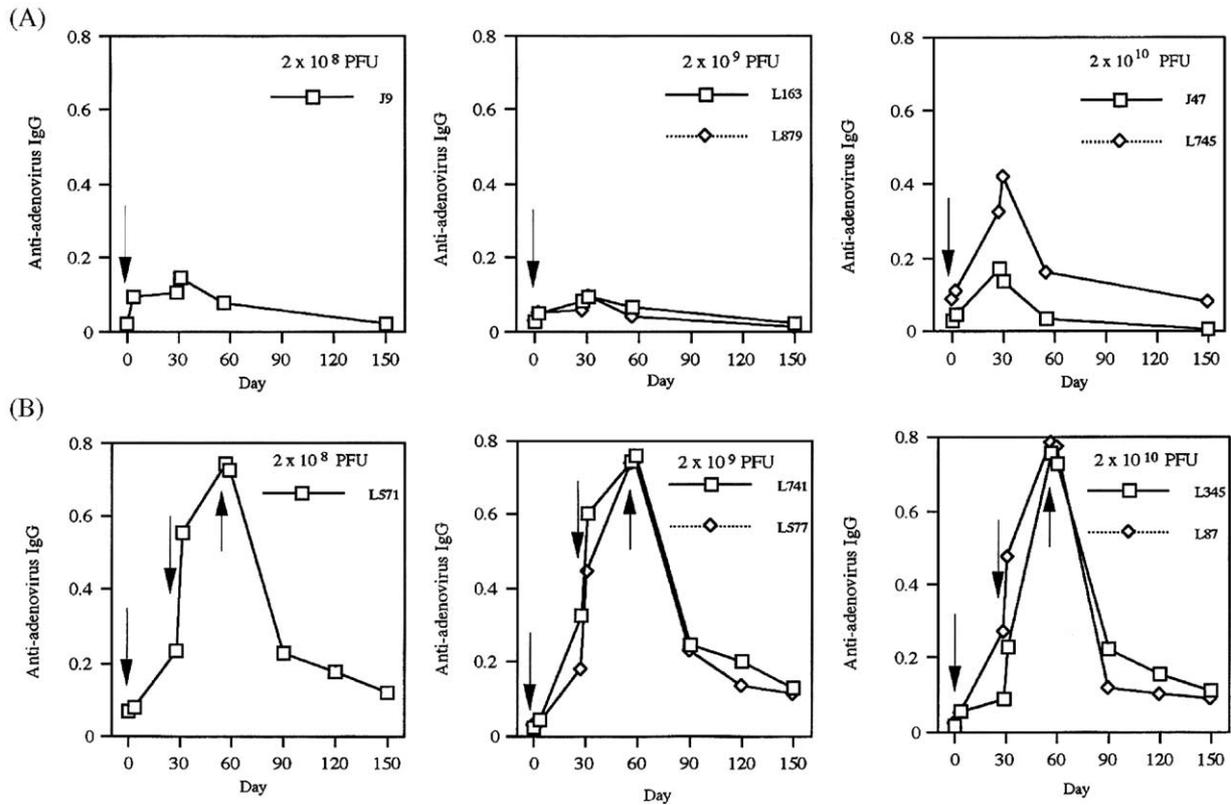


Fig. 2. Serum anti-adenovirus IgG levels in rhesus monkeys. (A) The cervixes of the rhesus monkey were abraded, and Ad5CMV-lacZ doses of 2×10^8 PFU for monkey J9, 2×10^9 PFU for monkeys L163 and L879, and 2×10^{10} PFU for monkeys J47 and L745 were topically applied against the abraded cervix on day 0. (B) Ad5CMV-lacZ doses of 2×10^8 PFU for monkey L571, 2×10^9 PFU for monkeys L741 and L577, and 2×10^{10} PFU for monkeys L345 and L87 were injected into the cervix on days 0, 28, and 56. Asterisk mark is the adenoviral application and injection.

with a computer-controlled stage according to the previous report [15]. The specific intensity of β -gal expression on each region was calculated as the total integrated absorbance divided by the area of that region minus the contribution of integrated absorbance of an equivalent area of background. The normalized value of β -gal expression or the relative staining intensity of β -gal expression represents the level of β -gal expression relative to that of SiHa cells infected with Ad5CMV-*lacZ* at 50 MOI.

Relative staining intensity of β -gal = (OI / OA – KB) / mean (OI / OA – KC) SiHa cells, where OI is the integrated signal, OA is the integrated area, KB is the mean absorbance of the background of subcutaneous tissue, and KC is the mean absorbance of non-infected SiHa cells.

Results

X-gal staining

To establish the treatment method and to determine the dose of adenovirus in gene therapy for cervical cancer, monkey cervixes were abraded and injected with Ad5CMV-*lacZ*. No β -gal-positive cells were noted in the monkeys who underwent abrasion and topical application of the vector (Fig. 1A). β -Gal-positive cells were noted in the monkey who received 2×10^8 PFU on the first injection (Fig. 1B). Additionally, progressive increases in staining were noted with increasing virus PFU (Figs. 1C and D). The second and third injection showed no β -gal-positive cells in the injected cervix (Figs. 1E and F). p53-positive cells were noted in the Ad5CMV-*p53*-injected cervix 3 days after the

first injection (data not shown). H&E staining did not show marked lymphocyte and neutrophil infiltration in the tissues abraded or injected with Ad5CMV-*lacZ* and Ad5CMV-*p53*.

Anti-adenovirus IgG and neutralizing antibody in rhesus monkey

To determine the humoral response in the abraded monkeys, the levels of anti-adenovirus IgG before and after exposure of Ad5CMV-*lacZ* are assayed. All five monkeys had some baseline IgG present (Fig. 2A). IgG levels in all five monkeys were significantly increased on days 3 and 28, peaked on day 31, started to decrease on day 56, and returned to basal levels on day 150. There was no significant difference between IgG levels in the monkey (J9) applied with 2×10^8 PFU Ad5CMV-*lacZ*, those of monkeys (L163, L879) applied with 2×10^9 PFU Ad5CMV-*lacZ*, and those of monkeys (J47, L745) applied with 2×10^{10} PFU Ad5CMV-*lacZ*.

To determine the relationship between the levels of anti-adenovirus IgG and doses of Ad5CMV-*lacZ*, five monkeys received 3 different doses of adenovirus. There was no significant difference between IgG levels of the monkey (L571) injected with 2×10^8 PFU Ad5CMV-*lacZ*, those of the two monkeys (L741, L577) injected with 2×10^9 PFU Ad5CMV-*lacZ*, and those of the two monkeys (L345, L87) injected with 2×10^{10} PFU Ad5CMV-*lacZ*. IgG levels of all five monkeys were significantly increased on days 3 and 28 after the first injection and on day 31 after the second injection, and the levels peaked on day 56 but were not significantly increased on day 59 after the third injection (Fig. 2B). IgG levels of all monkeys started to decrease 1

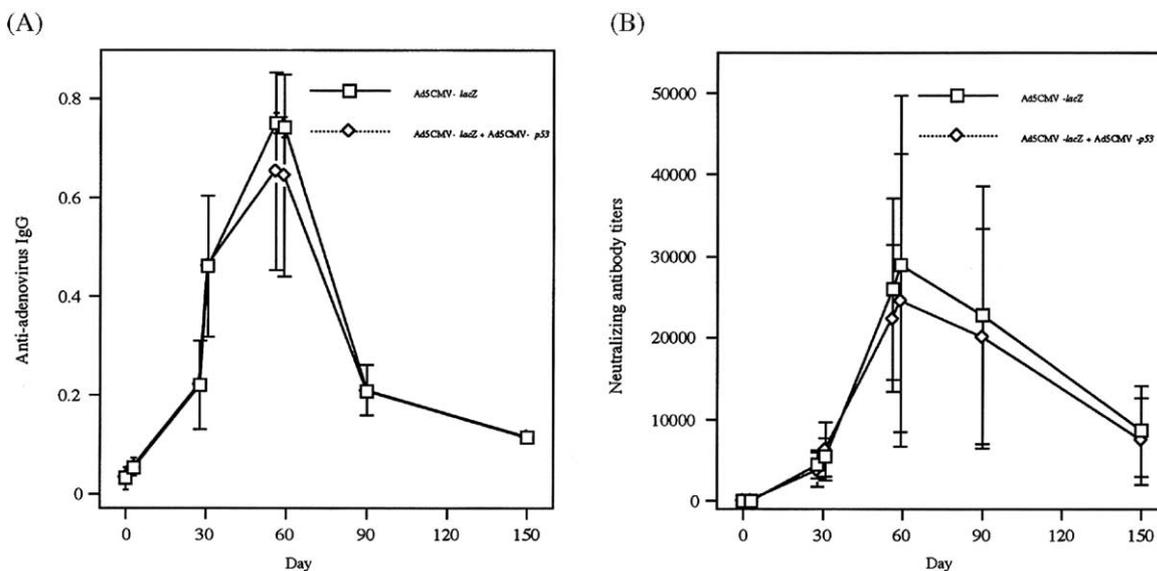


Fig. 3. Serum anti-adenovirus IgG levels and neutralizing antibody titers in the injected rhesus monkeys. Ad5CMV-*lacZ* doses of 1×10^{10} PFU and Ad5CMV-*p53* doses of 1×10^{10} PFU for monkeys L515, J23, L559, L57, and J3 were injected into the cervix on days 0, 28, and 56. (A) Serum anti-adenovirus IgG levels of the rhesus monkeys injected with Ad5CMV-*lacZ* and Ad5CMV-*p53* were compared with those of the rhesus monkeys injected with Ad5CMV-*lacZ*. (B) Serum neutralizing antibody titers of the rhesus monkeys injected with Ad5CMV-*lacZ* and Ad5CMV-*p53* were compared with those of the rhesus monkeys injected with Ad5CMV-*lacZ*.

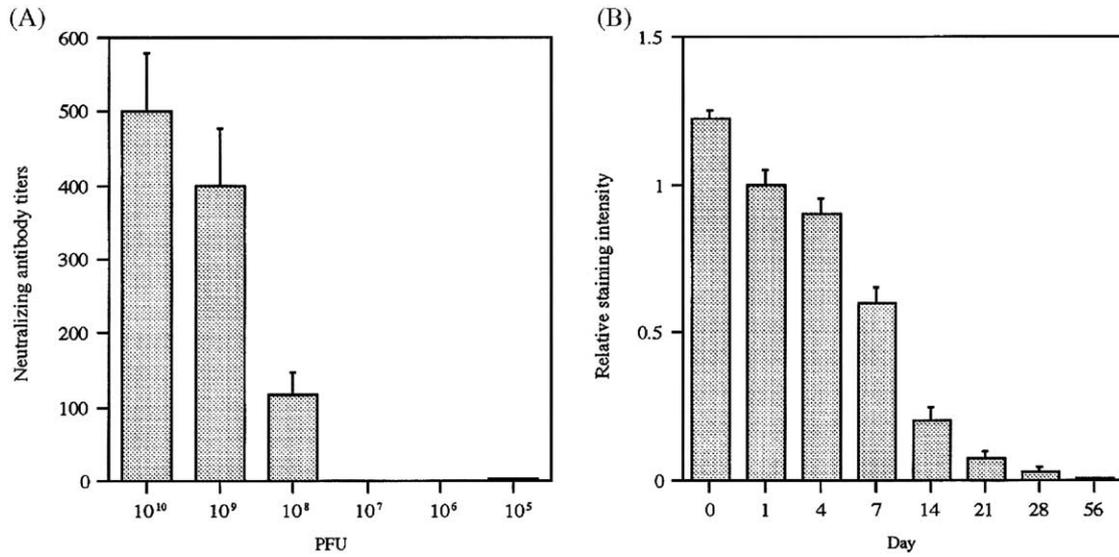


Fig. 4. (A) Neutralizing antibody titers of the C3H mice 4 weeks after the injection of Ad5CMV-*lacZ* at 1×10^{10} PFU, 1×10^9 PFU, 1×10^8 PFU, 1×10^7 PFU, 1×10^6 PFU, and 1×10^5 PFU. (B) Morphometric analysis of C3H mouse skin injected with Ad5CMV-*lacZ* at 1×10^{10} PFU. Ad5CMV-*lacZ* was injected into the mouse skin on day 0, day 0 + 1, day 0 + 2, day 0 + 3, day 0 + 4, day 0 + 5, day 0 + 6, day 0 + 7, day 0 + 14, day 0 + 21, day 0 + 28, and day 0 + 28 + 56. The injected mouse skin was resected 24 h after the injection of Ad5CMV-*lacZ* and stained with X-gal. The expression of *lacZ* gene in the injected skin was measured by morphometric analysis. Relative staining intensity of each C3H mouse skin after the second or third injection was compared with the mouse skin after the first injection.

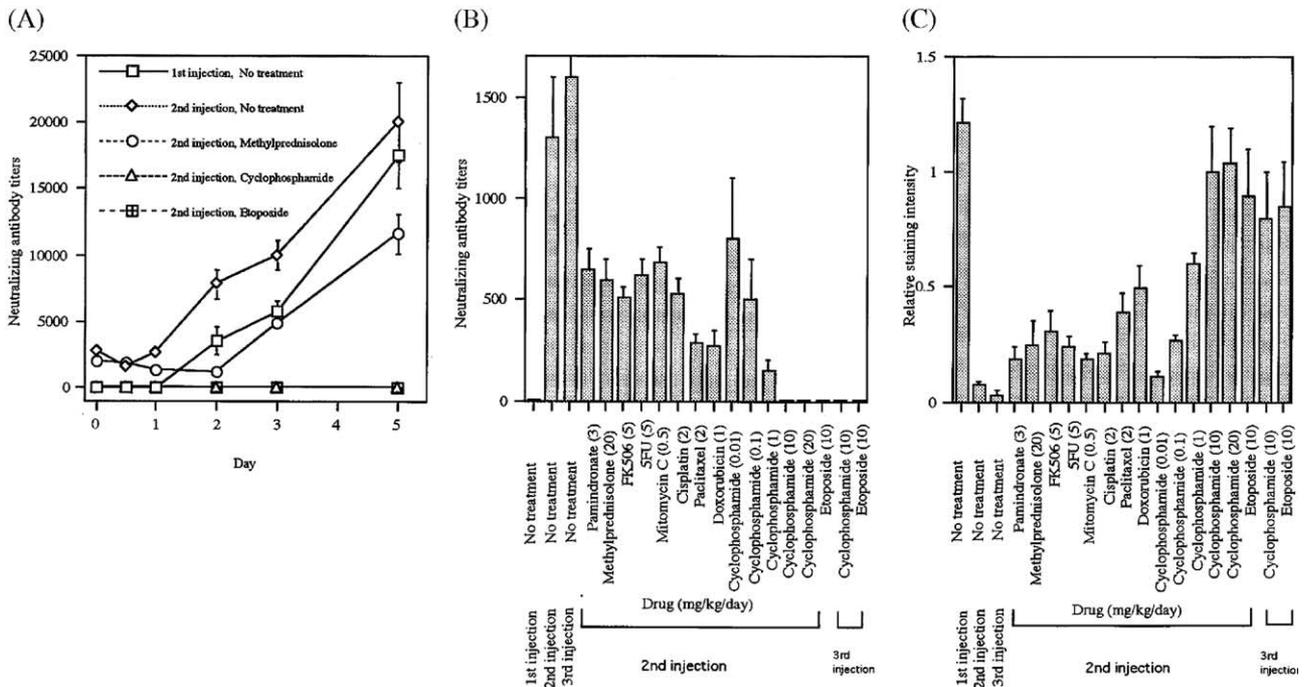


Fig. 5. (A) Time course of serum anti-adenovirus neutralizing antibody titers of C3H mice before and after the first or second injection with Ad5CMV-*lacZ* at 1×10^{10} PFU. Methylprednisolone at 20 mg/day, cyclophosphamide at 10 mg/kg/day, and etoposide at 10 mg/kg/day were injected every day from 3 days before the first injection to 5 days after the second injection. Each mouse was bled retro-orbitally at each time point. (B) Serum anti-adenovirus neutralizing antibody titers of C3H mice before the second or third adenoviral injection. Each mouse was treated with pamidronate at 3 mg/kg/day, methylprednisolone at 20 mg/kg/day, FK506 at 5 mg/kg/day, 5FU at 5 mg/kg/day, mitomycin C at 0.5 mg/kg/day, cisplatin at 2 mg/kg/day, paclitaxel at 2 mg/kg/day, doxorubicin at 1 mg/kg/day, cyclophosphamide at 0.01 mg/kg/day, 0.1 mg/kg/day, 1 mg/kg/day, 10 mg/kg/day, and 20 mg/kg/day, and etoposide at 10 mg/kg/day. Non-treated C3H mice before the first, second, and third injection were compared with treated mice. (C) Morphometric analysis of C3H mouse skin injected with Ad5CMV-*lacZ* at 1×10^{10} PFU. The injected mouse skin was resected 24 h after the injection of Ad5CMV-*lacZ* and stained with X-gal. Each mouse was treated with each drug as described in panel B. The expression of *lacZ* gene in the injected skin was measured by morphometric analysis. Relative staining intensity of non-treated C3H mice after the first, second, and third injection was compared with that of treated mice.

month after the third injection and gradually decreased by 2–3 months after that injection. IgG levels of all monkeys 3 months after the third injection were still significantly higher than the basal levels.

To determine the relationship between the levels of anti-adenovirus IgG and neutralizing antibody titers and to study the modification of humoral response by injection with adenovirus-*p53*, five monkeys were injected with 1×10^{10} PFU Ad5CMV-*lacZ* and 1×10^{10} PFU Ad5CMV-*p53* and the other five monkeys were injected with 2×10^{10} PFU Ad5CMV-*lacZ*. The levels of anti-adenovirus IgG and neutralizing antibody titers in five monkeys injected with 1×10^{10} PFU Ad5CMV-*lacZ* and 1×10^{10} PFU Ad5CMV-*p53* are shown in Figs. 3A and B. IgG levels and neutralizing antibody titers of five monkeys injected with Ad5CMV-*lacZ* and Ad5CMV-*p53* were not significantly different from those of five monkeys injected with Ad5CMV-*lacZ*. Neutralizing antibody titers of all 10 monkeys did not increase on day 3 but significantly increased on day 28 after the first injection. After the second injection, neutralizing antibody titers significantly increased on day 31 and furthermore increased and peaked on day 56. After the third injection, neutralizing antibody titers did not increase on days 59 and 90 and significantly decreased 3 months after the third injection. Neutralizing antibody titers of all 10 monkeys 3 months after the third injection were still significantly higher than the basal levels.

Anti-adenovirus neutralizing antibody titers and relative staining intensity in C3H mice

To determine the relationship between the doses of Ad5CMV-*lacZ* and neutralizing antibody titers, C3H mice were bled 4 weeks after the injection of each different dose of Ad5CMV-*lacZ*. Neutralizing antibody titers significantly increased in the mice which received more than 1×10^8 PFU Ad5CMV-*lacZ* but did not significantly increase in the mice which received less than 1×10^7 PFU Ad5CMV-*lacZ* (Fig. 4A). To determine the relationship between the gene expression and time point of second injection, each mouse received repetitive injection of 1×10^{10} PFU Ad5CMV-*lacZ*. Fig. 4B shows the morphometric analysis at time course of repetitive injection of Ad5CMV-*lacZ*. Gene expression was markedly suppressed when the second injection was done on more than day 14.

Anti-adenovirus neutralizing antibody titers, relative staining intensity, and X-gal staining in C3H mice treated with drugs

To obtain the repetitive gene expression, each mouse received every day anti-cancer agents and immunosuppressants 3 days before the first injection of Ad5CMV-*lacZ* until sacrifice. Time course of anti-adenovirus neutralizing antibody titers in the mice untreated or treated with methylprednisolone, cyclophosphamide, or

etoposide is shown in Fig. 5A. Neutralizing antibody titers started to increase 2 days after the first injection of Ad5CMV-*lacZ*. Before the second injection, the basal levels of neutralizing antibody markedly decreased compared with the levels on 5 days after the first injection. After the second injection of 1×10^{10} PFU Ad5CMV-*lacZ*, neutralizing antibody titers decreased on 12 h and started to increase on 24 h. Neutralizing antibody titers 3 and 5 days after the second injection significantly increased compared with the levels 3 and 5

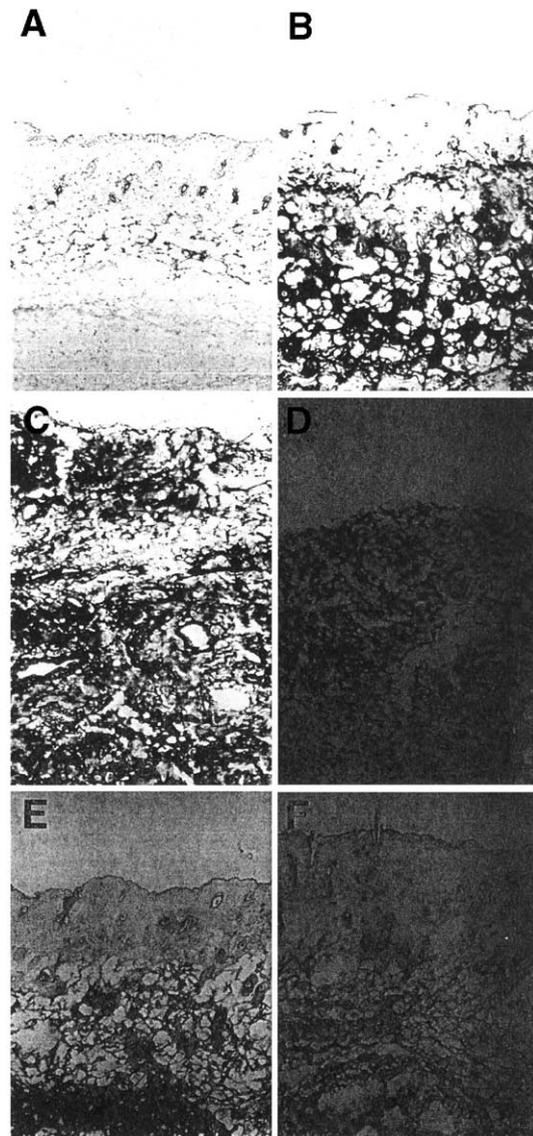


Fig. 6. Transgene expression in the C3H mouse skin transduced by Ad5CMV-*lacZ*. The mouse skin was stained with X-gal (A) after the first injection and (B) after the second injection with Ad5CMV-*lacZ*. The mouse was treated with cyclophosphamide at 10 mg/kg/day and the injected mouse skin was stained with X-gal after the second (C) and third (D) injection with Ad5CMV-*lacZ*. The mouse was also treated with etoposide at 10 mg/kg/day and the injected mouse skin was stained with X-gal after the second (E) and third (F) injection with Ad5CMV-*lacZ*. The injected mouse skin was resected 24 h after the injection of Ad5CMV-*lacZ* at 1×10^{10} PFU.

days after the first injection. Fig. 5A also shows the results of injection with methylprednisolone at 20 mg/kg/day, cyclophosphamide at 10 mg/kg/day, and etoposide at 10 mg/kg/day from 3 days before the first injection to 5 days after the second injection. Methylprednisolone suppressed the increase of neutralizing antibody titers for 2 days after the second injection, but not 3 or 5 days after the second injection. Cyclophosphamide and etoposide completely suppressed the increase of neutralizing antibody titers before and after the second injection.

Fig. 5B shows neutralizing antibody titers after treatment with pamidronate at 3 mg/kg/day, methylprednisolone at 20 mg/kg/day, FK506 at 5 mg/kg/day, mitomycin C at 0.5 mg/kg/day, cisplatin at 2 mg/kg/day, paclitaxel at 2 mg/kg/day, doxorubicin at 1 mg/kg/day, cyclophosphamide at 0.1 mg/kg/day, 1 mg/kg/day, 10 mg/kg/day, and 20 mg/kg/day, and etoposide at 10 mg/kg/day. Treatment for 3 weeks with each agent significantly decreased neutralizing antibody titers compared with non-treated mice on the second injection. Paclitaxel and doxorubicin moderately decreased neutralizing antibody titers compared with immunosuppressants, 5FU, mitomycin C, and cisplatin. Cyclophosphamide at 20 mg/kg/day and 10 mg/kg/day and etoposide at 10 mg/kg/day completely suppressed the increase of neutralizing antibody titers on the second injection. Cyclophosphamide at 10 mg/kg/day and etoposide at 10 mg/kg/day also completely suppressed the increase of neutralizing antibody titers on the third injection. Fig. 5C shows the results of morphometric analysis in the mice treated with pharmaceutical agents. Each drug significantly restored gene expression on the second injection 3 weeks after the first injection. Paclitaxel and doxorubicin moderately restored gene expression compared with immunosuppressants, 5FU, mitomycin C, and cisplatin. Especially, cyclophosphamide at 20 mg/kg/day and 10 mg/kg/day, or etoposide at 10 mg/kg/day, markedly recovered the relative staining intensity on the second injection as same as that on the first injection. Cyclophosphamide at 10 mg/kg/day or etoposide at 10 mg/kg/day also markedly recovered the relative staining intensity on the third injection. Fig. 6 shows the results of X-gal staining in the mice treated with cyclophosphamide at 10 mg/kg/day or etoposide at 10 mg/kg/day. The second injection with Ad5CMV-*lacZ* showed no β -gal-positive cells in the injected mice who were not treated with drugs. Chronic treatment with cyclophosphamide at 10 mg/kg/day or etoposide at 10 mg/kg/day showed β -gal-positive cells in the mice who received the second or third injection with Ad5CMV-*lacZ*.

Discussion

Animal studies are crucial for the development of therapeutic agents against human malignancies. Rhesus monkeys are a commonly adopted non-human primate animal model because of their close relatedness to humans.

In particular, rhesus monkeys provide an excellent model for the human female genital tract. The surface area was large enough to perform injection, abrasion and topical adenoviral application, cone biopsy, and punch biopsy without complications. Our study provides data on the safety of adenoviral vectors for gene transfer to cervix and an assessment of the effect of repeat administration. We found no evidence of viral replication, rather infectious viral particles were rapidly cleared from monkey sera. We observed significant increases in the levels of anti-adenovirus IgG, but not IgA, after exposure to Ad5CMV-*lacZ* or Ad5CMV-*p53*. This finding embodies the attributes of cervix where only systemic but not local antibody responses are induced. Zabner et al. [3] also reported that adenoviral instillation into the rhesus monkey airway did not induce the elevation of local IgA. Another major consideration for safety of an adenoviral vector in the treatment of cervical cancer is the possibility of an immune or toxic response. Our data showed no evidence of a marked systemic or local inflammatory response at the doses tested. The histology of epithelia in the cervix treated with adenovirus was indistinguishable from that of control epithelia. On the other hand, lung of rhesus monkey develops inflammation after intratracheal instillation of adenovirus at high dose of 10^{10} PFU [16]. These data suggest that cervix is more resistant to adenovirus-induced inflammation than lung tissue, and at least three sequential exposures of cervix to Ad5CMV-*lacZ* or Ad5CMV-*p53* do not cause a detrimental inflammatory response under the conditions used in this study.

Despite its ability to transduce the rhesus monkey cervix, one important consideration for utilizing adenoviral vectors is its immunogenicity. While abrasion and topical application caused a specific immune response, no positive β -gal staining could be detected in those cervix. Second and third injections of Ad5CMV-*lacZ* did not express any β -gal-positive cells in the cervix. In contrast to these animal studies, clinical trials demonstrated that anti-adenovirus antibodies do not block gene transfer into human organs [17–19]. An induced immune response to the vector might not impede repetitive gene expression in human cervical cancer. It is reported that anti- β -gal antibody elevates in the adenovirus-*lacZ*-injected mice and a non-constitutive protein β -gal is obviously recognized as foreign by the mice [8]. In addition to its immunogenicity, the elevated antibody response may also be attributed to its expression. Because the *lacZ* gene was driven by a cytomegalovirus promoter, high-level gene expression is obtained. Consequently, β -gal would be released further sensitizing the rhesus monkey. Thus, over time, the steady release of β -gal may represent a form of immunization. Our method to measure anti-adenovirus IgG is using the Ad5CMV-*lacZ*-infected 293 cell as antigens. Therefore, our anti-adenoviral IgG levels might include the levels of anti- β -gal antibodies. On the other hand, titers of anti- β -gal antibody may not contribute to titers of neutralizing anti-adenovirus antibody, since our

neutralizing antibody assay measured the antibody reactive with capsid proteins of adenoviruses, that is, penton, hexon, and fiber proteins. Ginkel et al. [8] reported that adenovirus-*lacZ* vector induced different serum IgG antibody responses between to the adenoviral vector and to β -gal. Our data showed the difference between anti-adenovirus IgG antibody levels and neutralizing antibody levels, especially 1 month after the third injection. This may be due to the difference of the IgG response between to the adenoviral vector and to β -gal.

Treatment with cyclophosphamide at 10 or 20 mg/kg/day completely suppressed the increase of neutralizing antibody titers and markedly increased the second gene expression. Cyclophosphamide is a nitrogen mustard-derived alkylating agent that interferes with DNA replication and RNA transcription. Cyclophosphamide is indicated for use against many types of cancer including lymphoma, leukemia, lung cancer, breast cancer, ovarian cancer, and cervical cancer. It is reported that low-dose cyclophosphamide inhibits B cell proliferation and high-dose cyclophosphamide completely suppressed B and T cell proliferation [20]. B cells are more than 200 times more susceptible than T cells to the cytotoxicity of cyclophosphamide [21]. Functional capacity and IgG synthesis of B cells are inhibited by cyclophosphamide at low dose [22,23]. Chronic low-dose cyclophosphamide at 2 mg/kg/day suppressed activation, proliferation, and differentiation of B cells in cyclophosphamide-treated patients [24]. Furthermore, cyclophosphamide inhibits immunoglobulin synthesis and suppressor T cells of immunoglobulin synthesis appear to be especially sensitive to cyclophosphamide [25]. It is also reported that the spontaneous secretion of immunoglobulin by peripheral blood B cells that is elevated in the patients with non-neoplastic immune-mediated diseases was suppressed back to normal levels during chronic low-dose (2 mg/kg/day) cyclophosphamide therapy [26]. In our study, plasma total IgG levels increased after Ad5CMV-*lacZ*-injected C3H mice but treatment with chronic low-dose cyclophosphamide (10 or 20 mg/kg/day) suppressed back elevated plasma total IgG levels to normal levels (data not shown); furthermore, anti-adenovirus neutralizing antibody production was completely suppressed.

Etoposide also completely suppressed anti-adenovirus neutralizing antibody production and markedly restored the gene expression on the second injection. Etoposide is a semisynthetic derivative of podophyllotoxin that causes an arrest at G₂ of the cell cycle. Etoposide is indicated in the treatment of small cell lung cancer, non-Hodgkin's lymphomas, leukemia, testicular tumor, non-small cell lung cancer, ovarian cancer, and cervical cancer. Because etoposide lacks significant extramedullary toxicity, it has also been useful in high-dose chemotherapy with autologous bone marrow transplantation. The role of etoposide in modulating immune response is not well documented. Although the underlying mechanism remains to be characterized, our findings suggest that etoposide can effectively suppress humoral immune

response at clinically acceptable low dose. Our findings may also have implications for patients who are being treated in anti-cancer gene therapy protocols.

The other drugs, pamidronate, immunosuppressants (methylprednisolone, FK506), and anti-cancer agents (cisplatin, paclitaxel, mitomycin C, 5FU, doxorubicin), showed some suppressive effects for neutralizing antibody formation, except restoration of gene expression, but these effects were not marked compared with that of cyclophosphamide or etoposide. However, paclitaxel and doxorubicin showed moderate suppressive effects of neutralizing antibody formation and restoration of repetitive gene expression. Combination treatments with these drugs in a single high dose also showed marked decrease of neutralizing antibody titers but did not show the marked restoration of gene expression compared with chronic low-dose cyclophosphamide or etoposide treatment (data not shown).

Chronic low-dose treatment with cyclophosphamide or etoposide restored the gene expression on the third injection in addition to the second injection. Jooss et al. [26,27] reported that one or two high-dose treatment with cyclophosphamide restored the gene expression on the second injection but not on the third injection. In our study, chronic low-dose treatment with cyclophosphamide restored the marked gene expression on the second injection compared with pulse treatment with high doses of cyclophosphamide; furthermore, pulse treatment with high doses of cyclophosphamide did not restore gene expression on the third injection (data not shown). Our findings suggest that chronic low-dose cyclophosphamide or etoposide treatment may effectively suppress the IgG production from memory B cells stimulated by the mitogens. The treatment with cyclophosphamide or etoposide has a lot of limitations for clinical application to get repetitive gene expression by adenovirus-mediated gene transfer because of its side effect of myelosuppression. Further treatment methods with low side effects and clinical accessibility should be developed to restore the adenovirus-mediated repetitive gene expressions.

The results of this study have several important implications for the use of adenoviral vectors for gene therapy of cervical cancer. First, the non-human primate as a model suggests that even at high doses, an adenoviral vector is well tolerated clinically and does not cause acute clinical signs commonly associated with adenoviral infection. Even in multiple adenoviral injections, monkeys did not show any clinical signs. Second, gene transfer can be detected in the injected cervix in a dose-dependent manner. Third, chronic low-dose administration of cyclophosphamide or etoposide restored gene expression in the mouse skin after the repetitive adenovirus-mediated gene transfer because the increase of neutralizing antibody titers induced by adenoviral administration was completely blocked by this treatment. Since cyclophosphamide and etoposide are potent anti-cancer agents for cervical cancer and other cancers, a combination of adenovirally mediated cancer gene therapy with low-dose cyclophosphamide or etoposide could be

incorporated into treatment regimens. By using such strategies, the immune responses to the adenoviral vector could be temporarily modulated resulting more effective anti-cancer gene therapy.

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