Long-Lasting Adenovirus Transgene Expression in Mice through Neonatal Intrathymic Tolerance Induction without the Use of Immunosuppression

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The major barrier to the clinical application of adenovirus gene therapy for diseases that require stable transgene expression is the immunogenicity of recombinant adenovirus, which ordinarily limits the duration of its effects to a period of about 2 weeks. We postulated that tolerance to adenovirus could be induced and transgene expression could be prolonged if T lymphocytes underwent thymic selection in the presence of adenovirus antigens. Mice were inoculated in the thymus with a recombinant adenovirus containing the lacZ marker gene during the neonatal period at a time before T-cell maturation had occurred. When the virus was administered intravenously to these mice in adulthood, they were found to have an impaired adenovirus-specific cytotoxic T-lymphocyte response which allowed prolonged hepatic lacZ expression, for up to 260 days. The ability to achieve unresponsiveness to a recombinant adenovirus after inoculation of the thymus in neonates extends the paradigm of intrathymic tolerance induction. Furthermore, this model will enable the study of stable adenovirus transgene expression in vivo without the use of immunosuppression and ultimately may have clinical utility.

Modifying the genetic composition of somatic cells offers intriguing potential for the prevention and therapy of inherited and acquired human disease (16). Several disorders would likely benefit from the simple replacement of a defective gene with its normal counterpart. In many diseases, the pathogenesis is understood and the relevant gene has been identified and cloned. However, gene therapy is currently thwarted by the lack of a suitable vector for gene delivery and stable transgene expression (3). A variety of gene transfer agents are presently under evaluation for human application, including plasmids, liposomes, and recombinant viruses (retrovirus, adenovirus, and herpesvirus). Of these, adenovirus possesses a number of attractive features: it infects a broad range of cell types, can be produced in high titer, accomplishes highly efficient gene transfer both in vitro and in vivo, and remains episomal, thereby reducing the risk of insertion mutagenesis (9, 14). Despite these advantages over other candidate vectors, the potential immune response to foreign viral proteins impedes stable adenovirus-mediated gene transfer in vivo (23) and renders adenovirus vectors useless when persistent transgene expression is required.

Several strategies have been employed to prolong adenovirus transgene expression in mouse liver. Modification of the adenovirus vector to produce fewer viral proteins has successfully extended expression (7) but does not alter the immunogenicity of the transgene itself, which has been shown to be substantial (22). Administration of CTLA4Ig (12), cyclosporine (7), and a monoclonal antibody that depletes CD4+ T lymphocytes (5, 24) each has effectively prolonged transgene expression. However, the side effects of immunosuppression make it less appealing for clinical application. Alternatively, the induction of tolerance to the adenovirus vector would allow for prolonged transgene expression. Previously, we have reported that intrathymic inoculation of antigen can achieve transplantation tolerance (17). Similarly, we have shown with adult mice that intrathymic injection of adenovirus-infected cells combined with brief immunosuppression can induce transient unresponsiveness (<7 weeks) to a recombinant adenovirus (6). Recently, this model of adenovirus tolerance has been utilized by others to correct a metabolic deficiency in rats (10). In these studies, we investigated whether direct intrathymic inoculation of adenovirus in neonatal mice could be used to induce specific immunologic tolerance and achieve long-lasting adenovirus transgene expression.

MATERIALS AND METHODS

Animal procedures. Animal procedures were in accordance with Institutional Animal Care and Use Committee guidelines. C57BL/6 (B6) neonatal mice (1 to 3 days old) were anesthetized with inhalational methoxyfluorane during viral inoculation. Intravenous injections (108 PFU of AdΔlacZ in 2 μl of phosphate-buffered saline [PBS]) were performed via the facial vein. For intrathymic inoculations, a midline incision was made in the lower neck, the strap muscles were brought laterally, and the manubrium was retracted inferiorly to expose the mediastinum. Each thymic lobe was injected with 1 μl of PBS containing 5 × 107 PFU of AdΔlacZ. The wound was closed with a single absorbable suture. Mortality from intrathymic injection was less than 5%.

Recombinant adenovirus. AdΔlacZ is a first-generation E1-deleted human type 5 adenovirus (23). The virus was propagated in 293 cells, which supply the E1 region in trans, enabling replication. Thirty hours after infection at a multiplicity of infection (MOI) of 5, the cells were harvested and lysed by freeze-thawing. Supernatants were banded twice by cesium chloride density ultracentrifugation, and the virus was purified over a Sephadex G50 column. Plaque assays were performed on 293 cells to determine the titer of the virus. Viral stocks were stored in 10% glycerol in PBS at −70°C until use.

Histochemistry. X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) histochemistry was used to detect β-galactosidase in frozen tissue sections. Tissue was placed in OCT medium and frozen over an isopentane dry-ice bath. Cryostat sections (6 μm) were fixed in 0.5% glutaraldehyde in PBS. Slides were incubated in X-Gal in 5 mM potassium ferricyanide–5 mM potassium ferrocyanide–1 mM magnesium chloride in PBS for 2 h in the dark at 37°C. Tissue sections were counterstained in hematoxylin and mounted.

CTL assay. Cytotoxic T-lymphocyte (CTL) activity was determined as described previously (5). Briefly, splenocytes (effectors) were harvested from test animals, plated at 5 × 105 cells per well, and infected with AdΔlacZ (MOI of 10) for 5 days. The assay was performed in RPMI supplemented with 2 mM l-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 20 μg of...
genticin per ml, and 5% heat-inactivated fetal bovine serum (FBS). Syngeneic target cells (the methylcholanthrene-induced fibroblast cell line MC37 [H-2b]) were transfected at an MOI of 50 or mock infected 1 day before the assay. On day 5, target cells were labeled with 100 μCi of sodium [3H]choline for 1 h and plated at 10^5 cells per well in 96-well V-bottom plates. Specific lytic activity was measured by the 50% plaques per well (% PPE) of HeLa cells, and the plates were incubated at 37°C in 5% CO₂ for 2 h. Then, 100 μl of Dulbecco modified Eagle medium with 10% FBS was added to each well. The next day, the cells were stained with X-Gal. The lowest dilution on average at which <50% of HeLa cells had lacZ expression was recorded.

RESULTS

The characteristic immune response of normal mice to recombinant adenovirus is readily demonstrated in normal adult B6 mice given an intravenous dose of 10⁹ PFU of an E1-deleted replication-defective adenovirus (AdlacZ) carrying the lacZ marker gene under control of the cytomegalovirus promoter-enhancer. As previously established (21), intravenously administered adenovirus localizes predominantly to the liver, where striking levels of transgene expression can be seen 3 and 7 days later (Fig. 1A). However, this hepatic lacZ expression is transient, and by day 14 it is undetectable (Table 1 and Fig. 1B). Several lines of evidence support the premise that the clearance of the virus is immunologic and predominantly T lymphocyte dependent. In normal mice, a mononuclear cell infiltrate appears in the infected liver shortly before the disappearance of transgene activity (23). Furthermore, expression of the transgene is substantially prolonged in T-cell-deficient mice (nu/nu or SCID) (23) and in normal mice treated with anti-T-cell monoclonal antibodies, as discussed above. In addition, prolonged transgene expression in mice with a selective deficiency of CD8⁺ T cells specifically implicates antiviral CTLs in viral clearance (25).

Hepatic transgene expression after intravenous inoculation of neonates. To determine whether immunologic tolerance to an adenovirus vector could be induced by the classic method of neonatal inoculation, newborn B6 mice were inoculated intravenously with 10⁶ PFU of AdlacZ. As others have shown, this procedure allowed highly efficient gene transfer, with more than 50% of hepatocytes strongly expressing the transgene when examined 3 or 7 days later (11, 21). Notably, there was no detectable transgene activity in the thymus (Fig. 1C). While hepatic transgene expression after intravenous inoculation exists only transiently in adult mice, we found it to be considerably prolonged in neonatal mice, as previously reported (21). Initially, almost all hepatocytes were infected in neonates. Transgene expression declined by day 30, though, and by 2 months only occasional hepatocytes stained positive (Table 1).

To investigate whether this gradual loss of hepatic transgene expression after intravenous injection of neonates was the result of an immune response, we performed parallel experiments with neonatal SCID mice that lack both cellular and humoral immunity. SCID neonates exhibited high levels of hepatic lacZ expression when studied after 14 and 30 days, but by 60 days expression declined, as it had in B6 neonates, to less than 10% of hepatocytes (Table 1). This pattern differed markedly from that seen in SCID mice first exposed to the virus as adults, in which strong transgene expression was maintained, with only a minimal reduction in intensity, for as long as the mice were monitored (>100 days). We hypothesize that the progressive loss of hepatic transgene expression after intravenous inoculation of SCID or normal neonates is the result not of immune clearance but of gradual dilution of the transgene as new, uninfected hepatocytes are generated through cell replication during normal growth of the liver. This hypothesis is further supported by the lack of a lymphocytic infiltrate in the liver (which precedes viral clearance in mice first injected in adulthood) in inoculated B6 neonates. Similarly, the potent neutralizing-antibody and CTL responses generated by adults within a few weeks after intravenous inoculation were not evident in B6 mice given virus intravenously as neonates (Table 2; Fig. 2).

Intravenous inoculation of neonates fails to induce tolerance. Collectively, these findings indicate that mice inoculated neonatally with adenovirus by the intravenous route do not mount typical humoral or cellular immune responses to the foreign viral proteins which accompany persistent hepatic viral gene expression. We therefore speculated that mice inoculated intravenously with adenovirus as neonates might actually have become immunologically tolerant to adenovirus, even though we had observed the gradual disappearance of transgene expression (a finding we attributed to dilution as the liver grew).

To evaluate this possibility, these mice were challenged intravenously at 30 to 60 days of age with a second dose of AdlacZ. Gene transfer was initially accomplished by this rechallenge, and high levels of lacZ activity were detected in the liver up to 7 days later. However, by day 14 these mice had no detectable hepatic lacZ expression and had lost even the low-level residual lacZ activity present from the neonatal inoculation (Fig. 1E). The time course of viral elimination in these mice was similar to that observed in naive mice first exposed to the virus in adulthood (Table 2). Thus, although neonatal exposure to AdlacZ by the intravenous route did not induce tolerance, neither did it result in immune sensitization. Neonatal recipients of intravenous virus responded to the viral challenge in adulthood as if immunologically ignorant of the vector that had persisted in their livers with a low level of transgene expression.

Thymic transgene expression after intrathymic inoculation of neonates. Since the thymus is the central locus for induction of cellular immune tolerance, we hypothesized that neonatal mice inoculated intravenously with adenovirus did not become tolerant because the virus failed to reach the thymus. Applying analogous reasoning, we had previously postulated that the well-known ineffectiveness of systemically administered nonlymphoid cells in induction of neonatal tolerance to allografts is a consequence of their inability to migrate to the thymus (17, 18). Previously, we had demonstrated that transplantation tolerance can, in fact, be induced with nonlymphoid donor cells even in adulthood, but only if these cells are inoculated directly into the thymus. Therefore, to test whether a similar strategy might also be successful for viral antigens, we treated neonatal B6 mice by intrathymic inoculation of 5 × 10⁶ PFU of AdlacZ per lobe. This procedure resulted in strong lacZ expression in the thymus 3, 7, and 10 days later (Fig. 1D). However, thymic lacZ activity waned with time and was detectable only in occasional thymic cells after 30 days. We suspected that the eventual loss of transgene expression may be caused by the very rapid turnover of cells in the neonatal thymus rather than by immune elimination. In these mice, expression of lacZ was not confined to the thymus but was also found in the liver, probably because some of the viral inoculum leaked out of the thymus upon injection. For adult recipients of intrathymic virus, we have previously noted that such extravasation was fol-
FIG. 1. lacZ expression following AdlacZ administration to adult and neonatal mice. Staining for β-galactosidase was performed on frozen sections of thymus, liver, and lung tissue by using X-Gal histochemistry. Strong hepatocyte staining was present in the liver at 7 days after intravenous infusion of $10^9$ PFU of AdlacZ to naive adult B6 mice (A) but was absent by day 14 (B). In neonates, intravenous injection of $10^8$ PFU of AdlacZ did not accomplish detectable gene transfer to the thymus at day 3 (C), whereas direct thymic inoculation resulted in significant thymic gene transfer at day 3 (D). Neonates treated with intravenous AdlacZ and then rechallenged as adults had no hepatic lacZ activity 14 days later (E). In contrast, mice conditioned with intrathymic virus as neonates demonstrated a high level of hepatic transgene expression when assessed 14 days after rechallenge in adulthood (F), and expression remained strong for more than 150 days (G). Tolerance achieved by neonatal intrathymic injection was also effective in prolonging transgene expression in tissues such as lung, examined at 70 days after rechallenge with virus via tracheal instillation when the animal was 4 weeks old (H). Magnification, ×180.
lowed by both humoral and cellular immunity to viral antigens (6).

**Intrathymic inoculation of neonates induces tolerance.** To test whether mice that had received an intravenous injection of virus as neonates were tolerant despite the loss of transgene expression at the site of the inoculum, we challenged them 30 to 60 days later with intravenous AdlacZ. Prolonged hepatic transgene expression was achieved (Table 2). In these mice strong hepatocyte transgene expression was evident by 3 days after the intravenous challenge, and consistently persisted, with only a slight loss of intensity, for 100 days. In several animals expression was evident beyond 150 days and for as long as 260 days (Fig. 1F and G). Thus, intrathymic inoculation of adenovirus in neonates induced an effective state of immunologic unresponsiveness in vivo.

T-cell responsiveness to viral antigens was studied in vitro by examining the antiviral CTL activity of splenocytes harvested from mice injected as neonates. Spleen cells from normal adult B6 mice immunized against AdlacZ from mice injected as neonates. Spleen cells from normal adult mice were used (data not shown). Moreover, normal nonimmunized mice did not generate significant CTL activity in this assay. In contrast to the sensitizing effect of exposure to the virus in adulthood, neonatal intravenous exposure to AdlacZ resulted in little CTL activity. However, if mice treated with intravenous AdlacZ as neonates were subsequently challenged at day 30 with intravenous AdlacZ, they mounted a CTL response comparable to that of naïve adults given a primary challenge. In contrast, mice rendered tolerant by direct thymic injection of the virus as neonates exhibited dramatically reduced adenovirus-specific CTL activity after intravenous challenge in adulthood, indicating a marked impairment of cellular immunity (Fig. 2).

There was no evidence of a generalized immunosuppressive effect of intrathymic virus inoculation. When these animals reached adulthood (4 to 8 weeks later), no differences between these mice and untreated littermates were detected with regard to (i) the immune profile of lymph node cells with respect to CD4+/CD8+ T cells, B cells, and T-cell receptor expression as measured by fluorescence-activated cell sorter analysis, (ii) the immune response to allogeneic skin or pancreatic islet cell grafts, and (iii) lectin-induced lymphocyte interleukin-2 production in vitro as measured by enzyme-linked immunoassay (data not shown).

Tolerant mice were also tested for the presence of serum neutralizing antibody before and after intravenous rechallenge. Although no detectable antiviral antibody was present in any of 19 mice treated intravenously or 15 mice inoculated intrathymically as neonates, a subsequent rechallenge with virus in adulthood provoked a normal antibody response in mice from both of these groups (Table 2). This suggests that mice exhibiting T-cell tolerance were capable of generating a strong humoral response to the virus. Interestingly, this did not prevent high-level hepatic transgene expression from lasting for more than 6 months.

To determine whether persistent expression of a transgene could also be accomplished in organs other than the liver, mice given an intrathymic inoculation of virus at birth were rechallenged in adulthood by direct injection of the adenovirus vec-

### Table 1. Time course of hepatocyte lacZ expression after intravenous administration of AdlacZ

<table>
<thead>
<tr>
<th>Recipient mice</th>
<th>Age at inoculation</th>
<th>Hepatocyte lacZ expression&lt;sup&gt;a&lt;/sup&gt; on day:</th>
<th>Prerechallenge</th>
<th>Postrechallenge</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>14–30</td>
<td>60–90</td>
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<td>600 (5)</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>B6 Neonate</td>
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<td>3, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2</td>
<td>540 (5)</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Animals received an intravenous inoculation of AdlacZ, and the duration of hepatocyte lacZ expression was determined. Adults were administered 10<sup>8</sup> PFU of AdlacZ, and neonates were injected with 10<sup>6</sup> PFU. Dosages were selected based upon dose-response experiments and represent the lowest dose that consistently produced maximal hepatocyte lacZ activity 3 days postinjection with minimal (<5%) associated mortality.

<sup>b</sup>Slices stained with X-Gal were scored in a blinded fashion by two independent observers (R. P. DeMatteo and J. F. Markmann) on a scale of 0 to 3: 3, staining in >50% of hepatocytes; 2, staining in 10 to 50% of cells; 1, staining in 1 to 10% of cells; 0, <1% positive staining. In cases in which scores were discrepant (<5% of slides), the lower of the two scores was recorded. Each number indicates a score from an individual mouse sacrificed at a given time point.
intravascular injection may allow for relatively prolonged periods, recipients appear to remain immunologically ignorant of its presence. Importantly, such mice fail to develop tolerance to the vector and remain capable of mounting a normal vigorous cellular immune response to the virus if rechallenged as adults by intravenous inoculation. We believe that the failure of intravenous virus to induce cellular tolerance rests with the inability of the virus to reach the thymus. Where we never observed transgene expression when inoculated intravenously in adulthood. Stable expression of the transgene can also be accomplished readily in other tissues, such as lung and muscle, by the same tolerance induction protocol. The reduced adenovirus-specific CTL activity in tolerant mice suggests that extended viral tissue expression may be due in part to the interruption of cellular immunity by interference with development of virus-specific CTLs.

Recently, several reports of failure to achieve tolerance by intravenous injection of cellular, viral, or protein antigens in neonatal mice have argued against the classical view that the neonatal period is uniquely advantageous for induction of tolerance. Instead of the traditional self-nonsel self-paradigm, Matzinger proposes a “danger theory” in which foreign (and self) antigens elicit immunity if they are perceived as dangerous by the host. However, none of these studies considered the possibility that failure to induce classical tolerance was attributable to the inability of the antigenic inoculum to reach the thymus. The present report and the prior studies by others reinforce the notion that development of a classic state of tolerance requires thymic exposure to the antigen. The response of the immune system, whether neonatal or not, to peripheral antigenic exposure is distinct from that which occurs in the thymus. In fact, the uniqueness of the neonatal period may depend in part on the absence of a significant peripheral response, thereby allowing tolerance to develop. Supporting this notion is our finding that in attempting to apply this strategy of intrathymic inoculation to adult recipients, we observed that concomitant peripheral sensitization to the virus interfered with tolerance. Success in the case of adult recipients was achieved only if the virus was specifically localized to the thymus and at the same time mature peripheral (and perhaps thymic) T cells were eliminated.

The paradigm of induction of tolerance to adenovirus in neonates has several advantages over our previous work with adult mice and the subsequent reports of others. First, the conditioning regimen is simplified, as direct intrathymic injection of virus is sufficient to accomplish tolerance. In adult mice, significant viral extravasation into the periphery occurred after intrathymic inoculation. A cellular carrier was needed to trap the virus in the thymus to avoid immune sensitization. Next, the current model does not require the administration of antilymphocyte serum to deplete existing T lymphocytes. Thus, the results are not confounded by the effects of transient immunosuppression. Finally, the duration of transgene expression in this model is markedly prolonged (up to 260 days), whereas expression lasted to about 7 weeks in our initial work.

This novel method for accomplishing stable adenovirus-mediated gene transfer offers a variety of potential applications. First, this model will enable the study of experimental adenovirus gene transfer to various tissues in normal immunocompetent recipients. This will permit validation of the efficacy of transgene expression in systems in which stable expression of the relevant transgene product is required. For instance, the long-term effects of adenovirus-mediated transfer of the cystic fibrosis transmembrane conductance regulator gene can now be assessed in vivo without the use of immunosuppression. Second, the ability to transfer genes to the thymus may allow for precise modification of the immune repertoire. By maintaining the appropriate virally encoded proteins in the thymus, it should be possible to favorably alter the immune response in states of autoimmunity and organ allograft rejection. Finally, this strategy for achieving long-term gene transfer may ultimately have clinical utility. Although it does not follow that inoculation of human neonates with a viral vector would achieve a similar state of specific tolerance, because of the relatively greater maturity of the human immune system than the rodent immune system at birth, recent advances in fetal diagnosis and in utero fetal manipulation may permit genetic intervention at a time sufficiently early for tolerance to develop.
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REFERENCES