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Protection Against Late-Onset AIDS in Macaques Prophylactically Immunized with a Live Simian HIV Vaccine Was Dependent on Persistence of the Vaccine Virus

Glenn A. Mackay,*‡‡ Zhenqian Liu,* Dinesh K. Singh,*‡ Marilyn S. Smith,‡ Sampa Mukherjee,* Darlene Sheffer,* Fenglan Jia,* Istvan Adany,* Kelvin H. Sun,* Sukhbir Dhillon,* Wu Zhuge,* and Opendra Narayan2*‡

This is a 5-year follow-up study on 12 macaques that were immunized orally with two live SHIV vaccines, six with V1 and six with V2. All 12 macaques became persistently infected after transient replication of the vaccine viruses; all were challenged vaginally 6 mo later with homologous pathogenic SHIVKu-1. Two of the V1 group developed full-blown AIDS without evidence of vaccine virus DNA in tissues. The data on the 10 vaccinated survivors showed that all 10 became infected with SHIVKu-1 and that DNA of both vaccine and SHIVKu-1 viruses were present 6 mo postchallenge, with minimal replication of SHIVKu-1. During the following 5 years, these animals remained persistently infected, but with only one of the two viruses. Six animals eliminated their vaccine virus after variable periods of time and four of these succumbed to reactivation of the challenge virus and AIDS. Five years after challenge, four latently infected animals, two with V2 and two with SHIVKu-1, were reinoculated with SHIVKu-1. This resulted in transient superinfection and the animals promptly returned to their prechallenge status. Immunosuppression of the four animals 1 year later with Abs to CD8+ lymphocytes resulted in transiently productive replication of their respective latent viruses, and upon recovery of CD8+ lymphocytes, they reverted to their latent virus status. The major finding was that of eight animals that eliminated the vaccine virus, six developed AIDS. The two others harboring SHIVKu-1 remain at risk for developing late-onset disease. The primary correlate against AIDS was persistence of the vaccine virus. The Journal of Immunology, 2004, 173: 4100–4107.

Disease manifestation in HIV-infected individuals results from the profound immunosuppression caused by highly productive replication of the virus in CD4+ T lymphocytes. The DNA intermediate phase of the viral life cycle enables the virus to persist indefinitely. Disease results from productive lytic viral replication in CD4+ T lymphocytes, a process that may begin at the time of exposure of the individual to the virus or after a prolonged period of clinical latency mediated by the proviral DNA. This long-term clinically asymptomatic phase of infection occurs naturally in a small number of individuals and correlates with development of antiviral immunity (1), and more predictably in infected persons undergoing HAART (reviewed in Ref. 2, 3). One of the enigmas of the pathogenesis of the infection is the unpredictable re-emergence of the virus from the latent state to its high replication phase with development of late-onset disease. The macaque models of HIV infection have confirmed both aspects of this pathogenesis. Use of this animal model system to evaluate efficacy of vaccines has shown that none predictably prevented initial infection by challenge viruses, although most succeeded in dampening the intensity of challenge-virus replication that normally occurs immediately following inoculation of the virus into immunized animals (4–7). Thus, latent or low grade infections by challenge viruses usually become established despite successful immunization against these agents. The onus on an efficacious vaccine is therefore 2-fold, the first being to minimize replication of the virus following inoculation, and the second, to prevent viral resurgence from persistently infected cellular reservoirs, with onset of disease after a prolonged period of clinical dormancy.

Live attenuated viruses have historically been the most efficacious of all antiviral vaccines. We and others had therefore explored whether administration of live attenuated SIV (4, 5, 8–10) and SHIV (4, 11, 12) vaccines to macaques could prevent infection by pathogenic viruses given as challenge, and if not, whether the vaccine-induced protection could prevent late-onset disease indefinitely. We had used two attenuated live SHIV vaccines to compare their efficacies. The first vaccine, ΔvpuΔnefSHIV-4 (V1) was derived from a molecular clone, SHIV-4, comprising env, tat, rev, and vpu of HIV-1, HXB2, in a background of SIVmac239 and kindly provided by Dr. J. Sodroski, Harvard University (Boston, MA) (13). As reported earlier, the parental virus, SHIV-4, was infectious but replicated poorly in macaques (14). The gene-deleted virus, V1, behaved similarly. The second vaccine ΔvpuSHIVppc (V2) was derived from progeny of SHIV-4 after three sequential passages in macaques (15). V2 replicated more robustly than V1 (4) but remained nonpathogenic. In a vaccination-challenge experiment, these two vaccines were each inoculated orally into six macaques, and all 12 animals became infected
with their respective viruses. Productive replication was transient and had ceased by 12 wk following inoculation. Both sets of animals and four unvaccinated controls were challenged 6 mo later by vaginal infusion of highly pathogenic SHIVKU-1 (4). The unvaccinated animals developed typical fulminant infections accompanied by precipitous loss of CD4+ T cells and progressive disease (4). All 12 of the vaccinated animals became infected with the challenge virus as evidenced by recovery of the virus from PBMC. Two of the six animals immunized with V1 developed fulminant infection similar to the controls, immediately following challenge and died of AIDS (4). Three others in the V1 group controlled replication of the pathogenic virus for between 1 and 3 years, after which the virus rebounded and this led to fatal disease (16). DNA of the vaccine virus was not detected in any of the tissues of these three animals. One of the six animals, PNA, has survived for more than 6 years and was used in a portion of the experiments described in this report.

In the V2 group, all six animals controlled replication of the pathogenic challenge virus and all six remained healthy. Examination of these animals at 19-wk postchallenge showed that DNA of the vaccine and the challenge viruses had both persisted in lymph nodes. A follow-up study on these animals ~135 wk (31 mo) postchallenge showed no evidence of replication of the pathogenic virus. PCR analysis of lymph node DNA from the six animals showed that vaccine virus DNA was present in all six, but DNA of SHIVKU-1 had become undetectable in four of the six. Thus, vaccine-induced protection had apparently caused a reduction in concentrations of the challenge virus in these four animals (12). Unfortunately one of the four died of causes unrelated to these studies.

In the present report, we describe a 5-year follow-up on this cohort of animals. We investigated whether their latent infection with vaccine and/or challenge viruses had become stable, and whether this state could be modified by superinfection of the animals with the same challenge virus, SHIVKU-1, paying special attention to the three that had apparently eliminated this virus. Following the resolution of the rechallenge experiment, we asked the same question about stability of infections by subjecting the animals to treatment with Abs that depleted CD8+ lymphocytes, a procedure that promotes reactivation of latent infections (17, 18).

Vaccine or challenge viruses were only transiently reactivated.

Materials and Methods

Animals

Animals were individually housed in the Laboratory Animal Resources building of the University of Kansas Medical Center (Kansas City, KS) under conditions approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. Two groups of six retired breeder female pigtail macaques had been used in a study begun in 1998 to determine the efficacy of V1 and V2 for protection against disease caused by SHIVKU-1. Four of the six animals vaccinated with V1 had developed late-onset disease within 3 years following challenge (4). The only survivor of this group, PNA, was added to the V2 group, one of which, 7024, died of unrelated reasons after failing to challenge the virus, and another, 8124, which died of late-onset AIDS after failing to eliminate the challenge virus, and another, 8124, which died of late-onset AIDS after failing to eliminate the challenge virus. At week 255, the remaining four V2 animals, PWV, PWL, PEY, and 42106, were rechallenged with the original virus, SHIVKU-1, by rectal infusion of the 1 ml of stock virus containing 10^9 TCID_50 PWV died after this study from unknown reasons. At week 304, four surviving animals, comprising PNA from the V1 group, and PEY, PWL, and 42106 from the V2 group, were given a mouse-human chimeric mAb against CD8+ T cells (CM-T807) at 10 mg/kg s.c. on day 0, and 5 mg/kg i.v. on days 3 and 7. This treatment followed the protocol originally described by Schmitt et al. (17).

Processing of blood samples

Peripheral venous blood collected in EDTA was centrifuged to separate plasma and buffy coats. Plasma was frozen for later determination of plasma viral RNA concentrations, determination of viral RNA genotype, and neutralizing Abs. PBMC were separated from buffy coats by centrifugation through Ficoll-Paque density gradients and portions were used for virus isolation, flow cytometry (FACS), detection of viral DNA, Ag-specific proliferation (ASP), and ELISPOT assays.

Cell cultures

Cell lines, PBMC, and lymph node cells. C8166 T cells and CEMx174 cells were cultured at a concentration ~0.5 x 10^6/ml in RPMI 1640 supplemented with 10 mM HEPES buffer, pH 7.3, 50 μg/ml gentamicin, 5 x 10^-5 M 2-ME, 2 mM glutamine, and 10% FBS (R-10). Peripheral lymph nodes were obtained by surgical biopsy of the animals under ketamine tranquilization at the time of blood collection. Cells were separated by disruption of the nodes and purified by differential centrifugation, as previously described (19).

For in vitro depletion of CD8+ T cells from PBMC and lymph node cell suspensions, the cells were centrifuged, the pellets resuspended in 100 μl of mouse-anti-CD8+ T cell Abs, incubated for 1 h on ice, and then washed twice with HBSS. The cells were then mixed with magnetic beads (Dyna-beads M-450) coated with sheep anti-mouse IgG. (Dynal Biotech, Oslo, Norway) incubated on ice for 45 min, and then the beads, with bound CD8+ T cells, were removed with a magnet. The remaining cells were stimulated with 10 μg/ml Con-A (Sigma-Aldrich, St. Louis, MO) for 2 days, washed twice with HBSS, and then cocultivated with C8166 T cells as previously described. Other portions of the remaining cells were used for FACS analysis and virus isolation.

Viruses. The biological properties of SHIVKU-1 were described in earlier reports. This virus replicated efficiently in CD4+ T cell lines (14, 15). An uncloned stock of SHIVKU-1 was prepared in macaque PBMC cultures and stored at -80°C.

Assessment of plasma viral RNA using quantitative real-time RT-PCR analysis

Plasma viral RNA loads were measured from RNA extracted from 800-1000 μl of EDTA anticoagulated plasma samples as previously described (20). Briefly, RNA samples were subjected to real-time RT-PCR using gag primers and a 5’-FAM- and 3’-TAMRA-labeled TaqMan probe (21) homologous to the SIVmac239 gag, which is identical in the challenge and vaccine viruses. Viral RNA copy numbers were calculated per milliliter of plasma.

Determination of the viral genotype in plasma

Plasma viral RNA was extracted from EDTA-anticoagulated plasma samples, as earlier described, and was subjected to RT-PCR and amplification of the vpu using a Promega Access RT-PCR kit (Promega, Madison, WI) using the primers described. The optimal assay conditions were as follows: 10 μl of AMV/Tth 5 × reaction buffer, 1 μl of dNTP mix (10 mM each), 50 pmol each of the forward primer and reverse primer, 2 μl of MgSO_4 (25 mM), 1 μl of AMV Reverse Transcriptase (5 U/ μl), 1 Tfi DNA Polymerase (5 U/μl) in 50 μl of total volume. RT-PCR was conducted under the following conditions: reverse transcription for 45 min at 48°C, 2 min at 94°C, followed by 40 PCR cycles of 30 s at 94°C, 1 min at 60°C, 2 min at 68°C, and the final extension was done for 7 min at 68°C.

The RT-PCR products were subjected to nested PCR to determine the presence of challenge or vaccine viral sequences. All primers are based on the oligonucleotide sequence according to SHIV sequences from GenBank accession no. NC001870. Initially, the vpu regions of viral genomes were amplified by nested PCR that produces fragments diagnostic for either vaccine virus DNA or challenge virus DNA as previously described (4). Primers for the first round of PCR amplification surround the deletion in vpu, and were previously described (4); these are designated as 111 and 114, respectively. For the second round of PCR amplification, we used forward primer 5′-CCTAGACTAGAAGGCTTTAAGCATCC (nt 5846 – 5871 of the HIV (Hb2)) and reverse primer 5′-GATCTCTGAGCATATGCTTTAAGCATC (nt 6394 – 6420 of HIV (HXB2)). The second round primers were designated as 112 and 113, respectively, and also are outside of the deletion in vpu. After the second round of amplification, challenge virus DNA yielded a fragment of 427 bp, whereas vaccine virus DNA, which has a deletion in the vpu gene, yielded a fragment of 367 bp. This assay is capable of detecting one viral genome in 10^3 cells.

A second, more sensitive assay for detecting challenge viral DNA was also performed using a different nested PCR. The outer PCR was performed as described, but for the nested set, we used the forward primer no. 3 Abbreviations used in this paper: ASP, Ag-specific proliferation; CMI, cell-mediated immune; SI, stimulation index.
ASP of CD4⁺ T cells
The ASP assay was previously described (4). Viral Ag consisted of infectious virus stock that was UV irradiated and heated at 86°C for 2 h. The Ag was mixed with freshly collected triplicate samples of PBMC, using 10⁴ cells/well in 96-well tissue culture plates in 200 μl of R-10 medium. The cells were cultured for 6 days at 37°C then pulsed with 1 μCi of [³H]thymidine/well. Eighteen hours later, the cells were harvested and the [³H]thymidine incorporation was determined by liquid scintillation counting on an Yper (Packard Instrument, Meriden, CT). Stimulation indices (SI) were calculated as mean cpm in stimulated wells, divided by the mean cpm in control wells. An SI value of >3 was considered significant.

ELISPOT assays
In the present study, we measured Ag-reactive cells using an ELISPOT assay that measured IFN-γ production by PBMC responding to groups of overlapping 15-mer peptides. This procedure provided a second assay of cell-mediated immune (CMI) responses. The peptides contained 11 amino acid overlaps, spanning the entire molecule of selected viral proteins. We used the following peptide groups represented in SHIV: HIV-1 MN Env (cat. no. 6451); consensus HIV Tat (cat. no. 5138); consensus HIV Rev (cat. no. 6445); SIVmac239 Gag (cat. no. 6204); and SIVmac239 Nef (former Cat. no. 6206), all kindly provided by the National Institutes of Health AIDS Research and Reference Reagent Program (McKesson Bio- Services, Germantown, MD). Pools of ~20 peptides were used in the assays. The Env peptides were divided into 10 pools, and the gag peptides into 5 pools. There were a total of 23, 27, and 21 peptides, respectively, for the Tat, Rev, and the Nef, and each was made into single pools. Peptide pools were aliquoted and stored at ~8°C at a concentration of 1 mg/ml.

Millipore multiscreen Immobilon-P opaque hydrophobic high-protein binding 96-well plates (0.45 μm; Billerica, MA) were coated overnight at 4°C with 50 μl/well of 5 μg/ml anti-monkey IFN-γ Abs (mAb G2-4; Mabtech, Stockholm, Sweden). The unbound Abs were removed on the following day by washing four times with PBS. The plates were blocked for 2 h at room temperature with R-10 medium. Each peptide pool (50 μl) was then added to one of each of two wells, except negative and positive control wells. R-10 culture medium and 0.5 μg/well of Con A were used for negative and positive controls, respectively. The PBMC suspension (50 μl; 2 × 10⁴/ml) was added to each well and the plates incubated for 18 h at 37°C. After discarding unbound cells, the plates were washed three times with PBS followed by three more washes with PBS-0.1% Tween 20. A total of 50 μl of 2-μg/ml biotinylated anti-IFN-γ Abs (7-B6-1; Mabtech) was then added to each well and the plates were incubated for 1 h at room temperature. The plates were then processed with six rinses with PBS-0.1% Tween 20, the addition of 50 μl/well Vectastain AB kit (Vector Laboratories, Burlingame, CA); incubation for 1 h at room temperature; six more rinses; color development by addition of 100 μl/well Nova-Red for 4 min at room temperature, and rinsing in running tap water. Spots were counted with a stereomicroscope, and reported as number of spots/10⁴ PBMC.

Results
Summary of studies during 5 years preceding the present study
In the original study described by Joag et al. (4), all 12 macaques inoculated orally with the two vaccine viruses became persistently infected with these agents. All 12 of the animals also became infected with the pathogenic challenge virus, SHIVKU1-1, that was inoculated vaginally 6 mo later. Five of the six macaques inoculated with V1 succumbed to AIDS, the time of onset of disease correlating with the duration of persistence of the vaccine viral DNA. All six of the animals in the V2 group supported latent infections by the two viruses for at least 1 year, but a follow up study on the group between week 63 and week 135 by Silverstein et al. (12) showed that although vaccine virus DNA had persisted in lymph nodes of all six animals (PEY, PWL, PWV, 7024, 8124, and 42106), the DNA of SHIVKU1-1 was detectable in only two of the six (8124 and 42106) (Table I). Animal 7024, one of the four animals in which only vaccine viral DNA was found, died at week 117 of causes unrelated to SHIV infection. Inexplicably, it gradually became anorectic and this resulted in cachexia that ended fatally. This macaque had no detectable infectious virus in its PBMC, no viral p27 in its plasma, and CD4⁺ T cell counts were maintained at concentrations in excess of 1000/μl of blood. No
proliferating opportunistic pathogens were found at the time of necropsy. In the present study, at week 224, macaque 8124, another animal in the V2 group, was diagnosed with anorexia, ataxia, and weight loss, and it died of AIDS, with neurological complications, at week 253. After the initial challenge at week 234 after immunization, this animal had developed the highest concentration of infectious PBMC (100/10⁶) compared with the other animals, before its infectious viremia was brought under control 3–4 wk postchallenge (4). This suggested that this animal had experienced the most severe infection of the group with the challenge virus, with implications for the most severe latent viral burden. As shown by Silberstein et al. (12) challenge and vaccine viral DNA were present in both PBMC of the animal at 91 wk and in its lymph nodes at week 63 and week 103. Virus isolation attempts performed at week 254 in both PBMC of the animal at 91 wk and in its lymph nodes at week 63 showed that although PEY and PWV, showing that in addition to the vaccine virus, the challenge virus must have still been present in a few cells of these animals. However, challenge virus could not be re-isolated from cultures of their PBMC. All four animals had neutralizing Ab titers of 1/320 to 1/1280 to the vaccine virus and 1/10 to 1/160 against SHIV KU-1. In all samples, titers were four times higher against the vaccine virus than corresponding titers to the challenge virus. ELISPOT analysis showed that all four animals had CMI responses with the highest titers of 150 spots in 42106, 95 in PEY, 480 in PWL, and 345 in PWV. Numbers of spots were on average two to three times higher against the gag than the env peptide group. Responses to rev, tat, and nef were sporadic, and when present, were <20. The animals also developed ASP T cell responses, but SI titers fluctuated widely at different sampling periods. SI values in 42106 ranged from 1 to 5, PEY from 0.5 to 2, PWL from 2 to 5, and PWV from 2 to 9.

Because the four surviving animals had maintained CMI responses and low viral RNA concentrations in plasma, and three of four animals had only low titers (1/20 to 1/160) of neutralizing Abs to SHIV KU-1 at 5 years following the initial vaginal challenge, we sought to determine whether these animals would become productively infected if reinoculated with the same virus, SHIV KU-1, and if reinfection occurred, whether this would lead to disease. At week 255, the four animals were therefore reinoculated rectally with 1 ml of virus inoculum containing 10⁶ TCID₅₀ of SHIV KU-1 twice, 1 day apart. The animals were bled at 1, 2, 3, 6, 8, 12, and 17 wk post-rechallenge and PBMC and plasma were collected. No infectious virus was recovered from PBMC of any of the animals at these time points. Plasma viral RNA concentrations, however, increased sharply at 1 wk as shown in Fig. 1. Unlike the challenged nonimmunized animals in which SHIV KU-1 plasma RNA concentrations typically range from 10⁵ to 10⁶ copies/ml, viral RNA copy numbers in the plasma of the rechallenged animals increased in three of the four animals from a pre-rechallenge level of ~1000–4000 copies/ml to only ~5000–45,000 copies/ml after rechallenge. Further, unlike virus control animals in which virus replication continued until death of the animals, concentrations of viral RNA in these animals declined to pre-rechallenge values by 6 wk after rechallenge as shown in Fig. 1. PWL did not develop a significant increase in viral RNA concentration, even though the animal did show evidence by selective PCR that the challenge virus was present 1 wk after rechallenge (Table I and Fig. 1).

Analysis of viral RNA during the post-rechallenge period showed that although PEY and PWL showed evidence of infection with SHIV KU-1, RNA or proviral DNA of this virus was no longer detectable at 6 wk (Table II). Thus, these two animals had reverted

| Table I. Detection of vaccine-DNA (V) and challenge-DNA (C) in PBMC- and viral genotype-specific RNA in plasma of macaques immunized with live vaccine and rechallenged with SHIV KU-1

<table>
<thead>
<tr>
<th>Macaque</th>
<th>LN (wk 18)</th>
<th>LN (wk 164)</th>
<th>Prior to Rechallenge</th>
<th>One Week Post-Rechallenge</th>
<th>Six Weeks Post-Rechallenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBMC (wk 254)</td>
<td>Plasma (wk 254)</td>
<td>PBMC (wk 256)</td>
<td>Plasma (wk 256)</td>
<td>PBMC (wk 261)</td>
</tr>
<tr>
<td>PEY</td>
<td>C,V</td>
<td>V</td>
<td>V,C,V</td>
<td>V</td>
<td>V</td>
</tr>
<tr>
<td>PWL</td>
<td>C,V</td>
<td>V</td>
<td>V,C,V</td>
<td>V</td>
<td>V</td>
</tr>
<tr>
<td>PWV</td>
<td>C,V</td>
<td>V</td>
<td>C,V,ND</td>
<td>V</td>
<td>C</td>
</tr>
<tr>
<td>42106</td>
<td>C,V</td>
<td>C,V</td>
<td>C,C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>8124</td>
<td>C,V</td>
<td>C,V</td>
<td>C,C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>7024</td>
<td>C,V</td>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Challenge viral DNA was also present in lymph node (LN), lung, spleen, spleen, and brain. Dash represents data not shown.
* ND. Neither vaccine nor challenge viral DNA was detected.
* This assessment was made at wk 63.
to their pre-rechallenge status showing persistence of only vaccine virus. Macaques PWV and 42106 had only challenge viral DNA at 6 wk, with the vaccine viral DNA becoming undetectable in PWV (Table II). None of the animals developed anamnestic immune responses in either ELISPOT titers or ASP indices, and only one, PWV, showed an increase in neutralizing Ab titer from 1/20 to 1/160, following rechallenge. Despite the increases in viral concentrations in plasma, the CD4$^+$ T cell counts remained stable throughout the 17-wk observation period after rechallenge (data not shown). Thus, all animals showed resistance to the pathogenic virus without any general change in their immune response profiles.

Approximately 10 mo after the rechallenge with SHIV$^{KU-1}$, macaque PWV was found dead in its cage (week 298). Three weeks earlier, the animal had a CD4$^+$ T cell count of 1379/μl and a plasma viral RNA copy number of 740 copies/ml, an Ab titer of 1/160 against challenge virus, and the animal appeared otherwise healthy. The only unusual finding was that the proviral DNA species found in tissues was now exclusively that of the challenge virus. Virus proviral DNA decreased to below detection at some point following rechallenge. As described earlier, this is one of the four animals that had maintained a persistent infection with the vaccine virus for 5 years. Presumably, the swarm of SHIV$^{KU-1}$ caused elimination of cells bearing the genome of the vaccine virus, although the mechanism is unknown. Whether exclusive presence of the pathogenic virus had anything to do with death of the animal is not known.

CD8$^+$ lymphocyte depletion

The foregoing study illustrated that despite confirmed reinfection with the challenge virus, macaques PWL and PEY reverted to their pre-rechallenge status, maintaining their persistent infection only with the vaccine virus. Thus, the challenge virus apparently did not establish persistence following rechallenge. Similarly, 42106 that had showed evidence of infection only with SHIV$^{KU-1}$ before rechallenge, had become reinfected with the same virus as evidenced by transient increase in numbers of viral RNA copies in plasma, but reverted to the latent infection with this virus. It is of interest that macaque PNA, the sole survivor of the V1 group, maintained the type of infection similar to that in macaque 42106, with evidence of latent infection only with SHIV$^{KU-1}$ (data not shown). To assess whether CD8$^+$ lymphocyte-mediated responses could have been involved in maintaining suppression of replication of SHIV$^{KU-1}$ of the vaccine virus, and whether PEY and PWL could still be harboring occult SHIV$^{KU-1}$, we depleted these cells in all of the surviving animals ~1 year after rechallenge.

Before initiation of the CD8$^+$ lymphocyte depletion, the four animals, PEY, PWL, PNA, and 42106 had plasma viral RNA concentrations of <1000 copies/ml. Viral RNA concentrations in plasma however increased significantly following administration of the anti-CD8$^+$ mAbs, with peak levels between 10$^4$ and 10$^6$ copies/ml plasma developing between week 1 and week 2 (Table II). Concomitantly, the CD8$^+$ T cell count had declined to its lowest point of 10–30 cells/ml blood between week 1 and week 2 after the initial treatment. Interestingly, there was no loss of CD4$^+$ T cells during this period of viral resurgence because all four animals maintain a stable CD4$^+$ T cell count exceeding 1000 cells/ml blood. By week 3, CD8$^+$ T cell counts began to rise again and this coincided with dramatic reduction in plasma viral RNA concentrations. By week 21, CD8$^+$ T cell counts had stabilized and the viral RNA concentrations in plasma became undetectable in all four.

ELISPOT and ASP titers changed dramatically during this period when the CD8$^+$ cell population in the animals was being modulated. Unfortunately, no assays were performed at the time when the CD8$^+$ cell count was at its lowest points. However, at 5 wk when the CD8$^+$ count was on the rebound, both CMI assays reached their highest titers, the ELISPOT titers greatly exceeding the CD8$^+$ cell counts. Because we were not assessing responses of separate CD4$^+$ and CD8$^+$ T cell populations in the PBMC at the time these studies were being performed, we speculate that the ELISPOT numbers represented a combined reactivity of both CD4$^+$ and CD8$^+$ T cell phenotypes. A suggestion that the CD8$^+$

### Table II. Correlation between plasma viral RNA concentration, CD8$^+$ T cell counts, and CMI responses following CD8$^+$ lymphocyte depletion in immunized macaques

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Viral RNA in Plasma (copies/ml)</th>
<th>CD8$^+$ T Cell Counts</th>
<th>ELISPOT Positive Cells</th>
<th>ASP to Vaccine$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>42106</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 290</td>
<td>360</td>
<td>695</td>
<td>10</td>
<td>3.8</td>
</tr>
<tr>
<td>Week 1</td>
<td>58360</td>
<td>9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Week 2</td>
<td>272000</td>
<td>9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Week 3</td>
<td>7760</td>
<td>481</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Week 5</td>
<td>360</td>
<td>359</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>Week 21</td>
<td>40</td>
<td>332</td>
<td>40</td>
<td>4.7</td>
</tr>
<tr>
<td>PEY</td>
<td></td>
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<tr>
<td>Week 290</td>
<td>360</td>
<td>2004</td>
<td>0</td>
<td>1.8</td>
</tr>
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<td>Week 1</td>
<td>173040</td>
<td>27</td>
<td>–</td>
<td>–</td>
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<td>Week 2</td>
<td>188588</td>
<td>181</td>
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<td>–</td>
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<tr>
<td>Week 3</td>
<td>1377</td>
<td>399</td>
<td>–</td>
<td>–</td>
</tr>
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<td>Week 5</td>
<td>360</td>
<td>589</td>
<td>1125</td>
<td>11</td>
</tr>
<tr>
<td>Week 21</td>
<td>40</td>
<td>1087</td>
<td>205</td>
<td>1.8</td>
</tr>
<tr>
<td>PWL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>120</td>
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<td>225</td>
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<td>–</td>
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<td>202</td>
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<td>–</td>
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<td>331</td>
<td>2640</td>
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<td>1520</td>
<td>1190</td>
<td>8</td>
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<td>PNA</td>
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<tr>
<td>Week 290</td>
<td>360</td>
<td>874</td>
<td>55</td>
<td>ND$^b$</td>
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<tr>
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<td>40660</td>
<td>54</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Week 2</td>
<td>24756</td>
<td>32</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Week 3</td>
<td>33960</td>
<td>447</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Week 5</td>
<td>9976</td>
<td>119</td>
<td>1275</td>
<td>26</td>
</tr>
<tr>
<td>Week 21</td>
<td>40</td>
<td>223</td>
<td>105</td>
<td>2.0</td>
</tr>
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$^a$ No anamnestic immune response developed in animals for ELISPOT titer or ASP index. Dash represents data not shown.
$^b$ ND, No vaccine detected.
T cells had a role in the response was supported by the heightened ASP titers of these cells. Titers of both assays declined by week 21, and this was associated with the decline in the concentration of viral RNA. Thus, the virus-host interaction had reverted to a status that was identical with the pre-CD8\(^+\) lymphocyte depletion period.

Analysis of the viruses during the virus resurgence period showed that the viruses isolated from plasma of macaques PEY and PWL were exclusively those of the vaccine genotype, whereas those isolated from macaques 42106 and PNA were of the challenge genotype (Table III).

The challenge viruses isolated from the animals during the period of depletion of CD8\(^+\) T cells were analyzed genetically to determine whether mutations had occurred during persistent infection, even though viral replication was completely controlled in the animals. A large portion of gp120 was amplified using primers in well-conserved regions of clade B env; both PNA and 42106 had env genes with two to four amino acid changes in the V1 region, plus in the case of PNA a deletion of an 11 amino acid block, which resulted in the loss of a predicted N-glycosylation site. PNA also contained three additional coding changes in the V2 region. Clones of the C2-V5 region amplified from 42106 all showed a new predicted N-glycosylation site at position 276 of the KU1 env, as well as a three amino acid deletion and one substitution in V4, and two substitutions in V5. In the nef gene, 42106 showed nine amino acid changes, and gained two predicted serine (positions 40 and 42) and one Thr\(^{106}\) phosphorylation sites, and lost different Ser\(^{54}\) and Thr\(^{68}\) sites. No phosphorylation changes were predicted for PNA clones, although there were six or more mutations in nef (data not shown; M. S. Smith, A. P. Kimotho, and K. Sun, manuscript in preparation). The biological significance of these changes is not clear because replication of the viruses remained controlled by host defense mechanisms.

**Discussion**

Persistence of lentiviruses in infected hosts is one of the taxonomic properties of these viruses, and is characteristic of infections caused by HIV, SIV, and SHIV in humans and macaques, respectively. Virus rebound after long periods of latency or immune restriction of replication is also well-known among these viruses, and is usually associated either with decline of immune responses or immune escape by the virus (16, 29–34). Live attenuated vaccine viruses share these general properties, undergoing productive replication during the acute phase of infection and persistence of the DNA thereafter, for variable periods. The original studies reported by Joag et al. (4) and Silverstein et al. (12) had showed that all 12 animals that had been immunized with live vaccine viruses became persistently infected with these viruses. Similarly, the pathogenic virus, SHIV\(_{KU-1}\), given as challenge, established infection in immunized animals, and even though replication of the virus was restricted to very low titers during the acute phase of infection in 10 of the 12 animals, the agent nevertheless established latent infection in all 10. The net result was latent infections by both vaccine and challenge viruses. This dual infection continued for variable periods in the immunized animals, but in this study, we showed that one genotype could supplant the other with time. Macaques PEY and PWL that have survived thus far, ~6 years postchallenge, clearly illustrated this phenomenon, showing that they eliminated the pathogenic virus but remained persistently infected with the vaccine virus. Both of these animals had developed persistent infection with both viruses for several months, but after 1–2 years of dual infection, they apparently eliminated the challenge virus and maintained the infection with the vaccine virus for at least 3 more years. Rechallenge with SHIV\(_{KU-1}\) at 5 years resulted in superinfection with the pathogenic virus but this agent could not be detected by PCR later. Further suggestion that the pathogenic virus had been eliminated was obtained by subsequent immunosuppression of the animals with anti-CD8\(^+\) lymphocyte Abs that resulted in rebound of only the vaccine virus.

Replacement of vaccine virus with the challenge virus has also been observed. In the V1 group, five of the six animals had experienced this phenomenon. Two apparently had eliminated their vaccine virus before challenge, and they succumbed to disease following challenge, similar to the challenged unvaccinated control animals (4). No vaccine DNA was detected in the tissues of these two animals when examined at necropsy. The other four animals in this group remained persistently infected with the vaccine virus for several months after challenge. However, three of the four developed fatal disease during the following 2 years and at the time of their necropsy, no vaccine viral DNA was detected in any of their tissues. Thus, development of fatal disease in five of the six animals immunized with V1 correlated with absence of the vaccine virus. Macaque 8124 in the V2 group followed this pattern, with loss of vaccine virus some time after week 164, and resurgence of challenge virus accompanied by late-onset fatal disease at week 253. It is of interest that this animal had the highest immune response titers of all the animals in this cohort in the weeks preceding its demise. In this case the CMI responses seemed to be an indicator of replication of rather than protection from the challenge virus. It is sobering that this animal maintained control of the pathogenic virus for nearly 5 years before losing its control. Taken together, these data, showing that all six of the vaccinated that died of AIDS lacked vaccine virus DNA, strongly suggested that loss of vaccine virus correlated with progression to disease. Macaques PNA, the only survivor of the vaccine 1 group, and 42106 in the vaccine 2 group also eliminated their vaccine viruses after several months of dual infection and have remained persistently infected with the challenge virus for nearly 5 years. They are healthy with low viral RNA burdens, normal CD4\(^+\) T cell counts, and moderate cellular immune responses to the virus. These two animals are the exception to the correlation observed above.

Previous studies have shown that treatment of SIV-infected macaques with Abs against CD8\(^+\) lymphocytes resulted in severe loss of CD8\(^+\) T cells and massive increases in virus replication and onset of disease (17, 35). One year after performing the rechallenge experiment on animals in the present study, we elected to use the Ab treatment on the four surviving animals, PEY, PWL, 42106, and PNA, to determine whether the elimination of CD8\(^+\) lymphocytes would result in similar re-emergence of latent virus on a massive scale. This was particularly relevant to macaques 42106 and PNA that harbored latent infection with only the pathogenic virus. Regarding macaques PEY and PWL, the question was whether the treatment would result in re-emergence of pathogenic virus even though PCR evidence had consistently shown infection with only the vaccine virus.

**Table III.** Plasma virus genotype, vaccine (V) or challenge virus (C), during virus resurgence after CD8\(^+\) lymphocyte depletion

<table>
<thead>
<tr>
<th>Week</th>
<th>PEY</th>
<th>PWL</th>
<th>PWV</th>
<th>42106</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>C</td>
<td>V</td>
<td>V</td>
<td>ND(^a)</td>
</tr>
<tr>
<td>1</td>
<td>C</td>
<td>V</td>
<td>C</td>
<td>V</td>
</tr>
<tr>
<td>6</td>
<td>V</td>
<td>V</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

\(^a\) Primer set 361, 113.
\(^b\) ND, No vaccine challenge detected.
Points of interest in the study were that the treatment did result in rapid and severe depletion of CD8+ T cells, and this was accompanied by virus rebound, confirming that these cells were responsible for controlling virus replication. Analysis of viruses cultivated from PBMCs of the four animals during the brief period of productive replication showed that the viruses isolated from PEY and PWL were indeed only vaccine viruses and those from 42106 and PNA were only of the challenge virus genotype. These results confirmed the validity of the earlier PCR results that showed selection of only one of the two viruses had become latent early after challenge. The magnitude of the viral rebound response, however, was only slight and was not accompanied by noticeable pathological effects. Control of virus replication was rapidly re-established following re-population of CD8+ T cells in the animals, and this was accompanied by a prominent anamnestic cellular immune response. Given the powerful anamnestic CMI response when the CD8+ T cell population had only partially recovered and when ELISPOT numbers greatly exceeded the number of CD8+ T cells in blood, it is possible that Ag-specific CD4+ T cells constituted a significant part of the CMI response and these cells could have modulated the response of the antiviral CD8+ T cells. The fact that there was no anamnestic neutralizing Ab response in three of four macaques suggested that the burst of virus replication provided enough viral Ag to stimulate the CMI response but this was not enough to trigger an anamnestic Ab response. Whether the existing Abs were responsible for preventing a massive burst of virus replication in the absence of CD8 lymphocytes is not known.

The single most prominent correlate of protection against AIDS was whether or not the vaccine virus persisted in the animals. All four of the animals in which only the vaccine virus persisted were protected against AIDS, even though two of the four died of non-AIDS related causes. Conversely, six of the eight animals that eliminated the vaccine virus succumbed to AIDS within months after DNA of the vaccine viruses became undetectable. We speculate that the vaccine viruses induced effector CD4+ and CD8+ T cell mediated CMI responses during the period of persistent infection but the responses were lost after the vaccine virus was eliminated, thereby leaving the animals at risk for late-onset disease caused by latent pathogenic virus. The anti-CD8 Ab treatment showed that virus replication correlated inversely with CMI responses, high viral RNA concentration in plasma correlating with low CD8+ T cell counts, and low viral RNA concentrations correlating with normal CD8 T+ cell counts and high CMI titers. Both CD4+ and CD8+ T cells responded correlated with control of virus replication, the CD8+ T cells being responsible for control of virus replication, and the CD4+ T cells presumably being responsible for regulating the CD8+ T cell response.

It is now clear that resurgence of pathogenic virus can be expected in individuals among any group of challenged immunized animals. The phenomenon has been documented in macaques that were immunized with DNA vaccines (36–38) and in studies reported with live vaccines. Unless new vaccines were to become significantly more protective than the viruses currently being used, the only hopeful strategy to prevent resurgence of pathogenic viruses may be to continue immunizations of infected individuals, including those on HAART, at selected intervals following exposure to the pathogenic virus. This would be one mechanism for replenishing or reinducing effector CD8+ T cells important for maintaining the pathogenic virus in a latent state for an indefinite period.

Acknowledgments

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References


