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The Role of Kupffer Cell Activation and Viral Gene Expression in Early Liver Toxicity after Infusion of Recombinant Adenovirus Vectors

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Systemic application of first-generation adenovirus induces pathogenic effects in the liver. To begin unraveling the mechanisms underlying early liver toxicity after adenovirus infusion, particularly the role of macrophage activation and expression of viral genes in transduced target cells, first-generation adenovirus or adenovirus vectors that lacked most early and late gene expression were administered to C3H/HeJ mice after transient depletion of Kupffer cells by gadolinium chloride treatment. Activation of NF-κB, and the serum levels of the proinflammatory cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6) were studied in correlation with liver damage, apoptosis, and hepatocellular DNA synthesis. While Kupffer cell depletion nearly eliminated adenovirus-induced TNF release, it resulted in a more robust IL-6 release. These responses were greatly reduced in animals receiving the deleted adenovirus. Although there were quantitative differences, NF-κB activation was observed within minutes of first-generation or deleted adenovirus vector administration regardless of the status of the Kupffer cells, suggesting that the induction is related to a direct effect of the virus particle on the hepatocyte. Early liver toxicity as determined by serum glutamic-pyruvic transaminase elevation and inflammatory cell infiltrates appeared to be dependent on adenovirus-mediated early gene expression and intact Kupffer cell function. Kupffer cell depletion had little effect on adenovirus-mediated hepatocyte apoptosis but did increase hepatocellular DNA synthesis. Finally, Kupffer cell depletion decreased the persistence of transgene (human α1-antitrypsin [hAAT]) expression that was associated with a more pronounced humoral immune response against hAAT. The elucidation of these events occurring after intravenous adenovirus injection will be important in developing new vectors and transfer techniques with reduced toxicity.

Viral proteins expressed in cells after transduction with first-generation adenoviruses elicit a host immune response leading to inflammation of the target organ and extinction of transgene expression (3, 5). The immune response in the liver following systemic adenovirus administration is divided into two phases (15, 28, 29). The first phase occurs between days 1 and 4 postinfection (p.i.) and is characterized in part by a periportal polymorphonuclear leukocyte infiltration and elevated liver enzymes, in some cases with lethal outcome, in response to high viral doses. The second phase begins 5 to 7 days p.i. and is associated with an antigen-dependent immune response specific to viral and/or transgene products. Substantial efforts have been undertaken to study and modulate the specific immune response against viral antigens (24, 29, 51, 52). However, little is known concerning the mechanisms behind the early, innate inflammatory response in the mouse liver following systemic application of first-generation adenovirus vectors. Possible elements of an antiviral innate immune response include the activation of macrophages, NK cells, complement, and cytokine release.

Kupffer cells (KC) are large liver macrophages. Because of their topological localization within the liver sinusoids, they represent the first line of defense against viruses entering the liver through the portal circulation. KC functions are activated by a variety of particles and substances, including viruses (Sendai virus and Newcastle disease virus), bacterial lipopolysaccharide (LPS), muramyl dipeptide, gamma interferon, and tumor necrosis factor alpha (TNF) (for a review, see reference 11). The phagocytosis of parasites by KC is accompanied by the release of proinflammatory cytokines that act primarily as a paracrine signal on neighboring hepatocytes and induce chemotaxis and aggregation of neutrophils. Furthermore, KC express class II major histocompatibility complex molecules, as well as processing and presenting antigens.

Recently, KC activation by first-generation adenovirus was reported, although active transduction of KC by adenovirus was not demonstrated (47, 48). In these reports, KC function was thought to be responsible for the elimination of ~90% of viral vector DNA within the first 24 h after intravenous application. Intravenous injections of gadolinium chloride ($GdCl_3$), a member of the rare earth metals, were able to prevent activation of KC by LPS (8, 18, 34). Hardonk et al. (18) speculated that $GdCl_3$ formed a colloidal precipitate in the bloodstream at neutral pH which is phagocytosed by KC and dissolved again in the acidic environment within the macrophage lysosomes, resulting in cell destruction. A single injection of $GdCl_3$ blocks phagocytosis in more than 90% of KC and selectively eliminates the large periportal macrophages (18). Repopulation of KC by immature macrophages and monocytes begins 4 days after injection (18). Macrophages in the red pulp of the spleen are less vulnerable to $GdCl_3$ (18, 34). There is no evidence that $GdCl_3$ exerts any direct toxic effects on hepatocytes, biliary epithelial cells, endothelial cells, Ito cells, circulating monocytes, or lymphocytes (34).

Activation of cytokines, especially TNF and interleukin-6

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(IL-6), is a central element of the innate immune response against viruses (7, 14, 17). TNF is produced primarily by activated KC as a biologically active membrane-bound precursor that is cleaved to produce the mature cytokine, which is released into the serum with systemic effects (17, 33). Released TNF binds to two different receptors on a variety of target cell types, including KC and hepatocytes. The two TNF receptors p55 and p75 have different intracellular domains involved in activation of different signal transduction pathways leading to cell cycle progression, apoptosis, or differentiation. Moreover, TNF activates KC and induces synthesis of leukotrienes that in turn attract cells involved in the inflammatory and/or immune response. TNF has at least two direct activities against wild-type adenovirus: inhibition of virus replication and killing of virus-infected cells and sensitization by E1a of cells to TNF-induced p53-mediated apoptosis (17, 19, 40, 46). Four of the ~25 early adenovirus proteins (E1b-19K and E3-14.7K and -10.4K/14.5K) prevent early TNF lysis (17). TNF is the principal mediator of endotoxemic shock, and in humans there is a good correlation between high TNF serum levels and the severity of adenovirus-induced pulmonary disease (31).

IL-6 is produced by a variety of cell types; a major source of IL-6 following virus infection or LPS stimulation is splenic macrophages (1, 35). A specific IL-6 receptor on hepatocytes is implicated in acute-phase reactions in the liver and in the regulation of liver regeneration after partial hepatectomy. IL-6 transcription is activated by TNF in KC, hepatocytes, and other cell types (32, 38). Its synthesis is rapidly induced after pulmonary adenovirus administration (14). Furthermore, IL-6 may be involved in the activation of cytotoxic T lymphocytes (CTL) that can affect immune responses against the vector, vector-transduced cells, and/or transgene product (16).

NF-κB is a ubiquitous transcription factor which is activated by a variety of stimuli, including viruses such as cytomegalovirus (CMV), human immunodeficiency virus, hepatitis B virus, Epstein-Barr virus and Sindbis virus (2, 30). It represents a master switch for the cellular immune response against viruses. This is achieved in part by its potential to coordinately transactivate transcription of inflammatory cytokine genes, including the TNF and IL-6 genes (32, 38). NF-κB is also involved in apoptotic pathways leading to elimination of virally infected cells independent of an immune response (2).

Recently, we generated recombinant adenoviruses lacking the genes for the immunogenic products encoded in the E1, E2, E3, and L1-L4 regions (DBP, polymerase, pTP, hexon, fiber, etc.) (29). The deleted adenovirus containing the human α1-antitrypsin (hAAT) expression cassette (ΔAd.hAAT) can be generated at high titers by a technique based on Cre/Lox recombination. The small 9-kb-deleted viral genome is packaged into capsids that are structurally similar to those of undeleted adenovirus vectors and can efficiently transduce mouse hepatocytes in vivo. However, transgene expression in vivo lasted only 2 days before the deleted genome was degraded. Expression from deleted genomes can be stabilized in *trans*, suggesting that viral proteins encoded in the excised region are needed for genome persistence.

MATERIALS AND METHODS

Adenoviruses. Ad/RSVhAAT and ΔAd.hAAT were produced as previously described (29). The viral titers given in transducing units were determined on HeLa cells by hAAT immunofluorescence (29). The number of E1-deleted helper viral particles was less than 5 plaques (on 293 cells)/10^6 transducing units in the ΔAd.hAAT preparations. Viruses with a titer of 5×10^{10} transducing units/ml were stored at −80°C in 10 mM Tris-Cl (pH 8.0)–1 mM $MgCl_2$–10% glycerol. Tests for replication-competent virus were performed as described previously (3). All adenovirus preparations were analyzed for endotoxin by using the *Mulus amebocyte* lysate (Pyrotell) test (Associates of Cape Cod, Inc., Falmouth, Mass.) according to the protocol of the manufacturer. The detection limit of the test was 0.05 endotoxin unit/10^{10} PFU/ml.

Animal studies. Animal studies were performed in accordance with the institutional guidelines set forth by the University of Washington. Five- to six-week-old female C3H/HeJ and C3H-SCID mice (Jackson Laboratory, Bar Harbor, Maine) were used. All animals were housed in specific-pathogen-free facilities. Adenovirus injection was performed via tail vein infusion with 200 μl of adenovirus. Generally 10^{10} transducing units, a dose that transduces ~100% of hepatocytes (27), was injected per mouse. Blood samples for analysis were obtained by retroorbital bleeding. Serum samples for cytokine and hAAT analysis were stored at −80°C; samples for serum glutamic-pyruvic transaminase (SGPT) and hAAT antibody analysis were stored at 4°C. For DNA replication studies, [*methyl*-^{3}H]thymidine (1 μCi/g of body weight) diluted in pyrogen-free physiologic saline was injected intraperitoneally in 200 μl at 12 and 1 h before sacrifice.

$GdCl_3$ injections. For transient KC blockage, a protocol described previously (18) was adapted. $GdCl_3$ was dissolved in H_2O, and 10 mg/kg of body weight was injected via the tail vein at 30 and 6 h prior to adenovirus administration in a total volume of 200 μl. Control animals were injected with 200 μl of saline. To test the phagocytic capacity of KC, colloidal carbon (0.8 ml/kg of body weight; Sigma, St. Louis, Mo.) was injected 30 min before sacrifice in mice treated with $GdCl_3$ or untreated at 0, 24, 48, and 72 h (18). The carbon uptake by liver macrophages, scored by light microscopy of liver sections was reduced by ~80% in mice that received the double $GdCl_3$ injection compared to untreated animals. Similar results were obtained when the dose of $GdCl_3$ was 20 mg/kg. This is consistent with results obtained in previous studies (8, 18, 34, 39).

Biochemical analysis of serum samples. Cytokine standards and antibodies were from Pharmingen. For the IL-6 enzyme-linked immunosorbent assay (ELISA), the rat anti-mouse IL-6 monoclonal antibody (MAb) MP5-20F3 was used as the capture antibody and the biotinylated anti-IL-6 MAb MP5-32C11 was used as the detecting antibody. Binding was detected with avidin D-horseradish peroxidase (A-2004; Vector Laboratories). For the TNF ELISA, anti-mouse TNF MAbs MP6-XT22 and Mp6-XT3 were used. The detection sensitivities were 15 and 50 pg/ml for IL-6 and TNF, respectively. Serum hAAT concentrations were determined by ELISA as previously described (22). A Sigma diagnostic kit was used for colorimetric determination of the SGPT activity, using 10 μl of serum (Sigma procedure no. 505).

NF-κB electrophoretic mobility shift assay (EMSA). Nuclear extracts were obtained from mouse livers as described previously (13) and stored at −80°C until used. Protein concentrations were measured by the Bradford method. The NF-κB binding sequence from the class I major histocompatibility complex enhancer element ($H2^k$) was used as a probe. Double-stranded oligonucleotides were end labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase. Ten micrograms of nuclear protein was incubated with 0.2 ng of labeled oligonucleotide probe for 30 min at room temperature and separated in 5% polyacrylamide-Tris-glycine-EDTA gels. For antibody supershift assays, anti-p50- and anti-p65 specific polyclonal antibodies (Santa Cruz Biotechnology) were used. One microgram of the corresponding antibody was added to the samples after 30 min of incubation with the labeled probe. Gels were dried and exposed to Kodak-AR film for 12 h.

Histological analysis. For histological analysis, liver samples (left half of the large upper lobe) were fixed in 10% neutral formalin, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin-eosin. Autoradiography after [*methyl*-^{3}H]thymidine labeling was performed on paraffin sections that were dip-coated with Kodak NTB-2 emulsion diluted 1:1 (vol/vol) with water and developed after a 2-week exposure as described previously (28). All slides were counterstained with hematoxylin-eosin.

For terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) analysis, liver tissue was frozen in OCT compound (Miles, Inc., Elkhart, Ind.) and cryosectioned in 10-μm sections. A Boehringer Mannheim in situ cell death detection kit was used to quantify apoptosis in hepatocytes as specified by the manufacturer. Liver sections were counterstained with hematoxylin and analyzed by light microscopy.

Southern blotting. For genomic DNA preparation, mouse livers were flushed with 5 ml of phosphate-buffered saline via the portal vein. After removal, livers were homogenized, and a 100-mg portion was used for DNA extraction as described previously (44). DNA concentrations were determined spectrophotometrically. Ten micrograms of genomic DNA was digested with *Bam*HI, run on a 0.8% agarose gel, and electrotransferred to a Hybond membrane (Amersham). The blots were hybridized in rapid hybridization buffer (Amersham) with an [α-^{32}P]dCTP-labeled hAAT probe, using a random priming kit (Gibco BRL). As a control, DNA from uninfected animals was spiked with 10 pg of purified Ad/RSVhAAT viral DNA and loaded on each gel. The relative amount of adenovirus DNA was determined by phosphorimager analysis as a ratio between sample signal and control signal. Small variations in DNA loading or transfer between lanes were adjusted by rehybridization of the blots with probes for the mouse metallothionein gene. The following plasmid DNA fragments were used for labeled probes: 1.4-kb hAAT cDNA (*Eco*RI fragment of pAd.RSVhAAT (23) and 2-kb mouse metallothionein I gene (*Hin*dIII/*Eco*RI fragment of pmMMT-I (44).

hAAT antibodies. Anti-hAAT antibodies were determined by ELISA as described previously (37). Briefly, ELISA plates coated with anti-hAAT capture MAb were blocked and then incubated with hAAT protein (calibrator serum no.

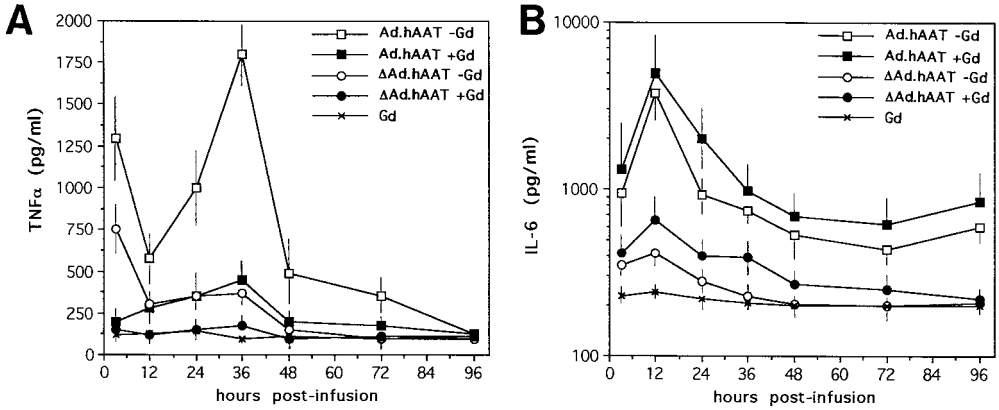

FIG. 1. Serum cytokine concentrations. Serum TNF (A) and IL-6 (B) serum concentrations after injection of Ad/RSVhAAT or ΔAd.hAAT in C3H mice with (+Gd) $GdCl_3$ or without (−Gd) $GdCl_3$ administration at 30 and 6 h before adenovirus ($n = 4$ animals per group). Gd, $GdCl_3$ without adenovirus.

4; Atlantic Antibodies, Stillwater, Minn.) diluted 1:50 in blocking buffer for 2 h at room temperature. For each two-sample well, two additional wells were mock loaded with blocking buffer to determine whether individual high serum hAAT levels would interfere with the assay. Mouse serum samples, diluted 1:1,000, were loaded onto the plate along with a similarly diluted naive serum as a negative control. Murine immunoglobulin G2b anti-hAAT MAb 178260 (Calbiochem, La Jolla, Calif.) serially diluted in blocking buffer from 10^{-2} to 10^{-6} was also included on each plate as a positive control. Following another 2 h of incubation at room temperature, the plates were incubated with horseradish peroxidase-labeled sheep anti-mouse immunoglobulin whole-molecule antibody (A-6782; Sigma) for another 2 h.

RESULTS

Block of KC function. $GdCl_3$ was extensively used in liver regeneration studies of rats and mice as an agent to block KC function (8, 18, 34, 39). In agreement with these studies, we confirmed that more than 75% of KC in the mouse liver can be blocked by two intravenous injections of $GdCl_3$ at 30 and 6 h before analysis (see Materials and Methods). The block lasted for at least 3 days after the second injection. $GdCl_3$ had no obvious side effects on the animals, as the levels of SGPT, TNF, IL-6, and NF-κB activation were indistinguishable from those for control mice at the time of analysis (data not shown).

Because bacterial LPS can copurify together with adenovirus particles and influence the parameters measured, only virus preparations that tested negative for LPS endotoxin were used in these studies. Furthermore, all animal studies were performed with C3H/HeJ mice, which are known to be resistant to bacterial endotoxin due to a mutation in the LPS-responsive gene in chromosome 4 (45).

Cytokine release. To determine the cytokine profiles in mice in response to adenovirus, Ad/RSVhAAT, a first-generation adenovirus, or ΔAd.hAAT, lacking E1, E2, E3, and late gene expression (29), were injected at a dose of 10^{10} transducing particles per mouse after $GdCl_3$ or saline injection. In mice that received Ad/RSVhAAT without $GdCl_3$ treatment, serum TNF concentrations were elevated by ~13-fold over baseline at 3 h after adenovirus administration, decreased at 12 h, and increased again, with a major peak at 36 h (~20-fold above normal) (Fig. 1A). KC depletion almost completely prevented the early TNF release at 3 h and significantly reduced the second peak (fourfold above baseline). ΔAd.hAAT injection induced early TNF release, but to a lesser degree (~7.5-fold) than Ad/RSVhAAT injection. Importantly, the second peak at 36 h was clearly less pronounced in animals injected with the vector depleted for viral gene expression. $GdCl_3$-pretreated mice that received ΔAd.hAAT had baseline TNF levels.

Serum IL-6 concentrations were elevated ~20-fold, with a peak about 12 h after infusion of Ad/RSVhAAT, and then gradually decreased during the next 4 days, reaching levels 4-fold above baseline (Fig. 1B). Infusion of ΔAd.hAAT caused a slight IL-6 elevation (~2 times normal) during the first 24 h. Interestingly, $GdCl_3$ pretreatment significantly stimulated IL-6 release after injection of Ad/RSVhAAT and ΔAd.hAAT.

NF-κB is a transcription factor present in an inactive cytoplasmic form in almost all cell types (2). Upon stimulation, the NF-κB dimer is released from its inhibitory subunit IκB and translocated to the nucleus, where it binds to specific DNA sites. Measuring the DNA binding activity in nuclear (liver) cell extracts by EMSA is a means to analyze the activation status of NF-κB (Fig. 2). Supershift analysis using antibodies to the NF-κB subunits p50 and p65 (not shown) designated the upper band as a p50/p65 heterodimer whose function is important in terms of transcriptional transactivation (2). The NF-κB binding activity in nuclear liver cell extracts was analyzed at different time points: 15 min, 3 h, and 3 days after adenovirus infusion. There was a remarkable NF-κB (p50/p65) activation immediately after infusion of Ad/RSVhAAT or ΔAd.hAAT in both $GdCl_3$-treated and untreated mice. The heterodimer p65/p50 binding activity was significantly lower by 3 h postinfusion. At day 3 after Ad/RSVhAAT injection, we observed a slight increase in NF-κB binding activity that was more pronounced in mice not receiving $GdCl_3$ treatment. NF-κB binding activity was almost absent at day 3 in animals receiving ΔAd.hAAT.

Liver toxicity. We reported earlier (29) that intravenous injection of 10^{10} PFU of Ad/RSVhAAT resulted in biphasic elevations in levels of SGPT, an early and sensitive marker for hepatocyte injury. Early elevations in SGPT were detected within the first 36 h after adenovirus administration, followed by chronic SGPT elevation lasting as long as viral and transgene expression were detectable. The second phase did not occur in immunodeficient animals, suggesting that it was the result of antigen-specific immunity. Here we focused on the early phase of adenovirus-induced liver toxicity (Fig. 3). In C3H mice, SGPT levels were elevated by ~15-fold during the first 2 days after infusion of Ad/RSVhAAT. In $GdCl_3$-pretreated mice, SGPT levels were increased only ~3-fold at 36 h postinfusion. Injections of ΔAd.hAAT resulted in an ~2-fold increase in the serum concentrations of the enzyme (with a small peak around 3 h). SGPT levels were normal in $GdCl_3$-treated mice that received ΔAd.hAAT. Taken together, these

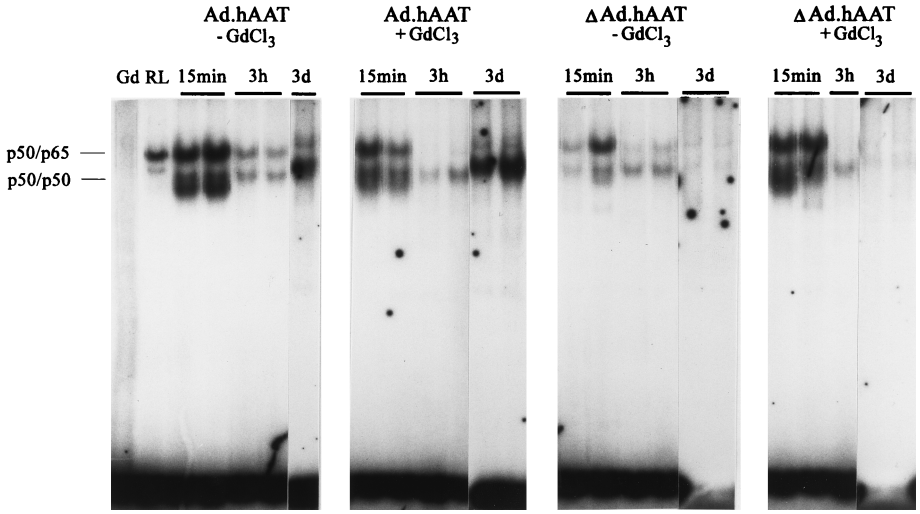

FIG. 2. NF-κB EMSA. Ad/RSVhAAT or ΔAd.hAAT was infused with or without $GdCl_3$ treatment. Controls (Gd) received $GdCl_3$ without adenovirus. Mice were sacrificed at 15 min, 3 h, and 3 days (3d) postinfusion, and nuclear extracts from livers were analyzed by EMSA, using 10 μg of nuclear protein per lane. The positions of the NF-κB p50/p65 heterodimer and p50 homodimer are indicated. Reticulocyte lysate (RL) was used as a marker to determine the position of the p50/p65 complex (13). Each lane represents an individual animal.

results suggest that adenovirus gene expression and interactions with KC are linked to hepatocyte injury.

To investigate whether the events triggered by adenovirus infection in vivo can lead to hepatocyte death by apoptosis, TUNEL studies were carried out on liver sections obtained at different times after adenovirus infusion. Representative microphotographs shown in Fig. 4b revealed that at 15 min and 3 h (data not shown), TUNEL signals were detected only in small nonparenchymal cells in mice treated with $GdCl_3$. Positive signals in hepatocytes were found at day 3 p.i., with ~1 to 3% apoptotic hepatocytes in livers of mice receiving Ad/RSVhAAT in both $GdCl_3$-treated and untreated animals (Fig. 4c to f). Fewer apoptotic cells ($\ll$1%) were found in mice infused with ΔAd.hAAT (Fig. 4g and h).

A neutrophil/lymphocyte infiltrate that was most pronounced in the periportal regions was found in liver sections from mice after infusion of Ad/RSVhAAT (Fig. 4c). Notably fewer inflammatory cells were present at day 3 in livers from $GdCl_3$-pretreated mice that received Ad/RSVhAAT and in mice after infusion of ΔAd.hAAT.

The liver responds to damage and cytokine release with regeneration, including hepatocellular DNA synthesis. The percentage of regenerating hepatocytes was quantified by use of [^{3}H]thymidine, the incorporation of which into DNA during replication can be detected in liver sections (Fig. 5). The peak number of replicating hepatocytes (69 and 37% in two different animals [Fig. 5b and d]) was found at day 4 after Ad/RSV hAAT infusion, with a trend toward greater DNA replication after $GdCl_3$ pretreatment (87 and 49% [Fig. 5c and e]). ΔAd.hAAT infusion induced DNA synthesis in less than 1% of hepatocyte nuclei in livers without $GdCl_3$ pretreatment and in ~2% in $GdCl_3$-injected mice (Fig. 5f and g). [^{3}H]thymidine incorporation before and after day 4 was significantly reduced in all animal groups, and naive controls had less than 0.01% labeling in hepatocytes (not shown).

The extent of hepatocyte replication after infusion of first-generation adenovirus is comparable to that observed during liver regeneration after partial hepatectomy. In both situations, NF-κB activation and/or high IL-6 levels could be among the initial triggers that induce hepatocellular DNA synthesis (50). Interestingly, KC depletion enhanced the adenovirus-induced DNA synthesis. This finding is in agreement with published data; treatment that inhibits TNF release from KC increases DNA replication after partial hepatectomy (9, 10, 34).

Effects on level and persistence of transgene expression. Innate immune mechanisms clearly influence the antigen-dependent responses by affecting antigen presentation and recruitment of immunologic effector cells. This may alter the final fate of adenovirus-transduced cells and transgene expression. We wanted to determine whether $GdCl_3$ treatment that depleted KC would influence antigen-specific immune mechanisms that limit transgene expression after adenovirus-mediated liver gene transfer. To explore this possibility, we followed serum hAAT concentrations after Ad/RSVhAAT injection in C3H mice with and without prior KC depletion. From earlier studies, it was known that this mouse strain represents a so-called short expressor because the transgene product, serum hAAT, declined to undetectable levels within the first 2 to 3

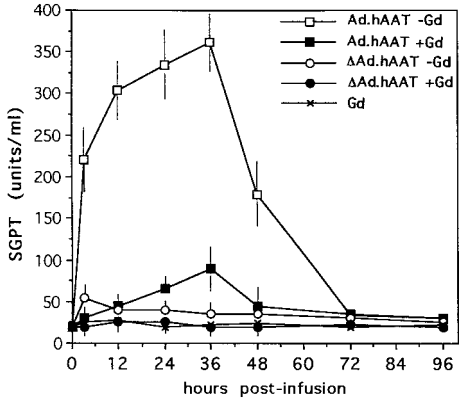

FIG. 3. Liver injury. SGPT concentrations were determined in mice infused with Ad/RSVhAAT or ΔAd.hAAT. +Gd, after $GdCl_3$ pretreatment; −Gd, without $GdCl_3$. Controls (Gd) received $GdCl_3$ without adenovirus infusion. n = 3 animals per point.

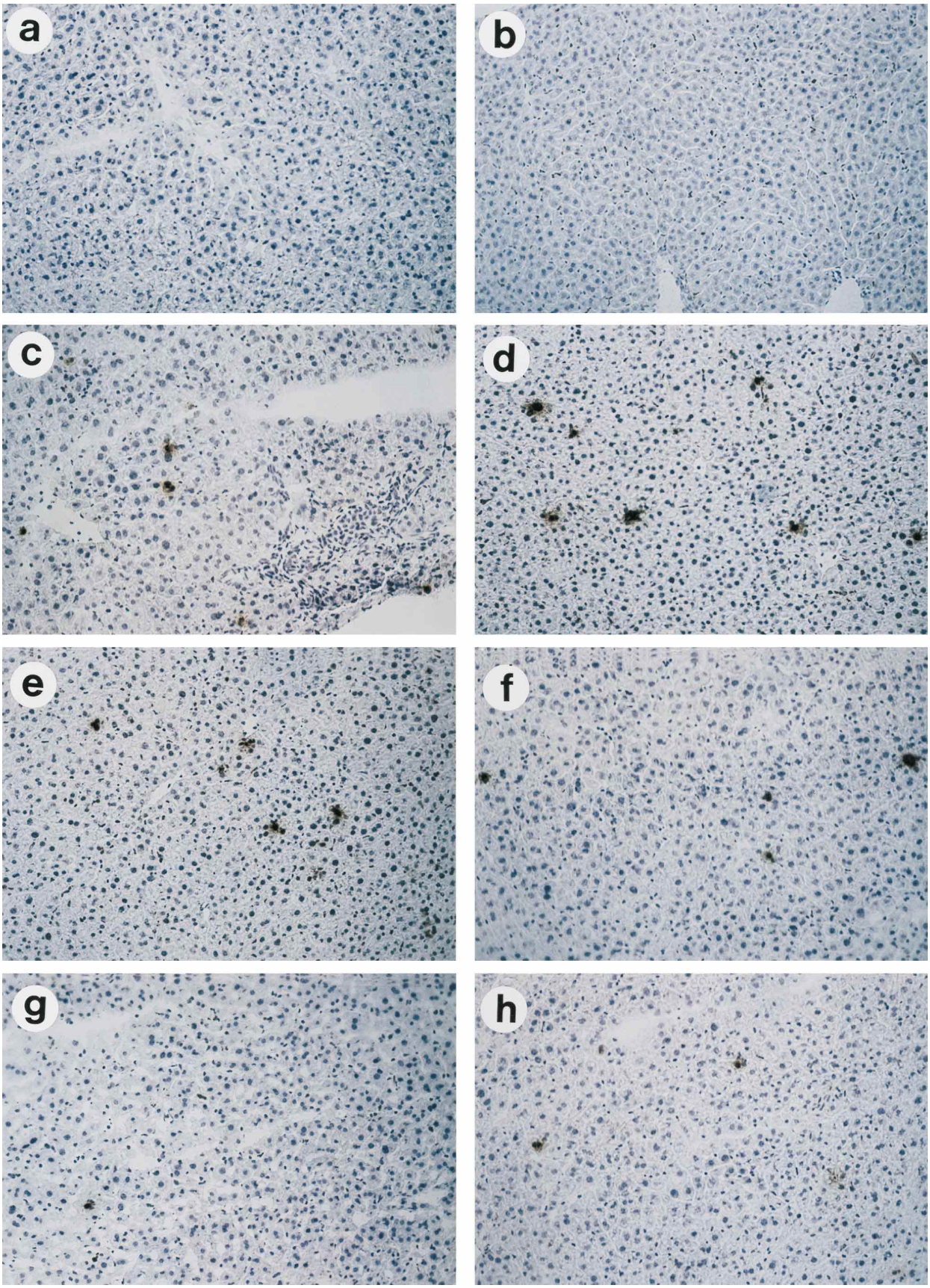

FIG. 4. TUNEL apoptosis assays. Apoptotic cell death was analyzed by the TUNEL technique in liver sections. (a) Liver section without adenovirus or $GdCl_3$ treatment; (b) $GdCl_3$ treatment only; (c and e) livers from two animals at day 3 after Ad/RSVhAAT without $GdCl_3$ pretreatment; (d and f) livers from two animals at day 3 after Ad/RSVhAAT with $GdCl_3$ pretreatment; (g) liver section after ΔAd.hAAT without $GdCl_3$; (h) liver section at day 3 after ΔAd.hAAT with $GdCl_3$. Slides are counterstained with hematoxylin. Note the neutrophil infiltration in panels c and e.

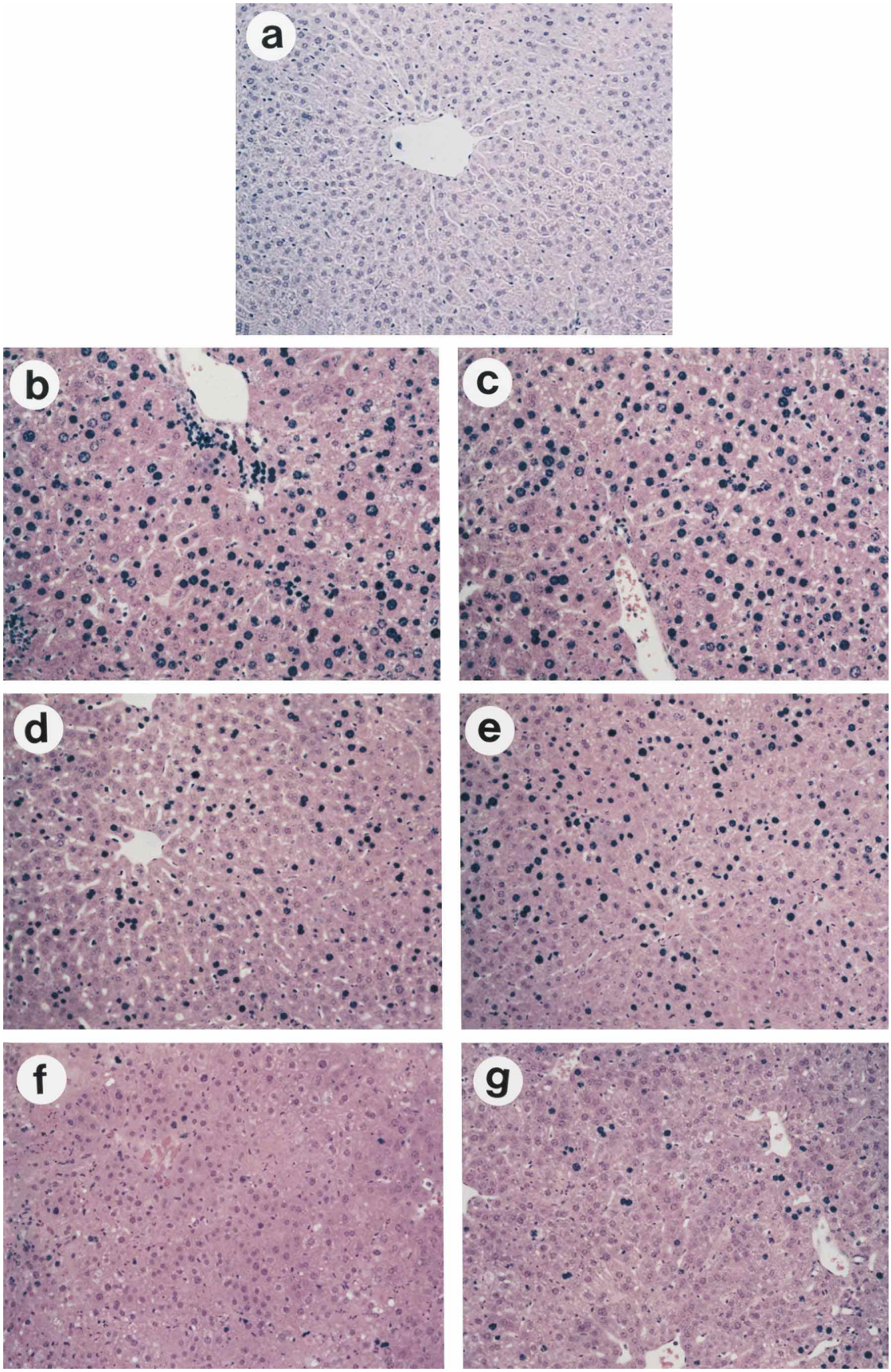

FIG. 5. Hepatocellular DNA synthesis. Analysis of hepatocellular DNA synthesis in liver sections at day 4 after adenovirus infusion. A total of 10^{10} transducing particles of Ad/RSVhAAT (b to e) or ΔAd.hAAT (f to g) were infused in mice after $GdCl_3$ pretreatment (c, e, and g) or without $GdCl_3$ pretreatment (b, d, and f). (a) Liver section after $GdCl_3$ administration only. At 12 and 1 h before sacrifice, the animals were infused with [*methyl*-^{3}H]thymidine. Liver sections were exposed to film emulsion for 2 weeks and counterstained with hematoxylin-eosin.

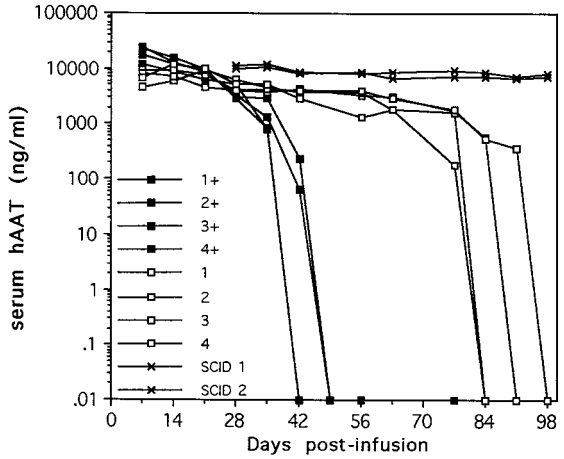

FIG. 6. Transgene expression in mice. Serum levels of hAAT after in vivo gene transfer. C3H mice were injected with 10^{10} transducing particles of Ad/RSVhAAT after $GdCl_3$ pretreatment (filled squares) or without $GdCl_3$ pretreatment (open squares). Serum samples were collected periodically and analyzed for hAAT by ELISA. For comparison, the same dose of Ad/RSVhAAT was injected into immunodeficient C3H-SCID mice without $GdCl_3$ (crosses). Each line represents an individual animal.

months after administration of Ad.RSVhAAT (3). In our studies, serum hAAT concentrations dropped after 80 to 100 days in C3H mice and were persistent in C3H-SCID mice (Fig. 6). Interestingly, in $GdCl_3$-pretreated mice, hAAT levels were initially two to three times higher but declined to zero ~50 days earlier than in nontreated C3H mice.

To investigate the source for these differences in transgene expression, viral DNA was quantified in preparations of genomic liver DNA at 5, 60, and 100 days after infusion of Ad/RSVhAAT (Fig. 7). In $GdCl_3$-treated mice, the concentration of Ad/RSVhAAT DNA was ~2.5-fold higher at day 5 than in untreated mice. This is in agreement with data from Worgall et al. (48), who reported that >90% of vector DNA is lost as a result of KC function soon after adenovirus administration. At 60 and 100 days postinfusion, viral DNA was still detectable at low levels in livers from C3H mice; these levels were comparable with vector DNA concentrations in livers from C3H-SCID mice. Thus, at time points when serum hAAT was no longer detectable, transduced vector DNA was still present in the livers of C3H mice. This finding suggests that CTL responses directed against the transduced cells are not responsible for loss of gene expression. One possible explanation is that hAAT gene expression is blocked at the level of transcription, translation, or posttranslational processing. Tsui et al. (42) reported that CTL-derived cytokines can induce posttranscriptional clearance of hepatitis B virus RNA in infected hepatocytes. We did not investigate this possibility but concentrated on an observation recently made by Schowalter et al. (reference 37 and unpublished results), who demonstrated that antibodies to the expressed transgene product (hAAT) reduce the level of detectable serum hAAT. To evaluate this possibility, we determined anti-hAAT antibody titers in the serum of C3H mice with and without prior $GdCl_3$ treatment (Table 1). Early production of antibodies against hAAT was suppressed in $GdCl_3$-treated mice, possibly due to inhibition of antigen presentation by KC. At later time points (weeks 4 and 10), the concentration of hAAT-specific antibodies was higher in $GdCl_3$-treated mice. We hypothesize that other antigen-presenting cells (e.g., splenic macrophages) were stimulated in KC-depleted mice or that viral antigens and/or transgene products synthesized in transduced hepatocytes were taken up by the replaced KC. As a result, perhaps an enhanced humoral immune response to the transgene products was responsible for the early falloff of serum hAAT concentrations in $GdCl_3$-treated C3H mice.

DISCUSSION

The pathologic changes in the liver that occur soon after adenovirus infusion result from a combination of a direct cytotoxic effect of expressed viral proteins and innate immune defenses to virus infection. We analyzed the changes in some of the important elements of the innate immune response, NF-κB, TNF, and IL-6, in correlation to liver damage and transgene expression after injection of a first-generation adenovirus (Ad/RSVhAAT) and a vector deleted for E1, E2, E3, and late gene expression (ΔAd.hAAT) in mice that received $GdCl_3$, an agent that selectively eliminates large periportal KC for 3 to 4 days after intravenous administration.

We observed a biphasic elevation of serum TNF, with the first peak occurring shortly after adenovirus administration and a second major peak at 36 h. We assume that the peak at 3 h represents TNF release as a result of KC activation occur-

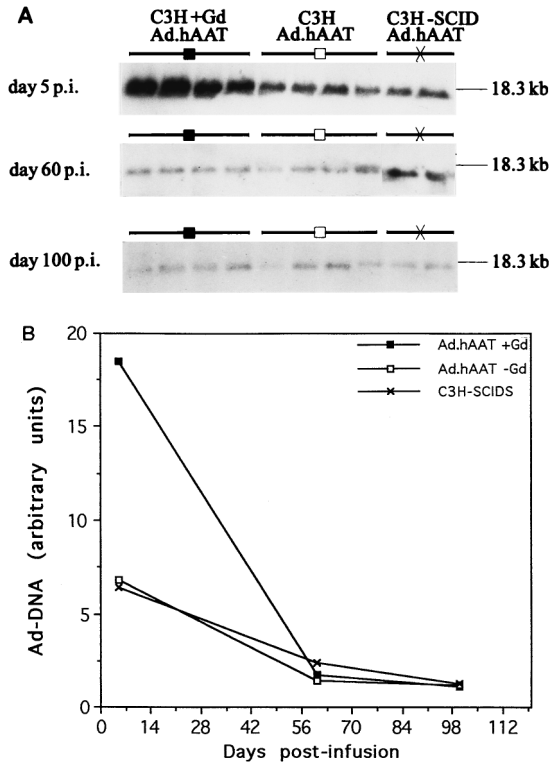

FIG. 7. DNA analysis. (A) Southern blot analysis of transduced adenovirus vector DNA in genomic liver DNA. Animals (C3H or C3H-SCID mice) were sacrificed at different time points (days 5, 60, and 100) after infusion of 10^{10} transducing particles of Ad/RSVhAAT after $GdCl_3$ pretreatment (filled squares) or without $GdCl_3$ pretreatment (open squares). Crosses represent SCID mice without $GdCl_3$. Ten micrograms of *Bam*HI-digested genomic DNA was loaded in each lane. Blots were hybridized with a ^{32}P-labeled 1.4-kb hAAT probe. The 18.3-kb band is a specific *Bam*HI fragment containing the hAAT expression cassette. Liver DNA for the 100-day time point was obtained from animals that were monitored for serum hAAT in Fig. 6. The filters for the day 5 and day 60 and 100 time points were exposed for 2 and 72 h, respectively. (B) DNA quantification. The relative amounts of adenovirus DNA were determined by phosphorimager analysis and expressed as a ratio between the sample signal and a control signal (10 pg of Ad/RSVhAAT DNA).

TABLE 1. Anti-hAAT MAb levels

Treatment	Serum anti-hAAT IgG[a]		
	Wk 2 p.i.	Wk 4 p.i.	Wk 10 p.i.
Ad/RSVhAAT − Gd	++, ++, ++	++, +++, ++	++, +++, ++
Ad/RSVhAAT + Gd	+, −, +	++, +++, +++	++++, +++, ++++

[a] Semiquantitative measurement for each animal in comparison to a standard curve of anti-hAAT MAbs and expressed as not detected (−) or detection similar to that of a standard MAb diluted 1:10,000 (+), 1:1,000 (++), 1:100 (+++), or 1:10 (++++).

ring during binding and internalization of adenovirus particles (Ad/RSVhAAT and ΔAd.hAAT) because it can be blocked by $GdCl_3$. The nature of the second TNF peak is correlated with the onset of early viral gene expression in Ad/RSVhAAT-transduced cells (29); it was significantly reduced when viral E2 gene expression was absent (ΔAd.hAAT). The major source of the second peak of TNF probably represents de novo production induced by early viral gene expression in cells transduced with Ad/RSVhAAT.

Liver damage measured by SGPT elevation is temporally correlated with serum TNF elevations and can be substantially prevented by $GdCl_3$ treatment. Thus, we conclude that TNF release/synthesis after KC activation is one of the etiologic factors for adenovirus-induced liver pathology. In this context, de novo synthesis of early viral antigens contributed, probably via TNF stimulation, to the observed hepatocellular injury, because intravenous infusion of ΔAd.hAAT particles did not lead to significant hepatocellular damage. Notably, hepatocellular apoptosis is not the major mechanism in TNF-induced liver injury after administration of first-generation adenoviruses. In contrast, infection with E1a-competent (wild-type) adenovirus sensitizes cells to apoptosis by TNF (17, 46). The infiltration of inflammatory cells observed in liver sections at day 3 after Ad/RSVhAAT infusion was efficiently blocked by $GdCl_3$, pretreatment. Reduced TNF release as a result of KC depletion could be among the factors that inhibit chemotaxis of neutrophils.

Intravenous administration of the first-generation adenovirus, Ad/RSVhAAT, resulted in substantial increases in serum IL-6 concentrations over the analyzed time period of 4 days, with a peak at 12 h postinfusion. $GdCl_3$ pretreatment did not block but rather enhanced IL-6 release, suggesting that the IL-6 production originated from cells other than KC. Possible candidates are splenic macrophages (not affected by $GdCl_3$) or endothelial cells, which may receive a greater viral load in KC-depleted mice. This could lead to their enhanced activation and cytokine production. High IL-6 levels affect hepatocyte metabolism (1) and stimulate CTL activation and infiltration (16). Further experiments are required to test this hypothesis.

We observed a strong NF-κB activation in the liver after intravenous adenovirus infusion. The mechanisms by which adenovirus activates NF-κB are not known. Since the activation occurs within 20 min after vector administration, it is unlikely that viral gene expression is involved. Moreover, ΔAd.hAAT exerts the same effect, indicating that the virus particle itself triggers the process. The same NF-κB activation pattern is observed in mice after KC depletion, suggesting that activation takes place in hepatocytes. We speculate that fiber binding to the adenovirus receptor or subsequent events such as the interaction of pentons with integrins or endosome lysis activate kinases that phosphorylate IκB. A possible candidate for such a kinase is the Raf/mitogen-activated protein kinase (6). There was no correlation between the observed NF-κB activation occurring in both control and KC-depleted mice and the increased TNF and SGPT levels. This is different than in other studies, where at later time points, the activation and release of inflammatory cytokines could be prevented by blocking NF-κB based on ectopic overexpression of a nucleus-localized IκB after adenovirus-mediated gene transfer (49).

To make space for cloning larger inserts, E3 deletions were introduced into first-generation adenovirus vectors, based on the premise that E3 proteins are not necessary for the adenovirus life cycle (4). However, studies by Ginsberg et al. (14) and Sparer et al. (40) in a mouse model for adenovirus pneumonia demonstrated that adenovirus lacking the E3-TNF resistance genes (14.7K, 10.4/14.5K, and gp19K) induce a more severe pulmonary disease, characterized by alveolar infiltration, than wild-type virus. Importantly, the E3 promoter is the only adenovirus promoter that contains binding sites for NF-κB, allowing for E1a-independent expression (12). Based on our results, it is appealing to consider that during adenovirus infection, NF-κB activates the E3 promoter and thereby induces the E3 proteins that block the antiviral and cytotoxic effect of TNF in hepatocytes. Moreover, a recent study (19) shows that antigen-dependent immunity may be reduced with constitutive E3 gene expression; however, the exact mechanism and its effects on early liver toxicity are not known. Thus, a restoration of E3 gene expression under the endogenous E3 promoter may reduce the hepatotoxicity of first-generation adenoviruses.

Another practical consequence of NF-κB activation in hepatocytes after adenovirus infusion is that certain (viral) promoters with NF-κB binding sites, for example, the CMV promoter (36), that were used to drive transgene expression were activated by NF-κB binding. The observation that after adenovirus-mediated liver gene transfer the CMV promoter is only transiently active in hepatocytes may be related to NF-κB activation (21, 22). This possibility should be taken into consideration when viral promoters are used for transgene expression.

The etiology of hepatocellular apoptosis observed at day 3 after adenovirus infusion is not clear. The number of apoptotic cells is relatively low in livers after ΔAd.hAAT or AdRSV hAAT. However, both vectors still contain the E4 region, and expression of E4 proteins in transduced hepatocytes could be responsible for the low level of apoptosis seen in liver sections at day 3 after vector infusion (41). On the other hand, apoptosis could be linked to NF-κB activation occurring immediately after adenovirus infusion. Although NF-κB activation has been shown for many viruses, only its activations by Sindbis virus and dengue virus (30) are definitive examples of virus-induced apoptosis.

Although we did not study the effects of $GdCl_3$ treatment on the development of an antigen-specific cellular immune response systematically, we observed an enhanced humoral response to the transgene product hAAT in KC-depleted mice at later time points. Thus, blocking elements of the innate immune response by depletion of KC while reducing early adenovirus-mediated liver toxicity shortens transgene expression due to an enhanced antigen-specific humoral immune re-

sponse. This is probably a result of virus spillover into other organs like the spleen or may be related to repopulation of KC and antigen presentation from viral proteins leaking from the hepatocytes into these cells.

Recently Wolff et al. (47) and Kuzmin et al. (26) analyzed in BALB/c mice the effect of macrophage depletion by intravenous injection of liposome-encapsulated dichloromethylene biphosponate (Cl_2MBP) on transgene expression after adenovirus-mediated gene transfer; however, its effect on the innate immune response and the correlation to liver pathology were not investigated. The authors found an inhibition of early antibody production and increased transgene expression level in KC-depleted mice compared with untreated mice. However, in contrast to our results, KC depletion by Cl_2MBP-liposomes in BALB/c mice extended the persistence of hAAT expression after adenovirus-mediated gene transfer (48). The humoral and cellular immune responses to viral and transgene product vary between different mouse strains (3). The decline in serum hAAT levels observed in C3H mice appears to be related to an antibody response against the transgene product, whereas in BALB/c mice, the loss of DNA in transduced hepatocytes is thought to be responsible for extinction of gene expression (36a). Second, the block of reticuloendothelial cell function by Cl_2MBP-liposomes and $GdCl_3$ is qualitatively different; whereas only large liver macrophages are vulnerable to $GdCl_3$, Cl_2MBP-liposomes affect other macrophage populations as well, including macrophages of the spleen, an organ that does receive adenovirus after intravenous adenovirus administration (29) and is known to secrete a different spectrum of cytokines (35). Third, macrophage studies of BALB/c mice may be problematic, because macrophages of this inbred strain have a reduced capacity to inactivate parasites such as *Mycobacterium bovis* BCG, *Mycobacterium smegmatis*, *Salmonella typhimurium*, or *Leishmania donovani* due to a mutation in the Bcg and/or Nramp gene (43). The C3H/HeJ mouse strain used in our studies represents a Bcg-resistant (wild-type) strain (43).

In general, the clinical application of KC-depleting agents such as $GdCl_3$ and Cl_2MBP is limited due to their pharmacological side effects (8, 20, 35) and their potential damage of organs other than liver resulting from viral spillover beyond the KC (47). We noticed that the treatment with $GdCl_3$, while preventing liver damage and cytokine release, made the animals more susceptible to high viral doses. When more than 4×10^{10} PFU of adenovirus per mouse ($n = 3$ or more per group) was injected, the mortality 24 h after infection was twofold greater in $GdCl_3$-treated mice than in untreated animals (not shown).

The interactions of recombinant adenovirus with the host liver are complex, and here we have started to uncover some of these early events. Clearly, host responses to adenovirus can vary greatly between animals with different genetic backgrounds, and they will be more difficult to unravel in a genetically diverse, human population. To reduce early toxicity induced by adenoviruses, further improvements of adenovirus-based gene delivery should focus on the use of vectors depleted for viral gene expression; on the restoration of E3 genes that protect cells from TNF-induced cytolysis; on the pursuit of other routes for vector delivery, such as infusion into the bile duct to reduce activation of KC; or on the transient repression of serum TNF or NF-κB, for example, by TNF antibodies, chimeric TNF receptors (25), or steroids.

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