Heterologous expression of adenovirus E3-gp19K in an E1a-deleted adenovirus vector inhibits MHC I expression *in vitro*, but does not prolong transgene expression *in vivo*

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An E1a-deleted adenovirus vector constitutively expressing native adenovirus E3-gp19K (Ad.RSV-gp19K) was constructed in order to determine whether or not E3-gp19K mediated interference with antigen presentation would result in prolonged transgene expression *in vivo*. Cultured fibroblasts infected with Ad.RSV-gp19K produced a native size gp19K protein and had decreased cell surface levels of MHC I as shown by immunoprecipitation and flow cytometry. The congenic mouse strains Balb/b (H-2^b MHC I with high gp19K affinity), Balb/k (H-2^k MHC I with no gp19K affinity), and Balb/c (H-2^d MHC I with moderate gp19K affinity) were chosen for *in vivo* experiments because of their range of gp19K affinities. Following transduction of mice from each strain with Ad.RSV-gp19K and Ad/RSV-hAAT (a reporter adenovirus), or Ad/RSV-cFIX (control

adenovirus) and Ad/RSV-hAAT, the level and duration of serum hAAT protein were unrelated to gp19K protein expression. Evaluation of MHC I abundance on hepatocytes following *in vivo* transduction demonstrated that recombinant adenovirus rapidly increased the abundance of surface MHC I molecules on hepatocytes, and surface MHC I molecules were reduced earlier and to a greater extent following wild-type adenovirus infection compared with hepatocytes transduced with control or Ad.RSV-gp19K recombinant adenovirus. This difference in surface MHC I down-regulation may be related to the different promoters (RSV-LTR versus the native E3 promoter), and will be an important consideration in the development of newer generation adenovirus vectors designed to evade host immune responses.

Keywords: adenovirus; hepatic gene therapy; E3 region; RSV-LTR; E3-gp19K protein; immune evasion; congenic mice strains

Introduction

The future success of adenovirus vectors for *in vivo* gene therapy depends upon our ability to understand and effectively evade the host's immune response towards: the virus, cells infected with virus, and the newly expressed transgene.^{1–4} Some success in 'down-regulating' responses towards the virus and cells infected with virus have recently been reported.^{5–8} These studies utilize the administration of systemic immunomodulatory drugs to achieve prolonged transgene expression by depleting T cells, interfering with T cell receptor function, or interfering with co-stimulatory signals required for T cell activation.

The adenovirus E3 region potentially encodes nine proteins, is well conserved between serotypes, and is not essential for viral replication.^{9–11} Four of these nine proteins have been characterized, and appear to function as immunoregulatory proteins.^{10,11} Three of these immunoregulatory proteins (14.7k, 14.5k and 10.4k) have been

found to prevent TNF-mediated cytotoxicity *in vitro*.^{12–14} The fourth, E3-gp19K, has been shown to inhibit target cell lysis by activated T lymphocytes (CTLs)¹⁵ by binding and retaining these molecules in the endoplasmic reticulum, and thus stopping the migration of MHC I alpha chain molecules to the cell surface.^{16,17} Decreasing cell surface levels of MHC I and the expression of proteins which interfere with TNF are two mechanisms that wild-type adenovirus utilizes in evading the host immune response.¹¹

Transgenic mice containing the adenovirus E3 region with its native promoter have been found not to express significant amounts of any E3 proteins in hepatocytes in the absence of E1a protein.¹⁸ Given this information, it seems unlikely that E3-gp19K is expressed from murine hepatocytes following infection with first generation adenovirus vectors, but this remains to be directly shown. It has also been demonstrated that constitutive expression of the adenovirus E3 protein gp19K with β -galactosidase in murine hepatocytes 'strongly' decreased the formation of antivector lymphocytes,¹⁹ but it is not clear from this study if this decrease in antivector lymphocytes would result in prolonged transgene expression.

To determine if constitutive expression of E3-gp19K

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protein would result in prolonged transgene expression following *in vivo* transduction of murine hepatocytes, a recombinant adenovirus (Ad.RSV-gp19K) was prepared containing the wild-type (Ad2) E3-gp19K gene 3' to the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. This promoter was chosen because we have previously found it to produce high levels of transgene expression in murine hepatocytes following *in vivo* transduction.^{1,5} This virus and another reporter adenovirus producing human alpha-1-antitrypsin (Ad/RSV-hAAT) were then injected into three congenic strains of mice with class I MHC molecules differing in affinity for gp19K, and the serum level of hAAT protein monitored over time.

Results

Characterization of recombinant adenovirus Ad.RSV-gp19K

The genomic structure of the recombinant adenovirus, Ad.RSV-gp19K was confirmed by restriction digestion. To ensure that Ad.RSV-gp19K produced a protein of the appropriate size, immunoprecipitation of gp19K protein was performed 48 h after infection of 1×10^7 SVB6 murine fibroblasts at an MOI (multiplicity of infection) of 300 with Ad.RSV-gp19K. An MOI of 300 was selected because preliminary experiments with Ad.RSV. β gal and SVB6 cells demonstrated approximately 80% blue nuclear staining at this MOI (data not shown). The results shown in Figure 1 demonstrate that the anti-gp19K immune serum precipitated a unique protein of approximately 26 kDa in size (lane A) from the cytosol of Ad.RSV-gp19K infected SVB6 fibroblasts, while pre-immune serum (lane B) did not. Additionally, immune serum did not precipitate any proteins of this size from uninfected cells (Figure 1, lane C). The migration of the gp19K protein in this size range is predicted from earlier studies and is likely due to abundant glycosylation.²⁰

While previous transgenic mice studies have determined that in murine liver, no or minimal expression is occurring from the E3 promoter in the absence of E1a,¹⁸ and it is generally felt that in the absence of the E1a protein gene expression from the E3 promoter is weak,²¹ it has not been definitively shown if E1a-deficient first generation recombinant adenovirus vectors produce any E3 protein products in SVB6 cells which were transformed by SV40 large T antigen. Since Ad.RSV-gp19K contains the native gp19K gene in the E3 region, a second immunoprecipitation experiment was performed to estimate the amounts of gp19K protein produced from the heterologous and endogenous promoters in SVB6 cells. To this end, Ad.RSV-gp19K or Ad/RSV-hAAT viruses containing the same endogenous E3 region were used to infect SVB6 fibroblasts. Twenty-four hours later, after immunoprecipitation, quantification of gp19K protein was performed on a phosphoimager. The results are shown in Table 1. For two different immunoprecipitations, Ad.RSV-gp19K infected SVB6 fibroblasts produced three times more gp19K protein than Ad/RSV-hAAT recombinant vector containing the native E3-gp19K sequences. It is of interest to note that Ad.RSV-gp19K produced about the same amount of gp19K protein as that produced following infection with wild-type adenovirus (Ad2) at one-third the MOI.

Figure 1 Immunoprecipitation analysis of gp19K. Immunoprecipitation of gp19K was performed on 10^7 c.p.m. of metabolically labelled cytosol prepared 48 h following infection with Ad.RSV-gp19K (lanes A and C), or mock infection (DMEM only) (lane B) with immune serum (lanes A and B) or pre-immune rabbit serum (lane C). Migration of molecular weight markers (69 kDa bovine serum albumin, 46 kDa ovalbumin, 30 kDa carbonic anhydrase, 22 kDa trypsin inhibitor, and 14 kDa lysozyme) and the band corresponding to gp19K (●) are shown to the left.

Table 1 Relative amounts of E3-gp19K protein

Recombinant Ad	MOI	Relative amount
Ad.RSV-hAAT	300	18
Ad2	100	66
Ad.RSV-gp19K	300	63
Ad.RSV-gp19K	300	53

E3-gp19K was immunoprecipitated from SVB6 fibroblasts that were infected 24 h before metabolic labeling. Relative amounts were determined as noted in the Materials and methods.

To demonstrate that Ad.RSV-gp19K produced a functional protein, infected SVB6 fibroblasts were subjected to staining with antibodies against MHC I proteins and quantified by fluorescence activated cell sorting (FACS) analysis (Figure 2). By 18 h after injection (Figure 2a), the intensity of MHC I on the fibroblasts was decreased in cells treated with wild-type adenovirus but not in Ad/RSV-hAAT, Ad.RSV-gp19K, or vehicle treated cells. By 72 h after infection (Figure 2b), there was an increase in MHC I staining in cells treated with Ad/RSV-hAAT or Ad/RSV-gp19K (E3-) compared with vehicle-treated controls. It is interesting to note that the presence of an intact, endogenous E3-gp19K gene in the vector

Figure 2 MHC I analysis of SVB6 murine fibroblast following *in vitro* transduction with adenovirus. (a) The fluorescence profiles of 20 000 fibroblasts stained with anti-MHC I (H-2^k) antibody, isolated 18 h after infection with vehicle only, 2×10^9 p.f.u. of wild-type Ad2 and 6×10^9 p.f.u. of Ad.RSV- β gal (Ad2), 8×10^9 p.f.u. of Ad.RSV-gp19K, or 8×10^9 p.f.u. of Ad/RSV-hAAT. The profile of fibroblasts treated with vehicle only and stained with anti-MHC I (H-2^k) antibody (vehicle H-2^k) is included as a negative control. (b) The fluorescence profiles of fibroblasts stained with anti-MHC I (H-2^b) antibody, isolated 72 h after infection with vehicle only, 8×10^9 p.f.u. of Ad.RSV-gp19K, 8×10^9 p.f.u. of Ad/RSV-hAAT, or 8×10^9 p.f.u. of Ad/RSV-hAAT (E3-). Note: The variation in fluorescence in the non-adenovirus-infected control SVB6 cells between panels a and b reflects slightly different fluorescence detector settings used in the two experiments.

(Ad/RSV-hAAT) did not affect MHC I patterns (Figure 2b). In contrast, the Ad.RSV-gp19K infected fibroblasts showed diminished intensity of MHC I staining compared with Ad/RSV-hAAT and Ad/RSV-hAAT(E3-) infected profiles at 72 h after infection. Fibroblasts infected with wild-type adenovirus (type 2) showed complete cytopathic effect (CPE) by 72 h and were not available for analysis. An experiment similar to that shown in Figure 2 using PC3H cells (H-2k haplotype) with surface MHC I molecules with no affinity for gp19K showed no significant decrease in surface MHC I abundance following wild-type and recombinant adenovirus infections (data not shown). This allowed us to conclude that the decreased abundance of surface MHC I was mediated by expressed gp19K protein and not the result of a general decrease in transcription following adenovirus infection.

The immunoprecipitation and FACS analysis taken together demonstrate that the exogenous E3-gp19K protein derived from the transgene in Ad.RSV-gp19K was functional, and in the absence of E1a, some endogenous E3-gp19K was produced in the transformed cell line SVB6, as previously noted in other cells,^{22,23} but that this level of gp19K expression did not significantly decrease cell surface MHC class I expression. However, down-regulation of surface MHC I levels was more rapidly achieved following wild-type adenovirus infection of SVB6 fibroblasts than compared with surface MHC I levels following infection with Ad.RSV-gp19K.

Constitutive expression of gp19K *in vivo*

E3-gp19K gene expression levels from a native *versus* heterologous promoter do not necessarily correlate with *in vivo* patterns of gene expression. However, functional studies related to a particular vector can be assessed in tissue culture. E3-gp19K has the highest affinity for MHC I molecules of the murine H-2^b haplotype (Balb/b and C57B1/6), an intermediate affinity for the H-2^d haplotype (Balb/c), and no affinity for H-2^k (Balb/k).^{9,24,25} To determine if constitutive expression of gp19K *in vivo* would result in a prolongation of hAAT reporter gene expression in permissive haplotypes, Balb/c, Balb/b, and

Balb/k mice were each divided into two groups, and each group was administered two viruses, Ad/RSV-hAAT and Ad.RSV-gp19K, or Ad/RSV-hAAT and Ad/RSV-cFIX (as a control). A vector expressing cFIX was used as a control because of the concordance of persistence of this protein with hAAT in similar inbred mouse strains.^{1,26} The ratio of the two viruses was 1:3, reporter virus to Ad.RSV-gp19K, and the total number of adenovirus particles transduced held constant at 8×10^9 p.f.u. because this represents at least 20–50 adenovirus genomes per hepatocyte on average, and has been shown not to produce severe liver damage.^{2,27,28} To ensure that administration of this amount of adenovirus would result in transfected hepatocytes containing multiple copies of adenovirus genome, the FISH analysis shown in Figure 3 was performed on hepatocyte nuclei isolated 72 h after infection with 5×10^9 p.f.u. Ad/RSV-hAAT. Hybridization was performed with the 30 kb adenoviral plasmid pBHG10 as noted in the Materials and methods. The two representative cells shown in Figure 3a demonstrate the multiple punctate spots of hybridization, representing 19 and 26 adenovirus genomes within each. This range of hybridization varied between two and 100 punctate spots per nuclei. However, the adenoviral signals seen in the majority of nuclei on this slide were similar to that shown in Figure 3, and there were no signals seen in any of the nuclei from uninfected animals as demonstrated in Figure 3b. Thus, a 1:3 excess of reporter to Ad.RSV-gp19K would assure that relatively few cells would contain only the reporter genome.

The serum hAAT levels determined by ELISA are shown in Figure 4 for both groups of Balb/c (a), Balb/k (b), and Balb/b (c) congenic mice. Within each strain of mice, there was no clear difference in the duration of hAAT expression between the mice which did and did not receive Ad.RSV-gp19K. However, there is a striking prolongation of serum hAAT levels in the Balb/b mice compared with their congenic parental strain Balb/c, regardless of E3-gp19K expression. This duration of hAAT expression is similar to that seen in C57B1/6 mice,¹ which is the H-2^b haplotype donor strain for Balb/b congenic mice.

Figure 3 Fluorescence in situ hybridization (FISH) of murine hepatocyte nuclei following adenovirus transduction. FISH was performed on murine hepatocyte nuclei isolated 72 h after tail vein injection of C57B1/6 mice with 5×10^9 p.f.u. of Ad/RSV-hAAT (a) or an untreated control (b). Hybridization and FITC staining was performed as noted in the Materials and methods with the 30 kb adenovirus plasmid pBHG10.

Determination of the origin of gp19K transcript in hepatocytes following in vivo gene transfer

While it is expected in the absence of the E1a protein that gene expression from the endogenous adenovirus E3 promoter will be weak following *in vivo* transduction of murine hepatocytes,^{18,21–23} this had not been previously evaluated using E1-deleted first generation adenovirus vectors. The gp19K cDNA used to make Ad.RSV-gp19K encodes the gp19K gene from type 2 adenovirus. While it has high homology at the amino acid level, differences in the nucleotide sequence result in the placement of a unique *Hind*III site in the middle of the Ad2 gp19K transcript. This sequence difference provides a means of identifying the relative amount of gp19K transcript from the E1 transgene *versus* the amount of transcript from the native gp19K gene in the E3 region using RT-PCR analysis of mRNA isolated from mouse liver following transduction with adenovirus (Figure 5).

A gp19K-amplified fragment of expected size was obtained only from the mRNA of the mouse which

received Ad/RSV-hAAT and Ad.RSV-gp19K, (Figure 5a, lane 3) and in the positive controls (Figure 5a, lanes 8 and 9). Cleavage of virtually all of this amplified fragment with *Hind*III (Figure 5b, lane 3) suggested that the vast majority of this gp19K transcript was produced from the E1-inserted transgene and not from the gp19K gene in the endogenous E3 region. A similar RT-PCR assay with PCR primers specific for hAAT demonstrated the presence of hAAT transcripts in only the mRNA from Ad/RSV-hAAT infected and Ad/RSV-hAAT + Ad.RSV-gp19K-infected animals (data not shown).

Down-regulation of MHC I following wild-type Ad2 infection but not Ad.RSV-gp19K infection

Fluorescence activated cell sorting (FACS) was chosen as a means to compare the ability of Ad.RSV-gp19K and wild-type Ad2 to down-regulate MHC I in murine hepatocytes at various times following transduction. C57B1/6 mice (H-2^b haplotype) were killed 1, 4 and 14 days after injection of recombinant adenoviruses, and fresh hepato-

Figure 3 Continued (c and d) The same nuclei following staining with DAPI, a chromatin-binding dye. The difference in chromatin staining intensity in c and d results from different photographic exposure times. Several of the multiple bright spots representing a specific signal in a are noted with an arrow head.

cytes prepared, stained and evaluated by FACS. Each of the panels in Figure 6 show one of two to three representative experiments. Figure 6a shows the profiles obtained for hepatocytes prepared 24 h after infection. The profiles of Ad.RSV-gp19K, Ad/RSV-hAAT and control mice were very similar, while the profile following transduction with wild-type Ad2 showed the largest decrease in MHC I abundance of cell surface MHC I by 24 h after transduction.

After 4 days (Figure 6b), the profiles of hepatocytes from animals infected with recombinant adenoviruses all showed a 10-fold increase in surface MHC I compared to hepatocytes from vehicle-treated mice. There was a minimal decrease in surface MHC I expression in mice given Ad.RSV-gp19K compared with the other recombinant vectors. Hepatocytes infected with wild-type Ad2 virus also had an increase in surface MHC I expression, but not to the extent seen with the recombinant adenoviruses. This increase in hepatocyte surface MHC I levels, similar to that seen in SVB6 murine fibroblasts following *in vitro*

infection (Figure 2b), was also seen previously in mouse livers in our laboratory⁵ and in others²⁹ and was mediated at least in part by interferon γ .

By 14 days after infection, MHC I expression levels in adenovirus-infected mice returned to baseline (Figure 6c) and were indistinguishable between groups receiving adenovirus (recombinant or wild-type) and controls. This normalization of MHC I expression was not related to loss of adenovirus-mediated gene expression, as the level of transgene expression was still robust (not shown), and the exact nature of this normalization remains unknown.

It is of interest that the kinetics of MHC regulation with the different adenoviruses were qualitatively similar and consistent between hepatocytes transduced *in vivo* and cultured fibroblasts. The MHC I studies of transduced hepatocytes from C57B1/6 mice (H-2^b haplotype) demonstrate that while E3-gp19K can substantially decrease the amount of cell surface MHC I molecules by 24 h after infection with wild-type adenovirus (both E1a and E3 present), insufficient E3-gp19K was produced from the

Figure 4 Persistence of human alpha 1-antitrypsin gene expression. Balb/c (a), Balb/k (b), and Balb/b (c) congenic mice were injected with 8×10^9 p.f.u. of Ad/RSV-hAAT and Ad.RSV-gp19K (solid circles) or 8×10^9 p.f.u. of Ad/RSV-hAAT and Ad.RSV-cFIX (open boxes) in a 1:3 ratio respectively. Each line represents data from an individual animal.

Figure 5 RT-PCR analysis of E3-gp19K expression in murine hepatocytes. Ethidium bromide staining of 10 µl undigested aliquots (a) resolved on a 2% agarose gel following RT-PCR of mRNA isolated 1 week after infection of animals with 8×10^9 p.f.u. of Ad/RSV-hAAT (lane 2), 2×10^9 p.f.u. of Ad/RSV-hAAT and 6×10^9 p.f.u. of Ad/RSV-gp19K (lane 3), or noninfected animals (lane 1). Reverse transcription was performed with random primers. Identical samples without reverse transcription are shown in lanes 4–6. Lane 7 is a PCR negative control using buffer in place of template. Lanes 8 and 9 are positive controls using Ad/RSV-hAAT and Ad2 genomic DNA, respectively. (b) A similarly prepared 2% agarose gel containing 10 µl aliquots of respectively amplified samples following digestion with HindIII. The BRL 100 bp ladder is shown in lane S, the expected full length gp19K amplification product is designated by a solid arrow, and the expected digestion fragments noted by the arrow heads on the right.

native E3 promoter (E1a absent) and the RSV-LTR promoter in recombinant adenovirus vectors to significantly alter the cell surface MHC I levels. This, in combination with the general induction of MHC I expression in hepatocytes with wild-type or recombinant adenovirus, makes it unlikely that E3-gp19K alone would be sufficient to

Figure 6 MHC I analysis of hepatocytes following *in vivo* transduction with adenovirus. Each fluorescence profile represents 20 000 hepatocytes isolated from C57B1/6 (H-2^b) mice 1 day (a), 4 days (b), or 14 days (c) after infection, stained with anti-MHC I (H-2^b) antibody. Shown are profiles of hepatocytes isolated from mice following transduction with vehicle only, 2×10^9 p.f.u. of wild-type Ad2 and 6×10^9 p.f.u. of Ad.RSV- β gal, 8×10^9 p.f.u. of Ad.RSV-gp19K, or 8×10^9 p.f.u. of Ad/RSV-hAAT.

prolong transgene expression by down-regulation of MHC I.

Discussion

It is the hypothesis of this study that constitutive expression of the native gp19K protein, which has been shown to down-regulate the development of class I MHC-restricted cytotoxic T lymphocytes directed towards adenovirus-infected hepatocytes,¹⁹ would result in prolonged transgene expression. Our current data suggest that while Ad.RSV-gp19K can modestly down-

regulate cell surface MHC-I levels *in vitro*, it has no effect on the duration of transgene expression *in vivo*. It appears that this is because adenovirus induces class I MHC expression on hepatocytes, and that insufficient protein is produced soon enough to produce a detectable decrease in cell surface MHC I levels *in vivo*. While we have found that the RSV promoter works well in hepatocytes, it typically takes days to obtain robust transgene expression following intravenous infusions.^{1,5} In contrast, previous studies with the native E3 promoter in the presence of E1a have demonstrated maximal gp19K expression only 8 h after infection of cells *in vitro*.³⁰ This *in vitro* data is consistent with our *in vivo* FACS data, and it will be important to consider in future efforts to constitutively express E3-gp19K, other native E3 proteins, or other viral immunomodulatory proteins such as adenovirus E3-14.7K and E1b 19K proteins.^{10,11}

Prolonged survival of pancreatic islet cells isolated from a transgenic mouse expressing the entire E3 region under the control of the insulin II promoter supports the notion that constitutive expression of E3 viral transgenes can have a favorable clinical outcome in tissue transplantation,³¹ and serves as a proof-of-principle study for this experimental design. In contrast to our study, these transgenic animals express more than the E3-gp19K protein within numerous tissues, while after adenovirus gene transfer just the transduced cells express only the E3-gp19K protein. Using a murine pneumonia model and a recombinant vaccinia virus constitutively expressing gp19K with a CMV promoter, the presence of gp19K had no effect on virus lethality, nor did it alter the development of a CD8⁺ infiltrate.³² These data, showing a similar lack of effect following constitutive expression of gp19K, may also be interpreted as a delay in expression of gp19K by the strong, yet non-native CMV promoter. In another study using various replication-competent adenovirus deletion mutants administered as aerosols to murine airways, these investigators found no difference in CTL generation or in pulmonary infiltrates formed following administration of viruses containing or deleted of gp19K.³³ Even in the absence of IFN- γ , a potent up-regulatory signal for MHC I, the presence or absence of gp19K had no effect on the immune response.³³ It should be noted that while these findings are consistent with the findings of our current study they contradict the previous study of gp19K function in murine liver,¹⁹ which may reflect a difference in the immune responses seen following transduction of the liver *versus* the lung.

Wild-type Ad2 virus, which expresses a number of immunomodulatory proteins from the E3 region, was used in both the *in vitro* and *in vivo* experiments as a point of reference for 'maximal' E3-gp19K expression in this murine model system. Thus, the non-gp19K gene products produced from a wild-type virus could in theory influence the inflammatory response and indirectly affect MHC I gene expression. One report examining the ability of various adenovirus deletion mutants to alter cell surface levels of MHC I using FACS analysis has shown that a particular mutant, dl327, which contains a specific E3 deletion removing the gp19K sequences, demonstrated near wild-type levels of cell surface MHC I in the cotton rat model system.³⁴ This strongly suggests that class I down-regulation seen with wild-type adenovirus was the result of gp19K expression.

Additionally, recent studies have shown that CTL

recognition of CMV infected cells occurs before endogenous gene (and thus transgene) expression.³⁵ If this were the case for recombinant adenoviruses, the ability to alter the CTL response by constitutive expression of proteins that must be synthesized *de novo* may be limited, and require the administration of immunomodulatory agents such as CTLA4Ig prior to recombinant adenovirus transduction.⁵

Unfortunately, whether or not MHC I presentation on hepatocytes, liver non-parenchymal cells, and/or non-liver cells are responsible for initiation of class I-mediated immune events is not known. A better understanding of both the induction and effector phase of the immune response may assist in designing more rational strategies for immune evasion by interference with class I presentation. One possibility would be to engineer vectors that express the herpes ICP47 protein which by interfering with peptide transporters (TAP), inhibits the transport of MHC I molecules to the cell surface.^{36,37} Because E3-gp19K functions distal to ICP47 perhaps coexpression of both proteins would be optimal for down-regulating class I expression.

An unexpected finding was the discordance of transgene expression between the three congenic strains used in the study. The alteration in duration of transgene expression between the three congenic Balb/c, Balb/k, and Balb/b mice strongly suggests that the H-2 locus is responsible for the prolonged transgene expression particularly in the Balb/b background. Our previous study¹ demonstrated that the duration of expression did not correlate with the H-2 locus for several major determinants (D, L and K), but also suggested that other genetic loci derived from a paternal founder strain may be the determinant for this strain variation. With this additional finding, the possibility arises that multiple loci are responsible for the murine strain variation in transgene expression. Additional studies are currently in progress to investigate the biological basis for this strain variability.

Materials and methods

Animal studies

Animal studies were performed in accordance with the institutional guidelines set out by the University of Washington. Female C57B1/6, C3H, Balb/b, Balb/k and Balb/c mice were all obtained from Jackson Laboratories (Bar Harbor, ME, USA) at 5–6 weeks of age and housed in SPF facilities. Mice were injected with recombinant adenovirus diluted to 150 μ l in DME medium (Gibco BRL, Gaithersburg, MD, USA) by tail vein injection, or by infusion through a portal vein catheter as previously described.²⁸ Previous studies have shown that infusion of 8×10^9 recombinant adenovirus particles results in each hepatocyte on average being transduced with 20 to 50 adenovirus genomes.^{2,28} Blood samples for serum protein analysis were obtained by the retro-orbital technique. Mice found to have serum hAAT levels less than 500 ng/ml on serum samples taken on days 3–7, and thought to have received poor injections, were removed from the study. The animals were killed by cervical dislocation.

Recombinant adenoviruses

Construction of the recombinant E1-deficient Ad5 vectors Ad/RSV-hAAT (endogenous gp19K '+'),³⁸ Ad/RSV-cFIX (endogenous gp19K '+'),²⁶ and Ad.RSV- β gal (produces β -galactosidase and is endogenous gp19K '-')³⁹ have been previously described. Wild-type adenovirus serotype 2 was obtained from ATCC (Rockville, MD, USA). The E3-gp19K cDNA clone from Ad2 was graciously provided by Dr W Wold from Washington University in St Louis, MO, USA. The recombinant adenovirus constitutively expressing E3-gp19K (Ad.RSV-gp19K) was prepared by cloning gp19K as an *Eco*RI fragment into the left-end adenoviral vector pXCJL directly downstream of the Rous sarcoma virus LTR promoter and directly upstream of the bovine growth hormone polyadenylation signal.³⁸ The appropriate orientation was ascertained by restriction digestion. Recombinant adenovirus was rescued following co-transfection of this construct with PJM17⁴⁰ in 293 cells.^{40,41} Recovered plaques were expanded and characterized by restriction digestion.⁴¹ Desired recombinants were grown in large scale and then purified and concentrated on two cesium chloride gradients.⁴¹ Each virus preparation was assayed for wild-type contamination,¹ and quantified both spectrophotometrically (optical density 260 nm) and by plaque assay.⁴¹ The recombinant adenovirus expressing human α -1-antitrypsin (hAAT) without an endogenous E3-gp19K gene Ad/RSV-hAAT(E3-) was prepared by cotransfecting the previously described left-end vector containing hAAT downstream from the Rous sarcoma virus promoter and upstream from the bovine growth hormone polyadenylation signal³⁸ with pBHG10.⁴² Recombinant plaques were characterized and expanded as described above.

Immunoprecipitation analysis of E3 gp19K protein

SVB6 and PC3H murine fibroblasts expressing H-2^b and H-2^k MHC I molecules respectively were graciously provided by Dr Linda Gooding of Emory University. Cells were metabolically labelled 4 h before harvesting in 150 μ Ci of ICN ³⁵S translabel as previously described.²⁰ The labeled cells were mechanically removed from the 10-cm tissue culture dishes, washed in PBS, and lysed for 30 min on ice in TEN (20 mM Tris, 1 mM EDTA, and 0.5% NP40 pH 7.5) buffer containing a 1:100 volume of $\times 100$ freshly prepared protease inhibitor mixture (77 μ g/ml aprotinin (Sigma, St Louis, MO, USA), 0.1 mM leupeptin (Sigma), 1.5 μ M pepstatin (Sigma), and 0.5 mM phenylmethylsulfonyl fluoride (Calbiochem)). Following a 15 min centrifugation at maximum speed in an Eppendorf microcentrifuge, the supernatant was removed, and two 2 μ l aliquots counted on a Beckman scintillation counter (Beckman, Palo Alto, CA, USA). The gp19K protein was immunoprecipitated from 10⁷ c.p.m. of cytosol with Ad2 gp19K terminal peptide antibody provided by Dr WSM Wold³⁰ preloaded on to protein A-sepharose resin at 4°C with rocking for 6–8 h. Following immunoprecipitation, the resin was washed three times in high salt buffer (TEN plus 400 mM NaCl), and then twice in 50 mM Tris pH 6.8. The washed resin pellet was then extracted into 20 μ l of 2 $\times$ SDS-PAGE sample buffer (0.5 M Tris, 4% SDS (w/v), 0.1% bromophenol blue, 40% glycerol (v/v), and 10% mercaptoethanol (v/v), pH 6.8), and diluted with 1 $\times$ sample buffer to a final volume of 60 μ l. The samples were denatured by boiling for 5 min after adding 5% BME, and then analyzed by SDS-PAGE with a 15% separating gel

as previously described.²⁰ Following electrophoresis, the gel was vacuum-dried, and visualized by autoradiography or phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA, USA). 'Relative amount' of gp19K was determined by subtracting the background from the phosphorimager raw data count for similar areas around the gp19K protein band, and then normalizing that number to the amount of a 46 kb nonspecific binding band.

Analysis of murine serum

Serum human alpha-1-antitrypsin (hAAT) levels were determined by ELISA⁵ on duplicate samples using multiple dilutions to assure that readings were made on the linear portion of the standard curve. Serum neutralizing antibody analysis was performed as previously described.^{2,5}

RT-PCR analysis of transduced murine hepatocytes

Messenger RNA from snap-frozen C3H murine liver samples transduced with various recombinant adenovirus constructs 1 week before liver harvesting was prepared using the Invitrogen mRNA isolation kit (Invitrogen, Carlsbad, CA, USA). Serum hAAT levels of blood samples taken before death were used to verify that the animal had been successfully transduced. RT-PCR was performed on 1 µg of mRNA, using the PCR buffer provided in the kit and an Ericomp thermocycler for 30 cycles of 94°C (1 min), 55°C (2 min) and 72°C (3 min) with the following gp19K specific primers: (5'-3')CTGCGTCTGCTTTTGTATT and TCAGCTTTTAAACGCTGG.

FACS analysis of murine hepatocytes and cultured fibroblasts

Hepatocytes were prepared for FACS analysis as previously described.⁴³ Following the last centrifugation step, the cells were counted and viability was assessed with trypan blue staining. Routinely, 1×10^7 hepatocytes were obtained with 70 to 80% viability.

SVB6 and PC3H fibroblasts were grown in DMEM containing 10% fetal calf serum (Gibco). Fibroblasts were harvested by washing twice in PBS followed by a 5-min incubation in PBS containing 0.1% EDTA. Staining for flow cytometry was performed immediately following fibroblast isolation.

Fluorescence activated cell sorting (FACS) analysis was determined essentially as previously described for cultured fibroblasts.²⁰ Hepatocytes were prepared on days 1, 4 and 14 following tail vein injection of recombinant adenovirus (days 4 and 14) or infusion via a portal vein catheter (day 1). Ad.RSV-βgal was used where indicated as a control in some experiments since it does not contain the native gp19K gene in the E3 region. The wild-type adenovirus was mixed with the control adenovirus Ad.RSV-βgal so that similar amounts of adenovirus would be transduced, and because severe immediate toxicity is seen in animals following large injections of wild-type virus. On day 3 serum samples were obtained from each injected mouse and serum hAAT levels determined to ensure adequate adenovirus-mediated hepatic gene transfer.

Two aliquots of 5×10^5 hepatocytes were washed twice in PBS/FCS (phosphate buffered saline containing 1% fetal calf serum both from GibcoBRL). One suspension was incubated 30 min on ice in the dark with PE-conju-

gated goat anti-murine H-2D^b MHC I (Pharmingen, San Diego, CA, USA), and the other with a PE-conjugated goat isotype control antibody (Pharmingen). Following the incubation, the cells were washed three times with 400 µl of PBS/FCS, and the final pellet fixed in 60 µl of 2% paraformaldehyde. FACS analysis was typically performed within 24 h of staining.

FACS analysis of SVB6 murine fibroblasts was performed essentially as described for hepatocytes except that the staining involved two sequential steps. First, the cells were incubated with either biotinylated rabbit anti-murine H-2D^b or H-2K^k MHC I primary antibody (Pharmingen) and then, after three washes with PBS/FCS, incubated with PE conjugated to streptavidin (Pharmingen).

The fluorescein profiles shown for both SVB6 fibroblasts and the murine hepatocytes were determined by analyzing 20 000 cells on a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), and were overlaid with Repoman II software (Truefacts Software, Seattle, WA, USA) using normalization and five-point curve smoothing function.

Fluorescein in situ hybridization (FISH) of murine hepatocyte nuclei

Hepatocytes were prepared for FISH analysis as outlined above for FACS analysis.⁴³ FISH analysis was performed as previously described⁴⁴ with the following modifications. After quantification of the hepatocytes, they were placed in 10 volumes of hypotonic saline (75 mM KCl) for 15 min, and then fixed twice in a freshly prepared solution of methanol and acetic acid (3:1 respectively). Cells were then either stored at -20°C or dropped on to microscope slides. The plasmid pBHG10,⁴² was labeled using the DIG-Nick translation mix from Boehringer Mannheim (Indianapolis, IN, USA). Fixed slides were hybridized for 12 to 18 h at 37°C in standard hybridization solution containing 10 µg Sigma Cot1 DNA and then blocked and washed as described. The immunocytochemical detection was performed in 4× SSC containing 1% BSA in three rounds with murine anti-digoxin, FITC rabbit anti-mouse, and FITC goat anti-rabbit antibodies respectively all from Sigma at 1:1000 dilutions separated by three, 3-min washes. After the final wash, the nuclei were placed in antifade and 2 µM DAPI (4',6'-diamidino-2-phenylindole), and stored at 4°C until viewing.

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