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## Evaluation of Biodistribution and Safety of Adenovirus Vectors Containing Group B Fibers after Intravenous Injection into Baboons

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### Abstract

Vectors containing group B adenovirus (Ad) fibers are able to efficiently transduce gene therapy targets that are refractory to infection with standard Ad serotype 5 (Ad5) vectors, including malignant tumor cells, hematopoietic stem cells, and dendritic cells. Preliminary studies in mice indicate that, after intravenous injection, B-group fiber-containing Ads do not efficiently transduce most organs and cause less acute toxicity than Ad5 vectors. However, biodistribution and safety studies in mice are of limited value because the mouse analog of the B-group Ad receptor, CD46, is expressed only in the testis, whereas in humans, CD46 is expressed on all nucleated cells. Unlike mice, baboons have CD46 expression patterns and levels that closely mimic those in humans. We conducted a biodistribution and toxicity study of group B Ad fiber-containing vectors in baboons. Animals received phosphate-buffered saline, Ad5-bGal (a first-generation Ad5 vector), or B-group fiber-containing Ads (Ad5/35-bGal and Ad5/11-bGal) at a dose of  $2 \times 10^{12}$  VP/kg, and vector biodistribution and safety was analyzed over 3 days. The amount of Ad5/35-bGal and Ad5/11-bGal vector genomes was in most tissues one to three orders of magnitude below that of Ad5. Significant Ad5/35- and Ad5/11-mediated transgene ( $\beta$ -galactosidase) expression was seen only in the marginal zone of splenic follicles. Compared with the animal that received Ad5-bGal, all animals injected with B-group fiber-containing Ad vectors had lower elevations in serum proinflammatory cytokine levels. Gross and histopathology were normal in animals that received B-group Ad fiber-containing Ads, in contrast to the Ad5-infused animal, which showed widespread endothelial damage and inflammation. In a further study, a chimeric Ad5/35 vector carrying proapoptotic TRAIL and Ad E1A genes under tumor-specific regulation was well tolerated in a 30-day toxicity study. No major clinical, serologic, or pathologic abnormalities were noticed in this animal.

**OVERVIEW SUMMARY**—B-group Ad fiber-containing vectors are promising tools for gene therapy, for example, for the treatment of metastatic cancer or cardiovascular diseases, or for vaccination/immunotherapy. However, only a few studies of vectors containing B-group Ad fibers in mice have been conducted so far, and little is known about the mechanisms and effects of B-group Ad vector delivery *in vivo*. Before these vectors can be considered for clinical application, this knowledge gap must be filled. We performed biodistribution and safety studies after intravenous injection of chimeric Ad5 vectors containing Ad35 and Ad11 fibers into baboons. Our study suggests

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that Ad vectors possessing B-group Ad fibers have a better safety profile after intravenous injection than do conventional Ad5-based vectors.

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## INTRODUCTION

HUMAN ADENOVIRUSES (Ads) are classified into six groups (A through F). All Ad vectors used clinically so far have been based on serotype 5. The main disadvantages of Ad5-based vectors appear to be preexisting immunity in the majority of humans, low transduction of important gene therapy target cells (because of low expression of the primary Ad5 receptor, the coxsackie-adenovirus receptor [CAR]), and innate toxicity induced on intravascular application. Toxicity associated with Ad5 injection is characterized by complement activation, cytokine release, and consequent vascular damage leading to a systemic inflammatory response that can be fatal to the host (Schnell *et al.*, 2001; Lozier *et al.*, 2002; Morral *et al.*, 2002; Raper *et al.*, 2002; Gaggar *et al.*, 2003). The innate response can be divided into two phases. The first phase of acute inflammation occurs within 24 hr of virus administration, and it depends entirely on virus capsid interactions with host cells (Liu and Muruve, 2003). This phase is therefore seen after administration of both first-generation and helper-dependent Ad vectors. The second phase, which is specific to first-generation Ad vectors, begins 3–4 days after Ad administration and requires expression of viral gene products. Ad5 uptake induces expression of a number of cytokines (including interleukin [IL]-6, IL-10, and IL-8, tumor necrosis factor  $\alpha$  [TNF- $\alpha$ ], and interferon  $\gamma$  [IFN- $\gamma$ ]) and chemokines (including macrophage inflammatory protein [MIP]-1 $\alpha$ / $\beta$  and MIP-2) (Lieber *et al.*, 1997, 1998; Muruve *et al.*, 1999), which, in turn, play a major causative role in inflammation, tissue damage, and the induction of an antiviral adaptive immune response. Induction of cytokine transcription is thought to be initiated by Ad interaction with cellular integrins and integrin-mediated signaling involving p38 MAPK (mitogen-activated protein kinase) and ERK1/2 (extracellular-signal regulated kinase 1/2), and subsequent activation of NF- $\kappa$ B (Borgland *et al.*, 2000; Bowen *et al.*, 2002; Liu and Muruve, 2003; Tamanini *et al.*, 2003). Kupffer cells, endothelial cells, and leukocytes are considered to be the primary source of these cytokines and chemokines (Liu and Muruve, 2003).

Data show that vectors containing fibers from B-group Ads (including serotypes Ad35 and Ad11) efficiently transduce human cell types that are relatively refractory to Ad5 infection. These cell types include CD34<sup>+</sup> cells (particularly subsets with potential stem cell activity) (Segerman *et al.*, 2000; Shayakhmetov *et al.*, 2000; Stecher *et al.*, 2001; Yotnda *et al.*, 2001; Mizuguchi and Hayakawa, 2002; Sakurai *et al.*, 2003), immature human dendritic cells (Rea *et al.*, 2001; Stone *et al.*, 2002; Vogels *et al.*, 2003; DiPaolo, 2004), primary tumor cells (Gaggar *et al.*, 2003; Sova *et al.*, 2004), bone marrow mesenchymal cells (Olmsted-Davis *et al.*, 2002; Gugala *et al.*, 2003), and myoblasts, amniocytes, and synoviocytes (Rea *et al.*, 2001; Havenga *et al.*, 2002). Furthermore, we found that intravenously injected vectors containing Ad35 or Ad11 fibers (which have short fiber shafts) only inefficiently transduce Kupffer cells (whose transduction requires long-shafted fibers) and therefore elicit significantly less innate toxicity compared with Ad5 vectors in mice (Shayakhmetov *et al.*, 2004). Therefore, Ad vectors containing B-group fibers show promise for *in vivo* gene transfer, particularly for the treatment of metastatic cancer or for vaccination/immunotherapy. Unfortunately, only a limited number of murine studies on vectors containing B-group Ad fibers have been conducted so far (Koizumi *et al.*, 2003; Seshidhar Reddy *et al.*, 2003; Barouch *et al.*, 2004), and little is known about mechanisms and effects of B-group Ad vector infection *in vitro* and *in vivo*.

We and others identified CD46 as a cellular receptor that is used by all B-group Ads and by B-group Ad fiber-containing vectors (Gaggar *et al.*, 2003; Segerman *et al.*, 2003; Sirena *et al.*, 2004). CD46 is also a receptor for measles virus laboratory strains, for human herpes virus 6, and for certain pathogenic bacteria (for a review see Cattaneo, 2004). CD46 is a membrane

protein that is expressed on all nucleated human cells. Initially, CD46 was identified as a regulator of complement activation that binds and cleaves C3b and C4b in concert with serine protease factor I and, consequently, protects cells from complement-mediated lysis. CD46 expression is greatly upregulated in malignant tumor cells (Hara *et al.*, 1992; Thorsteinsson *et al.*, 1998; Kinugasa *et al.*, 1999; Murray *et al.*, 2000) and hematopoietic stem cells (Cho *et al.*, 1991; Manchester *et al.*, 2002). CD46 is expressed at the blood–brain barrier and was suggested to promote passage of pathogens from blood to cerebrospinal fluid and particularly might be involved in the pathogenesis of meningococcal disease (Johansson *et al.*, 2003). Specific expression of CD46 on the inner acrosomal membrane of spermatozoa and human placental trophoblasts, as well as restricted expression of rodent CD46 in testis, suggests that CD46 also plays a role in reproductive biology (Cervoni *et al.*, 1992).

Whereas all human nucleated cells express CD46, expression of the murine CD46 homolog is restricted to the testis. In addition, there is no homology between mouse and human cytoplasmic domain sequences. This clearly limits the value of biodistribution and toxicity studies with B-group Ad fiber-containing vectors in mice. In contrast to mice, the CD46 expression profile in nonhuman primates is similar to that in humans (Hsu *et al.*, 1997). Nonhuman primates have been extensively used for studies with measles virus (which also uses CD46 as a receptor) (Sakaguchi *et al.*, 1986; McChesney *et al.*, 1997; Premenko-Lanier *et al.*, 2003).

In this study, we analyzed the biodistribution and safety of intravenously applied Ad5/35 and Ad5/11 vectors in a short-term study. We also conducted a 30-day toxicity study with an Ad5/35-based oncolytic adenovirus expressing E1A and TRAIL (TNF-related apoptosis-inducing ligand) in a tumor-specific manner.

## MATERIALS AND METHODS

### Adenovirus vectors

Ad5-bGal, Ad5/35-bGal, and Ad5/11-bGal are first-generation E1/E3-deleted adenovirus vectors expressing *Escherichia coli*  $\beta$ -galactosidase ( $\beta$ -Gal) under the control of the Rous sarcoma virus (RSV) promoter (Shayakhmetov and Lieber, 2000; Stecher *et al.*, 2001; Bernt *et al.*, 2002). Ad5/35.IR-E1A/TRAIL is a capsid-modified, conditionally replicating, oncolytic vector (Sova *et al.*, 2004). Physical vector titers were determined spectrophotometrically and expressed as vector particles (VP) per milliliter. Infectious titers were measured as plaque-forming units or  $\beta$ -Gal-expressing units on 293 cells. The genome titers of all vectors were between  $4 \times 10^{12}$  and  $8 \times 10^{12}$  genomes (viral particles) per milliliter and the ratio of genomes to infectious titer was 20:1.

Virus was stored in 10 mM Tris (pH 7.5), 1 mM MgCl<sub>2</sub>, 10% glycerol at  $-80^{\circ}\text{C}$ . Before infusion into animals, all viral preparations were dialyzed against 2000 volumes of phosphate-buffered saline (PBS) for 4 hr at  $4^{\circ}\text{C}$ . A preliminary study has shown that without this dialysis step, baboons develop nausea and hypotension during and immediately after intravenous infusion.

To measure contamination with E1<sup>+</sup> replication-competent adenovirus, real-time polymerase chain reaction (PCR) analysis was performed with primers for E1A (GACCGTTTAC-GTGGAGACTC [F; forward primer] and CAGCCAGTAC-CTCTTCGATC [R; reverse primer]) and with primers for a sequence in the E4 region (TAAGCATAAGACGGACTACG [F] and GTAAGGCTGACTGTTAGGC [R]). Quantitative PCR (qPCR) was performed with the SYBR Green kit for the LightCycler (Roche Applied Science, Indianapolis, IN) and external standards for E1 and E4 (15 sec at  $95^{\circ}\text{C}$ , 5 sec at  $57^{\circ}\text{C}$ , and 17 sec  $72^{\circ}\text{C}$ ). Only virus preparations that contained less than one E1<sup>+</sup> (wild-type) viral genome in  $1 \times 10^8$  genomes were used in these studies.

To measure contamination with bacterial endotoxin, the *Limulus* amoebocyte lysate Pyrotell test kit was used, which detects as little as 0.03 endotoxin unit/ml by a gel-clot technique. Only preparations that tested negative for endotoxin were used in baboon studies.

### Animals and procedures

All studies were performed at the Washington National Primate Research Center (Seattle, WA) in accordance with institutional guidelines of the University of Washington. The study was approved by the University of Washington Institutional Care and Use Committee. Six male baboons (*Papio cynocephalus*; age, 2.5 to 4.2 years) were used in this study. Animals were individually caged. Because of the age of the animals and contact with humans, serum samples from all animals were tested for neutralizing antibodies against Ad5-, Ad5/35-, and Ad5/11-bGal vectors, using an assay described elsewhere (Kay *et al.*, 1997). No infection-blocking antibodies were detected at the lowest serum dilution (1:2). All viral vectors (in PBS) were infused through the femoral vein at a dose of  $2 \times 10^{12}$  VP/kg in a volume of 4 ml/kg of saline ( $5 \times 10^{11}$  VP/ml) at a rate of 1 ml/min under sedation. Animals were sedated with ketamine (10 mg/kg). The animals were of the following ages and weights: mock injected: 4.2 years, 13.5 kg; Ad5-bGal injected: 2.5 years, 8.6 kg; Ad5/35-bGal injected: 5.0 years, 15.6 kg; Ad5/11-bGal injected: 2.7 years, 8.7 kg and 3.8 years, 12.1 kg; Ad5/35.IR-E1A/TRAIL injected: 3.2 years, 9.6 kg. Blood samples were collected from all animals before injection and 30 min, 60 min, 6 hr, 24 hr, and 72 hr postinjection. Mock-, Ad5-bGal-, Ad5/35-bGal-, and Ad5/11-bGal-injected animals were killed 72 hr postinjection. Tissue samples were collected as described elsewhere (Morrall *et al.*, 2002). The animal injected with Ad5/35.IR-E1A/TRAIL was monitored until day 30 postinjection and additional blood samples were drawn on days 3, 10, 14, 17, 21, 24, and 30 postinjection. During necropsy, animals were perfused with 3 liters of saline to remove blood from organs. Tissue samples were taken under conditions that avoid cross-contamination of material. Samples for DNA analysis were snap-frozen in liquid nitrogen. Isolation of mononuclear cells from peripheral blood and bone marrow was performed by Ficoll gradient centrifugation. Buffy coats of peripheral blood cells were collected by centrifugation at  $1000 \times g$ , collection of the leukocyte layer, and subsequent lysis of residual erythrocytes with ammonium chloride (PharmLyse; BD Biosciences Pharmingen, San Diego, CA).

### Quantitative PCR for vector genomes

Isolation of cellular DNA from organs was performed as described elsewhere (Lieber *et al.*, 1997). This method allows for efficient purification of the episomal 3–6 kb of Ad DNA together with cellular genomic DNA. Genomic DNA was isolated from 100 mg of tissue. Genomic DNA (500 ng) was used for real-time PCR, using vector-specific primers against the Ad5 L4 region and primers against the endogenous baboon  $\beta$ -globin gene as an internal standard (used to equalize vector genome numbers between different tissues). Samples were analyzed with a LightCycler and the SYBR Green kit, and the following primers were used: for viral DNA, F-L4 (5'-TGCAAGATACCCCTATCCTG-3') and R-L4 (5'-CCTGTTGCAGAGCGTTTGC-3'); for baboon  $\beta$ -globin, F- $\beta$ -globin (5'-CCTATCAGAAAGTGGTGGCTGG-3') and R- $\beta$ -globin (5'-TTGGACAGCAAGAAAGTGAGCTT-3').

### Blood analysis

Ca<sup>2+</sup>, Mg<sup>2+</sup>, amylase (Amy-Panc [pancreatic amylase] and Amy-Sal [salivary gland amylase]), alanine aminotransferase (ALT; glutamicpyruvic transaminase [GPT]), aspartate aminotransferase (AST; glutamic-oxaloacetic transaminase [GOT]), bilirubin (total and direct), lactate dehydrogenase (LDH), creatine kinase (CK), creatinine, white blood cell count (WBC), red blood cell count (RBC), hematocrit, hemoglobin, mean corpuscular volume (MCV), mean

corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were analyzed in the clinical laboratory of the University of Washington Medical Center (Seattle, WA) according to standard procedures (using assays developed for human blood biochemistry).

### Cytokines

Serum cytokines were measured with OptEIA monkey enzyme-linked immunosorbent assay (ELISA) kits for monkey IL-6 and TNF- $\beta$  (BD Biosciences Pharmingen).

## RESULTS

Baboons were injected via the femoral vein with PBS (Mock) or viral vector ( $2 \times 10^{12}$  VP/kg). All vectors had an Ad5-based genome deleted for E1 and E3 genes and expressed *E. coli*  $\beta$ -galactosidase ( $\beta$ -Gal) under the control of the RSV promoter. In the chimeric Ad5/35 and Ad5/11 vectors, the Ad5 fiber gene was substituted with a chimeric fiber that contains the Ad5 tail and the Ad35 or Ad11 shaft and knob domains.

In a short-term study, animals were injected with Ad5-bGal ( $n = 1$ ), Ad5/35-bGal ( $n = 1$ ), Ad5/11-bGal ( $n = 2$ ), or PBS ( $n = 1$ ) and monitored for 3 days. A long-term study (more than 30 days) was conducted with Ad5/35.IR-E1A/TRAIL ( $n = 1$ ), which is an E1/E3-deleted Ad5 vector containing the Ad35 fiber and expressing Ad5 E1A and human TRAIL in a tumor-specific manner on homologous recombination between inverted repeats (IRs) (Sova *et al.*, 2004). Transgene expression in Ad5/35.IR-E1A/TRAIL is under the control of the RSV promoter.

### Short-term study

**Biodistribution: vector genomes.**—The level of vector genomes in major organs was determined by qPCR, using Ad-specific primers directed against the L4 region and total cellular DNA as a substrate. Differences in quality of DNA preparations were equalized by qPCR using primers directed against an endogenous baboon gene ( $\beta$ -globin). The genome concentration was expressed as the number of viral genomes per  $10^4$  cells (assuming a diploid cellular genome) (Fig. 1). For the mock-injected animal, no viral genomes were detected in all organs analyzed. One to three orders of magnitude more Ad5-bGal than Ad5/35-bGal or Ad5/11-bGal genomes were found in liver, brain, stomach, duodenum, testis, bone marrow mononuclear cells, and lymph nodes. The levels of Ad5-bGal, Ad5/35-bGal, and Ad5/11-bGal vector genomes were comparable in heart, pancreas, and esophagus. More Ad5/35-bGal and Ad5/11-bGal than Ad5-bGal genomes were found in the spleen. The differences between Ad5/11-bGal and Ad5/35-bGal genomes were not significant.

**Biodistribution:  $\beta$ -Gal expression in organs.**—Tissue sections were analyzed for  $\beta$ -Gal expression by 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) staining (Table 1) and Ad5-mediated  $\beta$ -Gal expression was seen in most organs. More than 5% of hepatocytes were transduced (Fig. 2A). In the spleen, there were isolated  $\beta$ -Gal-positive cells in the red pulp that appeared to be endothelial cells lining the sinusoids (Fig. 2A). Areas with almost 100% transduction of arterial endothelium in lung were found (Fig. 2B). Furthermore, transduction of the arterial endothelium of the testis was observed with the Ad5-bGal vector (data not shown).

For Ad5/35-bGal- and Ad5/11-bGal-injected animals,  $\beta$ -galactosidase expression was detectable only in the marginal zone of splenic follicles (Fig. 2A). These  $\beta$ -Gal-expressing splenic cells also stained positive for S100, a marker for antigen-presenting cells (Fig. 2C). No Ad5/35-bGal- and Ad5/11-bGal-mediated  $\beta$ -Gal expression was seen in other organs.

**Biodistribution:  $\beta$ -Gal expression in bone marrow and blood cells.**—Mononuclear cells from bone marrow aspirates (BM-MNCs) were isolated by centrifugation in Ficoll gradients. Of  $10^7$  cells we found 40, 684, and 255  $\beta$ -Gal-positive BM-MNCs, respectively, for Ad5-bGal-, Ad5/35-bGal-, and Ad5/11-bGal-injected animals (Fig. 3). The transduction level of peripheral leukocytes (buffy coats) was even lower, with 1 and 0.1  $\beta$ -Gal-positive cells in  $10^7$  cells for Ad5/35-bGal- and Ad5/11-bGal-injected animals, respectively.

Taken together, biodistribution data from the three animals show that intravenous injection of Ad35 or Ad11 fiber-containing vectors resulted in low-level transduction of all major organs, including bone marrow and peripheral blood leukocytes. Significant transgene expression was found only in antigen-presenting cells of the spleen. In agreement with earlier reports (Morral *et al.*, 1999, 2002; Lozier *et al.*, 2002; Brunetti-Pierri *et al.*, 2004), our data from one animal injected with Ad5-bGal show transgene expression in most tissues, particularly in hepatocytes and vascular endothelial cells.

**Toxicity: blood analysis.**—We analyzed blood samples taken before Ad injection and 15 min, 30 min, 6 hr, 24 hr, and 72 hr postinfusion (Fig. 4). Serum levels of alanine aminotransferase (ALT; a marker of hepatocellular damage) were slightly increased for all Ad vectors at 30 and 60 min postinjection, particularly for Ad5-bGal (Fig. 4A). At 72 hr postinjection, ALT levels in the Ad5-bGal-injected animal increased further, whereas ALT levels in Ad5/35-bGal- and, to a lesser degree, Ad5/11-bGal-injected animals returned to normal. Serum aspartate aminotransferase (AST) levels were elevated only in the Ad5-bGal-injected animal at 72 hr. Markers of cholestasis,  $\gamma$ -glutamyltransferase ( $\gamma$ -GT) and bilirubin, were within normal ranges for all animals at all time points. Creatine kinase levels were elevated more than 10-fold in all Ad-injected animals at 30 and 60 min postinfusion. Kidney function as assessed by serum creatinine was normal for all animals at all time points.

We also analyzed serum IL-6 and TNF- $\alpha$  levels (markers of acute inflammation). Serum levels of these proinflammatory cytokines increased for all vectors and reached peak levels at 6 hr postinjection. For the Ad5-bGal vector, serum IL-6 increased from undetectable levels to 690 pg/ml at 6 hr postinfusion, whereas for the Ad5/35-bGal and Ad5/11-bGal-injected animals, IL-6 peak levels were 155 and 195 pg/ml, respectively. The peak TNF- $\alpha$  levels at 6 hr postinjection were 129, 11, and 14 pg/ml for Ad5-bGal-, Ad5/35-bGal-, and Ad5/11-bGal-injected animals, respectively. No clear signs of disseminated intravascular coagulation (DIC), as determined on the basis of changes in prothrombin time and platelet counts, were observed in all tested animals. Overall, all blood cell counts were within normal ranges for all animals (Fig. 4B).

**Histopathology.**—Necropsy was performed 72 hr after injection. Over the 3-day observation period the animal injected with Ad5-bGal was inactive, with decreased food intake and an elevated body temperature. At necropsy, this animal had acute multifocal hemorrhage in the lung accompanied by fibrin accumulation (Fig. 5). The absence of inflammatory cells and obstruction of bronchi and bronchioles indicated that the hemorrhage was near terminal (within 24 hr) and that blood was not inhaled. The pulmonary hemorrhage appears to be an extreme manifestation of vascular leakage potentially via activation of innate inflammatory responses. The liver demonstrated centrilobular, mild hydropic changes. Ascites was noted grossly during necropsy. Spleen, lymph nodes, and tonsils displayed mild lymphoid hyperplasia. Heart tissue showed subacute epicarditis. Other histopathological changes included tracheitis and sialoadenitis. No renal, CNS, or muscle abnormalities were noted. The mock-, Ad5/35-bGal-, and Ad5/11-bGal-injected animals showed reduced activity on the first day, but exhibited normal behavior on the following 2 days. Gross pathology and histology were normal for these animals.

The levels of serum markers indicated that mild tissue damage occurred rapidly after injection of all three vectors; however, liver damage was more pronounced for Ad5-bGal. A stronger acute inflammatory response after Ad5-bGal injection was reflected by 4- and 12-fold higher IL-6 and TNF- $\alpha$  levels, respectively, compared with the Ad5/35-bGal- and Ad5/11-bGal-injected animals. Severe gross and histopathological changes indicating vascular damage were observed at necropsy of the Ad5-bGal-injected animal.

### Long-term study

One animal was intravenously injected with Ad5/35.IR-E1A/TRAIL at  $2 \times 10^{12}$  VP/kg (Sova *et al.*, 2004). This vector has the same capsid and the same tropism as the Ad5/35-bGal vector used in the short-term study. Ad5/35.IR-E1A/TRAIL allows for expression of Ad E1A and TRAIL in a replication-dependent and tumor-restricted manner.

**Histopathology**—The animal was monitored for 30 days, until the planned time point for termination of the experiment. Over this period, the animal was healthy and active. Body temperature was measured twice per week and was normal. Blood samples were taken before injection and 1 min, 15 min, 6 hr, 24 hr, 72 hr, 8 days, 10 days, 14 days, 17 days, 21 days, 24 days, and 30 days after injection. Urine samples were taken on days 2 and 7 after injection. Tissue samples were harvested during necropsy as described above.

**Biodistribution.**—Distribution of Ad5/35.IR-E1A/TRAIL vector genomes was analyzed by qPCR in tissues on day 30 after vector infusion (Fig. 6A). Overall, the tissue distribution of vector genomes was comparable to that in the animal injected with Ad5/35-bGal (see Fig. 1); however, the total amount of vector genomes detected on day 30 postinfusion (compared with day 3 in the short-term study) was about 10-fold less in most tissues. E1A transgene expression was analyzed by immunohistochemistry of tissue sections and was absent in all tissues (liver, lung, spleen, kidney, lymph node, and heart). (As a positive control, we used tissue from mice injected with wild-type Ad5; Bernt *et al.*, 2002.) Activation of transgene expression in normal tissue is not expected from Ad.IR vectors (Steinwaerder *et al.*, 2001).

**Blood clearance.**—We used qPCR for vector genomes to assess the kinetics of Ad clearance from the blood. Ad5/35.IR-E1A/TRAIL genomes in blood cells reached preinjection levels by 6 hr postinfusion. When we compared the amount of viral DNA found in blood cells with the total amount of injected Ad, we found that at 1 min postinfusion, ~2% of the virus input dose was associated with blood cells. The number of blood cell-associated vector genomes remained constant over a period of 72 hr and declined afterward. On day 8 postinjection, viral genomes associated with blood cells reached undetectable levels (Fig. 6B).

In contrast to humans, baboons express CD46 on erythrocytes (Hsu *et al.*, 1997), which might affect vector distribution in blood cells. We therefore compared the number of genomes associated with white cells and total blood cells (white and red cells) on day 3 by qPCR. If Ad5/35.IR-E1A/TRAIL efficiently interacts with baboon erythrocytes, the number of Ad genomes found into total blood cell DNA should be ~3 orders of magnitude higher than the amount found in white cells as there are ~1000-fold more red cells than white cells (erythrocytes have no nuclei/nuclear DNA). We found, however, only 5-fold more vector genomes in total blood cells than in white cells (data not shown), indicating that Ad5/35 particles efficiently associated with white blood cells and not with erythrocytes.

No viral genomes were found at 48 hr in urine, analyzed at a 1:100 dilution.

**Biochemical markers, blood cells, and cytokines.**—After an initial slight increase, white blood cell counts decreased between 24 hr and day 8 postinfusion, after which they

increased again (Fig. 7A). The number of monocytes and lymphocytes increased between day 8 and day 17. Importantly, all changes in blood cell numbers were within normal ranges (based on human hematological parameters).

Serum transaminase levels increased between 6 and 24 hr after infusion and returned to normal afterward (Fig. 7B). Bilirubin and  $\gamma$ -GT levels remained within normal limits (Fig. 7C and D). Creatinine levels also remained within the normal range (Fig. 7D). IL-6 levels increased at 6 hr postinfusion (as seen in the short-term study) and returned to normal by 24 hr (Fig. 7E). TNF- $\alpha$  levels were elevated for 14 days after infusion (Fig. 7F).

## DISCUSSION

This is, to our knowledge, the first biodistribution and toxicity study of capsid-modified Ad vectors in nonhuman primates. In this study, Ad5-based chimeric vectors were used, in which the Ad5 fiber gene was replaced with either the Ad35 or Ad11 B-group virus sequence. We identified CD46 as the receptor used by B-group Ads for cell infection. In baboons, CD46 is expressed in a pattern and at a level comparable to humans (Hsu *et al.*, 1997). Nonhuman primates have been used in studies with measles virus vectors that also utilize CD46 as a receptor (Combredet *et al.*, 2003).

Biodistribution in this study was analyzed by two different methods: quantitative PCR and transgene expression. Quantitative PCR measures the number of viral genomes present in tissue DNA, but does not distinguish between virus that infects cells and virus that remains extracellular. Also, most tissues contain more than one cell type, and qPCR does not give information regarding the cell type targeted by the vector. Although we tried to minimize the presence of extracellular or intravascular viral DNA by aggressively flushing the vascular system with normal saline at necropsy, these potential confounding factors must be kept in mind when interpreting the results. Copy numbers measured by qPCR are therefore informative only in comparison. The total number must be taken with caution. This is exemplified by the results for viral DNA content in the liver where we found, on the basis of theoretical calculations, an average of five copies of Ad5-bGal per cell. In contrast, only approximately 5% of hepatocytes were found to express  $\beta$ -Gal. Transduced hepatocytes were found predominantly in the periportal area and probably represent cells that were exposed to the highest dose on intravenous vector injection. From published studies it is known that nonhuman primate hepatocyte transduction can be increased to ~50% by increasing the number of infused particles to  $5.6 \times 10^{12}$  VP (Morral *et al.*, 2002). In murine studies, a large portion of intravenously infused viral particles is taken up by the reticuloendothelial system (RES), and a dose-dependent increase in hepatocyte transduction is observed only at doses that are higher than the RES saturating dose (Liu and Muruve, 2003). Much of the extracellular or RES-associated viral DNA would be expected to degrade by the time of analysis (72 hr). Nevertheless, it is possible that a substantial portion of the viral genomes detected by PCR were taken up by cells of the reticuloendothelial system rather than by hepatocytes.

Transgene expression, as analyzed by X-Gal staining, better reflects active transduction. We chose the RSV promoter for this study, one of the strongest viral promoters, as it is active in a wide range of tissues. In particular, the main target cell population of Ad5-based vectors, hepatocytes, supports transcription from this promoter. (Notably, cytomegalovirus [CMV] promoter-driven expression is downregulated in liver; Loser *et al.*, 1998.)

Ad5-bGal transduction was found in all major organs, predominantly in liver and lung. Our data are in agreement with studies performed by others (Morral *et al.*, 1999, 2002; Lozier *et al.*, 2002; Brunetti-Pierri *et al.*, 2004), and although we infused only one animal, we believe that these data are representative.



Overall, transduction of baboon tissue with B-group fiber-containing vectors (Ad5/35-bGal and Ad5/11-bGal, a total of three animals), as assessed by the number of vector genomes and  $\beta$ -Gal-positive cells, was less efficient than with the Ad5 vector. The following observations made *in vitro* might account for the low *in vivo* transduction efficiency of chimeric vectors, despite the fact that CD46 is expressed on all cells in baboons: (1) Ad5/35 transduction of CD46-expressing CHO cells (at a multiplicity of infection [MOI] of 100 plaque-forming units [PFU]/cell) directly correlates with CD46 density on the cell surface and is inefficient when fewer than 100 CD46 molecules are expressed per cell (Anderson *et al.*, 2004). CD46 is expressed in normal cell only at low levels; and (2) the affinity of Ad35 and Ad11 fiber-containing Ads for CD46 is relatively low (Gaggar *et al.*, 2003). Notably, two of the main criteria that determine the efficiency of viral infection (in addition to MOI) are the receptor density and the affinity for a given receptor.

The highest numbers of viral genomes and  $\beta$ -Gal-positive cells in animals infused with Ad5/35-bGal or Ad5/11-bGal were found in the spleen. Transduced cells were located in the marginal zone of splenic follicles. Notably, transduction of marginal zone cells has also been observed for certain measles virus strains, which also use CD46 as a cellular receptor (Sakaguchi *et al.*, 1986; McChesney *et al.*, 1997; Premenko-Lanier *et al.*, 2003). The transduced cells were positive for S100, and negative for B cell markers. Further characterization of this cell population has been hindered by the limited availability of antibodies against baboon surface markers. Nevertheless, this limited analysis points toward an antigen-presenting cell as the main target for Ad5/35 and Ad5/11 transduction in the spleen. The immunological consequences of this effect are not clear. In theory, infection/transduction of splenic antigen-presenting cells can cause both an increased or decreased immune response against the viral vector and/or the transgene. A potential reduced immune response can be due to CD46 signaling that transiently affects the function of dendritic cells or T cells, in analogy to measles-induced immunosuppression (Marie *et al.*, 2001, 2002). Importantly, no opportunistic infections or WBC abnormalities were observed in the 30-day observation of the Ad5/35.IR-E1A/TRAIL-injected animal. A more in-depth analysis of the clinical and immunological consequences of splenic antigen-presenting cell transduction is ongoing.

In summary, the biodistribution studies show, on the basis of the presence of viral genomes and transgene expression, an overall decrease in levels of viral uptake and transduction for vectors carrying group B fibers when compared with Ad5. This was most evident in the liver, the main target of Ad5 transduction. Uptake into bone marrow and peripheral blood cells was also low, despite theoretical concerns over *in vivo* transduction based on the *in vitro* infectibility of these cell populations by Ad5/35 and Ad11 vectors (Shayakhmetov *et al.*, 2000; Stecher *et al.*, 2001). S100-positive cells in the marginal zone of splenic follicles represent an exception and are transduced at a fairly high level by vectors carrying group B fibers.

Differences in biodistribution between Ad5 and B-group fiber-containing vectors are likely the reason for the differences observed for the different vectors in short-term toxicity. Kupffer cells and other cells of the RES have been implicated as the main mediators of the cytokine “storm” triggered by the infusion of high doses of Ad5-based vectors (Liu *et al.*, 2003; Muruve *et al.*, 2004). Because Ad5/35 vectors possess short fiber shafts, uptake of Ad5/35 capsid-containing vectors into Kupffer cells (which is mediated by blood factors and requires long fiber shafts) is inefficient, as studies in mice have shown (Shayakhmetov *et al.*, 2004). On the basis of our data, we hypothesize that not only in mice, but also in baboons, a direct consequence of this is greatly reduced expression of proinflammatory cytokines and tissue damage after intravenous Ad5/35 and Ad5/11 vector injection, when compared with Ad5. The changes seen in histopathology and blood biochemistry after infusion of Ad vectors correlate with the levels of proinflammatory serum cytokines. Ad5-bGal infusion resulted in greatly elevated IL-6 and TNF- $\alpha$  levels and generalized inflammatory responses characterized by histopathological and

blood biochemistry abnormalities. In contrast, elevation of IL-6 and TNF- $\alpha$  levels on Ad5/35-bGal or Ad5/11-bGal infusion was markedly less and signs of acute inflammation were not detected.

Overall, the type and extent of clinical, serological, and pathological abnormalities in our Ad5-injected animal were consistent with other nonhuman primate studies (Morrall *et al.*, 1999, 2002; Lozier *et al.*, 2002; Brunetti-Pierri *et al.*, 2004). In a study by Morrall *et al.* (2002), infusion of  $1 \times 10^{12}$  VP/kg into a baboon caused elevation of transaminases (AST, 84 U/liter; ALT, 107 U/liter) at 48 hr, similar to the range in our study (where  $2 \times 10^{12}$  VP/kg was injected). In contrast, a second animal receiving a dose of  $1 \times 10^{13}$  VP/kg showed a massive increase in transaminases (AST, 7440 U/ml), thrombocytopenia, and DIC. In the low-dose animal, WBC numbers were similar to our study, and no coagulation abnormalities were observed. Organ distribution of  $\beta$ -Gal expression was similar to our study, with the exception of endothelial transduction, which was observed only in the high-dose animal. IL-6 and TNF- $\alpha$  levels were, respectively, 709 and 325 pg/ml at 48 hr in the high-dose animal and normal in the low-dose animal. However, a third animal (not otherwise discussed in this report) receiving  $0.6 \times 10^{12}$  VP/kg had an IL-6 level of 306 pg/ml at 24 hr, suggesting considerable variability and no strict dose dependency. We observed a peak of IL-6 and TNF- $\alpha$  at 6 hr, and it is possible that a small increase in these cytokines may have been missed in the low-dose animal studied by Morrall *et al.* because no 6-hr sample was obtained. Interestingly, in that study, IL-6 levels continued to rise even after 24 hr in the animal that received the lethal dose. In another study, infusion of an Ad5-based, first-generation vector expressing human factor IX at doses of  $0.34 \times 10^{12}$ ,  $1.7 \times 10^{12}$ , and  $3.8 \times 10^{12}$  VP/kg induced similar dose-dependent toxicity and inflammatory changes (Lozier *et al.*, 2002). IL-6 levels peaked at 6 hr and were 18.2, 297, and 238 pg/ml, respectively. Elevation of transaminases was also observed. Partial thrombin time (PTT) abnormalities may have been exacerbated by transgene expression, particularly at the later time points. Interestingly, CK abnormalities were reported in this study, with elevation to 800–2000 U/liter.

In addition to the short-term toxicity study, we have conducted a 30-day clinical observation and subsequent histopathology analysis of a baboon injected with Ad5/35.IR-E1A/TRAIL. This vector is in consideration for a phase I clinical trial in cancer patients, and this study is part of the preclinical evaluation. Ad5/35.IR-E1A/TRAIL has the same capsid, and hence tropism, as Ad5/35-bGal. In addition, this vector employs a new concept for tumor-specific gene expression that is based on homologous recombination in Ad genomes (Ad.IR system) (Steinwaerder *et al.*, 2001). Studies in primary normal human cells (fibroblasts, small airway epithelial cell, astrocytes, and mesothelial cells) (Steinwaerder *et al.*, 2001; Bernt *et al.*, 2002; Sova *et al.*, 2004), in mouse liver and baboon liver segments (Steinwaerder *et al.*, 2001), demonstrated the absence of activation of gene expression from Ad.IR vectors in nontumor cells. It was therefore not expected that E1A and TRAIL would be expressed in baboon tissues that were successfully transduced with Ad5/35 IR-E1A/TRAIL. Toxicity after infusion of Ad5/35.IR-E1A/TRAIL, at a dose of  $2 \times 10^{12}$  VP/kg, was minimal with a small, transient increase in transaminases during the first 24 hr, a transient increase in IL-6 (4-fold less than with the Ad5-based vector), and almost no change in TNF- $\alpha$  levels. The animal showed no signs of illness or distress during the observation period. On necropsy, no gross or histopathologic abnormalities were found. No expression of adenoviral E1A was observed in any of the tissues analyzed, including the spleen. This finding suggests that the second level of specificity, the tumor-specific Ad.IR expression system, efficiently prevents toxicity in nontransformed cells that are susceptible to Ad5/35 infection.

Overall, this study suggests that Ad vectors possessing B-group Ad fibers have a better safety profile after intravenous injection than conventional Ad5-based vectors. This makes them

potential candidates for gene therapy, for example, in the treatment of metastatic cancer or cardiovascular diseases.

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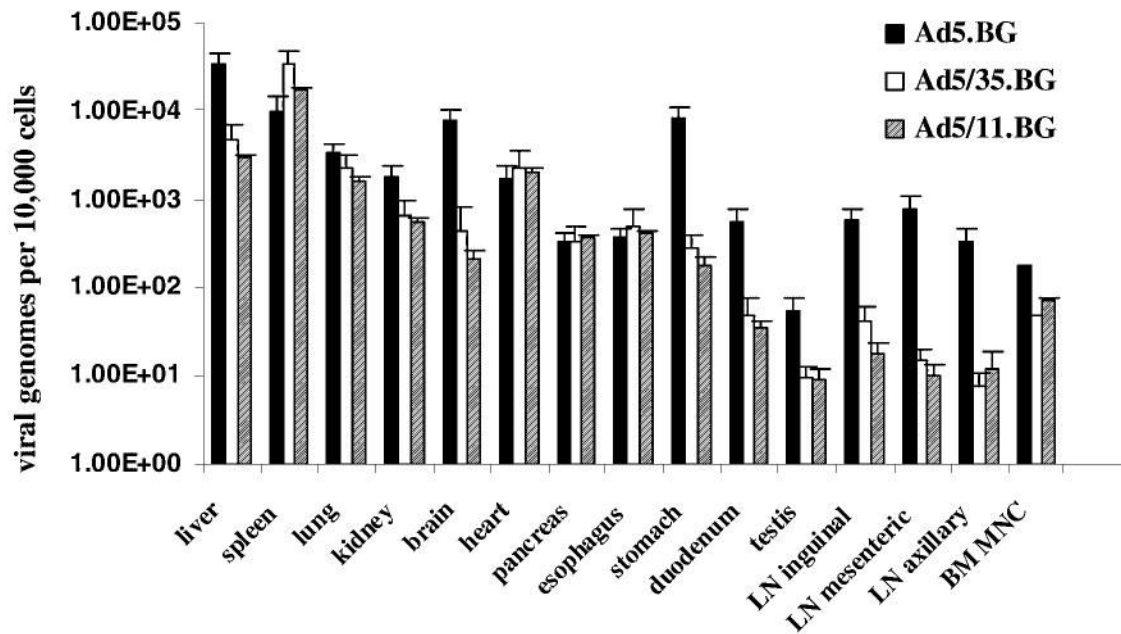
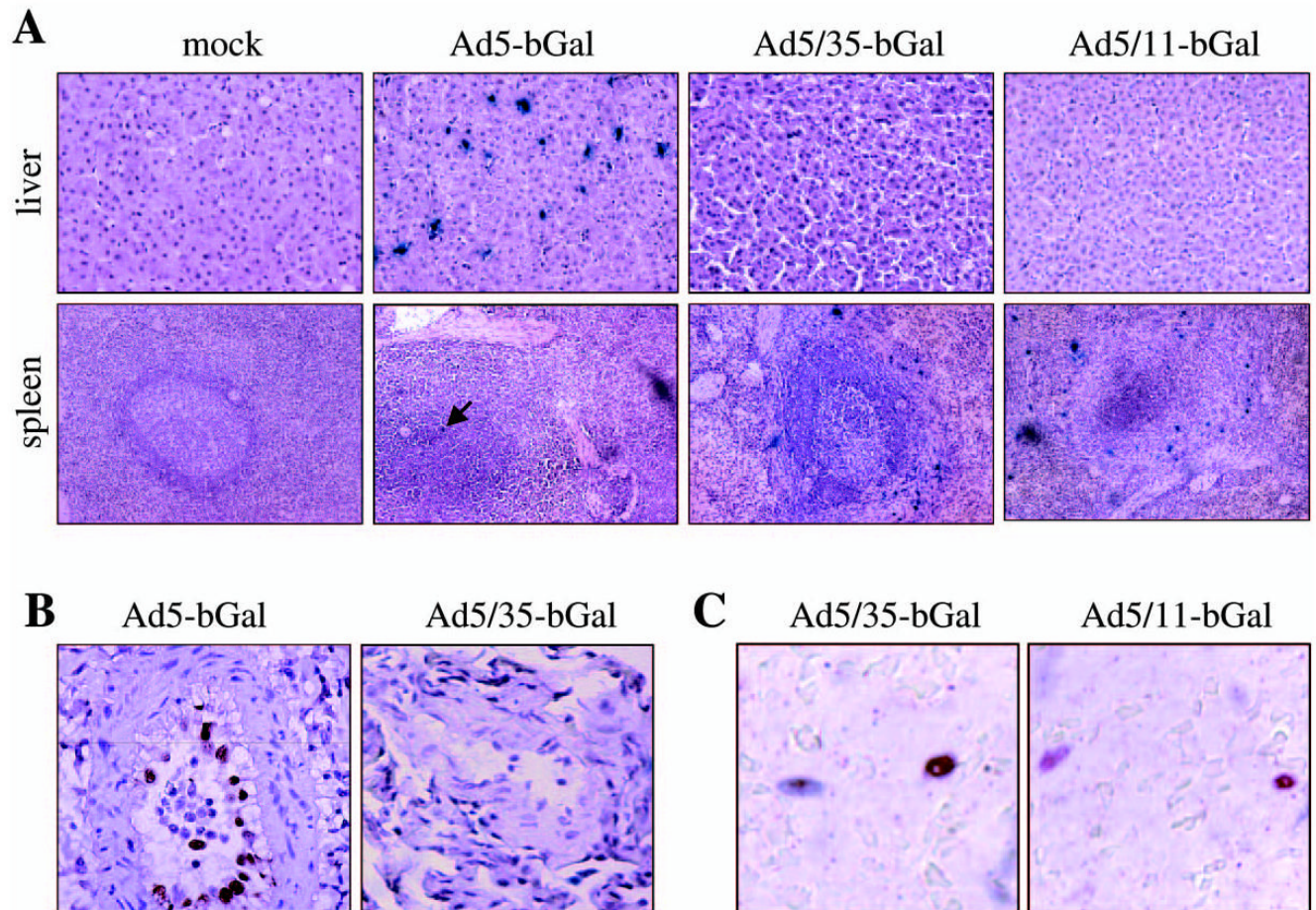


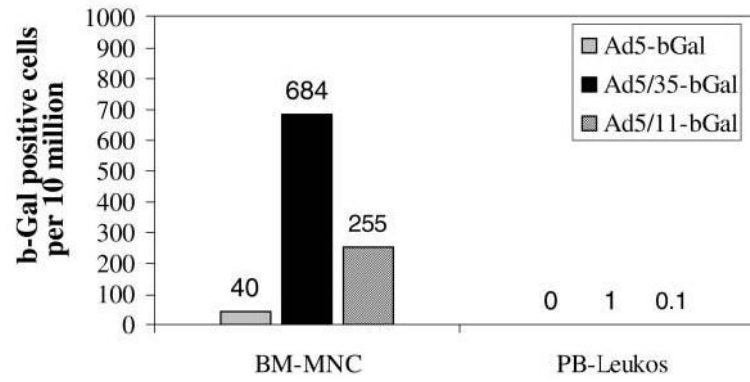
FIG. 1.

Quantitative comparison of viral genomes present in major organs 72 hr postinjection of Ad5-bGal, Ad5/35-bGal, and Ad5/11-bGal. Three independent tissue samples were analyzed and the SEM is shown. Genome concentration was expressed as the number of viral genomes per  $10^4$  cells (assuming that the mass of a diploid human genome is 6 pg). qPCR results for vector genomes were equalized on the basis of qPCR data for an endogenous (two copies per genome) monkey  $\beta$ -globin gene. Ad5-bGal (one animal), Ad5/35-bGal (one animal), Ad5/11 (two animals). BM-MNC, bone marrow mononuclear cells; LN, lymph node.

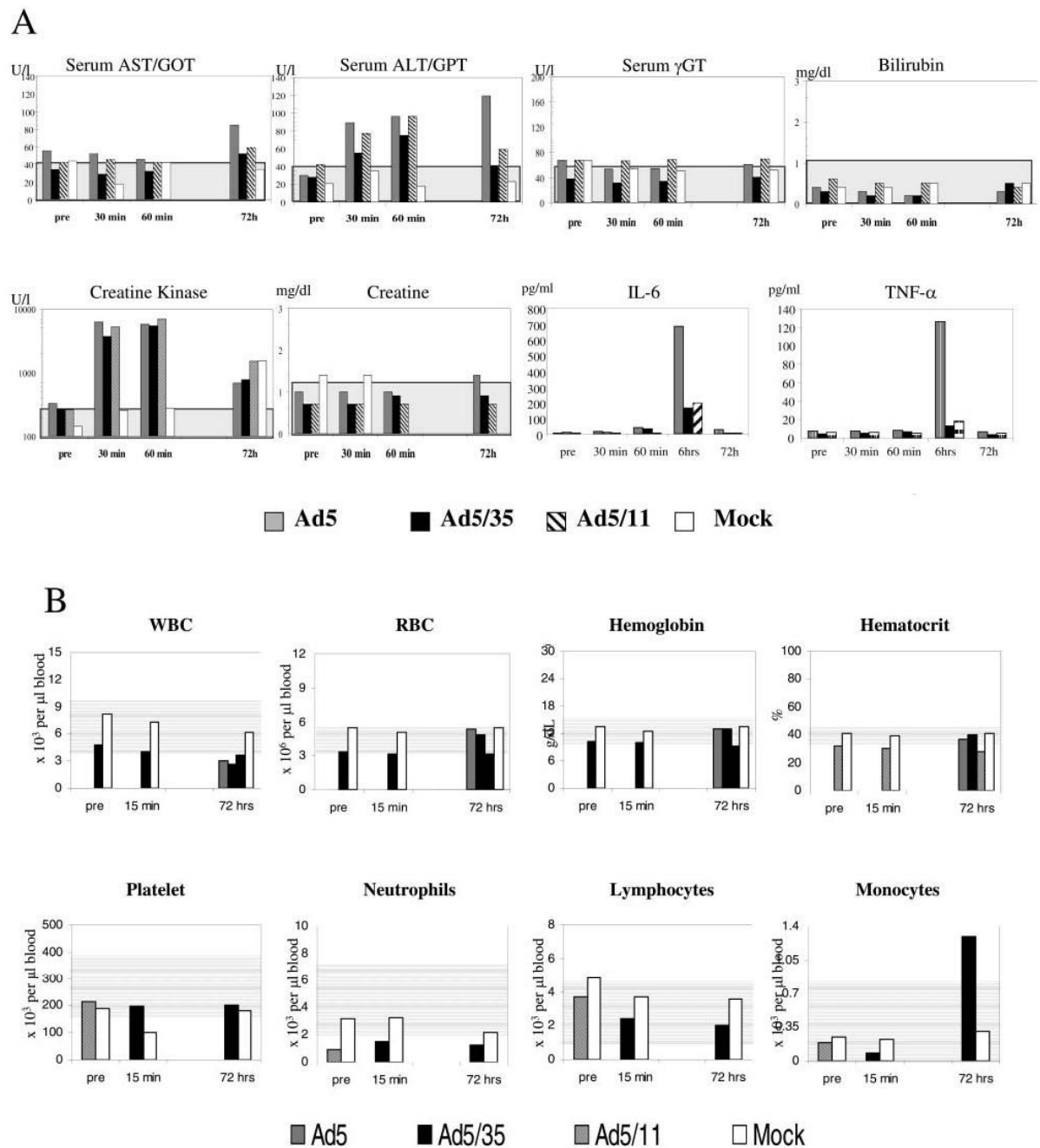
**FIG. 2.**

Analysis of  $\beta$ -galactosidase expression in selected tissues. **(A)** X-gal-stained sections of liver and spleen. **(B)** Immunohistochemistry for  $\beta$ -galactosidase in lung sections (positive staining appears brown). **(C)** Costaining of spleen section from the Ad5/35-bGal-injected animal with antibodies against  $\beta$ -Gal (blue) and S100 (red). Overlapping colors result in brown.

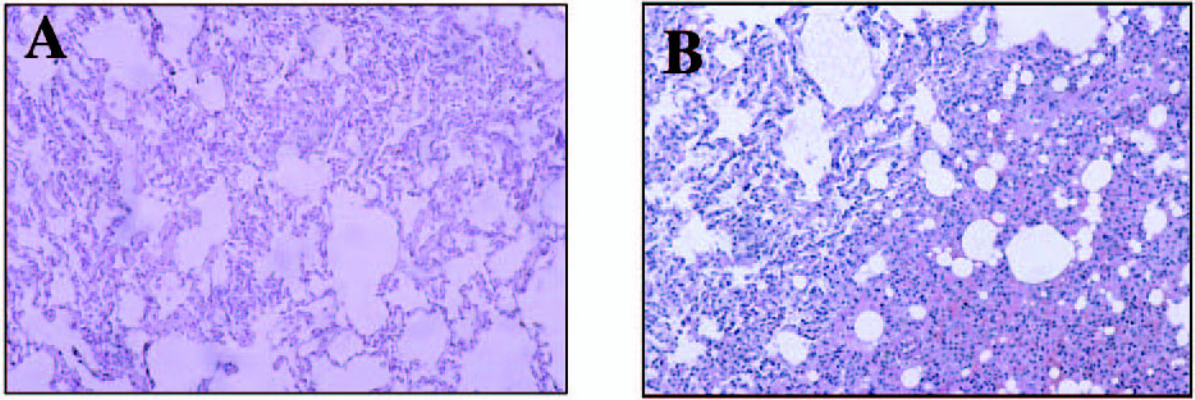


**FIG. 3.**

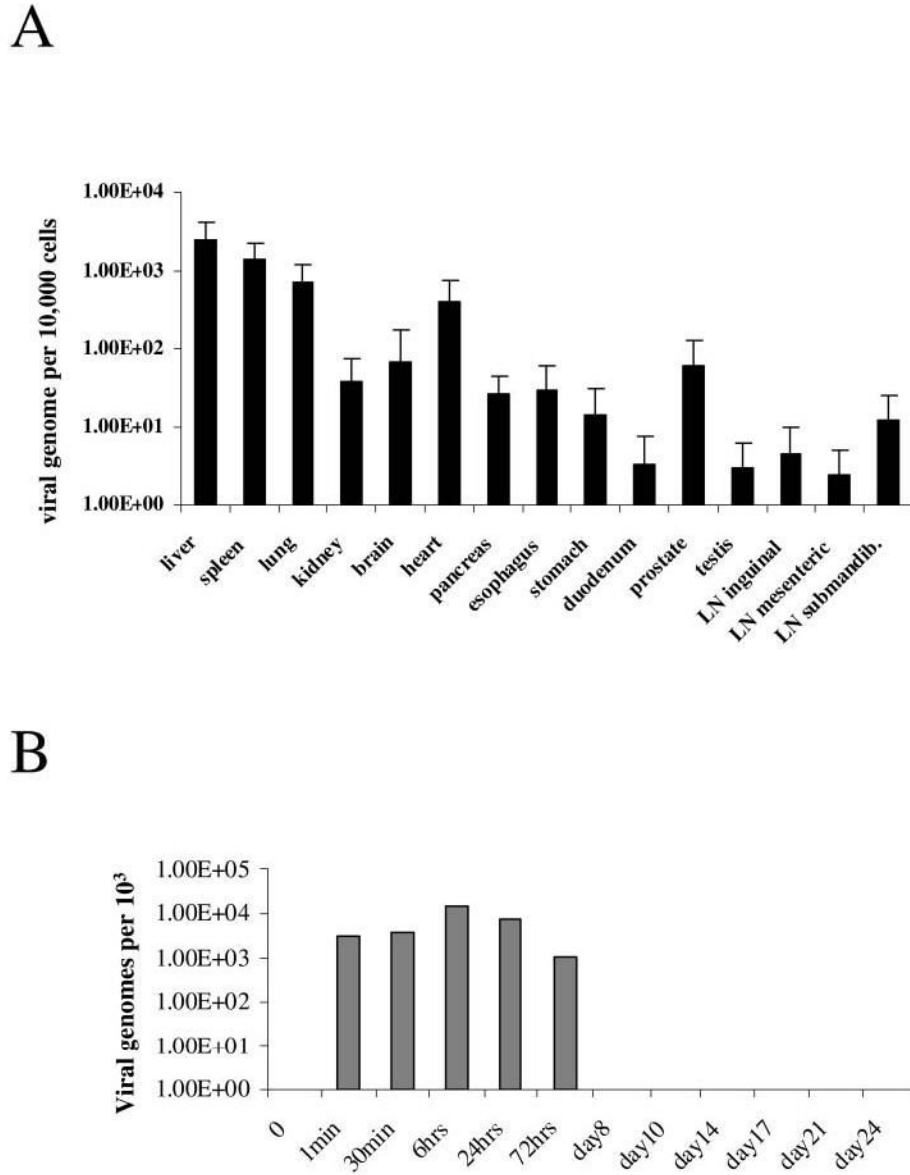
Transduction of peripheral blood and bone marrow cells after infusion of Ad vectors into baboons. A total of  $10^7$  bone marrow mononuclear cells (BM-MNCs) and peripheral blood cells (PB-leukos) from Ad-injected animals were analyzed for  $\beta$ -galactosidase expression by X-Gal staining (day 3 postinjection). Show is the total number of  $\beta$ -Gal-positive cells.



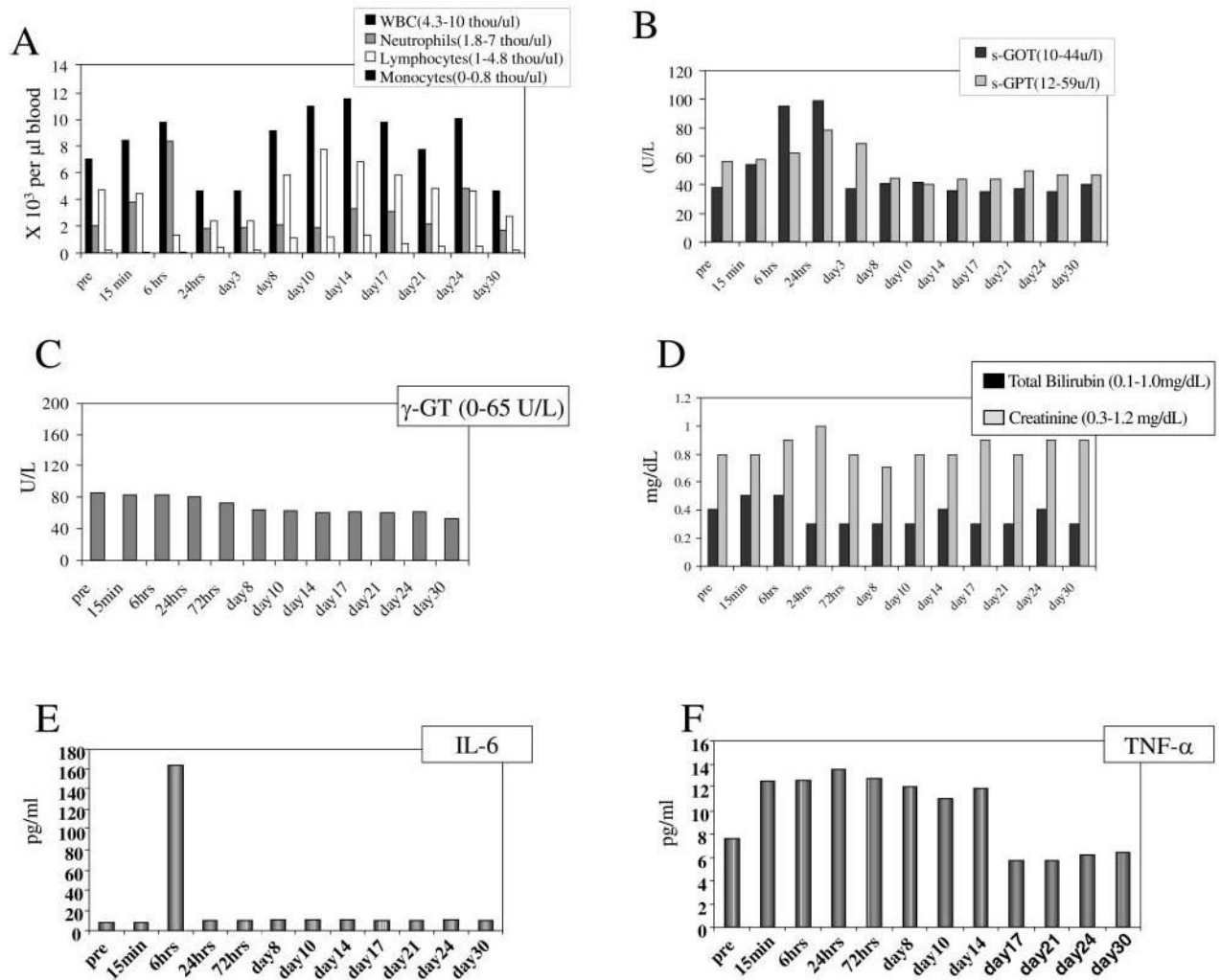
**FIG. 4.** Blood analyses. (A) Analysis of serum markers of liver damage (ALT,  $\gamma$ -GT, and bilirubin), tissue damage (AST), muscle damage (creatine kinase), and kidney damage (creatinine) as well as proinflammatory cytokines (IL-6 and TNF- $\alpha$ ) at various time points after Ad infusion. (B) Blood cell counts. Normal ranges are indicated by gray areas in the background of each panel.



**FIG. 5.** Lung histology at necropsy. (A) Mock injected; (B) Ad5-bGal injected. Note the diffuse hemorrhage. Lungs from Ad5/35-bGal- and Ad5/11-bGal-injected animals were normal.



**FIG. 6.** Quantitative analysis of Ad5/35.IR-E1A/TRAIL genomes. **(A)** Distribution of viral genomes in major tissues, 30 days after infusion. The genome concentration is expressed as the number of viral genomes per 10<sup>4</sup> cells (assuming that the mass of a diploid human genome is 6 pg). The average of three independent tissue samples ± SEM is shown. **(B)** Clearance of vector genomes from blood cells. Total blood cells were harvested at various time points. The average of three independent PCRs ± SEM is shown. The genome concentration is expressed as the number of viral genomes per 10<sup>3</sup> cells. qPCR results for vector genomes were equalized on the basis of qPCR data for the endogenous (two copies per genome) monkey β-globin gene.



**FIG. 7.** (A) Analysis of blood cell numbers. (B–D) Analysis of serum markers for liver damage (ALT,  $\gamma$ -GT, and bilirubin), tissue damage (AST), and kidney damage (creatinine). (E and F) Analysis of serum proinflammatory cytokine levels (IL-6 and TNF- $\alpha$ ) at various time points after Ad5/35.IR-E1A/TRAIL infusion.