Cutting Edge: Recombinant Adenoviruses Induce CD8 T Cell Responses to an Inserted Protein Whose Expression Is Limited to Nonimmune Cells

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*J Immunol* 2001; 166:4809-4812; doi: 10.4049/jimmunol.166.8.4809

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CD8 T cells (T\textsubscript{CD8+}) play a crucial role in immunity to viruses. Current understanding of activation of naive T cells entails Ag presentation by professional APCs (pAPCs). What happens, however, when viruses evolve to avoid infecting pAPCs? We have studied the consequences of this strategy by generating recombinant adenoviruses that express influenza A virus nucleoprotein under the control of tissue-specific promoters. We show that the immunogenicity of such viruses requires their delivery to organs capable of expressing nucleoprotein. This indicates that infection of pAPCs is not required for adenoviruses to elicit a T\textsubscript{CD8+} response, probably due to a cross-priming via pAPCs. While this bodes well for recombinant adenoviruses as vaccines, it dims their prospects as gene therapy vectors. The Journal of Immunology, 2001, 166: 4809–4812.

**Materials and Methods**

**Generation of rAdV**

All of the rAdV contained a full-length gene encoding the PR8 NP and the 0.4-kb SV40 intron/poly(A) signal derived from 3.7SPC/SV40a. In addition, AdSPCNP contained the 3.7-kb HindIII fragment of the surfactant promoter C promoter (SPC) derived from 3.7SPC/SV40 (1), AdCMVNP contained a 0.7-kb Bu/II-Xhol fragment containing the CMV immediate-early promoter derived from pCI, and AdK14NP contained the 2.4-kb BamHI-Xhol fragment containing the K14 promoter derived from pK14-luc (2) and inserted into the corresponding sites for pAdK14. rAdVs were generated by Quantum Biotechnologies (Montreal, Canada). Viral titers were determined by infecting 293A cells in 96-well plates and visually inspecting cells after 8 days for a cytopathic effect.

**Immunohistology**

Livers were removed from C57BL/6 (B6) SCID mice 3 days postinfection, frozen, and 15-μm sections were prepared. Sections were air dried on 12-mm diameter coverslips, fixed and permeabilized by incubation for 2 min with acetone:methanol (1:1), and then incubated with PBS-containing 5% (v/v) donkey serum followed by a mixture of anti-NP mAbs and finally fluorescein-conjugated donkey anti-mouse Ab with the DNA stain to-pro3. Dyes were localized by laser scanning confocal microscopy using the appropriate lasers and filters. NP expression was detected only in sections from mice that were infected with AdCMVNP.

**T\textsubscript{CD8+} functional assays**

Six- to 8-wk-old female BALB/cByJ B6 and I-Ab\textsuperscript{b} mice (Taconic Farms, Germantown, NY) were immunized by various routes with 10\textsuperscript{8}–10\textsuperscript{10} PFU of rAdV. The intracellular IFN-γ staining assay for activated T\textsubscript{CD8+}, in vitro stimulation, and microcytotoxicity assay were performed as described (3).
Viral inactivation

For inactivation, viruses were incubated in 1% (v/v) β-propiolactone at pH 7.9 overnight at 4°C, then for 1–2 h at 37°C (4), then frozen until use.

Results

We chose AdV to study the effects of tissue-specific expression on T\textsubscript{CD8\textsuperscript{+}} activation for a number of reasons. First, AdV infects a wide variety of cell types (including professional APCs (pAPC)) due to the widespread expression of cell surface receptors capable of enabling viral penetration. Second, the AdV genome is imported into the nucleus where inserted genes can be placed under the control of host cell transcription factors. Third, AdV vectors are able to accommodate relatively large segments of foreign DNA, a necessity given the size of tissue-specific promoters. Fourth, AdV is a widely used vector for gene therapy (6), so understanding its immunogenicity is of both basic and practical interest and importance.

We used a rAdV vector that has been a workhorse in gene therapy studies. This vector lacks the E1 region of the viral genome. Consequently, unlike wild-type AdV, the rAdVs used are replication incompetent, meaning that they only infect the cells they first encounter. There is limited expression of bona fide AdV gene products, reducing the potential impact of their immunodominance of the inserted gene. The rAdVs do not express a number of viral gene products with the capacity to modulate immune responses.

We created a panel of rAdV that express NP under the control of either the CMV promoter, which is active in all cell types, the surfactant promoter (SPC), whose known activity is limited to bronchiolar and alveolar epithelial cells (7), or the keratin promoter (K14), whose known activity is limited to keratinocytes and thymic epithelial cells (8). The specificities of these promoters were functionally established as described below. Additionally, we tested this by infecting mice i.v. and examining liver cryosections for NP expression by indirect immunofluorescence. We used SCID mice to minimize the immune-based elimination of infected cells and to prevent interfering effects of serum Ab on the indirect Ab-based detection of NP. As shown in Fig. 1, NP staining was easily detected in a high percentage of liver cells from SCID mice infected with rAdV encoding NP. Cells were located by staining with the DNA-binding dye topro3 (top), and NP was detected using a mixture of anti-NP mAbs (bottom).

We next examined the capacity of the rAdVs to prime memory T\textsubscript{CD8\textsuperscript{+}} by injecting BALB/c mice by different routes and stimulating splenocytes in vitro. T\textsubscript{CD8\textsuperscript{+}} activity was determined by a standard \textsuperscript{51}Cr release assay using target cells sensitized with the immunodominant K\textsuperscript{d}-restricted peptide from NP. As shown in Fig. 2, when rAdV was introduced i.v. a significant cytotoxic response to NP\textsubscript{147–155} could be detected using AdCMVNP, as expected. AdK14NP was also immunogenic, while AdSPCPN failed to detectably prime mice. Similar results were obtained when the rAdVs were introduced by s.c. infection. In contrast, following intranasal (i.n.) infection, priming was detected using AdCMVNP and AdSPCPN but not AdK14NP. Priming under these conditions required viral gene expression in vivo, because the immunogenicity of AdCMVNP introduced i.v. was abrogated by β-propiolactone inactivation of viral infectivity (data not shown). Because significant amounts of NP were present in AdCMVNP preparations (but not the other viruses, because the promoters are inactive in the 293 cells used for virus propagation (data not shown)), this indicates that exogenous NP is not immunogenic in the absence of viral gene expression.

To gain a more quantitative measure of murine T\textsubscript{CD8\textsuperscript{+}} (mT\textsubscript{CD8\textsuperscript{+}}) activation, we performed intracellular cytokine staining ex vivo on splenocytes obtained from mice immunized via different routes (Fig. 3). Following i.v. infection, the greatest number of IFN-γ-secreting mT\textsubscript{CD8\textsuperscript{+}} were induced by AdCMVNP. AdK14NP induced approximately one-fourth of this number of mT\textsubscript{CD8\textsuperscript{+}}. Consistent with the previous results, AdSPCPN failed to induce a detectable number of mT\textsubscript{CD8\textsuperscript{+}}. Following s.c. infection, AdK14NP generated the most IFN-γ-secreting T\textsubscript{CD8\textsuperscript{+}}, followed by AdCMVNP, with AdSPCPN once again failing to induce a detectable response. Intranasal infection resulted in best priming with AdSPCPN, with fewer cells triggered by AdCMVNP and no priming after infection with AdK14NP.

We next used MHC class II β-chain knockout mice (I-A\textsuperscript{b}β\textsuperscript{−/−}) to examine the role of CD4 T cells (T\textsubscript{CD4\textsuperscript{+}}) in rAdV-induced priming of NP-specific mT\textsubscript{CD8\textsuperscript{+}}. In B6 mice (the control for I-A\textsuperscript{b}β\textsuperscript{−/−} mice) the immunodominant determinant from NP is NP\textsubscript{366–374}. Initial experiments confirmed the dependence of priming of NP\textsubscript{366–374}-specific responses on the route of infection as observed for BALB/c mice (not shown). Mice were infected i.v. with AdCMVNP or AdK14NP and i.n. with AdSPCPN, and 4 wk later splenocytes were restimulated in vitro and tested for NP\textsubscript{366–374}-specific lytic activity. As seen in Fig. 4,
the response to AdCMVNP in I-Aβ−/− mice is slightly diminished relative to B6 mice. By contrast, there is a profound effect on the response to AdK14NP, which is no longer immunogenic. Similarly, AdSPCNP is no longer immunogenic in I-Aβ−/− mice following i.n. infection. An additional experiment (Fig. 4B) revealed that AdCMVNP retains immunogenicity when given i.n. to I-Aβ−/− mice, although as above immunogenicity is reduced. These findings demonstrate that placing control of rAdV-encoded NP under organ specific-promoters renders its immunogenicity completely dependent on TCD8+ cells.

Discussion

Two sets of findings demonstrate the effectiveness of the tissue-specific promoters we used. First and foremost is the dependence of immunogenicity on the route of immunization. The absolute requirement for i.n. immunization exhibited by AdSPCNP indicates that NP expression requires viral infection of lung cells. The failure of the virus to elicit responses following i.v. injection, a route that surely gives access to secondary lymphoid organs, demonstrates that its immunogenicity cannot be based on infection of pAPCs. Likewise, the absence of immunogenicity of AdK14NP by i.n. injection indicates that it too is under tissue-specific control. Unlike AdSPCNP, AdK14NP was immunogenic when administered by i.v. injection. Conceivably, this could be due to infection of pAPCs. However, we believe that it is far more likely that this is due to infection of epithelial cells, possibly skin cells at the site of injection, or thymic epithelial cells where the K14 promoter is known to be active. Second, there is a clear difference in the TCD8+ dependence of TCD8+ responses to AdCMVNP vs AdK14NP and AdSPCNP.

Taken together, these data indicate that limiting expression of rAdV-encoded NP to nonprofessional APCs does not prevent the capacity of rAdV to activate naive TCD8+ responses. However, it does force the immune system to enlist the help of TCD4+ in generating TCD8+ responses. This gives important insight into the generation of an immune response to viruses that do not infect pAPCs. Surprisingly little is known about the infection of pAPC by viruses in vivo, and it is likely that many viruses do not infect these sentinel cells in the course of their natural infections. Hepatitis B virus and human papilloma virus, for example, are notoriously finicky in their host cell range. Our results provide clear evidence that expression of endogenous Ags by pAPCs is not required to prime naïve TCD8+ following AdV infection.

There are two general explanations for induction of TCD8+ responses by pAPCs. First is direct priming, i.e., cells of the target organ present Ag on their own class I molecules. This has yet to be convincingly demonstrated for non-marrow-derived cells. Second is cross-priming (9, 10), i.e., Ag is taken up in some form by pAPCs that present peptides derived from the exogenous Ag. Using bone marrow chimera mice to distinguish presentation mediated by cells derived from adoptively transferred hematopoietic stem cells from that of nonhemopoietic cells, it has been demonstrated in numerous systems that bone marrow-derived cells are the predominant APCs for inducing naive TCD8+ responses (11). This was most elegantly shown for antiviral responses by Sigal et al. (12), who studied TCD8+ responses in poliovirus receptor transgenic mice. In this system, bone marrow-derived APCs lacking the viral receptor clearly activated naive TCD8+ specific for a nominal protein Ag expressed by poliovirus. Importantly, Carbone, Heath, and their colleagues demonstrated that cross-priming is absolutely dependent on TCD8+ cells (13), and along with other groups showed that this was probably due to a requirement for TCD8+ mediated activation of dendritic cells via the interaction of CD40 ligand on TCD8+ with CD40 on dendritic cells (14–16).

Based on these findings, it is most likely that the tissue-specific immunogenicity of rAdV-encoded NP is based on cross-priming by pAPCs. If this is true, then other strategies for limiting the expression of rAdV-encoded transgenes, such as altering the specificity of the viral receptor protein, will similarly fail to prevent TCD8+ activation. However, this is by no means an inevitable outcome of virus-mediated gene expression. In collaboration with Pinto and Hughes, we recently showed that retrovirus mediated expression of proteins controlled by a skeletal muscle-specific promoter occurs beneath the immune radar (17). Similarly, adenovirus-associated viruses have been shown to express proteins in muscle without eliciting TCD8+ activation (18, 19). We suspect that these viruses are intrinsically less immunogenic than rAdV, but it is possible that skeletal myocytes are less adept at providing Ags for cross-priming than bronchoalveolar or squamous epithelial cells.
Our findings indicate that the E1 region-deleted AdV vector we used is poorly suited for gene therapy of many, if not most, organs. However, this vector still expresses numerous AdV gene products that may contribute to rejection of transfected cells by either Ags or by triggering inflammation at the sites of infection. Second-generation AdV vectors expressing decreased amounts of viral proteins should be stealthier. Based on the observation that administration of the high doses of rAV needed for gene therapy results in priming of T_{CD8+}, responses to viral proteins in the inocula (20), it was suggested that rAdV is doomed as a gene therapy vector. This need not be, because infected cells will eventually lose class I molecules presenting peptides derived from incoming viral proteins. If this happens before the induction of T_{CD8+}, rAdV-infected cells could avoid detection. Indeed, due to the phenomenon of immunodomination (21), T_{CD8+} responses to incoming AdV Ags may even serve to depress responses to the transfected gene.

Acknowledgments
We acknowledge the excellent technical assistance of Beth Buschling and Andrea Weisberg. We thank Dr. Jeffrey Whitsett for the gift of the SPC promoter and Dr. Jeffrey Kudlow for the gift of the K14 promoter.

References