Highly Effective Control of an AIDS Virus Challenge in Macaques by Using Vesicular Stomatitis Virus and Modified Vaccinia Virus Ankara Vaccine Vectors in a Single-Boost Protocol

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Received 26 September 2003/Accepted 22 December 2003

Previous studies have shown that vaccination and boosting of rhesus macaques with attenuated vesicular stomatitis virus (VSV) vectors encoding Env and Gag proteins of simian immunodeficiency virus-human immunodeficiency virus (SHIV) hybrid viruses protect rhesus macaques from AIDS after challenge with the highly pathogenic SHIV 89.6P (23). In the present study, we compared the effectiveness of a single prime-boost protocol consisting of VSV vectors expressing SHIV Env, Gag, and Pol proteins to that of a protocol consisting of a VSV vector prime followed with a single boost with modified vaccinia virus Ankara (MVA) expressing the same SHIV proteins. After challenge with SHIV 89.6P, MVA-boosted animals controlled peak challenge viral loads to less than 2×10^6 copies/ml (a level significantly lower than that seen with VSV-boosted animals and lower than those reported for other vaccine studies employing the same challenge). MVA-boosted animals have shown excellent preservation of CD4⁺ T cells, while two of four VSV-boosted animals have shown significant loss of CD4⁺ T cells. The improved protection in MVA-boosted animals correlates with trends toward stronger prechallenge CD8⁺-T-cell responses to SHIV antigens and stronger postchallenge SHIV-neutralizing antibody production.

There are currently 42 million people worldwide living with human immunodeficiency virus (HIV)/AIDS, and more than 5 million people were newly infected with HIV type 1 (HIV-1) in 2003 (AIDS epidemic update, 2003; Joint United Nations Programme on HIV/AIDS [http://www.unaids.org]). While some attention has recently shifted away from the AIDS epidemic toward emerging diseases and potential bioterrorism, HIV-1 infection continues to cause enormous suffering and is contributing to social, economic, and political instability in many countries. It is now more urgent than ever that a safe, effective, and widely deliverable HIV vaccine be made available.

Within the past several years, significant progress has been made in the generation of vaccine candidates that are effective at preventing AIDS in nonhuman primate models (for reviews, see references 16 and 22). Several vaccine approaches have been developed that can protect rhesus macaques from AIDS caused by the highly pathogenic simian immunodeficiency virus (SIV)-HIV hybrid virus designated SHIV 89.6P (20), although they do not prevent infection by the challenge virus. These approaches include vaccination with plasmid DNAs encoding SHIV proteins and cytokines (4), vaccination with plasmid DNAs encoding SHIV proteins followed by boosting with modified vaccinia virus Ankara (MVA) vectors encoding SHIV proteins (1), vaccination with defective adenovirus vectors en-

coding SHIV Gag proteins (26), and vaccination with attenuated vesicular stomatitis virus (VSV) vectors encoding Env and Gag proteins (23). All of these studies employed a vaccination followed by two or more boosts to optimize immune responses. However, an ideal AIDS vaccine would not require extensive boosting.

In a previous AIDS vaccine study using VSV vectors, macaques were immunized with live-attenuated recombinant VSVs (rVSVs) expressing SIV Gag and HIV Env 89.6 (23). These animals were boosted twice with rVSVs expressing the same SIV and HIV antigens but with different VSV glycoproteins. These VSV glycoprotein exchange vectors evade VSV-neutralizing antibodies generated in the previous immunization and generate effective boosting (23, 24). All animals in the initial VSV vector studies were challenged intravenously (i.v.) with SHIV 89.6P virus (20). The seven vaccinees from this study remained healthy, with low or undetectable viral loads for up to 3 years after challenge, while all control animals progressed to AIDS with an average time of about 8 months and were euthanized (reference 23 and unpublished results). Analysis of immune responses in protected macaques in the VSV vector studies and in protected macaques in other studies (1, 4, 26) suggested that the initial protection relied upon (at least) a vigorous CD8⁺ cytotoxic T-lymphocyte (CTL) response.

One concern with protection based on CTLs is that if enough viral replication were to occur over time, mutations in the dominant CTL epitope(s) would be selected and would lead to vaccine failure. Such a failure was reported for one

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animal immunized with plasmid DNA and cytokine and challenged with SHIV 89.6P (2). Recently we experienced a vaccine failure in one of the seven vaccinees from our previous VSV-based vaccine study (23). This animal had a consistently detectable viral load after challenge and began to show an increasing viral load (along with decreasing CD4 T-cell counts) around 3 years postchallenge. Symptoms of AIDS were noted at 3.2 years postchallenge, and the animal was euthanized. The other six vaccinees from that study remain healthy, with undetectable virus loads. One possible way to overcome the CTL escape problem is to generate a maximal number of broadly reactive and highly activated CTLs during vaccination, resulting in a large memory CTL population. In theory, such a memory CTL population could respond rapidly, control the peak viral load after infection, and then limit replication of the virus so effectively that escape mutations would not be selected.

Exploratory studies have been directed at finding methods for inducing optimal memory CD8⁺-T-cell responses through the use of live viral vectors. These studies showed that mice immunized with an rVSV expressing HIV-1 Gag and boosted with a completely heterologous vaccinia virus recombinant expressing HIV-1 Gag generated a fivefold increase in peak Gag-specific CD8⁺ T cells relative to the levels seen in mice immunized with a VSV vector and boosted with a VSV G protein exchange vector expressing Gag (11). The population of Gag-specific T cells persisting long term was also increased fivefold by the heterologous vaccinia boost. The purpose of our present study was to determine whether a single boost with a heterologous vector also provided increases in CD8⁺-T-cell responses in rhesus macaques larger than those obtained with VSV G protein exchange vectors. We also wanted to determine whether a heterologous single-boost protocol could confer better protection from challenge with SHIV 89.6P than a single boost with VSV G protein exchange vectors.

In this study we report that macaques primed with rVSV vectors expressing SHIV proteins and boosted once with a MVA vector expressing SHIV proteins mount increased immune responses relative to animals boosted with VSV exchange vectors. Importantly, after SHIV challenge MVA-boosted monkeys had 10-fold-lower peak viral loads, cleared challenge virus faster, and preserved CD4⁺-T-cell counts better than those receiving VSV G protein exchange vectors. This represents a significant improvement in vaccine efficacy and delivery.

MATERIALS AND METHODS

Construction of VSV recombinants expressing SHIV 89.6P EnvG and SIVmac239 Pol proteins. Attenuated rVSVs encoding SHIV 89.6P EnvG (SHIV 89.6P Env with its cytoplasmic domain replaced with the VSV G cytoplasmic domain) were constructed using a method similar to that described previously for other EnvG hybrids (12). The 89.6P envelope gene was amplified by PCR using VENT polymerase (New England Biolabs) from the SHIV 89.6P KB9 3' plasmid (13) through the use of primers that introduced an XhoI site preceding the Env coding region (CCCGGGCTCGAGACAATGAGAGTGAAGGAGAAATAT CAG) and a BamHI site at the C-terminal end of the Env transmembrane domain (AACCTTGAAGGATCCTAACTCTATTTACTATAGAAAG). This fragment was ligated to a BamHI-NheI fragment encoding the 26 C-terminal amino acids of the VSV G cytoplasmic domain. The amino acid sequence at the junction of the SHIV 89.6P Env and VSV G is . . . IVNRVR/IHLCIK. . . , with the forward slash indicating the junction. The EnvG 89.6P hybrid gene was cloned between the XhoI and NheI sites of pVSVXN-1 (25) or

pVSV(GCh)XN-1 (24), and rVSVs expressing the hybrid protein were recovered using published procedures (15, 25). Expression of a protein from the recombinant was confirmed initially by indirect immunofluorescence microscopy on infected cells to verify expression on the cell surface and subsequently by metabolic labeling, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography as described previously (24) to verify the expected sizes and expression levels relative to those of VSV proteins.

RVSVs expressing the SIVmac239 Pol reading frame were constructed as follows. A PCR product was amplified from pSHIV-KB9-5' (AIDS Reagent Center) (13) through the use of VENT polymerase. The *pol* sequence from pSHIV-KB9-5' is derived from and identical to that of SIVmac239 (13, 19). The forward primer was 5'CCGGCTCGAGACAATGTTGG AATTGTGGGAAA GAGGGACAC (containing the underlined XhoI site); the reverse primer was 5'GGCCGGATCCGCTAGCCTAT GCCACCTCTCTAGCCTCTCCG (containing the indicated NheI site). The forward primer also introduced an ATG codon in frame with the Pol coding sequence. The 3,193-nucleotide XhoI-NheI fragment derived from the PCR product was cloned between the unique XhoI and NheI sites in pVSVXN-1 (25) or pVSV(GNJ)XN-1 (24). The complete sequence of the insert was verified to be identical to the published sequence. RVSVs expressing Pol protein were recovered from both constructs through the use of published procedures (15, 25). Expression of the Pol reading frame from both constructs was verified initially by indirect immunofluorescence microscopy on infected cells through the use of a primary antiserum (32H) from an SIVmac251-infected monkey (AIDS Reagent Program) to verify expression of Pol. Subsequently, metabolic labeling of virus-infected cells with (³⁵S)methionine, SDS-PAGE, and autoradiography were used to verify expression of an approximately 120-kDa protein from both viruses.

Construction of recombinant MVA virus expressing SHIV 89.6P Env and SIVmac239 Gag-Pol proteins. The plasmid pSHIV KB9 3' (obtained from the National Institutes of Health [NIH] AIDS Research and Reference Reagent Program) was the source of the SHIV 89.6P *env* gene. For efficient expression in vaccinia virus, two alterations were made. First, site-directed mutagenesis was employed to change the naturally occurring poxvirus transcription termination signal TTTTCT to TTCTCT while preserving the amino acid coding sequence (8). Second, the gene was truncated at the C terminus after nucleotide 2217 (ending amino acid sequence: GGERD). Removal of 115 to 130 amino acids from the cytoplasmic tail of *env* has been shown to result in enhanced cell surface expression and immunogenicity of *env* in recombinant MVA viruses (L. Wyatt and B. Moss, unpublished data). The *env* gene was then cloned into pLW17 (28), the MVA shuttle plasmid, under control of the mH5 promoter. The integrity of the gene was verified by DNA sequencing. The resulting plasmid, pLW17/KB9env, was recombined into the virus vJH4 (which expresses the SIVmac239 *gag-pol* gene) using standard methodology (9). *env*-expressing foci were selected by immunostaining with HIV-1 *env*-specific rabbit antiserum 2144. In the new recombinant virus, MVA-KB9env/SIVgp, 89.6P *env*, and SIVmac239 *gag-pol* are located in deletions II and III, respectively. Expression and processing of the SHIV antigens was confirmed by metabolic labeling of BSC-1-infected cells, radioimmunoprecipitation with specific antibodies, SDS-PAGE, and autoradiography (data not shown).

ELISA for total antibody to oligomeric HIV Env. Enzyme-linked immunosorbent assays (ELISAs) were performed essentially as described previously (21), with modifications published previously (23). Optical densities were determined at a wavelength of 415 nm in a Bio-Rad ELISA plate reader. Titers were determined from graphs of the data and are given as the reciprocal of the serum dilution that gave an absorbance of 0.2 after subtraction of background values obtained from an identical dilution series with preimmune sera.

VSV neutralization assays. We used VSV Indiana or VSV New Jersey to detect antibodies neutralizing the corresponding G proteins. To detect antibodies neutralizing the G of Chandipura protein (GCh), a rVSV Indiana expressing the (GCh) in place of the G of Indiana protein (GInd) was used. Neutralization assays were performed as follows. Monkey sera were diluted with phosphate-buffered saline (PBS) in a volume of 50 μ l in serial twofold dilutions in 96-well plates. A total of 50 μ l of each virus (approximately 100 PFU) in serum-free Dulbecco's modified Eagle's medium (DMEM) was added to the diluted serum in each well. The 96-well plates containing sera and virus were incubated at 37°C for 1 h. Approximately 4,000 BHK cells in 100 μ l of DMEM-10% fetal bovine serum were then added to each well. The plates were incubated at 37°C with 5% CO₂ for 2 to 3 days. Each assay was performed in duplicate. Neutralizing titers are given as the highest dilutions which correspond to complete inhibition of VSV cytopathic effect. All duplicate assays agreed within 1 dilution.

HIV-neutralizing antibody assays. Antibody-mediated neutralization of SHIV 89.6 and SHIV 89.6P was assessed in MT-2 cells by using a reduction in virus-induced cell killing as described previously (7). Briefly, 500 50% tissue culture

infective doses of virus were incubated with multiple dilutions of serum samples in triplicate for 1 h at 37°C in 96-well flat-bottomed culture plates. Cells were added, and the incubation was continued (usually for 4 to 6 days) until most but not all cells in virus control wells (cells with virus but with no serum sample) were involved in syncytium formation. Cell viability was then quantified by neutral red uptake. Neutralization titers are defined as the reciprocal serum dilution (before the addition of cells) at which 50% of cells were protected from virus-induced killing. A 50% reduction in cell killing corresponds to an approximately 90% reduction in p27 Gag antigen synthesis in this assay (6). The assay stocks of both SHIVs were prepared in human peripheral blood mononuclear cells (PBMCs).

Preparation of VSV and MVA recombinants and vaccinations. rVSV viruses used for inoculation of the macaques were grown for 18 to 20 h on BHK cells in serum-free DMEM followed by centrifugation to remove cell debris. Virus samples were frozen at -80°C; titers ranged from 2×10^7 to 10^8 PFU/ml after thawing. Macaques were inoculated intranasally with a total of 0.4 ml containing 5×10^6 PFU of each construct. Control animals received an equal amount of VSV-hemagglutinin intranasally.

MVA-KB9env/SIVgp and nonrecombinant MVA were grown in chicken embryo fibroblast cells and purified through a sucrose cushion (9). Titers were determined by immunostaining with vaccinia virus-specific antisera (9). For vaccination, the viruses were diluted to 10^9 infectious units/ml in PBS. Each animal was given two injections of 100 μ l each, one intradermal in the right lateral quadriceps and one intramuscular in the left lateral quadriceps, for a total of 10^8 PFU/monkey.

EliSpot assay. The EliSpot assay using recombinant vaccinia viruses was performed as described previously (18). Cryopreserved PBMCs were thawed and washed three times in medium plus 15% fetal calf serum. PBMCs (2×10^5) were resuspended in 200 μ l of medium-15% fetal calf serum and added to the wells of a microtiter plate. PBMCs were then infected (at a multiplicity of infection of 2) for 16 h at 37°C with recombinant vaccinia virus expressing HIV 89.6 Env, SIVmac239 Gag, control vaccinia virus (Tk⁻), or phytohemagglutinin as a positive control. PBMCs were transferred to a microtiter plate (U-CyTech, Utrecht, The Netherlands) coated with monoclonal antibody specific for rhesus gamma interferon (IFN- γ). After a 5-h incubation at 37°C, PBMCs were removed by washing with PBS-0.05% Tween. The wells were filled with an appropriate dilution of a biotinylated detector antibody and incubated for 1 h at 37°C. After incubation, the wells were washed with PBS-0.05% Tween and incubated with a goat anti-biotin antibody solution for 1 h at 37°C. The wells were then washed with PBS-0.05% Tween and filled with a chromogenic substrate. After color development, spots were visualized on an AID EliSpot reader system with EliSpot 2.5 software (Cell Technology, Inc., Columbia, Md.). Counts were adjusted to spot-forming cells/ 10^6 PBMC. The background of control vaccinia virus (Tk⁻) was subtracted from all samples.

Major histocompatibility complex class I (MHC-I) tetramer staining. Cryopreserved PBMCs were thawed and washed three times in RPMI medium-5% fetal calf serum. At least 5×10^6 PBMCs were placed in 15-ml polystyrene tubes for staining. The PBMCs were then incubated with Mamu-A*01-restricted phycoerythrin-labeled p11C or p41A tetramer (NIH tetramer facility) for 15 min at room temperature. Antibodies to CD3 and CD8⁺ (BD-Pharmingen, San Jose, Calif.) were then added for an additional 15 min. After staining, cells were washed 3 times in PBS-2% fetal bovine serum and then resuspended in PBS[2% paraformaldehyde for fixing. After 20 min, cells were washed 2 times in PBS and resuspended. Fluorescence-activated cell sorter analysis was performed on a FACSCalibur flow cytometer with FloJo software.

Determination of CD4⁺-T-cell counts and viral loads. CD4⁺- and CD8⁺-T-cell numbers were evaluated by flow cytometry with the following fluorochrome-labeled monoclonal antibodies: anti-rhesus monkey CD3-fluorescein isothiocyanate (clone SP34; Pharmingen), anti-human CD4-phycoerythrin (SK3; Becton Dickinson), and anti-human CD8-peridinin chlorophyll protein (SK1; Becton Dickinson). Antibodies were added to 50 μ l of whole blood collected on EDTA and incubated for 15 min at room temperature, after which erythrocytes were lysed by the addition of 450 μ l of fluorescence-activated cell sorter lysis solution (BDIS).

The concentration of SHIV RNA in plasma was measured at Chiron Corporation (Emeryville, Calif.) by a branched DNA signal amplification assay. The lower limit of detection was 125 SHIV RNA copy equivalents per ml.

RESULTS

Study design and antibody responses. In this study, twelve rhesus macaques were vaccinated, boosted, and challenged according to the schedule outlined in Fig. 1A. Group A and B

animals (four animals per group) were initially immunized with a mixture of three live-attenuated VSV recombinants (Indiana serotype G) expressing HIV EnvG 89.6P, SIVmac239 Gag, and SIVmac239 Pol. All animals were immunized intranasally with VSV vectors and, as in previous studies, there was no vaccine-associated pathology. After vaccination all animals generated serum-neutralizing antibody to the VSV (GInd) protein (Fig. 1B), indicating that immunization took in all vaccinees. At 56 days after vaccination, animals were boosted with a mixture of three VSV glycoprotein exchange vectors expressing VSV(GCh) Gag, VSV(GCh) EnvG89.6P, and VSV(GNJ) Pol (group A) or with recombinant MVA expressing HIV 89.6P Env and SIVmac239 Gag-Pol (group B). Only three animals expressing the Mamu-A*01 MHC-I allele animals (for which specific Gag and Env CTL epitopes are known) could be located for this study. One of these was placed in group A, and two were placed in group B.

We also had a nonconcurrent control group of four animals that were vaccinated with a VSV vector expressing an influenza antigen and boosted with an empty MVA vector. Animals from our previous study (23) vaccinated and boosted with VSV vectors expressing an influenza antigen and given an identical SHIV 89.6P challenge virus also served as controls. This control group also contained one Mamu-A*01⁺ animal (AP05). Although it has been reported that presence of the Mamu-A*01⁺ allele can retard progression to AIDS after SHIV 89.6P challenge (29), this animal was the most rapid progressor to AIDS among our controls (23).

At 1 month postboost, animals boosted with VSV exchange vectors generated antibodies to VSV G New Jersey (GNJ) and (GCh) (Fig. 1B), indicating that boosting was successful. As expected, animals boosted with MVA had antibodies only to VSV (GInd) and not to the VSV(GNJ) or VSV(GCh). The antibody responses to the HIV Env glycoprotein induced by vaccination and boosting were measured by ELISA (Fig. 1C), which detects antibody reactive with oligomeric 89.6 gp140 (21). By 8 weeks after initial immunization, five out of eight vaccinees had detectable serum antibody to gp140. At 5 weeks after boosting, antibody responses were increased in all animals except one. Antibody titers differed considerably (range, 190 to 2,100) among different monkeys, and there were no significant differences in antibody titers between VSV- and MVA-boosted animals.

Prechallenge CD8⁺-T-cell responses. We measured antigen-specific T-cell responses in all vaccinated animals by either MHC-I tetramer (Mamu-A*01-positive monkeys) or IFN- γ EliSpot (all monkeys) assays. After primary vaccination, seven out of eight monkeys had shown small but detectable responses to Gag or Env or both (Fig. 2A to E) by 3 weeks postvaccination. We did not detect significant responses to Pol in any animals prior to challenge (data not shown). Responses to Gag and Env were highly variable between individuals, with up to 235 or 375 spot-forming cells per million PBMC in response to the presence of Gag or Env, respectively. Tetramer staining levels were similar to those reported in other studies (4, 26), with preboost responses of less than 0.2% in all three Mamu-A*01-positive monkeys. After boosting, differences between MVA- and VSV-boosted animals became evident, with most MVA-boosted animals having a greater expansion of antigen-specific T cells than VSV-boosted monkeys. Responses to Env

FIG. 1. Timeline of study and prechallenge antibody responses. (A) All animals were primed intranasally with VSV(GInd) vectors encoding HIV EnvG 89.6P, SIVmac239 Pol, and SIVmac239 Gag. At 56 days after initial immunization, four monkeys per group were boosted either with VSV glycoprotein exchange vectors encoding HIV EnvG 89.6P, SIVmac239 Pol, and SIVmac239 Gag (group A) or with MVA encoding SIVmac239 Gag-Pol and HIV Env89.6P (group B). At 153 days after vaccination, all animals were challenged i.v. with 30 MID₅₀ of SHIV 89.6P. (B) Neutralizing antibody titers for the three VSV glycoproteins in the envelope exchange vectors. Titers for each vaccinee represent the reciprocal of the serum dilution that completely neutralized 100 PFU of VSV with the indicated glycoprotein. Titers for VSV(GInd) were determined 56 days after the primary vaccination. Titers for VSV(GNJ) and VSV(GCh) were determined 1 month after the boost. (C) ELISA titers for oligomeric HIV Env 89.6 gp140. ELISA titers represent the reciprocal of the serum dilution that gave an A₄₀₅ of 0.2 after subtraction of background values.

in some animals were of even greater magnitude than those to Gag, and animals having good responses to one antigen had good responses to the other. Monkey T424 (MVA boosted) was the best overall responder, while T694 and R445 (VSV boosted) were the poorest.

Viral loads and CD4⁺-T-cell counts following pathogenic challenge. At 3 months after the single boost, all vaccinees and controls were challenged i.v. with pathogenic SHIV 89.6P. Viral loads and CD4⁺-T-cell counts were monitored at frequent intervals as a measure of vaccine efficacy. Control ani-

FIG. 2. Prechallenge CTL responses to Gag and Env. Antigen-specific T-cell responses were quantified by MHC-I p11C-M Gag tetramer (A) and vaccinia-based IFN- γ EliSpot (B to E) assays. Tetramer analysis was restricted to those animals expressing the Mamu-A*01 allele (monkeys T694, V864, and AL86). In all plots, open and closed symbols represent VSV(G) exchange or MVA boosted animals, respectively. T-cell responses (determined by IFN- γ EliSpot assays) to SIVmac239 Gag are shown in panels B and C; responses to HIV 89.6 Env are shown in panels D and E.

mals displayed a high level of viremia, with viral loads reaching over 10^8 copies/ml in two animals by 2 weeks postchallenge (Fig. 3A and C). The average peak viral load for the control animals in this and the previous VSV vector study (23) was $10^{7.7} \pm 10^{0.7}$. In contrast, peak viremia was controlled in all vaccinees in this study, with average ($\pm$ standard deviation) peak viral loads of $10^7 \pm 10^{0.5}$ for VSV-boosted animals and $10^{6.2} \pm 10^{0.1}$ for MVA-boosted animals (Fig. 3B and C). All four MVA-boosted animals maintained significantly ($P < 0.03$;

Mann-Whitney test) lower viral loads than all four VSV-boosted animals from 14 to 21 days after challenge (peak viremia), with differences between the groups lessening as immune responses controlled the infection. In summary, viral loads among the four MVA-boosted animals were controlled earlier and were maintained at lower levels for more than 280 days after challenge compared to the results seen with either VSV-boosted or control monkeys.

Control animals challenged with SHIV 89.6P often show

FIG. 3. MVA-boosted animals have lower viral loads and higher CD4⁺-T-cell counts after challenge. (A to C) Viral loads for individual control monkeys (A) or vaccinees (B) boosted with either VSV vectors (open symbols) or MVA vectors (closed symbols). Average viral loads for each boost group are graphed in panel C. Average viral loads for eight previous control animals are displayed for comparison (C). Points represent averages $\pm$ standard errors of the means (SEM), with viral loads expressed as the number of copies of viral RNA/ml of plasma. (D to F) Postchallenge CD4⁺ counts for individual control monkeys (D) or individual vaccinees (E) boosted with VSV (open symbols) or MVA (closed symbols). Panel F shows average CD4⁺ counts $\pm$ SEM by boost group. In all graphs, CD4⁺ counts are expressed as percentages of the average prechallenge counts, with prechallenge counts being obtained on 12 separate days.

nearly complete elimination of CD4⁺ T cells by 14 to 21 days postchallenge, and this happened in all four control animals in this study (Fig. 3D). However, one animal (AR75) has made a partial recovery in CD4⁺ T cells following the initial drop. Animals AR74 and N535 developed symptoms of AIDS by days 53 and 88, respectively, and were euthanized.

In contrast, vaccinated macaques infected with SHIV 89.6P typically display transient drops in CD4⁺-T-cell counts in the 1

to 2 weeks after challenge; these resolve partially or completely as viremia is controlled (23). In this study, animals in both boost groups showed at least transient CD4⁺-T-cell-level declines. Figure 3E shows these data for the individual animals, and Fig. 3F shows the averages for both groups. The largest declines were in three of the VSV-boosted animals (L559, R445, and T694; Fig. 3E). MVA-boosted animals have maintained high T-cell counts out to 245 days postchallenge, with

FIG. 4. Postchallenge antibody responses. (A) The serum titers for oligomeric Env 89.6 gp140 were determined by ELISA for all vaccinees on the day of challenge and at 30 or 90 days after challenge. Bars represent the mean titers $\pm$ SEM of either VSV-boosted (open bars; $n = 4$) or MVA-boosted (closed bars; $n = 4$) animals. (B to E) Neutralizing antibody titers for SHIV 89.6P (B and C) or SHIV 89.6 (D and E) were determined at multiple days postchallenge. Neutralizing titers represent the reciprocal serum dilution at which virus infectivity was reduced by 50%. Graph symbols correspond to individual monkeys as described for previous figures, with open and closed symbols representing VSV vector and MVA vector boosting, respectively.

three of four monkeys at 80 to 100% of prechallenge levels and the remaining animal at 43% of prechallenge levels. Of the four VSV-boosted vaccinees, two (L559 and AK92) are at about 50% of prechallenge levels whereas the other two (R445 and T694) continue to have exhibit lower CD4⁺-T-cell numbers.

Postchallenge antibody responses. Animals were monitored after challenge for total antibody responses (ELISA analysis of oligomeric Env 89.6 gp140) and for levels of HIV-neutralizing antibody to SHIV 89.6P and SHIV89.6. None of the animals had detectable HIV-neutralizing antibody at the time of challenge. By 1 month after challenge, serum titers for gp140 increased at least 10-fold in all vaccinees, with these titers

declining significantly by 3 months postchallenge (Fig. 4A). There were no significant differences in antibody titers between the two vaccine groups. None of the four control animals produced detectable serum antibody titer to gp140 by 30 days postchallenge (data not shown).

ELISA titers did not correlate with clinical outcome in vaccinees, but the production of neutralizing antibody correlated better, since the neutralizing antibody titers for SHIV 89.6P in the MVA-boosted animals were higher on average than those in the VSV-boosted animals (Fig. 4B and C). However, in one of the MVA-boosted animals, AL86, there were no neutralizing antibodies detectable at day 14 after challenge (Fig. 4C) and yet this animal was controlling viral load at day 14 to levels

2 to 3 logs below the average seen with unvaccinated animals. Thus, the initial control of viremia, at least in this animal, cannot have been due to the presence of neutralizing antibody.

To determine whether neutralizing antibodies appearing in the vaccinees postchallenge were cross-reactive against viruses other than the SHIV 89.6P challenge virus, we also measured neutralizing antibody responses to SHIV89.6. This virus is the parent of SHIV 89.6P and has 12 amino acid differences in its ectodomain (13), causing major differences in its neutralization profile (17). Antibodies neutralizing SHIV 89.6 were detected in all animals by 2 to 4 weeks postchallenge, although these titers were generally lower than the titers for the challenge virus (Fig. 4D and E), as would be expected since the Env protein sequence used in immunization was derived from SHIV 89.6P. The very rapid appearance of these SHIV 89.6 cross-neutralizing antibodies was unexpected, however, because nonvaccinated macaques that survive challenge with SHIV 89.6P generate neutralizing antibodies to SHIV 89.6P as early as 11 weeks after infection but cross-neutralizing antibody to SHIV 89.6 does not appear until week 38 (17). In the VSV priming vector, the cytoplasmic domain of the SHIV 89.6P Env protein was replaced with the VSV G cytoplasmic domain. It is possible that this change alters the conformation of the 89.6P Env protein and primes for a broader response. A broader immune response would presumably be advantageous in protecting against natural infection.

Postchallenge T-cell responses. We measured postchallenge antigen-specific T-cell responses in all vaccinated animals with MHC-I tetramers recognizing either Gag (Fig. 5A) or Env (Fig. 5B) immunodominant epitopes (Mamu-A*01-positive monkeys) or IFN- γ by EliSpot assays (all monkeys). The results of EliSpot assays for SIV mac239 Gag are shown in Fig. 5C (VSV-boosted monkeys) and 5E (MVA-boosted monkeys). The results of EliSpot assays for HIV 89.6 Env are shown in Fig. 5D (VSV-boosted monkeys) and 5F (MVA-boosted monkeys). Expansion of antigen-specific (Gag and Env) CD8⁺ T cells occurred in all vaccinees after challenge. We also detected low-level postchallenge responses to Pol in some animals (data not shown). While higher-level prechallenge T-cell responses might be expected to predict a more positive clinical outcome after challenge, postchallenge T-cell responses may reflect a response to the degree of infection and exposure to antigen; i.e., greater expansion of T cells may occur in response to poor initial control of viremia. Consistent with such a model, some vaccinees generated consistently high numbers of antigen-specific T cells both pre- and postchallenge (i.e., MVA-boosted monkeys AL86 and T424) whereas other vaccinees generated much greater numbers of antigen-specific T cells after challenge (VSV-boosted monkeys T694 and R445). In the VSV-boosted monkey group, T-cell expansion followed rather than preceded peak viremia, suggesting that T-cell numbers expanded as necessary to control infection. Greater and more prolonged expansion of antigen-specific T-cell numbers postchallenge may therefore be an indicator of weaker rather than stronger vaccine-induced protection.

DISCUSSION

A previous study demonstrated that immunization of macaques with VSV-based vectors expressing SHIV Gag and Env

proteins followed by two VSV boosts generates immune responses capable of preventing AIDS in macaques for up to 3 years after challenge with SHIV 89.6P (reference 23 and unpublished results). In this study, we demonstrate that protection from AIDS can also be achieved with a single boost consisting of VSV vectors expressing SHIV Gag, Pol, and Env proteins (group A, VSV-VSV). Most importantly, however, animals receiving a single boost of the heterologous virus MVA expressing Gag, Pol, and Env generally raised stronger cellular immune responses prior to challenge than animals in the VSV-VSV group and were better protected after challenge, with significantly lower peak viral loads and better maintenance of CD4⁺-T-cell counts. All eight animals in the two vaccine groups have remained healthy, while two of the four controls have already progressed to AIDS and have been euthanized. A third control animal has very low CD4⁺-T-cell counts but has not yet shown symptoms of AIDS. If our previous controls that received the same challenge stock are included, we have already detected statistically significant protection from AIDS for both groups in this study ($P = 0.019$; Fisher's exact test). In addition, there are two other published reports of studies in which animals challenged i.v. with SHIV 89.6P were monitored for at least 250 days, as in our study (10, 29). When we pool the results seen with all control animals from these studies ($n = 19$, including 8 animals that expressed the Mamu-A*01 allele), we find that 15 of 19 animals progressed to AIDS by day 250 postchallenge. Comparing these controls to our vaccinees, we can again conclude that both of our vaccine groups show significant vaccine protection from AIDS ($P = 0.008$; Fisher's exact test).

One animal in the VSV boost group (T694) and two in the MVA group (V864 and AL86) express the Mamu-A*01 allele. We selected these animals for our study so that we could quantify their antigen-specific T-cell responses through the use of MHC-I tetramers. One concern with respect to the inclusion of Mamu-A*01⁺ macaques is that their progression to AIDS may be delayed relative to that of animals not expressing this allele (29). In our study, we find (as determined on the basis of comparisons of peak viral loads, CD4 counts, CTL responses, and neutralizing antibody responses) no evidence that the presence of the Mamu-A*01 allele conferred an advantage on any animal compared to the results seen with other animals in the same boost group.

We did not have enough animals available to include a group receiving an MVA prime and MVA boost. However, two publications have already described vaccination and two boosts with MVA encoding SHIV Gag-Pol and Env proteins followed by i.v. challenge with SHIV 89.6P (3, 10). The levels of CD8⁺-T-cell responses to SHIV antigens in those studies were lower than those we saw with the VSV prime and MVA boost protocol used in the present study, and the peak viral loads in all animals following challenge ranged from 10- to 100-fold higher than those seen in our VSV-MVA boost group. In addition, in the longer-term study in which progression to AIDS could be evaluated (10), two of five vaccinees developed high viral loads and progressed to AIDS within the time frame of our present study.

Infections with viral vectors typically induce strong immune responses. Why does the combination of two completely heterologous viral vectors work so well? We think this is probably

FIG. 5. Antigen-specific T-cell numbers expand in all animals after challenge. Antigen-specific T cells in the peripheral blood were identified by either MHC-I tetramer staining (A and B) or vaccinia-based ELISPOT (C to F) assays. Panels A and B show the expansion of CD8⁺ T-cell numbers among the three Mamu-A*01-positive monkeys via labeling with p11C-M Gag and HIV Env p41A Env tetramer, respectively. Panels C to F show levels of IFN- γ -secreting cells specific for SIV Gag (C and E) or HIV Env (D and F).

because the only antigens shared between the priming and boosting vectors are the SHIV Gag, Pol, and Env proteins. Thus, the boost focuses all the CD8⁺-T-cell recall responses on the SHIV proteins during the second infection. When the VSV envelope exchange vectors are used, the four internal VSV proteins (N, M, P, and L) in addition to Gag, Pol, and Env are identical between the priming and boosting vectors. The CTL responses to VSV proteins may limit vector replication and also compete with the CTL responses to the HIV proteins.

CTL directed to epitopes in the Gag Pol and Env proteins that are induced during the VSV prime may also limit MVA vector replication by killing MVA-infected cells. Because the MVA vector produces little or no replication-competent virus in human cells (5, 27), however, the effect on MVA may be less severe than on the replication-competent VSV.

Because of the highly differing immune responses in outbred animals such as rhesus macaques and the relatively small numbers available for these studies, it is difficult to quantitate the

degree to which the heterologous boost improves immune responses. In inbred mice, priming with VSV Gag and boosting with vaccinia virus Gag generated about five times more recall and long-term CD8⁺ T cells than a VSV Gag prime and VSV Gag (G-exchange) boost (11). In macaques a clear trend in the same direction is evident, but the differences in the immune responses do not reach statistical significance. However, the difference between the VSV-VSV and VSV-MVA groups with respect to peak viral loads following challenge is highly significant ($P < 0.03$; Mann-Whitney test). Indeed, the average peak SHIV 89.6P viral load ($10^{6.2} \pm 10^{0.1}$) in the VSV-MVA group is lower than that reported to date for other vaccine studies employing an i.v. SHIV 89.6P challenge. Only long-term infection of macaques with live, nonpathogenic SHIVs has yielded lower peak viral loads of SHIV 89.6P following superinfection (14). Based on the work reported here, we think that heterologous viral vectors used in a prime-boost protocol are likely to be an optimal method for providing protection from AIDS in human vaccine trials.

The attenuated VSV-based vectors have a major advantage of low-level seropositivity in the human population. In contrast, a significant fraction of the population has received vaccinia virus as a smallpox vaccine, and additional vaccination of some individuals has been undertaken in response to smallpox as a potential bioterrorism threat. These two considerations suggest that viral vectors other than MVA should also be tested in combination with the VSV-based vector in a prime-boost protocol.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI-45510, AI-40357, and AI-85343 and the TNPRC base grant A42616G1. Douglas F. Nixon is an Elizabeth Glaser Scientist of the Elizabeth Glaser Pediatric AIDS Foundation.

We thank Norman Letvin for providing the SHIV 89.6P challenge virus, and we are grateful to the veterinary staff at the Tulane Primate Center for their careful work supporting these studies. MHC-I tetramers were provided by the NIH tetramer facility.

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