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Repeated Intratracheal Instillations of Nonreplicating Adenovirus 2 Vector Attenuate CTL Responses and IFN-γ Production

David W. Pascual, Nancy Walters, and Peter Hillemeyer

The proposed usage of replication-deficient adenovirus (Ad) vectors for corrective gene therapy or for mucosal immunization has been limited in part by the host reactivity to the Ad vector, thus limiting repeated Ad instillations. We have recently shown that the reactivity to the Ad vector is in large part due to increased CD4+ Th1 and Th2 responses as well as elevated IgG and mucosal IgA responses. It has been recently proposed that the diminution of transgene expression in respiratory epithelia was due to increased CTL reactivity to expressed Ad proteins. Herein, we report that repeated intratracheal delivery of a second generation Ad vector into mice results in no detectable CTL activity in freshly isolated lymphoid cells from lungs, lower respiratory lymph nodes, or spleens or after in vitro restimulation. In contrast, a single dose of Ad vector did elicit a robust CTL response. This attenuation of CTL activity was long lived and was not affected by macrophage depletion or due to a reduction in CD4+ T cells. Examination of cytokine production via MHC class I or class II restimulation by lymphoid cells from three intratracheally treated mice showed an attenuation in the production of IFN-γ by as much as 110-fold. This reduction in IFN-γ could not be attributed to increased IL-4 or IL-10 production. Thus, this study shows that the CTL response to Ad vectors is attenuated upon repeated administration. The Journal of Immunology, 1998, 160: 4465–4472.

T he proposed usage of replication-deficient adenovirus (Ad) mutants either for gene therapy or as a mode of vaccination has been scrutinized in part by induced immunity reactivity toward the Ad vector (1–5). A number of studies has evaluated B and T cell parameters and can show increased Ab reactivity (3, 6, 7), increased CD4+ Th1- and Th2-type responses (6, 7), or increased CTL activity to the Ad vector (4, 5, 8, 9). Such elicited Ad-specific immunity has important implications if Ad vectors can be successfully used on respiratory epithelium. However, much of the earlier work evaluating anti-Ad immune responses has relied on the delivery of Ad vectors to sites other than into the lower respiratory tract (4, 10, 11). In addition, when Ad vectors were instilled into the lungs, many studies used only a single administration (3, 5, 9) and from that information inferred the consequences for repeated intratracheal (i.t.) administrations. Consequently, prediction of how these Ad vectors behaved when repeatedly instilled into the lungs was not always as evident, and until recently (6, 7), detailed analysis of associated respiratory lymphoid tissues had not been performed.

Such detailed analysis has revealed some important findings. In keeping with the idea that these Ad vectors are nonreplicating, assessment of anti-Ad Ab showed that repeated i.t. instillation of Ad into mouse lungs results in elevated serum IgG1 and mucosal IgA Abs (6, 7, 12). Upon analysis of CD4+ T cell responses, induction of IL-4 and other Th2-type cytokines could be demonstrated, supporting the idea that the induction of these cytokines supported the IgG1 and IgA anti-Ad Ab responses (7). However, significant production of IgG2a anti-Ad Ab was also observed (6, 7) suggestive of costimulated Th1-type responses to the Ad vector. This stimulation of CD4+ Th1 cells was suspected to have resulted from viral protein synthesis associated with Ad vectors (13). Thus, these studies indicate the importance of assessing CD4+ T cell reactivity to the Ad vector.

Additionally, understanding the importance of CD8+ T cell reactivity reveals the contribution of CTLs to the clearance of Ad vector-infected cells. Earlier studies have shown that infection with wild-type Ad elicited CTL responses largely due to induced viral early proteins associated with replication (E1) and to DNA-binding protein E2a and thus may impact upon their role in the development of immunity to Ad (14–17). Despite rendering the Ad vector replication deficient by E1 gene deletion, CTL responses to Ad were still observed (4, 5, 8, 9, 13), and the specificity of CTL responses to replication-deficient vectors could be in part accounted for by the expression of Ad proteins in the transduced cells (13). However, anti-Ad immune reactivity was not eliminated in mice deficient in MHC class I or class II expression (5). Collectively, these studies show that the duration of transgene expression is both CD4 and CD8 T cell dependent.

As proposed, the use of Ad vectors for gene therapy would require multiple instillations. Since the focus of our studies have been to understand the immune consequences following multiple Ad deliveries, this study has focused on the less well described, pulmonary-associated CD8+ T cell responses following i.t. delivery of Ad2/βGal-2 vector. We can demonstrate that repeated, and not single, i.t. instillations into C57BL/6N mice result in the attenuation of CTL responses. This attenuation is shown to be associated with diminished IFN-γ production. These studies have
important implications for the usage of Ad vectors for both gene therapy and mucosal vaccine delivery.

Materials and Methods

**Mice**

Specific pathogen-free male C57BL/6N mice were purchased from the National Cancer Institute at 5 to 6 wk of age and were maintained at the Montana State University Animal Resource Center. All mice were kept under specific pathogen-free conditions in horizontal laminar flow cabinets and were fed sterile food and water ad libitum. The mice were free of bacteri al and viral pathogens as determined by Ab screening and by histopathologic analysis of major organs and tissues. The mice were used at 8 to 12 wk of age in these experiments.

**Ad and virus administration**

The recombinant Ad2/Gal-2 vector and wild-type Ad2 virus were used in this study; they were provided by the Virus Production Unit of Genzyme (Framingham, MA). The Ad2/Gal-2 vector, which lacks the E1 region and has a modified E4 region (removal of all open reading frames except open reading frame 6), carries the *Escherichia coli lacZ* gene under control of the CMV immediate early promoter (8).

Intratracheal administration of the Ad2/Gal-2 vector was performed as previously described without anesthesia (6, 7). Each mouse (five mice per group) received either a single or three doses at 2-wk intervals of 1 × 10^10 infectious units (i.u.) in 50 µl and was sacrificed either 12 days after one instillation or 3 or 4 days following the third instillation. The lungs, lower respiratory lymph nodes (LRLN), and spleens were subsequently isolated.

**Lymphocyte isolation and culture**

Lymphocytes were isolated from the spleen and LRLN, which consist of the mediastinal and hilar lymph nodes, by mechanical disruption followed by Ficoll-Hypaque (Lymphocyte M, Accurate Chemical, Westbury, NY) density gradient centrifugation (6, 7). Lung mononuclear cells were isolated and subjected to collagenase (Worthington, Freehold, NJ) digestion as previously described (6, 7) with >95% cell viability.

To assess the cytokines produced following one or three i.t. instillations of Ad2/Gal-2, freshly isolated mononuclear cells from the LRLN and spleens were pooled from groups of five mice. Cells were cultured in complete medium containing RPMI 1640 with 0.2 mM L-glutamine (<0.1 ng/ml endotoxin; BioWhittaker, Walkersville, MD) and low endotoxin/10% FCS (HyClone, Logan, UT) plus the supplements (Life Technologies, Grand Island, NY), 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. Lymphocytes were cultured at 2 × 10^6/ml for 6 days in the presence of the equal number of Ad2-infected stimulator cells for MHC class I stimulation or without or with 5 × 10^5 i.u./ml of heat-inactivated wild-type Ad2 for MHC class II stimulation (7). Following in vitro restimulation, supernatants were collected for analysis in cytokine-specific ELISAs.

**CTL assay**

To assess CTL activity following a single or three i.t. doses of Ad2/Gal-2, a fluorescent-based CTL assay was adapted (18). Mononuclear cell suspensions were prepared from normal C57BL/6N mouse spleens and infected or mock infected with wild-type Ad2 at 100:1 i.u./cell. Cells were cultured overnight, washed, and treated with mitomycin C (50 µg/ml; Sigma, St. Louis, MO) for 2 h at 37°C. Ad2-infected and mock-infected cells were washed three times to remove residual mitomycin C. Mitomycin C-treated Ad2-infected and mock-infected cells were then loaded with 25 µM acetylated calcein (calcein-AM; Molecular Probes, Eugene, OR) for 30 min at 37°C, then washed twice in HBSS/5% FCS. Targets cells were incubated with varying ratios of lung, LRLN, or splenic lymphocytes for 2 h at 37°C in U-bottom 96-well microtiter dishes (Corning-Costar, Oneonta, NY). Following incubation, cells were spun down, and supernatants were transferred to Maxisorp Immunoplate II 96-well microtiter dishes (Nunc, Roskilde, Denmark) to measure the release of calcein-AM. Specific killing of target cells was determined by measuring the level of released fluorescence from Ad2-infected, but not mock-infected, cells using a Bio-Tek Instruments (Winooski, VT) FL 500 microtiter plate reader. Samples were excited at 495 nm, and emission was measured at 530 nm. The percent cytotoxicity was determined as the level of sample fluorescence corrected for spontaneous release divided by the level of fluorescence obtained by detergent (total) lysis corrected for spontaneous release. The observed cell lysis was Ag specific and not NK cell mediated. No lysis was obtained by incubating effector cells with calcein-AM-loaded YAC-1 cells (TIB 160, American Type Culture Collection, Rockville, MD) at any of the tested E/T cell ratios.

To determine whether alveolar macrophages contributed to the attenuation of the CTL response, mononuclear cells from lung, LRLN, and spleens were incubated with an excess (30 mg) of carbonyl iron (Sigma) to 1 × 10^10 cells in 5 ml of complete medium. The cell and carbonyl iron suspensions were incubated for 3 h at 37°C and were agitation every 30 min. Macrophages that phagocytized the carbonyl iron were separated in the presence of a magnetic field using a magnetic particle concentrator (Dynal, Oslo, Norway), and the remaining cell suspension was then removed and assayed for CTL activity. The effectiveness of carbonyl iron treatment on the removal of macrophages was assessed by flow cytometry using the biotinylated F4/80-like mAb (clone C1:A3-1, Biosource International, Camarillo, CA) and streptavidin-phycocerythrin (Southern Bio-technology Associates, Birmingham, AL). Greater than 95% of the F4/80^+ macrophages were depleted.

**Fluorescent immunolabeling of lymphocytes**

To determine the type of CD3^+ T cells induced following one or three i.t. administrations of Ad2/Gal-2 vector, lymphocytes from lungs, LRLN, and spleens were assessed. Fluorochrome-conjugated mAbs (PharMingen, San Diego, CA) for mouse CD3 (145-2C11), CD4 (RM4-5), and CD8α (53-6.7) were used, and immunofluorescent staining was measured by flow cytometry.

**Cytokine ELISA**

The ELISA method was modified from a previously described protocol (7, 19). Cytokine levels in culture supernatants were determined by a sensitive ELISA, using capture and detection mAbs (PharMingen) specific for the murine cytokines IFN-γ, IL-4, and IL-10 as previously described (19). An alkaline phosphatase (AP)-conjugated goat anti-biotin Ab (1/1000 dilution; Vector Laboratories, Burlingame, CA) was substituted for the horseradish peroxidase-conjugated goat anti-biotin Ab. The fluorescent substrate for AP, 4-methylumbelliferyl phosphate dicyclohexylaminonammonium salt (Molecular Probes), was used to develop the assay, and AP activity was measured using a Bio-Tek Instruments FL 500 microtiter plate reader. To determine the amount of cytokine present in test samples, varying dilutions of recombinant murine IFN-γ (Genzyme), IL-4 (Endogen, Boston, MA), or IL-10 (PharMingen) were run to establish standard curves from which values for the test samples could be extrapolated. Samples were assayed at 360 nm, and emission was measured at 460 nm. The values shown represent the mean of four experiments ± 1 SEM. The detection limits for IFN-γ, IL-4, and IL-10 were 40, 20, and 20 pg/ml, respectively.

**RT-PCR assay for detection of cytokine mRNA**

To assess the induction of mRNA specific for Th1 and Th2 cytokines, total RNA was isolated from ex vivo stimulated or in vitro restimulated mononuclear cells to determine the cytokine profiles subsequent to Ad2/Gal-2 administration. For in vitro restimulated cells, LRLN or spleen cells were lysed in Tri-Reagent (Molecular Research Center, Cincinnati, OH). The resulting RNA was reversed transcribed (45 min at 42°C), employing Superscript reverse transcriptase (Life Technologies), after which the resulting cDNA was amplified on a Perkin-Elmer (Norwalk, CT) model 9600 thermocycler for three cycles of 90 s at 95°C, 90 s at 65°C, and 120 s at 72°C followed by 33 cycles of 45 s at 95°C, 45 s at 65°C, and 60 s at 72°C using cytokine-specific primer pairs. Murine cytokine primer pairs were designed for optimal PCR amplifications using Oligo 4.0 primer analysis software (National Biosciences, Plymouth, MN). Oligonucleotides were checked for the lack of significant homology with all other sequences present in GenBank version 70 (MacVector Sequence analysis software, IBI, New Haven, CT). The following are the positive and negative strand oligonucleotide primer pairs used for PCR: IFN-γ, AAC TCA AGT GGC ATA GAT GTG GA and TCC TTT TCC GCT TCC TGA GGC TGG; IL-4, AGT GCA TCG GCA TTT TTA ACG AGG TC and CCA GTA ATC CAT TTG CAT GCT C; IL-10, GGA CAA CAT ACT GCT AAC CGA CTC and AAT TCA TTC ATG GCC TTA TAG TAC AG; β-actin, GTG GGC CGC TCT AGG CAC CA and CGG TGG GCC GTA GGG TTC AGG GGG G. cDNA PCR product sizes are in base pairs for IFN-γ (346 bp), IL-4 (326 bp), IL-10 (300 bp), and β-actin (349 bp).

**Statistical analysis**

Student’s t test was used to evaluate differences between experimental parameters in each experiment.
Results

Three i.t. instillations of Ad2/βGal-2 suppress the CTL responses to Ad2-infected target cells

Much of our current understanding of CTL responses to Ad vectors is based upon the data obtained with wild-type Ad infections (14–17) or following a single delivery of Ad vectors (3, 5, 9). Alternatively, where more than one dose of Ad vector was administered, T cell dependency was measured as the duration of transgene expression (10, 20). While such studies can provide insight into assessing immunity, it is essential to validate such conclusions if they represent true correlates of immunity following repeated Ad vector delivery. Thus, the basis of the work presented is to directly assess the immune consequences of multiple Ad instillations into the lungs. C57BL/6N mice received either one or three i.t. instillations of Ad2/βGal-2 vector at 2-wk intervals similar to what was previously described (7). Mice receiving a single dose of Ad2 vector were evaluated 12 days after receiving virus. For mice that received three i.t. doses of Ad2 vector, mice were sacrificed 4 days following the last instillation. Total mononuclear cells were obtained from the lungs, LRLN, and spleen and assessed for CTL activity at varying E:T cell ratios. The percent cytotoxicity was expressed as the level of cytotoxicity obtained at each E:T cell ratio corrected for spontaneous release divided by the total release of label corrected for spontaneous release. The results are representative of six separate experiments.

with the greatest CTL activity residing in the lung mononuclear cell fraction (Fig. 1A). In contrast, those mice receiving three i.t. doses of Ad2 vector failed to demonstrate CTL activity at any of the tested effector cell concentrations against Ad2-infected targets by any of the three tissues examined (Fig. 1B). In fact, this lack of CTL activity could not be reversed by in vitro culture for 6 days. LRLN and splenic cells from mice receiving either a single or three i.t. doses of Ad2 vector were cultured with Ad2-infected stimulator cells and analyzed for CTL activity. As anticipated, the LRLN and spleen cells from mice receiving the single dose showed CTL activity. The noted CTL activity was Ad2 specific and not NK cell mediated, since no lysis was obtained against YAC-1 cells (Fig. 2A). However, the mice receiving three i.t. doses showed no evidence of CTL activity (Fig. 2B). This lack of reversibility of CTL activity suggested that the anti-Ad2-specific CTL activity may be suppressed.

Lack of CTL activity by mice receiving three i.t. instillations of Ad2/βGal-2 vector is long lasting

We next questioned whether this lack of CTL activity was temporal or long lived. Mice received the first two i.t. instillations of Ad2 vector were evaluated 12 days after a single i.t. dose, which was found to be optimal for detection of CTL responses. Mononuclear cell suspensions were prepared from lungs, LRLN, and spleens to determine the level of ex vivo CTL activity. In a dose-dependent fashion, CTL responses were obtained for all three tissues tested,
Ad2/βGal-2 vector on days 0 and 14, and were then rested for 3 mo before receiving the last dose of Ad2 vector. Four days later, the mice receiving three i.t. doses of Ad2/βGal-2 vector were examined for ex vivo CTL activity in lungs, LRLN, and spleens. Again, no CTL activity could be detected in any of the tested tissues (Fig. 3A). In vitro culture with Ad2-infected stimulator cells also could not expand potential CTL precursors (Fig. 3B). Thus, this lack of CTL activity was long lived.

Lack of CTL activity by mice receiving three i.t. instillations of Ad2/βGal-2 vector was not due to suppressor tissue macrophages

Evidence from previous studies (21, 22) has shown that alveolar macrophages elicit suppressive activities for T cells. If such is the case, elimination of tissue macrophages should relinquish this inhibition. As before, mononuclear cells from lungs, LRLN, and spleens were obtained from mice receiving three i.t. doses of Ad2/βGal-2 vector. A portion of the cells was subjected to carbonyl iron loading to remove tissue macrophages. The CTL activity of freshly isolated cells subjected to this treatment was examined and compared with that of untreated samples. Removal of tissue macrophages had no effect on ex vivo lung, LRLN, or splenic CTL activity (Fig. 4A). Likewise, when macrophage-depleted cells were cultured with stimulator cells, no CTL activity could be detected (Fig. 4B).

The attenuated CTL response is not attributed to diminished numbers of CD4+ or CD8+ lymphocytes

One plausible explanation for the lack of CTL reactivity could be the reduced number of effector CD4+ or CD8+ T cells. Thus, lymphocytes isolated from the lungs, LRLN, and spleens of mice receiving either one or three i.t. instillations of Ad2/βGal-2 were immunostained for flow cytometry to determine relative levels of CD4+ and CD8+ T cells. Compared with normal mice, mice receiving a single i.t. dose of Ad2/βGal-2 vector showed no change in percentages of CD4+ and CD8+ T cells in the LRLN and spleens. However, noted elevations could be demonstrated in the lungs (Table I); there were approximately four- and sixfold increases in CD4+ and CD8+ T cells, respectively. Likewise, a similar increase in lung CD4+ and CD8+ T cells was noted in three i.t. treated mice, but for the LRLN, 40 and 50% increases over normal mice in the percentages of CD4+ and CD8+ T cells, respectively, were observed. In the spleen, there were no net increases in CD4+ and CD8+ T cells in mice receiving three i.t.

FIGURE 3. The lack of CTL activity from mice that received three i.t. doses of Ad2/βGal-2 vector is long lived. C57BL/6N mice (five per group) received the initial i.t. dose of Ad2 vector on day 0 and the second dose on day 14. Mice were rested for 3 mo and then received a final i.t. dose. CTL activity was measured 4 days later (A) and 6 days after in vitro restimulation with Ad2-infected stimulator cells (B). No lysis was obtained from freshly isolated cells or upon restimulation. Thus, the lack of CTL activity specific for Ad2 is long lived.

FIGURE 4. In vitro depletion of tissues macrophages does not restore CTL activity to Ad2. Mononuclear cells from lungs, LRLN, and spleen were obtained from mice (five per group) that received three i.t. instillations of Ad2/βGal-2 vector. Cells were incubated with excess carbonyl iron for 2 h at 37°C, and macrophages were removed in the presence of a magnetic field. This procedure resulted in >95% depletion of F4/80+ macrophages. When cells were assessed for CTL activity, none was detected (A). When LRLN and splenic cells were cultured in vitro with Ad2-infected stimulator cells for 6 days, no CTL activity could be measured (B).
doses of Ad2 vector (Table I). Thus, the attenuation in CTL activity could not be attributed to a loss of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells.

*The attenuated CTL response in mice receiving three i.t. instillations of Ad2/βGal-2 vector also exhibits a lack of IFN-γ production by both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets*

The loss of CTL activity observed in mice receiving three i.t. doses of Ad2/βGal-2 may have resulted from an attenuation of IFN-γ production. CD8<sup>+</sup> T cells have been shown to produce IFN-γ and to be important for CTL activity (23, 24). To establish whether such a correlation can be made for CTL activity to Ad2 vectors, IFN-γ production was assessed for mice receiving a single dose of Ad2/βGal-2 vector. To determine the elicited Th1- and Th2-type cytokines from both CD8<sup>+</sup> and CD4<sup>+</sup> T cells associated with the attenuated CTL response to the Ad2 vector, cytokine production by CD8<sup>+</sup> T cells was analyzed following in vitro stimulation with Ad2-infected stimulator cells. From mice receiving a single i.t. dose of the Ad2 vector following MHC class I stimulation, LRLN and splenic cultures showed elevated levels of secreted IFN-γ and IL-10, but no IL-4 (Fig. 6A) and induction of mRNA for IFN-γ and IL-10, but not IL-4, when evaluated by RT-PCR (Fig. 6B). Examination of the IFN-γ levels obtained for LRLN cell cultures showed 5-fold more IFN-γ than splenic cell cultures (p = 0.0055). Similar levels of IL-10 could be detected for both LRLN and splenic cultures. IFN-γ, IL-4, and IL-10 were below detectable levels for unstimulated cells. However, mice receiving three i.t. doses of Ad2 vector showed a dramatic attenuation in the production of IFN-γ and IL-10 (Fig. 6). The production of IFN-γ was reduced by approximately 110-fold (p = 0.001) in LRLN cells stimulated via MHC class I (Fig. 6A), and this was confirmed at the mRNA level (Fig. 6B, upper panel). No significant differences were observed for similarly treated splenic cell cultures (Fig. 6A), and this was confirmed at the mRNA level (Fig. 6B). Again, no IL-4 production was observed (Fig. 6). An approximately 28-fold (p = 0.016) reduction in IL-10 was observed for LRLN cell cultures stimulated with Ad2-infected stimulator cells (Fig. 6A) and was confirmed at the mRNA level (Fig. 6B, upper panel). No significant differences in IL-10 production were observed for splenic cell cultures as a result of mice receiving a single or three i.t. doses of Ad2 vector.

Following in vitro stimulation with heat-inactivated Ad2, LRLN and splenic cell cultures from mice receiving a single i.t. dose of Ad2/βGal-2 vector showed elevations in IFN-γ and IL-10 production (Fig. 7, A and B). This indicates that the mice were able to mount a CD4 T cell response to the Ad2 vector. In addition, differences in the amount of IFN-γ generated were noted between LRLN and splenic cell cultures. More IFN-γ was generated in splenic than in LRLN cultures (p = 0.049), suggesting that these two tissues differ in their responses to Ad2.

### Table I. Levels of CD4<sup>+</sup> and CD8<sup>+</sup> T cells obtained from lungs, LRLN, and spleens after three i.t. instillations of Ad2/βGal-2 vector<sup>a</sup>

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Normal % CD4&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Normal % CD8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>One i.t. instillation % CD4&lt;sup&gt;+&lt;/sup&gt;</th>
<th>One i.t. instillation % CD8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Three i.t. instillations % CD4&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Three i.t. instillations % CD8&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>5.33</td>
<td>2.29</td>
<td>20.94</td>
<td>12.75</td>
<td>17.72</td>
<td>11.57</td>
</tr>
<tr>
<td>LRLN</td>
<td>20.65</td>
<td>14.13</td>
<td>20.05</td>
<td>15.63</td>
<td>29.19</td>
<td>21.13</td>
</tr>
<tr>
<td>Spleen</td>
<td>18.26</td>
<td>11.78</td>
<td>19.20</td>
<td>11.53</td>
<td>17.82</td>
<td>11.44</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mononuclear cells were isolated from lungs, LRLN, or spleens obtained from normal mice or at 4 days after the first or third i.t. dose of Ad2 vector. Cells were stained for CD4 and CD8 and analyzed by flow cytometry.

As with cells stimulated via MHC class I, an attenuation of IFN-γ production following three i.t. doses of Ad2/βGal-2 vector was detected for LRLN and splenic CD4<sup>+</sup> T cells following MHC class II stimulation (Fig. 8A). When LRLN and splenic cultures were stimulated with the optimal dose of heat-inactivated Ad2 virus, as previously shown (7), approximately 65 and 83% (p = 0.0299) reductions in IFN-γ production, respectively, were obtained compared with those in mice receiving only a single i.t. dose of Ad2/βGal-2 vector. mRNA for IFN-γ and IL-10 could be detected for both LRLN and spleen; however, minimal mRNA was detectable for splenic IL-10 (Fig. 8B). As shown with cells stimulated via MHC class I, when cells were stimulated via MHC class II, no secreted IL-4 was detected by ELISA for either treatment regimen, although mRNA could be detected by RT-PCR (Fig. 8B). In addition, restimulation of LRLN and splenic cultures via MHC class II resulted in attenuation of the IL-10 response (Fig. 8A). A 14-fold reduction (p = 0.0444) in the amount of secreted IL-10 was observed for LRLN cultures, and approximately a 3-fold reduction (p = 0.017) in IL-10 was found for splenic cell cultures. Collectively, these results demonstrate that the loss of CTL activity may in part be attributed to the attenuated production of IFN-γ by CD4<sup>+</sup> or CD8<sup>+</sup> T cells, and this attenuation was not attributed to increased production of IL-4 or IL-10.
Discussion

Gene therapy provides an alternative for the phenotypic rather than the genomic correction of genetic deficiencies. As such, Ad vectors have been proposed as a means for correcting the aberrant cystic fibrosis transmembrane conductance regulator (CFTR) gene in CF patients by transducing their respiratory epithelia with the normal CFTR gene (25, 26). Several groups have used the nonreplicating Ad2 or Ad5 vectors for this purpose due to the viral tropism for the respiratory epithelium. Further, the relative ease of inserting foreign genes and the ability to transfect nonreplicating cells (27) have made Ad2 and Ad5 vectors attractive for gene transfer, although transgene expression mediated by Ad or any other gene delivery system will be limited by the rate of normal epithelial cell regeneration (28, 29). Consequently, multiple transductions would still be required to preserve corrective levels of CFTR expression.

This requirement for multiple gene transductions, in short, represents a form of immunization that can result in significant host immunity and inflammation to the Ad vector after a single dose. As a result, a number of studies have raised important questions about the induction of immunity and inflammation in these Ad vectors, which decreases the efficacy of transgene delivery and the duration of expression (4–6, 30–32). In fact, it could be shown that a substantial immune CTL response was elicited to the reporter genes (8, 33). Likewise, elevated Ab (6, 7) and CD4+ Th cell (7) responses could be induced by reporter genes.

In an effort to discern the mechanisms responsible for the reactivity to Ad vectors, we hypothesized that it is necessary to perform repeated Ad instillations into the lungs to represent the proposed human CFTR gene therapy. As evidenced in this study and were only correlated to the duration of transgene expression rather than to specific immune parameters. Albeit the ultimate answer is how to maintain transgene expression, insight into the specific immune mechanisms will dictate the feasibility of such treatments.

The approach of our studies is to understand the relevant mechanisms responsible for immunity to Ad vectors. It is clear from the earlier studies that the host does elicit both MHC class I-dependent CTL (4, 5, 8, 9, 13) and MHC class II-dependent T and B cells (5–7, 12) responses to Ad vectors after a single dose. LRLN and splenic mononuclear cells produced elevated levels of IFN-γ and IL-10, but not IL-4. IFN-γ levels were more prominent for the splenic cell cultures. IFN-γ, IL-4, and IL-10 levels for LRLN and splenic cells cultured without heat-inactivated Ad2 were below the level of detection. The indicated values are the mean of four experiments ± SEM.
as others have shown (4, 5, 8, 13, 19), a single i.t. instillation into the lungs does result in CTL activity against Ad-infected targets, which is not NK cell mediated. Most notably, the CTL activity resided with T cells obtained from the lungs, while lesser activities were observed in LRLN and spleens. However, following in vitro restimulation, enhanced LRLN and splenic CTL activities were detected compared with those of freshly obtained cells. How our results differ from previous studies is the observation that no CTL activity could be detected from mice receiving three i.t. doses of Ad2 vector. No CTL activity could be measured in freshly splenic nuclear cells from mice (five per group) that received three i.t. doses of Ad2 vector were cultured for 3 days with heat-inactivated wild-type Ad2. Supernatants were collected and assayed for the production of IFN-γ, IL-4, and IL-10 by cytokine-specific ELISA (A), and mRNA from LRLN (upper panel) and splenic (lower panel) cultures was assessed by RT-PCR (B). LRLN and splenic cells showed 65 and 83% reductions in IFN-γ, respectively. The observed attenuation of IFN-γ was not due to IL-4, since none was detected; however, mRNA for IL-4 could be detected by RT-PCR. Increased production of IL-10 was not observed, since the three i.t. doses of Ad2 vector resulted in a reduction of IL-10. The indicated values represent the mean of four experiments ± SEM.

FIGURE 8. Repeated i.t. instillation of Ad2/βGal-2 attenuated IFN-γ production following MHC class II stimulation. LRLN and splenic mononuclear cells from mice (five per group) that received three i.t. doses of Ad2 vector were cultured for 3 days with heat-inactivated wild-type Ad2. Supernatants were collected and assayed for the production of IFN-γ, IL-4, and IL-10 by cytokine-specific ELISA (A), and mRNA from LRLN (upper panel) and splenic (lower panel) cultures was assessed by RT-PCR (B). LRLN and splenic cells showed 65 and 83% reductions in IFN-γ, respectively. The observed attenuation of IFN-γ was not due to IL-4, since none was detected; however, mRNA for IL-4 could be detected by RT-PCR. Increased production of IL-10 was not observed, since the three i.t. doses of Ad2 vector resulted in a reduction of IL-10. The indicated values represent the mean of four experiments ± SEM.

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since their induction occurs early, elevated pre-existing, circulating anti-Ad Ab are sustained (39). Although elevated anti-Ad Ab titers occur in circulation, it is important to determine their neutralization capacities. In our recent study we could demonstrate a reduced neutralization capacity in mice receiving three i.t. doses of Ad vector. This would infer, then, that the neutralization activity may also be reduced as a result of multiple Ad doses. Finally, reduced transgene expression may be in part influenced by the type of transgene used for analysis, since the expression of self-proteins would not be expected to induce immune reactivity (31, 32).

In summary, the replication-deficient Ad vector, Ad2/ΔGal-2, can stimulate T cell reactivity in both the systemic and mucosal compartments of the lower respiratory tract. However, multiple i.t. instillations of the Ad2 vector attenuate both CD4 and CD8 T cell reactivities, and no CTL activity to Ad2-infected targets could be detected. This lack of CTL reactivity was enhanced by the attenuation of IFN-γ production, but not by increased IL-4 or IL-10 production. Thus, current Ad vectors may be suitable for proposed gene therapy, provided the initial reactogenicity to Ad vectors is acceptable.

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References


