

## REVIEW

# Inflammation and adaptive immune responses to adenoviral vectors injected into the brain: peculiarities, mechanisms, and consequences

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Gene Therapy (2003) 10, 946–954. doi:10.1038/sj.gt.3302048

*It has been repeatedly shown – first by Shirai (1921) and Murphy (1926), and most recently by Tansley (1946) – that foreign homologous tissues grafted to the brain either do not provoke an immunity reaction or, if they do, do not respond to it. It will be shown here that they do respond to an immune state of proved effectiveness called forth by a preliminary grafting of foreign tissue elsewhere. The two questions, relating as they do to quite distinct immunological properties, may thus have contrary answers. (taken from the introduction)*

*Skin homografts transplanted to the brains of specifically immunized rabbits respond by total breakdown to an immune state already in being. On the testimony of other workers, they enjoy prolonged or indefinite survival when grafted to the brains of non-immunized animals.*

*It is concluded that skin homografts transplanted to the brain submit to but cannot elicit an immune state. (taken from the final summary)*

Peter Medawar, 1948<sup>16</sup>

## Introduction

Most gene therapy vectors used for gene transfer into the CNS are derived from pathogenic viruses.<sup>1</sup> Therefore, inflammation and immune responses are one of the challenges facing successful and long-term gene therapy. Upon injection into the brain, viral vectors will encounter various cell types, for example, specific tissue cells (for example, neurons and glial cells), vascular endothelial cells, connective tissue fibroblasts, and local (for example, perivascular macrophages and microglial cells) and/or systemic circulating immune cells. These early interactions lead to a complex sequence of molecular and cellular responses that trigger innate and eventually adaptive immune responses.<sup>2</sup>

Innate immune responses are elicited almost immediately following vector–cell interactions, and consist of

stereotyped responses, such as cytokine release, and influx of inflammatory nonantigen-specific cells. Once stimulated, innate immunity's effector arm is represented by the activation of local astrocytes and microglial cells, and the influx of macrophages, NK-cells and dendritic cells. In systemic organs, such as the skin or liver, professional antigen-presenting cells (ie dendritic cells) will carry antigens to draining lymph nodes, where priming of naïve T cells occurs. Toll receptors, located on cells interacting with invading viruses or bacteria, can recognize pathogenic epitopes embedded within the viral capsids, or bacterial membranes themselves. Thus, viral vector capsids can *per se* elicit innate immune responses, and thus cause inflammation, stimulating an immune response.<sup>3</sup>

Primed T cells divide and exit the lymph nodes as activated or armed T cells and search systemic organs for the presence of the specific antigenic epitopes recognized by their unique T-cell receptor. Activated T cells, CD4+ and CD8+, will recognize viral antigenic epitopes on MHC molecules expressed by target tissues, and, upon antigen recognition, release proinflammatory cytokines, such as IL-1, IL-6, and TNF $\alpha$ . CD8+ cells can have a cytotoxic phenotype (CTL); CTLs can kill target cells infected with viruses, and express antigenic epitopes through a number of cytotoxic effectors, for example, perforin or Fas-L. Alternatively, activated T cells can eliminate virus replication using noncytolytic mechanisms, for example, through the secretion of IFN $\gamma$ .<sup>4</sup> Virus interactions with B cells lead to virus-specific and high titer anti-viral antibodies that recognize non-linear antigenic epitopes, and are thus able to detect intact viral capsids. Binding of antibodies to viral capsids activates a series of effector mechanisms that sequester virus from the circulation.

Through cellular and humoral mechanisms, the immune system constrains virus infection and replication. Similar mechanisms will constrain viral vector-mediated transduction of target cells. An important difference between viral vectors and pathogenic viruses is in the number of initial virus particles entering the organism. Low amounts of pathogenic viruses cause

disease, since they rely on intracellular multiplication. Viral vectors that do not replicate are usually administered at high doses. This leads locally to high multiplicity of infection, causing potentially different inflammatory and immune responses.

That, in spite of the immune system, viruses can cause life-threatening diseases indicate that viruses and the immune system must establish a delicate equilibrium, where neither virus infection nor immune responses are completely efficient. Viruses may have defective infectious pathways or the immune system may be unable to eliminate rapidly and completely virus infections, or viruses may have evolved strategies to overcome immune detection, and the immune system's sophisticated mechanisms to detect virus infection.<sup>5–8</sup> Examples of these strategies are downregulation of MHC molecules, inhibition of antigen-presenting pathways, and secretion of binding proteins for cytokines and chemokines, among others. To these challenges, the immune system has developed mechanisms to block virus entry to the body, a set of innate immune responses that have evolved throughout time and are fixed in the genome that can recognize specific or shared pathogen features, and an adaptive immune system that can rapidly evolve in the face of pathogen infection using mutation and adaptive molecular mechanisms not engraved in genome structure.<sup>9–11</sup> The removal of most if not all virally encoded proteins from the vector genomes also eliminates those viral functions, which, in the context of viral infection, allow viruses to escape deleterious immune responses. On the other side, once the target cell is infected, viral vectors will express few, if any, viral antigenic proteins.

Viruses used in gene therapy have been adapted for safe, nonpathogenic gene transfer. To convert a pathogenic virus into a vector, two minimum objectives need to be achieved. One, virus pathogenicity must be eliminated (eg viral functions encoding for replication, inhibition of cellular functions, etc, must be removed), and the potentially therapeutic transgene and transcriptional control elements cloned into the vectors. Details of systemic immune responses to different viral vectors are discussed further in other reviews in this issue.

### *How do immune responses in the brain differ from those in other organs?*

Immune responses to viral vectors injected directly into the CNS differ from those encountered following systemic vector administration.<sup>2,12</sup> The nervous system is constituted by specialized cell types such as neurons that are mostly postmitotic, and, if eliminated, cannot be replaced so far to any large extent. Support glial astrocytes, brain tissue macrophages, microglial cells, and blood vessels, on the other hand, can divide and be replaced. Although *a priori* it could have been postulated that the particular structure of the brain would necessitate specifically tailored immune responses, the particular nature of brain immune responses were indeed discovered between 1921 and 1923 by scientists studying organ and tumor transplantation.<sup>13</sup>

Shirai,<sup>14</sup> in 1921, and Murphy and Sturm,<sup>15</sup> in 1923, showed that tumor cells transplanted into the brain would grow, while subcutaneous or intramuscular

inoculations were rapidly rejected. This suggested that immune responses in the brain were blunted. This issue was addressed again in 1948, when Peter Medawar<sup>16</sup> enquired if extended transplant survival in the brain was because of failure to activate the immune system, or whether the effector arm of the immune system was impaired in the brain.

From a series of complex, yet elegant, technically challenging experiments, Peter Medawar concluded that brain immune privilege is because of immune ignorance, or failure to activate the immune system. His experiments demonstrated that preceding peripheral immunization against skin transplants eliminated these from the brain. This demonstrated that systemic immune responses were fully active against antigen placed into the brain. Of relevance to the present discussion, Medawar presented evidence that activated cells from the effector arm of the immune system could detect and eliminate foreign cells within the CNS. The lack of brain lymphatics was suggested to underlie the inability of transplants into the brain to elicit immune responses. Peter Medawar's<sup>16</sup> experiments ushered the modern exploration of the peculiar brain immune responses, referred to as the brain's immune privilege. This review of the immune responses against viral vectors will be made in the context of what is commonly referred to as the brain's immune privilege.<sup>12</sup>

### *Brain inflammation upon injection of viral vectors*

Although several virus-derived vectors have been shown to cause brain inflammation, most information is available on adenoviral vector-induced inflammation.<sup>17–19</sup> Cytokine<sup>20</sup> and cellular<sup>21</sup> inflammatory reactions are elicited upon injection of first-generation adenoviral vectors. This is reflected in a release of the cytokines IL-1, IL-6, TNF $\alpha$ , and the stimulation of a fever response,<sup>20</sup> when adenovirus is injected into the third ventricle. Cytokine levels increase very rapidly, with a peak observed at 1.5 h after virus injection, and a time course similar to the fever response, returning to normal within 24 h. Injection of adenovirus into the striatum elicits an increase of IL-1 and IL-6, but not TNF $\alpha$  or fever. Moreover, the fever response upon injection of adenoviral vectors into the lateral ventricle is blocked by antagonists of IL-1, demonstrating that the fever response is induced by IL-1, rather than TNF $\alpha$ .<sup>20</sup> Similar increases in IL-6 and TNF $\alpha$  are detected upon systemic injections of adenoviral vectors.<sup>22,23</sup>

Also, the fever response elicited was independent of the transgene encoded by the adenoviral vectors, but did depend on the integrity of the viral capsid.<sup>20</sup> The very rapid increase in cytokine secretion and fever makes viral protein expression an unlikely explanation for these responses. Similar very rapid increases in the levels of mRNA for IL-8, and stimulation of signal transduction pathways,<sup>24–28</sup> suggest that the earliest phase (1–2 h) of inflammatory response to adenoviral vectors, both in nervous tissue as well as in other tissues, is independent of viral vector expression. Thus, the adenoviral capsid on its own may cause, through direct interactions at the membrane of target cells, stimulation of pro-inflammatory signalling cascades resulting in cytokine release.

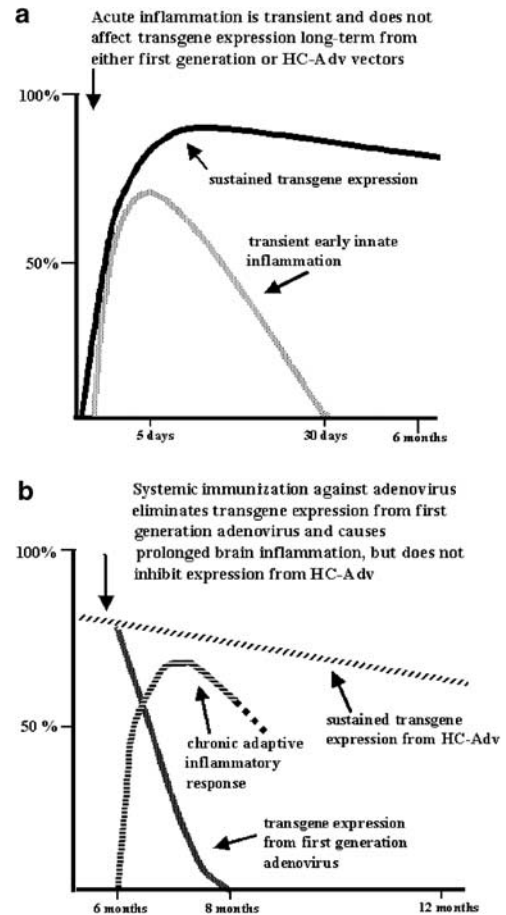
Cellular inflammatory infiltration also occurs in the brain, following the injection of adenoviral and herpesvirus derived vectors into the brain. Injection of adenoviral vectors induces the influx of macrophages, neutrophils, CD4+ and CD8+ lymphocytes, NK cells, as well as the appearance of cells immunopositive for dendritic cell markers.<sup>17,19,21,29–31</sup> Peak influx is around 3–7 days postviral vector injection. Adenoviral vector-induced inflammation is dose dependent, with cellular influx first detected at doses of  $1 \times 10^6$  infectious units (iu). Early inflammation is independent of the expression of adenovirally encoded transgenes, and is not observed in animals injected with heat-inactivated adenovirus.<sup>29,32</sup>

To test the hypothesis of whether the viral or vector genome *per se*, or through the expression of viral proteins encoded by first-generation viral vectors, could cause inflammation in the brain, inflammation in response to the injection of high-capacity helper-dependent adenoviral vectors (HC-Adv) was compared to that caused by first-generation vectors. HC-Adv are devoid of all viral sequences found within wild-type adenovirus, or remaining within adenoviral vectors deleted of restricted sequences (eg E1, E3, E4-deleted vectors). HC-Adv vectors only retain the ITRs, containing the sequences for vector genome replication, and the packaging signal  $\psi$ , to allow a helper virus to replicate and package the HC-Adv genome. Injection of HC-Adv caused an early cellular inflammatory response indistinguishable from that caused by first-generation adenoviral vectors. Transgene expression also remained stable, throughout and following early inflammation regression<sup>32</sup> (Figure 1a and b).

Up to  $1 \times 10^8$  iu of adenoviral vectors injected into a single brain site, inflammation is acute, transient, dose dependent, is reversible within 30 days, and accompanied by very limited cytotoxicity. This inflammatory response is unable to inhibit significantly transgene expression. Levels of transgene expression appear comparable at 3 days, 30 days, and 6 months (and can still be detected at 12 and 18 months) post-vector injection,<sup>29,32</sup> although a detailed quantitative analysis of protein levels and mRNA remains to be carried out.

Injection of  $1 \times 10^9$  iu, on the other hand, leads to massive cytotoxicity, chronic unremitting inflammation, that completely eliminates transgene expression as early as 30 days postvector injection. This can be interpreted as the existence of a threshold at  $1 \times 10^8$  iu of adenoviral vectors. Injections of adenovirus below this threshold cause reversible cytokine and cellular-mediated inflammation; injections above this threshold lead to chronic inflammation, significant cytotoxicity, and elimination of transgene expression by 30 days postinjection.<sup>2</sup>

Viral vector injections can lead to increases in the titer of neutralizing and non-neutralizing antibodies. Neutralizing antibodies can block viral vector infection upon administration of vectors into preimmunized experimental animals, or humans. Simultaneous monitoring of systemic antiadenoviral neutralizing antibodies indicates that injection of adenoviruses into the brain does not elicit a neutralizing humoral immune response (see Table 1),<sup>31</sup> although an increase in titer of non-neutralizing antibodies has been reported.<sup>33</sup> Further, titers of neutralizing antibodies, in response to an intradermal injection of adenovirus, are not increased



**Figure 1** Days postinjection of virus into the brain (a) or intradermal (b). The arrows at the top of (a) and (b) indicate the day of virus administration, the y-axis indicates brain inflammation or transgene expression as percentage of maximum, and time is given in the x-axis. The broken lines in (b) indicate that the ultimate longevity of expression from HC-Adv, and the full time course of the chronic adaptive inflammatory response in the brain remain to be elucidated.

or reduced if intradermal immunization follows intracerebral injection. This argues that, opposed to what occurs in the eye (reviewed in Bennett, this issue) previous intracerebral injection does not bias the immune response to systemically injected antigen. Systemic humoral neutralizing antibody responses cannot be detected even after injection of  $1 \times 10^9$  iu into the brain, a dose causing massive cell death and inflammation.<sup>29</sup> Systemic immune responses (that are easily detected upon injection of virus intradermally) remain undetectable even following injection of doses three-fold higher than those needed to stimulate a systemic immune response upon injection of vectors into the skin ( $1 \times 10^6$  iu, Gerdes *et al*, unpublished data).<sup>29</sup>

In studies of antivector immune responses, it is crucial to utilize well-characterized quality controls. Two main contaminants of adenoviral preparations are bacterial endotoxin (LPS) or replication competent adenovirus. Viral preparations may be contaminated by either, and quality control data need to be presented for each viral vector used. Contamination with either will render results uninterpretable because of the contaminating effects of either LPS or RCA.<sup>34</sup>

**Table 1** Neutralizing antibody responses following the injection of adenoviral vectors into the brain or intradermally

Inj. seq.	Inj. site								
1	Striatum	RAd35	RAd35	RAd35	HC-Adv	HC-Adv	HC-Adv	saline	saline
2	Skin	RAdHPRT	Ps-HPRT	saline	RAd-HPRT	Ps-HPRT	saline	RAd-HPRT	saline
<i>Ab. titer</i>		64–256	<2–8	<2	32–256	<2–4	<2	32–256	<2
1	Skin	Saline	HPRT	Saline	HPRT				
2	Striatum	HC-Adv	HC-Adv	RAd-35	RAd35				
<i>Ab titer</i>		<2	16–128	<2	16–128				

Vectors were injected either first into the brain, and secondly into the dermis, or *vice versa*. Antibody titer is given as 1/serum dilution neutralizing 50% of an adenovirus inoculum. Injection sequence indicates the order of injections, and injection site, whether the injection was done into the striatum or the dermis. RAd35, RAdHPRT are first-generation vectors expressing  $\beta$ -galactosidase or hypoxanthine guanine phosphorybosis transferase, respectively; HC-Adv is a high-capacity helper-dependent adenoviral vector expressing  $\beta$ -galactosidase; Ps-prefix indicates that the vector was inactivated with psoralen and UV, to eliminate expression from the vector genomes, while preserving capsid integrity. The amount of vectors injected into the brain was  $1.3 \times 10^7$  infectious units (iu) (first-generation vectors) or blue-forming units (HC-Adv). The amount of vectors used for the intradermal immunizations was  $5 \times 10^8$  iu of RAd-HPRT. Owing to the immunization paradigm used, immune responses are directed against elements of the viral capsid and genome, but not against the encoded transgene. The table illustrates that injection of either adenoviral vector injected into the brain does not cause a systemic increase in the titer of neutralizing antiadenoviral antibodies. Further, an injection of adenovirus into the brain does not alter significantly the titer obtained by a subsequent intradermal immunization, suggesting that the brain injection does not bias, either increasing or inhibiting, the outcome of the systemic immunization. The table is modified from Thomas et al<sup>31</sup> Data discussed in Thomas et al<sup>29</sup> show that even a dose of  $1 \times 10^9$  iu of a first-generation adenovirus that causes a massive cytotoxicity and substance loss in the injection site does not result in generation of a neutralizing anti-adenovirus antibody response.

Evidence discussed above suggests that the adenoviral capsid could interact with specific receptors on the plasma membrane to stimulate inflammatory responses. Adenovirus interacts with two main receptors for entry, through its fiber and penton base proteins. A domain in the adenoviral fiber knob binds the coxsackie and adenovirus receptor (CAR),<sup>35</sup> while RGD domains within the penton base protein bind to integrins, mainly  $\alpha_v \beta_5$ .<sup>36</sup>

Vectors mutated in the specific domains interacting with CAR and integrins were used to inject into the brain to test the hypothesis that early brain inflammation was dependent on the adenoviral capsid, possibly on interactions with the main adenoviral receptors identified so far.<sup>30</sup> Surprisingly, deletion of both the CAR-binding domain of fiber knob and the RGD domain of penton base, did not inhibit adenoviral-vector-induced brain inflammation, as detected by activation of macrophages and astrocytes within the brain.<sup>30</sup> In fact, as diffusion of adenoviral vectors within the brain likely depends on vector interactions with viral receptors, absence of binding to receptors could increase binding. Results suggest that, in the absence of CAR and integrin binding, diffusion of adenoviral vectors is increased concomitantly with inflammation being apparent over a larger area of the brain. Consequently, addition of polylysine to the fiber protein to increase interaction with tissue proteoglycans, reduced both the area of vector diffusion, vector-mediated transgene expression, and vector-induced inflammation. Detailed microarray studies may provide a larger picture of genes activated or inhibited during this early innate inflammatory response to adenoviral vectors.

In summary, injections of adenovirus at or below the threshold of  $1 \times 10^8$  iu, induce cytokine- and cellular-mediated brain inflammation. This early inflammatory response is acute, dose dependent, reversible within 30 days, and is unable to inhibit significantly transgene expression. Months after this early inflammation has

disappeared, transgene expression remains (summarized in Figure 1a and 1b). Various types of experiments suggest that early inflammation depends on the adenoviral capsid, rather than the adenoviral genome, or transgene expression thereof. Vector-induced cytokine secretion and fever are dependent on vector capsid integrity. Cellular immune responses are independent of transgene expression, and possibly of vector DNA, and, at least in the brain, appear to proceed independently of binding to the major adenoviral receptors, CAR and integrins. However, whether the adenoviral genome of first generation or HC-Adv cause inflammation *per se* remains to be rigorously tested (reviewed in Lowenstein and Castro<sup>2</sup>).

Of further importance to our understanding of immune responses in the brain is the fact, described for the first time in 1921, that the early inflammatory response does not lead to the activation of the efferent arm of the immune response. Thus, in the brain, and in distinction to what occurs in most other organs and tissues, innate acute innate inflammatory responses are dissociated from the stimulation of an adaptive immune response (illustrated in Figure 1). This demonstrates that in the brain the cellular and molecular mechanisms of early innate inflammation can be studied in isolation of their stimulation of an adaptive immune response. In other tissues, inflammation is closely followed by priming of a systemic immune response that rapidly leads to antigen-specific influx of lymphocytes of the adaptive efferent arm of the immune response.

### *Can an afferent arm to the immune response originate within the brain?*

How safe viral vector gene transfer into the brain is, depends on whether viral vector injection leads to a

systemic immune response. Dendritic cells, the central cells in stimulating the afferent arm of the immune response, are localized within the skin, heart, or lung, but not the naïve brain.<sup>12</sup> Under immune-stimulatory and inflammatory conditions, they take up antigen and transport it to secondary lymphoid organs where they prime naïve T cells. Whether this process of antigen transport to lymph nodes can occur in the brain is one of the central unresolved issues remained to be answered definitely, in order to understand completely the physiology of the brain's immune system.<sup>13</sup> However, brain perivascular macrophages and microglial cells originate from the bone marrow and are slowly turned over by circulating precursors originating in the bone marrow. It has been postulated that these cells could carry particulate antigens to the draining lymph nodes. However, even though some reports have argued that antigen can leave the brain, no convincing evidence has ever shown that *bona fide* cells localized to the brain can take up antigen in the brain and leave the brain to stimulate a systemic immune response.

Many immunological diseases affect the brain.<sup>37</sup> Although the initiation of multiple sclerosis is not well understood, a systemic immune response against myelin antigens results in progressive bouts of brain inflammation, with severe brain toxicity, and behavioral impairments.<sup>38</sup> In the case of paraneoplastic syndromes, the primary tumor can be located in the lung, for example, small cell lung carcinoma, and is sometimes first recognized through neurological symptomatology. In this case, the priming of the immune response occurs in the secondary lymphoid organs. Activated T cells, or antibodies, detect a crossreacting antigen in the brain, causing immune-mediated brain disease.<sup>39</sup> The experimental allergic encephalomyelitis (EAE) model reproduces aspects of brain autoimmune diseases. EAE is induced upon immunization of animals with various types of brain antigens. In this model, antigen is presented to the immune system, outside of the CNS, within a highly inflammatory context (eg the use of adjuvants),<sup>40</sup> leading to serious brain immune anatomical and behavioral pathology. The mechanisms underlying the initiation and pathological progression of human multiple sclerosis, however, remain to be elucidated.<sup>38</sup>

More recently, authors have demonstrated that inactivated bacillus Calmette-Guerin (BCG) injected into the brain will remain in the CNS, in the absence of a systemic immune response. In the presence of systemic immunization a chronic inflammatory response occurs that can cause pathological brain lesion, even if antigen cannot be eliminated from the brain.<sup>41,42</sup> Also, injection of wild-type (replicating, pathogenic) neurotropic influenza virus into the brain does not induce a systemic immune, and injection of nonreplicating viral vectors also does not induce a systemic immune response.<sup>43–46</sup> These antigens, moreover, can all be recognized by the adaptive immune system, but only after antigen has been made available systemically, or by injection into the brain ventricles, and thus has had an opportunity to prime efficiently the immune system.<sup>47</sup>

Note, however, that all antigens discussed above (eg bacterial products, viruses and viral vectors) are particulate. Different results have been obtained when the

antigen is soluble.<sup>48</sup> Injection of soluble antigen into the brain (eg ovalbumin) does elicit a systemic humoral immune responses.<sup>49</sup> Antigen characteristics, and their capacity to exit the brain, in the absence of a cellular carrier (eg dendritic cell), or outflow channel (eg lymphatics), could determine the immunogenicity of antigens delivered directly into the brain.

Of importance to the treatment of neurological diseases of the brain using viral vectors is the fact that the current knowledge of brain immune responses has been obtained from experiments performed on intact, for example non-inflamed, brains. Would the capacity of the brain to prime naïve lymphocytes be altered during acute and/or chronic inflammatory processes, such as those present during multiple sclerosis, Alzheimer's disease, and even Parkinson's disease? Evidence suggests that this may be the case.<sup>59</sup>

Cells displaying immunoreactivity towards markers of dendritic cells have been observed in several experimental brain inflammatory conditions.<sup>12,50–58</sup> In addition, the phenomenon of epitope spreading<sup>59,60</sup> also suggests that, once brain inflammation occurs, new antigens being released from the brain during inflammatory processes can stimulate additional immune responses. However, evidence against antigens released from the brain causing autoimmune diseases is strong, and includes the lack of autoimmunity following stroke, brain surgery, brain tumors, and brain infarcts. In all these conditions, large amounts of brain antigens are released into the bloodstream. Brain autoimmune responses are rarely, if ever, encountered. In comparison, up to one-third of patients surviving a heart attack will develop autoimmune pericarditis.

Viral vector delivery in the brain should thus follow certain rules to avoid stimulating a systemic adaptive immune response. Vectors should be carefully delivered to the brain parenchyma proper, with precautions taken for the vectors not to reach the brain ventricles. Delivery to the ventricles or the bloodstream will ensure an immune response to be avoided if stable gene expression is desired. When delivered carefully into the brain parenchyma, long-term transgene expression in the CNS has been obtained through the use of both nonintegrating vectors (such as adenovirus-derived) or integrating vectors (such as lentivirus-derived).

Indeed, delivery of viral vectors into the brain ventricles releases IL-1, IL-6, TNF $\alpha$ , causes fever, will induce an adaptive immune response, and only provides short-term transgene expression. The presence of dendritic cells within the brain ventricles, choroid plexus, and meninges, together with lymphatics present within these structures, could represent the cellular basis for these effects.<sup>52–54,56,61</sup>

Long-term transgene expression, in the presence of transitory inflammation, and in the absence of priming of either a humoral or cellular immune response, can be achieved in the brain. In other organs, this remains more difficult, since they contain, even in the resting stage, professional antigen-presenting cells with migratory properties, as well as lymphatics channels. The precise cellular and molecular basis of brain immune responses, however, remains to be elucidated. This will be central for successful long-term gene transfer to the brain for the treatment of the disease.

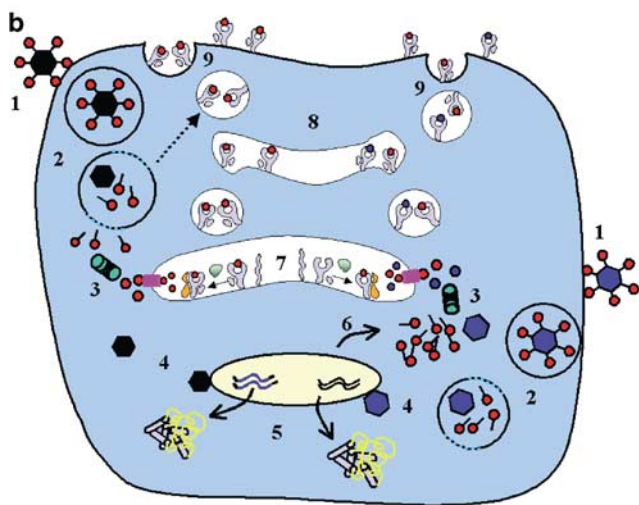
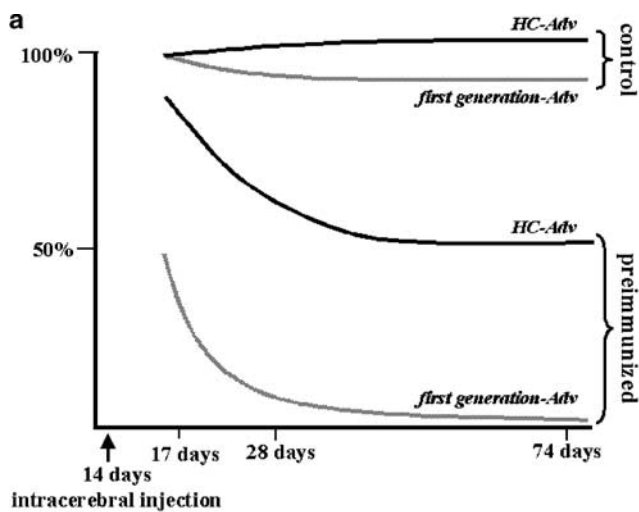
## Efferent responses

If systemic priming of the immune response occurs, activated T-lymphocytes can detect antigen in the brain. The effects of the peripheral immunization against an antigen within the brain are likely to depend on the precise type of brain antigen. In the case of skin transplants into the brain, these are eliminated by activated lymphocytes, presumably through cytotoxic mechanisms, that disappear once the transplant has been rejected. Immunization against brain antigens causes inflammatory brain disease that is either limited in time, or relapsing, depending on the antigen utilized and the strain of animals immunized. If BCG, an antigen that cannot be removed from the brain by immune cells, has been injected into the brain, activation of the immune system causes a chronic, although anatomically limited, cytotoxic inflammatory response.<sup>41,42,47</sup> Systemic immunization of animals following the injection of adenovirus or herpesvirus vectors into the brain leads to inflammation, and elimination of transgene expression.<sup>17-19,21,29,31-33,30,62</sup>

In particular, first-generation adenoviral-vector-mediated expression in the brain is stable in the absence of systemic priming. In its presence, transgene expression is eliminated within 1–2 months, depending on various factors including the nature of the transgene and

its levels of expression. Expressions from HC-Adv that are devoid of any sequences from the wild-type adenovirus genome, and thus, cannot present any antigen epitopes to activated T cells, remain stable, without brain inflammation<sup>32</sup> (Figure 1). HC-Adv vectors also allow stable expression when injected into already immune-primed animals, a paradigm that eliminates transgene expression from first-generation vectors in 14 days<sup>32</sup> (Figure 2a).

Activated T-cells need to recognize antigenic epitopes on antigen-presenting cells within the brain, in order to enter the brain and inhibit adenoviral-mediated transgene expression. This can explain why expression from first-generation adenoviral vectors is systematically eliminated by the adaptive immune response, since viral proteins encoded by these vectors, even when expressed at very low levels, are immunogenic, and are detected in minute amounts by activated T cells. Once in the brain, activated T-cells eliminate transgene expression through mechanisms that remain to be elucidated. In distinction, HC-Adv's genome does not express any viral proteins,



**Figure 2** Injection of first-generation adenovirus (black lines), or HC-Adv (grey lines) into the brains of control or pre-immunized animals. Animals were pre-immunized intradermally on day 0, and injected intracerebrally on day 14. In pre-immunized animals, expression of transgenes from a first generation adenovirus is eliminated completely, while that from a high-capacity adenoviral vector is only partially reduced, and then remains constant. (b) compares antigen presenting pathways of first generation adenoviral vectors (on the right half of the figure) with that of high-capacity adenoviral vectors (on the left half of the figure). First generation adenoviral vectors are shown in blue, and high-capacity adenoviral vectors are shown in black, to illustrate their different genome structure. Capsid proteins are shown in the same colors for each vector since they are identical. Numbers indicate the steps leading to antigen presentation: (1) vector attachment to the plasma membrane; (2) internalization into endosomes, initial stages of viral capsid disassembly, endosomolysis, and release of viral capsids and proteins into the cytoplasm; (3) proteasome degradation of viral proteins; (4) transport of capsids to the nucleus, and intranuclear release of vector genome; (5) expression of transgenic proteins; (6) low level synthesis of viral proteins that do not assemble into virions and are targeted for intracellular degradation; (7) loading of peptides onto MHC-I molecules; (8) transport of MHC-I through the endoplasmic reticulum, Golgi complex, and plasma membrane bound vesicles; (9) display of MHC-I molecules on the plasma membrane. The broken arrow indicates an alternative route for viral peptides to access plasma membrane bound vesicles containing MHC-I molecules. In the case of first generation adenoviral vectors, the vector genome will direct expression of the transgenic protein, and also continue to direct the production of a low level of virion proteins that provide antigenic peptide epitopes that are displayed on cell surface MHC-I and can be detected by CD8+ T-lymphocytes. The high-capacity adenoviral vectors however, will only provide antigenic capsid protein epitopes from the input virions infecting the cell. These proteins can be presented on MHC-I too, but only transiently, since after the input capsids are degraded and plasma membrane MHC-I are turned over, no more antigenic peptide epitopes will be made available to be presented on MHC-I proteins. The high-capacity adenoviral vector's genome only encodes for the transgenic protein, but not for any viral peptides. Thus, even several days after infection, first generation vectors will continue to provide antigenic peptides to be presented on MHC-I and can thus be recognized by T-cells. Cells infected by high-capacity adenoviral vectors will only transiently present antigenic peptides on MHC-I, thus explaining the partial inhibition of transgene expression detected in this experimental paradigm. The proteasome is shown in green; TAP, the protein that transports peptides into the endoplasmic reticulum, is shown in purple. In the secretory pathway empty MHC-I molecules are shown being loaded with peptides (small black arrow in (7)), and being transported to the plasma membrane. Some of the proteins associated with the loading of MHC-I molecules in the endoplasmic reticulum (e.g. tapasin, calreticulin, and calnexin) have been illustrated schematically.

and thus does not provide any antigenic epitopes that are recognized by the activated T-cells. Therefore, transgene expression mediated by HC-Adv persists fully following systemic immunization administered following brain injections, and persists partially when vector is injected into pre-immunized animals (Figure 1a and b, 2a).

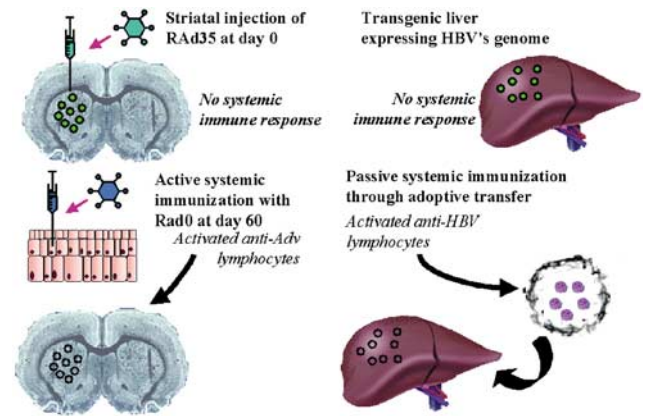
Upon infection, adenoviral capsids enter endosomes, from which they are released into the cytoplasm.<sup>63</sup> Cross-presentation of vector antigens on MHC-I could provide the necessary antigens to the activated T lymphocytes. In this paradigm, availability of antigenic epitopes from the HC-Adv is limited to the amount of antigenic capsid proteins present in incoming vectors (Figure 2b). This postulated mechanism could explain differences obtained on the effect of preimmunization on either vector system, the limited reduction in transgene expression from HC-Adv in preimmunized animals, and further predicts that using HC-Adv expression will remain physiologically relevant, even in the presence of an anti-adenoviral immune response. Similar mechanisms occur in muscle, where injection of HC-Adv in pre-immunized animals also leads to a reduction, but not elimination, of transgene expression.<sup>64</sup>

Cross-presentation of adenoviral capsid antigens has been demonstrated,<sup>65,66</sup> and supports this hypothesis. Furthermore, activation of CTLs against capsid protein antigens has been reported to occur upon injection of inactivated adenoviral vectors<sup>67</sup> in some experiments, although in others, neutralizing antibodies or an immune response capable of eliminating brain transgene expression was not detected upon injection of inactivated adenovirus.<sup>31</sup> Whether this discrepancy is caused by differences in species immunized, immunization schedules, or immunization sites, remains to be determined.

Humoral responses are unlikely to play a major role in the downregulation of gene expression in preimmunized animals, since, at 3 days, postvector administration expression from HC-Adv is indistinguishable from controls. Antibodies recognize capsids of either vector with equal affinity (Thomas *et al*, unpublished results); thus, antibodies would inhibit transgene expression equally for both vectors. If this would be the case, at 3 days, expression from either vector would have been significantly reduced to the same level.

However, the exact mechanisms by which activated immune cells inhibit transgene expression from viral vectors in the brain have not yet been elucidated, and could be due to cytotoxicity, transcriptional inhibition, or both. In peripheral tissues, direct cytotoxicity of transduced cells has been described in some,<sup>68–71</sup> but not in all experimental paradigms.<sup>72</sup> Thus, the phenomenological response detected in the brain upon activation of systemic immunity depends on the antigen. Whether a single lymphocyte displays all the different effector mechanisms, or whether different lymphocytes carry out different functions in the brain, remains to be determined.

In elucidating the immune mechanisms responsible for inhibiting transgene expression in the brain, work by Chisari and collaborators in a transgenic model of Hepatitis B (HBV) viral infection of the liver provides a testable blueprint for further research. In this model, CD8+, cytotoxic, antiviral-activated T cells, administered through adoptive transfer, eliminate viral genome



**Figure 3** This figure compares how the activated adaptive immune system eliminates adenoviral-mediated transgene expression in the brain (left hand side), with the predominantly non-cytolytic CTL-mediated mechanisms that inhibit hepatitis B virus (HBV) replication in a transgenic model of HBV infection of the liver.<sup>73</sup> The left side illustrates the injection of vector into the brain, brain cells expressing transgene (green circles), skin immunization against adenovirus, and the inhibitory effect of systemic immune activation on transgene expression in the brain (empty circles). The right side illustrates the HBV transgenic model in which activated HBV-specific CTL cells are provided by adoptive transfer, leading to the elimination of HBV replication on the liver.

replication in the liver expressing transgenic HBV.<sup>73</sup> Although there is an initial limited cytotoxic elimination of liver cells, it is the secretion of interferon- $\gamma$  by CTLs that inhibits HBV replication in the liver. This experimental paradigm tests the efferent arm of the immune response. Whether it is a predictive model helpful to understand immune responses to viral vectors injected into the brain remains to be determined. This model is summarized in Figure 3. This model predicts a certain outcome, and thus puts forward a number of testable hypotheses, regarding the mechanisms and consequences of the antivector immune response in the CNS. Figure 3 illustrates this hypothesis by comparing the transcriptional inhibition of Hepatitis B replication in the liver by virus-specific CTLs, and those underlying the inhibition of transgene expression in the brain of animals injected with viral vectors, and immunized systemically against adenovirus (Figure 3).

### Consequences and future

The consequences of the activation of an anti-vector or anti-transgene immune response need to be taken into consideration *vis-à-vis* the implementation of clinical gene therapy trials for the treatment of neurological diseases. Although it has been proposed that the immune privilege of the brain may pose a lesser challenge to the implementation of neurological gene therapy, experiments discussed above indicate that the activated immune system can eliminate transgene expression in the brain with very high efficiency. The predictions are that this will occur, either if the immune response is directed against the vector itself or the transgene.

Alternatives postulating that low levels of viral vectors injected into the brain may remain undetected are

challenged by data demonstrating that the adaptive immune response can recognize as little as 1000 infectious particles in the brain. This, plus the ease with which an injection into the brain, especially in a clinical context, could be made available to the ventricles and thus stimulate an immune response. Further, a thorough understanding of the molecular and cellular basis of brain immune responses to viral vectors is paramount for the further safe and efficient development of human neurological gene therapy.

## Acknowledgements

We would like to thank the Board of Governors at Cedars-Sinai Medical Center for their vision and very generous creation and funding of the GTRI. We would also like to thank Dr. Shlomo Melmed for his strong support of our program, Ms. Cheryl Cathcart for her first class administrative support, Mr. Danny Malaniak for his enthusiasm in dealing with the creation of a new place, Mrs. Semone Muslar for her excellent secretarial skills, and Mr. Nelson Jovel for the skillful and top quality editing and preparation of the figures and manuscript for publication. Work in the GTRI is funded by the NIH grants 1 RO1 NS42893 01 (to PRL), 1 RO1 NS44556 01 (to MGC) and U54 4 NS04-5309 (to PRL).

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