

Adenovirus Expression of IL-1 and NF- κ B Inhibitors Does Not Inhibit Acute Adenoviral-Induced Brain Inflammation, but Delays Immune System-Mediated Elimination of Transgene Expression

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Despite their ability to provide long-term transgene expression in the central nervous system of naïve hosts, the use of first-generation adenovirus (Ad) vectors for the treatment of chronic neurological disorders is limited by peripheral immunization, which stimulates anti-adenovirus immune responses and causes severe inflammation in the central nervous system (CNS) and elimination of transgene expression. The purpose of this study was to investigate the roles of NF- κ B and interleukin-1 (IL-1) during inflammatory responses to Ads in the CNS of naïve and preimmunized rats. We assessed activation of macrophages/microglia, up-regulation of MHC I expression, infiltration of leukocytes, and transgene expression following delivery of Ads to the rat striatum. After delivery of increasing doses of adenoviral vectors expressing various anti-inflammatory agents (e.g., NF- κ B or IL-1 inhibitors) to naïve rats, no reduction in Ad-mediated CNS inflammation was seen 1 week after delivery of Ads, compared to a control Ad.hCMV. β -galactosidase (RAd.35) virus. We then assessed CNS inflammation and transgene expression at a time when control transgene expression would be completely eliminated, i.e., 1 month post-vector injection into the brain. This would optimize the assessment of an anti-inflammatory agent expressed by an adenoviral vector that could either delay or diminish immune system-mediated elimination of transgene expression. As expected, at 1 month postinfection, control preimmunized rats receiving Ad.mCMV. β -galactosidase (RAd.36)/saline or RAd.36/Ad.null (RAd.0) showed complete elimination of β -galactosidase expression in the brain and levels of inflammation comparable to those of naïve animals. However, animals injected with RAd.36 in combination with Ads expressing NF- κ B or IL-1 inhibitors showed a delayed elimination of β -galactosidase compared to controls. As predicted, the extended presence of transgene expression was accompanied by increased levels of CNS inflammation. This suggests that blocking NF- κ B or IL-1 delays, albeit partially, transgene elimination in the presence of a preexisting systemic immune response. Prolonged transgene expression is predicted to extend concurrent brain inflammation, as noted earlier. Taken together these data demonstrate a role for NF- κ B and IL-1 in immune system-mediated elimination of Ad-mediated CNS transgene expression.

Key Words: interleukin-1, nuclear factor- κ B, gene transfer/therapy, adenoviral vectors, brain inflammation

INTRODUCTION

Although recombinant adenoviral vectors (RAds) are able to mediate both widespread and long-term gene transfer

in the central nervous system (CNS), their use is limited by vector-mediated cytotoxicity and inflammation, which is elicited at doses of $>10^8$ iu. Delivery of doses of $<10^8$ iu to the CNS of naïve animals results in transduc-

tion of multiple neural cell types [1–3], persistent transgene expression lasting up to 12 months [4–7], and induction of transient acute innate inflammation that does not inhibit transgene expression [8–11]. In contrast, when the same doses of RAd are delivered to the CNS parenchyma of animals with preexisting systemic antiadenoviral immune responses, a rapid influx of CD8⁺ and CD4⁺ T cells, NK cells, and macrophages occurs, leading to a rapid elimination of transgene expression within 2 weeks post-RAd delivery [6,12–14]. Importantly, whereas acute immune responses are identical upon injection of first-generation or high-capacity helper-dependent adenoviral vectors, the systemic anti-adenovirus immune response eliminates only expression mediated by first-generation vectors, not high-capacity helper-dependent adenoviral vectors. In contrast to the response to doses of adenovirus of <10⁸ iu, delivery of high doses of RAd (>10⁸ iu) to the CNS induce a long-term inflammatory response, extensive neuronal and glial cytotoxicity leading to brain substance loss, and complete elimination of transgene expression [9].

Upon direct injection of adenoviral vectors into the brain of naïve animals, a dose-dependent inflammation is detected. This inflammatory response is characterized by activation of neural cells and influx of circulating immune cells. Brain astrocytes up-regulate expression of GFAP, and microglial cells up-regulate expression of MHC-I and MHC-II. It is also likely that other cell types may up-regulate MHC proteins. Also, an influx of lymphocytes and macrophages is detected. Macrophages are also activated, as evidenced by their immunoreactivity for the lysosomal protein recognized by antibodies to ED1.

Importantly, this acute inflammation appears to depend on the integrity of the adenoviral capsid. This is supported by the following data. Acute inflammation is independent of the transgene encoded by the adenoviral vectors and independent of whether the vector is a first-generation or a high-capacity helper-dependent adenoviral vector [6,9]. Also, acute inflammation leads to an increase in IL-1 and IL-6 in the brain and is eliminated by heat inactivation of the adenovirus virions [15]. Also, we have determined that this early inflammation is not mediated by binding of adenovirus to its two main receptors, i.e., CAR and integrins. Vectors unable to bind either of these proteins maintain their capacity to cause brain inflammation [16]. It is also crucial to consider that this early inflammation does not compromise long-term transgene expression and is transient, with all signs of inflammation having disappeared by 30 days post-adenovirus injection [6]. Also, this early inflammation does not lead to a systemic priming of an adaptive immune response against adenovirus vectors. This is supported by direct and indirect evidence. Direct evidence is provided by the absence of neutralizing anti-adenovirus antibodies in serum, even following the injection of high doses of Ad into the brain [17]. Indirect evidence is provided by the

presence of long-term transgene expression in the CNS, which is completely eliminated in the presence of a systemic antiadenoviral immune priming [6].

Systemic immunization against adenoviruses has different effects on adenovirus-mediated transgene expression in the brain, depending on whether expression is mediated by first-generation vectors or high-capacity helper-dependent adenovirus vectors. Also the temporal sequence of vector injection and immunization is crucial. Systemic immunization can be administered either preceding or following the injection of adenoviral vectors into the brain. Both paradigms lead to different experimental outcomes.

Systemic immunization following injection of adenoviral vectors into the brain causes brain inflammation and transgene elimination, if transgene expression is mediated by first-generation adenoviral vectors. Expression mediated by high-capacity helper-dependent adenovirus vectors is neither eliminated nor a cause of the influx of inflammatory cells into the brain [6].

Systemic immunization preceding the injection of adenoviral vectors into the brain leads to complete elimination of transgene expression mediated by first-generation vectors within 2–4 weeks, accompanied by brain inflammation during this period. Expression mediated by high-capacity helper-dependent adenovirus is reduced to approximately 50% within 2 weeks and remains stable thereafter [14]. In this case brain inflammation coincides with the period of transgene elimination. Thus, brain inflammation correlates with transgene elimination. Infiltrating immune cells need to recognize antigens to enter the brain. Once these antigens stop being available to immune cells, they cannot be detected in the brain anymore.

Previous data obtained by ourselves [15] and others [18] indicate that IL-1 and NF- κ B are important mediators of adenovirus-induced inflammation. In this article we studied the effects of anti-inflammatory inhibitors of IL-1 and NF- κ B on two paradigms of adenoviral immune responses. First, we tested the hypothesis that first-generation adenoviral vectors expressing inhibitors of IL-1 and NF- κ B would inhibit acute innate adenovirus-induced inflammation. Second, we tested the hypothesis that adenoviral vectors expressing inhibitors of IL-1 and NF- κ B signaling pathways could delay the immune-mediated elimination of transgene expression in animals in which systemic antiadenoviral immunization precedes the injection of viral vectors into the brain. Given that many potential patients may have been previously infected with adenovirus, this experiment will test whether expression from first-generation vectors could be extended during clinical gene therapy trials using first-generation adenoviral vectors.

To investigate the roles of IL-1 and NF- κ B during adenovirus-mediated CNS brain inflammation we used adenoviruses expressing inhibitors of both IL-1 and NF- κ B.

Both the receptor antagonist (IL-1ra) and type II decoy receptor (IL-1RII) were used to inhibit IL-1 signaling. IL-1ra is able to interact directly with the type I signaling receptor (IL-1RI) without initiating signal transduction [19], while IL-1RII, in either a membrane-bound or a secreted form, is able to bind directly with both IL-1 α and IL-1 β and prevent them from signaling through IL-1RI [19]. To inhibit NF- κ B transactivation we used the natural inhibitor of NF- κ B (I κ B α) and a dominant negative C-terminal truncation mutant of p65 (p65RHD). I κ B α is able to block transactivation by binding to cytoplasmic NF- κ B complexes and preventing their nuclear localization [20], while p65RHD is able to compete for DNA binding and dimerization to other NF- κ B family members, through the N-terminal Rel homology domain (RHD), without causing transactivation due an absence of the C-terminal p65 transactivation domain [21].

Our results indicate that expression of inhibitors of IL-1 and NF- κ B is unable to inhibit early innate adenovirus-induced brain inflammation. However, in the presence of systemic immunization preceding the injection of adenoviral vectors into the brain, inhibitors of IL-1 and NF- κ B can delay the immune-mediated elimination of transgene expression. Our data suggest that expression of anti-inflammatory molecules from the viral vectors themselves will not be a useful strategy to eliminate early innate adenovirus induced inflammation. However, optimizing expression of anti-inflammatory molecules could significantly extend transgene expression from first-generation vectors in the presence of preexisting anti-adenovirus immunity.

RESULTS

Based on the ability of IL-1 to induce activation of NF- κ B in HeLa cells [22], we developed an *in vitro* bioassay to assess whether vectors encoding inhibitors of IL-1 (i.e., IL-1ra, IL-1RII) or NF- κ B (i.e., I κ B α , p65RHD) inhibit IL-1 signaling or NF- κ B-mediated transcriptional transactivation. We transfected HeLa cells with the plasmid pNRE-Luc, which contains three NF- κ B binding sites from the human ICAM-1 promoter and a minimal thymidine kinase promoter upstream of a luciferase reporter gene. After transfection of pNRE-Luc into cells, activators of NF- κ B, including the phorbol ester PMA and cytokines TNF- α or IL-1, can induce expression of luciferase. Following transfection, we assessed HeLa cells for luciferase activity 12 h after incubation with increasing concentrations of rhIL-1 β . An induction of luciferase activity was seen at all rhIL-1 β concentrations and the stimulation reached a plateau at 100 pg/ml (Fig. 1A). We assessed the possibility that infection with RAds per se could induce luciferase activity in pNRE-Luc-transfected HeLa cells 48 h after infection with increasing m.o.i. of RAd.GFP, since *in vitro* activation of NF- κ B has previously been shown following adenovirus infection using an m.o.i. of 1000 [23].

An increase in luciferase activity was seen with increasing viral doses (Fig. 1B), although the level of induction seen with 100 pg/ml rhIL-1 β was 13.4-fold higher than that seen with the highest dose of RAd.GFP tested (m.o.i. 300; already higher than that achievable *in vivo*) (Fig. 1B). We then assessed the luciferase activity of pNRE-Luc-transfected HeLa cells after 48 h of infection with RAd.GFP, RAd.IL-1RII, RAd.IL-1ra, RAd.I κ B α , or RAd.p65RHD (m.o.i. 200), followed by a 12-h incubation with rhIL-1 β (30 pg/ml) (Fig. 1C). Infection with RAd.GFP followed by incubation with rhIL-1 β resulted in a 15-fold induction in luciferase activity. Infection with RAd.IL-1RII followed by incubation with rhIL-1 β , however, resulted in only a 3.5-fold induction in luciferase activity, while infection with RAd.IL-1ra, RAd.I κ B α , or RAd.p65RHD followed by incubation with rhIL-1 β resulted in no significant induction of luciferase activity (Fig. 1C), indicating that all these vectors could inhibit *in vitro* activation of the NF- κ B transcriptional activation pathway elicited by IL-1. We then assessed the luciferase activity of pNRE-Luc-transfected HeLa cells following 12 h of incubation with rhIL-1 β after pretreatment with conditioned medium from RAd.GFP-, RAd.IL-1RII-, and RAd.IL-1ra-infected Cos-7 cells to confirm that the inhibitory activities encoded by these vectors were effectively secreted, as expected (Fig. 1D). In previous experiments we have found Cos-7 cells to be readily infectable by RAds and able to secrete biologically active RAd-expressed proteins very efficiently into the culture medium (Millan, Castro, and Lowenstein, unpublished results). Incubation with rhIL-1 β following pretreatment with RAd.GFP conditioned medium resulted in a 13.3-fold induction of luciferase activity. Incubation with rhIL-1 β following pretreatment with RAd.IL-1RII and RAd.IL-1ra conditioned medium, however, resulted in only 3.5- and 2.2-fold inductions in luciferase activity, respectively (Fig. 1D), indicating that secreted forms of vector-derived IL-1ra or IL-1RII inhibited *in vitro* activation of NF- κ B mediated by IL-1.

Effect of RAds Encoding Inhibitors of IL-1 and NF- κ B on Early Acute Innate Inflammation Caused by Adenovirus Injection into the CNS

Macrophage/microglial activation following adenovirus delivery to the CNS. Having shown that RAds encoding I κ B α , p65RHD, IL-1ra, or IL-1RII could inhibit NF- κ B or IL-1 signaling *in vitro*, we wished to test whether acute inflammation could be reduced by RAds encoding inhibitors of IL-1- and NF- κ B-mediated proinflammatory signaling *in vivo* in the central nervous system. We determined activation of macrophages/microglia (as assessed by ED1-positive immunoreactivity), expression of MHC I, and infiltration of leukocytes (as assessed by CD43-positive immunoreactivity). All markers were studied at 7 days after delivery of 10^7 , 10^8 , and 10^9 iu of RAd.35, RAd.I κ B α , RAd.p65RHD, RAd.IL-1ra, or RAd.IL-1RII to the rat striatum. At each dose and for each virus group we injected a

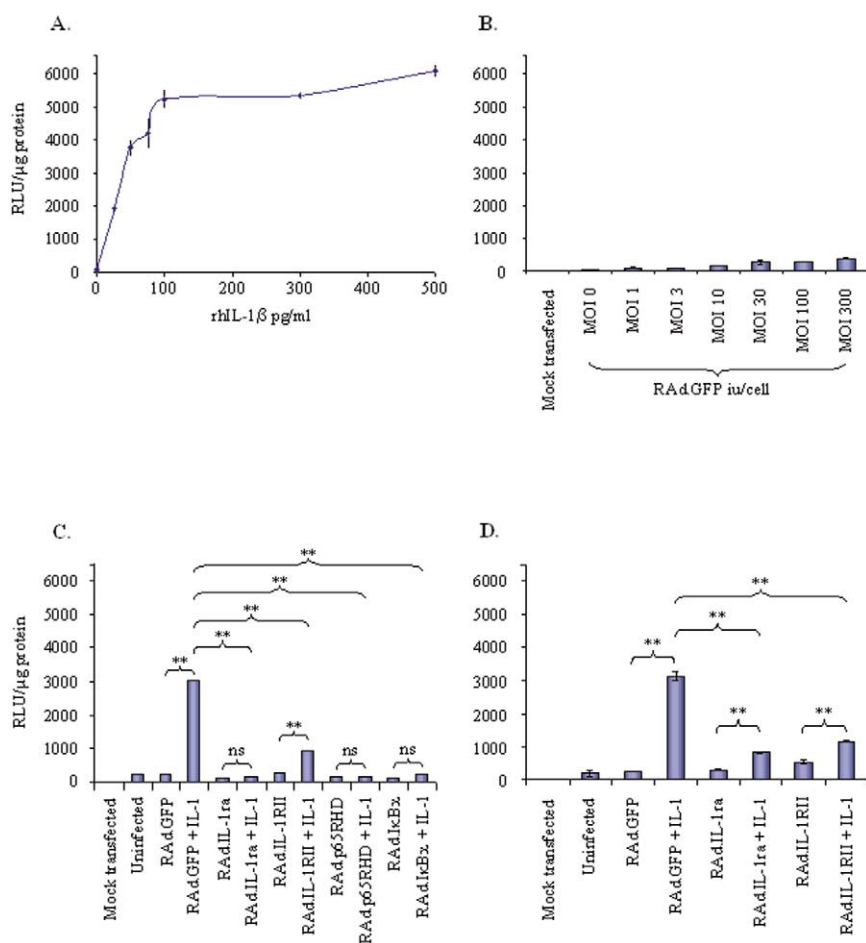


FIG. 1. *In vitro* inhibition of NF- κ B activation or IL-1 signaling. (A) Luciferase activity in pNRE-Luc-transfected HeLa cells following activation of NF- κ B by administration of rhIL-1 β . (B) Luciferase activity in pNRE-Luc-transfected HeLa cells following infection with RAAd.GFP. (C) Inhibition of NF- κ B activation in pNRE-Luc-transfected HeLa cells (\pm rhIL-1 β 30 pg/ml) following infection with RAAd.IL-1ra, RAAd.IL-1RII, RAAd.p65RHD, and RAAd.I κ B α (m.o.i. 100). (D) Inhibition of NF- κ B activation in pNRE-Luc-transfected HeLa cells (\pm rhIL-1 β 30 pg/ml) following incubation with conditioned medium from RAAd.IL-1ra- and RAAd.IL-1RII-infected Cos-7 cells. ns, not significant; **, $P < 0.0001$ ($n = 3$).

total of three rats. Seven days after delivery of 10^7 iu of virus ED1-positive cells were restricted to the needle track and the striatum immediately surrounding the site of injection (Fig. 2). Upon quantification no significant difference was seen in the area of ED1 immunoreactivity between any of the virus-injected groups (Fig. 5A). Following delivery of 10^8 iu of virus ED1-positive cells were seen along the needle track, throughout the ipsilateral striatum and white matter, and at lower levels in the cortex of each virus-injected group (Fig. 2). When the area of ED1 immunoreactivity was quantified, the number of ED1-positive cells was significantly higher than in animals receiving 10^7 iu of virus but there was no significant difference between any of the virus-injected groups (Fig. 5A). After delivery of 10^9 iu of virus, large lesions were seen at the site of injection, while ED1-positive cells were seen along the needle track and throughout the ipsilateral striatum, the white matter, and the cortex in each virus-injected group of animals (Fig. 2). The number of ED1-positive cells at 10^9 iu visually appeared greater in all virus

groups than at 10^8 iu of virus (Fig. 2). Upon quantification of the area of immunoreactivity only the RAAd.IL-1ra- and RAAd.IL-1RII-injected brains showed significantly higher ED1 levels than at 10^8 iu (Fig. 5A). Also, at this dose there was no significant difference between any of the virus-injected groups except RAAd.IL-1RII vs RAAd.I κ B α and RAAd.IL-1RII vs RAAd.p65RHD (Fig. 5A). Thus, at the highest dose tested, inflammation in response to IL-1ra and IL-1RII was increased in comparison to the other groups.

MHC I expression following adenovirus delivery to the CNS.

We assessed MHC I expression in serial sections of all animals monitored for macrophage/microglia activation by monitoring MHC I-positive immunoreactivity. After delivery of 10^7 iu of virus the MHC I immunoreactivity profile was similar to that of ED1, with positive cells restricted to the needle track and areas immediately surrounding the injection site (Fig. 3). Quantification showed no significant difference in immunoreactivity between any virus-injected groups (Fig. 5B). Expression profiles at 10^8 iu were also similar to those of ED1 (Fig. 3),

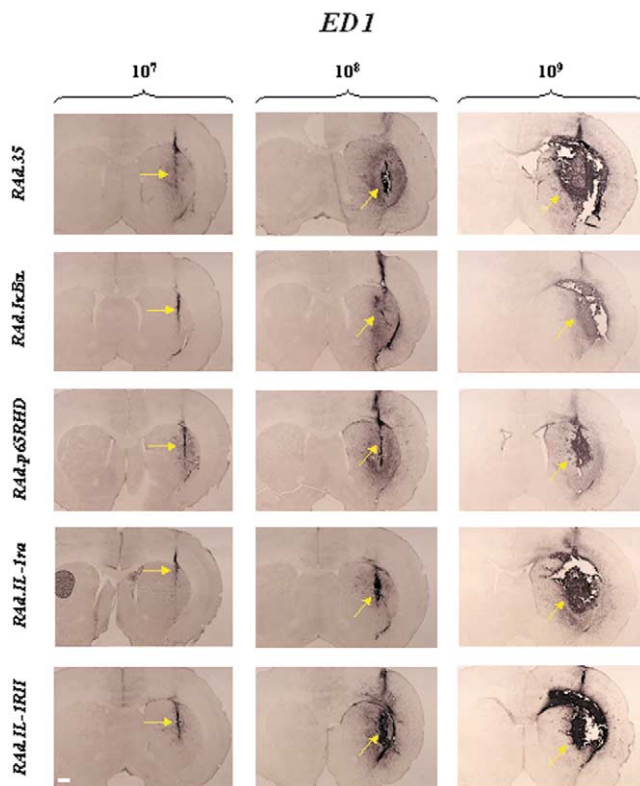


FIG. 2. ED1 immunoreactivity following delivery of 10^7 , 10^8 , and 10^9 iu of RAd.35, RAd.IkBa, RAd.p6SRHD, RAd.IL-1ra, and RAd.IL-1RII to the rat striatum. Areas of activated macrophages/microglia in the ipsilateral hemisphere are indicated by yellow arrows. Scale bar (bottom left), 1 mm.

with no significant difference in immunoreactivity seen between any of the virus-injected groups after quantification (Fig. 5B), and levels of MHC I were higher than those seen at 10^7 iu in the RAd.IkBa-, RAd.IL-1ra-, and RAd.IL-1RII-injected groups (Fig. 5B). Likewise, at 10^9 iu expression profiles were similar to those of ED1 (Fig. 3), with no difference between any of the virus groups seen upon quantification (Fig. 5B). Levels of immunoreactivity in all groups, except RAd.IL-1RII, were significantly higher than those seen at 10^7 iu. Morphologically, differences in immunoreactivity were detected (as illustrated in Fig. 3), and in many cases, these were not statistically significant. These differences are likely due to variability in expression of MHC proteins and the lesions detected following the injection of 10^9 iu of adenovirus that could bias increases obtained (Figs. 3 and 5B).

Leukocyte infiltration following adenovirus delivery to the CNS. We also assessed leukocyte infiltration in serial sections of animals monitored for macrophage/microglia activation and MHC I expression through monitoring CD43 immunoreactivity. At a dose of 10^7 iu a few CD43-positive cells were restricted, mainly to the needle track

and surrounding ipsilateral striatum (Fig. 4), with no significant difference in CD43 immunoreactivity seen between any of the virus-injected groups upon quantification. At 10^8 iu CD43-positive cells were predominantly seen in the ipsilateral striatum (Fig. 4), and evidence of leukocytes infiltrating the CNS microvasculature was seen, with CD43-positive perivascular cuffing (Fig. 4). Quantification revealed no significant difference between any of the virus-injected groups, while only the RAd.IkBa- and RAd.IL-1RII-injected groups showed a significant increase over those receiving 10^7 iu (Fig. 5C). Delivery of 10^9 iu resulted in a more pronounced leukocyte infiltration into the ipsilateral striatum, with evident CD43-positive perivascular cuffing also seen (Fig. 4). Quantification of CD43 immunoreactivity showed no significant difference between any of the virus-injected groups. Although CD43 immunoreactivity appeared higher in groups injected with 10^9 iu, due to data variability and possibly the induced brain lesion, only the RAd.35-, RAd.IkBa-, and RAd.IL-1ra-injected groups showed significantly higher levels than those seen at both 10^8 and 10^7 iu (Fig. 5C).

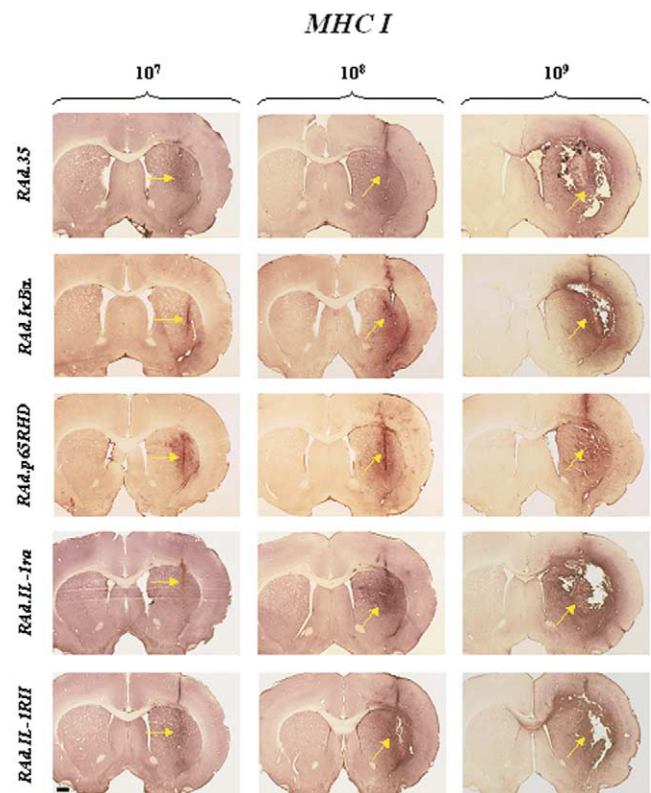


FIG. 3. MHC I immunoreactivity following delivery of 10^7 , 10^8 , and 10^9 iu of RAd.35, RAd.IkBa, RAd.p6SRHD, RAd.IL-1ra, and RAd.IL-1RII to the rat striatum. Areas of MHC I-positive cells in the ipsilateral hemisphere are indicated by yellow arrows. Scale bar (bottom left), 1 mm.

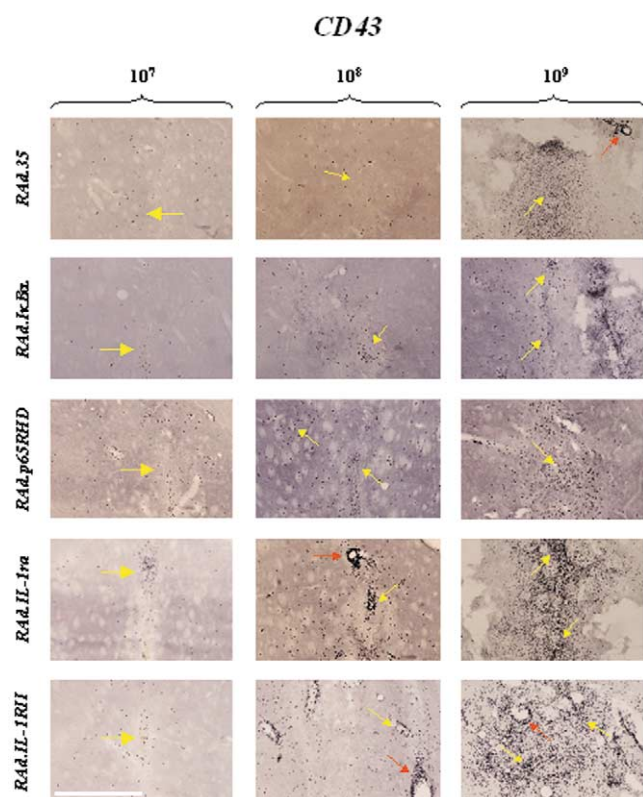


FIG. 4. CD43 immunoreactivity following delivery of 10^7 , 10^8 , and 10^9 iu of RAd.35, RAd.IkBa, RAd.p65RHD, RAd.IL-1ra, and RAd.IL-1RII to the rat striatum. Areas of CD43-positive cells are indicated by yellow arrows. Red arrows indicate perivascular cuffing of leukosialin-positive cells. Scale bar (bottom left), 1 mm.

Effect of RAds Encoding Inhibitors of IL-1 and NF κ B on Transgene Expression Following the Injection of Adenovirus into the Brains of Animals Previously Immunized Systemically against Adenovirus

To determine the effect of NF- κ B or IL-1 inhibition in CNS inflammation of animals with preexisting antiadenoviral immunity we carried out CNS injections in animals that had already been immunized systemically with a recombinant adenoviral vector. Five animals per group received an intradermal injection of 5×10^8 iu of a vector expressing no transgene (RAd.0), a dose that has previously been shown to prime a systemic adenovirus-specific immune response [6], or saline. We confirmed the presence of anti-adenovirus neutralizing antibodies in reciprocal blood serum dilutions of 1:8–1:256 (data not shown), indicating that an adenovirus-specific immune response had been mounted. Fourteen days after the intradermal injection, we injected rats in the striatum with 1×10^7 iu of a reporter virus expressing β -galactosidase from the strong major immediate early murine cytomegalovirus

promoter (mCMV) in combination with saline or with an equal vector dose of RAd.0, RAd.IkBa, or RAd.IL-1ra. Previous experiments have shown that 30 days after the injection of adenovirus into the brain following systemic immunization, transgene expression from first-generation adenovirus vectors has been completely eliminated. Thus, we examined the brains of animals 30 days after the intrastriatal injection to determine if the inhibitors used could prolong transgene expression. We perfused-fixed the animals and assessed the levels of β -galactosidase expression and CNS inflammation.

In nonimmunized rats, high levels of β -galactosidase expression were seen throughout the ipsilateral hemisphere 30 days after delivery of RAd.36 (Fig. 6A), without any significant inflammation. Staining of serial sections revealed low levels of ED1-positive cells around the needle track and very sparse numbers of MHC I- or CD8-positive T cells (Fig. 6A). In preimmunized rats injected with RAd.36/saline, expression of β -galactosidase had been eliminated and no inflammation was present (Figs. 6A and 6B). This indicates that, in either case, brain inflammation is undetectable 30 days after vector injection into the brains of naïve rats or rats previously preimmunized systemically against adenovirus. Preimmunized rats injected with RAd.0/RAd.36 also had very few β -galactosidase-positive cells and also displayed very little remaining brain inflammation (Fig. 6A). Interestingly, the group of preimmunized animals injected intracranially with RAd.IkBa/RAd.36 had a significantly higher number of β -galactosidase-positive cells than either of the preimmunized animals injected with saline/RAd.36 or RAd.0/RAd.36, while the group injected with RAd.IL-1ra/RAd.36 had a significantly higher number of cells than all the other groups (Fig. 6B). Even though the RAd.IkBa/RAd.36 and RAd.IL-1ra/RAd.36 groups showed significantly higher β -galactosidase immunoreactivity levels than the other preimmunized animals, these levels were low compared to those detected in the nonimmunized group.

In contrast to the nonimmunized group, the immunized groups showed higher levels of inflammation, correlating directly with the levels of β -galactosidase-positive cells, as predicted by our earlier experiments [6,14]. In the group showing the lowest β -galactosidase expression (saline/RAd.36 and RAd.0/RAd.36) ED1 expression was limited to the needle track, while very few MHC I- or CD8-positive cells were found (Figs. 6A and 6B). In the RAd.IkBa/RAd.36 and RAd.IL-1ra/RAd.36 groups a significant increase in both ED1 and MHC I immunoreactivity was seen over the RAd.0/RAd.36 and nonimmunized groups, while the increase in CD8-positive cells was statistically significant only in the animals injected with RAd.IL-1ra/RAd.36 (Figs. 6A and 6B). Thus, the preimmunized group, showing the highest levels of β -galactosidase

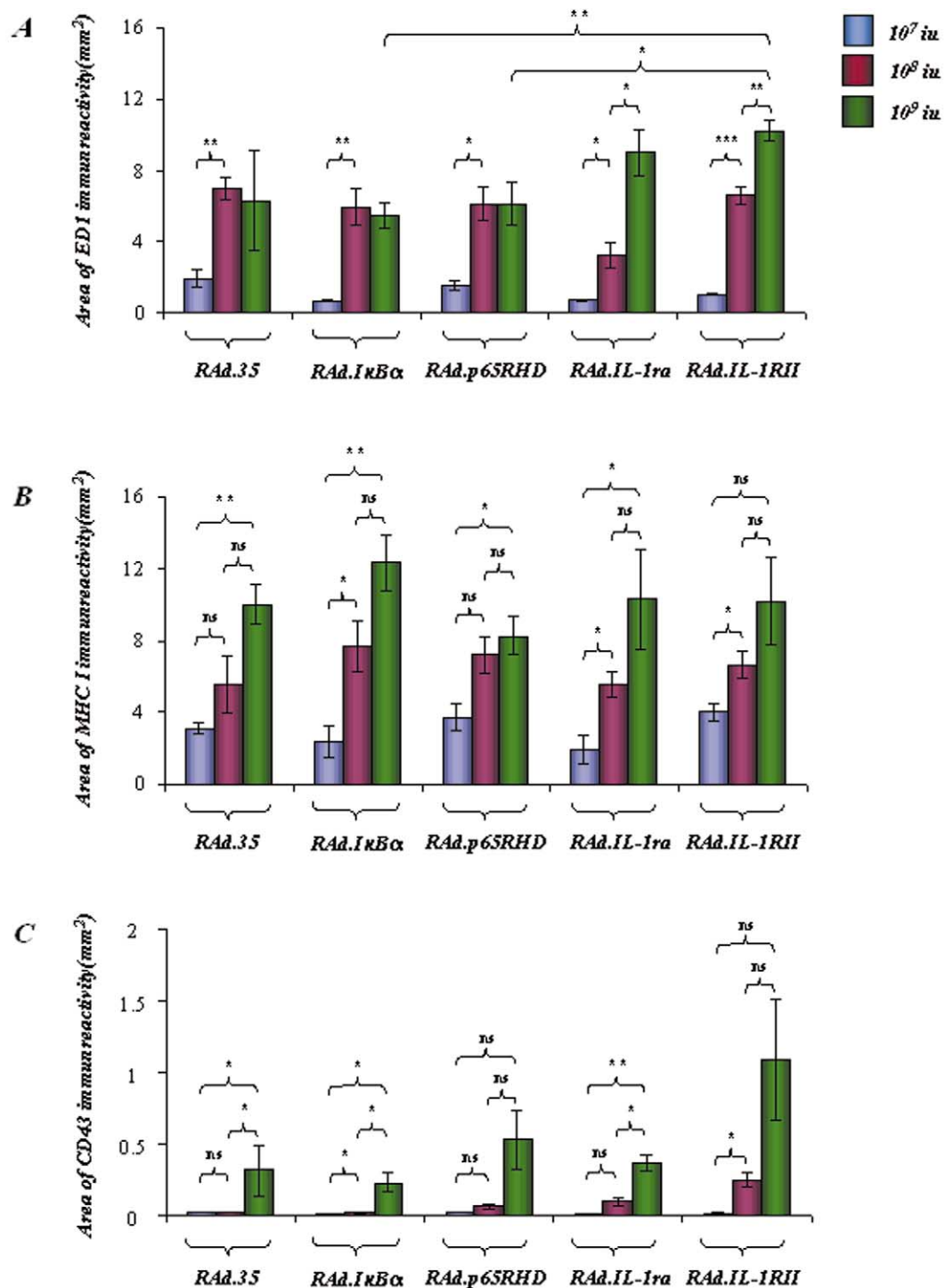


FIG. 5. Quantification of ED1, MHC I, or CD43 immunoreactivity following delivery of 10^7 , 10^8 , and 10^9 iu of RAd.35, RAd.IκBα, RAd.p65RHD, RAd.IL-1ra, and RAd.IL-1RII to the rat striatum. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$).

immunoreactivity (RAd.IL-1ra/RAd.36), also showed the highest levels of inflammatory markers ED1, MHC I, and CD8 compared to all other groups (Figs. 6A and 6B). These

data suggest a prolongation in transgene expression that is accompanied by a prolonged inflammatory response, as would be predicted by our previous data.

a.

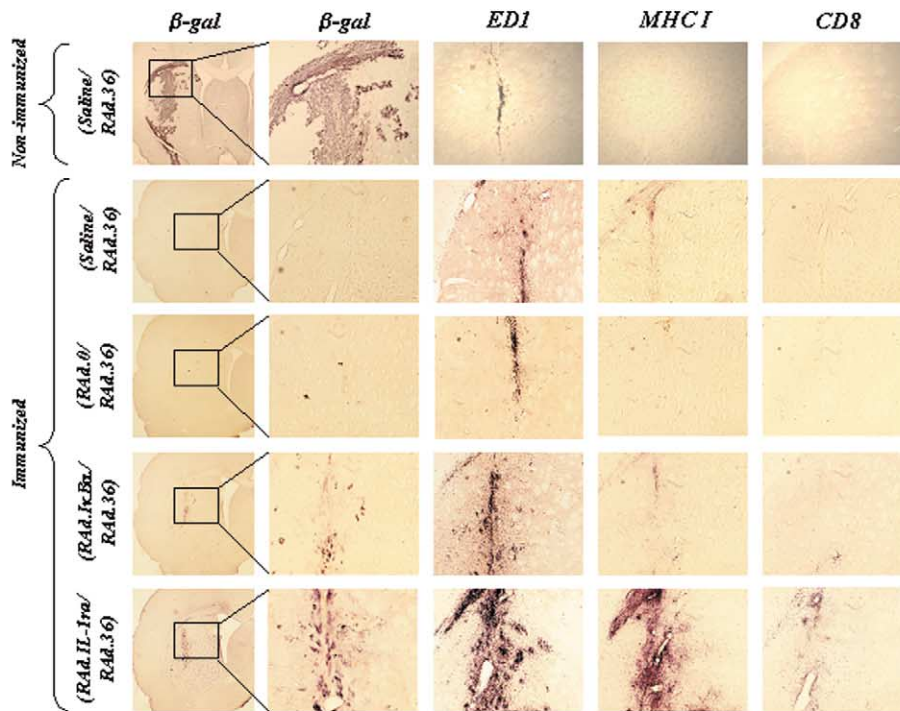
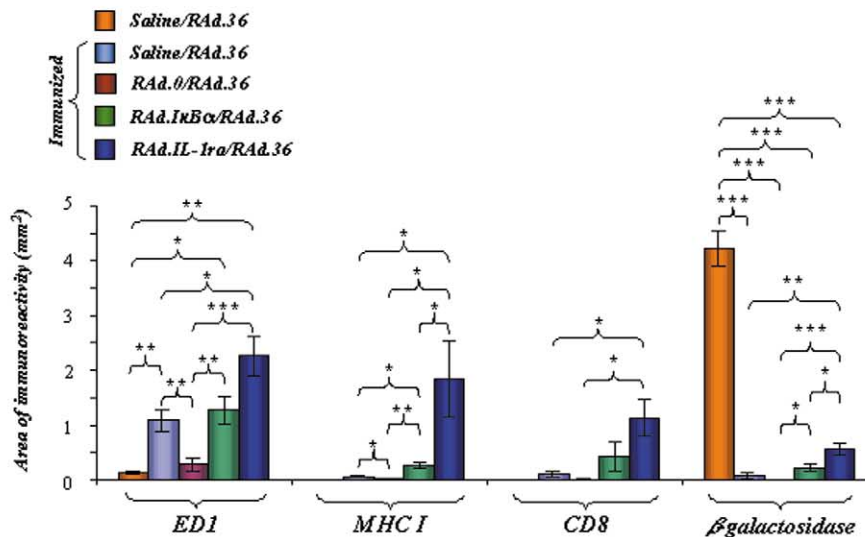


FIG. 6. Transgene expression and inflammation following delivery of RAd to the striatum of adenovirus-sensitized rats. RAd were delivered to the striatum 14 days after intradermal priming and rats were sacrificed after a further 30 days. RAd were delivered at a dose of 1×10^7 iu per virus. (A) Low-power β -galactosidase expression in the ipsilateral hemisphere is shown on the left. High-power composite images show β -galactosidase-positive cells plus ED1-, MHC I-, and CD8-positive cells in serial sections. Scale bars (bottom left two images), both 1 mm. (B) Quantification of ED1, MHC I, and CD8 immunoreactivity 30 days after delivery of saline/RAd.36, RAd.0/RAd.36, RAd.IkBa/RAd.36, and RAd.IL-1ra/RAd.36 to the striatum of immunized or nonimmunized rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ($n = 5$).

b.



DISCUSSION

It was previously demonstrated that RAd-mediated acute innate inflammation is accompanied by increases in IL-1 that mediate its inflammatory effects through activation of NF- κ B. In this study we examined the hypothesis that inhibitors of IL-1 and NF- κ B would be able to inhibit inflammatory and immune responses to adenoviral vectors. First, we examined whether these inhibitors would block the acute innate and dose-dependent inflammatory

response that follows injection of adenoviral vectors into the brain and second, if the same inhibitors could block immune-mediated inhibition of transgene expression following the injection of adenoviral vectors into the brains of preimmunized animals.

To examine these hypotheses, we injected into the brain of naïve rats increasing doses of RAd expressing IL-1ra and IL-1RII (two inhibitors of IL-1) and I κ B α and p65RHD (two inhibitors of NF- κ B-mediated transcrip-

tional activation). The activation of macrophages/microglial markers (ED1), upregulation of MHC-I, and infiltration of lymphocytes were monitored as markers of local inflammatory responses.

Following the delivery of RAd to the brain, a rapid, dose-dependent increase in inflammatory markers occurs. The delivery of NF- κ B or IL-1 inhibitors did not reduce the increase in ED1, MHC I, or CD43 immunoreactivity, compared to a control virus expressing β -galactosidase. This indicates that three markers of adenovirus-induced brain inflammation, e.g., activation of macrophages and microglia (ED1), upregulation of MHC I expression in the brain, and infiltration of circulating lymphocytes, are not reduced when IL-1 or NF- κ B inhibitors are expressed by first-generation adenoviral vectors.

These experiments, however, cannot negate a role for NF- κ B or IL-1 in mediating the very early inflammatory responses following RAd delivery. The reason for this is that the expression of NF- κ B and IL-1 inhibitors needs 12 to 24 h after RAd delivery to achieve high levels of expression. As macrophage/microglial activation is already evident 6 h after delivery of Ads into the CNS [8], the inhibition of NF- κ B or IL-1 expressed from the adenoviral vectors themselves may become effective too late to inhibit the activation of macrophage/microglial cells, the upregulation of MHC I, and the infiltration of circulating lymphocytes. These results also indicate that the acute inflammatory responses are triggered very early upon adenoviral injection and that they cannot be inhibited by concomitant expression of inhibitors of IL-1 and/or NF- κ B.

Available data, moreover, suggest that NF- κ B and IL-1 are rapidly activated following RAd delivery [15,24–26]. Thus, whether inhibition of NF- κ B or IL-1 preceding RAd delivery would inhibit subsequent brain inflammation remains to be determined. This would be supported by previous data indicating that LPS-induced secretion of proinflammatory cytokines by normal human macrophages can be inhibited by RAd-mediated delivery of a NF- κ B inhibitor, when the adenoviral vector is delivered to the cells prior to LPS exposure [27,28].

Following RAd delivery to the brain, infiltrating leukocytes can be seen in the injection site after 6 h and in the marginal blood vessels of the striatum by 12 h. For activated leukocytes to infiltrate the CNS they require the upregulation of adhesion molecules to enable them to cross the brain capillary endothelium. Because IL-1 is known to play a role in activating inflammatory cells and can affect blood–brain barrier permeability [29], and NF- κ B upregulates several adhesion molecules involved in leukocyte migration, we decided to monitor the levels of leukocyte infiltration. The delivery of NF- κ B or IL-1 inhibitors did not reduce leukocyte infiltration. These data indicate that with this experimental design NF- κ B and IL-1 inhibitors do not block leukocyte infiltration following RAd delivery to the brain; however, it does not pre-

clude that the delivery of these inhibitors preceding adenovirus administration may do so. Following RAd delivery to the tail vein of mice NF- κ B activation can be detected in the liver after 10 min [24]. If such a rapid activation of NF- κ B were to occur in the brain, administration of these inhibitors would need to precede the injection of adenovirus vectors.

Since expression of MHC I is rapidly upregulated in response to adenoviral injection [8], especially during microglial activation, and NF- κ B is able to transactivate transcriptionally MHC I expression, the effects of NF- κ B and IL-1 on MHC I immunoreactivity were monitored. The delivery of NF- κ B or IL-1 inhibitors resulted in no significant difference in the area of MHC I immunoreactivity at any of the doses of adenovirus vectors tested.

In summary, the early innate acute inflammatory response appears to be triggered very early after adenoviral injection, and the expression of immune-modulatory transgenes from adenovirus vectors is unable to inhibit the ongoing inflammatory response. This conclusion is further supported by many experiments in which detection of inflammation inhibition in response to IL-1- and/or NF- κ B-mediated inflammatory responses required inhibitors to be administered before exposure to the inflammatory stimulus [30]. Data showing that pretreatment with recombinant IL-1ra inhibited RAd-induced fever following delivery to the CSF [15] strongly support the hypothesis that prior inhibition of NF- κ B- or IL-1-mediated inflammatory signal transduction may be necessary to reduce subsequent RAd-induced brain inflammation.

In contrast, when the NF- κ B or IL-1 inhibitor was delivered into the brains of preimmunized animals the results were encouraging. In control animals, no transgene expression or brain inflammation remained 30 days after the injection of adenovirus. However, in those animals injected concurrently with vectors expressing β -galactosidase and IL-1ra or I κ B α , transgene expression, albeit low, was significantly higher than in controls, while inflammation, especially in animals treated with IL-1ra, was also higher than in controls. Given that our previous data indicate that transgene expression under the control conditions will be eliminated by >90% 14 days after vector injection [14], this indicates that these inhibitors effectively delay transgene elimination by activated immune cells. It also suggests that IL-1ra and I κ B α can prolong transgene expression when adenoviral vectors are injected into preimmunized animals. However, prolonged transgene expression is accompanied by prolonged brain inflammation. We, and others, have shown previously that following the priming of the systemic adaptive immune response following the injection of first-generation adenoviruses into the brain, brain inflammation will subside only once transgene expression is eliminated [6,10–14]. Thus, the extended transgene expression and prolonged inflammation are part of the same phenomenon. Even though these results are interesting in theory, the

small effect obtained reduces the practical applications of using this strategy *in vivo* to prolong transgene expression from first-generation adenoviral vectors. Given that it may be very difficult to prolong expression of transgenes from first-generation adenoviral vectors in the brains of preimmunized experimental animals, and possibly humans, the availability of high-capacity helper-dependent adenoviral vectors [6] that allow substantial and continued transgene expression even when injected into preimmunized animals [14] will make these new powerful vectors the carriers of choice in future application of adenoviral vectors for the treatment of brain diseases.

MATERIALS AND METHODS

Recombinant adenoviruses. RAD.IL-1RII is an E1/E3-deleted first-generation adenovirus from which the expression of IL-1RII is driven by a small IE-hCMV promoter. The human IL-1RII cDNA was excised from the plasmid pCEP4 β IL-1RII (provided by Dr. Alberto Mantovani, Istituto Ricerche Farmacologiche, Milan, Italy) as a *Hind*III/*Bam*HI fragment and was adapted to a *Bam*HI fragment using an oligonucleotide linker. IL-1RII was then cloned into the unique *Bam*HI site of the shuttle plasmid pAL119 (pMV35 in [31]) to create the shuttle vector pAL119/IL-1RII. RAD.IL-1RII was then generated by homologous recombination in 293 cells following cotransfection of the shuttle vector pAL119/IL-1RII with the plasmid pJM17 (Microbix Biosystems, Inc.). The presence of IL-1RII in the viral genome was confirmed by Southern blotting *Hind*III-digested RAD.IL-1RII viral DNA with an IL-1 RII-specific probe (data not shown). RAD.0 was generated by homologous recombination of the pAL119 shuttle plasmid (containing no transgene) and pJM17 in 293 cells. RAD.35 (RAD.hCMV.LacZ), RAD.36 (RAD.mCMV.LacZ), RAD.GFP, RAD.IL-1ra, RAD.Ik β , and RAD.p65RHD are E1/E3-deleted first-generation adenoviruses whose generation has been described previously [30,32–36]. Production of high-titer stocks, purification by double cesium chloride density gradient separation, and titration of viruses were carried out as previously described [37,38]. Stocks were found to be free of replication-competent adenovirus using a supernatant rescue assay able to detect 1 replication-competent virus within 10^9 recombinant viruses [39]. Adenovirus preparations were ascertained to be endotoxin (lipopolysaccharide) free, according to the criteria of Cotten *et al.* [40], using the E-TOXATE assay (Sigma–Aldrich, Dorset, UK).

Tissue culture. 293 cells were obtained from Microbix Biosystems (Ontario, Canada), HeLa and Cos-7 cells were obtained from the American Tissue Culture Collection (Manassas, VA). Media and maintenance of cells were as described previously [38].

In vitro NF- κ B/IL-1 bioassay. HeLa cells were plated at 2×10^5 cells per well in six-well plates. Twenty-four hours later cells underwent a modified calcium phosphate transfection procedure [41] with 6 μ g per well of the plasmid pNRE-Luc. Construction of the plasmid pNRE-Luc has been described in detail elsewhere as 3 \times NF- κ B(IC)tkLuc [42] and luciferase expression from this plasmid can be induced through transactivation of NF- κ B. The following day cells were infected with recombinant adenovirus at an m.o.i. of 200 in fresh medium and then left for a further 48 h. NF- κ B was then activated with recombinant human IL-1 β , at a final concentration of 30 pg/ml, and after 12 h the luciferase activity of cell lysates was analyzed. To test the ability of conditioned medium to inhibit IL-1 signal transduction Cos-7 cells were plated at 1×10^5 cells per well in six-well plates. The following day cells were infected with recombinant adenovirus at an m.o.i. of 200 in fresh medium and left for 48 h and then medium was collected. HeLa cells were transfected as described with pNRE-Luc and after 24 h they were incubated with conditioned medium, either 1 h before the addition of rhIL-1 β (30 pg/ml) or after the conditioned medium had been incubated with rhIL-1 β (30 pg/ml) at 37°C for 1 h. The transfected HeLa cells

were then left for a further 12 h before the luciferase activity of cell lysates was analyzed. Luciferase activity was assessed using the Luciferase Assay System (Promega). The relative light units of each sample were assayed over 10 s in a Lumat LB 9501 luminometer (Berthold) and the luciferase activity of each lysate was then standardized to the protein content of each sample.

Delivery of RAds to the CNS. All animal experiments were conducted according to the United Kingdom Animal (Scientific Procedures) Act of 1986. All animals had free access to food and water, a 12-h light/dark cycle, and constant housing temperature and humidity. Adult male Sprague-Dawley rats weighing 250 g were anesthetized with 4% halothane gas vaporized with an oxygen:nitrous oxide mix of 66% oxygen:33% nitrous oxide. Animals were then placed in a stereotaxic frame and injected using a 26-gauge Hamilton syringe in the left striatum at coordinates 0.6 mm forward and 3.4 mm lateral from the bregma and 5.0 mm vertical from the dura. Doses of 10^7 , 10^8 , or 10^9 iu of virus were administered in a total volume of 3 μ l, diluted with PBS (pH 7.4), at a rate of 1 μ l per minute, after which the needle was left in for a further 5 min before withdrawal. After 7 days animals were overdosed by ip injection of pentobarbitone and transcardially perfused-fixed with heparinized saline and 4% paraformaldehyde in PBS. Brains were removed, postfixed in 4% paraformaldehyde for 5 h, and stored in PBS. In the preimmunization experiment, animals were anesthetized, shaved, and injected intradermally in the back with 5×10^8 iu of RAD.0 in 100 μ l of sterile saline. Fourteen days later, RAds were injected in the striatum as described above. A dose of 1×10^7 iu of RAD.36 was delivered in combination with saline or 1×10^7 iu RAD.0, RAD.Ik β , or RAD.IL-1ra. Thirty days after intrastriatal injection, animals were overdosed by ip injection of pentobarbitone, blood was withdrawn by cardiac puncture, and animals were then transcardially perfused-fixed as described above.

Immunohistochemistry. Serial 50- μ m coronal sections were cut using a Leica VT100S Vibratome and free-floating immunohistochemistry was performed to detect inflammatory and immune cell markers as described previously [6,9,14]. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide, and sections were blocked with 10% horse serum 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 mouse monoclonal anti- β -galactosidase (Promega; 1:1000), mouse monoclonal anti-rat ED1 antibody (Serotec; 1:1000; activated macrophages and microglial cells), mouse monoclonal anti-rat CD43 antibody (Serotec; 1:1000; T cells, monocytes, and granulocytes), mouse monoclonal anti-rat CD8 antibody (Serotec; 1:500; cytotoxic T lymphocytes and natural killer cells), and mouse monoclonal anti-rat MHC I antibody (Serotec; 1:1000). Secondary antibody was biotinylated rabbit anti-mouse (DAKO; 1:200) diluted 1:200 in 0.5% Triton X-100 with 1% horse serum and was detected using the Vectastain Elite ABC horseradish peroxidase method (Vector Laboratories). After being developed with diaminobenzidine and glucose oxidase, sections were mounted on gelatinized glass slides and were dehydrated through graded ethanol solutions before coverslipping.

Quantification. Quantitative analysis of areas of immunoreactivity to antibodies against ED1, MHC I, CD43, CD8, and β -galactosidase within single 50- μ m brain sections was performed by using a Leica Quantimet 600 Image Analysis System controlled by QWIN software (Leica Microsystems, Cambridge, UK) using a Leica RMD8 microscope. Sections containing the highest levels of immunoreactivity (around the needle track) were used for quantitative analysis. Student's *t* test was used to determine the degree of statistical significance between values from different experimental groups.

Anti-adenovirus neutralizing antibody assay. To confirm that intradermal injections had successfully primed an adenovirus, specific immune response levels of anti-adenovirus neutralizing antibodies were monitored. Levels of circulating neutralizing antibodies in serum were measured as described previously [6]. Briefly, blood serum was inactivated at 56°C for 30 min and serially diluted twofold (final dilutions range from 1:2 to 1:4096) in MEM containing 2% FCS. One hundred microliters of each

dilution was incubated with 10^6 iu of RAAd.35 (10 μ l) at 37°C for 90 min. Then, 50 μ l of each mixture was added to 293 cells (4×10^4 cells/well) in 96-well plates in duplicate at 37°C for 60 min. A further 50 μ l of MEM containing 10% FCS was then added and cells were incubated at 37°C for 20 more hours before being stained with 5-bromo-4-chloro-indolyl- β -D-galactosidase. The neutralizing antibody titer is expressed as the reciprocal serum dilution at which 50% of RAAd.35 β -galactosidase transduction was inhibited.

ACKNOWLEDGMENTS

Work in the laboratories of Dr. Maria G. Castro and Dr. Pedro R. Lowenstein is supported by grants from the NINDS, NIH 1 RO1 NS44556.01 (Maria G. Castro) and 1 RO1 NS42893 01 and US4 4NS 04-5309 (Pedro R. Lowenstein), and by a generous donation from the Board of Governors at Cedars-Sinai Medical Center. We acknowledge the support received from the Wellcome Trust (UK), the BBSRC (UK), the Royal Society, the Parkinson's Disease Society (UK), and EU-Biomed Programmes (Contracts BMH4-CT98-3277, BMH4-CT98-0297, and QLK3-CT-1999-00364). We thank Dr. David Ray (Endocrine Sciences Research Group, Schools of Medicine and Biological Sciences, University of Manchester, UK) for providing the plasmid pNRE-Luc, Dr. Beverley Davidson (Program in Gene Therapy, Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA) for providing RAAd.II-Ira, Dr. Rainer de Martin (Department of Vascular Biology and Thrombosis Research, University of Vienna, Vienna International Research Cooperation Center, Vienna, Austria) for providing RAAd.IkBa, and Dr. Hans Winkler (AstraZeneca, Alderley Park, Macclesfield, Cheshire, UK) for providing RAAd.p65RHD. P.R.L. was a fellow of The Lister Institute of Preventive Medicine and D.S. was a Ph.D. student funded by BBSRC (UK). We are grateful to Ms. Semone N. Muslar for superb administrative assistance, to Mr. Ian K. Williamson for his excellent managerial skills, and to Mr. Nelson Jovel who was responsible for editing and preparing the manuscript for publication. We also thank Dr. Shlomo Melmed and Ms. Cheryl Cathcart for their continued support and encouragement.

RECEIVED FOR PUBLICATION MARCH 24; ACCEPTED MAY 8, 2003.

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