

# Preexisting Antiadenoviral Immunity Is Not a Barrier to Efficient and Stable Transduction of the Brain, Mediated by Novel High-Capacity Adenovirus Vectors

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## ABSTRACT

The utility of first-generation adenovirus vectors for long-term gene transfer in humans is limited by preexisting antiadenoviral immunity. We demonstrate here that new-generation high-capacity adenovirus vectors (HC-Ads) can efficiently transduce the brain and mediate stable transgene expression for at least 2 months, even in the presence of a preexisting antiadenoviral immune response. First-generation vector-mediated transduction was almost completely abolished in preimmunized animals within 60 days of the vector injection. Levels of HC-Ad-mediated transduction by 3 days postinjection were not significantly affected by preimmunization, were reduced within 14 days to 56% of those levels seen in nonimmunized animals, and remained stable until day 60 postinjection. Acute brain inflammation elicited by the HC-Ad vector injection was more transient, and was reduced in intensity compared with brain inflammation elicited by the first-generation vector injection in immunized animals. Inflammation was significantly higher in all immunized animals than in nonimmunized animals. Our results show that preexisting antiadenoviral immunity does not significantly reduce initial HC-Ad-mediated infection of the brain and is not a barrier to stable HC-Ad vector-mediated transduction of the CNS. Although input HC-Ad capsid proteins injected into the brain may contain transient targets for a brain-infiltrating cellular adenovirus-specific immune response, this fails to eliminate transgene expression. Thus HC-Ads show promise for gene therapy of chronic brain disease.

## OVERVIEW SUMMARY

Adult rats were injected intradermally with the first-generation adenovirus vector RAdHPRT (immunized animals) or saline (non-immunized animals). Two weeks after the intradermal injections, the animals were injected in the brain with either the first-generation vector RAd35 or the high-capacity vector AdGS46, both expressing the transgene  $\beta$ -galactosidase. We show that HC-Ads can mediate stable transduction of the brain, even in preimmunized animals. Transgene expression from first-generation vectors was eliminated within 60 days in preimmunized animals. HC-Ad-mediated transgene expression in the brains of preimmunized animals was maintained at 56% of the levels seen in nonimmunized animals. We suggest that HC-Ad-infected neural cells presenting input capsid protein-derived epitopes may be transient targets for a preexisting antiadenoviral cellular immune response, which infiltrates the brain,

but fails to eliminate HC-Ad vector-mediated transgene expression.

## INTRODUCTION

SUCCESSFUL HUMAN GENE TRANSFER using adenoviruses has been hampered by the intrinsic inflammatory potential of first-generation adenovirus vectors, particularly when patients have preexisting antiadenoviral immunity (Recombinant DNA Advisory Committee, National Institutes of Health, 2000). Although first-generation vectors may prove efficacious for the treatment of cancer, their suitability for the treatment of chronic disease remains more controversial. Adenoviruses are, however, extremely powerful tools for transducing nondividing glial cells and neurons and, in this respect, remain extremely attractive vehicles for neurological gene therapy. We and others have demonstrated that adenovirus vectors injected into the brains of

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naive animals can sustain transgene expression for up to 12 months (Geddes *et al.*, 1997; Ghodsi *et al.*, 1998; Navarro *et al.*, 1999; Thomas *et al.*, 2000a; and C.E. Thomas *et al.*, unpublished), a situation markedly different from that in peripheral organs, where adenovirus vector-mediated transgene expression declines rapidly (Morral *et al.*, 1999; Southgate *et al.*, 2000a).

Moderate doses of adenovirus vectors injected into the brains of naive animals elicit a transient innate inflammatory response that is resolved within 2 weeks without eliminating vector-mediated transgene expression (Byrnes *et al.*, 1995; Thomas *et al.*, 2000). In the presence of systemic antiadenovirus immunization, however (occurring either through natural infection with wild-type adenovirus, vector administration to peripheral organs, or through substantial leakage of vector from the brain), an antiviral T cell response is activated that can target first-generation adenovirus vectors in the CNS, causing a chronic inflammatory response in the brain and eliminating vector-mediated transgene expression (Byrnes *et al.*, 1996). For adenovirus-mediated gene therapy of chronic disorders to become a reality, vectors will need to mediate long-term transduction of brain cells even in the presence of antiadenovirus immunity generated through previous natural infection, or vector readministration.

New-generation high-capacity adenovirus vectors (HC-Ads), which are deleted of all adenoviral genes, have been shown to display reduced toxicity and prolonged transgene expression compared with first-generation vectors after administration to peripheral organs of immunologically naive animals (Chen *et al.*, 1997; Morsy *et al.*, 1998; Schiedner *et al.*, 1998; Morral *et al.*, 1999; Majone *et al.*, 2000; O'Neal *et al.*, 2000). We have previously investigated the stability of HC-Ad-mediated transgene expression in the brain after induction of peripheral immunization. We demonstrated that HC-Ad vectors injected into the brain were able to completely evade an antiadenovirus immune response elicited through a subsequent systemic infection with adenovirus; no reactivation of brain inflammation was observed after immunization and HC-Ad-mediated transgene expression remained stable (Thomas *et al.*, 2000a).

Many human patients, however, have already been infected with wild-type adenovirus prior to gene therapy and it is widely documented that preexisting antiadenovirus immunity can severely compromise the efficacy of subsequent adenovirus vector administration, masking both gene transfer and vector toxicity (Parks *et al.*, 1999; Moffatt *et al.*, 2000; Stallwood *et al.*, 2000). In addition, it has been reported by others that *de novo* expression of viral genes is not a prerequisite for the elicitation of an antiadenoviral cytotoxic T lymphocyte (CTL) response (Kafri *et al.*, 1998). Further, CTLs derived from human patients have been shown to recognize viral input capsid-derived protein epitopes presented by HLA class I molecules in an *in vitro* assay (Molinier-Frenkel *et al.*, 2000). Thus, the possibility exists that prior systemic infection with adenovirus could elicit the activation of CTLs, which could target epitopes derived from HC-Ad vectors subsequently administered to the brain. To address these questions, we have now investigated the efficacy of HC-Ad infection and the stability of HC-Ad-mediated transgene expression in the brains of preimmunized animals.

Our data suggest that HC-Ads injected into the brain do indeed contain targets for cellular elements of an immune re-

sponse elicited through prior systemic infection with adenovirus. This cellular immune response in the brain is, however, transient and does not eliminate HC-Ad-mediated transgene expression; although expression from first-generation vectors was abolished within 60 days in immunized animals, HC-Ad-mediated expression remained robust and was decreased by less than 50% compared with nonimmunized animals. Our data show that high-capacity adenovirus vectors should prove efficient and safe tools for gene transfer for the potential treatment of chronic disease, even in patients who have strong preexisting antiadenoviral immunity.

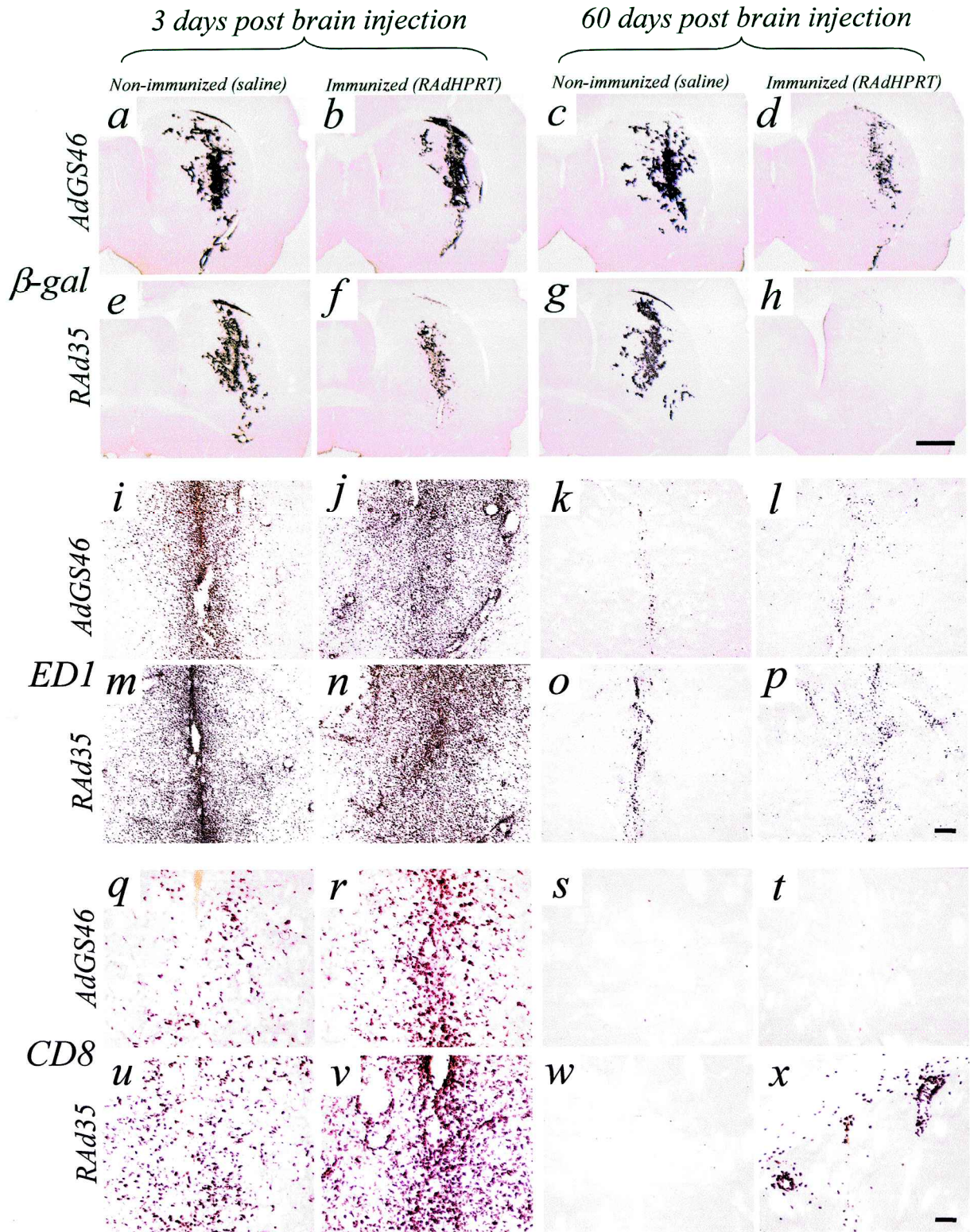
## MATERIALS AND METHODS

### *Adenoviral vectors*

RA35 and RAHPRT are first-generation E1a-deleted recombinant adenovirus vectors based on adenovirus type 5. The construction of RA35 and RAHPRT has been described elsewhere (Wilkinson and Akrigg, 1992; Southgate *et al.*, 1999). Briefly, expression cassettes containing the *lacZ* gene (RA35) or the hypoxanthine-guanine phosphoribosyltransferase gene (RAHPRT) were inserted into the adenoviral genome in place of the E1 region. Both vectors expressed *lacZ* or HPRT from the human cytomegalovirus (hCMV) intermediate-early promoter. RA35 and RAHPRT were propagated on 293 cells, purified by double cesium chloride gradient centrifugation followed by dialysis, and finally titered for infectious units by end-point dilution of 293 cells (Southgate *et al.*, 2000b). AdGS46 is a high-capacity adenovirus vector expressing *lacZ* from the hCMV promoter, the construction of which is described in Thomas *et al.* (2000a). The propagation, purification, and titration of AdGS46 was performed as described for AdSTK109 by Schiedner *et al.* (1998). The level of helper virus contamination in the AdGS46 preparation was less than 0.05%, as assessed by titration on 293 cells, using end-point dilution (Southgate *et al.*, 2000b). The concentration of physical particles in all vector preparations was determined by optical absorbance at 260 and 280 nm as described in Mittereder *et al.* (1996). The ratio of particles to infectious units was 15:1 for RA35, 20:1 for RAHPRT, and 20:1 for AdGS46. All vector preparations were screened for replication-competent adenovirus contamination by serial amplification on HeLa cells as described by Dion *et al.* (1996) and Schiedner *et al.* (1998). Replication-competent adenovirus contamination was below  $1:1 \times 10^9$  IU for all virus preparations. Adenoviruses were diluted in sterile saline solution prior to injection.

### *Animals and surgical procedures*

Thirty adult Sprague-Dawley rats weighing 200 g (Charles River Breeding Laboratories, Wilmington, MA) were anesthetized with halothane and were immunized against adenovirus by injecting  $5 \times 10^8$  infectious units of RAHPRT into the skin of the back. A second control group of 30 animals received an intradermal injection of saline. All intradermal injections were performed with a volume of 100  $\mu$ l. Fourteen days after the intradermal injection, the animals were reanesthetized with halothane and were injected in the left striatum with 2  $\mu$ l of virus solution. Half the animals in each group (immunized or



**FIG. 1.** Immunohistochemical detection of transgene expression and inflammatory responses in the brains of immunized versus nonimmunized animals, 3 and 60 days after intrastriatal injection of the HC-Ad vector AdGS46, or the first-generation vector RAd35. (a–h)  $\beta$ -Galactosidase immunoreactivity in the ipsilateral striatum (scale bar shown in [h] = 1 mm). (i–p) ED1 immunoreactivity in the area of the needle injection (macrophages and activated microglial cells; scale bar in [p] = 200  $\mu$ m). (q–x) CD8 immunoreactivity, also in the area of the needle track (natural killer cells and cytotoxic T lymphocytes; scale bar in [x] = 100  $\mu$ m).



nonimmunized) received a brain injection containing  $1 \times 10^7$  IU of the first-generation vector RAd35 and the remaining animals received a brain injection containing  $1 \times 10^7$  IU of the HC-Ad vector AdGS46. Intrastratial injections were performed with a 10- $\mu$ l Hamilton syringe with a removable 26-gauge needle at the following coordinates: 0.6 mm forward and 3.4 mm lateral from bregma, and 0.5 mm vertical from the dura. The procedures used for both the intradermal and intrastratial injections are described in Thomas *et al.* 2000b). At 3, 14, or 60 days after the intrastratial injection, animals were injected intraperitoneally with pentobarbitone (five animals per virus group, per time point) and blood was removed from the heart before transcardially perfusing with heparinized saline and 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed, postfixed overnight in 4% paraformaldehyde, and stored in PBS.

### Immunohistochemistry

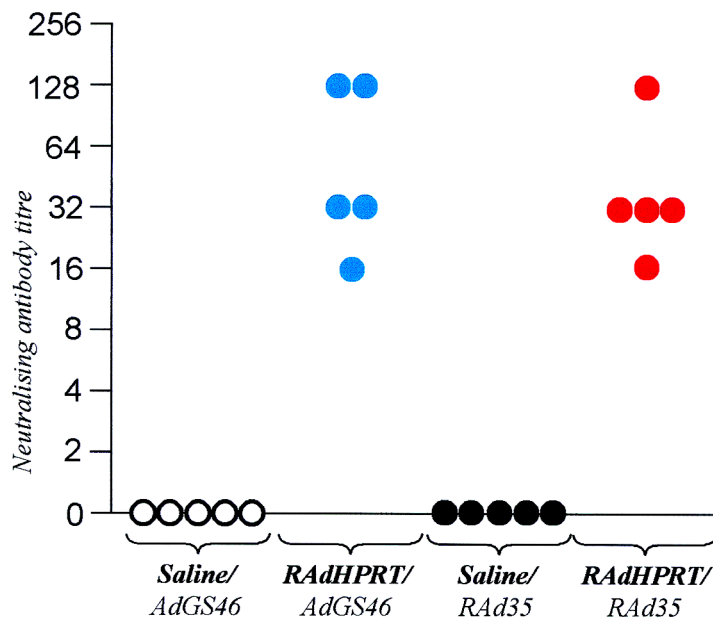
Coronal brain sections, 40  $\mu$ m thick, were cut through the striatum with a tissue sectioner (Vibratome, St. Louis, MO). Immunohistochemistry to detect transgene product and immune markers was performed as described in Thomas *et al.* (2000a), using anti  $\beta$ -galactosidase antibody (Promega, Madison, WI) at a dilution of 1:1000, ED1 antibody (Serotec, Kidlington, Oxford, UK; activated macrophages and microglial cells) at a dilution of 1:1000, and anti-CD8 antibody (Serotec; cytotoxic T lymphocytes and natural killer cells) at a dilution of 1:500.

### Quantification

Quantitative image analysis to determine the area occupied by cells immunohistochemically stained with anti- $\beta$ -galactosidase, ED1, or anti-CD8 antibodies within 40- $\mu$ m brain sections was performed with a Leica Quantimet 600 image analysis system controlled by QWIN software (Leica Microsystems, Cambridge, UK) as described previously (Thomas *et al.*, 2000a). To quantitate transgene expression in each of the brains, we measured the area of  $\beta$ -galactosidase immunoreactivity in five 40- $\mu$ m sections, spaced at regular 360- $\mu$ m intervals through the injected striatum. The five sections included a single section corresponding to the center of the injection site (i.e., the needle track), two sections posterior to the needle track, and two sections anterior to the needle track. The area of  $\beta$ -galactosidase immunoreactivity in each of the five sections was summed for each brain. Thus the value obtained for each animal corresponded to a sampling of an area spanning 1.4 mm through the injected striatum. This sampling method covered >90% of the transduced area from each brain. Single brain sections containing the needle track (and thus displaying the highest levels of immunoreactivity) were used to quantitate levels of ED1 or CD8 immunoreactivity. The Student *t* test was used to determine the degree of statistical significance between values from different experimental groups.

### Adenovirus-neutralizing serum antibody assays

Titers of neutralizing antibodies raised in response to the intradermal injection of adenovirus were measured in serum sam-



**FIG. 3.** Neutralizing serum antibody titers from animals killed 3 days after the intrastratial injection of RAd35 or AdGS46 (i.e., 17 days after-intradermal injection of RAdHPRT or saline). Open circles and blue circles show the neutralizing antibody titers from the sera of each of the five nonimmunized (open) and the five immunized (blue) animals injected in the brain with AdGS46, and black circles and red circles show the titers from the sera of each of the five nonimmunized (black) and the five immunized (red) animals injected in the brain with RAd35. The neutralizing antibody titer is expressed as the highest dilution of serum that inhibited 50% of adenovirus transduction in an *in vitro* assay.

ples from all 20 animals killed 3 days after the intrastriatal injection (i.e., 17 days after the intradermal injection), using an *in vitro* assay described in Thomas *et al.* (2000a). The neutralizing antibody titer for each sample was described as being the reciprocal of the highest dilution of serum that inhibited RAD35-mediated transduction of 293 cells by 50%.

## RESULTS

To determine the effect of preexisting systemic immunity on the efficiency and stability of HC-Ad vector-mediated transduction of the brain, a strong antiadenoviral immune response was raised in adult Sprague-Dawley rats by injecting a first-generation vector (RADHPRT) into the skin. This was previously shown to abolish first-generation adenovirus vector-mediated transduction of the brain. A second control group of animals was intradermally injected with saline. Two weeks after the intradermal injections, the HC-Ad vector AdGS46, or the first-generation vector RAD35, was stereotaxically injected into the left striatum. Both AdGS46 and RAD35 express  $\beta$ -galactosidase from the hCMV promoter. RADHPRT expresses hypoxanthine-guanine phosphoribosyltransferase but is otherwise identical to RAD35, both in terms of capsid composition and the vector genome. RADHPRT shares only its capsid with AdGS46.

Animals were killed 3, 14, and 60 days after the intrastriatal injection of virus and the brains were analyzed by immunohistochemistry for transgene expression and inflammation (Fig. 1). Levels of  $\beta$ -galactosidase expression and inflammation in all animals were quantified by computer-assisted image analysis and values were compared between immunized and nonimmunized animals at all time points (Fig. 2). Three days after the brain injection, all animals injected with AdGS46 exhibited comparable levels of  $\beta$ -galactosidase ( $\beta$ -Gal) expression, regardless of whether they had received a previous intradermal injection of RADHPRT or saline (Fig. 1a and b and Fig. 2a). By 14 days, levels of AdGS46-mediated  $\beta$ -Gal expression in immunized animals were reduced to 56% of those seen in nonimmunized animals (Fig. 2a), but thereafter were stable; analysis of transgene expression at 60 days showed no further decline (Fig. 1c and d and Fig. 2a). Analysis of RAD35-mediated  $\beta$ -Gal expression in immunized versus nonimmunized animals showed a different time course; 3 days after the brain injection, immunized animals already displayed only 57% of the levels of expression seen in nonimmunized animals (Fig. 1e and f and Fig. 2a). Thereafter, expression declined rapidly, such that by 14 days, expression in immunized animals was down to 12.5% of controls (Fig. 2a), and was almost eliminated by 60 days (Fig. 1g and h and Figure 2a).

To confirm that animals injected intradermally with RADHPRT had successfully raised a systemic immune response to adenovirus, we measured the titers of adenovirus-neutralizing antibodies in serum samples from animals killed 3 days after the intrastriatal injection. All animals injected with RADHPRT, including those injected in the brain with AdGS46, had high titers of circulating neutralizing antibodies (Fig. 3). The neutralising antibodies were equally effective at inhibiting AdGS46 infection compared with RAD35 infection in an *in vitro* assay (data not shown). Thus, the finding that levels of initial brain trans-

duction by HC-Ad vectors were unaffected by preimmunization indicates that, unlike systemic vector readministration, pre-existing antiadenovirus immunity does not seem to play a major role in inhibiting subsequent adenovirus infection of the brain.

Levels of brain inflammation elicited in response to the intrastriatal vector injections were analyzed by immunohistochemical detection of ED1-positive cells (activated macrophages and microglial cells) and CD8-positive cells (cytotoxic T lymphocytes and natural killer cells). By 3 days after vector injection, inflammation elicited in response to injection of AdGS46 was significantly higher in immunized animals compared with nonimmunized animals (Fig. 1i, j, q, and r and Fig. 2b and c). ED1<sup>+</sup> and CD8<sup>+</sup> cells were principally confined to the region of the needle injection in nonimmunized animals, but were far more numerous and more diffused through the area of transduction in immunized animals (Fig. 1i, j, q and r). By 14 days, the inflammation was much reduced in all brains injected with AdGS46 and was more restricted to the needle track; however, levels were still significantly higher in immunized animals compared with nonimmunized animals (Fig. 2b and c). By 60 days after the intrastriatal injection, inflammation was totally resolved in all animals injected with AdGS46; no CD8<sup>+</sup> cells could be detected in any of the brains, and only very few ED1-positive cells could be detected within the scar remaining at the site of the needle injection (Fig. 1k, l, s and t and Fig. 2b and c).

Brain inflammation 3 days after intrastriatal injection of RAD35 was significantly higher in immunized animals than in nonimmunized animals (Fig. 1m, n, u, and v and Fig. 2b and c). Levels of brain inflammation in immunized animals were significantly higher after injection of RAD35 compared with injection of AdGS46 (levels of brain inflammation in response to AdGS46 or RAD35 injection in nonimmunized animals were not significantly different [Fig. 2b and c]). By 14 days after the intrastriatal injection of RAD35, immunized animals still exhibited high levels of brain inflammation. Even 60 days after RAD35 injection, brain inflammation was still significantly higher in immunized animals than in nonimmunized animals; ED1-positive cells were not restricted solely to the site of the needle injection and scattered CD8-positive cells could still be detected within the ipsilateral striatum. Thus, brain inflammation elicited in immunized animals in response to an intrastriatal injection of first-generation vector was significantly more severe and more prolonged than the inflammation induced by a similar dose of a high-capacity vector.

## DISCUSSION

One of the most significant barriers to the successful implementation of gene transfer and potential therapies using adenoviruses is the fact that most individuals will have previously encountered a natural infection with wild-type adenovirus and will therefore possess an immune armory ready to battle with the invading adenovirus vector. This armory will include circulating adenovirus-neutralizing antibodies and activated or memory T cells specific for adenoviral proteins, many of which are shared by wild-type virus and adenovirus vectors of identical serotypes. Our data demonstrate that these preexisting im-

munological defense mechanisms are not sufficient to successfully ablate HC-Ad vector transduction of the brain.

Although initial levels of HC-Ad infection were not affected by preimmunization (suggesting that HC-Ad-mediated transduction of the brain is not affected by preexisting adenovirus-neutralizing antibodies), transgene expression in immunized animals declined within 14 days to approximately 50% of the levels in nonimmunized animals. Since inflammation was higher in immunized animals, it is likely that this decline in transgene expression is brought about (directly, or through cytokine release) by CNS-infiltrating immune cells that specifically recognize HC-Ad capsid-derived epitopes. A previous *in vitro* study by Molinier-Frenkel *et al.* (2000) demonstrated that adenovirus-specific CTL recognition of capsid epitopes does not require *de novo* adenoviral gene expression. Thus, these authors postulated that use of deleted adenovirus vectors would not reduce the antiadenoviral CTL response. Importantly, in this study, we show that in comparison with first-generation vectors, the immune response to HC-Ads injected into the brain is reduced both in intensity and in duration in the brains of preimmunized animals. The *in vitro* study of Molinier-Frenkel *et al.* (while providing extremely important evidence that input adenovirus capsid epitopes can be presented by MHC class I and recognized by CTLs) did not reflect the consideration that the presentation of HC-Ad input capsid epitopes will be transient. Unlike first-generation vectors, the supply of target HC-Ad capsid protein epitopes is limited to those that were initially injected; cells infected by HC-Ads will not synthesize *de novo* any adenoviral proteins. Thus in our *in vivo* study, we see only short-lived brain inflammation in response to the HC-Ad vector injection. This explains why in our experiments, the brain inflammatory response reduces HC-Ad vector-mediated transgene expression by less than 50%. Presumably, the more chronic inflammation seen in immunized animals after brain injection of first-generation vectors reflects the continued synthesis of adenoviral proteins by infected cells (both capsid and noncapsid proteins).

We have shown that use of extremely strong promoters can result in as much as a 100 to 1000-fold increase in levels of transgene expression and can dramatically increase the anatomical area of adenovirus vector-mediated transduction, in the absence of inflammation (Gerdes *et al.*, 2000). Since preimmunization results in only an approximately 2-fold decline in HC-Ad-mediated transduction, use of stronger promoters should greatly increase the potential for extremely efficient and widespread transduction using HC-Ad vectors. Furthermore, our previous findings demonstrate that once the acute inflammatory response to the initial HC-Ad injection has resolved, the HC-Ad-mediated transgene expression in the brain is totally resistant to any subsequent reactivation of systemic antiadenoviral immunity. Taken together, our data therefore demonstrate that HC-Ads show promise for realistic gene therapy of neurological disease, and indeed for other disorders that require long-term and stable gene expression.

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