Heterologous Human Immunodeficiency Virus Type 1 Priming-Boosting Immunization Strategies Involving Replication-Defective Adenovirus and Poxvirus Vaccine Vectors

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We compared the human immunodeficiency virus type 1 (HIV-1)-specific cellular immune responses elicited in nonhuman primates by HIV-1 *gag*-expressing replication-defective adenovirus serotype 5 (Ad5) or poxvirus vectors, used either alone or in combination with each other. The responses arising from a heterologous Ad5 priming-poxvirus boosting regimen were significantly greater than those elicited by homologous regimens with the individual vectors or by a heterologous poxvirus priming-Ad5 boosting regimen. The heterologous Ad5 priming-poxvirus boosting approach may have potential utility in humans as a means of inducing high levels of cellular immunity.

There is increasing evidence that human immunodeficiency virus type 1 (HIV-1)-specific cellular immune responses, particularly those associated with CD8⁺ cytotoxic T lymphocytes (CTL), play an important role in controlling persistent viral replication. A decline in acute HIV-1 viremia was found to be coincident with the appearance of virus-specific CTL (11). Various rates of disease progression in infected humans have been associated with specific major histocompatibility complex class I alleles, suggesting control mediated by defined CTL epitopes (7, 10, 13). In addition, immunization of macaques with vectors expressing internal viral gene products (e.g., Gag and Pol) yields a reduction of viremia and slower disease progression upon subsequent challenge with the simian-human immunodeficiency virus 89.6P (2, 18), while experimental depletion of CD8⁺ cells in simian immunodeficiency virus-infected macaques results in increases in steady-state viremia (9, 17). Finally, there is evidence that enhancement of host cellular immunity, through either vaccination or therapy interruption, could lead to enhanced immunodeficiency virus control (12, 14, 16, 20).

Recently, we evaluated the ability of several vaccine vectors, DNA, replication-defective adenovirus type 5 (Ad5), and modified vaccinia virus Ankara (MVA) to induce specific cellular immune responses against the HIV-1 Gag protein (5, 6). The Ad5-based vaccine proved to be very potent in eliciting such immune responses in nonhuman primates. However, the potency of the Ad5 vector in humans is likely to be compromised by preexisting vector-specific neutralizing antibodies, as was noted in earlier animal model studies (5, 21). The antivector

immunity will also likely attenuate booster immunization responses when the same vector is used for both immune priming and boosting. Accordingly, in the present study, we assessed the ability of poxvirus vectors (3, 8, 15) to boost Ad5-primed responses as a means of enhancing the levels of vaccine-elicited responses.

Construction of an optimized E1[−] E3⁺ replication-defective Ad5 vector expressing a codon-biased HIV-1 (strain CAM1) *gag* gene has been described previously (22). An Ad6 vector was also constructed by use of a homologous bacterial recombination strategy similar to that described for the Ad5 construct; the *gag* gene was placed under the control of the same human cytomegalovirus early promoter and bovine growth hormone polyadenylation signal as in the Ad5-*gag* construct. Ad6 and Ad5 represent closely related human Ad group C serotypes. The construction and generation of the MVA vector expressing the codon-optimized HIV-1 CAM1 *gag* were also described previously (5). In addition to evaluating MVA, we evaluated the canarypox virus ALVAC vector, as well as the replication-defective NYVAC poxvirus vectors (4, 8, 19). Both vectors were constructed by following established protocols (4, 19) to express the same codon-optimized CAM1 *gag* gene.

Indian rhesus macaques (*Macaca mulatta*) were given intramuscular injections of the test vaccines under the following regimens: (i) 10⁹ vector particles (vp) of Ad5-*gag* at months 0, 1, and 6; (ii) 10⁹ PFU of MVA-*gag* at months 0, 1, and 6; or (iii) 10⁹ vp of Ad5-*gag* at months 0 and 1, followed by 10⁹ PFU of MVA-*gag* at month 6. In all cases, the total vaccine dose was suspended in 1.0 ml of phosphate-buffered saline. The macaques were anesthetized (ketamine-xylazine), and the vaccines were delivered intramuscularly in 0.5-ml aliquots into both deltoid muscles by use of tuberculin syringes (Becton-Dickinson, Franklin Lakes, N.J.). Animal studies were performed in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (13a) and with the approval of the Institutional Animal Care and Use Commit-

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FIG. 1. Frequencies of Gag-specific IFN- γ -secreting cells from macaques immunized with homologous or heterologous priming-boosting regimens of Ad5-gag and/or MVA-gag. Two priming immunizations were given at weeks 0 and 4, followed by a single booster inoculation at week 26 or 27. The frequencies are expressed as the number of SFC/10⁶ PBMC and were calculated as the differences in responses between the PBMC stimulated with the Gag 20-aa peptide pool and the mock-treated PBMC; animal identification numbers are indicated along the x axis. The responses were determined prior to immunization (pre), at weeks 4 (post-dose 1) and 8 (post-dose 2), at time of boosting (pre-boost), and at 4 and 8 weeks postboosting. NA, not available.

tees of the New Iberia Research Center and Merck Research Laboratories.

Peripheral blood mononuclear cells (PBMC) collected during the course of immunization were analyzed for levels of Gag-specific T-cells by using the gamma interferon (IFN- γ) enzyme-linked immunospot assay (1). Antigen stimulation of cytokine production was achieved by adding to the PBMC a pool of 20-amino-acid (aa) peptides that encompassed the entire HIV-1 CAM1 Gag sequence with 10-aa-length overlaps (Synpep Corp., Dublin, Calif.). Figure 1 shows the number of IFN- γ spot-forming cells (SFC) per 10⁶ PBMC for each animal following the priming and booster doses. For comparative analyses of enzyme-linked immunospot assay data, the 95% confidence intervals (95% CI) of the cohort geometric means (GM) were calculated. The difference between two data sets was statistically significant if the 95% CI of the GM of the mock-subtracted SFC/10⁶ PBMC values were nonoverlapping or, alternatively, if the 95% CI of the ratio of the GM was above 1.0. Homologous priming-boosting immunization with Ad5-gag elicited relatively high levels of detectable Gag-specific T lymphocytes (cohort GM, 241 SFC/10⁶ PBMC [95% CI, 129 to 449 SFC/10⁶ PBMC] at 4 weeks postbooster), whereas the homologous priming-boosting vaccination with MVA-gag produced responses close to background (<40 SFC/10⁶ PBMC). Importantly, the response levels elicited by the homologous Ad5 booster were not better than the respective peak priming responses. Heterologous boosting with 10⁹ PFU of MVA-gag resulted in a mean level (GM, 1,186 SFC/10⁶ PBMC [95% CI, 901 to 1,562 SFC/10⁶ PBMC]) which was 10.5-fold higher (95% CI, 5.5- to 20-fold) than that of homologous Ad priming-Ad boosting cohort at 8 weeks postbooster. Moreover, these levels were 2.4-fold higher (95% CI, 1.6- to 3.8-fold) than the peak priming responses.

To address whether other poxvirus vectors can similarly

serve as boosters for Ad-primed responses, monkeys which were previously immunized with three doses of either Ad5- or Ad6-gag (at either 10⁷ or 10⁹ vp) at weeks 0, 4, and 26 were inoculated with 10⁸ PFU of either ALVAC-gag or MVA-gag at weeks 56 and 119. Results (Fig. 2) showed that ALVAC is also notably effective in boosting Ad-primed responses. At the time of administration of the first booster, the levels of Gag-specific T-cells were very low (<114 SFC/10⁶ PBMC); the levels rose, on average, 16-fold (95% CI, 6.2- to 37-fold) at 4 weeks following administration of the poxvirus booster (GM, 1,191 SFC/10⁶ PBMC [95% CI, 760 to 1,867 SFC/10⁶ PBMC] for the ALVAC-boosted cohort; GM, 378 SFC/10⁶ PBMC [95% CI, 149 to 957 SFC/10⁶ PBMC] for the MVA-boosted cohort). While the responses declined afterwards, the levels at week 118 remained 4-fold higher (95% CI, 1.6- to 10.5-fold) than those levels observed just prior to the boosting (week 53), indicating an immunological benefit of the booster.

A second poxvirus booster was administered over a year (week 119) after the first poxvirus immunization in order to explore the utility of these vaccine vectors for maintenance boosters. At the time of the booster administration, no serum-neutralizing activity against either the ALVAC or the MVA vectors could be detected in *in vitro* assays (data not shown). At 4 weeks after the second booster, the Gag-specific immune responses were comparable if not slightly improved (though not statistically significant) relative to the responses present 4 weeks after the first booster was administered (Fig. 2). This suggests that poxvirus vectors can be used as boosters for maintaining cellular immunity, although more administrations will be necessary to fully evaluate this utility.

The sequence in which the vaccines are used also determines the effectiveness of the heterologous priming-boosting approach. Cohorts of three monkeys were immunized with two priming doses of either ALVAC-gag (at 10⁹ PFU/dose) or

FIG. 2. ALVAC and MVA vectors as boosters, following priming with Ad vectors. Macaques were immunized at weeks 0, 4, and 26 with either 10⁹ vp of Ad5-gag (animals 99C117 and 99D227), 10⁷ vp of Ad5-gag (animals 99D021 and 99D156), 10⁹ vp of Ad6-gag (animals 99D126 and 99D128), or 10⁷ vp of Ad6-gag (animals 99D147 and 99D151). At weeks 56 and 119, animals were given either 10⁸ PFU of ALVAC-gag or 10⁸ PFU of MVA-gag. Numbers of SFC/10⁶ PBMC were calculated as noted in the legend to Fig. 1.

NYVAC-gag (at 10⁹ PFU/dose), followed by a booster at week 27 with 10⁷ vp of Ad5-gag. This low dose of Ad5-gag is used to mimic the effect of preexisting Ad5 immunity in the general population; this dose has been previously shown to boost responses in animals primed with a DNA vector vaccine (5). Animals that had received three doses of MVA-gag from the experiment described in Fig. 1 were also given a booster of 10⁷ vp of Ad5-gag. The levels of Gag-specific T-cells induced by priming with the various poxvirus vectors were consistently weak, never exceeding 100 SFC/10⁶ PBMC at any given sampling time (Fig. 3). Boosting with a dose of 10⁷ vp of Ad5-gag

yielded only a slight elevation in responses, with only two to nine animals having Gag-specific T-cell levels between 100 and 200 SFC/10⁶ PBMC. A separate cohort of three monkeys was primed with the same low dose of Ad5-gag (10⁷ vp/dose) at weeks 0 and 4 and given a booster of 10⁹ vp of MVA-gag at week 27. Priming responses resulting from this small Ad5 dose were relatively low, never exceeding 190 SFC/10⁶ PBMC at any time prior to the MVA booster, and ranged from 10 to 110 SFC/10⁶ PBMC at the time of the booster (Fig. 3). However, unlike the poxvirus priming-Ad5 boosting cohorts, the post-boosting Gag-specific T-lymphocyte levels rose to above 500

FIG. 3. Comparison of the Gag-specific cellular immune response elicited by heterologous poxvirus priming-Ad5 boosting and Ad5 priming-poxvirus boosting. Priming doses of the gag-expressing ALVAC and NYVAC vectors were given at weeks 0 and 4, followed by the Ad5 booster at week 27; dose levels are indicated. MVA-gag was given at week 0, 4, and 27, and the Ad5 booster was given at week 65. In the final group, the Ad5 vector priming inoculations were delivered at weeks 0 and 4, with the MVA vector boosting delivered at week 27. The frequencies are expressed as the numbers of SFC/10⁶ PBMC and were calculated as the differences in responses between the PBMC stimulated with the Gag 20-aa peptide pool and the mock-treated PBMC. These values were determined at the start of the treatment (pre), at week 8 (post-prime), at the time of the boosting (pre-boost), and at 4 and 8 weeks postboosting; animals are indicated along the x axis.

FIG. 4. Percentages of Gag-specific T-cells that are CD3⁺ CD8⁺ in rhesus macaques immunized with either the Ad priming-Ad boosting (Ad/Ad) or Ad priming-poxvirus boosting (Ad/pox) regimen. The Ad/Ad cohort consisted of animals given two priming doses of either Ad5 or Ad6 (at a 10⁹- to 10¹¹-vp dose) followed by a homologous Ad booster. This cohort includes four of six Ad5-primed-Ad5-boosted animals that are represented in Fig. 1 (open squares) and for which responses were detectable by the IFN- γ cytokine staining method. The data for Ad priming-poxvirus boosting cohorts were collected for animals at 4 weeks following boosting with ALVAC (gray circles) or MVA (black circles, data for macaques from Fig. 1; black diamonds, data for macaques from Fig. 2; black triangles, data for macaques from Fig. 3). Also shown are the cohort arithmetic means and the associated standard errors.

SFC/10⁶ PBMC (GM, 1,023 SFC/10⁶ PBMC [95% CI, 293 to 3,563 SFC/10⁶ PBMC] at 8 weeks postboosting).

The CD4⁺ CD8⁺ distribution of the Gag-specific T-cell population was determined by flow cytometric intracellular IFN- γ cytokine staining by using the 20-mer peptide pool (5). Analyses of PBMC collected after the poxvirus booster for all animals described Fig. 1 through 3 revealed that the percentage of Gag-specific T-cells that were CD3⁺ CD8⁺ ranged from 20 to 90%, with a mean of 70% (Fig. 4). In contrast, for the animals that received multiple doses of Ad5-gag only, which include those described in Fig. 1, as well as animals from a previous report (5), these values were more tightly confined to >70%. The distribution did not change notably with time after the booster inoculation (data not shown). It would appear that compared to the Ad booster, the poxvirus booster produced more detectable helper responses when the 20-aa peptide pool was used as the stimulus. This relative difference in CD4⁺ CD8⁺ T-cell distribution is consistent with a trend previously observed when poxvirus and Ad5 boosters were compared to DNA vector-primed responses (6).

In conclusion, we have presented data demonstrating the potential utility of poxvirus vectors as booster immunogens to enhance responses initially elicited by Ad vector-based vaccines. ALVAC and MVA, two of the most extensively studied vectors in HIV-1 vaccine human trials, were shown here to be effective boosters of Ad-primed T-cell responses. The immunogenicity of Ad5 vector priming followed by ALVAC poxvirus vector boosting is currently undergoing evaluation in a human phase I trial.

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