

# Recombinant adenovirus vectors for cytokine gene therapy in mice

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**Background:** Adenoviruses have several specific features useful for gene therapy. They infect various lineages of cells irrespective of cell cycle status. However, the exact mechanism of their infection and in vivo kinetics as a gene expression vector have not been elucidated.

**Objective:** Using adenovirus vectors expressing marker genes, we examined the infectivity of these vectors (including cellular and tissue tropism), the duration and intensity of transgene expression, and the side effects.

**Methods:** Various cells were infected with adenovirus expressing LacZ gene at various doses, and  $\beta$ -galactosidase activity was measured and compared in relation with dose, time course, and cellular vitronectin receptor. Mice were injected with adenoviruses expressing LacZ, luciferase and GM-CSF, and in vivo gene expression was examined.

**Results:** Adenovirus infection induced viral dose-dependent transgene expression that persisted for 2 weeks. Adherent cells were infected much more efficiently than nonadherent cells, probably because the former expressed much higher levels of the vitronectin receptor, one of the main receptors for adenovirus. Studies performed in mice with luciferase-expressing adenovirus revealed that the liver was the main target organ after intravenous injection and showed that the intravenous route was superior to other routes with regard to transgene expression. After intravenous injection of adenovirus expressing human GM-CSF, there was a transient and dose-dependent increase in the serum level of this cytokine. Administration of adenovirus expressing mouse GM-CSF enhanced hematopoiesis in the spleen and bone marrow.

**Conclusion:** These results indicated that adenoviruses can be used for in vivo cytokine gene therapy but suggested the necessity of taking into consideration the route of administration, the duration of transgene expression, the toxic dose, and host immune reactions. (*J Allergy Clin Immunol* 1999;103:S471-84.)

**Key words:** Adenoviral vector, cytokine, gene therapy, vitronectin receptor, luciferase, hematopoiesis.

Several trials of cytokine gene therapy have been performed with viral vectors. Among the viral vectors, retroviruses and adenoviruses have been used most frequently.<sup>1</sup> Adenoviruses have certain features that are useful for gene therapy:<sup>2</sup> (1) a broad host range and the ability to infect cells in any stage of cell cycle, (2) no tumorigenicity in humans (at least type 2 and type 5 adenoviruses), (3) high viral titers with a permissive 293 cell line, (4) the viral genome rarely integrates into host chromosomes and remains extra-chromosomal, resulting in transient gene expression in replicating cells. These features of adenoviruses seem to make them useful for relatively short-term gene therapy. However, adenoviruses have some critical disadvantages because infection is directly cytotoxic and induces several types of cellular and humoral immune responses that limit the effectiveness of gene therapy.

Adenoviruses have several natural target organs, such as the lungs (acute febrile respiratory syndrome), conjunctiva (epidemic keratoconjunctivitis), gastrointestinal tract (gastroenteritis), and urinary bladder (acute hemorrhagic cystitis).<sup>2</sup> On the basis of the natural tropism of adenoviruses, the first clinical trial of gene therapy with an adenoviral vector involved the respiratory disease.<sup>3,4</sup> Because this vector can also deliver transgenes efficiently to the liver,<sup>5</sup> liver-targeted gene transfer has been tried for several disorders, including  $\alpha$ 1-antitrypsin deficiency, coagulation factor deficiency,<sup>6,7</sup> and hyperlipidemia.<sup>8,9,10</sup> Furthermore, this vector has also been applied to other cells and tissues, including neuronal cells,<sup>11-16</sup> muscle cells,<sup>17-20</sup> endothelial cells,<sup>21,22</sup> synovial cells,<sup>23</sup> and cancer cells.<sup>24-30</sup> In contrast, there have been only few reports on the use of adenoviral vectors in the lymphoid and hematopoietic systems.<sup>31-35</sup> Accordingly, the application of this vector to lymphoid and hematopoietic cells was examined in the present study.

We investigated factors influencing the infectivity of adenoviruses in vitro and in vivo, performed adenovirus-mediated cytokine gene transfer, and assessed the possibility of immunomodulation by such gene therapy.

## METHODS

### Recombinant adenoviral vectors

Adenoviral vectors encoding the LacZ gene under the control of SR $\alpha$ <sup>36</sup> (Adex1SRLacZL) and CAG promoters<sup>37,38</sup> (Adex1-CAL-acZL), an adenoviral vector containing no expression unit (Adex1w), expression cassette cosmid (pAdex1w, pAdex1pCAw

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**Abbreviations used**

|          |                                                    |
|----------|----------------------------------------------------|
| FDG:     | Fluorescent di- $\beta$ -galactopyranoside         |
| hGM-CSF: | Human GM-CSF                                       |
| mGM-CSF: | Mouse GM-CSF                                       |
| MOI:     | Multiplicity of infection                          |
| VNR:     | Vitronectin receptor                               |
| X-gal:   | 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside |

and pAdex1CAwt, pAdex1pCA-hGM-CSF), and helper virus Ad5-dlX were provided by Dr I. Saito (Institute of Medical Science, the University of Tokyo).<sup>39-41</sup>

pAdex1CAw and pAdex1CAwt are 42- and 45-kb cosmids containing the adenovirus type 5 genome lacking the E1a region, part of E1b and part of E3, with the expression cassette in the E1 region.<sup>40,41</sup> The expression cassette (CAG promoter) contained the cytomegalovirus early/intermediate enhancer, chicken  $\beta$ -actin promoter, and rat  $\beta$ -globin poly-adenylation signal<sup>37,38</sup> but no transgene. To construct pAxCA-Luc, the *SalI*-*DraI* 1819-base pair fragment from pUHC13-3 (Stratagene, La Jolla, Calif), which contains a whole firefly luciferase cDNA, was inserted into *SwaI*-digested pAdexCAwt. To construct pAdex1SR $\alpha$ -mGM-CSF, the *SalI* 2196-base pair fragment from pCD-SR $\alpha$ -mouse GM-CSF (mGM-CSF), which contained the SR $\alpha$ -promoter and the complete mGM-CSF cDNA, was inserted into *SwaI*-digested pAdex1w.

Recombinant adenovirus vectors were generated in 293 cells (American Type Culture Collection; CRL1573) through homologous recombination between the cosmid DNA and *EcoT22I*-digested DNA-terminal protein complex of Ad5-dlX;<sup>41</sup> the resulting viral plaques were harvested, amplified, banded in CsCl, dialyzed,<sup>42</sup> and stored in aliquots at  $-80^{\circ}\text{C}$ . All viral stocks were shown to be free of replication-competent wild-type adenoviruses by restriction enzyme digestion of DNAs from virus-infected 293 cells and infectious virus amplification test with HeLa cells.<sup>43</sup>

### Cell cultures and virus infection

Jurkat, EOL3, HL60, U937, KG-1, and Raji cells were maintained in RPMI-1640 medium with 10% FCS. TF-1 cells were also cultured in RPMI-1640 medium with 10% FCS and 1 ng/mL human GM-CSF (hGM-CSF). BA/F3 cells were cultured in RPMI-1640 medium with 10% FCS, 50  $\mu\text{mol/L}$  2-mercaptoethanol, and 1 ng/mL murine IL-3. COS7, 293, HeLa, and NIH3T3 cells were cultured in Dulbecco's minimal essential medium with 10% FCS.

For all inoculations, the virus stocks were diluted to the appropriate concentrations, added to cell monolayers or cell pellets in a 1.5-mL Eppendorf tube, and kept in contact with the cells at  $37^{\circ}\text{C}$  for 1 hour with occasional agitation. The cells were then washed and cultured at a density of  $1 \times 10^6/\text{mL}$  at  $37^{\circ}\text{C}$ .

### In vitro detection of LacZ gene expression ( $\beta$ -galactosidase activity)

LacZ gene expression was detected by 2 methods: (1) cytochemical staining with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal),<sup>44,45</sup> and (2) flow cytometry with fluorescent di- $\beta$ -galactopyranoside (FDG).<sup>46</sup> In flow cytometry, cells positively stained with propidium iodide were eliminated from further analyses.

### Assessment of vitronectin receptor expression

The level of vitronectin receptor (VNR) expression in several cell lines was examined by flow cytometry with an anti-VNR polyclonal antibody (MAB1904; Chemicon, Temecula, Calif), which reacted with both the  $\alpha_V$  and  $\beta$  subunits of the receptor. Expression

of the  $\alpha_V$  subunit was also detected with an anti- $\alpha_V$  subunit mAb (MAB1980, Chemicon). The level of  $\alpha_V$  subunit expression almost paralleled the VNR expression detected with the polyclonal antibody (data not shown).

### Preparation of bone marrow cells

Heparinized bone marrow samples were obtained from healthy volunteers after they gave informed consent. Mononuclear cells were prepared by Ficoll-Hypaque density gradient centrifugation and were then washed with PBS. These cells were infected with adenovirus in the same way as mentioned, cultured in RPMI-1640 medium with 10% FCS and 1 ng/mL hGM-CSF and 20 U/mL of human interleukin-2 (hIL-2) for 3 days, and analyzed for  $\beta$ -galactosidase activity.

### Culture and stimulation of human T-cell clones

The human T-cell clone TA23, which is specific for tetanus toxin, was established from the peripheral blood of a patient with severe combined immunodeficiency who had been successfully reconstituted with fetal thymus and fetal liver transplantation.<sup>47</sup> It is a CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup>, TCRV $\beta$ 8<sup>+</sup> T-cell clone and produces a TH1-type cytokine response when stimulated with anti-CD3 mAb. The cells were cultured and given bimonthly stimulation with a feeder cell mixture consisting of irradiated (3000 rad) allogeneic peripheral blood mononuclear cells and an irradiated (5000 rad) JY cells, an Epstein-Barr virus-transformed B-cell line, in the presence of 0.1  $\mu\text{g/mL}$  PHA.<sup>48</sup> The cells were stimulated with hIL-2 (20 U/mL), PMA (1 ng/mL), and A23187 (500 ng/mL), or plate-coated anti-CD3 antibody for 1 day. Then cells were infected with AdexCALacZL at a multiplicity of infection (MOI) of 100 or 1000, cultured for 3 days with the same stimulation as mentioned earlier, and analyzed for  $\beta$ -galactosidase activity.

### Animals

Six-week-old C57BL/6 and C3H/HeJ male mice (Nihon SLC, Shizuoka, Japan) were used in these experiments. All studies were performed in accordance with the guidelines of the Institute of Medical Science at the University of Tokyo.

### In vivo detection of adenovirus-mediated LacZ gene expression ( $\beta$ -galactosidase activity)

AdexCALacZL was injected into the tail veins of 6-week-old male C57BL/6 mice, which were killed 3, 7, 14, and 21 days later. After perfusion with 4% paraformaldehyde in PBS, organs were removed, fixed, and stained with X-gal.<sup>44</sup>

### Tissue luciferase assay in mice infected with AxCA-Luc

Different amounts of adenoviruses containing firefly luciferase gene AxCA-Luc were diluted in 500  $\mu\text{L}$  of PBS, and injected intravenously (into the tail vein), intraperitoneally, and intramuscularly (into the calf muscle), or subcutaneously. Luciferase activity was examined with a luciferase assay system (Promega, Madison, Wis) in the following organs: liver (the average of right, middle, and left lobes), spleen, kidney, adrenal, lung, heart, thymus, small intestine, testis, lumbar and calf muscles, femoral bone marrow, brain, mediastinum (aorta and esophagus), peritoneum, and tail. The protein concentrations of the tissue homogenates were measured with a Protein Assay kit (Bio-Rad, Richmond, Calif). Then tissue luciferase activity was expressed as relative light units (rlu) per micrograms of tissue protein.

The purified viral solution of AxCA-Luc contained a slight amount of luciferase activity (0.1 rlu/pfu [relative light unit/plaque forming

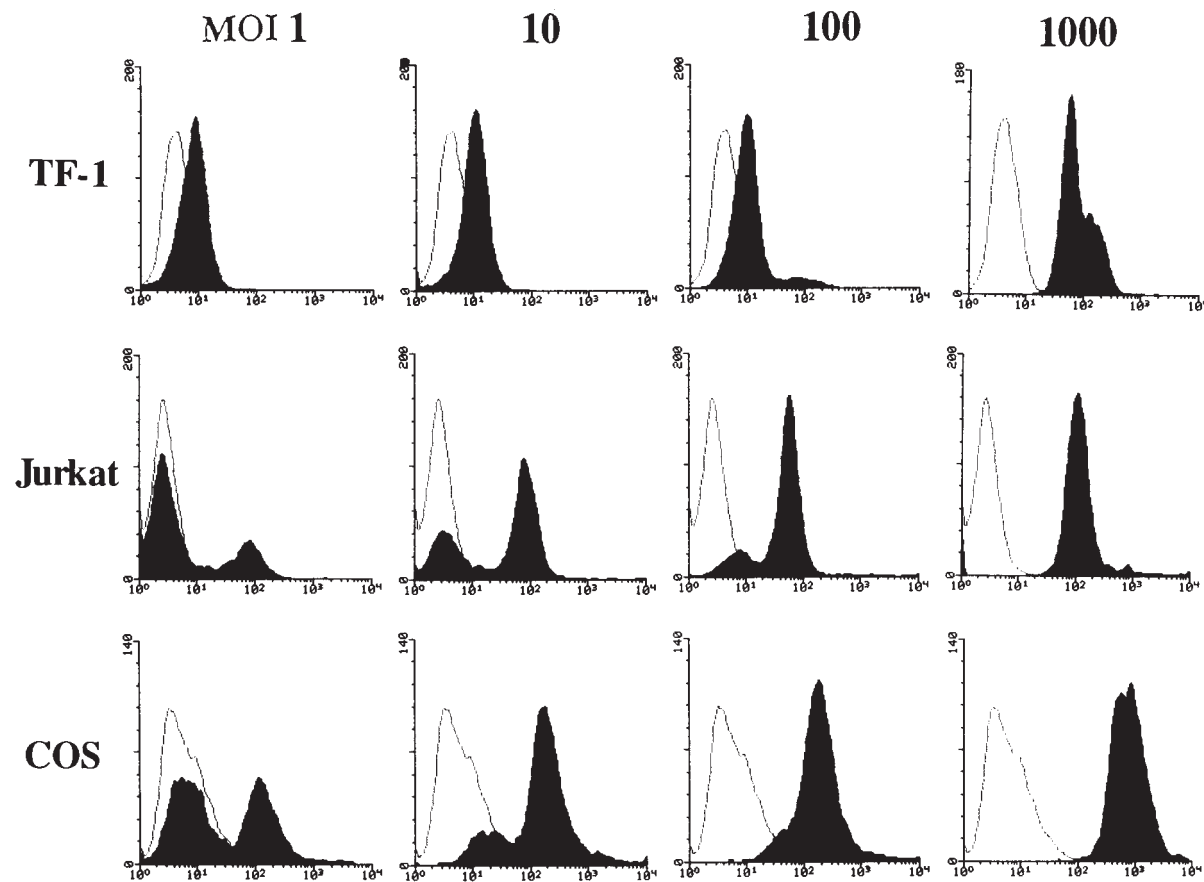


**FIG 1.** MOI and LacZ gene expression ( $\beta$ -galactosidase activity) TF-1, Jurkat, and COS-7 cells were infected with Adex1CALacZL at an MOI of 1, 10, 100, and 1000 and then cultured for 3 days. Cellular  $\beta$ -galactosidase activity was analyzed by FCM after being stained with FDG (black histograms). Uninfected cells (control cells) were stained in the same way, and their histograms were overlaid (white histograms).

unit]). However, intravenous injection with an equivalent amount ( $10^8$  rlu/0.11 mg) of luciferase protein (Wako, Japan) resulted in only marginal tissue luciferase activity.

#### Measurement of serum hGM-CSF in mice injected with AxCA-hGM-CSF

C3H/HeJ mice were injected with different amounts of AxCA-hGM-CSF through various routes. At the specified times, blood was drawn from the tail vein, stored at 4°C overnight, and centrifuged to collect serum. The sandwich ELISA for hGM-CSF was performed with BVD2-23B6 as the coating antibody and BVD2-21C11 as the detecting antibody.<sup>49</sup>

#### Administration of Adex1SR $\alpha$ -mGM-CSF and histologic examination

C3H/HeJ mice were injected intravenously into the tail vein with  $10^9$  pfu of Adex1SR $\alpha$ -mGM-CSF. Seven days later, mice were killed and perfused with 4% paraformaldehyde in PBS. Then, organs were removed, fixed, and stained with hematoxylin and eosin.

#### Statistics

All statistical analyses were performed with StatView J-4.5 (Abacus Concepts Inc, Berkeley, Calif). All the experiments were performed multiple times as indicated. The levels of tissue luciferase were analyzed with the Student *t* test.

## RESULTS

### Adenovirus-mediated LacZ gene expression at different MOI

TF-1, Jurkat, and COS7 cells were infected with Adex1CALacZL, and  $\beta$ -galactosidase activity was examined 3 days later by flow cytometry with FDG staining (Fig 1). The  $\beta$ -galactosidase activity was MOI-dependent. In TF-1 and Jurkat cells, expression showed 2 peaks, probably as the result of heterogeneity of the susceptibility to adenovirus infection. In COS7 cells,  $\beta$ -galactosidase activity also showed two peaks, but the overall level of expression was higher than in TF-1 and Jurkat cells. Colorimetric assay with o-nitrophenyl- $\beta$ -D-galactopyranoside also confirmed these findings (data not shown). In TF-1 and Jurkat cells, the level of expression was dose-dependent until an MOI of 1000, but expression in COS7 cells reached a plateau at an MOI of approximately 100.

### Time course of adenovirus-mediated LacZ gene expression

We examined the time course of  $\beta$ -galactosidase activity in TF-1 cells over 14 days after infection with Adex1CALacZL at an MOI of 10, 100, and 500 (Fig 2).  $\beta$ -

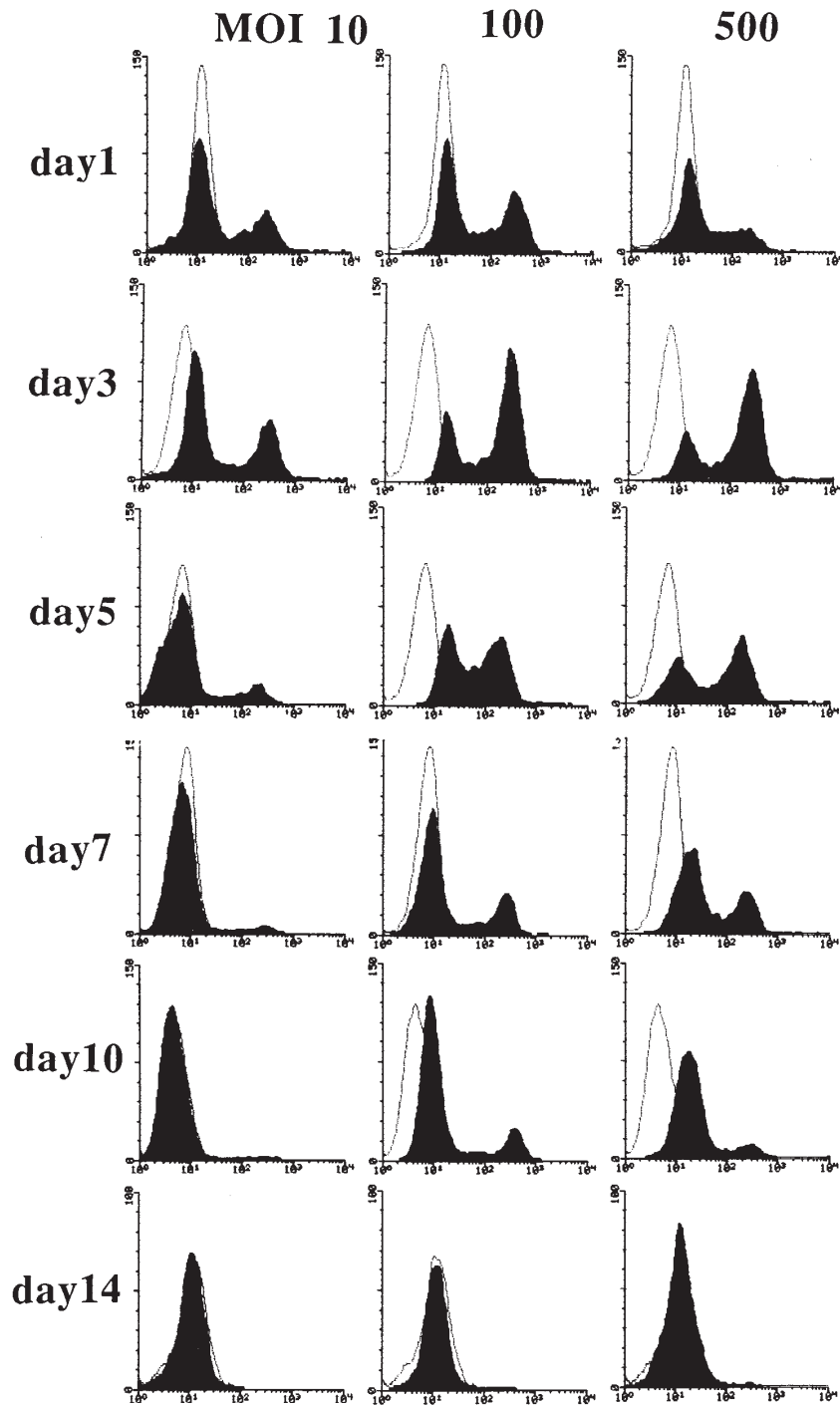


**FIG 2.** Time course of LacZ gene expression ( $\beta$ -galactosidase activity) in TF-1 cells. TF-1 cells were infected with AdexCALacZL at an MOI of 10, 100, and 500 and then cultured for 2 weeks. Cells were harvested on the indicated days, and  $\beta$ -galactosidase activity was analyzed by FCM after being stained with FDG (black histograms). Uninfected cells (control cells) were stained in the same way, and their histograms were overlaid (white histograms).

galactosidase activity appeared from day 1, reached a maximum on days 3 to 5, and disappeared before day 14. The proportion of higher peaks increased as the dose of adenoviruses increased (Fig 2). Assays with AxCA-Luc in Jurkat cells yielded a similar outcome (data not shown).

#### Vitronectin receptor expression and LacZ gene expression

Entry of adenovirus into the cytoplasm is thought to be mediated by an interaction between viral capsid penton bases and cellular VNRs ( $\alpha_V$  and  $\beta_3$  or  $\beta_5$ ).<sup>50</sup> We exam-

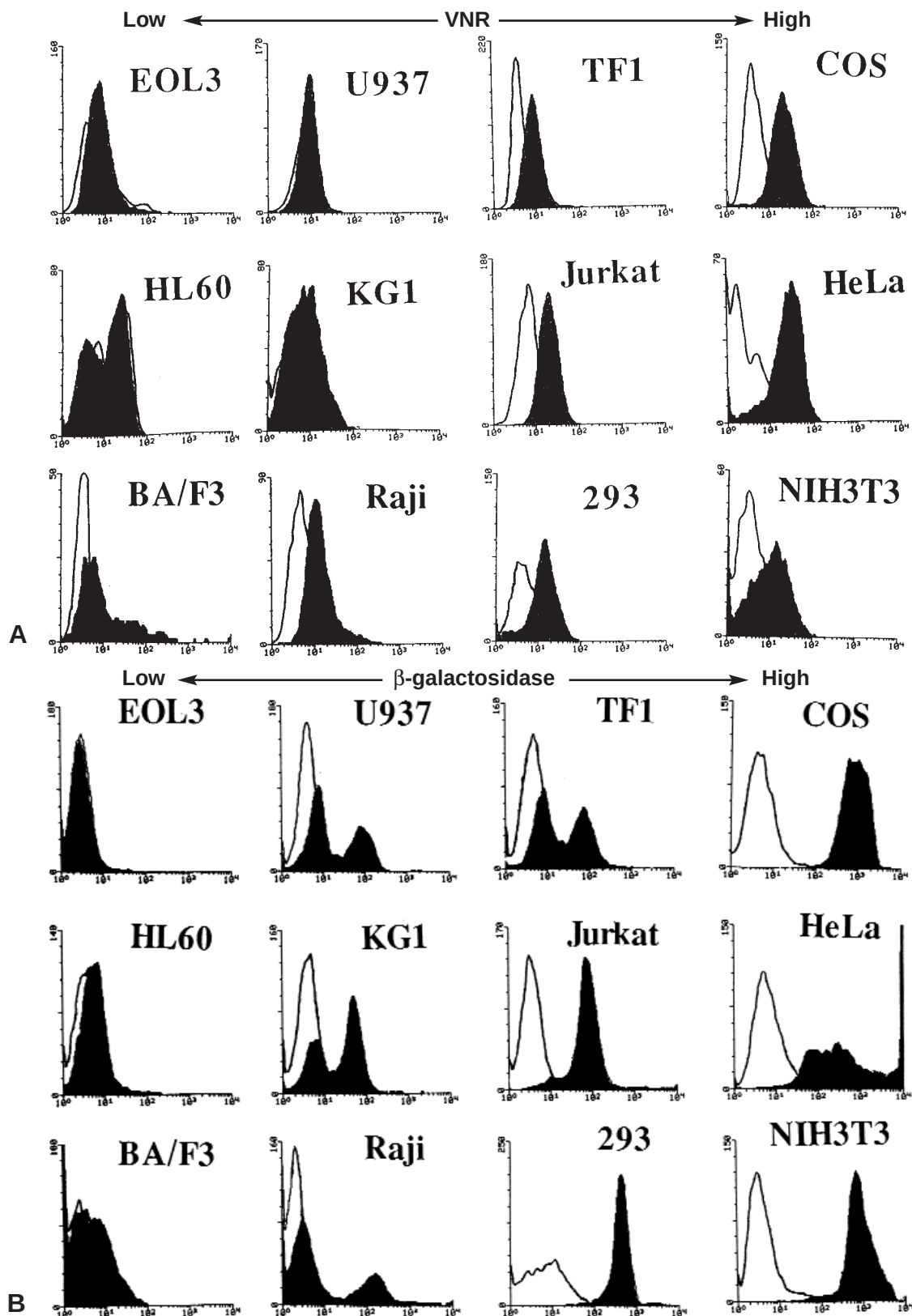


**FIG 3.** Cellular VNR expression and adenovirus-mediated LacZ gene expression. **A**, Cell lines were stained with an anti-VNR polyclonal antibody and an FITC-labeled anti-rabbit antibody and then examined with FCM. **B**, The same cell lines that were used in **A** were infected with AdexCALacZL at an MOI of 100 (*black histograms*), incubated for 3 days, and examined by FCM after FDG staining. Uninfected cells (control cells) were stained in the same way, and their histograms were overlaid (*white histograms*).

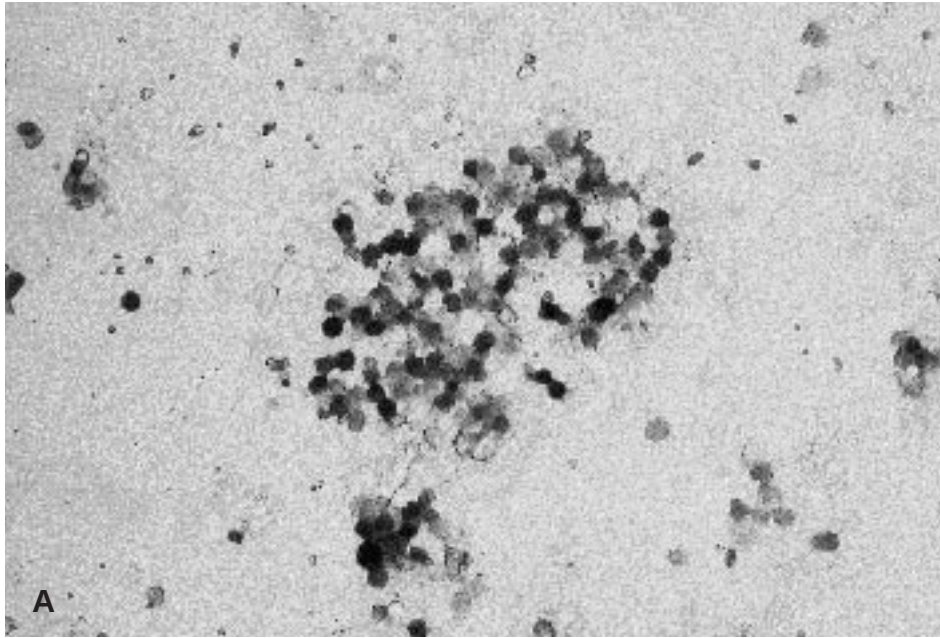


**FIG 4.** LacZ gene expression ( $\beta$ -galactosidase activity) in mononuclear cells. **A**, Bone marrow mononuclear cells were infected with AdexCALacZL at MOI 100, cultured with hGM-CSF and human IL-2 for 3 days, and stained with X-gal.

ined the VNR expression and adenovirus-mediated LacZ gene expression in several cell lines. Generally, adherent cells (such as COS7, 293, HeLa, and NIH3T3) showed high levels of VNR expression, although nonadherent cells (such as K562, KG1, U937 and BA/F3) showed much lower or undetectable levels of VNR expression (Fig 3, A).

LacZ gene expression in these cell lines was examined by the method shown in Fig 1<sup>46</sup> at 3 days after infection with Adex1CALacZL at an MOI of 100 (Fig 3, B).  $\beta$ -galactosidase activity was correlated well with the level of cell surface VNR. Nonadherent cell lines with little VNR expression had low levels of  $\beta$ -galactosidase activity, although adherent cell lines showed much higher  $\beta$ -galactosidase activity. Some of the nonadherent cell lines with moderate VNR expression (such as Jurkat, TF-1, and Raji cells) also showed moderate levels of  $\beta$ -galactosidase activity.

#### **LacZ Gene expression by primary bone marrow cells and lymphocytes**

To examine the infectivity for primary hematopoietic cells, human bone marrow mononuclear cells were infected with Adex1CALacZL at an MOI of 10 and 100. After culture for 3 days with GM-CSF and IL-2,  $\beta$ -galactosidase activity was examined by X-gal staining.<sup>17</sup> The cells showed a dose-dependent increase in X-gal-positivity and staining density (Fig 4, A; at an MOI 100).

To examine infectivity for lymphocytes, a human lymphocyte cell line (TA23) was infected with Adex1CALacZL at an MOI of 100 and 1000, and  $\beta$ -galactosi-

dase activity was examined by the method shown in Fig 1<sup>46</sup> at 3 days after infection. The cells were prestimulated with IL-2, PMA and A23187, or anti-CD3 for 1 day before infection. However, the population of positively stained cells was small (less than 3%) even with such stimulation and similar to that under unstimulated conditions (Fig 4, B). Only after stimulation with anti-CD3 antibody were 7% to 8 % of the cells positively stained at an MOI of 1000.

#### **In vivo examination of LacZ gene expression**

To allow us to examine the efficiency of in vivo gene transfer of adenoviruses, mice were injected intravenously with Adex1CALacZL at  $1 \times 10^9$  pfu. After 7 days, organs were stained with X-gal (Fig 5). The liver was stained most densely and showed multiple positive foci (Fig 5, A and B), which contained densely stained hepatocytes, Kupffer cells, or vascular endothelial cells. The spleen also showed positive staining especially in the parafollicular areas, although there was also relatively high background staining (Fig 5, C and D). The lungs showed several faintly stained spots that were presumably of vascular endothelial cells or macrophages. The intensity of staining was much weaker than observed in the liver or spleen (data not shown). The kidneys showed dense staining of the glomeruli; the proximal convoluted tubules showed only background staining (data not shown). Other organs, including the thymus, heart, muscle, and brain, showed no detectable staining. The intensity of X-gal staining was maximal on day 7 and decreased thereafter (data not shown).

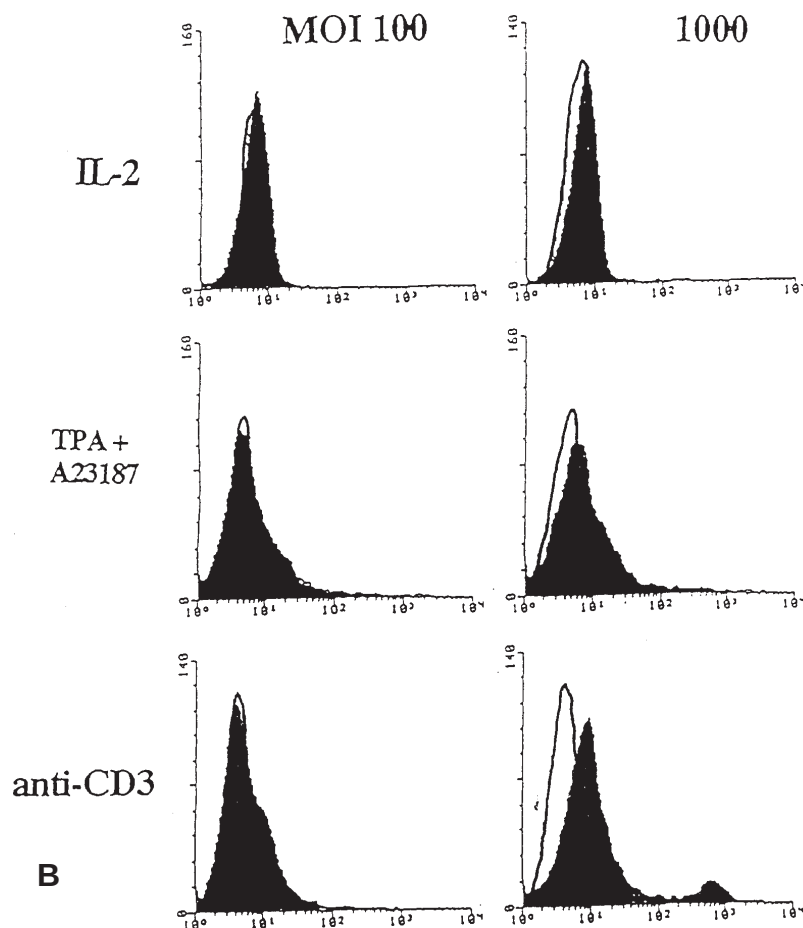


**FIG 4. B.** A human lymphocyte cell line (TA23) was stimulated with human IL-2, PMA + A23187, and plate-coated anti-CD3 antibody for 1 day, infected with AdexCALacZL at a MOI of 100 and 1000 (*black histograms*), incubated for 3 days, and examined by FCM after FDG staining. Histograms of unstained cells (control cells) were overlaid (*white histograms*).

#### Tissue distribution and viral dose dependency of adenovirus-mediated luciferase gene expression

To allow us to examine transgene expression quantitatively, AxCA-Luc was administered at a dose of  $10^9$ ,  $10^8$ , and  $10^7$  pfu, and the luciferase activity was examined in several organs at 3, 7, and 14 days later (Fig 6). The highest level of luciferase activity was observed in the liver, although much lower activity was seen in the spleen, tail (injection site), adrenals, and lungs (the significance of differences in luciferase activity between liver versus spleen and spleen versus heart on day 3 at  $10^9$  pfu was  $P = .07$  and  $.02$ , respectively, by the Student  $t$  test). There was almost no expression in the other organs studied. Expression was dependent on the viral doses at least between  $10^7$  and  $10^9$  pfu per mouse ( $10^9$  versus  $10^8$  and  $10^8$  versus  $10^7$  pfu on day 3;  $P = .07$  and  $.06$ , respectively; Fig 6, A). The expression pattern on day 7 was similar to that on day 3 (Fig 6, B). By 14 days after infection, the liver showed almost undetectable levels of luciferase activity (Fig 6, D).

#### Efficiency of adenovirus-mediated luciferase gene expression after administration by different routes

The efficiency of luciferase gene transfer was compared among different routes of administration by assessing luciferase activity in several organs after 7 days. Only intravenous injection resulted in significant levels of luciferase activity in the liver, tail (injection site), spleen, mediastinum, adrenals, and lungs, although no significant activity was detected in mice infected by the other 3 routes (intraperitoneal, intramuscular, and subcutaneous; Fig 6, C). By day 14, there was almost complete disappearance of expression in the liver, but there was persistence of low levels of expression in the spleen (intravenous), adrenals (intravenous and intraperitoneal), the tail injection site (intravenous), and the back muscles just beneath the injection site (subcutaneous; Fig 6, D). This comparison revealed that the intravenous route was superior to the other routes for adenovirus-mediated transgene expression.

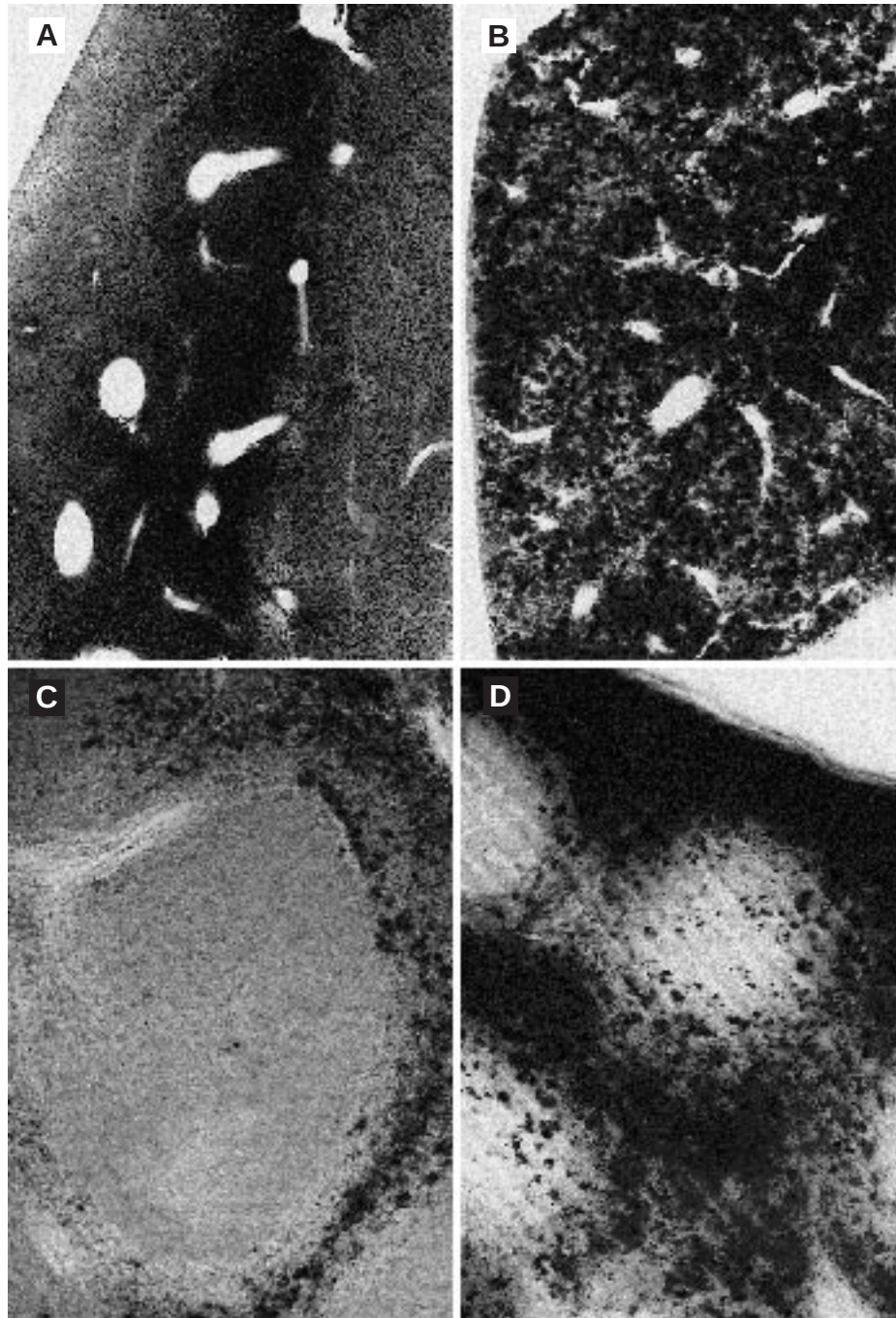


**FIG 5.** In vivo adenovirus-mediated LacZ gene expression ( $\beta$ -galactosidase activity). Six-week-old male C3H/HeJ mice were intravenously administered  $10^9$  pfu of AdexCALacZL, and 7 days later organs were stained with X-gal. **A**, Uninfected mouse liver (original magnification,  $\times 10$ ). **B**, AdexCALacZL-infected mouse liver (original magnification,  $\times 10$ ). **C**, Uninfected mouse spleen (original magnification,  $\times 10$ ). **D**, AdexCALacZL-infected mouse spleen (original magnification,  $\times 10$ ).

### Efficiency of adenovirus-mediated hGM-CSF gene expression

Next, we compared the efficiency of gene expression using AxCA-hGM-CSF. We chose human GM-CSF as the marker cytokine transgene because there is no cross-

activity on murine cells and because ELISA can discriminate between murine and human GM-CSF. Intravenous injection resulted in the highest serum level of hGM-CSF, and the level was dose-dependent. The other routes of administration only achieved a slight increase of serum hGM-CSF (subcutaneous > intraperitoneal >

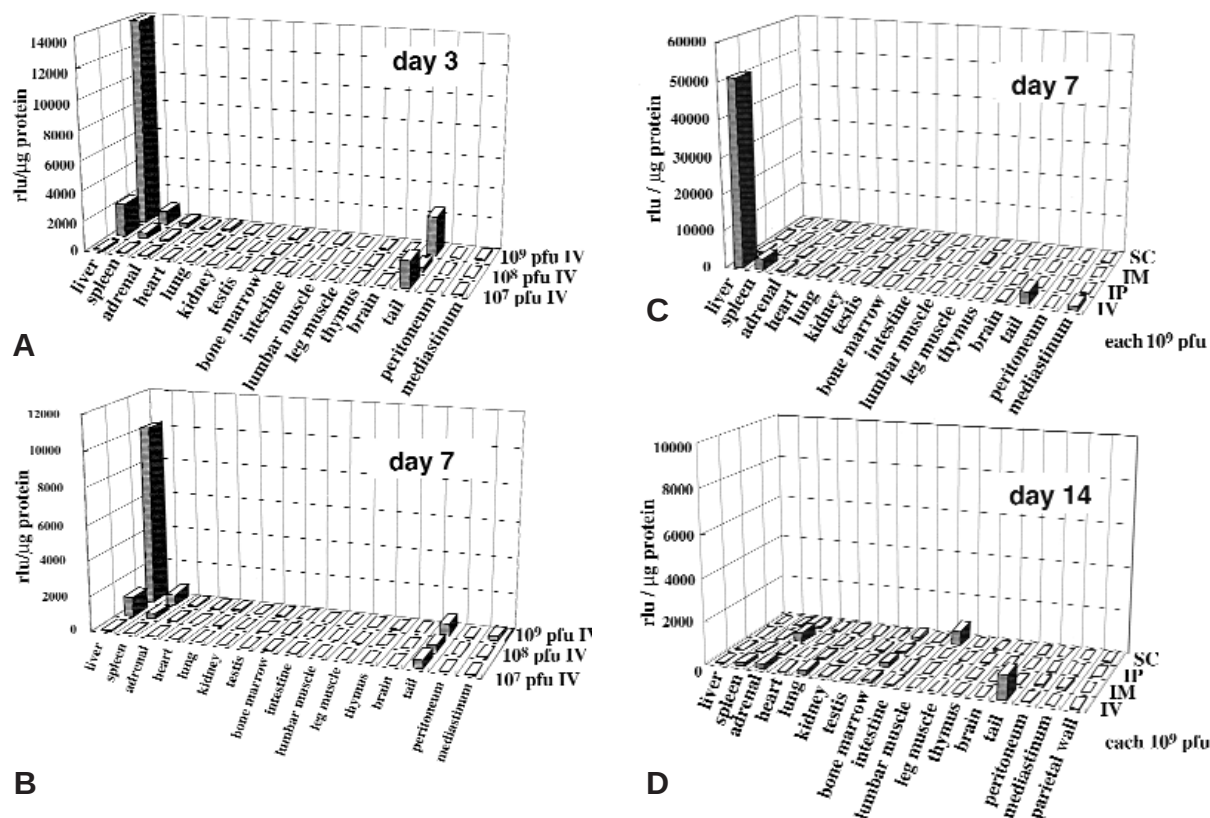


**FIG 6.** Adenovirus-mediated in vivo luciferase gene expression. Six-week-old male C3H/HeJ mice were injected intravenously with  $10^7$ ,  $10^8$ , and  $10^9$  pfu of AxCA-Luc. Three days (A) and 7 days (B) later, organs were lysed and analyzed for luciferase activity. The same mice were injected with  $10^9$  pfu of AxCA-Luc into the tail vein intravenously (IV), intraperitoneally (IP), intramuscularly (IM), and subcutaneously (SC). Seven days (C) and 14 days (D) later, luciferase activity was examined in the same way ( $n = 4$  to 5).

intramuscular; Fig 7). Serum hGM-CSF was no longer detectable by day 14 after viral infection by any route.

### In vivo effect of adenovirus-mediated mGM-CSF expression

To examine the biologic effect of adenovirus-mediated cytokine gene expression, AdexSR $\alpha$ -mGM-CSF was administered intravenously to 6-week-old C3H/HeJ mice, and organs were examined histologically after 7 days (Fig 8). The bone marrow showed hypercellularity with increased granulopoiesis and megakaryopoiesis (Fig 8, A and B); the spleen was enlarged with enhanced hematopoiesis in the parafollicular regions (Fig 8, C and D).

## DISCUSSION

### Infectious mechanism and tropism of adenoviruses

Although the natural targets of adenoviruses are the airway epithelium, conjunctiva, and gastrointestinal tract, these viruses can infect a broad spectrum of target cells under the right conditions. The target organ infected

depends mainly on the route of administration. Generally, the level of adenovirus-mediated transgene expression is much lower in hematopoietic cell lines than in adherent cell lines (Figs 3, B, and 4, B). This might be ascribable to differences in the infectivity of the vectors for these cells, the stability of viral DNA in host cells, and the efficiency of the cellular machinery for protein synthesis.

We found a clear parallel between cellular VNR expression and adenovirus-mediated transgene expression (Fig 3), compatible with a previous report.<sup>50</sup> Generally, adherent cell lines expressed much higher VNR level than hematopoietic cells and expressed much higher levels of transgene products.

Accordingly, our initial trial of adenovirus-mediated gene transfer into lymphoid cells was not promising<sup>51</sup> (Fig 4, B) because lymphocytes show very low or undetectable VNR levels (data not shown).<sup>52</sup> Only after activation and infection at a high MOI, a small population of cells revealed  $\beta$ -galactosidase activity (Fig 4, B), a result compatible with the previous report that human T cells can only be infected with adenovirus after integrin  $\alpha_V$  upregulation by stimulation with PHA and PMA.<sup>53</sup> In contrast, monocytes,<sup>31,53</sup> and dendritic cells<sup>54</sup> can be

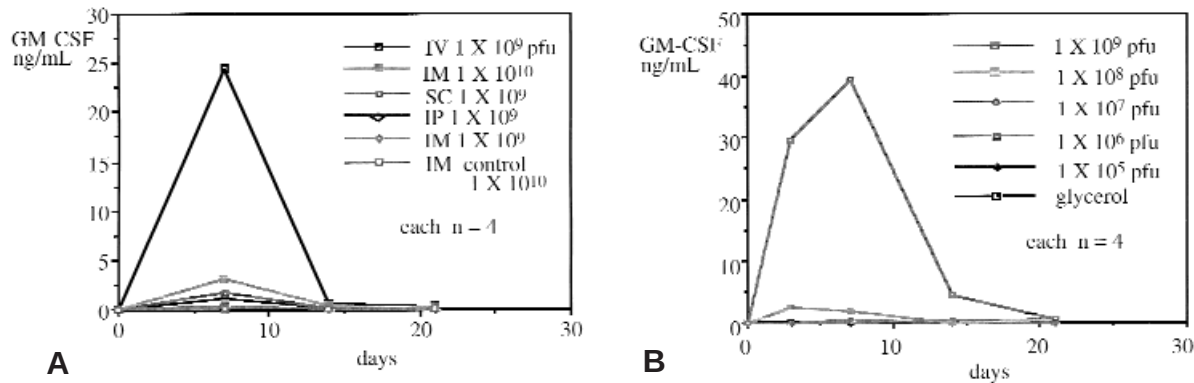


**FIG 7.** Adenovirus-mediated in vivo hGM-CSF expression. Six-week-old male C3H/HeJ mice were injected intravenously with AxCA-hGM-CSF. **A**, Serum hGM-CSF levels were examined by ELISA in mice injected intravenously (IV), subcutaneously (SC), intraperitoneally (IP), or intramuscularly (IM). Control mice were injected intramuscularly with AxCA-NI and 10% glycerol in PBS. Four mice were examined for each route of injection. **B**, Serum hGM-CSF levels in mice injected with 10<sup>5</sup> to 10<sup>9</sup> pfu of AxCA-hGM-CSF.

efficiently infected by adenoviruses. We also found that bone marrow mononuclear cells expressed  $\beta$ -galactosidase after infection with AdexCALacZL (Fig 4, A), but the transduction efficiency for bone marrow precursor cells has been shown to be much lower than that for adherent cells.<sup>32,34</sup>

#### Tissue distribution of adenovirus-mediated transgene expression and routes of administration

In addition to the cell susceptibility, anatomic and immunologic barriers modify the infectivity of adenoviruses. Our study revealed that the liver is the main target after intravenous injection based on the measurement of transgene (luciferase) expression (Figs 7 and 8). Adenovirus was effectively taken up by the liver, spleen, kidney, and other organs, and expression persisted for at least 2 weeks (findings were compatible with other reports).<sup>55-61</sup>

Quantification of tissue luciferase activity in vivo revealed that, after intravenous injection of adenovirus, transgene expression almost exclusively occurred in the liver (Fig 6). Worgall et al<sup>61</sup> also showed that 96% of the total adenoviral DNA was detected in the liver within 10 minutes after injection, but only 8% was detected at 24 hours without any increase in other organs.

#### Toxic side-effects of adenovirus infection and host immune response

Adenovirus infection causes cellular damage in a viral dose-dependent manner. Adherent cells infected in vitro at an MOI of more than 1000 showed cytopathic effect and detachment (data not shown). These toxicities may be explained by the binding of cellular VNRs by adenovirus capsid proteins and leaky expression of viral proteins, such as E2a and E4, which inhibit several host cellular functions.<sup>62</sup>

Several investigators have reported that pneumonitis<sup>63-65</sup> and hepatitis<sup>62,66-68</sup> were induced by cytotoxic T

lymphocytes directed against viral proteins expressed by the cells of these organs. Clinical trials of adenovirus containing cystic fibrosis conductance regulator cDNA have also shown a transient systemic and pulmonary syndrome, such as leukocytosis, fever, fatigue, tachycardia, hypotension, and pneumonitis,<sup>69</sup> which hinders the clinical application of this virus.

To attenuate the host immune response and prolong the duration of adenovirus-mediated transgene expression, several strategies have been tried, including anti-CD4,<sup>70</sup> anti-TCR,<sup>71</sup> and anti-CD40L<sup>72</sup> monoclonal antibodies; soluble CTLA4-Ig<sup>73,74</sup>; macrophage depletion<sup>75</sup>; induction of tolerance by intrathymic injection of adenovirus into neonatal mice<sup>76</sup>; immunosuppressants such as FK506,<sup>77</sup> cyclosporin A,<sup>78,79</sup> dexamethazone,<sup>80</sup> cyclophosphamide<sup>79,81</sup>; and other methods.<sup>82</sup>

#### Feasibility of adenovirus-mediated cytokine gene therapy for the intervention of immunologic status

Adenovirus-mediated cytokine gene therapy trials have proved successful in several situations, including systemic cytokine gene therapy<sup>57,83-88</sup> and local therapy.<sup>89-94</sup> Our data showed that adenoviruses can be applied for in vivo cytokine gene therapy at least for a period of 2 weeks (Fig 7).

In addition to systemic cytokine gene therapy, several other applications of adenoviruses for diseases of the immune and hematopoietic systems are promising, including tumor vaccination with cytokine genes; stem-cell amplification with cytokine receptor genes or signaling-molecule genes; and control of the growth of leukemic and cancer cells with tumor-suppressor genes, toxin genes, and antisense or dominant negative molecule genes.<sup>95</sup> In some of these fields, adenovirus may provide a convenient and effective method of expressing transgenes in a transient fashion. The feasibility of such applications of adenoviruses is the subject of our ongoing studies.

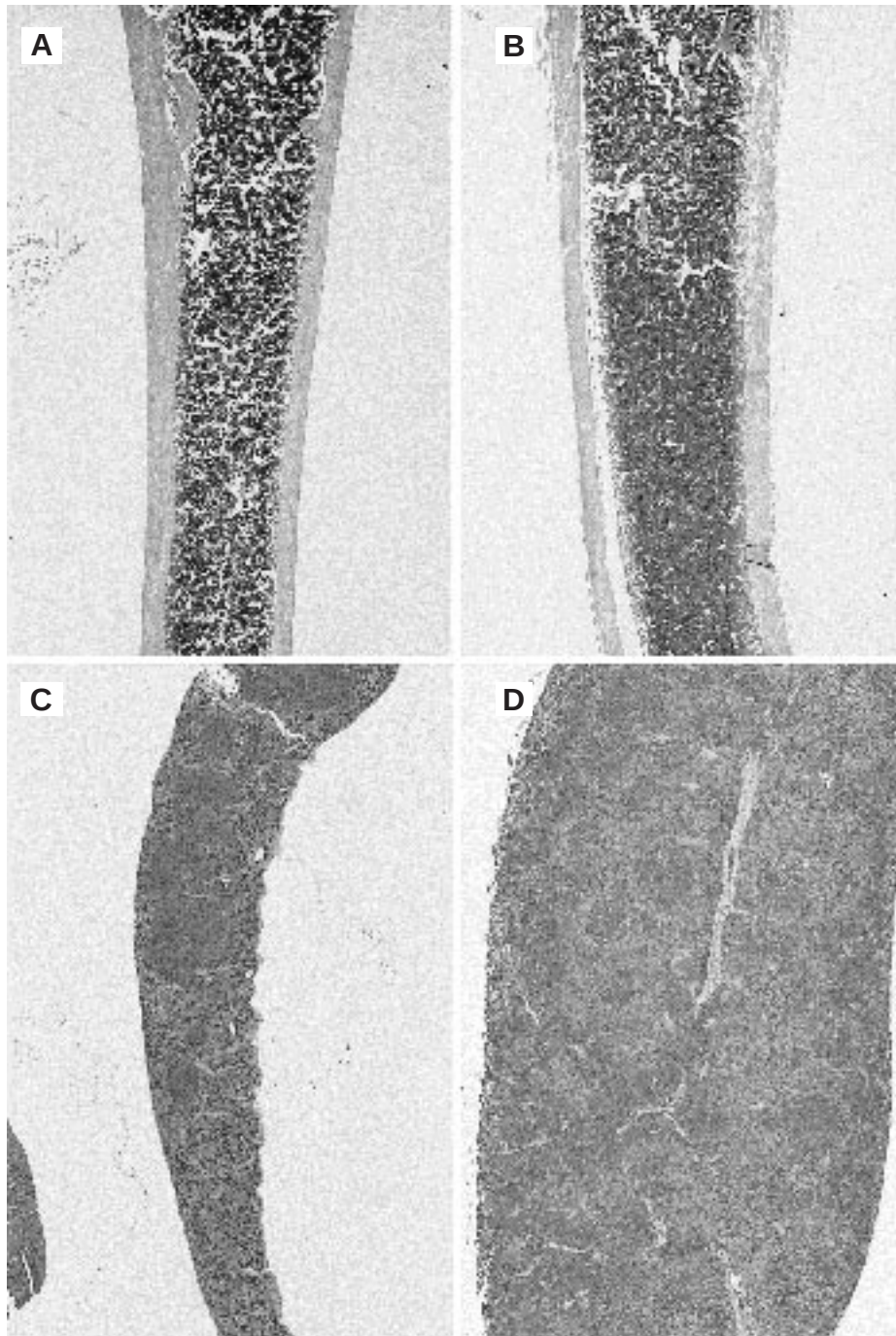


**FIG 8.** The biologic effects of adenovirus-mediated mGM-CSF expression. Six-week-old male C3H/HeJ mice were injected intravenously with  $10^9$  pfu of AdexSRα-mGM-CSF. Seven days later, organs were examined histologically after hematoxylin and eosin staining. Uninfected (**A**) and infected (**B**) mouse bone marrow (original magnification,  $\times 40$ ); uninfected (**C**) and infected (**D**) mouse spleen (original magnification,  $\times 40$ ).

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