Ability of herpes simplex virus vectors to boost immune responses to DNA vectors and to protect against challenge by simian immunodeficiency virus

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Abstract

The immunogenicity and protective capacity of replication-defective herpes simplex virus (HSV) vector-based vaccines were examined in rhesus macaques. Three macaques were inoculated with recombinant HSV vectors expressing Gag, Env, and a Tat-Rev-Nef fusion protein of simian immunodeficiency virus (SIV). Three other macaques were primed with recombinant DNA vectors expressing Gag, Env, and a Pol-Tat-Nef-Vif fusion protein prior to boosting with the HSV vectors. Robust anti-Gag and anti-Env cellular responses were detected in all six macaques. Following intravenous challenge with wild-type, cloned SIV239, peak and 12-week plasma viremia levels were significantly lower in vaccinated compared to control macaques. Plasma SIV RNA in vaccinated macaques was inversely correlated with anti-Rev ELISPOT responses on the day of challenge (P value <0.05), anti-Tat ELISPOT responses at 2 weeks post challenge (P value <0.05) and peak neutralizing antibody titers pre-challenge (P value 0.06). These findings support continued study of recombinant herpesviruses as a vaccine approach for AIDS.

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Introduction

Development of an effective vaccine against HIV/AIDS faces a number of severe difficulties (Desrosiers, 2004; Garber and Feinberg, 2003). In the absence of blind luck in stumbling upon a successful vaccine, one important obstacle is the lack of knowledge regarding the characteristics of a protective immune response that we should be looking for from a vaccine. Once initial infection is established, HIV has an uncanny ability to replicate continuously in the face of readily measurable immune responses to the virus. HIV has evolved a number of specific immune evasion strategies that allow this continuous, unrelenting viral replication (Desrosiers, 1999). These characteristics raise concern whether long-lived memory cells that may be present months or years after immunization can respond in time to provide sufficient blunting of viral replication during the initial days and weeks following exposure.

A defining feature of all herpesviruses is that they persist for the life of the infected individual. Replication-competent and replication-defective herpesviruses have been used experimentally as vectors for gene therapy (Burton et al., 2005) and for vaccine purposes. Vaccine applications of herpesvirus vectors have included both AIDS (Murphy et al., 2000) and non-AIDS (Hoshino et al., 2005; Morrison and Knipe, 1994; Spector et al., 1998) settings. Herpesvirus vectors may differ fundamentally from other viral vectors that

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have been tried experimentally, for example, poxviruses (Amara et al., 2001; Dale et al., 2004; Horton et al., 2002; Pal et al., 2002), adenoviruses (Patterson et al., 2003; Santra et al., 2005; Shiver et al., 2002) and rhabdoviruses (Ramsburg et al., 2004; Rose et al., 2001), regarding the nature and persistence of the immune responses that are generated. Since herpesviruses may periodically or chronically reactivate, the numbers of memory cells and the numbers of actively responding cells at any one time after the last exposure may be significantly higher than that which is observed with viruses that do not have the potential to persist. Consequently, we have been interested in the ability of recombinant herpesviruses to provide vaccine protection against AIDS in an experimental setting.

Here we describe the immune responses and protective capacity of replication-defective herpes simplex virus (HSV) vectors with and without prior priming by recombinant DNA.

Results

Experimental design

To improve upon our earlier studies that utilized replication-competent and replication-defective HSV vectors (Murphy et al., 2000), we constructed new experimental HSV-SIV vaccines based on HSV-1 Δ106, a replication-defective vector with multiple HSV genes deleted (Samaniego et al., 1998). The lack of expression of ICP4 and ICP27 prevents the shut-off of host cell transcription and translation and allows prolonged expression of the transgene. The lack of host shut-off and early and late HSV protein synthesis prevents MHC downregulation and ICP47-associated blockage of the TAP peptide transporter. Separate HSV-1 Δ106 vectors were engineered to express gag, env and a rev–tat–nef fusion protein. High levels of SIV gag and env expression were achieved by use of codon-modified cassettes that were modified to achieve maximal levels of expression (Nasioulas et al., 1994; Schneider et al., 1997; Schwartz et al., 1992a,b). The nef reading frame in the Tat-Rev-Nef fusion protein was modified to render it incapable of MHC class I downregulation (Swigut et al., 2004). This vector expresses significantly higher levels of SIV protein for more prolonged periods than we have been able to achieve previously in the absence of HSV-induced cytopathic effect (Watanabe et al., 2007).

We also included a group of monkeys in these studies that were primed by DNA immunization prior to recombinant HSV-1 Δ106 immunization. The recombinant DNA constructs that were used have been described recently (Rosati et al., 2005). Gag and Env expression plasmids were fused to MCP-3 to facilitate humoral immune responses. Gag, Env, Pol, Nef, Tat and Vif genes were fused with the β-catenin peptide in order to target the viral proteins to the proteasomal degradation pathway and enhance MHC class I antigen presentation. SIV239 sequences were used for all immunogen constructions.

One group of three Indian-origin rhesus macaques (Mm 136-90, 450-91 and 349-91) received the mixture of HSV/SIV recombinant Δ106 strains at weeks 0, 4, 12 and 20 (Fig. 1). Another group of three Indian-origin rhesus macaques (Mm 205-87, 297-91 and 240-91) was immunized with the mixture of DNAs at weeks 0 and 4 and the mixture of HSV/SIV recombinant Δ106 strains at weeks 12 and 20 (Fig. 1). Two macaques from each group were Mamu-A*01-positive (Mm 136-90, 450-91, 205-87 and 297-91). One macaque (Mm 450-91) was Mamu-B*17-positive. All of the rhesus monkeys were obtained from the herpes B-free, specific pathogen free breeding colony at the NEPRC (Desrosiers, 1997) and were negative for antibodies to HSV at the time of enrollment. All six vaccinated monkeys were challenged intravenously with WT SIV239 at week 26 (Fig. 1).

Antibody responses—vaccine phase

Antibodies to HSV were measured by ELISA using plates covered with whole lysed HSV virions. All six monkeys were negative for anti-HSV antibodies at the time of the first immunization (Fig. 2A). The three monkeys that received recombinant HSV-SIV Δ106 at week 0 responded with anti-HSV antibodies within weeks of this first immunization. Anti-HSV antibody levels appeared to be boosted in this group of animals upon each subsequent receipt of recombinant Δ106 inoculations (Fig. 2A). The initial inoculations of recombinant DNA, in contrast, did not result in anti-HSV reactivity in this assay, as expected. However, inoculation of this group of monkeys with the HSV-SIV recombinant Δ106 strains at week 12 did promptly result in readily detectable levels of anti-HSV antibodies (Fig. 2A). These persisted in all six monkeys through challenge.

Antibodies to SIV assayed again by whole virus ELISA using methodologies that have been extensively described and

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Fig. 1. Schedule of immunizations and SIV challenge in six rhesus macaques. The DNA immunization consisted of five plasmid constructs: MCP3p39 gag, MCP3p39 env, CATEDX gag, CATE239 env and CATEpolNTV. One milligram of each plasmid DNA was administered via the intramuscular route. The HSV immunization included three recombinant HSV-1 Δ106 constructs, Δ106env, Δ106gag and Δ106rev-tat-nef, each administered at a dose of 1.25 to 3 × 10^9 pfu per dose. Two-thirds of the HSV vaccine dose was inoculated via the intramuscular route while one-third of the dose was administered via the subcutaneous route. Ten animal infectious doses of cloned homologous pathogenic SIV239 inoculated via the intravenous route were used for challenge.
validated previously (Daniel et al., 1988; Wyand et al., 1996) were not so readily detected (Fig. 2B). However, anti-SIV antibodies were measured in all six monkeys with this assay by 2 weeks after the final vaccine boost (Fig. 2B).

We next measured the ability of plasma to neutralize SIV infectivity. None of the plasma was able to neutralize infectivity of SIV239 detectably. This is not surprising because SIV239 is difficult to neutralize, and even monkeys infected experimentally with SIV239 make neutralizing antibodies of low levels when measured against this strain (Johnson et al., 2003). Neutralizing activity was detected, however, when the laboratory-passaged, neutralization-sensitive SIV251 strain (SIV251-lab) was used (Fig. 3A and Table 1). Neutralizing activity against SIV251-lab was detected at multiple time points in five of the six monkeys prior to challenge (Table 1). The highest titers of

<table>
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<tr>
<th>Sample time</th>
<th>50% neutralization titers to lab-adapted SIVmac251</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mm 205-87</td>
</tr>
<tr>
<td>0</td>
<td>&lt;1:20</td>
</tr>
<tr>
<td>2</td>
<td>1:20</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1:20</td>
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<td>1:20</td>
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<tr>
<td>28</td>
<td>1:20</td>
</tr>
<tr>
<td>34</td>
<td>1:20</td>
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</tbody>
</table>

Table 1

Ability of plasma from immunized monkeys to neutralize SIV infectivity


Fig. 2. Antibody responses to (A) HSV and (B) SIV in immunized monkeys by ELISA. Closed symbols denote the DNA–DNA–HSV–HSV vaccinated macaques, while open symbols denote the HSV–HSV–HSV–HSV vaccinated macaques. Arrows show the time of vaccination. Anti-HSV antibody responses in plasma were tested at 1 in 200 dilution, while anti-SIV antibody responses are shown at 1 in 20 plasma dilutions.

Fig. 3. Neutralizing antibody responses to laboratory-passaged SIV251 in six vaccinated rhesus macaques. (A) At week 26, on day of challenge. (B) At week 28, 2 weeks post SIV challenge. Serial two-fold dilutions of plasma were incubated with SIV251-lab for 1 h before addition of $3 \times 10^5$ CEMx174SIV-SEAP reporter cells. One pool of negative rhesus monkey plasma was used as a negative control, and two separate pools of SIV+ rhesus monkey plasma were used as positive controls at the time of each assay. In the assays shown 50% neutralizing antibody titers in the positive pool plasma ranged between 1:3200 and 1:5120. Negative control plasma was negative.
neutralizing activity that were achieved against SIV251-lab prior to challenge were 1:160. This contrasted with titers of 1:3200 to 1:5120 in pooled sera obtained from unvaccinated rhesus macaques infected with SIV239 and used as a positive control in the neutralization assay. Thus, the vaccine regimens did induce neutralizing activity but the titers were low and they did not detectably neutralize cloned SIV239 whose sequences were used in the immunogen constructions.

**Cellular immune responses—vaccine phase**

Cellular immune responses to SIV were measured by the IFN-γ ELISPOT and ICS assays using overlapping peptides of individual SIV239 proteins as immunogens. Additionally, Mamu-A*01/Gag CM9 tetramer-reactive CD8+ T lymphocytes were measured in the Mamu-A*01-positive rhesus macaques. IFN-γ ELISPOT responses to the SIV proteins expressed by both DNA and HSV-1 d106 vectors (Gag, Env, Nef and Tat) were detected in all six vaccinated monkeys (Fig. 4A). From the time of the HSV booster inoculation at week 12, the magnitude of ELISPOT responses to shared SIV immunogens remained significantly higher in the DNA–DNA–HSV–HSV immunized macaques compared to the HSV–HSV–HSV–HSV immunized macaques (Table 2). At its peak, Gag-specific responses as high as 1442 spot forming cells (SFC)/10^6 PBMC and Env-specific responses as high as 3208 SFC/10^6 PBMC were observed in the DNA–DNA–HSV–HSV group of rhesus macaques (Fig. 4A). Such levels are comparable to or higher than those achieved after wild-type SIV infection of unvaccinated rhesus macaques. In contrast to Gag and Env, positive ELISPOT responses to the SIV regulatory proteins Nef, Tat and Rev were of considerably lower magnitude (Fig. 4A).

Mamu-A*01/Gag CM9 tetramer-reactive CD8+ T lymphocytes were detected in all four Mamu-A*01-positive vaccinated rhesus macaques and reached a peak level of 2.3% of CD8+ T lymphocytes in the peripheral blood of Mm 205-87 (Fig. 4B). There was a trend for increasing affinity of Gag CM9-specific CD8+ T lymphocytes with each HSV booster inoculation.
inoculation in the DNA primed macaques as evidenced by an eight-fold or greater decrease in the peptide concentration required for T cell activation (Fig. 4C). One week after the last vaccine inoculation (week 21), tetramer-positive CD8+ T lymphocytes with both effector memory (CD28−, CD62L−, CCR7−) and central memory (CD28+, CCR7+, CD62L+) phenotype were observed (Fig. 5 and Table 3). At this time point, the gut-homing molecule α4β7 was expressed on 13–50% of tetramer-positive CD8+ T lymphocytes in the peripheral blood (Table 3). The frequency of Gag CM9 tetramer-reactive CD8+ T lymphocytes correlated positively with Gag ELISPOT responses, suggesting that a significant portion of the Gag-specific T lymphocyte response in Mamu-A*01-positive rhesus macaques consisted of CD8+ T lymphocytes responding to the Gag CM9 peptide (Fig. 6A). ELISPOT responses to the whole SIV239 Gag p55 antigen were also detected and correlated positively with Gag peptides (Fig. 6B). Because whole proteins predominately stimulate CD4+ T lymphocytes in vitro, the positive p55-specific ELISPOT response suggested that SIV-specific CD4+ T lymphocytes were also induced in vaccinated macaques. Induction of both CD4+ and CD8+ T lymphocyte responses to SIV was confirmed by the ICS assay (Fig. 7 and Table 4). CD4+ T lymphocytes responding to SIV Gag and/or Env were detected in four vaccinated macaques (Table 4).

Results of challenge

All six vaccinated macaques were challenged intravenously with 10 animal infectious doses of SIV239 at week 26, 6 weeks after the last vaccine dose (Fig. 1). All vaccinated monkeys became infected with SIV (Fig. 8). In the vaccinated monkeys, the median viral load at peak 2 weeks after SIV challenge was 2.4×10^6 (range 4.0×10^5 to 2.7×10^7) plasma SIV RNA copies/ml; at 12 weeks, it was 8.7×10^2 copies/ml (range 2.1×10^2 to 1.3×10^6 copies/ml); and at 24 weeks it was 1.6×10^3 copies/ml (range 7.0×10^1 to 4.5×10^6 copies/ml). One HSV–HSV–HSV–HSV vaccinated macaque (Mm 136-90) died suddenly 3 weeks after SIV inoculation and was likely a rapid progressor. The set-point plasma SIV RNA at 24 weeks post SIV challenge in three of the five surviving vaccinated macaques was <5000 copies/ml (Fig. 8).

To examine whether there was a vaccine effect on control of viremia, we compared plasma viral load in the vaccinated macaques with historical controls consisting of 31 unvaccinated SIV-infected Indian rhesus macaques, 14 from NEPRC and 17 from the Wisconsin Primate Research Center. All had been inoculated with the same stock of cloned SIV239 used for SIV challenge in the vaccinated macaques. While there is animal to animal variation, there have not been changes in the behavior of this virus stock in groups of unvaccinated control Indian-origin rhesus macaques over the course of several years. The median plasma SIV RNA level at peak (week 2) was 0.9 log lower, and at 12 weeks it was 1.7 log lower in the vaccinated as compared to the historical control rhesus macaques, and these differences were statistically significant by the nonparametric Mann–Whitney U test (Fig. 9). At later time points (20–24 weeks), the difference in plasma viral load between vaccinated and historical control macaques failed to reach statistical significance (Fig. 9).

Four of six vaccinated macaques (67%) and 23 of 31 controls (74%) were Mamu-A*01-positive (Table 5). Because the presence of Mamu-A*01 may be an independent predictor of SIV viral control (Mothe et al., 2003; Muhl et al., 2002; Pal et al., 2002; Zhang et al., 2002), we compared viral loads in vaccinated and control macaques stratified on the basis of their Mamu-A*01 status (Table 5). Vaccinated Mamu-A*01-positive macaques continued to demonstrate a significant reduction in peak and 10–14 week time point viral loads as compared to control Mamu-A*01-positive macaques (Table 5). Although there was a trend for lower viral loads in vaccinated Mamu-A*01-positive macaques at 17–28 weeks, this difference did

Table 2
Pre-challenge cellular immune responses in the two group of vaccinated macaques

<table>
<thead>
<tr>
<th>Weeks post vaccination</th>
<th>Mean (range) SFC/10⁶ PBMC</th>
<th>P value</th>
<th>DNA–DNA–HSV–HSV group (n = 3)</th>
<th>HSV–HSV–HSV–HSV group (n = 3)</th>
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<tbody>
<tr>
<td>0³</td>
<td>15 (4–32)</td>
<td>13 (0–30)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>186 (16–390)</td>
<td>317 (34–536)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>4³</td>
<td>121 (36–176)</td>
<td>361 (276–488)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>514 (462–558)</td>
<td>236 (130–448)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>12³</td>
<td>181 (88–318)</td>
<td>184 (88–236)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1389 (1280–1512)</td>
<td>357 (102–818)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>20³</td>
<td>487 (318–724)</td>
<td>121 (18–236)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>1489 (854–2464)</td>
<td>329 (270–412)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>26³</td>
<td>899 (430–1788)</td>
<td>288 (132–400)</td>
<td>&lt;0.05</td>
<td></td>
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</table>

³ Sum of IFN-γ ELISPOT responses to shared SIV immunogens (Gag, Env, Tat and Nef) at all time-points and Rev included after week 12 after subtraction of background.


Fig. 5. Phenotype of vaccine-induced Mamu-A*01/Gag CM9 tetramer-positive CD8+ T lymphocytes in the peripheral blood of one immunized monkey (Mm 205-87). Representative dot plots of four-color flow cytometry gated on CD3+CD8+ T lymphocytes shown. Percentages in upper right quadrant show the frequency of Gag CM9 tetramer-positive CD8+ T lymphocytes that express the molecule shown on the horizontal axis.
not reach statistical significance (Table 5). It is to be noted that one vaccinated macaque (Mm 450-91) contained two MHC Class I alleles, Mamu-A*01 and Mamu-B*17, a combination that is particularly associated with low SIV viral loads (O’Connor et al., 2003). On exclusion of the Mamu-B*17-positive monkeys (one vaccinated and five controls), the difference between vaccinees and controls continued to be statistically significant ($P$ value <0.05) for peak plasma viremia but did not reach statistical significance ($P$ value 0.07) for the 10–14 week time point. Given the small number of vaccinated macaques, we cannot definitively evaluate vaccine efficacy independent of the attenuating effect of MHC Class I alleles in the current study.

Immune responses post challenge

Neutralizing activity in plasma against SIV251-lab, presumably neutralizing antibodies, increased abruptly in all six animals following challenge, consistent with the viral load measurements demonstrating that all six vaccinated monkeys had become infected with SIV (Fig. 3B and Table 1). Neutralizing activity was dramatically increased in five of the six vaccinated monkeys by 2 weeks post challenge consistent with an anamnestic antibody response. Neutralizing activity was not detected against SIV239 even by 8 weeks post SIV infection. Dramatic increases in Mamu-A*01/Gag CM9 tetramer-reactive CD8+ T lymphocytes and ELISPOT responses to Gag, Env and Nef were observed in all vaccinated macaques in the first 2 to 4 weeks after SIV challenge (Fig. 10A and data not shown).

Since three of the six vaccinated macaques had set-point plasma SIV RNA levels of <5000 copies/ml, we investigated whether there was a correlation between vaccination-induced anti-SIV immune responses and the level of SIV viremia post challenge. Peak, day of challenge and 2 weeks post challenge anti-SIV immune responses were examined for inverse correlation with the area under curve (AUC) plasma SIV RNA values between 0 and 24 weeks of SIV infection (Figs. 10B, C and data not shown). Although Gag- and Env-specific cellular immune responses dominated the anti-SIV IFN-γ ELISPOT response prior to challenge, their magnitude at peak response or at day of challenge or at 2 weeks post challenge did not correlate with control of viral load (Figs. 10B, C and data not shown). Instead, cellular immune responses targeting accessory SIV proteins appeared to be important. The magnitude of the Rev-specific IFN-γ ELISPOT response on the day of challenge (Fig. 10B), as well as the magnitude of the Tat-specific IFN-γ ELISPOT response 2 weeks post challenge (Fig. 10C) showed a significant inverse correlation with viral load (Rho −0.86 and −0.87, respectively, $P$ values <0.05, Spearman Rank Correlation test). Neutralizing antibody titers on the day of challenge and 2 weeks post challenge did not correlate significantly with viral load (Figs. 10B, C). However, the maximal titer of neutralizing antibodies detected during the vaccine phase showed a strong trend towards an inverse correlation with AUC plasma SIV RNA (Rho −0.83, $P$ value 0.06, Spearman Rank Correlation test; data not shown).

Death of Mm 136-90

One vaccinated macaque, Mm 136-90, died 23 days after intravenous challenge with SIV239 with no premonitory signs of illness. Gross and microscopic abnormalities were confined to the brain. Cut sections of the formalin-fixed brain showed extensive tan discoloration with multiple red-brown foci.
confined to the white matter of the left occipital lobe (Fig. 11A). On light microscopic examination, multiple and randomly distributed ring hemorrhages and inflammatory cell infiltrates dotted the otherwise pale white matter; extension into the inner layers of the gray matter was infrequent (Fig. 11B). On higher magnification, the white matter was rarefied with angiocentric hemorrhage and/or inflammatory cell infiltrates (Fig. 11C). At high magnification, vascular walls were necrotic and surrounded by degenerate inflammatory cells primarily composed of neutrophils (Fig. 11D). Sections of the left occipital lobe immunostained with anti-myelin basic protein showed perivascular loss of myelin (Fig. 11E). Rare glial nodules were observed within the white matter of the cerebellum and the corpus striatum (Fig. 11F). The histopathologic findings are most consistent with a necrotizing and suppurative vasculitis culminating in secondary ischemic necrosis of the white matter with perivascular ring hemorrhages and angiocentric loss of myelin in the foci of inflammation. Light microscopic examination of multiple sections of the left occipital lobe stained with H&E, and several special stains did not detect viral inclusion bodies, protozoa, bacteria, acid fast bacilli or fungal organisms. A battery of immunohistochemical markers for herpes simplex virus, cytomegalovirus, adenovirus, SV40 and SIV did not establish a viral etiology. Furthermore, electron microscopic examination of several sites from the brain lesion did not identify an infectious etiology, including herpes simplex. Co-cultivation of trigeminal and sacral ganglia from Mm 136-90 onto Vero cells and the E-11 complementing cell line did not recover any virus.

Discussion

The ability of herpesviruses to persist and induce durable immune responses in their infected hosts makes them an

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Table 4

<table>
<thead>
<tr>
<th>Animal</th>
<th>Mamu-A*01 Vaccine</th>
<th>% CD4 Gag</th>
<th>% CD4 Env</th>
<th>% CD8 Gag</th>
<th>% CD8 Env</th>
<th>% CD8 Gagp11c</th>
</tr>
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<tbody>
<tr>
<td>Mm205-87 + DNA–DNA–HSV–HSV</td>
<td>0.52</td>
<td>0.53</td>
<td>0.65</td>
<td>0.07</td>
<td>0.68</td>
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<tr>
<td>Mm297-91 + DNA–DNA–HSV–HSV</td>
<td>0.10</td>
<td>0.25</td>
<td>0.32</td>
<td>0.12</td>
<td>nd</td>
<td></td>
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<tr>
<td>Mm240-91 − DNA–DNA–HSV–HSV</td>
<td>nd</td>
<td>0.20</td>
<td>nd</td>
<td>0.02</td>
<td>nd</td>
<td></td>
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<tr>
<td>Mm136-90 + HSV–HSV–HSV–HSV</td>
<td>0.05</td>
<td>nd</td>
<td>0.05</td>
<td>nd</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Mm450-91 + HSV–HSV–HSV–HSV</td>
<td>nd</td>
<td>0</td>
<td>0.05</td>
<td>nd</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Mm349-91 − HSV–HSV–HSV–HSV</td>
<td>0.03</td>
<td>0.06</td>
<td>0.03</td>
<td>0.01</td>
<td>nd</td>
<td></td>
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</table>

nd— not done.

a Data on week 24 shown.

b Frequency of CD4+ or CD8+ T lymphocytes co-expressing CD69 and interferon-γ on 6 h stimulation with SIV peptides. SIV-specific frequencies are shown after subtraction of background responses with medium. Responses >0.05% above background were considered positive.
attractive viral vector for delivery of an AIDS vaccine. We have previously reported on the efficacy of recombinant replication-deficient and replication-competent herpes simplex virus (HSV) vaccine vectors expressing SIV Env and Nef to protect rhesus macaques against a mucosal challenge with pathogenic SIV239 (Murphy et al., 2000). Using a new generation of HSV-SIV recombinants based on the replication-deficient HSV vector HSV-1 d106 (Samaniego et al., 1998), we now demonstrate considerable increase in vaccine immunogenicity as compared to the previous HSV vaccine vectors (Murphy et al., 2000).

The previous HSV recombinants induced weak humoral and cellular immune responses against SIV. Binding antibodies were not readily detected by ELISA-based assays, and neutralizing activity against lab-adapted SIV251 was not detected (Murphy et al., 2000). Using the traditional CTL assay, weak CTL activity was detected at a single time point in only two of seven immunized monkeys (Murphy et al., 2000). In contrast, the HSV-1 d106 recombinants used in the current study induced readily detectable anti-SIV antibodies and a robust cellular immune response in all six vaccinated monkeys. A single inoculation of HSV-1 d106 SIV recombinants expressing Gag, Env and a Rev-Tat-Nef fusion protein was sufficient to induce anti-SIV T cell responses that were detectable ex vivo by the IFN-γ ELISPOT assay and by tetramer staining. Vaccination with recombinant HSV-SIV in the absence of DNA priming gave rise to peak anti-SIV T cell responses to the vaccine immunogens ranging between 528 and 818 SFC/10⁶ PBMC, with more than half of this response being directed towards the Gag and Env proteins. These levels are higher or comparable in magnitude to those induced by several other recombinant viral vector SIV vaccines in rhesus macaques including attenuated poxviruses, vaccinia virus, vesicular stomatitis virus, semliki forest virus, and adenov-associated virus, given alone or in combination with DNA priming or as heterologous virus boost regimens (Dale et al., 2004; Doria-Rose et al., 2003; Johnson et al., 2005; Koopman et al., 2004; Pal et al., 2002; Ramsburg et al., 2004; Santra et al., 2002). The magnitude of SIV-specific cellular immune responses in the DNA primed macaques was even higher and in the range of other highly immunogenic DNA prime/viral vector boost SIV vaccination regimens in rhesus macaques consisting of Modified Vaccinia Ankara (MVA) or replication-deficient Adenovirus Ad5 (Amara et al., 2001; Casimiro et al., 2003; Horton et al., 2002; Shiver et al., 2002), as well as with heterologous prime-boost immunization regimen using MVA and replication-defective Adenovirus vectors (Casimiro et al., 2004) and replication-competent Adenovirus-based SIV vaccines (Patterson et al., 2003). Both CD4+ and CD8+ T lymphocyte responses to SIV were elicited in the vaccinated macaques. Furthermore, up to 50% of the vaccine-induced tetramer-positive CD8+ T lymphocytes in peripheral blood expressed the gut-homing molecule, α4β7, suggesting that systemic administration of recombinant HSV may be able to elicit mucosal immunity. These data are consistent with the finding of a recombinant NYVAC-SIV vaccine being able to induce anti-SIV CD8+ T cells in the rectal and vaginal mucosa.

Table 5
Comparison of viral load in vaccinated and control Mamu-A*01-positive and Mamu-A*01-negative rhesus macaques

<table>
<thead>
<tr>
<th>Group</th>
<th>Mamu-A*01 status</th>
<th>Mean plasma SIV RNA copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak (n)</td>
<td>10–14 weeks (n)</td>
</tr>
<tr>
<td>Controls Positive</td>
<td>2.2×10⁷ (23)</td>
<td>8.5×10⁵ (22)</td>
</tr>
<tr>
<td>Vaccine Positive</td>
<td>8.0×10⁶ (4)</td>
<td>3.4×10⁵ (3)</td>
</tr>
<tr>
<td>P value a</td>
<td>0.047</td>
<td>0.040</td>
</tr>
<tr>
<td>Controls Negative</td>
<td>4.2×10⁷ (8)</td>
<td>3.7×10⁵ (8)</td>
</tr>
<tr>
<td>Vaccine Negative</td>
<td>4.4×10⁶ (2)</td>
<td>6.5×10⁵ (2)</td>
</tr>
<tr>
<td>P value</td>
<td>0.037</td>
<td>0.117</td>
</tr>
</tbody>
</table>

a Number of macaques in each group.
b Mann–Whitney U test.
c Significant P value.
even when administered via the intramuscular route (Stevceva et al., 2002). Since mucosal priming can further accentuate mucosal immunity (Bertley et al., 2004; Egan et al., 2004; Evans et al., 2003), the ability to deliver recombinant HSV vectors via mucosal routes may be an added advantage of this vaccine approach. In all, our finding of robust vaccine-induced cellular immune responses, combined with the potential of HSV vectors to elicit durable immune responses and induce mucosal immunity, makes recombinant HSV-based AIDS vaccine worthy of further study.

Relative to the magnitude of SIV-specific cellular immune responses, anti-SIV antibody responses elicited by recombinant HSV were modest. Binding anti-SIV antibodies were elicited in all vaccinated macaques, and neutralizing antibodies to the neutralization-sensitive lab-adapted strain of SIVmac251 were detected at several time points prior to challenge in five of the six vaccinated macaques. A similar range of neutralizing antibody titers to HXB2, a lab isolate of HIV-1, were recently reported in rhesus macaques immunized with DNA and recombinant Adenovirus Ad5 vectors encoding HIV Env proteins (Mascola et al., 2005). The neutralizing antibody titers against lab-adapted SIVmac251 generated in the immunized macaques in our study are 100- to 1000-fold lower than following wild-type SIV infection and at least 10-fold lower than that induced by live attenuated SIVΔ3 vaccination (Wyand et al., 1999). The low levels of antibody responses are perhaps not surprising. In SIV vaccination approaches used thus far in rhesus macaques, high titer neutralizing antibodies during the vaccination phase have generally only been seen with regimens that incorporate a booster inoculation of envelope protein (Patterson et al., 2004; Peng et al., 2005; Zhao et al., 2003), or AT2-inactivated SIV particles (Willey et al., 2003), and even in these instances neutralizing antibodies to primary SIV isolates are not detected.

It is of interest that the enhanced immunogenicity of the current HSV-1 Δ106 SIV recombinants as compared to the earlier generation of HSV vectors (Murphy et al., 2000) did not translate to better protection against SIV239 challenge. In the previous study, two of seven vaccinated macaques were solidly protected while one macaque had reduced viral load (Murphy et al., 2000). In this study, three of six vaccinated macaques had reduced viral loads. However, both studies differ in important aspects, particularly with regard to the route of SIV239 challenge (intrarectal versus intravenous), and the timing between immunization and challenge (22 weeks versus 6 weeks after the last vaccination), making direct comparison between the two studies difficult. A discrepancy between vaccine immunogenicity and protective efficacy was apparent on analysis of the six vaccinated macaques in this study. Thus, the magnitude of vaccine-induced SIV-specific cellular immunity to immunodominant antigens (Gag and Env), as measured by the frequency of IFN-γ-secreting or tetramer-positive CD8+ T lymphocytes in peripheral blood, did not correlate with post challenge viral load. On the contrary, Mm 205-87, the rhesus macaque with the highest responses during the vaccine phase, showed no evidence of viral control post challenge. Interestingly, immune parameters that significantly correlated with lower viral loads post challenge included the presence of Rev-specific T cell responses on the day of challenge, the rapidity of

![Fig. 10. IFN-γ ELISPOT responses post SIV challenge in vaccinated rhesus macaques. (A) IFN-γ ELISPOT responses to SIV Gag, Env, Nef, Tat and Rev from day of challenge to week 12 post challenge. Closed symbols show DNA–DNA–HSV–HSV vaccinated macaques, open symbols show HSV–HSV–HSV–HSV vaccinated macaques. Mm136-90 died 3 weeks post challenge. (B) Regression plots showing relationship between area under curve (AUC) plasma SIV RNA and cellular and humoral immune responses on the day of challenge. (C) Regression plots showing relationship between AUC plasma SIV RNA and cellular and humoral immune responses 2 weeks post challenge. AUC was calculated for plasma SIV RNA values until 24 weeks post challenge. Correlation coefficient (Rho) and P values shown in each plot were calculated by the nonparametric Spearman Rank Correlation test. Plots with an asterisk (*) denote statistically significant correlations. 'SFC'—spot forming cells.
emergence of the anamnestic Tat-specific T cell response post challenge and the magnitude of neutralizing antibody activity prior to challenge. These findings suggest that a successful AIDS vaccine may need to prime for T cell responses to accessory HIV proteins in addition to inducing neutralizing antibodies.

The vaccinated monkey that died 3 weeks after SIV challenge, likely a rapid progressor, had a unilateral lesion in the left occipital lobe. The microscopic findings were characterized by a necrotizing and supplicative vasculitis with secondary necrosis of the white matter. In addition, rare glial nodules were present in the cerebral white matter and brainstem but were unrelated to the occipital lobe lesion. Glial nodules are nonspecific lesions that usually result from viral infection (An et al., 2002; Prineas et al., 2002); in this case, the nodules were negative by IHC for viruses, including SIV, and of unknown significance. Differential diagnoses for the occipital lobe lesion included a septic lesion of hematogenous origin (Kennedy, 2004; Moore, 2000), HSV encephalitis (Kennedy, 2004, 2005) and acute hemorrhagic leukoencephalitis (AHL). An extensive histopathologic workup failed to identify an infectious etiology, including HSV, as the cause of the vasculitis. Encephalitis related to reversion of the replication-deficient HSV vaccine to replication-competent HSV was considered unlikely for several reasons. In vivo replication of HSV-1 Δ106 vectors could hypothetically occur in two scenarios; one, in the presence of host cellular proteins able to compensate for the deleted immediate early HSV genes; and two, by recombination with herpes B virus naturally infecting rhesus macaques. Rhesus macaques in this study were seronegative for herpes B. Although a seronegative status for herpes B does not preclude herpes B
infection in macaques (Ward and Hilliard, 1994), the absence of herpes virions by electron microscopy and herpes antigen by immunohistochemistry makes the diagnosis of HSV encephalitis highly unlikely. In the absence of an infectious etiology, the morphologic changes of perivascular hemorraghes, perivascular myelolysis and neutrophilic infiltration in the white matter are consistent with a diagnosis of AHL as the cause of the encephalitis (An et al., 2002; Bennetto and Scolding, 2004; Hart and Earle, 1975; Prineas et al., 2002). AHL is a rare, fulminant and often fatal disorder of unknown etiology that is thought to be immune-mediated or a sequela of the acute perivascular inflammation (Bennetto and Scolding, 2004; Hart and Earle, 1975; Prineas et al., 2002). A febrile or nonspecific respiratory infection often precedes onset of both AHL and postinfectious encephalitis with a temporal relationship of 1 to 4 weeks between the precipitating cause and onset of neurologic disease. While there was a temporal relationship (3 weeks) between experimental SIV infection and death, it cannot be ascertained that SIV was the precipitating cause. Finally, the morphology of the lesion and the long interval (8 weeks) between the last vaccination and death argue against a diagnosis of postvaccinal encephalomyelitis.

In this study, historical controls were used for assessment of vaccine efficacy. Although not ideal, the use of historical controls allowed us to include a large number of animals that would not otherwise have been possible with concurrent controls. The historical controls consisted of 31 SIV naive rhesus macaques that had been infected with the same challenge stock of SIV239 that was used for infecting the vaccinated macaques in the present study. Peak height and set-point viral loads with this SIV239 are reasonably consistent from animal-to-animal and study-to-study (Johnson et al., 2003). Furthermore, we were able to compare the vaccinated and control groups as a whole as well as stratified based on their Mamu-A*01 status. A significant vaccine effect on viral load persisted even when only the Mamu-A*01-positive macaques were examined. Although these results are encouraging, the small number of vaccinated macaques in the current study precludes definitive conclusion of vaccine efficacy independent of other attenuating MHC Class I alleles such as Mamu-B*17 and Mamu-A*1303 (Muhl et al., 2002; O’Connor et al., 2003). Furthermore, relative efficacy of the two vaccine approaches cannot be assessed with the small number of vaccinated monkeys in this study.

It is somewhat disappointing that stronger levels of protection were not observed in these experiments, particularly given the breadth and nature of the immune responses that were generated by the vaccines. The disappointing levels of protection occurred despite attempts to use unrealistic vaccine/challenge conditions that favor the possibility of protection. The challenge virus, SIV239, is not only a homogeneous clone, it is exactly matched in sequence to the sequences present in the vaccine. Furthermore, challenge occurred 6 weeks after the fourth vaccine administration. The challenge virus, like primary isolates of HIV-1, principally uses CCR5 as its second receptor, is difficult to neutralize and induces a chronic, progressive disease course following long-term persistent infection. It could be argued that IV inoculation of 10 monkey infectious doses may be uncharacteristically higher than the 1–2 infectious doses that typify natural infection. However, in the absence of sterilizing immunity, it is still disappointing that the cellular responses could not do a more effective job in controlling the sequence-matched challenge virus. It seems likely that a vaccine will need to be able to provide much better levels of protection against homologous SIV239 challenge to have any hope of working in human field trials. Among factors that could improve protective efficacy are efficient induction of mucosal immunity, induction of long-lived memory T cells with good proliferative capacity, i.e., central memory T cells (Vaccari et al., 2005; Wherry et al., 2003), and induction of higher levels of potently neutralizing antibodies. In DNA/viral vector vaccine approaches, priming with viral vector via the mucosal route followed by systemic administration of DNA can induce excellent mucosal and systemic T cell and humoral immune responses (Eo et al., 2001). Whether priming with recombinant HSV via the mucosal route will similarly improve induction of mucosal immunity and improve protective efficacy against the AIDS virus remains to be determined.

Materials and methods

DNA plasmids

The plasmids used for DNA vaccination contain the CMV promoter without an intron, the bovine growth hormone polyadenylation site and the kanamycin resistant gene. The RNA (codon) optimized SIV genes were generated introducing multiple silent point mutations not affecting the sequence of the encoded proteins, as previously described for HIV-1 gag and env (Nasioulas et al., 1994; Schneider et al., 1997; Schwartz et al., 1992a,b) using synthetic DNAs. The secreted and intracellularly degraded variants of the SIV antigens were generated by fusion of either IP10-MCP3 or of a beta-catenin (CATE)-derived peptide, respectively, at the N-terminus of Gag and Env, as previously described (Rosati et al., 2005) or by fusion of the CATE-derived peptide to polNTV, encoding a fusion protein comprising of an inactive pol, nef, tat and vif (von Gegerfelt et al., in preparation).

Recombinant HSV vaccine

HSV recombinant vectors were constructed in the HSV-1 d106 mutant strains (Samaniego et al., 1998). HSV-1 d106 contains mutations that inactivate expression of four immediate early genes, ICP4, ICP27, ICP22 and ICP47, and thus was grown in the E-11 cell line containing the ICP4 and ICP27 genes. The vaccine vector properties of d106 and the isolation of d106 based recombinant vectors expressing SIV env, SIV gag and a rev-tat-nef fusion protein is described elsewhere (Watanabe et al., 2007). Expression cassettes for SIV env and SIV gag utilized RNA-optimized ORFs described above, and the rev-tat-nef cassette was constructed from sequences in the p239SpE3/nef-open plasmid (Regier and Desrosiers, 1990) and the nefA plasmid (Kestler et al., 1990). The expression cassettes were inserted into transfer plasmids and recombined...
into the d106 genome at the UL54 (ICP27) gene locus by homologous recombination. Stocks for the immunization were prepared by growth on the E-11 complementing cell line.

**Animals, vaccine regimen and SIV challenge**

Six Indian rhesus macaques housed in the herpes B-free specific pathogen free colony of the New England Primate Research Center (NEPRC) were enrolled in the study. Animals were maintained in accordance with federal and institutional guidelines mandated by the Animal Care and Use Committee of Harvard Medical School. Three rhesus macaques received a DNA prime, recombinant HSV boost vaccine regimen (Group DNA–DNA–HSV–HSV) while three rhesus macaques were only vaccinated with recombinant HSV (Group HSV–HSV–HSV–HSV).

The vaccine regimen consisted of four inoculations administered at 0, 4, 12 and 20 weeks (Fig. 1). The DNA inoculum included five plasmid DNA constructs: MCP3p39 gag, MCP3p39 env, CATEDX gag, CATE239 env and CATepolNTV. One milligram of each plasmid DNA (total 5 mg DNA) was injected at two to three sites via the intramuscular route. The HSV d106 vaccine inoculum included three recombinant HSV constructs, d106env, d106gag and d106rev-tat-nef, each administered at a dose of 1.25 to $3 \times 10^9$ pfu per dose. Two-thirds of the HSV vaccine dose was inoculated via the intramuscular route, while one-third of the dose was administered via the subcutaneous route. All six vaccinated animals were challenged at 26 weeks with 10 animal infectious doses of cloned homologous pathogenic SIV239 via the intravenous route.

**Analysis of humoral immunity**

ELISA plates for HSV were obtained commercially (Viral Antigens, Memphis TN); SIV ELISA plates were constructed as previously described (Daniel et al., 1988; Wyand et al., 1996). Serum was assayed at a dilution of 1:20 or 1:200 in phosphate buffered saline (PBS)-Tween20 0.05% supplemented with 5% inactivated goat serum or 5% inactivated fetal bovine serum for the SIV and HSV ELISA, respectively. Plates were incubated at room temperature for 1 h, washed three times with PBS–Tween20 0.05% and incubated with 100 μl diluted goat anti-human IgG-conjugated alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Following a 1 h incubation at room temperature, plates were washed three times and color was developed at room temperature with 200 μl p-nitrophenyl substrate solution (KPL) per well. After 30 min, the color reaction was stopped by the addition of 50 μl 3 N NaOH per well, and absorbance was determined at 410 nm on a microplate reader (Dynex Technologies, Chantilly, VA).

Neutralizing antibody titers to SIV239 and lab-passaged SIV251-lab were determined by the ability to block infection of CEMx174SIV-SEAP cells harboring a Tat-inducible SEAP reporter construct as described previously (Means et al., 1997). Serial two-fold dilutions of plasma were incubated with virus at 100 μl per well in 96-well plates for 1 h prior to addition of $3 \times 10^4$ CEMx174SIV-SEAP cells (100 μl) for a final volume of 200 μl per well. After 72 h, 18 μl of supernatant was removed and assayed for SEAP activity using the Phospha-Light SEAP detection kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions, with analysis on a Wallac multilabel microplate reader (Perkin Elmer, Boston, MA). Control wells for mock-infected cells and virus treated with pooled SIV-negative plasma were used to determine background and maximal SEAP production, respectively. The percent neutralization was calculated from the mean SEAP counts at each plasma dilution divided by the maximal SEAP counts after subtracting background activity. Fifty percent neutralization titers were calculated as the reciprocal of the plasma dilution at which infectivity was reduced by 50%.

**Antibodies for flow cytometry**

The following anti-human monoclonal antibodies (mAbs) were obtained from BD Biosciences Pharmingen (San Jose, CA) unless otherwise indicated: anti-CD3 (clone SP34) fluorescein isothiocyanate (FITC) or phycoerythrin (PE), anti-CD4 (clone L200) alkaline phosphatase (APC), anti-CD8 (clone SK1) peridinin chlorophyll protein (PerCP), anti-CD11a (clone H111) FITC, anti-CD28 (clone CD28.2) FITC or PE, anti-CD62L-selection (clone SK1) PE, anti-β7 integrin (clone FIB 504) PE, anti-CCR7 (clone 150503, R&D System, Inc., Minneapolis, MN) PE and α4β7 (clone ACT-1; a gift from Leukosite, Inc.) PE. Anti-IFN-γ (clone B27) APC or FITC, anti-TNF-α (clone Mab11) APC or FITC, anti-CD69 (clone FN50) PE, anti-Granzyme B (clone GB12) PE and anti-Ki67 (clone B56) FITC were used for intracellular staining. Purified anti-CD28 (clone 28.2, 10 μg/ml) and anti-CD49d (clone 9F10, 10 μg/ml) were used for costimulation in cytokine staining assays (ICS). Affinity-purified F(ab’2) fragments of goat anti-mouse IgG (H+L) (GAM, Kirkegaard and Perry laboratories, Gaithersburg, MD) were used for cross-linking the stimulatory antibodies in ICS assays.

**SIV antigens**

Fifteen amino acid (aa) long peptides with 11 aa overlaps spanning the entire SIV239 proteome were obtained through the NIH AIDS Research and Reference Reagent program and synthesized at the Massachusetts General Hospital peptide core facility (Charlestown, MA) by F-moc chemistry using an automated peptide synthesizer (MBS 396; Advanced Chemtech, Inc., Louisville, KY). Peptide pools for individual SIV proteins were made up and used for stimulation in ELISPOT and ICS assays. The individual peptide concentration used for stimulation ranged between 1 and 2 μg/ml. SIVmac251 P55 Gag antigen derived from the supernatant of baculovirus was obtained from Quality Biological, Inc (QBI, Gaithersburg, MD) through the Vaccine Research and Development Branch, Division of AIDS, NIAID, NIH and used at 5 μg/ml for stimulation.
Analysis of cellular immunity

Three complementary assays, the interferon-γ (INF-γ) ELISPOT assay, intracellular cytokine staining (ICS) assay and MHC tetramer analysis, were used for quantitation and phenotypic characterization of SIV-specific CD4+ and CD8+ T lymphocytes.

(i) IFN-γ ELISPOT was performed on PBMC freshly isolated from heparinized blood by density gradient centrifugation over Ficoll-Hypaque (ICN Biomedicals Inc, Aurora, OH). PBMC were suspended in RPMI (Gibco/Invitrogen Corp., Carlsbad, CA) containing 2 mM L-glutamine (Gibco/Invitrogen), 10 mM HEPES buffer (Gibco/Invitrogen), 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO) and 50 IU/ml each of penicillin and streptomycin (henceforth referred to as R-10 medium) and placed in wells of a 96-well polyvinylidene difluoride membrane bottom ELISPOT plate (Millipore, Bedford, MA) coated with the anti-human IFN-γ monoclonal antibody (mAb) clone GZ-4 (Mabtech, Sweden) along with SIV peptides or p55 antigen or R-10 medium. After overnight incubation at 37 °C in a 5% CO2 incubator, cells were removed by extensive washing and the wells incubated serially with biotinylated anti-human IFN-γ mAb clone 7-B6-1 (Mabtech, Sweden) followed by streptavidin/alkaline phosphatase. Spots were developed by addition of alkaline phosphatase conjugate substrates nitro blue tetrazolium (NBTr)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) in color development buffer (Biorad, Hercules, CA). Spots were counted with a KS ELISPOT Automated Reader (Carl Zeiss Inc, Thornwood, NY) using KS ELISPOT software 4.5 (performed by Zellnet Consulting, Inc., Fort Lee, NJ). Cut-off values for positive SIV-specific ELISPOT responses were established by testing responses to SIV peptide pools in SIV-negative rhesus macaques. Using a negative cut-off value of three standard deviations above the mean in SIV-negative rhesus macaques, spot frequencies greater than 50 spot forming cells (SFC)/10⁶ PBMC after subtraction of background were considered positive.

(ii) Intracellular cytokine staining (ICS) was performed on cryopreserved PBMC using a protocol optimized for rhesus macaques as previously described (Kaur et al., 2002). Briefly, PBMC were stimulated with 1–2 μg/ml of peptides in the presence of 10 μg/ml each of costimulatory mAbs anti-CD28 and anti-CD49d which had been cross-linked to affinity purified positive. The anti-human IFN-γ ELISPOT was performed for 6 h at 37 °C in a 5% CO2 incubator in polytyrene tubes placed 5° above horizontal, and Brefeldin A (10 μg/ml) was added for the final 5 h of stimulation. At the end of stimulation, cells were washed, incubated with 0.02% EDTA for 15 min, washed and then surface stained with anti-CD3 and anti-CD8 mAb for 39 min at 4 °C. After surface staining, cells were subjected sequentially to fixation and permeabilization using a commercial medium (FIX and PERM Medium A and B; Caltag Labs, Burlingame, CA). Permeabilized cells were incubated with anti-CD69 and anti-IFN-γ at room temperature for 29 min for intracellular staining. After washing, cells were kept overnight at 4 °C in fresh 2% paraformaldehyde. At least 200,000 events were collected on a FACSCalibur (BD Bioscience) and data analyzed using CELLQuest software (BD Biosciences).

(iii) Mamu-A*01/CM9 Gag tetramer analysis was performed on whole blood using four-color flow cytometry. Freshly drawn heparin blood (100 μl) was incubated with the tetramer APC conjugate for 15 min at 37 °C and followed by surface staining with anti-CD3, anti-CD8 and additional antibodies for phenotypic characterization. After surface staining, cells were washed and red cells lysed with FACSlyse solution (BD Biosciences). Washed cells were fixed overnight in 2% paraformaldehyde at 4 °C. At least 200,000 events were collected on a FACSCalibur and data analyzed with CELLQuest software.

MHC typing

DNA was extracted from PBMC or B-lymphoblastoid cell lines using the QIAamp DNA blood kit (Qiagen, Valencia, CA) and subjected to PCR with rhesus macaque MHC Class I sequence specific primers for Mamu-A*01 and Mamu-B*17 as described (Knapp et al., 1997; Muhl et al., 2002).

Measurement of plasma SIV RNA

Blood collected in tubes containing EDTA was spun at 2000 rpm for 10 min within 3 h of drawing blood and stored at −80 °C for quantitation of SIV RNA. SIV RNA was quantified using a real time RT PCR assay, as previously described (Cline et al., 2005). As used in the present study, the assay has a threshold sensitivity of 10 SIV gag RNA Copy Eq/ml of plasma.

HSV isolation

Trigeminal and sacral ganglia obtained at necropsy were minced and co-cultured onto Vero cells and the E-11 complementing cell line. Co-cultures were visually monitored twice daily for 1 week for evidence of cytopathic effects.

Histopathologic examination

At necropsy, 5-μm sections of formalin-fixed, paraffin-embedded tissues were stained with hematoxylin and eosin. Special stains included: Brown and Brenn Gram, modified Steiner’s silver, Ziehl-Neelsen acid fast, Grocott’s methenamine silver and periodic acid–Schiff. Monoclonal antibodies used for immunohistochemistry included: anti-SIV nef (clone KK75, Centralised Facility for AIDS Reagents, National Institute for Biological Standards and Control, Potters Bar, U.K.), anti-HIV1-p24/SIVp27 (clone 183-H12-5C, NIH AIDS Research and Reference Reagent Program, Germantown, MD), anti-SV40T Ag Ab-2 (clone PA5416, Oncogene, Boston, MA), anti-adenovirus (clone 20/11, Chemicon, Temecula, CA); polyclonal antibodies included: anti-rhesus cytomegalovirus IE1 (gift from Dr. Peter Barry, University of California, Davis), anti-human myelin basic protein (Dako, Carpinteria, CA) and anti-herpes simplex virus 1 (Dako), which also immunolabels herpes B. Five-μm sections of paraffin-embedded sections of the left
occipital lobe were deparaffinized and rehydrated, and hydrogen peroxide was applied for 5 min to quench endogenous peroxidase. Antigen retrieval was achieved by microwaving for 20 min in citrate buffer with the exceptions of anti-adenovirus, which was treated with proteinase K for 5 min, and anti-herpes simplex, which required no antigen retrieval. Dako Protein Block was applied for 10 min, and duplicate sections were incubated with either the primary antibody or the appropriate irrelevant antibody for negative controls (irrelevant IgG1 for monoclonal antibodies, and anti-rabbit immunoglobulin fraction for polyclonal antibodies). Next, biotin block was applied for 20 min followed by incubation with the secondary antibody (biotinylated horse anti-mouse for monoclonal antibodies and biotinylated goat anti-rabbit for polyclonal antibodies) for 30 min. Vectastatin avidin–biotin complex (Vector, Burlingame, CA) was applied for 30 min, and the reaction was visualized with DAB chromogen (Dako) and counterstained with Mayer’s hematoxylin.

Electron microscopy

Formalin-fixed tissue from the left occipital lobe was processed for electron microscopy. Specimens were washed in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide. The specimens were dehydrated, embedded in Epon araldite and stained with uranyl acetate and sato’s lead stain for examination using a Jeol 1010 Electron Microscope (Jeol, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Statview software (Abacus Concepts, Inc., Berkeley, CA). The nonparametric Mann–Whitney U test was used for comparison of unpaired groups. Correlations between viral load and immune parameters were performed by the Spearman Rank Correlation test. Area under curve (AUC) for plasma SIV RNA was calculated using the GraphPad Prism software (GraphPad software Inc., San Diego, CA).

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