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Immune parameters affecting adenoviral vector gene therapy in the brain

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Gene therapy utilizing replication deficient adenoviral vectors represents a potentially promising approach to the treatment of brain tumors. Limited duration of systemic transgene expression and inefficient transduction following repeat systemic vector administration secondary to an effective anti-vector immune response limits the potential application of first generation adenoviral vectors. Whether host immune responses will significantly limit the use of these vectors within the immunopriviledged environment of the central nervous system remains to be elucidated. Following a single intravenous injection of a β-galactosidase expressing adenoviral vector (Ad.CMV-βgal), we found maximal βgal transgene expression in systemic sites (i.e. liver) at day 4, with almost complete disappearance by day 7. In contrast, significant β-galactosidase activity was seen for greater than 28 days following a single intracerebral inoculum of virus. Rechallenge experiments demonstrated complete protection against repeat systemic vector administration, whereas intracerebral transgene expression was not affected by prior systemic or intracerebral exposure to adenoviruses. These data suggest that systemic anti-adenoviral vector immune responses are attenuated within the central nervous system and may not pose as significant a problem for the treatment of brain tumors as for other systemic indications.

Keywords: adenovirus; gene therapy; β-galactosidase; neuroimmunology; brain; inflammation

Introduction

Replication-deficient, recombinant adenoviral vectors are highly attractive gene transfer vehicles secondary to their relative ease of construction and propagation, high initial transgene expression, and ability to transduce a wide variety of cell types (Kozarsky and Wilson, 1994; Stratford-Perricaudet *et al*, 1990). Recent concern has been raised over the potential clinical utility of these vectors, however, secondary to the generation of a robust anti-vector immune response (Tripathy *et al*, 1996; Worgall *et al*, 1997; Yang *et al*, 1995a). Detection of this response first came to light when it was noted that systemic adenoviral vector-mediated transgene expression, although initially high, diminished to undetectable levels over days in immunocompetent animals (Yang *et al*, 1994a, 1995b). In contrast, the duration of transgene expression was prolonged in immunodeficient animals (Yang *et al*, 1994b). Cytotoxic T lymphocytes (CTL) appear to be responsible for the limited duration of transgene expression through the recognition and subsequent elimination of viral infected cells (Yang, 1994a, 1995b,c). Recognition of infected cells presumably occurs following presentation of viral and/or transgene peptides in the context of major histocompatibility complex (MHC) class I surface proteins. Support for a CTL-mediated mechanism of limited transgene expression comes from experiments demonstrating that natural (i.e. Nu/Nu or SCID mice) and/or iatrogenic (i.e. glucocorticoids, cyclosporin, Anti-CD4/8 IgG) (Wilson and Kay, 1995) perturbations of T-cell function significantly prolong adenoviral mediated transgene expression. In addition to a cellular mediated limit on the duration of transgene expression, the humoral immune response significantly limits the ability to effectively deliver repeated vector administration

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secondary to the presence of virus neutralizing antibodies (Yang, 1995a,b).

Adenoviral vectors have shown particular promise for gene transfer strategies involving the central nervous system (CNS) secondary to their ability to infect post-mitotic cells. A number of recent reports demonstrate the ability of adenoviral vectors to transduce both glial as well as neuronal cell types *in vivo* (Akli *et al*, 1993; Bajocchi *et al*, 1993; Davidson *et al*, 1993). Additionally, the ability to directly transduce CNS tissue using highly concentrated adenoviral vectors, without the need for *in vivo* transplantation of producer cell lines, offers a distinct advantage over standard retroviral vectors. Although it has been demonstrated that adenoviral vectors elicit an inflammatory response within the brain, much like that seen in systemic tissue (Byrnes *et al*, 1995, 1996; Kajiwara *et al*, 1997; Wood *et al*, 1996), it is far less clear whether this anti-adenoviral immune response will significantly impede the use of these vectors within the CNS. We have, therefore, performed a series of experiments designed to assess the impact of the immune system on both the efficiency and duration of adenoviral vector-mediated transgene expression within the CNS. We demonstrate that the anti-adenoviral immune response is significantly attenuated within the CNS, allowing for extended duration of transgene expression and repeated vector administration suggesting that adenoviral vectors may yet have a role in gene therapy strategies in the CNS.

Results

Duration of adenoviral vector-mediated transgene expression

In order to evaluate whether the immune response limits the duration of vector-mediated transgene expression within the CNS to the same extent it does systemically, we performed a series of *in vivo* viral transduction time course experiments. Ad.CMV-βgal (10^9 plaque forming units, or PFU) was injected into the portal vein or stereotactically injected into the right caudate nucleus of the brain, and at various time points the level of β-galactosidase (β-gal) expression was assessed. As can be seen in Figure 1A and B, β-gal expression in the liver was robust on day 4, but had totally dissipated by day 7. In contrast, stereotactic injection of Ad.CMV-βgal into the brain resulted in high level of β-gal expression on days 14 and 28 (Figure 1C and D). In fact, β-gal activity could still be observed in the brains of rats 70 days after vector injection (the latest time point we have evaluated), although the level of expression was only 5–10% of that seen in the first 2 weeks.

Despite the prolonged duration of transgene expression, an acute inflammatory reaction was seen within the area of transduced brain (Figure 1E and F), not unlike that seen in adenoviral vector transduced systemic tissues. This inflammatory reaction consists of a mixed population of cells including T and B lymphocytes, NK cells, monocytes, and granulocytes. Which of these cells is responsible for the eventual elimination of transgene expression within the CNS is not clear. As discussed, it is largely believed that T-cells mediate the principle cellular response against adenovirus transduced systemic cells. In order to evaluate how selective down-regulation of the anti-adenoviral T-cell response would impact on duration of vector-mediated transgene expression in the brain, we performed a series of experiments utilizing CTLA4Ig induced immunosuppression. Immediately following intravenous injection of a control immunoglobulin (Ig) or CTLA4Ig, Ad.CMV-βgal was injected into the opposite femoral vein or into the caudate nucleus. Co-administration of the control immunoglobulin with Ad.CMV-βgal had no effect on the duration of transgene expression in the liver or the brain compared to that seen with β-gal virus alone (Figure 1). Co-administration of CTLA4Ig with Ad.CMV-βgal virus, however, resulted in prolongation of β-gal expression in the liver, with hepatic levels of β-gal expression on days 7 and 14 that were nearly identical to that seen on day 4 (Figure 2A and B). In contrast, co-administration of the same dose of CTLA4Ig immediately prior to stereotactic intracerebral injection of Ad.CMV-βgal had no effect on the duration of transgene expression within the brain (Figure 2C and D). Additionally, co-administration of CTLA4Ig did not impact on the level or timing of the acute vector-mediated inflammatory process seen in the brain (Figure 1F). Although these data support the hypothesis that T-cell responses constitute a major component of the immune response directed against adenoviral vector transduced tissues systematically, their role in eradicating adenoviral vector-transduced CNS tissue cannot be conclusively demonstrated. It is possible that increased dose, altered timing or different routes of administration of CTLA4 or other specific immunosuppressive agents could indicate a role for some level of anti-adenoviral T-cell response in the brain.

Repeated vector administrations

Many of the potential clinical applications of gene therapy will undoubtedly require one or more repeat administrations of the therapeutic vector, and thus the development of protecetive humoral immunity could significantly limit the clinical utility of these vectors. Additionally, since most adults have been previously exposed to wildtype adenovirus, and have circulating anti-adenoviral antibodies, even first time vector administration could be problematic. Indeed, previous experimental data suggests a potent anti-viral humoral

response can significantly limit repeat systemic vector administration (Kay *et al*, 1995; Mack *et al*, 1997; Yang *et al*, 1995c). We were, therefore, interested in examining the effect of anti-adenoviral antibodies on the efficiency of repeat intracerebral vector administration.

To experimentally address this question, animals were pre-immunized with either the virus vehicle control or Ad.CMV-CD, a vector identical to Ad.CMV-βgal except that it encodes a different transgene so that vector specific immune responses could be evaluated independent of immune responses directed against the transgene. As demonstrated in Table 1, all animals systemically exposed to Ad.CMV-CD or Ad.CMV-βgal developed high titers of adenoviral neutralizing antibody between days 4 and 7. Consistent with the finding of circulating adenovirus neutralizing antibodies, Figure 3 demonstrates that the livers of animals which have been previously immunized with Ad.CMV-CD cannot be effectively transduced by Ad.CMV-βgal. These results are in agreement with previously published reports demonstrating protective humoral immunity following prior vector

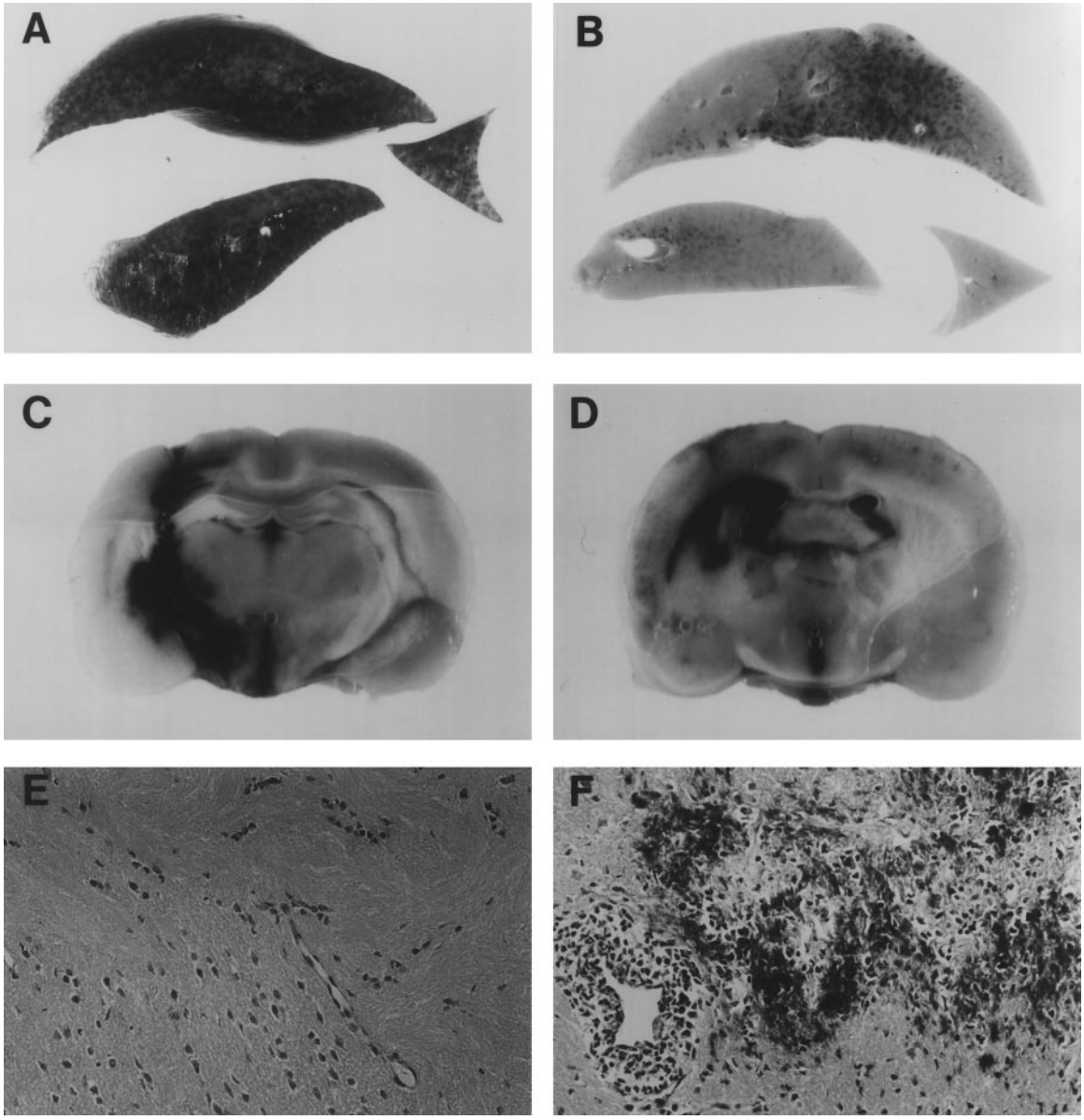

Figure 1 Extent and duration of β-galactosidase expression after single intravenous (i.v.) or intracerebral (i.c.) injections of a βgal-expressing adenoviral vector. Animals were i.v. or i.c. injected with Ad.CMV-βgal (1×10^9 p.f.u.) and then sacrificed at day 4 and 7 (i.v. injected vector) or day 14 and 28 (i.c.). X-gal histochemistry was carried out on liver and coronal brain whole amounts. (**A**) Ad.CMV-βgal i.v., liver day 4; (**B**) Ad.CMV-βgal, liver day 7; (**C**) Ad.CMV-βgal, brain day 14; (**D**) Ad.CMV-βgal, brain day 28. Also shown are thin sections of normal brain (**E**) or brain following Ad5.CMV-βgal injections (**F**).

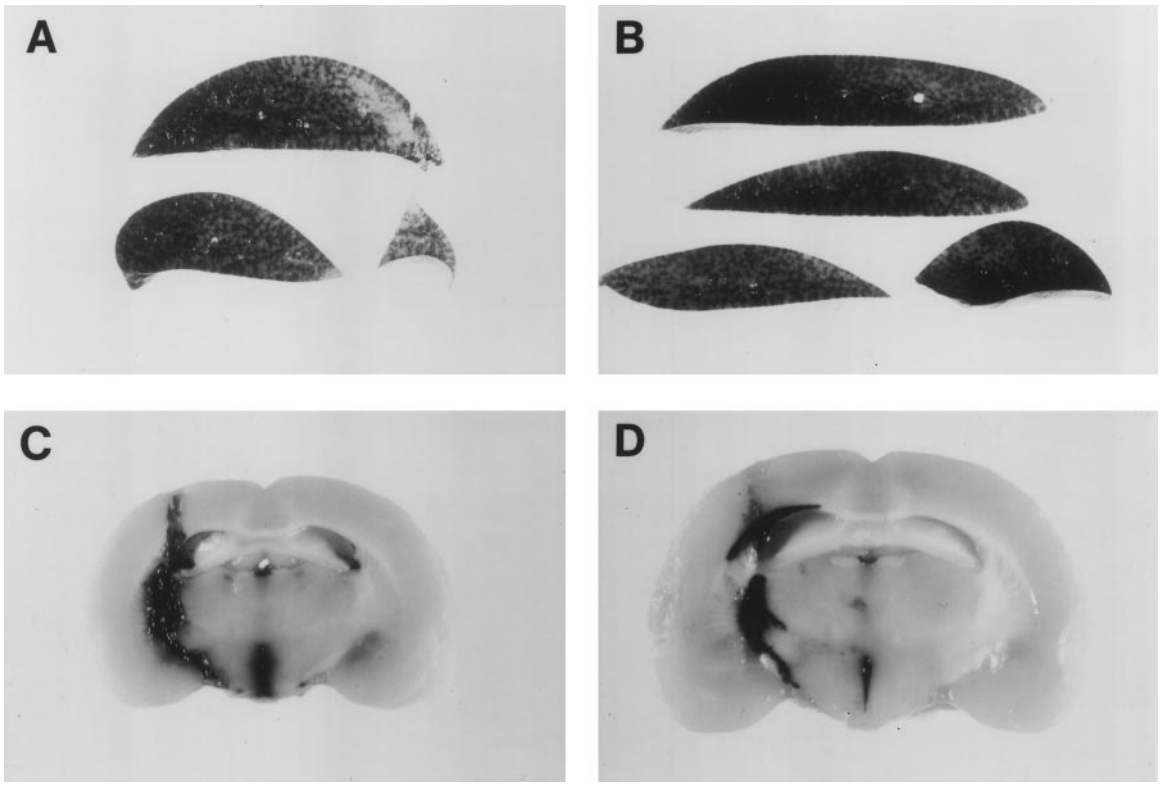

Figure 2 Co-administration of Ad.CMV-βgal with CTLA4Ig. A bolus femoral i.v. injection of 0.5 mg murine CTLA4Ig or a control murine Ig was given 5 min prior to i.v. or i.c. injection of Ad.CMV-βgal (1×10^9 p.f.u.). Animals were sacrificed at day 6 and 14 (i.v. injected vector) or day 14 and 28 (i.c.) and X-gal histochemistry carried out on liver and coronal brain whole mounts. (**A**) CTLA4Ig/Ad.CMV-βgal i.v., liver day 6; (**B**) CTLA4Ig/Ad.CMV-βgal, liver day 14; (**C**) CTLA4Ig/Ad.CMV-βgal, brain day 14; (**D**) CTLA4Ig/Ad.CMV-βgal, brain day 28.

exposure (Kay *et al*, 1997; Mack *et al*, 1997; Yang *et al*, 1995c).

The situation in the brain, however, is clearly different. Animals pre-immunized by prior systemic exposure to Ad.CMV-CD demonstrated high levels of β-gal staining in the brain on day 14 that was indistinguishable from that seen in vector-naive animals despite the presence of high titer circulating neutralizing antibodies (Figure 3B). Additionally, the duration of transgene expression did not seem to be effected by prior vector immunization (data not shown). To control for the possibility that an effective humoral response in the CNS requires prior CNS vector exposure, we also immunized animals by intracerebral injections of Ad.CMV-CD, and then re-challenged with Ad.CMV-βgal. Intracerebral injections of adenoviruses also resulted in the development of circulating neutralizing antibodies, although the titers were lower than that achieved following systemic immunization (Table 1). Similar to that seen in the systemically immunized animals, repeat intracerebral administration of Ad.CMV-βgal in CNS immunized animals resulted in β-gal expression identical to that seen in vector-naive animals (Figure 3C).

Discussion

Adenoviruses hold significant promise as potential vectors for clinical applications of gene therapy. The ultimate therapeutic utility of these vectors, however, may be limited by potent anti-adenoviral immune responses. Various strategies have been experimentally explored to overcome adenoviral vector immunogenicity. These strategies can generally be grouped into those aimed at altering the viral vector itself, or modulating the host immune response.

Adenoviral vectors possess three different potential sources of foreign antigen, including the expressed transgene (Tripathy *et al*, 1996), expressed viral proteins (Yang *et al*, 1995a), and the viral particle itself (Byrnes *et al*, 1995; Worgal *et al*, 1997). Future improvements in vector development should help eliminate unwanted viral gene expression which contribute to the CTL response against transduced host cells. Examples of such vector improvements include mutations in the E2a promoter (Engelhardt *et al*, 1994) and deletions of the E4 genes (Armentano *et al*, 1995; Krougliak and Graham, 1995), both of which are involved in

Table 1 Neutralizing antibody assay

Dilution 1/X	*<20*	*20*	*40*	*80*	*160*	*320*	*640*	*1280*	*2560*	*5120*	*10240*	*20480*
SYSTEMIC/SYSTEMIC												
day 4												
saline/βgal					X	X	X		X			
CD/βgal									X	XX	X	
day 7												
saline/βgal						X	XX	X				
CD/βgal								X	X		X	X
SYSTEMIC/BRAIN												
day 14												
saline/βgal		X		X			X	X				
CD/βgal									X	XX	X	
day 28												
saline/βgal		XX				X	X					
CD/βgal							X	X	X	X		
BRAIN/BRAIN												
day 14												
saline/βgal					XX				X	X		
CD/βgal								XXX	X			
day 28												
saline/βgal				X		XX	X					
CD/βgal								X	X		X	X
CTLA4Ig/BRAIN												
day 14												
control Ig/βgal				XX		XX						
CTLA4 Ig/βgal	XXXX											
day 28												
control/βgal						XX			XX			
CTLA4 Ig/βgal	XXXX											

Serum samples from animals used in experiments shown in figures above were analyzed for neutralizing antibody titer by mixing various dilutions of serum with Ad.CMV-βgal before infection of 293 cells. Titer was scored as the highest dilution (i.e. lowest serum concentration) which inhibited infection and the resulting expressed β-galactosidase activity by 75% as measured by ONPG color development.

regulation of late viral protein expression. While E3 was originally found dispensable with regard to viral infection (Bett *et al*, 1994), it now appears that E3 encodes several proteins which protect against CTL responses *in vivo* through down-regulation of MHC expression and resistance to tumor necrosis factor-mediated cell death (Wold and Gooding, 1991). Accordingly, newer generation vectors are being built that reconstitute the ability of the vector to express these protective genes (Lee *et al*, 1995). Finally, the latest generation of adenoviral vectors have the entire viral genome replaced with non-viral DNA flanked only by the viral terminal repeats and the adenoviral packaging signal, thus totally eliminating the possibility of vector-mediated expression of viral proteins. Viral structural proteins are provided *in trans* by a packaging defective helper virus (Haecker *et al*, 1996; Kochanek *et al*, 1996; Parks *et al*, 1996; Parks and Wilson, 1997). Although preliminary reports suggest that such vectors may allow prolongation in the duration of transgene expression, the true degree of improvement these vectors offer over older generation vectors has yet to be studied in full. Such vectors, however, do not address the problem of the potent humoral response to the viral particle itself, limiting ones ability to effectively re-administer the vector since the outer capsid proteins are still present in all these vectors. Although one report suggests that switching the serotype of adenovirus may circumvent the problem of serotype-selective neutralizing antibody (Mack *et al*, 1997), practicality of this approach for repeated vector administrations appears limited.

In addition to designing less immunogenic vectors, altering the host immune system is another approach to improving the efficiency of adenoviral vectors. Non-selective immunosuppressive agents can significantly prolong adenoviral vector-mediated transgene expression (Kay *et al*, 1995; Stratford-Perricaudet *et al*, 1990; Yang *et al*, 1995a; Yang and Wilson, 1995), but the long term use of such agents in patients with chronic diseases is problematic. More recent attempts have been directed toward more selective types of immunosuppression including CD4 and/or CD8 T-cell depletion and attempts to induce tolerance to adenoviral antigens (DeMatteo *et al*, 1995; Guerette *et al*, 1996). Whether any of these strategies will ultimately be clinically successful or practical

remains to be seen. In either case, it is clear that significant progress will need to be made in countering the anti-adenoviral immune response if adenoviral vectors are to ultimately be clinically useful for systemic indications.

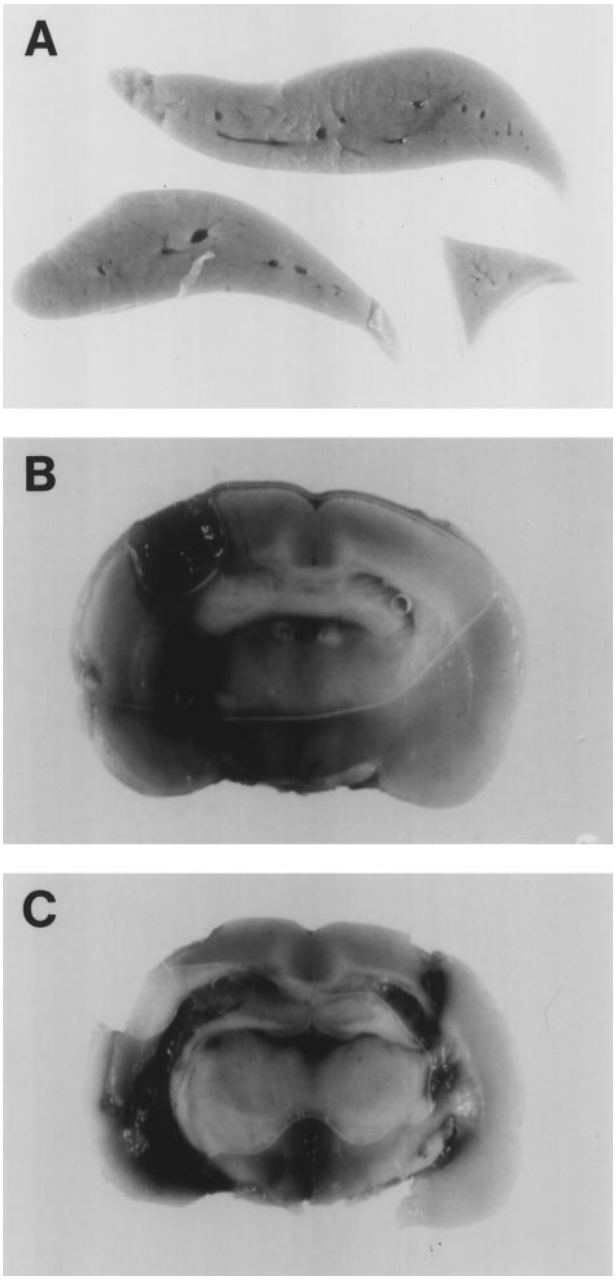

Figure 3 β-gal expression after i.v. or i.c. injections of Ad.CMV-βgal following prior exposure to adenoviral vectors. Rats were injected with saline or Ad.CMV-CD (1×10^9 p.f.u.) 14 days prior to the i.v. or i.c. injection of Ad.CMV-βgal (1×10^9 p.f.u.). Animals were then sacrificed 4 (i.v. injected animals) or 7 (i.c. injected animals) days after Ad.CMV-βgal administration, and X-gal histochemistry carried out on liver and brain whole mounts. (**A**) prior i.v. Ad.CMV-CD followed by i.v. Ad.CMV-βgal, liver day 4; (**B**) prior i.v. Ad.CMV-CD followed by i.c. Ad.CMV-βgal, brain day 14; (**C**) prior i.c. Ad.CMV-CD (opposite hemisphere) followed by i.c. Ad.CMV-βgal, brain day 14.

It is becoming increasingly clear that the immune response may limit many of the potential sytemic clinical applications of early generation adenoviral vectors. What remains less clear, however, is whether the immune response will also limit the therapeutic utility of these vectors for gene transfer applications within the CNS. It is generally accepted that the CNS is at least a relative immunologic sanctuary such that systemic immune responses are partially or fully attenuated within the brain (Fabry *et al*, 1994). Our data, in agreement with several other reports, demonstrate that the duration of adenoviral-vector transgene expression in the brain, following direct stereotactic injection, is significantly prolonged compared to that seen following systemic vector administration (Akli *et al*, 1993; Davidson *et al*, 1993). This extended duration of transgene expression occurs despite the fact that an acute inflammatory reaction occurs within several days of vector administration. It has been demonstrated that this inflammatory infiltrate consists of a heterogeneous population of cells including T and B-lymphocytes, NK cells, monocytes, and granulocytes (Byrnes *et al*, 1995, 1996; Kajiwara *et al*, 1997). Why these inflammatory cells do not effect a significant and rapid destruction of target cells within the CNS to the same extent they do systemically remains unclear. The fact that these infiltrates appear very similar to those seen in systemically transduced tissue, however, suggests that the defect in immune clearance of transduced cells is more a function of inhibition of cell-directed cytotoxicity rather than immune effector cell migration within the CNS (Byrnes *et al*, 1995).

Despite the prolonged transgene expression, we as others, have noted a diminishment of transgene expression with time (weeks to months) that is associated with the loss of some of the transduced cells within the CNS. Whether this effect represents a direct long-term cytotoxic effect from the adenoviral vector itself, or is immune mediated, remains unclear. It is known, however, that when inflammatory responses do occur in the brain, they are often mediated by effector cell infiltration from outside of the CNS as is seen in experimental autoimmune encephalomyelitis (EAE) (Khoury *et al*, 1995). In EAE, foot pad immunization with myelin basic protein (MBP) leads to formation of MBP-specific CD4 helper T-cells resulting in a potent anti-MBP response within the CNS, which can be prevented by CTLA4Ig. The immunosuppressive mechanism of CTLA4Ig, a chimeric protein of murine immunoglobulin Cγ2a fused to murine CTLA4, is thought to be related to its ability to bind to B7, thereby inhibiting B7-mediated co-stimulatory signals. Blocking T-cell co-stimulatory signals, in the presence of T-cell receptor activation by antigenic peptides, results in antigen-specific an-

ergy. Thus, we hypothesized that if systemic T-cells were responsible for the destruction of vector-transduced cells within the CNS, induction of selective systemic T-cell anergy by CTLA4Ig should result in even more prolonged transgene expression within the brain. Thus, it was interesting to observe that under the limited experimental conditions we utilized, co-administration of CTLA4Ig did not change the time course of transgene expression and cell loss within the CNS, in contrast to its profound effect on systemic transgene expression. These data are consistent with previous reports that have shown that depletion of CD4+ or CD8+ cells, while greatly reducing the local inflammatory response, did not change the pattern of transgene expression in brain (Byrne *et al*, 1996; Wood *et al*, 1996). Nevertheless, more detailed studies are clearly warranted before we can entirely dismiss a significant contribution from T-cell responses to the progressive loss of adenoviral vector-mediate transgene expression within the CNS.

Our data demonstrates that all animals systemically exposed to Ad.CMV-CD or Ad.CMV-βgal developed high titers of adenoviral neutralizing antibody between days 4 and 7, consistent with the hypothesis that the humoral response is largely responsible for protective immunization. Even animals exposed only to intracerebral adenoviral vectors developed neutralizing antibody, although the titers were lower than that seen following systemic exposure. Whether the lower titers reflect a fundamental difference in antigen presentation within the CNS or merely represents a lower systemic viral innocula, following 'spillage' of the vector into the blood stream during the trauma of intracerebral injection, remains unclear. In either case, it is notable that even in animals with high titers of circulating neutralizing antibodies, successful intracerebral vector rechallenge could be accomplished demonstrating that systemic protective immunity is ineffective for preventing efficient re-administration of adenoviral vectors to the CNS.

It is interesting to note that co-administration of CTLA4Ig with the adenoviral vectors completely prevented the development of neutralizing antibodies. This suggests that CTLA4Ig immunosuppression may be potentially useful both for inhibiting cellular immunity, thereby prolonging systemic vector-mediated transgene expression, and repressing the development of an effective humoral response, thus allowing systemic vector re-administration. Nevertheless, it appears that this type of immunosuppression may not be necessary for increasing the efficacy of adenoviral vector-mediated transgene expression and re-application within the CNS.

In summary, our observations support the hypothesis that the potent systemic immunologic response to adenoviral vectors is attenuated within the CNS such that prolonged transgene expression, and repeat vector administration can be achieved. Although attenuated, it does appear that some level of immune response is mounted against CNS tissue transduced by adenoviral vectors ultimately resulting in late down-regulation of transgene expression and cell loss. Thus, for therapeutic indications requiring long-term stable transgene expression, such as for replacement gene therapy, further advances in adenoviral vector technology and selective immunosuppression may still be necessary. For other indications, such as the delivery of cytotoxic genes to brain tumors, where repeat vector administration may be necessary, but long-term transgene expression may not be necessary nor optimal, adenoviral vectors may ultimately play an important role in future gene therapy strategies.

Materials and methods

Recombinant adenoviral vectors

The construction of the recombinant adenoviral vectors Ad.CMV-CD and Ad.CMV-βgal have been described previously (Dong *et al*, 1996; Hirshowitz *et al*, 1995). Briefly, vectors were constructed by homologous recombination between pJM17, a plasmid containing the genome of adenovirus type 5 (Ad5) with deletions in the E1 and E3 regions, and a shuttle plasmid that includes Ad5 sequences (map units 0.0–1.3 and 9.2–17.3) and either the *E. coli* lacZ (β-galactosidase)(βgal) or cytosine deaminase (CD) gene, both driven by the cytomegalovirus (CMV) early gene enhancer/promoter, with the SV40 p(A) signal terminating the transgene construct. The recombinant viruses were plaque purified and propagated in 293 cells as previously described (Graham *et al*, 1977). The structure of the resulting recombinant vectors were confirmed by restriction enzyme digestion and sequencing. The virus stocks were purified by two cesium chloride ultracentrifugations, dialyzed against 10% glycerol, 10 mM Tris (pH 8.0) and 1 mM $MgCl_2$, and stored at -80°C. Virus titers were determined as plaque-forming units (PFU) assayed in semisolid cultures of 293 cells. Both viral stocks were prepared at 1×10^{11} ml.

Animal studies

For single systemic (i.v.) or intracerebral (i.c.) vector injections, Ad.CMV-βgal (10^9) was injected into the femoral vein of 200 g Fisher 344 rats (Harlan Sprague-Dawley) in a volume of 0.5 ml saline, or into the right caudate nucleus in a volume of 10 μl. For repeated systemic vector administrations, Ad.CMV-CD (10^9 PFU) was injected i.v., whereas control rats were injected with 0.5 ml saline only. Fourteen days later, both control and virus injected rats were injected with Ad.CMV-βgal (10^9 PFU) in the opposite vein. Repeat i.c. injections, or systemic i.v. injections followed by i.c. injections, were also carried out (Ad.CMV-CD followed 14 days later by

Ad.CMV-βgal in both experiments). In experiments evaluating the effectiveness of CTLA4Ig immunosuppression, 0.5 mg of murine CTLA4Ig (Bristol-Myers-Squibb, Seattle) or 0.5 mg control murine Ig was injected into the right femoral vein 5 min prior to injection of Ad.CMV-βgal i.v. or i.c. All animals were sacrificed at days 4, 6, 7 or 14 (for liver) or days 4, 14, 28, 56 and 70 (for brains) after Ad.CMV-βgal administration. Stereotactic injections were carried out using a modification of the method of Kobayashi *et al*. (1980). In brief, rats were anaesthetized with an intraperitoneal injection of Ketamine/Xylazine (40 mg/kg Ketamine, 10 mg/kg Xylazine) (Ketaset, Rompun, from JA Webster Veterinary Supply, MA) and placed in a small animal stereotaxic frame (Kopf Instruments). A sagittal incision was made through the scalp to expose the skull and a small burr hole was made 1.3 mm posterior and 4 mm to the right of the bregma. Injections were made with a 701 Hamilton syringe over 30 s to a sub-dural depth of 4.5 mm. The needle was left in place for 1 min and then withdrawn slowly. The hold in the skull was plugged with bone wax and the incision was closed with surgical clips (Ethicon Plus).

Tissue preparation

Animals were sacrificed by an intraperitoneal injection of Ketamine/Xylazine overdose followed by cardiac perfusion, first with ice cold PBS, followed by 4% paraformaldehyde/PBS. Serum was collected just prior to perfusion by cardiac puncture, centrifuged and stored at -20°C for later analysis. Brain and liver tissue was harvested whole and further fixed in 4% paraformaldehyde/PBS for 2 h at 4°C, and then sequentially washed over three nights in 10%, 20% and 30% sucrose in PBS/2 mM $MgCl_2$. Brain was then cut coronally along the needle track, liver lobes cut lengthwise, and slices used directly for whole amounts or embedded in O.C.T. compound (Tissue Tek) and frozen for thin sections.

For β-gal staining of whole mounts, the fixed samples were rinsed once with cold PBS and incubated in PBS solution containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% NP-40 for 10 min at 4°C. Samples were taken stained for 6 h at 37°C in the same solution containing 5 mM $K_3(Fe(CN)_6$, 5 mM $K_4(Fe(CN)_6$ and 1 mg/ml β-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside), washed in PBS, mounted and photographed.

For thin sections, 6 μm sections were cut using an IEC Minotome cryostat and placed on poly-lysine coated slices. Slices were allowed to air dry and stored at -20°C. Slides were briefly washed in PBS, fixed with cold acetone and allowed to air dry. β-gal staining was then carried out overnight (18 h) with the β-gal staining solution as described. Standard hematoxylin and eosin (H+E) staining of sections was also carried out on β-gal stained sections.

Neutralizing antibody assay

Serum samples were analyzed for neutralizing antibody titer as previously described (Kay *et al*, 1995; Yang *et al*, 1995a). Briefly, 100 μl heat inactivated samples were serially diluted in serum free DMEM (Sigma). This was mixed with a 20 μl aliquot of Ad.CMV-βgal (10^4 PFU) for 1 h at 37°C, and 100 μl of this mixture used to replace the media covering 80% confluent 293 cells (ATCC, Rockville, MD) in 96-well culture dishes (Falcon). After an additional 1 h, 100 μl DMEM containing 20% FBS was added and the cells assayed for expression of β-gal activity 24 h later by ONPG (O-nitrophenyl β-D-galactopyranoside) color spectrophotometric micro-assay (Stratagene). Titer was scored as the highest dilution (i.e. lowest serum concentration) which inhibited staining by 75%. Serum from control animals was used to establish appropriate maximum assay values for ONPG color development.

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