HIV-1 DNA/MVA vaccination reduces the per exposure probability of infection during repeated mucosal SHIV challenges

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Abstract

Historically, HIV vaccines specifically designed to raise cellular immunity resulted in protection from disease progression but not infection when tested in monkeys challenged with a single high virus exposure. An alternative approach, more analogous to human sexual exposures, is to repetitively challenge immunized monkeys with a much lower dose of virus until systemic infection is documented. Using these conditions to mimic human sexual transmission, we found that a multi-protein DNA/MVA HIV-1 vaccine is indeed capable of protecting rhesus monkeys against systemic infection when repeatedly challenged with a highly heterologous immunodeficiency virus (SHIV). Furthermore, this repetitive challenge approach allowed us to calculate per-exposure probability of infection, an observed vaccine efficacy of 64%, and undertake a systematic analysis for correlates of protection based on exposures needed to achieve infection. Therefore, improved non-human primate models for vaccine efficacy can provide novel insight and perhaps renew expectations for positive outcomes of human HIV clinical trials.

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Introduction

Effective control of the AIDS epidemic will require vaccines capable of limiting the sexual transmission of a spectrum of HIV-1 isolates representing different subtypes and dissimilar circulating recombinant forms. Given that greater than 5 million people were newly infected with HIV-1 in 2004 (AIDS epidemic update, 2004; Joint United Nations Programme on HIV/AIDS [http://www.unaids.org]), it is imperative that such a vaccine is developed and made available for worldwide distribution.

Currently, there are approximately 35 preventive HIV vaccines in human trials worldwide (IAVI Report, 2005; IAVI [http://www.iavireport.org]). Among these, several approaches have been developed that raise cellular immunity and protect rhesus monkeys from AIDS caused by high dose challenges with SHIV-89.6P, although they do not prevent infection by the challenge virus. These approaches include: vaccination with...
plasmid DNA encoding 8 SHIV proteins followed by boosting with a recombinant MVA encoding matched SHIV Gag, Pol and Env proteins (Amara et al., 2001); vaccinations with plasmid DNA encoding SHIV Gag and Env proteins and plasmid encoding IL-2/Ig (Barouch and Letvin, 2000); vaccination with defective adenovirus vectors encoding SHIV Gag protein (Shiver et al., 2002); and vaccinations with attenuated vesicular stomatitis virus vectors encoding Env and Gag proteins (Rose et al., 2001).

In the past, HIV vaccine regimens, including vaccines designed to specifically induce cellular immunity (Allen et al., 2000; Barouch et al., 2001a; Vogel et al., 2002), have been tested in non-human primates by administering a single high dose of SIV or SHIV either by intravenous injection (Barouch and Letvin, 2000; Rose et al., 2001; Seaman et al., 2005; Shiver et al., 2002) or intrarectal or intravaginal challenges (Amara et al., 2001; Lena et al., 2002). This historical approach uses doses of virus that are much higher than those observed during natural transmissions of HIV where multiple exposures generally occur for infection (Chakraborty et al., 2001; Gray et al., 2001; Tachet et al., 1999). Furthermore, the per-exposure risk of infection is 100% in monkeys with this high dose of virus, whereas the per-exposure risk of HIV infection in humans is generally estimated to be in the range of 0.01–10%. The highest level of vRNA detected in semen of men with acute HIV-1 subtype B infections (without sexually transmitted diseases) is $10^{5.24}$ copies/mL (mean ± SD for that study, $4.1 ± 1.14 \text{log}_{10}$ copies/mL (Pilcher et al., 2004)). The minimal transmission rate for the average heterosexual male with acute HIV-1 infection has been predicted to be 0.0047 (1 transmission event/213 episodes of intercourse), suggesting that the male would transmit virus to 2%–6% of female sex partners within the first 2 months of the acute infection (Pilcher et al., 2004).

An alternative, albeit more demanding, approach than the single standard high virus dose is to repetitively challenge with a lower dose of virus similar to that observed in human semen. Several groups have demonstrated that repetitive mucosal SIV and SHIV challenges result in successful transmission and systemic infection of macaques, and these investigators are using different routes of exposure, including vaginal, rectal and oral (Ma et al., 2004; McDermott et al., 2004; Otten et al., 2005; Van Rompay et al., 2005). For comparison to the single higher dose, Otten et al. have shown that 10 TCID of SHIV162P3 was successfully transmitted 33% of the time (3 infections after a total of 9 intravaginal exposures) in female pig-tailed macaques (Otten et al., 2005). Moreover, this challenge dose was approximately 5-fold higher than HIV-1 RNA levels in human semen during acute infection, a period of accepted high transmission risk (Cohen and Pilcher, 2005). Lastly, Otten et al. have shown that the repetitive-exposure, non-human primate model can be used successfully to conduct preclinical studies focused on interventions to prevent transmission (Otten et al., 2005).

The purpose of our present study was to determine whether an HIV-1 DNA/MVA vaccine could provide protection from infection using repetitive intrarectal exposures with doses of a heterologous challenge virus (SHIV) that more closely parallel viral loads observed in semen. We report that vaccinated macaques were significantly less likely to be infected than naïve control animals and 25% of the vaccinated monkeys (4/16) showed protection from infection after 18 or more virus exposures. These findings provide the first direct evidence that a multi-protein DNA/MVA vaccine can successfully prevent highly heterologous transmissions in a model that more closely mimics human sexual transmission.

Results

Repetitive intrarectal challenges

Vaccinated monkeys were challenged 7–11 months after completing an immunogenicity trial of an HIV-1 CRF02_AG DNA prime-MVA boost regimen (Ellenberger et al., 2005) to investigate whether the monkeys would be protected from heterologous SHIV challenges using repetitive intrarectal exposures. Two naïve monkeys were included; one monkey became systemically infected after 2 challenges and the second monkey after 8 exposures. Historical rhesus macaque naïve controls ($n = 12$) when challenged under identical conditions, including the same virus stock of SHIV162P3 were shown to be infected after an average of 4.6 exposures (range 1–14 exposures) with only a single animal remaining uninfected after 14 exposures (data not shown). Monkeys were considered systemically infected by documented evidence of plasma viremia and confirmed by provirus detection in PBMC. Among the vaccinated monkeys, nine were infected with 6 or fewer exposures and one was infected after 10 exposures. However, quite remarkably, 6 of these 16 (37.5%) vaccinated monkeys remained protected from systemic infection after 17 challenges; well beyond what is necessary to achieve infection of the naïve control monkeys (Fig. 1). Furthermore, 4 of these 6 monkeys that remained protected from systemic infection were not infected by 18 or more exposures and in fact, one animal (99H) was selected to receive 8 additional challenges for a total of 26 without evidence of systemic infection. With the inclusion of the 12 additional rhesus naïve controls, as a group, the 16 vaccinated animals were 2.6-fold less likely to be infected than 14 naïve control monkeys (Cox hazard model, $P = 0.027$; hazard ratio is 0.391).

Assessment of vaccine efficacy

Based on a per-exposure transmission probability model, the outcome of the challenge study indicated a significant “leaky vaccine” effect ($P = 0.007$). Under this partial protection model, the maximum likelihood estimate of vaccine efficacy was 0.64, indicating a 64% reduction in the probability of infection per exposure among vaccinated animals compared to the 14 naïve control animals. The corresponding 95% confidence interval for vaccine efficacy was (0.26, 0.83). Comparison of the log likelihood values for the leaky model and an all-or-none model suggests that the leaky model demonstrates superior fit. Further evidence that the partial protection model fit the animal challenge data well is provided by the excellent agreement between the observed survival curve and an expected survival curve from the fitted
leaky vaccine model (Fig. 1). The expected curve is based on the maximum likelihood estimates of vaccine efficacy (0.64) and the per-exposure transmission probability (0.203) from the fitted leaky vaccine model to the SHIV challenge study data. A goodness-of-fit assessment also indicated adequate fit of the leaky model \( (P = 0.35) \).

Viremia as a secondary endpoint

Most successful HIV vaccine regimens have controlled viremia following infection by either SIV or SHIV high-dose exposure. Therefore, the plasma viral load profiles of the vaccinated animals that became systemically infected \( (n = 12) \) were compared to that of 14 naïve control monkeys (Fig. 2). There was a significant difference in the viral load peak when the vaccinated animal group (geometric mean, \( 2.1 \times 10^5 \); range \( 1.1 \times 10^5 \)–\( 1.8 \times 10^6 \) RNA copies/mL plasma) was compared to the naïve control group (geometric mean, \( 5.5 \times 10^5 \); range \( 2.2 \times 10^5 \)–\( 2.9 \times 10^6 \) RNA copies/mL plasma), \( P \) value = 0.0002. However, the viral load for the vaccinated group over time (area under the curve) was not significantly lower than the naïve control group (geometric mean, \( 5.5 \times 10^3 \); range \( 7.5 \times 10^2 \)–\( 8.2 \times 10^4 \) versus geometric mean, \( 1.9 \times 10^5 \); range \( 2.7 \times 10^5 \)–\( 4.1 \times 10^7 \) RNA copies/mL), \( P \) value = 0.244.
Analysis for correlates of protection

Based on the number of exposures needed to achieve infection (Table 1), 10 animals were considered most susceptible to infection while 6 animals, remaining uninfected after 17 or more exposures (~4-fold beyond the average exposures for non-vaccinated animals), were considered least susceptible. These groupings of relative susceptibility were then used to investigate correlates of protection by comparing vaccine-induced pre-challenge immunogenicity data. At the peak cellular response, 1 week post-MVA, the mean Gag and Env CRF02_AG temporal ELISPOT responses for each group (most susceptible vs. least susceptible) were essentially identical (Figs. 3A and B). At memory time point, 8 weeks after the MVA boost, the mean Gag ELISPOT responses among the least-susceptible group of animals was approximately 2-fold greater than the most-susceptible animals (Fig. 3C). The Env ELISPOT responses at the memory time point were too low to reasonably evaluate (data not shown). To test for an association between these ELISPOT responses and risk of infection, a Cox proportional hazards model was fit using data from vaccinees between these ELISPOT responses and risk of infection, a Cox proportional hazards model was fit using data from vaccinees between these ELISPOT responses and risk of infection (data not shown). To test for an association between these ELISPOT responses and risk of infection, a Cox proportional hazards model was fit using data from vaccinees between these ELISPOT responses and risk of infection (data not shown).

In addition, other immune response parameters were examined to investigate correlates of protection. In the memory phase, the mean cross-subtype cellular responses to Gag peptides from diverse HIV-1 subtypes A, AE, and B (Fig. 4) were essentially identical (Figs. 3D, E and F). However, none of the associations achieved statistical significance, but there was a trend for memory Gag ELISPOT responses and risk of infection (P = 0.06).

In addition, other immune response parameters were examined to investigate correlates of protection. In the memory phase, the mean cross-subtype cellular responses to Gag peptides from diverse HIV-1 subtypes A, AE, and B (Fig. 4) were essentially identical. These data were also tested for an association between the immune response and risk of infection with the Cox proportional hazards model (as described above) but statistical significance was not achieved (data not shown). In addition, pre-challenge humoral immunity was investigated by measuring neutralizing antibody, binding antibody, and anti-Env antibody to a CRF02_AG virus (Ellenberger et al., 2005), but no differences were revealed between the two groups of immunized animals based on susceptibility to infection (data not shown).

Major histocompatibility complex-typing of animals

Since genetic haplotypes of the major histocompatibility complex can influence mammalian immune responses and susceptibility to infection, genetic typing of the 16 vaccinated monkeys was completed (Table 1). As expected in an outbred population, the genetic haplotypes among this animal cohort were unique. Importantly, neither Mamu-A*01 nor Mamu-B*17 animals were among those studied, as both these Mamu alleles have been previously associated with control of SIVmac239 infection (O’Connor et al., 2003). We compared the genetic haplotypes of the most susceptible animals to the least susceptible group and no significant associations with susceptibility were found except that all 3 Mamu-A*02-positive animals fell within the most susceptible group and were infected by 6 or less virus challenges.

Tests for subclinical infection

Inguinal lymph node biopsies were performed on 2 monkeys that had no evidence of systemic infection after 18 exposures. By using a quantitative DNA-PCR assay sensitive to 1 proviral copy per 10^7 cells, lymph node extracts from these monkeys showed no evidence of SHIV provirus; thereby supporting their virus-free status (data not shown). Inguinal lymph node biopsies were also taken from two vaccinated animals that had become systemically infected after 6 exposures and as expected, provirus was detected at a significant level in both macaques (data not shown).

The possibility remained that the 4 immunized animals without evidence of systemic infection (after 18 exposures) harbored a low-level viral infection. Therefore, 3 months after the animals received their last viral challenge 3 of these animals were administered antibody cM-T807 to ablate the CD8+ T-cell population while the other received a control antibody (IVIg). The cM-T807 antibody treatment resulted in the near total depletion of CD8+ T cells within 72 h (Fig. 5A). However, no systemic viremia emerged in these treated animals over the course of the 28-day follow-up (Fig. 5B). Two systemically infected vaccinated animals that had controlled plasma viremia to below detection were also administered cM-T807 (Fig. 5C).

In these animals, plasma viremia increased by three to four orders of magnitude immediately upon CD8+ cell ablation and decreased rapidly following the reemergence of the CD8+ T cell population (Fig. 5D).

Upon euthanasia, gut-associated lymphoid tissue, spleen, lymph nodes, and testes were harvested from all macaques.

Table 1

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Animal</th>
<th>Mucosal challenge</th>
<th>Infection status</th>
<th>MHC class I</th>
<th>MHC class II</th>
</tr>
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<td>Infected</td>
<td>A*02</td>
<td>W201</td>
</tr>
<tr>
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<td>A*08</td>
<td>W201</td>
<td></td>
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<td>AC83</td>
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<td></td>
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</tr>
<tr>
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<td>5</td>
<td>Infected</td>
<td>A*02</td>
<td>W201</td>
<td></td>
</tr>
<tr>
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<td>5</td>
<td>Infected</td>
<td>A*08</td>
<td>W201</td>
<td></td>
</tr>
<tr>
<td>M509</td>
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<td>A<em>02, A</em>08</td>
<td>W201</td>
<td></td>
</tr>
<tr>
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<td>W201</td>
<td></td>
<td></td>
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<td>W201</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Infected</td>
<td>W201</td>
<td></td>
<td></td>
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<tr>
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<td>10</td>
<td>Infected</td>
<td>A<em>08, B</em>01</td>
<td>W201</td>
<td></td>
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<tr>
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<td>18</td>
<td>Infected</td>
<td>A*08</td>
<td>W201</td>
</tr>
<tr>
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<td>W201</td>
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<tr>
<td>99H</td>
<td>26</td>
<td>No infection</td>
<td>W201</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Number of mucosal challenges received.

b Cell-free and cell-associated virus load assays were positive.

c Rhesus MHC Class I: A*01, A*02, A*08, A*11, B*01, B*03, B*04 and B*17.

d Rhesus MHC Class II: DR*W201 and *0401/06.

e Ten vaccinated animals that were infected by 10 or less mucosal challenges.

f Six vaccinated animals that resisted systemic infection for 17 or more mucosal challenges.
From these various tissues, lymphocytes were recovered and co-cultured. There was evidence of spreading viral infection in 26% (23/87) of the infected macaque (n = 12) tissue lymphocyte co-cultures including approximately 50% (15/31) of the lymph node-derived tissue co-cultures, but none (0/34) from the 4 immunized but protected animals (Fig. 6), indicative of uninfected status.

**Discussion**

Our results demonstrate that a multi-protein HIV-1 DNA/MVA vaccine is capable of protecting animals from infection by repeated intrarectal challenges with a highly heterologous SHIV. These results would clearly not have been expected based on many previous studies where similar vaccine regimens eliciting cellular immunity protected animals from disease progression but not infection by homologous virus challenge (Amara et al., 2001; Barouch and Letvin, 2000; Barouch et al., 2001b; Rose et al., 2001; Shiver et al., 2002). Therefore, the application of the repeated dose exposure approach to vaccine evaluation may uncover aspects of protection and efficacy not appreciated by single higher dose exposure approaches. Furthermore, this method of expanding the exposure window through multiple challenges permitted the evaluation of vaccine efficacy beyond simply comparing the number of animals infected between the control and vaccinated groups. These data provide valuable insight into how HIV-1 vaccines eliciting cellular immunity may behave in human trials and suggest...
designing trials to assess efficacy within a framework of partial protection.

The predictive value of non-human primate models for HIV-1 vaccine efficacy hinges on how well the animal system represents human circumstances (Haigwood, 2004; Nathanson et al., 1999). The strategy of using an R5 HIV-1 envelope-based virus, a more realistic physiologic inoculum dose, and repeated mucosal exposures to achieve infection was employed in this study to more closely mimic human sexual HIV-1 transmission. Other investigators are also pursuing the development of repeat exposure models to infect non-human primates using different routes of exposure and different viral challenge strains (Ma et al., 2004; Otten et al., 2005; Van Rompay et al., 2005). The challenge dose used in this study is approximately 5-fold higher.

Fig. 5. Effect of CD8+ cell depletion on plasma viral loads. Monoclonal antibody cM-T807 was administered to rhesus monkeys 99H, M562, 94J, AC82 and M509. Monkeys 86H and 60J received a control antibody. The injection of antibody is indicated by arrows. (A and C) CD8+ T cells were enumerated by four-color flow cytometric analyses. (A and B) Monkeys protected beyond 17 intrarectal challenges. (C and D) Systemically infected macaques that had controlled plasma viremia to below detection. (B and D) Plasma levels of viral RNA. As marked on each graph, the sensitivity limits are 100 copies/mL for plasma.

Fig. 6. Tissue lymphocyte co-cultures. Comparison of the number of positive co-cultures demonstrating evidence of spreading viral infection in infected monkeys and uninfected vaccinees. Virus was propagated for 28 days in culture and analyzed on the basis of p27 values obtained from culture supernatants. Quantification of Gag was done using p27 antigen capture kits according to the manufacturer’s directions. White bars represent gut-derived tissues, filled dark bars represent spleen and testes, and light-colored gray bars represent lymph nodes.
than HIV-1 RNA levels in human semen during acute infection (Pilcher et al., 2004), a period of presumed high transmission risk (Cohen and Pilcher, 2005). Other animal challenge models may use in excess of $10^3$ to $10^5$ 50% tissue culture infective doses in single or multiple high dose challenges when evaluating a potential vaccine regimen (Allen et al., 2002; Nixon et al., 2000; Van Rompay et al., 2005). The combination of a highly transmissible inoculum dose and repeated exposures results in a rigorous challenge system in which an average of only 4.6 exposures was necessary to achieve infection among all our real-time and historic control animals combined (total $n = 14$, data not shown). While high-dose single exposure systems may still be advantageous for issues related to pathogenesis, repeat exposure systems can provide distinct advantages for the pre-clinical evaluation of interventions designed to prevent infection as recently concluded by Regoes et al. using virtual experimental simulations (Regoes et al., 2005).

Understanding correlates of protection from HIV-1 infection would dramatically advance progress towards vaccine implementation and ideally requires a system of study, such as the repeat exposure system, that provides a clear distinction among vaccinated individuals based on susceptibility to infection. The model described here is among the first to provide such a distinction. Furthermore, the repeated exposure approach allows for more sophisticated hazard models to be applied for direct evidence of an association between immune response parameters and susceptibility to infection. However, no obvious specific or non-specific correlate of protection was evident in this study between animals based on susceptibility. In addition to comparing pre-challenge cellular (Figs. 3 and 4) and humoral immune responses, we also measured cellular and humoral anamnestic responses and found no significant differences to distinguish animals based on their susceptibility (data not shown). We also found no significant differences in the ability to infect PBMCs in vitro with the challenge virus regardless of the animal’s susceptibility to infection in the repeat exposure model (data not shown). Such findings suggest that specific mechanisms of immune protection against HIV-1 infection are complex and may not necessarily involve only these cellular responses previously associated with protection from disease progression (Allen et al., 2000; Barouch et al., 2001a; Vogel et al., 2002). A similar conclusion was reached in studies of animal re-challenge after antiretroviral intervention during initial acute infection (Lifson et al., 2001) and oral challenge of vaccinated infant macaques (Van Rompay et al., 2005) where little or no viral-specific cellular and humoral immunity was associated with reduced pathogenesis.

Moreover, a combination of local and systemic immune mechanisms, including nonspecific barriers, adaptive immune responses and innate host defense mediators, may be necessary to provide full protection against HIV-1 exposure. Characterizing these resistance factors present at the mucosal site of exposure will be important adjuncts to understanding vaccine-elicted protection. Furthermore, the system we describe here may be adapted to efforts aimed at uncovering natural resistance or other yet undefined mechanisms as has been implicated in highly exposed persistently seronegative individuals which remain uninfected despite repeated exposure to virus (Kulkarni et al., 2003). Frequent and repeated exposure to virus may be important in maintaining a combination of specific and non-specific mucosal resistance necessary to prevent infection (Kaul et al., 2001).

The repeat exposure method expands the challenge window and, in theory, may allow the vaccine-elicited immune memory an opportunity to be recalled and recruited to the site of mucosal exposure. Examination of the mucosal lymphocytes found in the lamina propria of protected animals upon necropsy will also provide meaningful insight into mechanisms of protection as the gut CD4/CCR5-expressing effector lymphocytes are demonstrated crucial sanctuaries for SIV and HIV-1 production (Brenchley et al., 2004; Li et al., 2005; Mattapallil et al., 2005; Veazey et al., 1998). However, had these animals actually harbored subclinical infection as the basis for their apparent protection, we would have expected some measurable viremia following CD8 depletion as observed by others (Jin et al., 1999; Lifson et al., 2001; Schmitz et al., 1999). The resulting burst of viremia and subsequent control in the systemically infected animals (Fig. 5D) does indeed support the importance of cellular immunity in suppressing HIV and SIV infections (Borrow et al., 1994; Cranage et al., 1997; Gallimore et al., 1995; Greenough et al., 1997; Koup et al., 1994; Matano et al., 1998, 2004; Schmitz et al., 1999).

Ultimately, the efficacy of HIV-1 vaccines eliciting cellular immunity will most likely be determined in human trials. Current presumptions that these vaccine regimens will only impact HIV-1 disease progression are largely derived from non-physiological high-dose and/or intravenous challenges of non-human primates that may overwhelm moderately efficacious vaccines and provide little or no protection from infection (Cranage et al., 1997; Gallimore et al., 1995; Jin et al., 1999). This report advocates that an HIV-1 DNA/MVA vaccine can provide reasonable protection from infection against a highly heterologous challenge virus, should help guide future vaccine evaluation, provide insight into protection/resistance mechanisms, and renew expectations for potential outcomes of clinical trials.

Methods

Immunogens

The construction of the DNA and MVA immunogens has been previously described (Ellenberger et al., 2005). Briefly, the DNA vaccine prime expressed HIV-1 Gag, Pol, Tat, Rev, Vpu and Env (GenBank accession no. AY227361 and AY227362) and the MVA boost expressed matched Gag, Pol and Env proteins from a circulating recombinant form CRF02_AG (Ellenberger et al., 2002, 2004).

Immunizations

The immunization protocol has been previously described (Ellenberger et al., 2005). All animal protocols were approved.
by the Institutional Animal Care and Use Committees at both the Caribbean Primate Research Center and Centers for Disease Control and Prevention.

**Challenge virus stock and animal inoculations**

SHIV SF162P3 was obtained from the NIH AIDS Research and Reference Reagent Program (catalog no. 6526) for use in the present study. The in vitro tissue culture infectious dose (TCID) was verified on Con-A stimulated rhesus macaque PBMC (CD8-depleted). Endpoint TCID was used as a guide for the inoculum’s level (Otten et al., 2005). The virus stock was diluted with RPMI to 10 TCID/mL (3.8 × 10^5 viral particles) for the inoculum's level. The in vitro tissue culture infectious dose (TCID) was verified on Con-A stimulated rhesus macaque PBMC (CD8-depleted). Endpoint TCID was used as a guide for the inoculum’s level (Otten et al., 2005). The virus stock was diluted with RPMI to 10 TCID/mL (3.8 × 10^5 viral particles) for in vivo rectal inoculations.

All virus exposures involved atraumatic inoculation of cell-free virus (1 mL) into the rectal cavity via a sterile gastric feeding tube of adjusted length. Anesthetized macaques remained recumbent for 15 min following each IR inoculation that occurred on an approximate weekly basis.

Seven months after MVA boost; the initial SHIV challenge of two vaccinated and two naïve control monkeys was completed. Eleven months after the MVA boost, the remaining 14 vaccinated monkeys were challenged weekly. Using the same SHIV162P3 challenge stock, a total of 14 naïve rhesus monkeys (including 12 naïve control monkeys from past and current intervention studies) have received weekly challenges.

**Evidence of systemic infection**

Cell-free and cell-associated virus load assays were completed as previously described (Otten et al., 2005). Briefly, the cell-free virus load (plasma viremia) was assessed by quantifying viral RNA in blood plasma by use of a reverse transcriptase polymerase chain reaction (PCR) assay system. The Taqman real-time S' nucleicase technique was used to detect, amplify, and quantify cell-associated proviral DNA from PBMC samples. A synthetic-peptide EIA (Genetic Systems Core Antigen kit (Beckman Coulter, Fullerton, CA). Stained PBMC were washed with 3 ml of PBS, resuspended in 0.5 ml of PBS containing 1.5% paraformaldehyde and analyzed by using a FACSCalibur flow cytometer and the CELLQUEST (Becton-Dickinson) software. Absolute values of CD4, CD8, and NK cells were calculated from complete blood count results.

**Peptide pools**

HIV-1 subtype CRF02_AG peptides were 15mers overlapping by 11 matched to the vaccine strain. HIV-1 subtype A and CRF01_AE peptides were similarly 15mers overlapping by 11. Consensus B peptides were obtained from the NIH AIDS Research and Reference Reagent Program (catalog #5107). Peptides pools contained 25 sequential peptides. Peptides were dissolved in DMSO at 50–100 μg/ml and stock solutions maintained at −70 °C. Peptide working solutions were kept at −20 °C for 1 week.

**T-cell assays**

ELISPot assays were done as previously described (Ellenberger et al., 2005). Ten μl of antibodies were mixed and added to 100 μl of whole blood then incubated for 1 h in the dark. Red blood cells were lysed by incubation for another 10 min at room temperature with an Immunoprep Reagent (Beckman-Coulter). Stained PBMC were washed with 3 ml of PBS, resuspended in 0.5 ml of PBS containing 1.5% paraformaldehyde and analyzed by using a FACSCalibur flow cytometer and the CELLQUEST (Becton-Dickinson) software. Absolute values of CD4, CD8, and NK cells were calculated from complete blood cell count results.

**Tissue lymphocyte isolation and co-culture**

Isolation of mononuclear cells from gut-associated tissue necropsies (jejunum, duodenum, ileum, cecum and colon) were done using the collagenase type II method, and lymph nodes (peripheral, axillary and mesenteric) and spleen cells were teased into single cell suspensions as previously described (Shacklett et al., 2003). Co-cultures were established with Con A-stimulated CD8 T cell depleted macaque feeder PBMC and monitored for 28 days for the presence of Gag by using SIV Core Antigen kit (Beckman Coulter, Fullerton, CA).

**Statistical analysis and modeling of outcome data**

All statistical analyses were done using SAS. Inferences regarding vaccine efficacy were based on a discrete time transmission probability model which allows for both “leaky” and “all-or-none” effects of vaccination (Longini and Halloran, 1996). This model treats the number of exposures as the time.
scale and assumes the probability of infection is independent of the number of prior exposures. Maximum likelihood estimates were obtained using standard numerical techniques. Likelihood ratio tests were used to compare nested models and profile likelihood was employed to obtain confidence intervals. Goodness-of-fit assessment was completed using the Kolmogorov–Smirnov test.

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Use of trade names is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention.

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References


