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Immunological thresholds in neurological gene therapy: highly efficient elimination of transduced cells might be related to the specific formation of immunological synapses between T cells and virus-infected brain cells

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Abstract

First-generation adenovirus can be engineered with powerful promoters to drive expression of therapeutic transgenes. Numerous clinical trials for glioblastoma multiforme using first generation adenoviral vectors have either been performed or are ongoing, including an ongoing, Phase III, multicenter trial in Europe and Israel (Ark Therapeutics, Inc.). Although in the absence of anti-adenovirus immune responses expression in the brain lasts 6–18 months, systemic infection with adenovirus induces immune responses that inhibit dramatically therapeutic transgene expression from first generation adenoviral vectors, thus, potentially compromising therapeutic efficacy. Here, we show evidence of an immunization threshold for the dose that generates an immune response strong enough to eliminate transgene expression from the CNS. For the systemic immunization to eliminate transgene expression from the brain, $\geq 1 \times 10^7$ infectious units (iu) of adenovirus need to be used as immunogen. Furthermore, this immune response eliminates >90% of transgene expression from 1×10^7 – 1×10^3 iu of vector injected into the striatum 60 days earlier. Importantly, elimination of transgene expression is independent of the nature of the promoter that drives transgene expression and is accompanied by brain infiltration of CD8⁺ T cells and macrophages. In conclusion, once the threshold for systemic immunization (i.e. 1×10^7 iu) is crossed, the immune response eliminates transgene expression by >90% even from brains that receive as little as 1000 iu of adenoviral vectors, independently of the type of promoter that drives expression.

Keywords

Gene therapy; neuroimmunology; adenovirus; promoter; threshold

INTRODUCTION

First-generation adenovirus are powerful vectors for gene transfer into the brain *in vitro* and *in vivo*, and are employed currently in clinical trials for gene therapy. Experimentally, they are

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successful in gene therapy models of many brain diseases, including Parkinson's disease (Bilang-Bleuel *et al.*, 1997; Bjorklund *et al.*, 2000; Do Thi *et al.*, 2004; Hurtado-Lorenzo *et al.*, 2004), Huntington's disease (Bemelmans *et al.*, 1999; Mittoux *et al.*, 2000; Mittoux *et al.*, 2002), brain tumors (Dewey *et al.*, 1999; Sandmair *et al.*, 2000; Maleniak *et al.*, 2001; Biglari *et al.*, 2004; Chiocca *et al.*, 2004; Gondi *et al.*, 2004; Immonen *et al.*, 2004) and various pain syndromes (Cope *et al.*, 2006). In comparative clinical trials of gene therapy for malignant brain tumors, first-generation adenoviral vectors are significantly more effective than retroviral vectors, utilizing the conditional cytotoxic gene HSV₁-TK and ganciclovir (Immonen *et al.*, 2004). Numerous clinical trials for glioblastoma multiforme using first generation adenoviral vectors have been performed or are ongoing (Sandmair *et al.*, 2000; Trask *et al.*, 2000; Eck *et al.*, 2001; Ehteshami *et al.*, 2004; Immonen *et al.*, 2004), including an ongoing Phase III multicenter trial in Europe and Israel (see <http://www.virtualtrials.com/news3.cfm?item=3785>) by Ark Therapeutics, Inc.

However, there is controversy on the extent of adenoviral-mediated transgene expression in the brain. Short-term expression (e.g. <1 month) results from the use of very high titers that are cytotoxic because they stimulate a strong innate immune response (Thomas *et al.*, 2001a), the injection of vectors into the brain ventricles, which primes the systemic immune system (Lowenstein, 2002; Tada *et al.*, 2005), or contaminated vector preparations. However, if injections keep the amount of injected vectors to $\sim 10^7$ iu, are delivered carefully into the brain parenchyma, and vector preparations are devoid of lipopolysaccharide and replication competent adenovirus, expression lasts between 6–18 months (Byrnes *et al.*, 1995; Geddes *et al.*, 1997; Kajiwarra *et al.*, 1997; Ideguchi *et al.*, 2000; Thomas *et al.*, 2000b; Zou *et al.*, 2000; Amalfitano *et al.*, 2002; Glover *et al.*, 2003).

However, if animals become immunized against adenovirus, either before or following vector administration, stimulation of the adaptive immune response either silences or significantly reduces transgene expression (Byrnes *et al.*, 1995; Byrnes *et al.*, 1996a; Byrnes *et al.*, 1996b; Thomas *et al.*, 2000b; Thomas *et al.*, 2001a; Thomas *et al.*, 2001c). Loss of transgene expression is accompanied by infiltration of CD8⁺- and CD4⁺-T cells, NK cells and macrophages, and activation of local astrocytes and microglia. The role of these cells in eliminating transgene expression, and their molecular mechanisms of action are unknown.

Many different types of viruses infect the brain (Griffin, 2003). The pathological, functional and long-term consequences depend on the type of virus, route of infection, and the capacity of the immune system to clear the virus from the brain. In those cases in which virus is effectively cleared from the brain by the immune system, for example LCMV (Rall *et al.*, 1995; Kagi *et al.*, 1996) and Sindbis virus (Binder *et al.*, 2003; Griffin, 2003), most authors postulate that viral clearing from the brain *in vivo* is achieved by non-cytolytic mechanisms, even if T cells are able to kill cells *in vitro*. The molecular details of such non-cytolytic mechanisms have not been elucidated. However, even the most accomplished researchers in the field, have not yet demonstrated that *in vivo*, CTLs effectively kill target brain cells. The inability to detect cell death of brain cells by CTLs *in vivo* despite their capacity to do so *in vitro* (Rall *et al.*, 1995) remains unexplained. Further, the quantitative aspects of immunemediated elimination of transgene expression from the brain have never been studied. We believe these studies will aid development of a model to examine neuroimmune interactions at the single cell level *in vivo*.

OBJECTIVES

Herein, we address the quantitative analysis of systemic immune-mediated elimination of adenoviral transgene expression from the brain. Furthermore, we also evaluate the formation of immunological synapses, the microanatomical structure that mediates intercellular

communication between T cells and transduced astrocytes. The quantitative aspects of loss of transgene expression from the brain have not been addressed before (Thomas *et al.*, 2000b; Thomas *et al.*, 2001c).

METHODS

Adenoviral vectors

All adenoviruses used in this study are first-generation E1a-deleted recombinant adenovirus vectors based on adenovirus type 5. The construction of Ad-hCMV- β gal and Ad-hCMV-HPRT have been described elsewhere (Akrigg *et al.*, 1985; Wilkinson *et al.*, 1992; Dewey *et al.*, 1999; Cowsill *et al.*, 2000; Southgate *et al.*, 2000). Briefly, expression cassettes containing the gene for HSV1 thymidine kinase (RAdTK), β -galactosidase (Ad-hCMV- β gal) or hypoxanthine-guanine phosphoribosyltransferase (Ad-hCMV-HPRT) were inserted into the adenoviral genome in place of the E1 region. RAdTK, Ad-hCMV- β gal and Ad-hCMV-HPRT all expressed their transgenes from the human cytomegalovirus intermediate-early promoter. The adenovirus vector RAdRSVlacZ was constructed similarly to all other vectors in this study, and it expressed β -galactosidase from the Rous Sarcoma Virus (RSV) promoter. The adenovirus vector Ad-synapsin-lacZ (obtained from Dr. James Uney, University of Bristol, UK) expressed β -galactosidase from the synapsin promoter (Ralph *et al.*, 2000). The vector Ad- β Actsp1-lacZ was obtained from Dr. Frank Graham, McMaster University, Canada. Construction of this vector is described in Bett (1995). Ad- β Actsp-1lacZ expressed lacZ from the human β -actin promoter.

All adenovirus vectors were propagated on 293 cells, purified by double caesium chloride gradient centrifugation followed by dialysis and finally titered for infectious units by end point dilution of 293 cells (Southgate *et al.*, 2000b). All vector preparations were screened for replication-competent adenovirus contamination by serial amplification on HeLa cells as described (Dion *et al.*, 1996). Replication-competent adenovirus contamination was below $1:1 \times 10^9$ iu for all virus preparations. Adenoviruses were diluted in sterile saline solution before injection.

Animals and surgical procedure

Adult, male, Sprague-Dawley rats of 250 g body weight (Charles River Breeding Laboratories) were used in all the experiments described below. All experiments were conducted according to the Declaration of Helsinki. All animal experiments were performed at either the University of Manchester, UK, under the authority of The Home Office, or following approval by the IACUC at Cedars-Sinai Medical Center.

To determine the threshold immunization dose necessary to silence transgene expression, three animals per experimental group were anaesthetized with halothane and were placed in a stereotactic apparatus that was modified for use with inhalational anaesthesia. Animals were injected unilaterally in the left striatum (0.6 mm forward and 3.4 mm lateral from bregma, and 5.0 mm vertical from the dura) with 1×10^7 iu of Ad-mCMV- β gal in a volume of 3 μ l. Each injection was performed over 10 min. One month after the intrastriatal injections, rats were anaesthetized with halothane and injected intradermally in the back with increasing doses of Ad-hCMV-HPRT (10^1 – 10^8 iu of Ad-hCMV-HPRT). Sixty days after the immunization animals were injected intraperitoneally with pentobarbitone and transcardially perfused-fixed with heparinized and oxygenated tyrode solution followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde for 12 hours and stored in PBS, before sectioning and immunohistochemical analysis.

To determine the minimal dose of adenoviral vector injected in the brain that avoids immune-mediated elimination three animals per experimental group were anaesthetized with halothane and injected unilaterally in the left striatum, using the conditions described above, with increasing doses of Ad-mCMV- β gal (10^1 – 10^7 iu) in a volume of 3 μ l. One month after the intrastriatal injections, rats were anaesthetized with halothane and injected intradermally in the back with 5×10^8 iu of Ad-hCMV-HPRT). Sixty days after the intradermal injection, animals were perfused-fixed and their brains removed and post-fixed as above before sectioning and immunohistochemical analysis.

To determine the stability of adenovirus-mediated β -galactosidase expression driven by different promoters vis-à-vis the immune system, rats were injected unilaterally in the left striatum with Ad-hCMV- β gal ($n = 10$), Ad-mCMV- β gal ($n = 6$), RAdRSVlacZ ($n = 10$), RAdsynapsinlacZ ($n = 10$) or Ad β Actsp1lacZ ($n = 10$). Six weeks after the intrastriatal injection, half the animals in each group were injected intradermally with 5×10^8 iu of Ad-hCMV-HPRT. Sixty days after the intradermal injection, animals were perfused-fixed and their brains removed and post-fixed as above before sectioning and immunohistochemical analysis.

To illustrate the formation of immunological synapses between virally infected astrocytes and T cells that infiltrate the brain, animals were injected unilaterally in the left striatum as described above with 1×10^7 iu of Ad-hCMV-TK. Four weeks after the intrastriatal injection animals were immunized intradermally with 5×10^8 iu of Ad-hCMV-HPRT. Fourteen days after the intradermal injection, animals were perfused-fixed and their brains removed and post-fixed as above before sectioning and immunohistochemical and confocal analysis.

Immunohistochemistry

Coronal brain sections (50 μ m thick) were cut through the striatum using a vibratome. Free-floating immunohistochemistry was performed on serial brain sections as previously described (Thomas *et al.*, 2000a), using the following primary antibodies: anti- β -galactosidase (1:1000, Promega); ED1 (1:1000, Serotec); anti-CD8 (1:500, Serotec); anti phosphorylated ZAP-70 (1:100, Cell Signaling); anti phosphorylated Lck (1:50, Cell Signaling); anti LAT (1:100, Cell Signaling); and anti-GFAP (1:500, Advanced Immunochemical), as described earlier (Barcia *et al.*, 2006). The antibodies against TK were generated against peptides derived from HSV1-TK in chicken (raised by Aves Labs) and used at 1:100.

Immunostaining

Serial sections were used to detect transgene expression using either immunofluorescence or DAB. Immunofluorescence was carried out as described in detail previously (Barcia *et al.*, 2006). For DAB staining, endogenous peroxidase activity was quenched with 0.3% H_2O_2 in PBS, non-specific Fc-binding sites were blocked with 10% horse serum and sections incubated 48 hours at room temperature with primary antibody diluted in PBS containing 1% horse serum and 0.5% Triton-X-100 (antibody solution). Sections were then incubated for 4 hours with appropriate biotin-conjugated secondary antibodies (DAKO, Cambridge, UK). Antibody binding was detected using the avidin-biotin peroxidase with diaminobenzidine as chromogen. These sections were mounted on gelatinized glass slides and dehydrated before coverslipping.

Quantification of immunohistochemical staining

Quantitative image analysis of (1) the area occupied by cells immunohistochemically stained with anti β -galactosidase, or ED1 antibodies and (2) the estimation of the number of β -gal and CD8 cells were performed with stereology software (StereoInvestigator®) using a computer-assisted image analysis system with a Zeiss microscope connected to a digital camera through a Zeiss zoom set, as described by us previously (Suwelack *et al.*, 2004). (1) The area of transgene immunoreactivity (β -galactosidase) was quantified in five 50 μ m sections spaced

at regular 250- μ m intervals through the injected striatum as described (Thomas *et al.*, 2000a; Thomas *et al.*, 2001b; Thomas *et al.*, 2001c). The area of transgene immunoreactivity in each of the five sections was summed for each brain. 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 quantify the area of ED1 immunoreactivity. (2) The stereological estimation of the number of CD8-immunoreactive cells was performed in the striatum and external capsule overlying the striatum, and anatomical locations were identified according to the atlas of the rat brain (Paxinos, 1986) in 50 μ m brain sections spaced at regular, 250- μ m intervals in immunized animals versus non-immunized animals. The estimation of the number of cells was performed using the principle of the optical fractionator (Sterio, 1984). The number of cells was measured in 200 μ m-sided disectors covering the whole surface area of the analysed regions (50 counting frames in each section). Positive cells were counted only when they cut the superior and left limit of the square. Results were expressed as an absolute number of positive cells in the anatomical regions analyzed. Data are expressed as the mean $\pm$ SEM.

Neutralizing antibody assay

Neutralizing antibody titers were measured in serum samples taken from animals that were killed at specific time-points following intraperitoneal injection of Ad-hCMV-HPRT. Serum was heat inactivated at 56°C for 30 min and diluted from 1:2-1:4096 in MEM containing 2% FBS. Serum dilutions were incubated with 3×10^5 iu of Ad-mCMV- β gal in a volume of 10 ml for 90 min at 37°C. 50 μ l of each serum dilution was then added to wells of a 96-well plate in duplicate for each animal. An additional 50 μ l of 10% FBS was added to each well and the cells incubated overnight (18 hours) at 37°C. Cells were fixed with 4% paraformaldehyde in PBS and stained with 5-bromo-4-chloro-indolyl- β -D-galactosidase (X-gal). Anti-adenoviral neutralizing antibody titer for each animal is given by the reciprocal of the highest dilution of serum at which 50% of Ad-hCMV- β gal-mediated staining was inhibited.

Statistical analysis

Viability data were expressed as mean $\pm$ SEM and evaluated by either two- or one-way ANOVA (followed by either Dunnett or Tukey multiple comparisons tests). Differences were considered significant if $P < 0.05$. When the ANOVA test was not possible Kruskal-Wallis non-parametric test was used.

Confocal analysis

Brain sections were examined using a Leica DMIRE2 confocal microscope as described previously (Barcia *et al.*, 2006). A series range for each section was determined by setting an upper and lower threshold using the Z/Y Position for Spatial Image Series setting. Confocal microscope settings have been established and maintained by Leica and local technicians for optimal resolution.

RESULTS

What is the threshold dose of RAd necessary to induce a systemic immunization that is sufficient to eliminate transgene expression from the CNS?

1×10^7 iu of Ad-mCMV- β gal (previously referred to as RAd36), were injected into adult striata of Sprague-Dawley rats. Double immunofluorescence staining demonstrated that β -galactosidase expressing cells were mostly astrocytes (Fig. 1A). Thirty days later, animals were immunized intradermally through a single injection of Ad-hCMV-HPRT (previously referred to as RAdHPRT), in doses of 10^4 to 10^7 iu. Ad-hCMV-HPRT was used as the immunogen so that the immune response would be generated against the viral vector, not the transgene. This

paradigm has been described and published in detail previously (Thomas *et al.*, 2000b). One month after immunization brains were processed to detect β -galactosidase expression by immunohistochemistry. Animals immunized intradermally with doses of $\leq 10^5$ Ad-hCMV-HPRT did not eliminate expression of β -gal from the striatum (Fig. 1B, a,b). Quantification of the number of β -gal expressing cells showed no reduction in the level of striatal β -gal immunoreactivity when animals were immunized with doses of Ad-hCMV-HPRT of up to 10^5 (Fig. 1B, i); these doses did not increase serum neutralizing antibody titers (Fig. 1B, j). Further, animals injected intradermally with 10^7 iu of Ad-hCMV-HPRT lost all β -gal expression in the striatum (Fig. 1B, g,h), and these animals displayed high titers of neutralizing antibodies (Fig. 1B, j). Animals immunized intradermally with 1×10^6 iu of Ad-hCMV-HPRT showed variable expression of β -gal in the striatum (Fig. 1B, c–f), and variable levels of neutralizing antibodies (Fig. 1B, j). This demonstrates that the peripheral threshold of RAd needed to induce a systemic immune response that is strong enough to eliminate RAd-mediated expression from the CNS in 100% of animals is $\geq 1 \times 10^7$ iu.

Is there a minimum dose of RAd injected into the brain that escapes elimination by the systemic immune response?

Animals injected in the brain with increasing doses of Ad-mCMV- β gal (i.e. 1×10^1 – 1×10^7 iu) have a proportional, dose-related increase in expression of β -galactosidase in the striatum of naïve animals (injected systemically with saline) (Fig 2 and Fig 3). However animals immunized systemically with 5×10^8 iu of Ad-mCMV-HPRT showed a reduction of β -galactosidase expressing cells even when very low amounts of vector (i.e. 10^3 iu) were injected into the brain (Fig 2 and Fig 3). No statistically significant reductions were detected at either 10^1 iu or 10^2 iu doses (Fig 2 and Fig 3).

Systemic anti-RAd immunization silences adenoviral β -gal expression from RAd driven by neuronal, housekeeping, and/or viral promoters

Experiments illustrated in Fig 1–Fig 3 demonstrated decreased expression of transgene expression from Ad-mCMV- β gal following systemic immunization. Therefore, we examined whether the immune response to adenovirus would selectively inhibit expression from the mCMV promoter, a viral promoter, or whether other viral, housekeeping and/or cellular promoters would not be affected by the systemic immune response. To this end, we injected into the striatum RAds encoding β -gal under the control of the hCMV promoter (Ad-hCMV- β gal; previously named RAd35), mCMV (Ad-mCMV- β gal), the RSV promoter (RAd122), the β -actin promoter (RAd- β actin), and a recombinant adenovirus expressing β -gal under the control of the neuronal-specific synapsin promoter (RAdsyn).

Expression of β -gal was observed in the striatum of all control animals (sham immunized with saline). Maximum expression, as expected, depends on individual promoters (Fig. 4a–c). However, all animals immunized systemically with Ad-hCMV-HPRT showed a dramatic decrease in expression (Fig. 4a–c). The decrease in transgene expression was independent of the promoter utilized to drive transgene expression. ED1 (lysosomal membrane antigen specific for brain macrophages and microglial cells) and CD8 immunostaining of serial adjacent sections revealed an increase of both cell types surrounding the injection site in all immunized animals. Quantification of the striatal area occupied by ED1 immunoreactivity was increased significantly in animals immunized systemically with Ad-hCMV-HPRT compared with non-immunized rats (Fig. 4c3). Stereological quantification of the number of CD8⁺ T cells infiltrating the striatum in immunized animals also revealed a significant increase compared with controls (Fig. 4c2). Although animals were injected with the same amount of recombinant adenovirus, the level of expression detected depended on the individual promoter activity. Nevertheless, the inflammatory and immune response was identical in all animals

because it is directed against viral-vector proteins rather than the amount of transgene expressed.

Activated T cells expressing tyrosine kinases form immunological synapses with virally infected cells

Recently, we have demonstrated that activated antiviral T cells form specific immunological synapses with virally infected astrocytes. Immunological synapses are thought to mediate intercellular communication in the immune system and, thus, might mediate antiviral immune responses in the brain, as described above. The anatomical substrate of these interactions is illustrated in Fig. 5; these confocal images demonstrate the formation of immunological synapses between virally infected astrocytes and CD8⁺ T cells. This figure demonstrates, for the first time, that phosphorylated ZAP 70 (Fig. 5a), and phosphorylated LCK (Fig. 5b) are indeed polarized in T cells establishing immunological synapses with infected astrocytes (they are shown to express TK), thus, demonstrating that T cell receptor (TCR) signaling has been stimulated in response to antigen encounter. LAT (Fig. 5c), an adaptor protein that is involved in TCR signaling, is also localized within the contact area between T cells and infected astrocytes.

CONCLUSIONS

- The minimum dose of replication-defective adenovirus type 5 required to induce a systemic immune response that can eliminate transgene expression from the brain in 100% of Sprague-Dawley rats is 1×10^7 infectious units.
- The maximum dose of a replication-defective adenovirus type 5 injected into the striatum of Sprague-Dawley rats that **avoids** immune-mediated elimination is 100 infectious units. Transgene expression in the brain from all vector doses **above** 10^2 infectious units is eliminated.
- The primed/activated adaptive immune response abolishes transgene expression in the brain driven by viral, housekeeping, or cell-type specific promoters.
- Specific TCR cell signaling at the immunological synapse between CTLs and infected adenovirally infected brain cells induces the targeting of phosphorylated tyrosine kinases and LAT to the T cell:astrocyte interface.

DISCUSSION

First-generation adenoviral vectors are powerful vectors for gene transfer and gene therapy in the CNS (Bilang-Bleuel *et al.*, 1997; Bemelmans *et al.*, 1999; Dewey *et al.*, 1999; Bjorklund *et al.*, 2000; Mittoux *et al.*, 2000; Sandmair *et al.*, 2000; Maleniak *et al.*, 2001; Mittoux *et al.*, 2002; Biglari *et al.*, 2004; Chiocca *et al.*, 2004; Do Thi *et al.*, 2004; Gondi *et al.*, 2004; Hurtado-Lorenzo *et al.*, 2004; Immonen *et al.*, 2004). As a result, recent trials have shown adenoviral vectors encoding HSV1-TK to be effective in randomized clinical trials for brain glioblastoma multiforme, compared to patients treated with standard treatment. These encouraging clinical data have led to a large, European, Phase III, multicenter, clinical trial of adenovirus-TK (Shand *et al.*, 1999).

In naïve rats not exposed previously to adenovirus, transgene expression from adenoviral vectors is stable for up to 13 months (Byrnes *et al.*, 1995; Geddes *et al.*, 1997; Kajiwara *et al.*, 1997; Ideguchi *et al.*, 2000; Thomas *et al.*, 2000b; Zou *et al.*, 2000; Amalfitano *et al.*, 2002; Glover *et al.*, 2003). However, systemic immunization of animals injected previously with adenovirus into the CNS with first-generation adenoviral vectors leads to immune responses in the CNS that substantially reduce and, in some cases, eliminate transgene

expression from the brain (Byrnes *et al.*, 1995; Byrnes *et al.*, 1996a; Byrnes *et al.*, 1996b; Wood *et al.*, 1996; Kajiwara *et al.*, 1997; Thomas *et al.*, 2000; Zermansky *et al.*, 2001). However, transgene expression from high-capacity adenoviral vectors remains stable; further, three days after intracranial injection of vector in animals previously immunized with a peripheral injection of adenoviral vector was identical to non-immunized animals. This indicates that neutralizing antibodies are ineffective at blocking adenoviral infection of brain tissue (Thomas *et al.*, 2001c).

In these studies we determined the dose of adenovirus needed for systemic immunization to eliminate transgene expression from the brain. Systemic injections of increasing amounts of viral vectors demonstrated that doses of $>1 \times 10^6$ iu eliminate transgene expression from the brains of all animals. In some cases, loss of transgene expression has been attributed to the shut-off of viral promoters. To ascertain whether expression from a viral promoter in our vectors was inhibited non-specifically, we injected into the brain vectors that express the transgene under various viral, housekeeping and neuronal promoters. In all cases, immunization reduced transgene expression, demonstrating that this phenomenon is not a consequence of promoter shut-off, but represents a general mechanism that abrogates expression from any promoter within the viral vector.

Previous experiments describing the shut-off of transgene expression utilized recombinant adenovirus vectors encoding the hCMV promoter. This promoter is influenced by several regulatory mechanisms, including pro-inflammatory cytokines such as IFN- γ (Shering *et al.*, 1997). Our experiments indicate that the immune system downregulates expression from several promoters, including viral, housekeeping and cell type-specific promoters. However, the alternative hypothesis, that limited cytotoxicity occurs throughout the CNS, needs to be considered equally at this stage.

Specific downregulation of transcription from viral promoters might operate through inhibition of viral genome-specific mechanisms. These might, for example, involve reorganization of ND-10 domains (viral DNA docking sites) and sequestration of transcription factors in response to IFN γ (Griffin, 2003; Khanna *et al.*, 2004), phosphorylation of the translation regulator eIF α , and activation of PKR-related multi-viral mechanisms (Scheuner *et al.*, 2003; Baltzis *et al.*, 2004; Bonizzi *et al.*, 2004; Hsu *et al.*, 2004; Isler *et al.*, 2005). However, it remains to be seen whether more specifically gene expression is affected in transduced cells. It is interesting to note that regulation of transgene expression is mediated mostly by cellular elements of the adaptive immune response because the early innate inflammatory immune responses do not affect gene expression (Thomas *et al.*, 2000b; Thomas *et al.*, 2001b).

Furthermore, other mechanisms have been described by which the adaptive immune response might silence gene expression. In the CNS, antibodies and IFN- γ mediated mechanisms have been postulated to clear virus infection from neurons through non-cytolytic pathways (Patterson *et al.*, 2002; Griffin, 2003). Non-cytolytic mechanisms might be more frequent in clearing virus from infected neurons, whereas cytotoxic mechanisms might be more common in clearing virus from infected astrocytes and oligodendrocytes (Griffin, 2003). Using quantitative PCR, we have previously demonstrated a reduction in vector genome copy numbers in animals immunized against adenovirus (Barcia *et al.*, 2006). Although the copy numbers of the vector genome were reduced, our data did not rule out promoter shut-off completely. We believe that most elimination of transgene expression could be attributed to cytotoxic effects and that transcriptional down-regulation, if it occurs, is likely to make a minor contribution to the loss of transgene expression.

To date, however, cytotoxicity has only been demonstrated conclusively in *in vitro* models; *in vivo* direct killing and elimination of brain cells by lymphocytes is difficult to demonstrate

(Rall *et al.*, 1995). Immunological synapses are the anatomical substrate that underlies intercellular communication in the immune system, and recently we have demonstrated their existence *in vivo* (Barcia *et al.*, 2006). Here, we provide further evidence that anti-viral CD8⁺ cytotoxic T cells that express phosphorylated tyrosine kinases form immunological synapses with virally infected brain cells preceding the elimination of transgene expression from virally infected astrocytes. We also demonstrate that LAT, the crucial linker between TCR engagement and T-cell activation also accumulates at the immunological synapses. Currently, we are evaluating a novel system to explore the consequences to virally-infected astrocytes of the formation of immunological synapses with anti-viral CD8⁺ cytotoxic T cells.

In summary, we have demonstrated that a primed adaptive immune response specifically inhibits transgene expression in the CNS independently of the promoter that drives transgene expression. Cellular elements of the systemically activated immune response infiltrate the CNS and cause the shut-off of expression from viral, housekeeping and cell-type specific promoters encoded with adenoviral vectors.

Understanding the mechanisms of action by which the immune system regulates transgene expression in the brain is crucial for safe, effective clinical trials. This is especially important if patients have been exposed previously to viruses from which the vectors used in gene therapy are derived (e.g. adenovirus, AAV and HSV-1). If the immune system regulates gene expression in the CNS mainly through noncytotoxic mechanisms, any downregulation of gene expression will block the efficiency of clinical trials that involve gene therapy. However, if the immune response physically eliminates transduced cells, it might make conditions in which the therapy aims to protect neurons from ongoing degeneration, such as Parkinson's disease, worse. An optimal animal model of the immunological challenges associated with human clinical trials for gene therapy would involve immunization with a wild-type, replicating, human adenovirus. Unfortunately human adenovirus does not replicate in rodents and so we use a replication-deficient viral vector as the immunization agent.

These studies also establish the threshold input of non-replicating adenovirus vectors needed to prime a systemic immune response, and demonstrate that a threshold must be crossed before a systemic, anti-adenoviral immune response is unleashed. Furthermore, we demonstrate that once a systemic immune response has been primed it is highly efficient in recognizing and eliminating even very small numbers of transduced cells. We believe that our data demonstrate the need to establish vector dose thresholds for all viral-derived vector platforms that are used in gene therapy for neurological diseases.

Of specific interest to this special issue, pain is a potential target for gene therapy strategies, especially by modifying the function of either dorsal root ganglion neurons or neural pathways along the pain circuits. Preclinical studies of chronic pain have used several gene therapy vectors (Cope *et al.*, 2006). Adenoviral, AAV, and HSV vectors have been used to deliver therapeutic genes including β -endorphin (Finegold *et al.*, 1999), proopiomelanocortin (Lu *et al.*, 2002), interleukin 2 (IL-2) (Yao *et al.*, 2003), proenkephalin-A (Braz *et al.*, 2001), IL-10 (Milligan *et al.*, 2005a; Milligan *et al.*, 2005b), glial-derived growth factor (Hao *et al.*, 2003) and neurotrophin-3 (Pradat *et al.*, 2001).

Currently gene therapy approaches for chronic pain are yet to be utilized in a human clinical trial but several research groups are in the early stages of implementation. It is important to consider the immunological consequences of delivering viral vectors systemically. Immune responses might, in some cases, be deleterious to either the transduced cells or the long-term expression of the therapeutic gene, an effect that is mediated through the establishment of immunological synapses (Barcia *et al.*, 2006).

Our data indicate that the primed systemic adaptive immune response inhibits expression from any type of promoter, and efficiently suppresses expression from as few as 1000 transduced brain cells. The mechanisms by which the immune system regulates gene expression in the CNS needs to be explored and developed further to make clinical trials in neurological gene therapy safer and more effective. In addition, steps must be taken to avoid priming the systemic adaptive immune response.

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Fig. 1. (A) RAD36 induces expression of β -galactosidase in astrocytes

Rat brain sections injected with RAD36 were stained for β -galactosidase (green) and GFAP, a marker of astrocytes (magenta) and analyzed with confocal microscope. Confocal analysis demonstrated that β -galactosidase is expressed in astrocytes. Note the colocalization of β -galactosidase and GFAP in the merged image. Scale bar, 30 μ m. (B) Determination of the dose of systemic immunization needed to silence transgene expression in the CNS. Panels a–h show β -gal immunohistochemistry in rats injected with 10^7 iu Ad-mCMV- β gal in the striatum and immunized 30 days later with 10^1 – 10^8 iu Ad-hCMV-HPRT. Only doses 10^5 to 10^7 iu are illustrated. Peripheral immunization with $\leq 10^5$ iu did not reduce brain transgene expression (a,b,i) or increase serum neutralizing antibodies (j). Immunization with 10^6 iu induced loss of

transgene expression in some animals and one animal from each group is illustrated (c–f,i,j). Following peripheral immunization of $\geq 10^7$ iu transgene expression was lost in all animals (g,h,i), with all animals showing significant titers of neutralizing antibodies (j). Each dot in i and j represents one animal.

Fig. 2. β -gal immunohistochemistry of rat brain injected with increasing doses of Ad-mCMV- β gal in the striatum

Animals were injected with 10^1 – 10^7 iu Ad-mCMV- β gal in the striatum and 30 days later systemically with either saline (left) or 10^8 iu Ad-hCMV-HPRT (right). Peripheral immunization with 10^8 iu Ad-hCMV-HPRT reduced brain transgene expression independently of the dose of adenovirus injected into the brain.

Fig. 3. Quantification of β -gal-positive cells after injection with Ad-mCMV- β gal in the striatum and Ad-hCMV-HPRT systemically

Rats were injected with increasing doses of Ad-mCMV- β gal in the striatum (10^1 – 10^7 iu) and injected systemically 30 days later with either saline or 10^8 iu Ad-hCMV-HPRT. Expression of β -gal was always reduced following immunization with 10^8 iu Ad-hCMV-HPRT despite the different doses injected intracranially.

Fig. 4. Immunohistochemical detection of β -gal, CD8 and ED1

β -gal, CD8 and ED1 in brain sections from rats injected in the striatum with different virus (Ad-hCMV- β gal, Ad-mCMV- β gal, RAd122, RAdsyn and RAd β act) containing different promoters (hCMV, mCMV, RSV, syn and β act, respectively) to drive expression of β -gal. Thirty days after injection animals were injected intradermally with either Ad-hCMV-HPRT (immunized animals) or with saline (control group). Sixty days after intradermal injection, animals were sacrificed and perfused. The expression of β -gal, CD8 and ED1 is shown in immunized (Ad-hCMV-HPRT) and in control animals (saline). (A,B) 1st, 3rd and 5th columns show low magnification of β -gal, CD8 and ED1, respectively, and the boxed areas are shown at higher magnification (right columns). (C) The area occupied by β -gal is reduced significantly

in immunized animals (Ad-hCMV-HPRT) compared to controls (C1). Stereological estimation of the number of CD8 cells increased significantly in animals immunized with Ad-hCMV-HPRT compared to controls (C2). The area occupied by ED1 also increased significantly in immunized animals compared to controls (C3). * $P < 0.05$, ** $P < 0.01$ Student's *t*-test.

Fig. 5. Activated T cells that express tyrosine kinases form immunological synapses with virally infected cells

Confocal images show three immunological synapses formed between T cells and virally infected astrocytes in the striatum of rats injected with Ad-hCMV-TK and immunized systemically 30 days later with Ad-hCMV-HPRT. Activated T cells show expression, phosphorylation and polarization of tyrosine kinases when in close apposition with virally infected astrocytes, indicating the activation and signaling of TCR in response to antigen encounter. Top row: a T cell (red, stained for TCR) that expresses phosphorylated ZAP-70 (green) forms an immunological synapse with a virally infected cell (white, stained for TK). Middle row: a T cell (red, stained for TCR) expressing phosphorylated Lck (green) polarized to the area of contact with the target cell (white, stained for TK). Bottom row: a CD8⁺ T cell that expresses LAT (green) forms an immunological synapse with a virally infected cell (white, stained for TK). White arrows indicate the areas of close apposition between T cells and infected brain cells. Scale bar, 20 μ m.