Adenovirus–human immunodeficiency virus (HIV) envelope recombinant vaccines elicit high-titered HIV-neutralizing antibodies in the dog model


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ABSTRACT Recombinant human adenoviruses (Ads) (types 4, 5, and 7) expressing the HIV-1 envelope membrane glycoprotein (gp160) were tested for immunogenicity in the dog. Administration of recombinant Ad7-env by intratracheal inoculation resulted in a low serum antibody response to gp160, which developed over several weeks. A strong neutralizing antibody response to the Ad7 vector developed within 1 week of infection. A subsequent booster inoculation 12 weeks later with the heterotypic Ad4–env recombinant virus resulted in significantly enhanced humoral responses directed at the envelope antigen, as measured by both ELISA and Western blot analysis as well as high-titer type-specific neutralizing antibodies, with some animals achieving neutralization titers approaching 1000. Recombinant HIV envelope glycoprotein derived from Ad–HIV-infected cell cultures was used as a subunit booster injection for dogs that had previously received sequential immunizations with heterotypic recombinant Ads. Significant immune responses against the envelope developed as measured by ELISA, Western blot analysis, and neutralization assays. These data indicate that live recombinant Ad–HIV vaccines are capable of inducing high-titer type-specific neutralizing antibodies to gp160 in vivo. Recombinant HIV envelope glycoprotein subunit vaccines, prepared from Ad–env-infected cells, are capable of boosting these responses.

AIDS has been linked etiologically with the HIV-1 by various investigators (1–3). Numerous approaches to producing a safe and efficacious vaccine for HIV have been pursued by several laboratories, ranging from employment of whole inactivated virus (4) to using recombinant protein subunit preparations (5). The development and progress of various HIV vaccines has recently been reviewed (6). The major HIV antigen targeted for use in vaccines is the membrane-associated envelope glycoprotein gp160 or one of its cleavage products, gp120. Recombinant virus-vectorized vaccines, primarily using vaccinia virus, have been tested in chimpanzees (7) and in human clinical studies (8). These recombinant viruses were demonstrated to induce seroconversion to the HIV envelope antigen and cell-mediated immune responses, as measured by antigen-specific proliferation assays, in both chimpanzees and humans. However, they failed to produce high-titer neutralizing responses and did not protect chimpanzees from challenge with infectious HIV-1 (7).

Human adenovirus (Ad) has received increasing attention as a potential vector for recombinant viral vaccines. Recently, Ad-vectorized recombinant vaccines have been constructed using viral genes from a variety of animal viruses (9, 10). Advantages of recombinant Ad-vectored vaccines include (i) safety [human Ad vaccines, types 4 and 7, have proven safe and efficacious during use in military recruits for >30 years] and (ii) the antigenic diversity of Ads (>45 serotypes described), which may allow exploitation of antigenically distinct vectors for multiple sequential booster immunizations.

In vivo analysis of recombinant Ad vaccines has been difficult due to the extreme host-range restriction of human Ads. Ad5-vectorized vaccines have been tested in hamsters and cotton rats, but these models are not permissive for Ad4 and Ad7 recombinants. Ad5–HIV vectors expressing the gp160 were tested for immunogenicity in cotton rats (11). We have since reported the enhancement of envelope expression mediated by coexpression of the rev gene by using recombinant Ad7 vectors in vitro (12). In the present study, in addition to the Ad7 recombinant, we have also made use of an Ad4–env and an Ad5–env recombinant. All recombinant viruses described in this report contain the HIV rev regulatory gene. Two additional animal models have been described for evaluation of recombinant Ad4 and Ad7: the chimpanzee, the only permissive model for oral-enteric Ad infection (13), and the laboratory beagle, which undergoes a semipermissive or abortive infection after deposition of virus in the lung by intratracheal inoculation (14). Unfortunately, the chimpanzee is not a practical animal model for initial testing and screening of recombinant viruses due to a lack of availability and high expense. In contrast, the beagle has proven useful for evaluating humoral immune responses to foreign viral antigens in spite of its low permissivity for human Ads (14). In this report, the dog model has been employed to show that sequential administration of serologically distinct recombinant Ads was effective in eliciting strong humoral responses to HIV envelope glycoprotein, including high-titer neutralizing antibodies. Furthermore, we demonstrate that recombinant envelope produced in the Ad system effectively boosts immune responses primed by live recombinant Ads.

MATERIALS AND METHODS

Construction of Recombinant Ads. The procedure for generation of recombinant Ads has been described (12). To confirm the DNA structure of each recombinant, DNAs were extracted by the method of Hirt (15) and analyzed by restriction enzyme digestion.

Purified HIV-1 Envelope Antigen Preparations. Ad–HIV-infected A549 cells were suspended in 10 vol of 0.05 M Tris-HCl/0.15 M NaCl, pH 7.4, and centrifuged for 30 min at 7777

Abbreviations: HIV, human immunodeficiency virus; Ad, adenovirus; T-MDP, threonylmuramyl dipeptide.

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25,000 × g. The pellet fraction was suspended in 0.05 M Tris-HCl/0.15 M NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.2 mM phenylmethylsulfonyl fluoride, pH 8, shaken gently for 30 min, and centrifuged for 30 min at 25,000 × g. The supernatant was collected, and CaCl₂ and MnCl₂ were added to final concentrations of 0.5 mM. The solution was applied to a column of lentil lectin-Sepharose 4B, equilibrated with 0.05 M Tris-HCl/0.5 M NaCl, pH 8. The column was washed with 500 ml of equilibration buffer and bound glycoproteins were eluted with 0.05 M Tris-HCl/0.5 M NaCl/1 M α-methylmannopyranoside, pH 8. The eluted fraction was dialyzed extensively against 0.02 M Tris-HCl/0.15 M NaCl/0.1 M betaine, pH 8, and applied to an anion-exchange column of Fractogel EMD TMAE-650 (E. M. Separations, Gibbstown, NJ) equilibrated with the above buffer. The flow-through from the column was dialyzed against phosphate-buffered saline and used for immunization. This fraction, referred to as gp120/gp160, was 95% pure env proteins, composed of 90% gp120 and 10% gp160. It was resuspended at 45 μg/ml in 0.2% alum, 0.2% alum/0.25% deoxycholate, or Syntex conjugate containing 200 μg of threonyl-Ala-threonyl-Ala-threonyl-Lys (T-MDP).

gp120 from isolate MN was purified as described (16). Baculovirus-derived gp120 was purchased from the Repligen (Cambridge, MA).

Inoculation of Dogs. Beagles obtained from Marshall Farms (North Rose, NY) were used in studies at 6–12 months of age. Recombinant Ads in 3–5 ml of medium were inoculated intratracheally. Subunit env preparations (50 μg per dog) were administered subcutaneously in 1.1 ml containing adjuvant as indicated. Dogs were bled from the cephalic vein at the indicated times and serum was prepared.

ELISA for Antibodies to HIV-1 Envelope. An HIV ELISA kit (DuPont) was used according to the manufacturer’s instructions except that affinity-purified and alkaline phosphatase-labeled goat anti-dog IgG and IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were substituted for detection of dog antibodies.

A peptide ELISA (17) was used to monitor antibody binding to the V3 loop peptide of HIV-1/HTLV-IIIIB envelope (amino acids 306–328). The peptide was obtained from Multiple Peptide Systems (San Diego, CA). Binding of serial dilutions of dog sera was detected using a goat anti-dog IgG peroxidase conjugate. Binding titer was defined as the reciprocal of the serum dilution at which absorbance of the test serum equaled the absorbance of a normal control serum diluted 1:100.

Western Blot Analysis to Detect Anti-HIV-1 Envelope. A commercial Western blot kit (DuPont) or Western blot strips (DuPont) were used according to the manufacturer’s instructions with the following modifications: affinity-purified and biotin-labeled goat anti-dog IgG antibody (heavy and light chain specific, 1:500 dilution; Kirkegaard and Perry Laboratories) or alkaline phosphatase-labeled goat anti-dog IgG antibody (heavy and light chain specific, 1:500 dilution; Kirkegaard and Perry Laboratories) was substituted for labeled anti-human IgG; avidin-peroxidase (1:200 dilution; Boehringer Mannheim) was used in place of the avidin-peroxidase reagent supplied in the kit.

HIV-1 Neutralization. The assay for neutralization of cell-free HIV has been described (18). H9 cells (1) were used as targets for infection, and either a frozen titered stock of HIV-1/HTLV-IIIIB (1) or fresh H173-1/HTLV-IIIIMN (2, 19) from culture supernatant were the infecting viruses. Endpoint titers were defined as the reciprocal of the serum dilution at which infectivity levels were 60% of control values after normalization of the data to control infectivity levels.

Adenovirus Vaccine Neutralization Assay. Type-specific neutralizing antibody activity was determined using A549 cell monolayers as described (14). Titers were expressed as the reciprocal of the lowest dilution at which 50% cytopathic effect was observed.

RESULTS

Virus Construction and Expression of HIV-env in Tissue Culture. Recombinant Ads were generated that contained the gp160 HIV envelope gene along with a copy of the rev gene. These recombinant viruses maintained the foreign DNA in a stable fashion. All constructs expressed roughly the same amount of recombinant envelope antigen, 2–5 μg per 10⁶ cells. There were no significant differences in the growth kinetics of these viruses (data not shown).

Humoral Responses to Ads. Intratracheal inoculation of dogs with recombinant Ad7-env viruses (1–5 × 10⁹ plaque-forming units per dog) resulted in generation of type-specific serum neutralizing antibody responses to the Ad vectors with titers >1000 by 2 weeks after infection. Antibodies were not induced in animals that received UV-inactivated viruses, indicating that de novo expression of viral proteins was required for generation of neutralizing antibodies.

Humoral Responses to HIV Envelope Glycoprotein. Primary infection of the dog lower respiratory tract with the Ad7-env recombinant viruses induced detectable serum antibody responses to the HIV envelope antigen in most animals. A weak anti-gp160 response was detected 6 weeks after infection by Western blot analysis (Fig. 1). Upon administration of the Ad4–env recombinant 13–18 weeks after primary immunization, the secondary antibody response to HIV envelope antigens was significantly augmented in terms of magnitude of the response and fine specificity (detection of gp120 and gp41 antibodies, Fig. 1).

Induction of HIV Neutralizing Antibodies by Ad Recombinants. In a second experiment (Table 1), dogs were injected with Ad4–env, Ad5–env, and Ad7–env viruses at 12-week intervals (10⁹ plaque-forming units per dose). Again, a strong immune response to the HIV envelope was not observed by ELISA or Western blot analysis until after the first heterotypic booster injection. This booster injection, however, also elicited strong HIV-neutralizing antibody in seven of eight dogs ranging from 35 to 930. Administration of a second heterotypic booster injection failed to significantly enhance immune responses generated against the HIV envelope compared to the first booster injection.

Humoral Responses by Ad-Derived Recombinant HIV-env Subunit. Recombinant HIV-env produced by Ad–HIV was next tested for its ability to enhance immune responses in dogs immunized with Ad–HIV recombinant viruses. The gp120/gp160 (50 μg per dog) was evaluated in the context of three adjuvants: alum, alum plus deoxycholate (0.25%), or

![Fig. 1.](image-url) Western blot analysis of dog serum antibody responses to HIV antigens. Dogs 211 and 215 were injected intratracheally with Ad7-env virus and given a booster injection 14 weeks later with Ad4–env virus. Dog sera collected at the indicated weeks after infection (as shown by lane labels) were tested at 1:100 dilution. A control human serum (C) was used to detect the positions of gp160, gp120, and gp41. Arrows indicate time of booster immunization.
Syntex supplemented with T-MDP. All recombinant envelope subunit preparations were demonstrated by Western blot analysis to boost antibody responses to gp160, gp120, and gp41 (Fig. 2). Syntex adjuvant induced the highest immune responses, with gp160 reactivity detected at a serum dilution of 1:10,000.

After the subunit booster injection, neutralizing antibody titers also rose significantly (Table 2). In dogs inoculated with Ad7-env recombinant, after the first booster injection with Ad4-env, neutralizing antibodies were elicited in 9 of 11 dogs with titers ranging from 30 to 780. These titers declined over the subsequent 10 weeks, although in general neutralizing activity could still be detected. The booster injection with gp120/gp160 subunit resulted in restoration of neutralizing antibody activity, with 7 of 11 dogs exhibiting higher titers than those seen after the Ad4-env booster injection. In fact, dogs 226 and 168, which failed to display significant neutralizing activity after the Ad4-env booster injection, demonstrated good neutralizing activity after the booster injection with envelope subunit, indicating that they had been primed by the recombinant immunizations. Dogs that received the subunit booster injection in Syntex plus T-MDP (dogs 211, 183, 169, and 226) or in alum plus deoxycholate (dogs 215, 187, 170, and 168) exhibited greater increases in neutralizing antibody titers compared to dogs who received the envelope booster injection formulated in alum alone (dogs 196, 204, and 208). Dog 206, which received a primary immunization of Ad7-env recombinant followed by the envelope subunit in alum with no intervening Ad4-env recombinant booster injection, failed to seroconvert to envelope antigen (Fig. 2) or to develop neutralizing activity (data not shown).

Table 2 also illustrates that after immunization with two Ad recombinants and an envelope subunit booster injection, all 11 dogs exhibited antibodies by a peptide ELISA to the V3 loop of HIVIIIB. The V3 region has been shown to elicit predominantly type-specific neutralizing antibodies (20), and in fact type-specific neutralization of only the IIIB isolate and not MN was observed (data not shown).

After a period of ~7 months, these same animals were given another booster injection with an envelope subunit in alum consisting of either gp120 of the IIIB strain, expressed in the Ad or the baculovirus system, or of the MN strain, purified from cultures of productively infected human T cells. At the time of this inoculation, neutralizing antibodies could be detected in only 5 of the dogs. Two to 4 weeks after this booster, however, neutralizing antibodies could be observed in 9 of the 11 dogs, although they did not reach levels observed previously (Table 2). The best boosting effect was seen in dog 196, which received as subunit MN gp120. Not only did this heterologous protein boost the neutralizing antibody titer against the IIIB isolate, it also elicited low neutralizing activity (titer of 1:20) to the MN strain itself.

A comparison of the humoral immune responses elicited by various schedules of inoculation with Ad recombinants and envelope subunit is shown in Table 3. No detectable antibody to HIV envelope was elicited in dogs injected only with subunit preparations. In two of three dogs immunized with subunit prior to recombinant virus administration a weak anti-envelope response was detected. Three of five dogs that received a single immunization with Ad4–HIV recombinant virus exhibited significantly higher immune responses after administration of the subunit preparation, as measured by ELISA and Western blot analysis. However, this immunization regimen elicited only low-titer HIV-neutralizing antibodies. As before, dogs treated with two heterotypic recombinant Ad–HIV envelope viruses produced antibody responses to envelope at high titer as measured by ELISA, Western blot analysis, and HIV-neutralization assays.

**DISCUSSION**

In this study recombinant HIV vaccines were constructed using as vectors Ad4, Ad7, and Ad5 vaccine strains that had
been safety-tested in humans. Recombinant viruses contained the entire envelope gene and a copy of the rev regulatory gene, which has been demonstrated to enhance expression of gp160 in the recombinant Ad system (12).

Other investigators have reported that the Ad5–HIV recombinant virus that expresses HIV envelope gp160 (E3 region deletion and insertion) induces anti-envelope antibodies in cotton rats after intranasal inoculation (11). Although antibody responses were detected by ELISA and Western blot analysis, neutralizing antibody responses were not reported. However, in the present study, Ad–HIV recombinant viruses in the dog have elicited potent type-specific neutralizing antibody responses. Heterotypic booster immunizations with a second Ad–HIV recombinant virus were required to obtain optimal immune responses. We also demonstrated that gp120/gp160 preparations derived from Ad7-env recombinant virus-infected cells effectively boosted anti-envelope antibody responses in dogs previously immunized with Ad–HIV recombinants. However, anti-HIV neutralizing antibody activity was boosted significantly only in dogs that received two immunizations with Ad–HIV recombinants prior to subunit administration. In addition, the timing of these booster injections was important. While a schedule of immunization every 3 months resulted in high neutralizing antibody activity, a hiatus of 7 months before a second subunit inoculation resulted in generally low neutralizing antibody titers. Human Ads have a very restricted host range and infect the dog poorly, undergoing an abortive infection. It will be crucial to test whether improved priming by Ad recombinants with resultant improvement of immunologic memory occurs in a more permissive host such as the chimpanzee and ultimately humans.

The immunological mechanism(s) responsible for protection against HIV infection continues to remain uncertain. Whereas some aspect of cell-mediated immunity (such as cytotoxic T-cell activity) would be expected to be essential for elimination of HIV-infected cells, the development of humoral immunity to HIV would be expected to play a critical role in protection against infection, spread, and clearance of cell-free virus. Due to the demonstrated antigenic diversity among HIV isolates worldwide, it is obvious that induction of a neutralizing antibody response that recognizes group-specific epitopes would be highly desirable. The envelope glycoprotein has been identified as the primary target of neutralizing antibodies and has been shown to contain both group- and type-specific determinants. Others have shown that type-specific neutralizing antibody can protect chimpanzees from challenge with cell-free HIV (21). The same investigators demonstrated protection of chimpanzees that were immunized with recombinant gp120. A principal neutralization determinant has been identified in the third variable loop of the gp120 portion of the envelope gene (22). Neutralizing antibodies directed against the principal neutralization determinant appear for the most part to be type-specific, although 60% of HIV-1 isolates examined in one study were demonstrated to contain a conserved sequence of Gly-Pro-Gly-Arg-Ala-Phe within the principal neutralization determinant (23). Certain strains, such as the MN isolate, appear to be serologically more related to the majority of U.S. field isolates than other strains, such as the LAV isolate (24). It therefore might be possible to use a single isolate such as the MN strain to produce an effective vaccine that will protect across a wide spectrum of HIV strains. In this regard, it is of interest that in one dog, inoculation of the MN envelope protein boosted the neutralizing antibody against the original immunizing strain IIIB and also resulted in low-titer neutralizing antibody to the MN isolate itself (Table 2). This may reflect immunologic recognition in this animal of the shared tip of the V3 loop, resulting in broadening of immunologic specificity. Alternatively, a mixture of several strains possessing antigenically diverse envelope antigens may be necessary to induce broad protection.

![Fig. 2. Enhancement of humoral responses to HIV-env by subunit booster injection. Dogs that had been immunized with Ad7-env and given a booster injection (14 weeks later) with Ad4-env recombinant virus received a subcutaneous booster injection with Ad-derived recombinant gp120/gp160 (50 μg per dose) 12 weeks after the first booster immunization. Sera collected 1 week prior to (−1) and 2 weeks after subunit booster immunization were tested as described in Fig. 1. A human Ad HIV anti-serum was used to detect the positions of the HIV glycoproteins gp160, gp120, and gp41. Lane labels indicate the time (weeks) after the booster injection, the number of the dog, and the adjuvant used.](image_url)

## Table 2. Neutralizing antibody responses in dogs sequentially immunized with Ad7– and Ad4–env recombinants and HIV–env subunits

<table>
<thead>
<tr>
<th>Dog</th>
<th>Primary immunization (Ad4–env)</th>
<th>First subunit booster immunization (Ad gp120/gp160; 2 wk)</th>
<th>Subunit injected</th>
<th>HVIIB neutralization titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 wk</td>
<td>12 wk</td>
<td>gp120/gp160 IIIB</td>
<td>gp120/gp160 IIIB</td>
</tr>
<tr>
<td>168</td>
<td>&lt;10</td>
<td>10</td>
<td>225 (900)</td>
<td>Ad gp120</td>
</tr>
<tr>
<td>169</td>
<td>310</td>
<td>70</td>
<td>455 (1700)</td>
<td>Ad gp120</td>
</tr>
<tr>
<td>170</td>
<td>30</td>
<td>85</td>
<td>170 (840)</td>
<td>Ad gp120</td>
</tr>
<tr>
<td>215</td>
<td>55</td>
<td>15</td>
<td>15 (550)</td>
<td>Ad gp120</td>
</tr>
<tr>
<td>208</td>
<td>65</td>
<td>&lt;10</td>
<td>95 (1700)</td>
<td>Bv gp120</td>
</tr>
<tr>
<td>211</td>
<td>630</td>
<td>95</td>
<td>190 (830)</td>
<td>Bv gp120</td>
</tr>
<tr>
<td>226</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>425 (6100)</td>
<td>Bv gp120</td>
</tr>
<tr>
<td>183</td>
<td>290</td>
<td>15</td>
<td>65 (700)</td>
<td>Native gp120 MN</td>
</tr>
<tr>
<td>187</td>
<td>60</td>
<td>15</td>
<td>565 (1850)</td>
<td>Native gp120 MN</td>
</tr>
<tr>
<td>196</td>
<td>780</td>
<td>200</td>
<td>390 (4200)</td>
<td>Native gp120 MN</td>
</tr>
<tr>
<td>204</td>
<td>55</td>
<td>15</td>
<td>185 (570)</td>
<td>Native gp120 MN</td>
</tr>
</tbody>
</table>

Dogs were immunized with Ad7–env recombinant, 13 weeks later with Ad4–env recombinant, and 13 weeks later with gp120/160 subunit. Seven months later they received an additional subunit boost as indicated. Weeks (wk) after immunization are shown; the binding titer to the IIB V3 loop peptide is shown in parentheses. All preimmune dog sera had neutralizing titers of <10. Bv, baculovirus.
recombinant Ad system can be adapted to express numerous envelope antigens by insertion of different envelope genes into separate vectors of the same serotype. Furthermore, this system has the potential advantage of boosting the immune response to recombinant antigen by administration of heterotypic recombinant Ads.

In summary, we have examined the potential of the recombinant Ad system for delivery and presentation of the HIV envelope antigen. This system can be manipulated to elicit high-titer type-specific antibody responses to HIV isolates by using booster injections of heterotypic recombinant viruses expressing the same recombinant envelope glycoprotein. We have also demonstrated that recombinant Ads are capable of inducing high-titer neutralizing antibodies directed at the envelope antigen. However, in addition to the generation of a potent humoral antibody response to HIV antigens, we believe that it is also likely that replicating recombinant Ad will effectively induce cell-mediated immune responses. However, further work is required to address the problem of inducing a broader neutralizing antibody response that will recognize a variety of HIV isolates.

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