Overcoming Immunity to a Viral Vaccine by DNA Priming before Vector Boosting

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Replication-defective adenovirus (ADV) and poxvirus vectors have shown potential as vaccines for pathogens such as Ebola or human immunodeficiency virus in nonhuman primates, but prior immunity to the viral vector in humans may limit their clinical efficacy. To overcome this limitation, the effect of prior viral exposure on immune responses to Ebola virus glycoprotein (GP), shown previously to protect against lethal hemorrhagic fever in animals, was studied. Prior exposure to ADV substantially reduced the cellular and humoral immune responses to GP expressed by ADV, while exposure to vaccinia inhibited vaccine-induced cellular but not humoral responses to GP expressed by vaccinia. This inhibition was largely overcome by priming with a DNA expression vector before boosting with the viral vector. Though heterologous viral vectors for priming and boosting can also overcome this effect, the paucity of such clinical viral vectors may limit their use. In summary, it is possible to counteract prior viral immunity by priming with a nonviral, DNA vaccine.

Increased understanding of the molecular genetic regulation of viral gene expression has led to a variety of new viral vectors for gene-based vaccination (reviewed in references 6, 18, 19, and 21). In addition, recombinant DNA technology has facilitated the use of naked DNA as a vaccine modality that has proven particularly effective in animal models (12). An appreciation of the need to vaccinate against a variety of infectious pathogens has recently increased. Because a number of vaccines for diverse microbes may utilize the same vector, prior immunity to the viral vaccine vector has become a concern. For example, immunity to vaccinia virus could reduce its ability to serve as a vector for the delivery of recombinant genes used for other infectious diseases or cancer, although this may be partially alleviated by using the mucosal route (5). As widespread smallpox vaccination is again considered, the prevalence of immunity to vaccinia virus could increase substantially. Protection against disease progression has been observed in simian-human immunodeficiency virus (SHIV) infection models in nonhuman primates with modified vaccinia virus Ankara (MVA) alone or as part of a prime-boost combination (see, for example, references 1, 2, and 10), which could be affected by prior vaccinia immunization. The success of replication-defective adenovirus (ADV) vectors (25, 26) used in prime-boost combinations for Ebola virus or HIV also suggests considerable promise for this approach; however, a significant percentage of the population has been exposed to natural ADV infection (7, 14), which could potentially limit the efficacy of ADV-based vaccines.

To address these concerns, we have evaluated the effect of prior viral immunity on ADV and vaccinia virus vector immunization by using Ebola virus glycoprotein (GP) immunization as a model. Cellular and humoral immune responses to Ebola virus GP in different vectors were examined. For DNA priming, 8- to 10-week-old BALB/c mice (Taconic, Germantown, N.Y.) were injected three times at 2-week intervals with 50 μ g of DNA [pCMV-GP(Z)] (29) intramuscularly. A single viral vector injection was made at week 6 with an ADV [10^{10} particles in 200 μ l of phosphate-buffered saline (PBS; pH 7.4)] (30) or MVA poxvirus vector (2×10^7 PFU in 200 μ l of PBS [pH 7.4]) to boost the primary immunizations. MVA-GP(Z) was prepared by transfecting pLW-17-GP(Z) into MVA-infected secondary chicken embryo fibroblasts. The MVA-GP(Z) recombinants were isolated by immunostaining (9) with anti-sGP/GP(Zaire,76) rabbit antiserum (A. Sanchez, personal communication), purified through a 36% sucrose cushion, and stored at -80°C . For viral vector priming and boosting, injections were performed at day 0 and week 6. Cellular immune responses were assessed at 6 to 8 weeks after the boosting.

In the absence of viral vector immunity, DNA immunization induced measurable cytotoxic T lymphocyte (CTL) immune responses to Ebola virus GP, but GP DNA priming followed by ADV boosting induced higher responses more consistently than DNA alone or DNA plus MVA (Fig. 1A). Animals injected repeatedly with MVA-GP or ADV-GP also showed measurable CTL responses, with ADV again providing more consistent and stronger responses than MVA. There were no statistical differences between the CTL responses induced by DNA-GP/ADV-GP and ADV-GP/ADV-GP or between those induced by DNA-GP/MVA-GP and MVA-GP/MVA-GP. Although a humoral response was observed after immunization

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FIG. 1. Immunological response against the Ebola virus GP (Zaire subtype) after vaccination in the absence of viral vector immunity. (A) Ebola virus GP-specific CTL responses 6 to 8 weeks after boosting. A ^{51}Cr release assay was performed using CT26 and CT26-GP at the indicated effector-to-target ratios as previously described (29). Splenocytes from immunized BALB/c mice were sensitized by using cells from the mouse tumor cell line CT26-GP for 7 days with 10 U of human interleukin-2/ml. Percent specific lysis was calculated as previously described (29). Each symbol reflects the cytotoxic T-cell response at the indicated ratios for an independent animal ($n = 4$). (B) ELISA titers to Ebola virus GP 2 weeks after the last priming or 2 weeks after boosting. Titers were obtained by endpoint dilution of the antiserum by using plates coated with Ebola (Zaire, 76) virion membrane preparation (17). The reactive immunoglobulin G was detected using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. Data are presented as averages $\pm$ standard deviations of results from five animals in each group. The starting dilutions of all sera were 1:100.

with DNA alone, the enzyme-linked immunosorbent assay (ELISA) titers were low, generally less than 1:25,000, in contrast to viral vector boosting of DNA or MVA alone, where antibody titers were increased up to 50-fold (Fig. 1B).

To evaluate the effect of prior exposure to the ADV vector on the vaccine response, animals were injected with 10^{10} particles of E1-deleted, replication-defective ADV (ADV- ΔE1). Two months following the exposure, immunization was performed with DNA-GP priming and viral vector boosting, and the results were compared to those for ADV-GP or MVA-GP vector priming and boosting. These data can be directly compared with those in Fig. 1, as they were collected at the same time. Prior exposure to ADV did not inhibit the CTL response after DNA priming and ADV vector boosting but dramatically reduced the CTL response induced by ADV priming and boosting (Fig. 2A). Fisher's exact test was used with an α value of 0.05 to compare the proportion of mice whose percentages of specific lysis exceeded 10% for at least two of the four effector-to-target cell (E:T) ratios among DNA-primed viral vector-boosted versus ADV-GP-primed and -boosted groups. According to this test, there is a statistically significant difference between the DNA-primed, ADV-GP-boosted mice and the ADV-GP-primed and -boosted mice ($P = 0.029$). As expected, immunity to ADV did not affect the CTL response induced by DNA priming and MVA-GP boosting (compare Fig. 1A and 2A). The difference between the DNA-primed, MVA-GP-boosted mice and the ADV-GP-primed and

-boosted mice was also statistically significant ($P = 0.029$) by Fisher's exact test (Fig. 2A).

Prior ADV vector exposure caused a substantial reduction in the humoral immune response induced by DNA priming and ADV-GP boosting and slightly reduced the already lower response to ADV-GP priming and boosting (compare Fig. 1B and 2B). The Wilcoxon rank sum test with an α value of 0.05 was used to compare the ELISA titers of each of the two DNA-primed groups to that of the ADV-GP-primed and -boosted group. When immunity to ADV was previously induced, the DNA-GP-primed, MVA-GP-boosted mice had statistically significantly higher ELISA titers than the ADV-GP-primed and -boosted mice ($P = 0.017$), but there was no significant difference between the titers of the DNA-primed, ADV-GP-boosted mice and those of the ADV-GP-primed and -boosted mice ($P = 0.517$), as can be seen in Fig. 2B. In each case, prior exposure to the ADV vector elicited potent neutralizing ADV antibodies (Fig. 2C) at levels greater than or equal to those observed in humans naturally exposed to ADV (24). The neutralizing antibody responses for the three groups are indistinguishable and hence do not warrant a formal statistical test.

The effects of vaccinia virus immunity on MVA immunization were similarly tested after prior exposure to 10^7 PFU of the Wyeth strain of vaccinia virus in mice inoculated by tail scratch 8 weeks prior to vaccination. DNA priming and viral vector boosting was compared to MVA priming and boosting.

FIG. 2. Prior immunity to ADV inhibits immune responses to an ADV-GP(Z) priming and boosting regimen in contrast to DNA priming and viral vector boosting. (A) The Ebola virus GP-specific CTL response was evaluated as described in the legend for Fig. 1A. Each symbol reflects the cytotoxic T-cell response at the indicated ratios for an independent animal ($n = 4$). (B) ELISA titers to GP(Zaire) from sera were determined as described in the legend for Fig. 1B ($n = 5$). (C) Quantitation of neutralization antibody to ADV. Serum samples were collected 6 weeks after intramuscular injection of ADV for pre-exposure and assayed for their ability to neutralize infection by ADV in A549 cells. The titer represents the endpoint dilution for inhibition of cytopathic effect on A549 cells. Data are presented as averages with standard deviations of results from five animals in each group. The starting dilutions of all sera were 1:5. To measure ADV-neutralizing antibody titers, serially diluted pre- and postexposure sera were mixed with human Ad5 derivative Sub360. The mixtures were then added to cells from the A549 human lung carcinoma cell line in 96-well dishes at a multiplicity of infection of 25 (25 particles/cell) and incubated at room temperature for 1 h. After 1 h, the mixtures were discarded. The cells were washed three times with normal medium and cultured for 72 h. ADV neutralization titers were determined as the highest dilutions of the anti-serum where no cytopathic effect was observed.

Similar to the response to ADV preexposure, the immune response to vaccinia virus dramatically inhibited the CTL response elicited by MVA-GP priming and boosting but did not affect that elicited by DNA priming followed by boosting with either ADV vector or MVA vector (Fig. 1 and 3A). Fisher's exact test was again used (with an α value of 0.05) to compare

FIG. 3. Prior immunity to vaccinia virus inhibits immunity to MVA priming and boosting, in contrast to DNA priming and viral boosting regimens. (A) The Ebola virus GP-specific CTL response was evaluated as described in the legend for Fig. 1A. Each symbol reflects the cytotoxic T-cell response at the indicated ratios for an independent animal ($n = 4$). (B) ELISA titers to GP(Zaire) from sera were determined ($n = 5$). (C) Vaccinia neutralization titers in mice preexposed to vaccinia virus. Serum samples were collected 6 weeks after vaccinia virus infection by scarification. Data are presented as averages with standard deviations. Vaccinia virus-specific neutralizing antibodies were determined by a plaque reduction assay by using vaccinia virus strain WR in BS-C-1 cells. Sera were diluted twofold in 96-well plates in 100 μ l of PBS plus 0.1% bovine serum albumin. An equal volume of PBS containing approximately 50 to 150 PFU of vaccinia virus was added to each serum dilution. The virus-serum mixture was incubated for 1 h at 37°C; then 100 μ l was plated onto a confluent monolayer of BS-C-1 cells. After 2 h of adsorption, fluid was removed and replaced with 2 ml of Eagle's minimal essential medium containing 0.5% methyl cellulose and 2% fetal bovine serum. At 48 h postinfection, the monolayers were stained with crystal violet and plaques were counted. The neutralization titers of the sera are given as the reciprocals of the last dilutions giving a >50% reduction of plaque number compared to controls.

the proportion of mice whose percentage of specific lysis exceeded 10% for at least two of the four E:T ratios among DNA-primed and viral vector-boosted versus MVA-GP-primed and -boosted groups. According to this test, there is a statistically significant difference between the DNA-primed,

ADV-GP-boosted mice and the MVA-GP-primed and -boosted mice ($P = 0.029$). The difference between the DNA-primed, MVA-GP-boosted mice and the MVA-GP-primed and -boosted mice was also statistically significant ($P = 0.029$) by Fisher's exact test. As expected, vaccinia virus immunity had no effect on the CTL response due to DNA priming and ADV-GP boosting (compare Fig. 1 and 3).

Prior immunity to vaccinia virus reduced the antibody response after DNA-GP/MVA-GP but not after MVA/MVA immunization (Fig. 1B and 3B). The Wilcoxon rank sum test (with an α value of 0.05) was used to compare the ELISA titers of each of the two DNA-primed groups to those of the MVA-GP-primed and -boosted group. The DNA-primed, MVA-GP-boosted mice had statistically significantly lower ELISA titers than the MVA-GP-primed and -boosted mice ($P = 0.0092$), but there was no significant difference between the titers of the DNA-primed, ADV-GP-boosted mice and those of the MVA-GP-primed and -boosted mice ($P = 0.8271$), as can be seen in Fig. 3B. We confirmed that the prior inoculation with vaccinia virus induced a substantial neutralizing antibody response in the animals used for all three groups (Fig. 3C).

Increasing knowledge of viral replication strategies has led to the use of a variety of new vectors for vaccination against infectious diseases and cancer (6, 20, 21). Several promising replication-defective viruses that have recently been developed and tested in models of Ebola virus and HIV infection can elicit potent immune responses that confer protective effects in relevant preclinical models (1, 3, 23, 25, 26). As immunization strategies using these vectors are tested in humans, the immune responses to naturally occurring infections, such as infection by ADV, for example, may limit their efficacy. In addition, if a viral vector is employed for different vaccines (for example, as vaccinia virus or modified vaccinia virus vectors may be used for smallpox vaccination), the possibility exists that vector immunity induced by prior immunization may interfere with the efficacy of a different vaccine using the same vector.

In this study, the efficacy of different heterologous prime-boost combinations was assessed in an effort to understand and overcome the effects of prior immunization on vaccine-induced responses. Prior immunity to the cognate viral vector was found to significantly reduce cellular and humoral immune responses, though the effect of vaccinia virus on the humoral immune response was less pronounced. This effect could be overcome to a large extent by using heterologous priming strategies, particularly with naked DNA. The levels of immunity achieved in the presence of prior viral exposure with this immunization strategy were similar to those achieved in the absence of prior immunity. Given the likelihood of exposure to these viruses, either through natural infection or immunization, this approach may prove useful in the development of vaccines in humans.

DNA priming has been shown to be highly effective in stimulating a primary immune response based on T-cell recognition of diverse subdominant epitopes (2). This response is presumably based on the ability of antigen-presenting cells to take up and present endogenously synthesized antigens. In the absence of proteins from the viral vector, the primary immune response presumably can focus on the antigen of interest and facilitate the generation of memory T cells specific for the

relevant antigen, Ebola virus GP. Though the CTL and antibody responses have been the focus of study here, previous studies of Ebola virus vaccine responses have suggested that CD4 responses are concordant with the CTL and antibody responses, both of which are dependent on helper T-cell function. Once these memory cells are present, viral vector proteins do not interfere with the recall response, allowing a robust immune response to develop and suggesting a mechanism for the responses reported here.

These findings have implications for the use of different viral vectors in human studies. It is known from previous studies that prior vector immunity to vaccinia virus has limited the efficacy of related poxvirus vectors (8, 22). For this reason, poxvirus vectors from alternative species have been analyzed. For example, canarypox or fowlpox have been analyzed in numerous preclinical and clinical models for HIV vaccines (4, 15, 27). For ADV vectors, there are more than 50 ADV serotypes (13) that provide an abundant source for alternative ADV vectors. In addition, ADV vectors from different species, including canine or chimpanzee-derived viruses, have shown promise in gene transfer (11, 16) and other viral vaccine models (26, 28). Because it will take significant time and effort to understand the efficacy and toxicities of alternative viral vectors, the ability to prime immune responses with DNA vaccines provides a viable and generally applicable approach to overcome viral vector immunity, especially if it can be made to work efficiently in humans. This approach deserves further study in clinical studies in which it will be possible to analyze the specific immune exposures of diverse human subjects.

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