

# Strong Promoters Are the Key to Highly Efficient, Noninflammatory and Noncytotoxic Adenoviral-Mediated Transgene Delivery into the Brain *in Vivo*

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Using the major immediate early murine cytomegalovirus (MIECMV) promoter to drive expression of  $\beta$ -galactosidase, we have demonstrated that, following adenoviral-mediated transduction of brain cells *in vivo*, a single viral infectious unit is capable of producing detectable levels of transgene expression and that gene transfer into the brain is close to 100% efficient. By reducing 100-fold the amount of virus needed to detect large numbers of transduced brain cells, we were able to completely eliminate the cellular inflammation and viral cytotoxicity associated with the delivery of adenoviral vectors into the brain compared to saline-injected controls. These results demonstrate that a strong promoter is necessary to allow the use of low concentrations of adenoviral vectors for gene transfer into the brain, thereby eliminating deleterious side effects and increasing the potential efficacy of gene therapy.

## INTRODUCTION

Gene therapy using viral vectors is currently being developed to replace a missing protein product in inherited diseases, to deliver therapeutically active proteins to diseased tissues, or to eliminate tumor cells (1). In spite of their importance to the potential clinical success of gene therapy, neither the efficiency of viral vector-mediated gene transfer *in vivo* nor the number of viral infectious particles (iu) needed to transduce a single cell *in vivo* has been previously determined. Although viral vector-mediated gene transfer *in vitro* is believed to be very efficient, *in vivo* gene transfer normally requires the use of high numbers of viral infectious units to achieve anatomically detectable and physiologically relevant levels of transgene expression. Similar data have been reported with all different types of viral vectors utilized to date (2).

In addition to the low efficiency of transgene expression *in vivo*, we and others have recently demonstrated important side effects of first-generation adenovirus vector used to transfer genes to the CNS. Direct cytotoxicity and strong persistent brain inflammation were seen following injection of functionally effective doses of first-generation adenoviral vector into the brain (3–5). Acute inflammation (macrophage/microglial and CD8+ lymphocyte/NK cell infiltration) follows injection of  $10^7$  iu,

and direct cytotoxicity (astrocyte and neuronal cell death) occurs when doses above  $10^8$  iu are injected into the adult rat brain (4). Using an adenoviral vector expressing  $\beta$ -galactosidase from the very strong MIECMV promoter (6–8), we now demonstrate close to 100% gene transfer efficiency and expression in the adult rat brain. At very low doses of this vector ( $10^1$ – $10^3$  infectious units per injection site), the number of detectable transduced brain cells is equal or very close to the number of viral infectious units injected. This vector allows transgene expression throughout relatively large areas of the brain at high levels, in the complete absence of any cellular inflammation or cytotoxicity compared to saline-injected controls.

## MATERIALS AND METHODS

*Gene transfer in vitro.* Rat CNS-1 cells, mouse Neuro2A cells, hamster Chinese hamster ovary (CHO) cells, nonhuman primate COS7 cells, and human HeLa cells were grown using the cell growth medium described previously (9). Cell lines were plated in 24-well plates at a density of  $2 \times 10^4$  cells/well 1 day prior to infection. On the day of infection, 2 wells were counted, and plates were infected at appropriate m.o.i. values for each virus (m.o.i. values used: 10, 30, 100, and 300). Forty-eight hours later cells were stained for  $\beta$ -galactosidase histochemistry, or  $\beta$ -galactosidase enzyme assays were carried out on cell lysates as described previously (10). Expression of the *lacZ* gene under transcriptional control of the 1.4-kb MIECMV promoter (–1336 to +36) was compared to the expression of  $\beta$ -galactosidase from RA35, which uses a short 0.4-kb MIEhCMV promoter (–299 to +72), in cell lines from different species. In all cell lines used the levels of  $\beta$ -galactosidase activity, driven by the MIECMV promoter, were significantly higher, in agreement with Refs. 6–8 and 11. The

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difference of expression varied from ~9-fold in human HeLa cells to ~500-fold in rat CNS-1 cells, at m.o.i. = 10. The glioblastoma cell line (CNS-1) was kindly provided by Dr. William F. Hickey (Pathology Department, Dartmouth Medical School, NH). The murine neuroblastoma cell line (Neuro2A), the SV40 transformed African green monkey kidney cell line (COS7), the human cervical epitheloid carcinoma cell line (Hela), and the chinese hamster ovary (CHO) cell line were purchased from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK).

**Generation of recombinant adenoviruses RAD 36 and RAD 35.** A HindIII fragment was excised from the pMCMV 3 plasmid (kindly provided by Dr. Martin Messerle, Max von Pettenkofer Institute, Munich, Germany), containing 1.4 kb from the major immediately early murine cytomegalovirus promoter (MIEhCMV) and a 0.15-kbp SV40 polyadenylation signal was subcloned into HindIII-digested pAL119 (pMV35 in Ref. 12) from which the sMIEhCMV promoter and SV40 polyadenylation signal were deleted. The resulting plasmid was called pAL120. The shuttle vector pAL120-*lacZ* was constructed by blunt-end ligation of a 3.4-kb *EcoRV-Sall lacZ* fragment with blunt-ended *Sall*-linearized pAL120. Plasmid pAL120 was used to generate RAD36. RAD 35 (an adenovirus encoding *lacZ* under the control of the MIEhCMV promoter) was originally described by Wilkinson and Akkrigg (13) and has been used previously (3, 4, 9, 14). The methods for adenoviral generation, production, characterization, scale up, and viral vector purification have been previously described (10, 14). Titrations were carried out in triplicate and in parallel for all viruses by end-point dilution, cytopathic effect (cpe) assay, with or without centrifugation of infected 96-well plates as described in detail by Nyberg-Hoffman *et al.* (15). Titers obtained using the standard accepted calculations, from plates with or without centrifugation, were identical. The only difference we found following centrifugation of infected plates was that titers could already be read 3 days after infection (and did not change even when examined at 10 days postinfection), while in the absence of centrifugation titers could only reliably be read 8 days after infection. The titer, determined by standard calculations routinely utilized in our laboratory and elsewhere, was  $8.2 \times 10^{10}$  iu/ml for RAD36, with a particle:pfu ratio of 17. The titer of RAD35 was  $6.55 \times 10^{11}$  iu/ml, and the particle:pfu ratio was 27. If, in addition to centrifugation of infected plates, we applied the mathematical correction described by Nyberg-Hoffman *et al.* (15), the infectious titer obtained for RAD36 becomes  $2.3 \times 10^{11}$ , and for RAD35 it becomes  $1.8 \times 10^{12}$ . [The mathematical correction used was Eq. [6] described in Ref. 15:  $V = -\ln(1 - P_W/n) / \phi C_T(d + Nt)$ , where  $I = 2.38 \times 10^{-4}$  cm/s<sup>1/2</sup>,  $\phi$  (average cell area for 293 cells) =  $6.3 \times 10^{-6}$  cm<sup>2</sup>,  $C_T$  is total number of cells per well,  $t$  is duration of the assay,  $P_W$  is positive wells,  $n$  is total wells,  $V$  is virus concentration, and  $d$  is displacement (by centrifugation) or depth of the well, whichever is less. Calculations are in centimeters for distances and seconds for time; adjusted titers must be multiplied by the dilution factors to obtain the titers of the preparation.] Throughout the description of our experiments we refer to vector titers as calculated from the standard method of titration, because this is the accepted international standard used in the estimation of adenoviral titers, and this allows comparisons to be made across all previously published reports. The human embryonic kidney 293 cell line (293) used to produce recombinant adenoviral vectors was obtained from Microbix Biosystems Inc. (Toronto, Canada). All viral preparations were tested for the presence of replication-competent adenovirus (16) and for LPS content (17), and preparations used were negative for both. All relevant adenoviral methods and quality control procedures are described in detail in Ref. (10).

**Stereotactic surgery and immunohistochemistry.** Male Sprague-Dawley rats (200–250 g) were anesthetized with a combination of halothane (1.5–4%) using an O<sub>2</sub> flow of 1l/min and nitrous oxide (0.6l/min) and then placed in a stereotactic frame. Different infectious units of each virus were injected directly into the striatum in a total volume of 3  $\mu$ l [coordinates from bregma: anterior 0.6 mm, lateral 3.4 mm (for Rad 36) or -3.4 mm (for Rad 35), ventral -5 mm] using a 10- $\mu$ l Hamilton syringe as described previously (3, 5). At doses of 10<sup>4</sup>–10<sup>8</sup>, both vectors were injected into the same animal, one vector into each hemisphere, to improve the comparison between both vectors, while in other experiments (i.e., RAD36 10<sup>4</sup>–10<sup>3</sup>) only one virus vector was injected per animal. A different Hamilton syringe was used for each vector. Five days after

virus inoculation, animals were perfusion-fixed. After removing the brain from the skull, one hemisphere was marked with a small cut throughout the neocortex to differentiate the right from the left hemisphere and thus identify which virus had been injected into either side of the brain. All practical aspects of gene delivery into the brain using adenoviruses are described in detail in Ref. (18). The following number of injection sites ( $n$ ) were analyzed for each dose of virus: RAD36,  $1 \times 10^4$ – $1 \times 10^3$  iu,  $n = 4$ ;  $1 \times 10^4$ – $1 \times 10^5$  iu,  $n = 3$ ;  $1 \times 10^6$ – $1 \times 10^8$  iu,  $n = 6$ ; RAD35,  $1 \times 10^4$ – $1 \times 10^5$  iu,  $n = 3$ ;  $1 \times 10^6$ – $1 \times 10^8$  iu,  $n = 6$ ; saline injection,  $n = 6$ . Five days after surgery, the rats were perfusion-fixed with 300 ml of Tyrode's buffer, followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Immediately following perfusion, the brains were removed and postfixed for 6 h. Fifty-micrometer-thick sections were cut using a vibratome (3, 4). Immunohistochemistry was performed on these sections as described in detail elsewhere (3, 4, 18). The primary antibodies used were mouse anti-rat CD8, Serotec Ltd. (1:500); mouse anti-rat ED1, MCA341, Serotec Ltd. (1:800); and mouse anti- $\beta$ -galactosidase, Z3781, Promega (1:1000). A biotinylated rabbit anti-mouse, E0464, DAKO (1:200), was used as secondary antibody. To detect specific antibody binding, we used the Vectastain ABC detection kit from Vector Laboratories following the manufacturer's instructions. In brains injected with 10<sup>1</sup>–10<sup>3</sup> infectious units of RAD36, the viral injection site was cut in serial vibratome sections, and immunoreactive cells were counted throughout each site in one of each of three sections. The diameter of  $\beta$ -galactosidase-positive cells was estimated, and total immunoreactive cell counts were corrected using Abercrombie's correction (19), according to the formula  $(\Sigma x) f st / (st + cd)$ , where  $\Sigma x$  is the total number of cells counted,  $f$  is the frequency of sections sampled,  $st$  is the section thickness, and  $cd$  is the cell body diameter. Cell body diameter was estimated experimentally and was found to be 12  $\mu$ m. Sites injected with doses of 10<sup>4</sup> infectious units and above were cut serially, and one of six sections was stained for the immunocytochemical detection of  $\beta$ -galactosidase, ED1, or CD8.

**Transduced area quantification.** The analysis to determine the area of striatum occupied by cells immunoreactive with antibodies against  $\beta$ -galactosidase was performed using a Leica Quantimet 600 image analysis system (Leica Cambridge Ltd., UK) controlled by QWin software using a Leica RMD8 microscope. The equipment was calibrated using standard micrometer slides for each lens and then used to measure the area of transduced cells.

## RESULTS

Transgene expression and acute inflammation were compared between vectors expressing  $\beta$ -galactosidase under the control of the major immediate early human cytomegalovirus (MIEhCMV) promoter (RAD35) or of the major immediate early murine cytomegalovirus (MIEhCMV) promoter (RAD36). Prior to their use *in vivo*, both viruses were tested *in vitro*; in all cell lines tested, including nonhuman primate and human cell lines, expression from RAD36 was consistently and substantially higher than expression from RAD35.

### Highly Efficient Transgene Expression in the Brain *in Vivo*

*In vivo*, viral doses from 10<sup>4</sup> to 10<sup>8</sup> infectious units of RAD 35 and RAD 36 were injected into adult rat striata. Five days later, the brains were sectioned and the presence of immunoreactive  $\beta$ -galactosidase enzyme was assessed by immunohistochemistry. At all doses used, the levels of immunoreactive  $\beta$ -galactosidase expressed from RAD36 were higher than those detected following injection

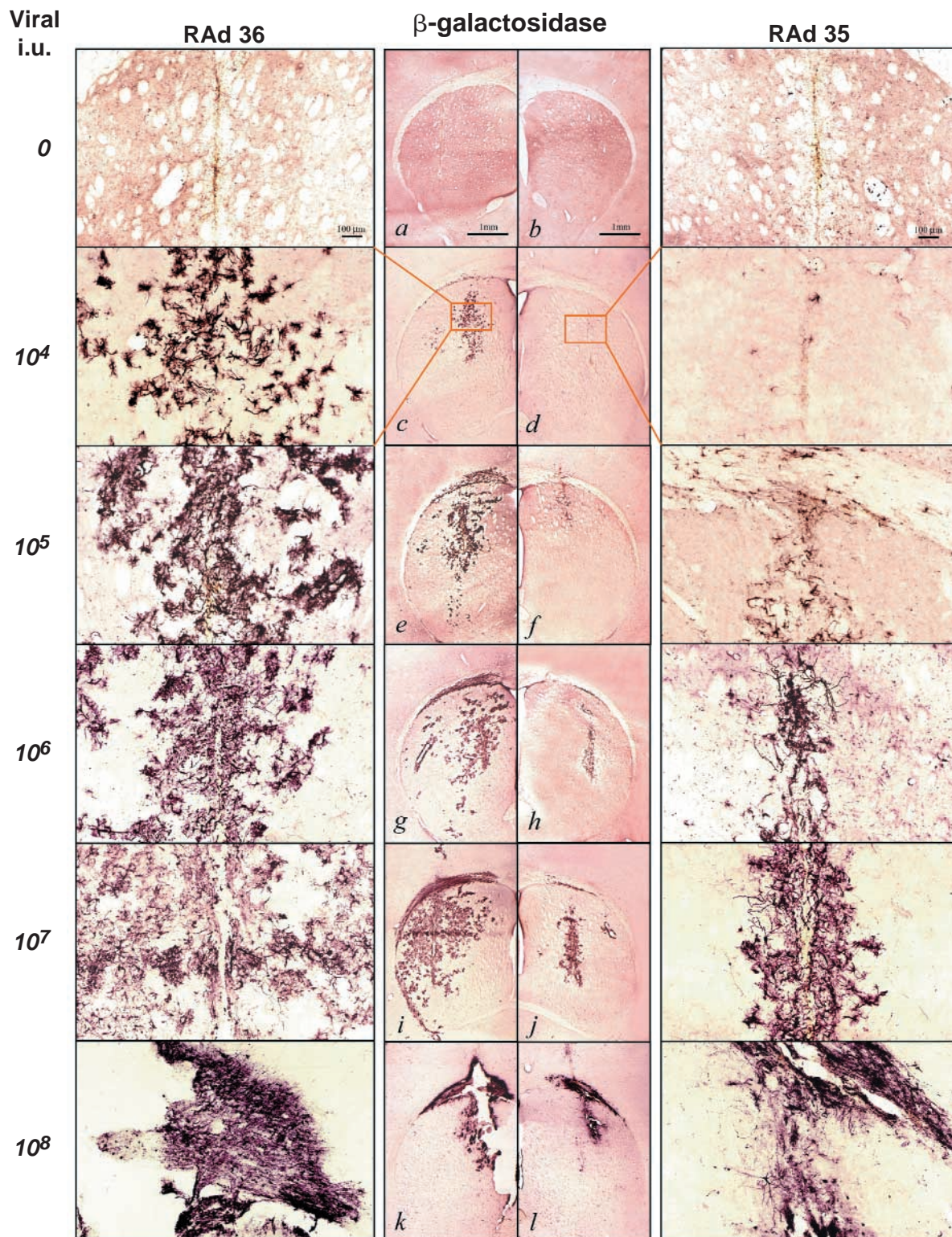


FIG. 1. Increasing doses of vectors were injected into the striatum of adult rats. Three microliters containing the appropriate dose of either RAd36 or RAd35 was injected into the brain, and animals were perfusion-fixed 5 days later. Brains were processed as described in Ref. (18). Note that already at 10<sup>4</sup> i.u. substantial transduction can be seen with RAd36, while expression is barely detectable in the site injected with RAd35. The area of striatum encompassing transduced cells is always larger in those sites injected with RAd36, compared with those injected with RAd35. The central panels show low-magnification views of the centers of each injection site; the lateral panels at either side of the central panels show higher magnification of boxes, as indicated in c and d. Scale bars for the central panels are shown in a and b, while scale bars for the lateral panels are shown in the top left and top right images. Viral i.u. = 0 represents injection of saline.

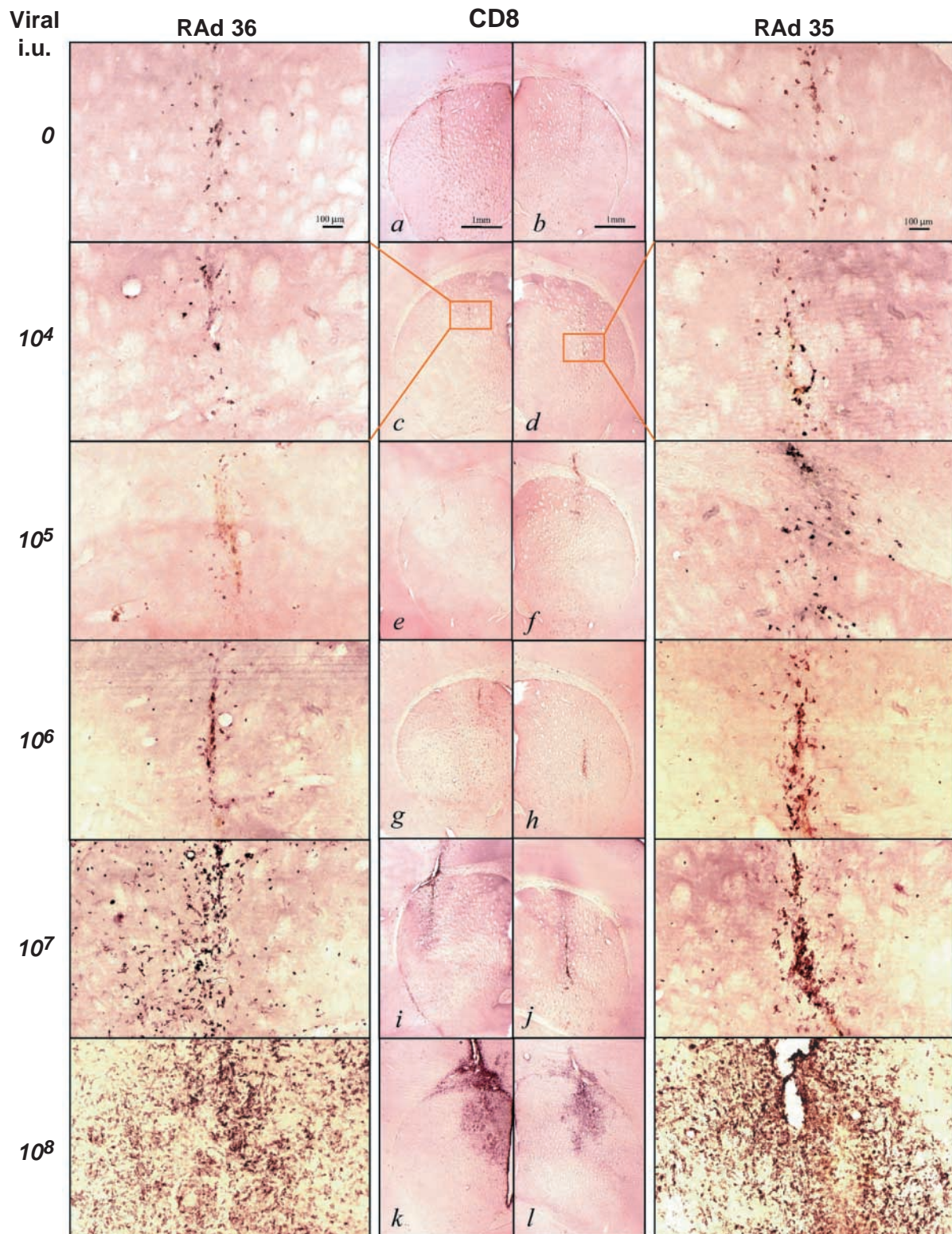


FIG. 3. Increasing doses of vectors were injected into the striatum of adult rats, and serial sections to those illustrated in Fig. 1 were analyzed for the presence of CD8+ inflammatory NK and T cells and are shown. Increased influx of CD8+ cells is only seen at 10<sup>7</sup> and 10<sup>8</sup> i.u. of RAAd36 and 10<sup>8</sup> i.u. of RAAd35. The central panels show low-magnification views of the centers of each injection site; the lateral panels at either side of the central panels show higher magnification of boxes, as indicated in c and d. Scale bars for the central panels are shown in a and b, while scale bars for the lateral panels are shown in the top left and top right images. Viral i.u. = 0 represents injection of saline.

tion of RAd35 (Fig. 1). A low dose of  $10^4$  infectious units of RAd 36 allowed transduction of a relatively large area of the rat striatum (Fig. 1c), comparable to the area transduced by  $10^7$  iu of RAd 35 (Fig. 1j; Fig. 2). A dose of  $10^4$  iu of RAd35, however, produced only very few immunoreactive cells (Fig. 1d).

A quantitative analysis of the total area of  $\beta$ -galactosidase immunoreactivity in the center of the injection sites demonstrated that the area transduced by RAd36 is significantly larger than that transduced by RAd35 following injection of vector doses of  $10^4$ – $10^7$  (Fig. 2; see Figs. 1c–1j). At  $10^8$ , the difference in transduction between both vectors was not significant due to the neurotoxicity associated with the high dose of vector and high levels of transgene expression. Maximal transduction for RAd36 and RAd35 was achieved at  $10^7$  iu. At  $10^8$  iu there was significant toxicity associated with both vectors (Figs. 1k and 1l; Fig. 2; Figs. 3k and 3l; Figs. 4k and 4l). Following infection with  $10^8$  iu of RAd35,  $\beta$ -galactosidase expression appeared to be higher than at  $10^7$  iu, but not statistically different due to a high variability in  $\beta$ -galactosidase levels in animals injected with  $10^8$  iu. This was possibly a result of direct vector cytotoxicity observed following the injection of high vector dose (Figs. 1j and 1l; Fig. 2) (4). Further, when comparing the efficiency of expression, statistical analysis demonstrated that a dose of  $10^4$  infectious units of RAd36 expresses  $\beta$ -galactosidase throughout an area equivalent to that achieved by  $10^7$  infectious units of RAd35 (Fig. 2). This indicates that RAd36 is approximately 1000 times more effective than RAd35 in expressing  $\beta$ -galactosidase in the brain *in vivo*.

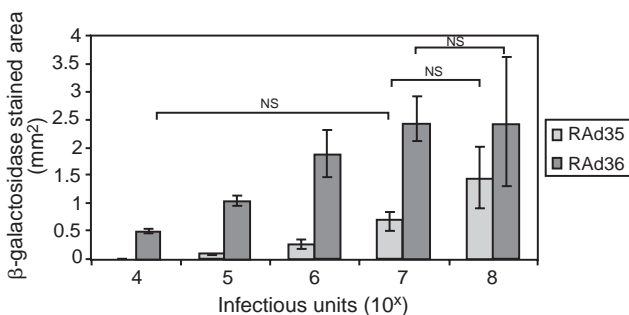


FIG. 2. Quantification of the area of the brain encompassing  $\beta$ -galactosidase immunoreactive cells using a semiautomatic Quantimet imaging system. Expression of  $\beta$ -galactosidase following injection of RAd36 (green bars) was always significantly higher than expression from RAd35 (blue bars) at all doses ( $P < 0.001$ ), except at  $10^8$  iu, at which the difference was not statistically significant. The area of  $\beta$ -galactosidase immunoreactivity after injecting  $10^4$  iu of RAd36 was not statistically different from that obtained following infection of RAd35 at  $10^7$  iu. This shows the expression from RAd36 to be approximately 1000-fold more effective than expression from RAd35. The difference in expression from RAd36 did not differ between  $10^7$  and  $10^8$  iu, demonstrating that with RAd36 maximal transgene expression is achieved at  $10^7$  iu. The difference in expression between  $10^7$  and  $10^8$  iu of RAd35 was not statistically significant, probably due to the increased toxicity at  $10^8$  iu, which can eliminate transduced cells.

### Absence of Virus-Induced Brain Cytotoxicity and Inflammation at Viral Doses Achieving High-Level Transgene Expression

Inflammatory cell infiltration in response to viral vector injection was studied in serial brain sections from animals injected with either RAd35 or RAd36 and stained for the presence of CD8+ NK and T-cells (Fig. 3) or ED1+ macrophages/microglia (Fig. 4) by immunohistochemistry. The inflammatory response to injection of either viral vector was greater than that observed following the injection of saline, only at doses of  $10^7$  iu and above. At these doses ( $10^7$  iu and above), inflammation was higher in animals injected with RAd36 (Figs. 3i and 3k; Figs. 4i and 4k) compared to animals injected with RAd35 (Figs. 3j and 3l; Figs. 4j and 4l). This is probably due to inflammatory effects caused by higher levels of  $\beta$ -galactosidase expression from RAd36 (Figs. 1i and 1j).

### One Infectious Event Is Enough to Allow Transgene Detection in the Brain *in Vivo*

To determine the lower limit of detection of transgene expression from RAd36 in the brain *in vivo* and to assess whether transduction *in vivo* was linear with respect to viral vector dose, we injected into the brain viral doses ranging from 10 to 1000 total infectious units. Serial brain sections taken throughout the injection sites were immunohistochemically stained for  $\beta$ -galactosidase and positive cells were counted throughout the injection sites. In animals injected with 10 infectious units ( $n = 4$ ), we detected  $7 \pm 2$   $\beta$ -galactosidase immunoreactive cells; in animals injected with 100 infectious units ( $n = 4$ ), we detected  $102 \pm 9$   $\beta$ -galactosidase immunoreactive cells; and in animals injected with 1000 infectious units ( $n = 4$ ), we detected  $1046 \pm 23$  immunostained cells (Fig. 5). Thus, gene transfer into the brain appears to be 100% efficient following injection of low doses of RAd36. The correlated linear increase in the number of positive cells with increasing dose of RAd36 (in the  $10^1$ – $10^3$  iu range) shows that *in vivo* infection by a single infectious unit of RAd36 is sufficient to express enough  $\beta$ -galactosidase to detect transgene expression in an infected cell by immunohistochemistry. Such a linear relationship is predicted by Poisson's distribution if, and only if, a single infectious viral unit directs the expression of sufficient transgene to be reliably detected (20, 21). This linearity is lost at doses of  $10^4$  iu and above. It is only at these doses that transduction becomes detectable in brains injected with RAd35, an effect we attribute to the occurrence of multiple infectious events.

### Method of Titer Determination Does Not Affect the High Transduction Efficiency

The calculated efficiency of transgene expression obtained in our experiments depends on the infectious titer assigned to the virus stock used. The worldwide

accepted standard method, for determining adenovirus titers, gives a titer which is calculated from the highest dilution of virus which results in productive infection of cells and complete cytopathic effect within infected wells. Recently, Nyberg-Hoffman *et al.* (15) have proposed that titers obtained by this method may be an underestimate of the actual number of infectious units present in any individual vector batch. Using the standard method of titration, we obtained a titer for RAd36 of  $8.2 \times 10^{10}$  iu/ml (with a particle:pfu ratio of 17) and an efficiency of gene transfer of 100% (Fig. 5). Applying the theoretical corrections to the titer, according to Eq. [6] of Nyberg-Hoffman *et al.* (15), we obtained a titer of  $2.3 \times 10^{11}$  iu/ml (with a particle:iu ratio of 6) and a corresponding efficiency of 33%. Regardless of whether the standard titer or the corrected titer is used for the calculations, the efficiency of RAd36-mediated gene transfer to the brain remains very high. In this work, we refer to vector titers as calculated from the standard method of titration. With either titer, the linear increase in the number of  $\beta$ -galactosidase-expressing cells obtained at increasing viral doses from  $10^1$ – $10^3$  proves that each stained cell is infected by a single viral particle, demonstrating that one infecting physical particle is enough to produce detectable levels of  $\beta$ -galactosidase in a cell *in vivo* (20, 21).

## DISCUSSION

Efficiency of gene delivery and expression is likely to depend on a number of factors: possessing the appropriate viral receptors at high density, the processes of viral internalization and delivery of the vector genome from endosomes to the nucleus of infected cells, and transcription–translation efficiency of the transgene will all play a role. *In vivo*, one must further consider the role played by the diffusion of virus throughout brain tissue and the effects of inflammatory and immune cells. Single viral particles are very efficient in infecting their preferred target cells *in vitro* during the determination of viral titers. However, *in vivo* gene transfer, as assessed by the number of detectable transduced cells, appears to be inefficient. This has led to the use of high titers of vectors to transduce the brain *in vivo* and to obtain either anatomically detectable cells or a therapeutic effect in animal models of disease.

When using adenoviral vectors encoding marker transgenes under the transcriptional control of very powerful promoters (i.e., an adenovirus vector encoding  $\beta$ -galactosidase under the transcriptional control of a powerful short MIEhCMV promoter), no transgene expression is seen if fewer than  $10^4$  infectious units is injected into the target brain area. Similar data have been reported by many other laboratories, but the reasons for the apparently inefficient gene transfer and expression *in vivo* have never been properly explored. In most papers doses of  $10^4$ – $10^{10}$  total infectious units have been inject-

ed into the brain of rodents. All studies available so far report that at least  $10^6$ – $10^8$  or higher total infectious units need to be injected into the brain to detect anatomically or physiologically relevant transgene expression. Significantly, similar titers ( $10^6$ – $10^8$  infectious units) have been used when transducing the brain with AAV, lentiviral, or herpes simplex virus type 1 vectors (2).

The reasons underlying the apparently inefficient gene transfer *in vivo* could be attributed to any of the factors listed above. In a previous paper (14), we demonstrated that, while RAd35 was able to infect neocortical neurons in primary cultures, transgene expression was not seen until cells were superinfected with other viruses, an experimental paradigm which was found to activate the sMIEhCMV promoter sequence in RAd35. This was taken to indicate that although neurons in primary culture were infected with RAd35, the MIEhCMV promoter remained silent. Our current work demonstrates that *in vivo* gene expression from viral vectors depends on the promoter element employed. Using very low doses of a viral vector expressing a transgene from a very strong promoter, we have demonstrated for the first time that a single viral infectious unit is sufficient to transduce a single brain cell *in vivo*. This in turn allowed us to determine that the efficiency of Ad-mediated gene transfer into the rat brain *in vivo*, under our experimental conditions, is 100%. The reason for the generally low efficiency of gene transfer and expression hitherto observed *in vivo* appears thus to be due to the low activity of promoters used previously.

Viral vector diffusion throughout brain tissue has so far been regarded as very poor, but has not been evaluated experimentally. Our data show that RAd36 at each viral vector dose transduces an area of the striatum which is much larger than the area transduced by the same dose of RAd35. We suggest that this is due to the fact that a single viral particle of RAd36 leads to detectable  $\beta$ -galactosidase expression, while coinfection with a high number of particles of RAd35 is necessary to achieve detectable levels of transgene expression in the brain. RAd35 transgene expression can only be detected at  $10^4$  iu. Since experiments with RAd36 show that virus does indeed enter brain cells after delivery of lower doses, multiple hits must be necessary for detectable transgene expression following infection with RAd35. Results with RAd36 demonstrate that adenoviral vectors can diffuse relatively large distances in the brain, even in the absence of special physical delivery methods such as high-pressure injections into the brain (22).

Much work has been devoted to the development of cell-type-specific promoters, inducible promoter elements, or even combined cell-type-specific and inducible transcriptional regulatory systems. Such cell-type-specific promoters are at least  $10^3$ - to  $10^4$ -fold weaker than the MIEhCMV promoter we used in our experiments (unpublished data from our laboratory; Refs. 11 and 23), and thus at least  $10^3$ - to  $10^4$ -fold more virus would be required to detect equivalent numbers of transduced cells

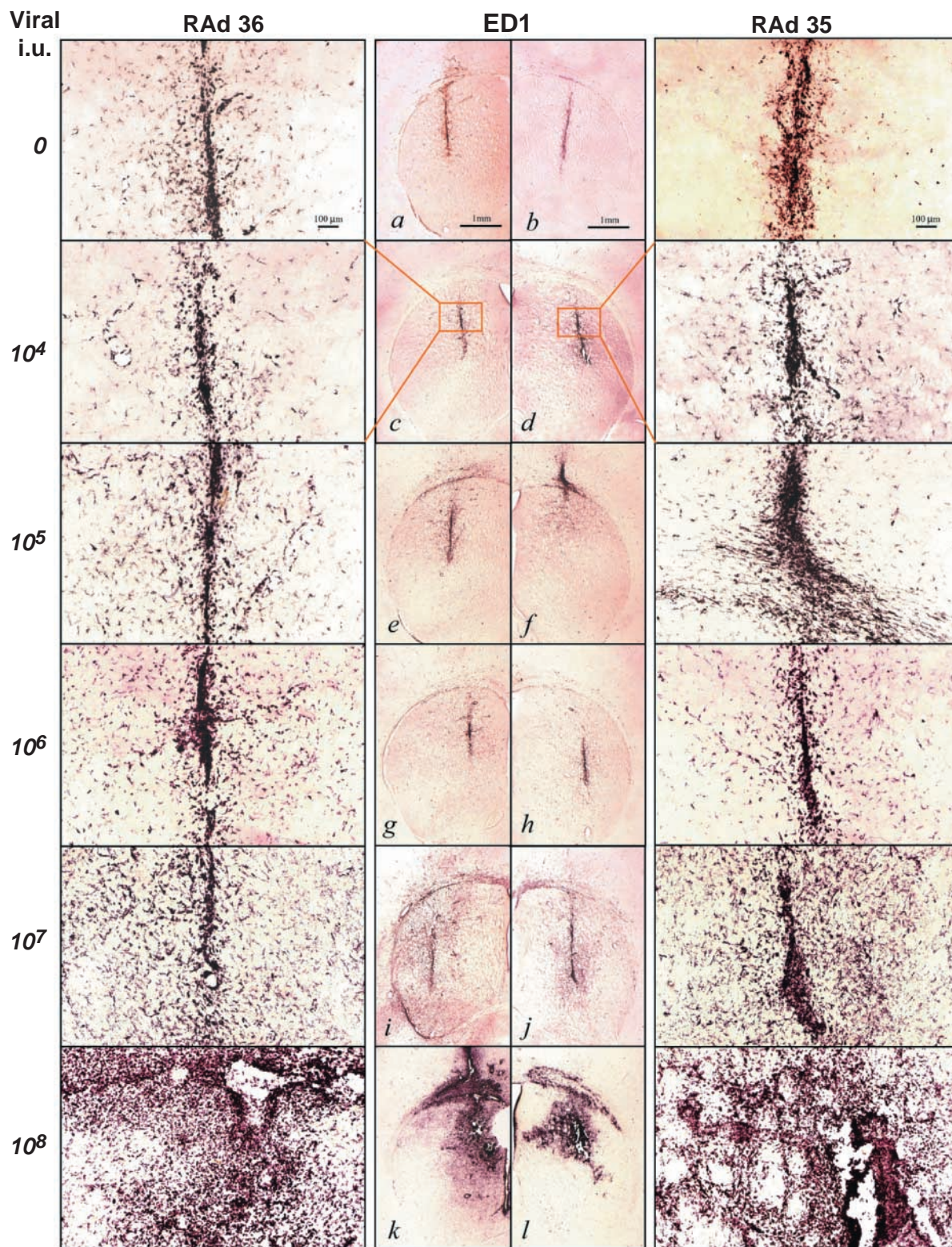


FIG. 4. Increasing doses of vectors were injected into the striatum of adult rats, and serial sections of those illustrated in Fig. 1 were analyzed for the presence of ED1+ activated macrophages/microglial cells and are shown. Increased influx of ED1+ cells is only seen at  $10^7$  and  $10^8$  iu of both RAAd36 and RAAd35. The central panels show low-magnification views of the centers of each injection site; the lateral panels at either side of the central panels show higher magnification of boxes, as indicated in c and d. Scale bars for the central panels are shown in a and b, while scale bars for the lateral panels are shown in the top left and top right images. Viral iu = 0 represents injection of saline.

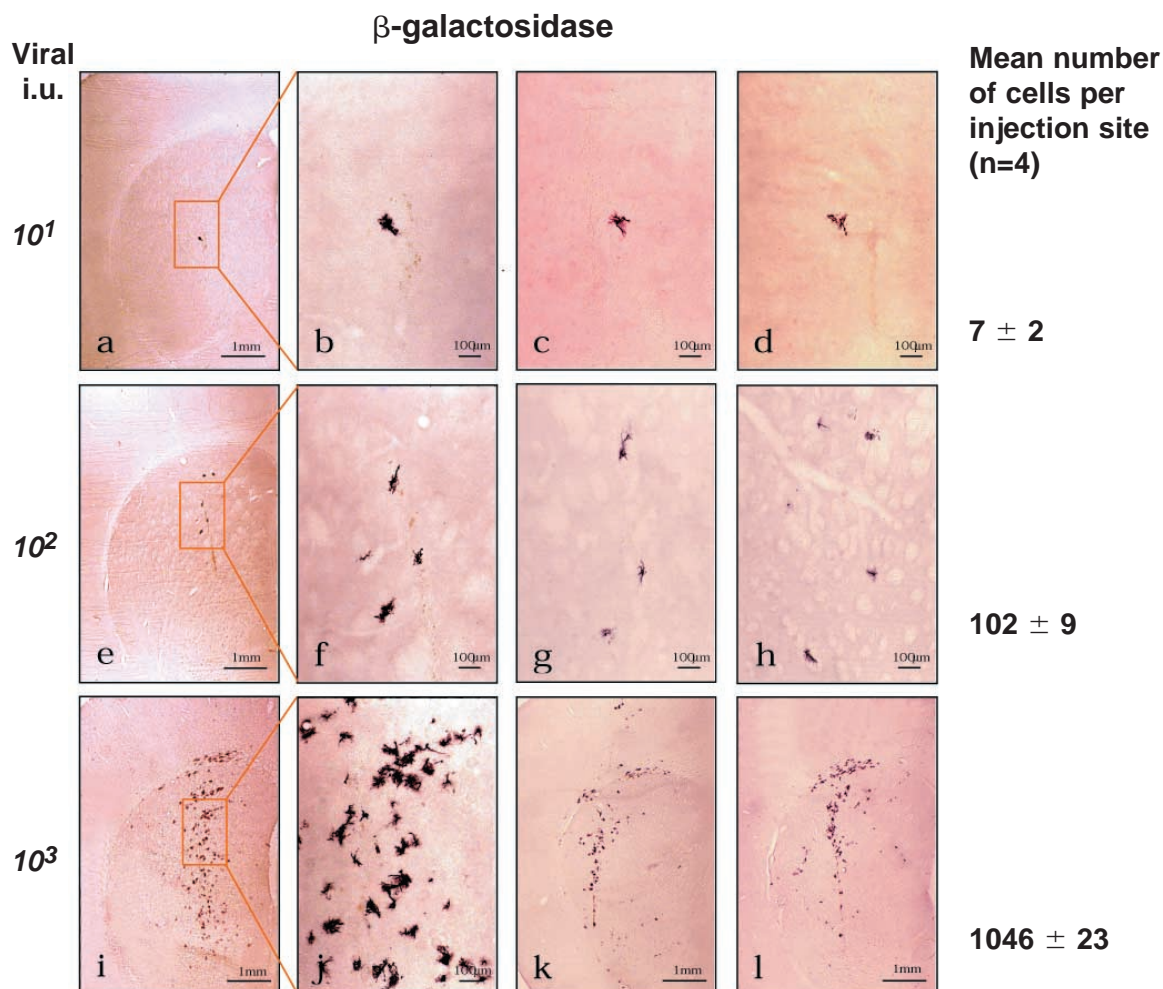


FIG. 5. Low doses of RAd36 were injected into the adult rat striatum:  $10^1$ ,  $10^2$ , and  $10^3$  i.u. a, e, and i show low-power views through the middle of an injection site at one of the doses. b, f, and j show enlarged views of the injection site, as outlined in the boxes in a, e, and i. c, d, g, h, k, and l each show further examples of different injection sites. In c, d, g, and h, higher power views are shown to illustrate the morphology of transduced cells, while k and l are shown at lower power to demonstrate the full extent of distribution of RAd36 transduced cells at this viral vector dose. Scale bars and magnifications are shown for each panel. The mean numbers of cells per injection site ( $n = 4$ ) are shown to the right of the figure. All transduced cells detected at these low doses displayed a characteristic astrocyte-like morphology.

in the brain. Investigators have so far been forced to choose between accepting a high degree of inflammation and cytotoxicity accompanying administration of higher doses of vector, which will in turn curtail the duration of transgene expression, or using lower doses and achieving a more limited transduction (4, 17, 24). We now demonstrate for the first time that high-level expression in the brain can be obtained in the complete absence of cellular inflammation and using very low doses of adenoviral vectors. The activity of cell-type-specific or inducible promoters will have to be improved substantially in order to achieve efficient and safe gene therapy approaches for the treatment of human neurological diseases.

We suggest that, concomitantly with further optimization of viral vectors (25), including the basic knowl-

edge necessary for successful retargeting of viral vectors (26, 27) and engineering of appropriate transcriptional regulation it will be possible to maximize transgene expression and eliminate direct cytotoxicity and inflammation. In conclusion, our data demonstrate that gene delivery using adenoviruses *in vivo* can be extremely efficient and devoid of any direct cytotoxicity or acute brain inflammation.

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