

Acute Direct Adenoviral Vector Cytotoxicity and Chronic, but Not Acute, Inflammatory Responses Correlate with Decreased Vector-Mediated Transgene Expression in the Brain

Clare E. Thomas, Darren Birkett, Ijeoma Anozie, Maria G. Castro, and Pedro R. Lowenstein¹

Molecular Medicine and Gene Therapy Unit, School of Medicine, University of Manchester, Room 1.302, Stopford Building, Oxford Road, Manchester M13 9PT, United Kingdom

Received for publication May 17, 2000; accepted in revised form November 9, 2000

The potential utility of adenoviruses for the treatment of chronic neurological disease is controversial due to reports of vector-associated toxicity, inflammation, and transient transgene expression. To focus upon the mechanism by which transgene expression is lost, we injected increasing doses [1×10^6 to 1×10^9 infectious units (iu)] of a first-generation adenovirus vector expressing β -galactosidase into the brains of immune-competent adult rats. Transgene expression was evaluated simultaneously with acute neuronal and glial cell cytotoxicity, and acute and chronic inflammation using immunohistochemistry, at 3 and 30 days post-vector administration. Our results show a clear threshold effect of viral dose upon the amount of transgene expression persisting by 30 days after vector administration. Below 10^8 iu, transgene expression remained stable over the 30-day period. Following infection of more than 10^8 iu, the extent of transgene expression at 30 days was inversely correlated with increasing viral dose. The severity of acute inflammation increased proportionally with increasing vector dose from 10^6 to 10^9 infectious units. In contrast, acute vector-mediated cytotoxicity and chronic inflammation were observed only above the threshold level of vector dose. Above 10^8 iu both the extent of the acute toxicity and the severity of the chronic inflammation were inversely correlated with transgene expression at 30 days. Thus, our data suggest that both an acute loss of cells through direct vector-mediated toxicity and the elicitation of chronic inflammation (but not acute inflammation) may account for the decline in transduction persistence at high vector doses.

Key Words: adenovirus; gene transfer; vector-mediated direct cytotoxicity; acute inflammation; chronic inflammation; dose response.

INTRODUCTION

Replication-defective recombinant adenoviruses are attractive candidate vectors for gene therapy of CNS disease as they can infect nondividing neurons and glial cells with high efficiency (1, 2). Adenovirus vectors are currently being used in clinical trials for the treatment of brain tumors (3); however, their suitability for the treatment of chronic neurological disease remains contentious due to reports of vector-associated toxicity and inflammation. It is well documented that adenovirus vectors in-

jected into the brain parenchyma cause acute cellular (4) and cytokine-mediated inflammatory responses (5). Additionally, significant toxicity both *in vivo* and *in vitro* has been reported when these vectors are used to infect cells at high multiplicities of infection (6–11). We have recently shown that a chronic inflammatory T cell response, accompanied by neurotoxic demyelination, results from successful inhibition of glioma growth in the brain using adenoviruses encoding HSV1-TK with peripheral administration of ganciclovir (1).

Successful gene therapy of chronic neurodegenerative disorders such as Parkinson's disease will necessitate long-term transgene expression (years), compared with tumor therapy, which requires shorter term therapeutic inter-

¹ To whom correspondence and reprint requests should be addressed.
Fax: 44-0-161-275-5672. E-mail: lowenstein@man.ac.uk.

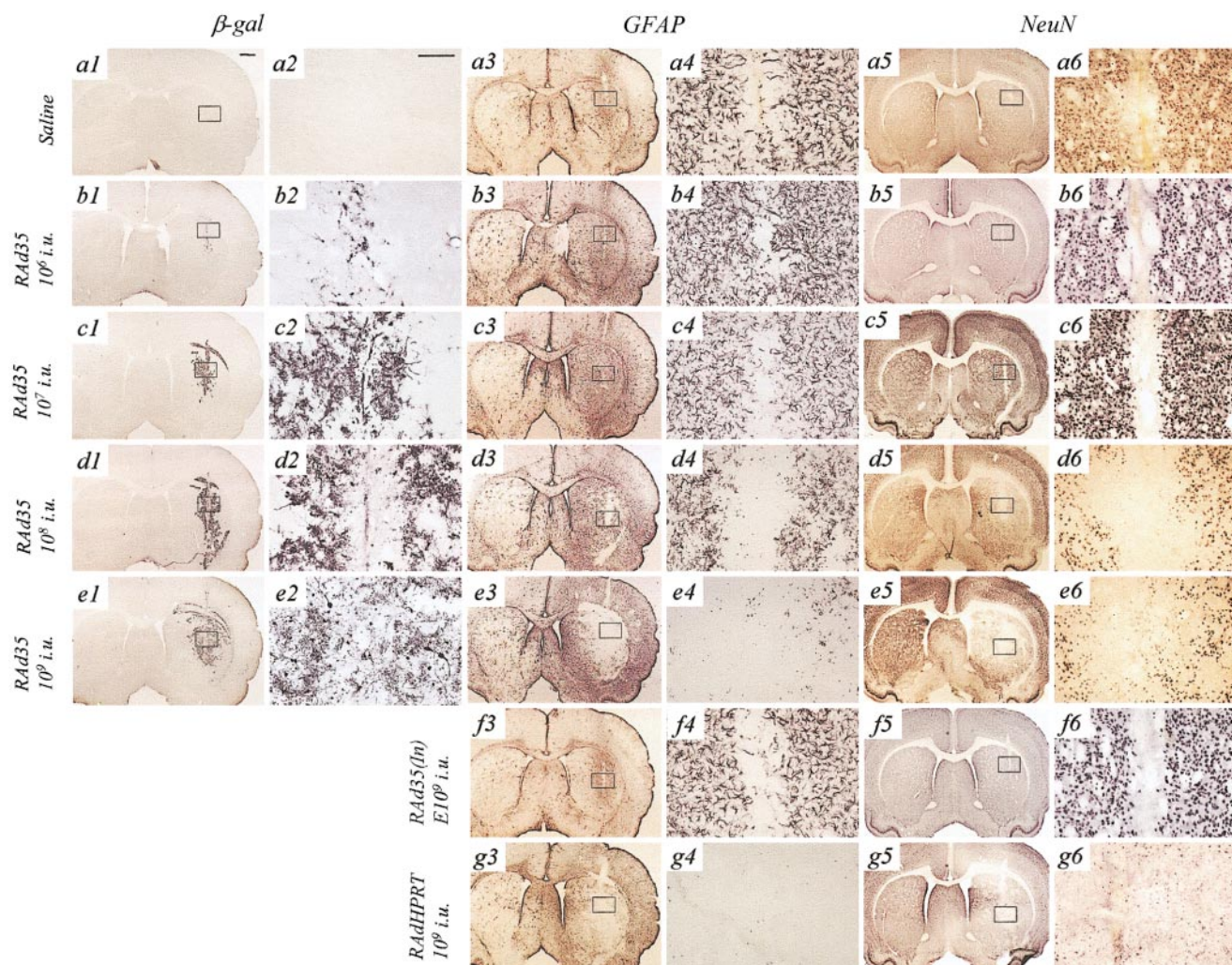


FIG. 1. β -Galactosidase expression and acute toxicity seen 3 days postinfection. Rows a–g show brains from animals injected intrastrially with saline (row a); 1×10^6 – 1×10^9 i.u. of the first-generation vector RAd35, expressing β -galactosidase (rows b–e); a dose of heat-inactivated RAd35 [RAd35(In)] equivalent to 10^9 i.u. ($E10^9$) (row f); or 1×10^9 i.u. of an alternative first-generation vector expressing hypoxanthine-guanine ribosyltransferase, RAdHPRT (row g). Brains were perfused-fixed 3 days post-vector administration, cut into serial 40- μ m sections, and analyzed for expression of β -galactosidase (columns 1 and 2, low- and high-magnification images, respectively), the astroglial marker GFAP (columns 3 and 4), and the neuronal marker NeuN (columns 5 and 6). The small box in the low-magnification images delineates the region shown in the high-magnification images. Administration of increasing doses of RAd35 led to an increase in the anatomical area transduced by 3 days postinjection. Administration of 10^8 and 10^9 i.u. of vector was increasingly toxic to the astroglial and neuronal cells. Equivalent cytotoxicity was observed after administration of RAdHPRT, but not RAd35(In). The scale bar for the low-magnification images is shown in row a, first column, and represents 1 mm. The scale bar for the high-magnification images is shown in row a, column 2, and represents 200 μ m.

vention (weeks–months). Previous work from our laboratory has demonstrated that first-generation adenovirus vectors injected into the CNS of unprimed immune-competent animals are able to sustain transgene expression for up to 12 months [(2) and unpublished observations], a prolonged period of time compared with first-generation vector-mediated transduction of peripheral organs such as the liver, in which transgene expression is eliminated within 2–3 weeks. Other investigators studying adenovirus-mediated transduction of the rat facial nucleus (12), the rat spinal cord (13), and the primate caudate nucleus (14) have demonstrated that persistent transduction cannot be achieved with high doses of vector. The mechanism(s) responsible for the lack of persistence of

transgene expression in the brain remains unclear. Different researchers have so far correlated the decline in transgene expression observed over time when high vector doses are administered, with direct vector-mediated toxicity (13), acute inflammation, and/or the elicitation of a chronic T cell response (12, 14–16), but have not examined these factors in combination.

In this study, we have sought to define the factors responsible for the elimination of transgene expression by injecting different doses of adenovirus vector (10^6 – 10^9 i.u.) into the brain parenchyma of immune-competent rats and comparing the persistence of transgene expression, direct vector-mediated toxicity, and acute and chronic inflammation, *simultaneously and at all viral doses*. We

show that prolonged transgene expression is not achieved after administration of more than 10^8 i.u. of vector. Our data suggest that acute inflammatory responses to adenovirus injection are not responsible for the eventual loss of transgene expression. Rather, expression is lost following administration of high doses of vector, through a combination of direct vector-mediated acute cytotoxicity and the elicitation of a chronic inflammatory response.

MATERIALS AND METHODS

Adenoviral vectors. RAd35 (expressing β -galactosidase) and RAdHPRT (expressing hypoxanthine-guanine phosphoribosyl transferase) are first-generation replication-defective recombinant adenovirus type 5 vectors expressing transgenes under the transcriptional control of the human cytomegalovirus intermediate early promoter, within the E1 region. The construction of RAd35 and RAdHPRT has been described earlier (17–19). Both vectors were propagated on 293 cells, purified, and titered for infectious units (iu) by end-point dilution (20) and for particle concentration by optical absorbance (21). The ratio of particles to infectious units was 13:1 for RAd35 and 8:1 for RAdHPRT. Virus preparations were screened for replication-competent adenovirus (RCA) contamination by serial amplification on HeLa cells as described in Dion *et al.* (22) and for lipopolysaccharide (LPS) contamination, using the *Limulus* amoebocyte gel clot assay (BioWhittaker, UK). Virus preparations used were free from RCA and LPS contamination. RAd35 was inactivated by incubating 20 μ l of purified stock in a 500- μ l microcentrifuge tube at 56°C for 20 min. No plaques were detected on 293 cells following titration of inactivated virus by end-point dilution, indicating that the inactivation had been efficient and there were less than 10^2 infectious units per milliliter of virus. Viruses were diluted in sterile saline solution for injection.

Animals and surgical procedures. Adult Sprague–Dawley rats of 250 g body weight (Charles River, UK) were anesthetized with halothane and placed in a stereotaxic apparatus which was modified for use with inhalational anesthetic. Animals were injected in the left striatum (0.6 mm forward from bregma and 3.4 mm lateral and 5.0 mm vertical from the dura) with 1×10^6 , 1×10^7 , 1×10^8 , or 1×10^9 infectious units of RAd35; 1×10^9 infectious units of RAdHPRT; or a dose of heat-inactivated RAd35 [RAd35(In)], equivalent to 1×10^9 infectious units. Virus was administered in a volume of 2 μ l using a 10- μ l Hamilton syringe and each injection was performed over a period of 3 min, with the needle being left in place for a further 5 min before withdrawal. Anesthesia was maintained throughout surgery with 1% halothane in 67% medical O_2 and 33% medical NO_2 . Three or thirty days after virus administration, rats were terminally anesthetized with an intraperitoneal injection of pentobarbitone and transcardially perfused-fixed with heparinized Tyrode solution followed by 4% paraformaldehyde in PBS ($n = 4$ per virus dose per time point). Brains were removed, postfixed in 4% paraformaldehyde for 5 h, and stored in PBS containing 0.01% sodium azide.

Immunohistochemistry, TUNEL, and Nissl staining. Serial coronal sections, 40- μ m-thick, were cut through the whole extent of the striatum using a Vibratome. Free-floating immunohistochemistry was performed to detect transgene or inflammatory and immune cell markers. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide and sections were blocked with 10% horse serum (Life Technologies, Paisley, UK) before being incubated overnight with primary antibody diluted in PBS containing 1% horse serum and 0.5% Triton X-100. The primary antibodies and the dilutions at which they were used were anti- β -galactosidase (1:1000; Promega, Southampton, UK), anti-MHC class I (OX-18, 1:200; Serotec, Kidlington, UK), anti-CD8 (1:500; Serotec), anti-CD4 (1:200; Serotec), ED1 (1:1000; Serotec), anti-GFAP (1:200; Roche, UK), NeuN (1:50; Autogen Bioclear, UK), and anti-myelin basic protein (MBP, 1:2000; DAKO, Cambridge, UK). All primary antibodies were mouse monoclonal anti-rat, except for anti-MBP, which was rabbit polyclonal anti-human. Secondary antibodies were biotinylated rabbit anti-mouse or biotinylated swine anti-rabbit (DAKO), diluted 1:200 in 0.5% Triton X-100 with 1% horse serum, and were detected using the Vectastain Elite ABC horseradish peroxidase method (Vector, Bretteon, UK). After being developed with diaminobenzi-

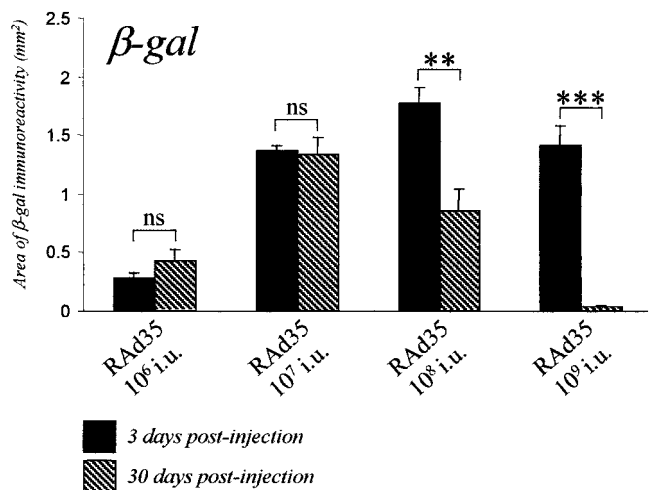


FIG. 2. Quantification of the area of 40- μ m brain sections occupied by β -galactosidase immunoreactivity 3 and 30 days after administration of increasing doses of adenovirus vector.

dine and glucose oxidase, sections were mounted on gelatinized glass slides and were dehydrated through graded ethanol solutions and xylene before being coverslipped with DPX mountant (Sigma, UK).

TdT-mediated dUTP nick end labeling (TUNEL) was performed on sections mounted on gelatinized glass slides, using the *in situ* death detection kit, AP (Roche), according to the manufacturer's instructions. Histological staining of Nissl substance was performed by incubating slide-mounted sections in 0.1% cresyl violet in 1% acetic acid for 15 min at room temperature, before washing in distilled water and differentiating in 70% alcohol for approximately 30 s.

Quantification. Quantitative image analysis to determine the area occupied by cells immunohistochemically stained with anti- β -galactosidase, ED1, or anti-CD8 antibodies, or to determine the area of loss of GFAP immunoreactivity within single 40- μ m brain sections was performed using a Leica Quantimet 600 Image Analysis System controlled by QWIN software (Leica Microsystems, Cambridge, UK) as described previously (2). Brain sections containing the needle track (and thus displaying the highest levels of immunoreactivity) were used for the quantitative analysis. Student's *t* test was used to determine the degree of statistical significance between values from different experimental groups.

Neutralizing antibody assay. Titers of adenovirus neutralizing antibodies in the serum of all animals killed 30 days after the intrastriatal injection were measured using an *in vitro* assay as described in Thomas *et al.* (2).

RESULTS

Administration of Increasing Doses of First-Generation Adenovirus Vector to the Rat Striatum Correlates with Increased Astroglial and Neuronal Cytopathogenicity

Administration of increasing doses of RAd35 (1×10^6 to 1×10^9 i.u.) in a small, fixed volume to the striatum of adult rats led to an increase in the anatomical area transduced at 3 days post-vector injection (Figs. 1 and 2). β -Galactosidase immunoreactivity was seen only close to the site of the needle injection after administration of 10^6 i.u. of vector (Figs. 1b1 and 1b2), but was progressively more widely distributed throughout the ipsilateral striatum and corpus callosum after injection of 10^7 , 10^8 , and

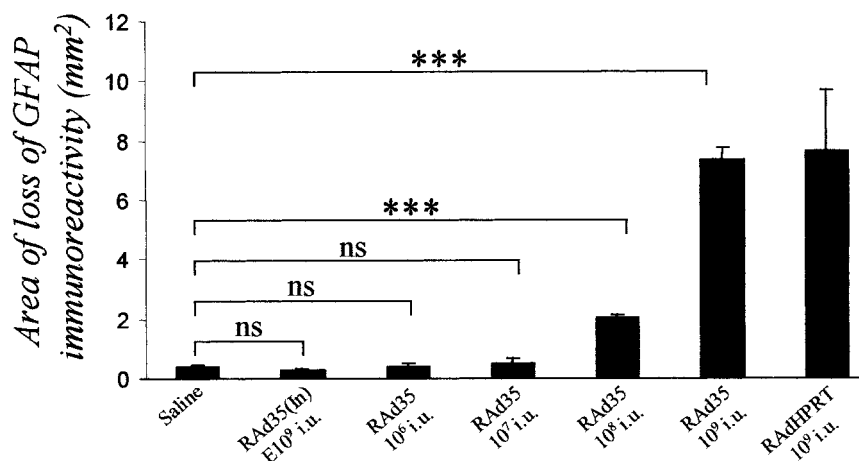


FIG. 3. Quantification of the area of loss of GFAP immunoreactivity in the ipsilateral striatum of 40- μ m brain sections, from animals sacrificed 3 days after intra-striatal injection of increasing adenovirus vectors.

10^9 iu (Figs. 1c1, 1c2, 1d1, 1d2, 1e1, 1e2). Transduced cells appeared to be predominantly of astroglial-like morphology.

Administration of all doses of virus caused astrocyte activation (GFAP upregulation) throughout the ipsilateral striatum (Fig. 1, columns 3 and 4). An absence of GFAP immunoreactivity directly within the needle track was evident after injection of saline (Figs. 1a3 and 1a4), indicating glial cell loss as a result of mechanical injury. A similar "lesion" within the needle track was also seen after injection of 10^6 and 10^7 iu of RAd35 (Fig. 1, columns 3 and 4). The area of glial cell loss increased proportionally with administration of increasing doses of 10^8 and 10^9 iu of RAd35 (Figs. 1d3, 1d4, 1e3, and 1e4). Administration of 10^9 iu of vector caused GFAP-immunoreactive cell loss throughout the ipsilateral striatum and white matter, in an area overlapping with the area transduced by the viral vector (compare e3 and e4 with e1 and e2 of Fig. 1). The area of loss of GFAP immunoreactivity in the ipsilateral striatum after injection of the different doses of vector was quantified using image analysis software (Fig. 3). Injection of 10^8 or 10^9 iu of RAd35 caused a loss of GFAP immunoreactivity which was significantly greater than the loss observed after injection of saline. At doses below 10^8 iu, the "lesion" areas were not significantly different from the saline controls.

A similar dose-dependent pattern of neuronal cell loss was reflected in sections stained with the neuronal marker NeuN (Fig. 1, columns 5 and 6). This loss of GFAP and NeuN immunoreactivity possibly reflects cell death through vector-mediated toxicity at high multiplicities of infection. Cell death in brains injected with the highest dose of vector was confirmed by the profusion of TUNEL-positive cells detected throughout the ipsilateral striatum and white matter (Fig. 4).

Injection of 10^9 iu of a different first-generation vector, RAdHPRT, expressing a potentially less inflammatory transgene, resulted in a pattern of glial and neuronal cell loss similar to that of RAd35 (Figs. 1g3–1g6), whereas

brains injected with the equivalent dose of heat-inactivated RAd35 exhibited patterns of GFAP and NeuN immunoreactivity comparable to the saline control (Figs. 1f3–1f6 vs 1a3–1a6). These results indicate that the constituents of the vector particles per se appear to be non-toxic; rather the cytopathogenicity is mediated by viral infection and/or viral gene expression and it does not seem to depend upon the type of transgene being expressed.

Increased Viral Dose Is Not Correlated with Increased Transduction 30 Days after Vector Administration

By 30 days after vector administration, β -galactosidase-immunoreactive cells could still be detected in all animals (Fig. 5). Brains injected with 10^6 and 10^7 iu of RAd35 (Figs. 5a1, 5a2, 5b1, and 5b2) showed no decline in the area of transduction at 30 days postinfection (p.i.) compared with 3 days p.i. (Fig. 2). Conversely, β -galactosidase expression 30 days after injection of 10^8 iu had declined from 3 days p.i., and a dramatic decrease in transgene expression was observed 30 days after injection of 10^9 iu (Figs. 5c1, 5c2, 5d1, 5d2, and 2). Brains injected with 10^9 iu of vector showed massive ventricular enlargement (particularly on the ipsilateral side) and a large lesion in the ipsilateral striatum; however, scattered β -galactosidase-positive cells could still be detected around the lesion (Figs. 5d1 and 5d2).

Acute Inflammation Increases Proportionally with Increasing Viral Dose from 10^6 to 10^9 iu

A viral dose-dependent increase in acute brain inflammation was seen 3 days after intra-striatal administration of RAd35 (Figs. 6 and 7). The inflammatory responses to the injection of saline (Figs. 6a1–6a5) and heat-inactivated RAd35 (Figs. 6f1–6f5) were negligible, with microglial activation (evidenced by increases in ED1 and MHC class I immunoreactivity) and CD8⁺ cell immunoreactivity restricted to the needle track. After injection of 10^6 iu

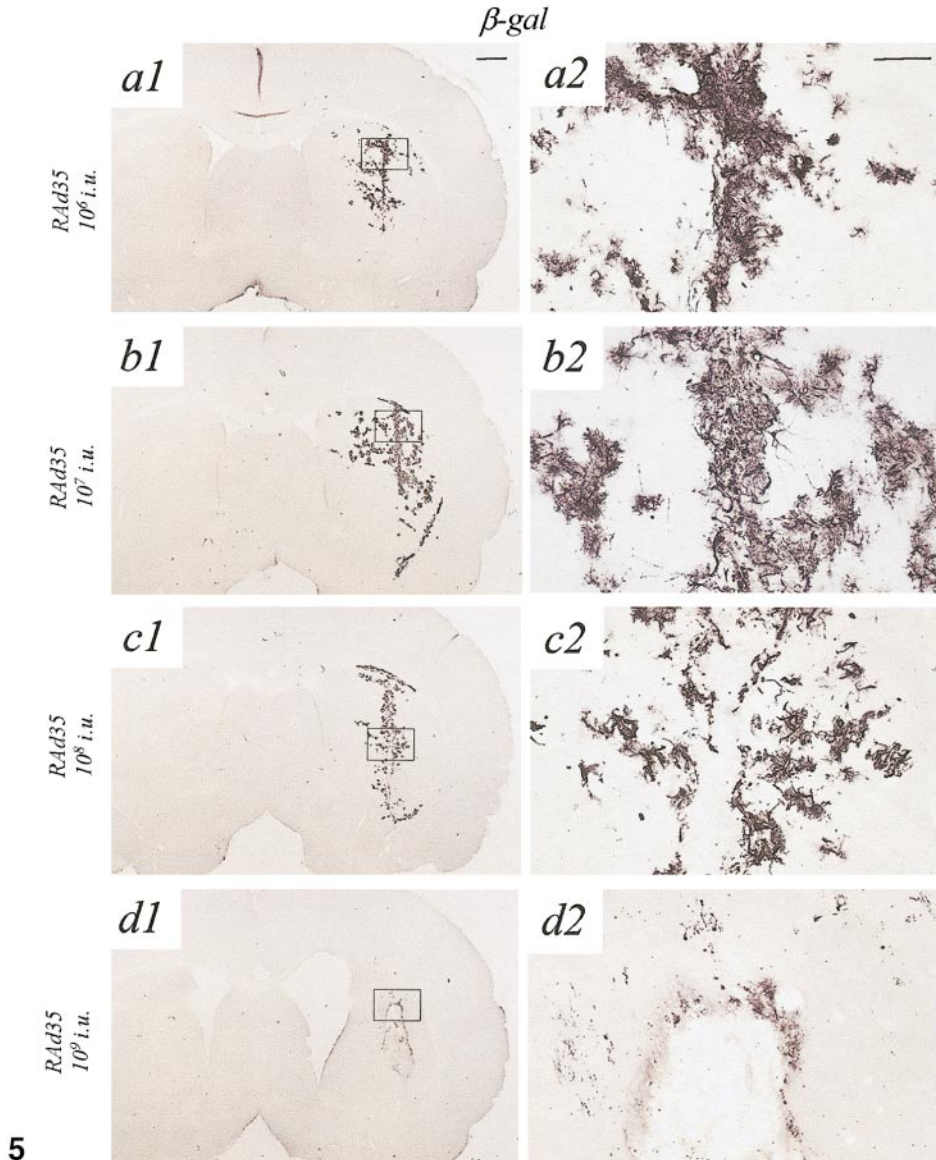
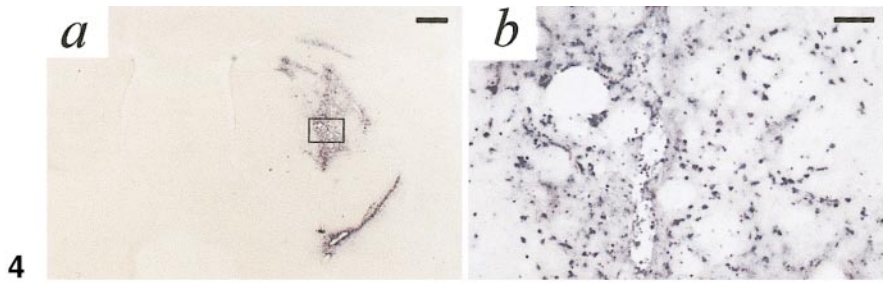


FIG. 4. Cell death seen 3 days after administration of 1×10^9 iu of RAd35. 40- μ m sections from brains injected with 1×10^9 iu of RAd35 were evaluated using TUNEL for cell death. TUNEL-positive cells were found throughout the ipsilateral side of the brain, particularly localized to the site of virus administration. The scale bars for the low- and high-magnification images represent 1 mm and 50 μ m, respectively.

FIG. 5. Persistence of β -galactosidase expression, 30 days postinfection. Rows a–d show brains from animals injected with increasing doses of RAd35 from 1×10^6 to 1×10^9 iu and analyzed for expression of transgene 30 days post-vector administration. Low- and high-magnification images are shown in columns 1 and 2, respectively. β -Galactosidase-immunoreactive cells could be detected in all animals, but those brains injected with 10^8 or 10^9 iu of vector showed a decline in transgene expression compared to the 3-day time point, the magnitude of which was correlated with increasing vector dose. Scale bars are shown in row a and represent 1 mm and 200 μ m for the low- and high-magnification images, respectively.

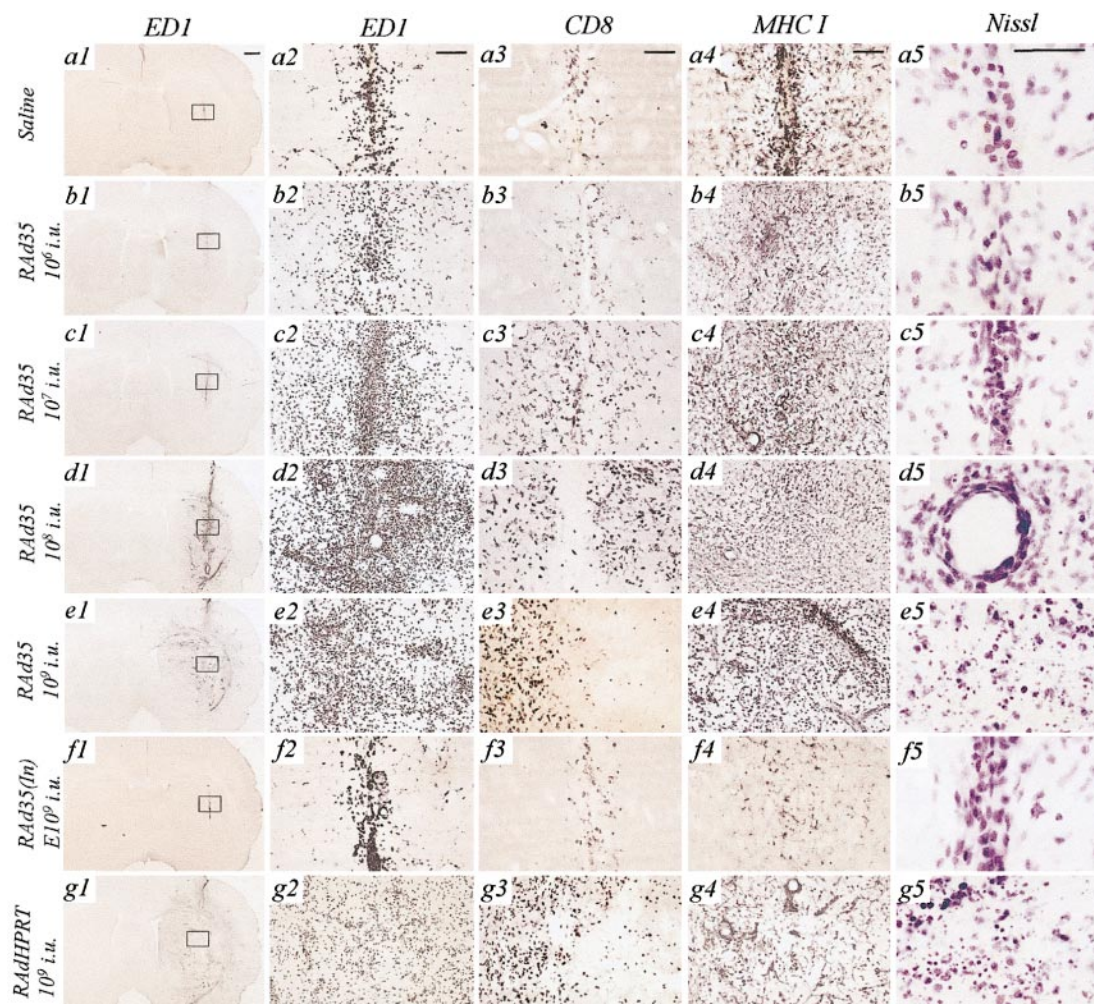


FIG. 6. Acute brain inflammation, 3 days after vector administration. Rows a–g show sections from brains which were perfused-fixed 3 days after intrastriatal injection of saline (row a), 1×10^6 – 1×10^9 i.u. of RAD35 (rows b–e), a dose of heat-inactivated RAD35 [RAD35(In)] equivalent to 10^9 i.u. (row f), or 1×10^9 i.u. of RADHPRT (row g). Sections were stained for ED1 (shown at low magnification in column 1 and at high magnification in column 2), CD8 (column 3), and MHC class I immunoreactivity (column 4) and were stained with cresyl violet to visualize nuclei and Nissl substance (column 5). The small box in the low-magnification images in column 1 delineates the region shown in the higher magnification images in columns 2–4. A linear increase in the severity of the inflammatory response to adenovirus vector was seen with increasing vector dose. The inflammatory response to 10^9 i.u. of RADHPRT was the same as that elicited by the equivalent dose of RAD35. An absence of CD8⁺ immunoreactivity was observed in the striata of animals that were injected with 10^9 i.u. of RAD35 or RADHPRT, in an area corresponding to the cytotoxic lesion indicated by loss of GFAP immunoreactivity. Numerous CD8⁺ cells were, however, seen around the periphery of this lesion. Panels e3 and g3 show images taken at the border of this acute lesion. Acute inflammation elicited by administration of RAD35(In) was comparable to that of the saline control. Scale bars are shown in row a and represent 1 mm (column 1), 200 μ m (columns 2–4), and 50 μ m (column 5).

of RAD35, CD8⁺ cell immunoreactivity remained localized to the needle track (Fig. 6b3), but microglial activation was more widespread, with ED1 staining spreading from the needle track (Figs. 6b1 and 6b2) and MHC class I upregulation extending throughout the ipsilateral striatum (Fig. 6b4). Acute brain inflammation became progressively more severe and more widely distributed throughout the ipsilateral striatum with increasing vector dose (Figs. 6 and 7).

Histological staining of Nissl substance revealed an accumulation of cell nuclei within the needle track after injection of saline (Fig. 6a5), heat-inactivated RAD35 (Fig. 6f5), and 10^6 and 10^7 i.u. of RAD35 (Figs. 6b5 and 6c5), with minimal cellular infiltration of the brain paren-

chyma. Extensive perivascular cuffing was observed throughout the ipsilateral striatum, with a dense accumulation of inflammatory cells throughout the injection site after injection of 10^8 of vector (Fig. 6d5). Perivascular cuffs were also seen in brains injected with 10^9 i.u. of vector, but the pattern of Nissl staining in these brains was more heterogeneous, with many small punctate spots of Nissl staining suggestive of apoptotic nuclei within the injection site (Fig. 6e5). Injection of 10^9 i.u. of RADHPRT elicited levels of acute inflammation comparable to those of the equivalent dose of RAD35 (Figs. 6g1–6g5 and 7). Levels of acute inflammation following injection of the equivalent of 10^9 i.u. of heat-inactivated RAD35 were comparable to those seen after injection of saline (Figs. 6f1–

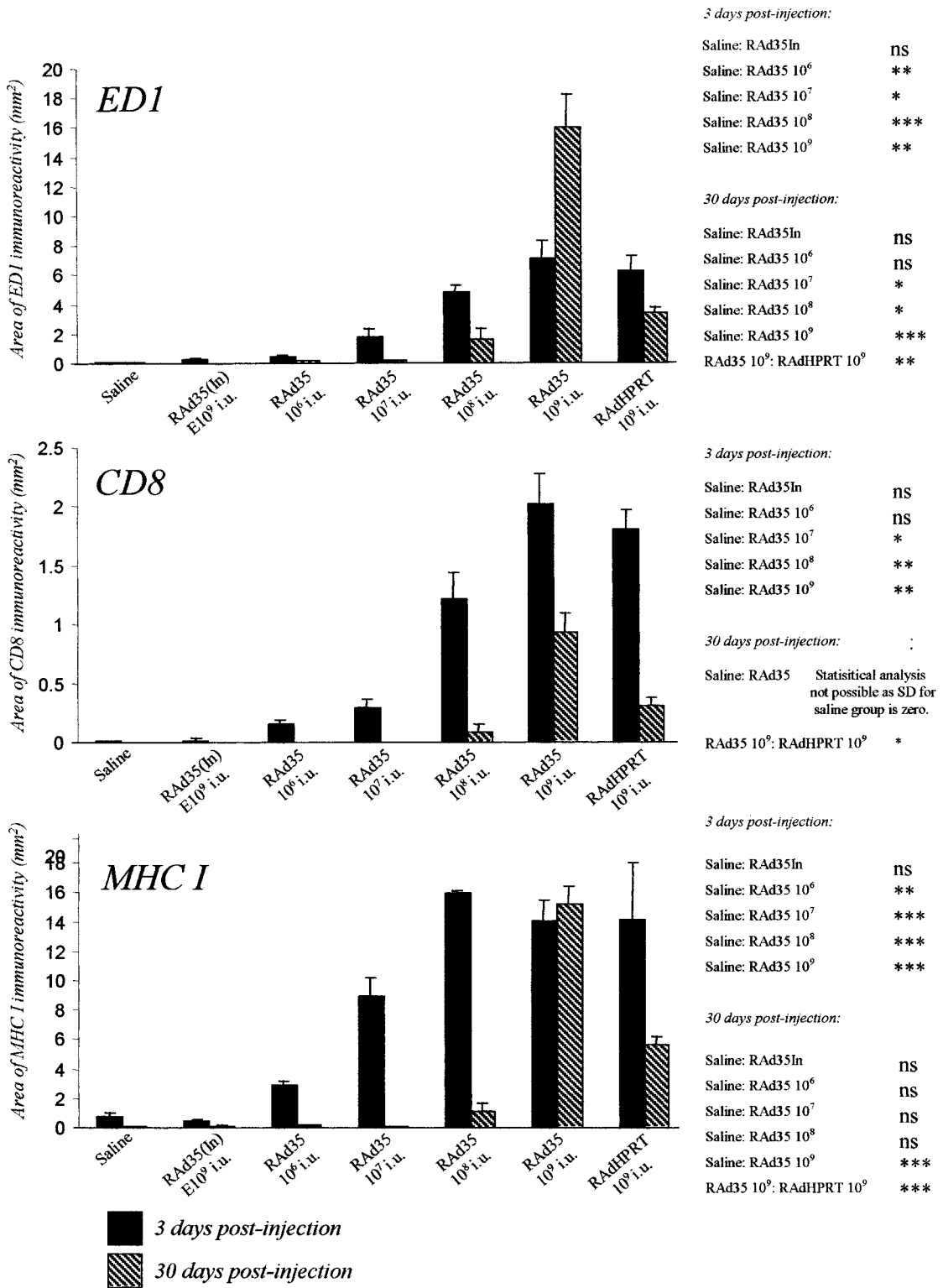


FIG. 7. Quantitative analysis of acute and chronic inflammation in 40- μ m brain sections from animals sacrificed 3 (acute) and 30 (chronic) days after intrastriatal injection of increasing doses of adenovirus vector. A linear dose-dependent increase in acute inflammation (ED1, CD8, and MHC I immunoreactivity) was observed over increasing doses of RAd35, from 10⁶ to 10⁹ i.u. Administration of the amount of heat-inactivated adenovirus vector equivalent to 10⁹ i.u. of RAd35 [RAd35(In) E10⁹] failed to elicit acute inflammation that was significantly different from the saline control. Administration of 10⁹ i.u. of RAdHPRT elicited levels of inflammation comparable to those of the same dose of RAd35. Chronic inflammation (30 days p.i.) was only observed at viral doses of 10⁸ i.u. and above and was significantly greater in animals that had been injected with 10⁹ i.u. of RAd35, compared with the same dose of RAdHPRT.

6f5 and 7). Thus, these data show that, like vector-mediated direct cytotoxicity, vector-induced acute inflammation appears to be independent of the transgene expressed by the vector, but is mediated by the intact virus capsid, virus infection, and/or viral gene expression.

Chronic Inflammation and T Cell Infiltration Is Elicited Only above a Threshold Level of Viral Dose

By 30 days after virus administration, brains which had been injected with saline, heat-inactivated RAd35, or 10^6 or 10^7 iu of RAd35 showed negligible inflammation (Figs. 7 and 8). No CD8⁺ cells could be detected in any of these brains (Figs. 8a3, 8b3, 8c3, and 8f3), and microglial/macrophage activation (ED1 and MHC I immunoreactivity) was confined to the site of the needle injection (Figs. 8a1, 8a2, 8a4, 8b1, 8b2, 8b4, 8c1, 8c2, 8c4, 8f1, 8f2, and 8f4). Nissl staining showed an accumulation of cell nuclei within the needle track, but no evidence of cellular infiltration of other parts of the parenchyma. In contrast, brains that had been injected with 10^8 iu of RAd35 showed more severe inflammation; CD8⁺ cells could be detected within the injection site and ED1 and MHC class I immunoreactivity was more widely distributed throughout the ipsilateral striatum (Figs. 8d1–8d5). Animals injected with 10^9 iu of RAd35 showed extensive inflammation throughout the ipsilateral side of the brain and extending through the contralateral side. Very strong ED1 and MHC class I immunoreactivity and large numbers of CD8⁺ cells could be detected in the ipsilateral striatum, particularly densely localized around the large lesion remaining in the site of injection (Figs. 8e1–8e4). Microglial activation (ED1 and MHC class I staining) and scattered CD8⁺ cells could also be detected throughout the cortex and corpus callosum on both sides of the brain. TUNEL-positive cells were detected throughout the ipsilateral striatum and the cortex and corpus callosum of both sides of the brain, i.e., in all regions where CD8⁺ cells were detected (not shown). Perivascular cuffs and tightly packed inflammatory cells were detected within the lesions by Nissl staining (Fig. 8e5). A similarly large lesion containing perivascular cuffs and tightly packed inflammatory cells was also detected within the injection site of brains injected with 10^9 iu of RAdHPRT (Fig. 8g5). These brains also showed intense microglial/macrophage activation and CD8⁺ cell infiltration (Figs. 8g1–8g4), although this was restricted to the ipsilateral striatum and white matter and did not extend into the contralateral side of the brain as seen in those animals injected with the equivalent dose of RAd35. Quantitation of the levels of ED1, CD8⁺, and MHC I immunoreactivity showed that chronic inflammation in brains injected with 10^9 iu of RAdHPRT was significantly less severe than in brains injected with the equivalent dose of RAd35.

The integrity of brain myelination was evaluated in all animals by immunohistochemical detection of myelin basic protein (Fig. 9). Thirty days after vector administration, no damage to myelin integrity could be detected in any of the brains injected with saline, heat-inactivated

RAd35, or 10^6 – 10^8 iu of RAd35 (10^7 and 10^8 doses shown in Figs. 9a1, 9a2, 9b1, and 9b2). In contrast, the architecture of the white matter tracts in the ipsilateral side of the brains injected with 10^9 iu of either RAd35 or RAdHPRT was severely disrupted; the white matter fibers appeared pulled apart and paled (suggesting edema), and in some brains, loss of MBP immunoreactivity was also evident around the lesion in the striatum (Figs. 9c2 and 9d2). Consistent with our earlier report of vector-induced neurotoxic demyelination following adenovirus-mediated inhibition of tumor growth in Lewis rats (1), the localization of the damage observed in this study indicated that loss of MBP immunoreactivity was also most likely due to vector-mediated cytotoxicity, rather than to autoimmune demyelination.

To assess whether high doses of adenovirus vector injected into the brain could leak to the periphery and prime an anti-adenoviral neutralizing antibody response, we measured titers of virus-neutralizing antibodies in the sera of two of the four animals, per virus dose group, that were sacrificed 30 days after the intrastriatal injection. Although we have previously detected high neutralizing-antibody titers in the serum of animals which had received an intradermal injection of adenovirus (2), no neutralizing antibodies could be detected in any of the serum samples from this study. Thus, intrastriatal injection of even extremely high doses of adenovirus (10^9 iu) does not succeed in priming an adaptive anti-adenovirus neutralizing antibody response.

DISCUSSION

Although adenoviruses possess numerous characteristics that make them attractive candidate vectors for gene therapy of neurological disease, their administration to the brain is associated with inflammation, cytotoxicity, and transient transgene expression, particularly after injection of high vector doses. To determine the processes responsible for the short-lived transgene expression, we have injected increasing adenoviral vector doses into the brain and analyzed in the same anatomical site the effects of vector dose on direct brain cell toxicity, acute and chronic brain inflammation, and acute and long-term transgene expression. Our results show a clear threshold effect of viral dose upon the amount of transgene expression persisting by 30 days after vector administration. Up to 1×10^8 iu, levels of vector-mediated transgene expression increased proportionally with increasing viral dose and remained stable over the 30-day period. Above 1×10^8 iu, viral dose was inversely correlated with levels of transgene expression remaining by 30 days postinfection.

This article demonstrates that both the loss of cells through vector-mediated toxicity and the elicitation of chronic, but not acute, inflammation may account for the decline in transduction persistence seen at high vector doses. Acute inflammatory responses to vector injection are unlikely to account for the loss of transduction at 30 days postinfection: administration of increasing vector doses up to 1×10^8 iu elicited a dose-dependent increase

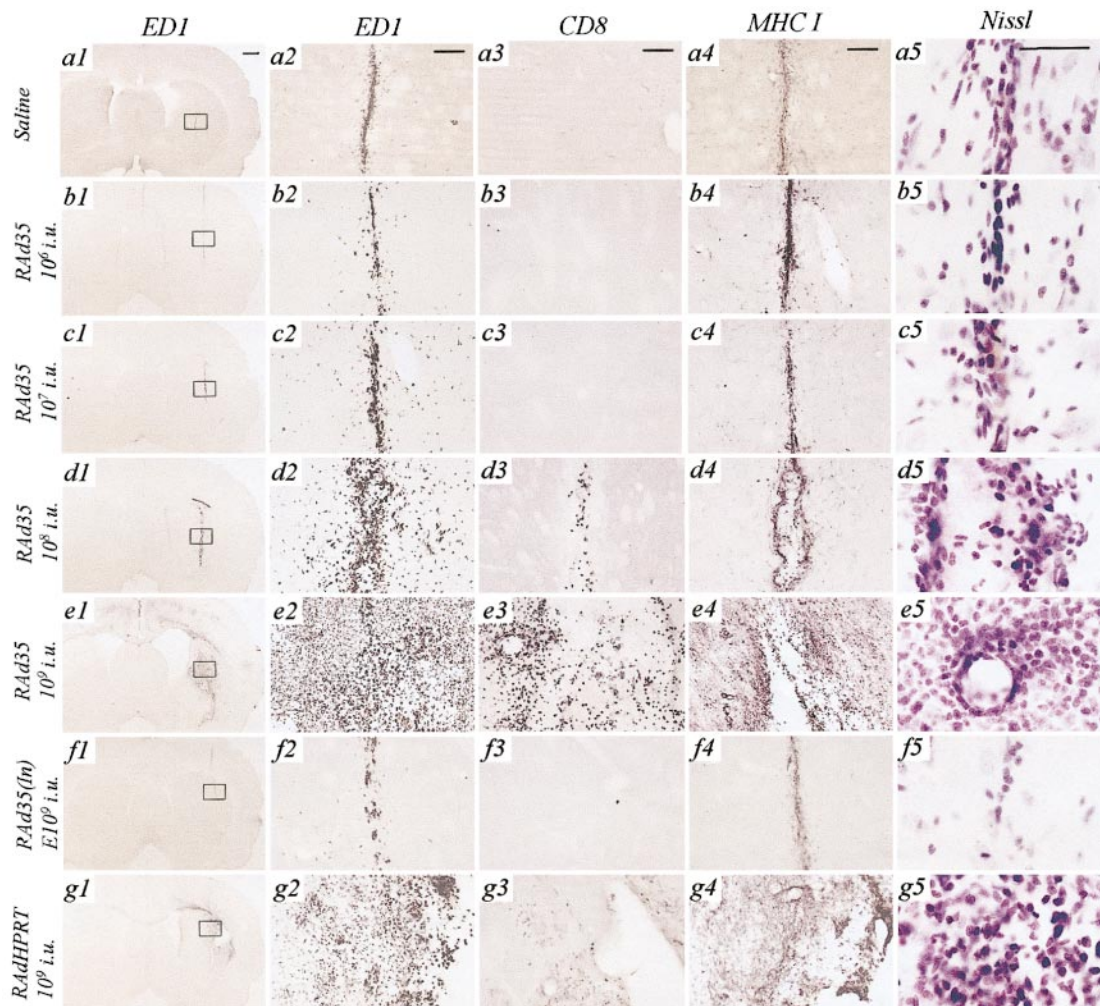


FIG. 8. Chronic inflammation and T cell infiltration, 30 days after adenoviral vector injection. Rows a–g show sections from brains which were perfused-fixed 30 days after intrastriatal injection of saline (row a), 1×10^6 – 1×10^9 iu of Rad35 (rows b–e), a dose of heat-inactivated Rad35 [Rad35(In)] equivalent to 10^9 iu (row f), or 1×10^9 iu of RadHPRT (row g). Sections were processed for ED1, CD8⁺, and MHC class I immunoreactivity and stained for Nissl substance as previously. A threshold of vector titer was observed (around 10^8 iu) above which chronic inflammation was observed. The chronic inflammatory response to 10^9 iu of Rad35 was more severe than that elicited by the equivalent dose of RadHPRT. Scale bars are shown in row a and represent 1 mm (column 1), 200 μ m (columns 2–4), and 50 μ m (column 5).

in acute brain inflammation but also resulted in stable transgene expression over the 30-day period. Direct virus-mediated acute neuronal and glial cell toxicity and chronic inflammation/CD8⁺ cell infiltration were observed only above the threshold dose of 1×10^8 infectious units. Above this dose both the magnitude of the acute direct cytotoxicity and the severity of the chronic inflammation increased with increasing vector dose.

In support of our conclusions, Lawrence *et al.* (16) reported that administration of the anti-inflammatory agent dexamethasone immediately before, and for only 8 days following, vector delivery had no effect upon the duration of adenovirus-mediated transgene expression in St. Kitts green monkeys. In rats, Hermens *et al.* (15) showed that acute dexamethasone administration for 3 days post-vector injection prolonged adenovirus-mediated transgene expression in astrocytes (but not neurons)

of the rat facial nucleus. Interestingly, Hermens *et al.* observed reduced infiltration of macrophages and $\alpha\beta^+$ T cells by 14 days after injection of relatively high doses of vector, which may suggest that dexamethasone suppresses the extent of the chronic inflammation, rather than the initial acute inflammatory response. Further, Durham *et al.* (23) reported that administration of the immunosuppressant FK506 (which specifically inhibits T cell function) succeeded in prolonging transgene expression after administration of very high doses of adenovirus vector to the mouse striatum.

Although we demonstrate in this study that both direct adenoviral vector-mediated toxicity and chronic inflammatory and immune responses contribute to the decline in vector-mediated transgene expression in the brain, their relative contributions remain to be determined. Further, it also remains to be determined whether the elicit-

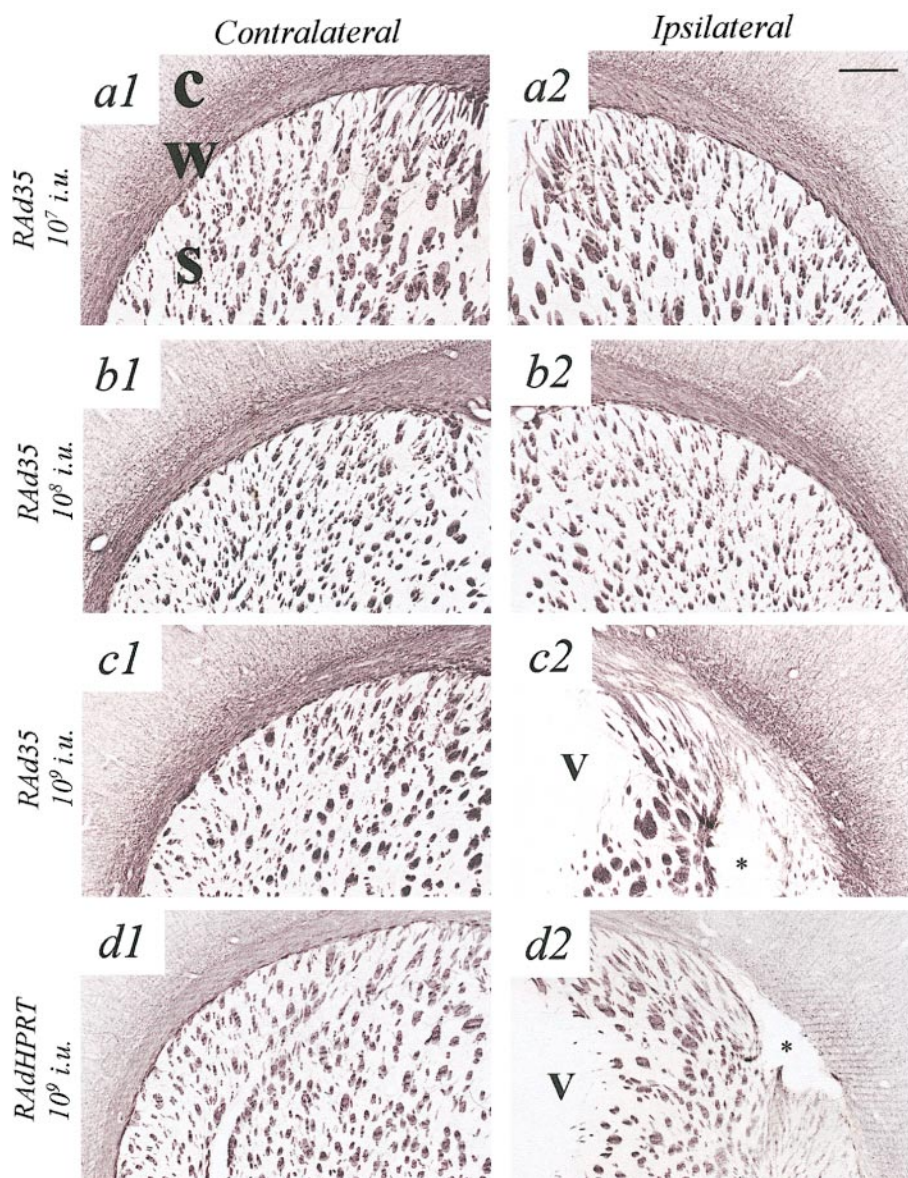


FIG. 9. Myelin integrity, seen 30 days post-vector administration. MBP immunoreactivity was analyzed in brain sections from animals perfused 30 days post-vector administration. Rows a–d show brains from animals injected with increasing doses of RAD35 from 1×10^7 to 1×10^9 i.u. (a–c) or 1×10^9 i.u. of RADHPRT (d). The letters “c”, “w”, and “s” in row a, column 1, represent cortex, white matter, and striatum. No observable differences in myelin integrity between the contralateral and the ipsilateral sides of the brains could be detected in brains injected with 10^7 or 10^8 i.u. of RAD35. Brains injected with 10^9 i.u. of either RAD35 or RADHPRT showed severe white matter damage and myelin pallor in the ipsilateral side of the brain compared with the contralateral side. The letter “v” indicates the enlarged ipsilateral ventricles seen in the brains injected with 10^9 i.u. of vector; the ventricular ependyma was destroyed during sectioning, as was the fragile lesioned area of the ipsilateral striatum (indicated by *). The scale bar is shown in row a, column 2, and represents $500 \mu\text{m}$.

tation of chronic CD8⁺, possibly T cell, infiltration at high vector titers occurs as a response to the increased number of vector particles per se or whether the chronic inflammation is a secondary consequence of the large direct cytotoxicity of the high vector dose. The finding that intrastriatal injection of even 10^9 i.u. of vector did not succeed in raising an anti-adenovirus neutralizing antibody response suggests that adenovirus-specific adaptive immunity is not primed, even by high vector doses injected into the brain.

The finding that injection of RAD35 elicited more severe

chronic inflammation compared to RADHPRT suggests that some of the chronic inflammatory responses may depend on the transgene being expressed by the vector, as suggested previously (11). Our results demonstrate that persistent and noncytotoxic transduction of the rat CNS can be achieved with moderate doses (below 10^8 i.u.) of first-generation adenoviral vectors. Importantly, we have recently demonstrated that the use of stronger promoters can vastly increase levels of transgene expression over large anatomical areas, from low doses of vector [10^4 – 10^6 i.u., i.e., doses which are below the cytotoxicity threshold

described in this article (24)]. Secreted transgene products should further increase the anatomical area available for genetic manipulation (25).

New-generation high-capacity adenovirus vectors (HC-Ads), deleted of all viral genes, have been shown to be less toxic in the rat liver, allowing higher viral doses to be administered compared with first-generation vectors (26). We have recently shown that unlike first-generation vectors, HC-Ads mediate stable transgene expression in the brain without eliciting any chronic inflammation, even in the presence of an anti-adenoviral immune response elicited through peripheral infection with adenovirus (2). Thus, novel HC-Ads, combined with powerful transcriptional elements, will be able to transduce clinically significant areas of the brain in the absence of deleterious inflammatory and direct cytotoxic responses.

ACKNOWLEDGMENTS

This work was supported by the Parkinson's Disease Society, UK (Project Grants 3087 and MAP96/28), the Wellcome Trust (Project Grants 051248 and 050283), and the EU-Biomed Programme (QLK3-CT-1999-00364 and BMH4-CT98-3277). We are very grateful to Ms. Tricia Maleniak and Miss Emma Jones for LPS measurements, Mr. Peter Stanley for technical assistance during the preparation of RAD35, and Mrs. Ros Poulton for expert secretarial assistance. We also acknowledge Professors A. M. Heagerty, F. Creed, and D. Gordon for their continuous support and encouragement. We also acknowledge the support which our laboratory receives from Action Research, the MRC, the British Heart Foundation, the Royal Society, the BBSRC (UK), REMEDI, and the Lister Institute for Preventive Medicine. P.R.L. is a Research Fellow of the Lister Institute of Preventive Medicine.

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