Vaccination of rhesus macaques with a \textit{vif}-deleted simian immunodeficiency virus proviral DNA vaccine

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Abstract

Studies in non-human primates, with simian immunodeficiency virus (SIV) and simian/human immunodeficiency virus (SHIV) have demonstrated that live-attenuated viral vaccines are highly effective; however these vaccine viruses maintain a low level of pathogenicity. Lentivirus attenuation associated with deletion of the viral \textit{vif} gene carries a significantly reduced risk for pathogenicity, while retaining the potential for virus replication of low magnitude in the host. This report describes a \textit{vif}-deleted simian immunodeficiency virus (SIV)\textit{mac239} provirus that was tested as an attenuated proviral DNA vaccine by inoculation of female rhesus macaques. SIV-specific interferon-γ enzyme-linked immunospot responses of low magnitude were observed after immunization with plasmid containing the \textit{vif}-deleted SIV provirus. However, vaccinated animals displayed strong sustained virus-specific T cell proliferative responses and increasing antiviral antibody titers. These immune responses suggested either persistent vaccine plasmid expression or low level replication of \textit{vif}-deleted SIV in the host. Immunized and unvaccinated macaques received a single high dose vaginal challenge with pathogenic SIV\textit{mac251}. A transient suppression of challenge virus load and a greater median survival time was observed for vaccinated animals. However, virus loads for vaccinated and unvaccinated macaques were comparable by twenty weeks after challenge and overall survival curves for the two groups were not significantly different. Thus, a \textit{vif}-deleted SIV\textit{mac239} proviral DNA vaccine is immunogenic and capable of inducing a transient suppression of pathogenic challenge virus, despite severe attenuation of the vaccine virus.

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Introduction

The incidence of AIDS worldwide is rising, and the need continues for the development of an effective and safe vaccine against the human immunodeficiency virus (HIV), with the primary objective of arresting the spread of the AIDS epidemic.

Studies in non-human primates with simian immunodeficiency virus (SIV) and simian/human immunodeficiency virus (SHIV) demonstrated efficacy for live-attenuated virus vaccines based on non-virulent cloned viral variants or clones engineered with deletions in viral various accessory genes including nef (Abel et al., 2003; Almond and Stott, 1999; Daniel et al., 1992; Lohman et al., 1994). However, subsequent studies revealed the potential for pathogenicity for live-attenuated SIV vaccine viruses for both neonatal and adult macaques (Baba et al., 1995, 1999; Hofmann-Lehmann et al., 2003). More recent studies have revealed vaccine induction of limited protection against pathogenic SIV or SHIV challenge with protection defined as
suppression of challenge virus loads, increased survival, and preservation of memory CD4 T cell populations. Vaccine approaches generating this type of efficacy include multi-gene DNA expression plasmids boosted with either recombinant viral vectors encoding viral genes or with cytokine adjuvants (Hokey and Weiner, 2006; Letvin, 2006; Lori et al., 2006). Nevertheless, optimization of different components of current vaccine strategies, or development of additional novel approaches, will be required to generate a truly effective AIDS vaccine.

Several reports have also shown immunogenicity with limited efficacy for single modality DNA vaccines that are based on a replication-defective SIV or SHIV provirus encoding mutations within reverse transcriptase, integrase, or nucleocapsid genes (Akahata et al., 2000; Gorelick et al., 2000; Liu et al., 2006; Singh et al., 2005; Wang et al., 2000). Similarly, a previous study in our laboratory revealed some success for a highly attenuated vif-deleted feline immunodeficiency virus (FIV) proviral DNA vaccine for inducing protection in cats against challenge with a wild type FIV, despite the severe restriction imposed on virus replication by deletion of the viral vif gene within the vaccine viral DNA (Lockridge et al., 2000). Although reports describing animal infection studies with vif-deleted lentiviruses have been limited, all have shown the absolute requirement for vif for detectable virus replication in vivo (Desrosiers et al., 1998; Gabuzda et al., 1994; Harmache et al., 1996; Inoshima et al., 1996; Kristbjornsdottir et al., 2004; Lockridge et al., 1999). Inoculation with lentiviral vif mutants resulted in infections characterized by undetectable virus loads in peripheral blood and lymph nodes and by antiviral antibody responses in some animals that were measurable only by very sensitive antibody assays. These findings suggested the possibility that inoculation with a vif-deleted virus or proviral DNA results in either an abortive infection or a persistent infection with exceptionally low level replication. Importantly, observations from in vivo inoculation studies confirmed the severe attenuation associated with vif-deletion. In addition, mechanisms by which HIV-1 and SIV vif promote viral infectivity and replication in cell culture, were recently characterized by reports that revealed HIV-1 vif targets cellular cytidine deaminases of the human APOBEC3 family for proteosomal degradation by the vif gene product, thereby preventing virion incorporation of these antiviral cellular proteins (see review) (Cullen, 2006). These recent studies not only clarified the role of vif in the viral replication cycle, but also confirmed the critical requirement of this viral protein for HIV-1 and SIV infectivity and replication.

The use of a vif-deleted provirus, as the basis for a DNA vaccine for AIDS, holds several potential advantages. Similar to replication-defective proviral DNA vaccines, a vif-deletion mutant provides a vector capable of expressing all viral genes, with the exception of vif, necessary for the assembly of virus particles. Moreover, virus produced from a vif-deletion mutant may not be truly defective, but instead may be highly restricted for replication, allowing for a slightly higher expression of viral immunogens than that observed with proviruses deleted for genes absolutely required for virus replication such as reverse transcriptase. Also, proviral DNA inoculation, in contrast to inoculation with virus particles, allows direct delivery of the viral genome to host cells for those attenuated viruses of considerably lower infectivity as displayed by vif-deletion mutants.

Lastly, immunization with a vif-deleted provirus plasmid combines the immunogenicity associated with plasmid DNA (see reviews) (Giri et al., 2004; Letvin, 2006) and attenuated virus vaccines. Based on these underlying principles, female rhesus macaques were immunized with a vif-deleted SIVmac239 provirus by DNA inoculation for the present study. Immunogenicity of this proviral DNA vaccine was examined by assays for SIV-specific cellular and humoral immune responses after DNA vaccination. Efficacy of this vif-deleted SIVmac239 proviral DNA vaccine was examined by challenge with pathogenic SIVmac251 by the vaginal route. This study revealed that a vif-deleted SIVmac239 proviral DNA vaccine was immunogenic, especially for virus-specific T cell proliferative responses, regardless of severe virus attenuation. Furthermore, immunization with this vif-deletion mutant resulted in restriction of challenge virus replication during the acute stage of infection with SIVmac251.

Results and discussion

Construction and replication of SIV/CMOVΔvif

To enhance expression of the proviral genome, the 5′ LTR of SIVmac239 was modified by the replacement of the U3 promoter domain with the enhancer region of the human cytomegalovirus (CMV) immediate early (IE) gene promoter. The vif-deleted SIVmac239 provirus encoding a 5′ hybrid LTR (CMV-LTR) is illustrated in Fig. 1A; construction of this clone utilized a previously described vif-deleted provirus (Zou and Luciw, 1996). To assess transcriptional activity of the chimeric LTR, transient expression assays were performed to compare luciferase reporter plasmids containing the hybrid LTR (pCMV-LTR/Luc) and wild type SIVmac239 LTR (SIV-LTR/Luc). As a result of luciferase activity from transfected 293T cells showed a five-fold increase in activity directed by the hybrid LTR compared to that of wild type SIV-LTR (data not shown). Based on these findings and previous reports showing that expression vectors containing the CMV IE promoter or enhancer domains exhibit higher levels of expression (Galvin et al., 2000; Manthorpe et al., 1993), the genome of the SIVmac239Δvif provirus was modified by replacement of the 5′ LTR with that of the hybrid CMV-LTR. The provirus plasmid clone with this hybrid LTR was designated SIV/CMOVΔvif and was used as the vaccine DNA in this study. Virus produced from the vaccine provirus SIV/CMOVΔvif was assayed for replication in cell types either permissive or nonpermissive for vif-deleted SIVmac isolates, including SupT1 cells and rhesus peripheral blood mononuclear cells (PBMC), respectively. Virus production from PBMC infected with SIV/CMOVΔvif virus was severely restricted although not completely ablated, with SIV p27Gag concentrations peaking at 3.5 to 3.9 ng per ml of cell culture supernatant (Fig. 1B). Similarly, SIV/CMOVΔvif virus replication in rhesus MM221 cells (Fig. 1C) was also severely restricted compared to wild type SIVmac239, although this cell line appeared slightly more permissive for vif-deleted SIV replication.
compared to rhesus PBMC. In contrast, replication of SIV/CMVΔvif and wild type SIVmac239 was comparable in SupT1 cells (Fig. 1D). These findings confirmed that virus produced by plasmid SIV/CMVΔvif replicated in vitro similar to that previously shown for the progenitor provirus SIVmac239Δvif (Zou and Luciw, 1996).

**Cellular immune responses induced by vaccination with SIV/CMVΔvif proviral DNA**

The first objective of this study was to test the immunogenicity of the vaccine plasmid SIV/CMVΔvif by intradermal and intramuscular inoculation of six female rhesus macaques. An interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay, testing PBMC responses specific to four different SIV immunogens, revealed variable and relatively low activity at multiple time points after priming immunization (Fig. 2). This recombinant vaccinia-based ELISPOT assay was previously shown to reflect predominantly CD8 responses, as antigen is presented by the MHC class 1 pathway (Larsson et al., 1999). Responses were observed predominantly at early time points including two and six weeks after priming immunization and at two weeks after the first booster immunization. In addition, responses were detected in five of the six vaccinated macaques for at least one of the four immunogens tested. A higher frequency and magnitude of IFN-γ ELISPOT responses specific to SIV Gag and Nef were measured, whereas responses to Env and Pol were low or absent. Overall, IFN-γ ELISPOT responses were weak with the exception of one macaque (27101) who exhibited moderate to strong responses specific to Gag and Nef, respectively.

T cell proliferative responses specific to inactivated purified SIV preparations were robust over most time points tested with all animals testing positive at two or more time points after immunization (Fig. 3A). These proliferative responses, presumably reflecting antigen-specific CD4 T cell activity, persisted after priming and the first booster immunizations. Interestingly, two vaccinated macaques showed stimulation indices (SI) of 45 and 65 at sixteen weeks after the first booster. Proliferative responses were high and surprisingly sustained, given that plasma viremia was not detected by real-time PCR assay for SIV RNA at various time points after proviral plasmid DNA inoculation (data not shown). Similarly, proviral DNA was also not detected in PBMC by a real-time PCR assay for SIV DNA (data not shown). Although proliferative responses measured by this particular assay have previously been shown to predominantly represent proliferating CD4 T cells, proliferation of antigen-specific CD8 T cells may also be measured by this assay based on aldrithiol-2 (AT-2)-inactivated SIVmac239 (Frank et al., 2003; Rutebemberwa et al., 2007). Nevertheless, the difference in magnitude between IFN-γ ELISPOT and T cell proliferative responses was considerable and possibly suggested that SIV/CMVΔvif proviral DNA
CMVΔvif DNA immunization induced more potent virus-specific CD4 responses compared to CD8 responses, although specific cellular phenotypes associated with SIV-specific IFN-γ ELISPOT and proliferation were not tested. The SIV-specific T cell proliferative responses observed with inoculation of a vif-deleted SIV provirus were notable and as high, or greater than, those observed for macaques infected with wild type SIV-mac251, and tested by the same assay (Ma et al., 2004). Induction of strong virus-specific CD4 responses by cloned attenuated SIVmac239 isolates was reported previously, although the strength of responses was shown to be reduced with more severely attenuated SIV isolates compared to nef-deleted SIVmac239 (Gauduin et al., 1999, 2006). Vaccine-induced clonal expansion of CD4 T cells without full differentiation into mature CD4 effector cells may provide one possible explanation for the disproportion of antigen-specific responses observed with CMVΔvif DNA immunization.
IFN-γ ELISPOT and proliferation responses detected after immunization with SIV/CMVΔvif DNA (Bayry et al., 2007; Spörri and Reis e Sousa, 2007). Similarly, recent studies have reported induction of antigen-specific CD4 T cells without helper function as a result of CD4 T cell activation by incompletely matured dendritic cells (Spörri and Reis e Sousa, 2007). A definitive elucidation of mechanisms for the differential induction of cellular responses by the SIV/CMVΔvif proviral DNA vaccine will require additional studies focused on phenotypic and polyfunctional characterization of virus-specific CD4 and CD8 T cells.

Antiviral antibody responses elicited by immunization with SIV/CMVΔvif proviral DNA

Low to undetectable antibody responses have been detected in infection studies with vif-deleted lentiviruses (Desrosiers et al., 1998; Gupta et al., 2007; Harmache et al., 1996; Inoshima et al., 1996; Lockridge et al., 2000). Therefore a sensitive concanavalin A (ConA) ELISA for detection of antibody to native SIV Env was used to assay antiviral antibodies after immunization with SIV/CMVΔvif proviral DNA. Results of the ConA ELISA revealed that only one animal was positive for Env antibody at six weeks after priming immunization, whereas five of six animals demonstrated either transient, low (1:100) to higher (1:800) antibody titers at two weeks after the first booster inoculation (Fig. 3B). Env antibody titers ranging from 1:800 to 1:3200 were detected for all six vaccinated macaques by two weeks after the booster inoculation. Low titer antiviral antibodies were also detected by commercial HIV-1/HIV-2 antibody ELISA for two vaccinated macaques after the first booster immunization and for all vaccinees after the second booster vaccination (Fig. 3C). Although antibody titers measured for vaccinated macaques by both assays were considerably lower than those reported for animals infected with wild type and some attenuated SIVmac isolates, detection of antibody by the commercial assay was unexpected based on previous reports of infection studies with vif-deleted lentiviruses (Desrosiers et al., 1998; Harmache et al., 1996; Inoshima et al., 1996; Kristbjomsdottir et al., 2004; Lockridge et al., 2000). However, previous studies of vif-deleted lentiviruses frequently used a single inoculation of virus particles for infection rather than multiple proviral DNA inoculations. Therefore, differences in route and number of inoculations, and use of the a provirus directed by the hybrid CMV-LTR, may have accounted for the emergence of increasing antibody titers observed for SIV/CMVΔvif-immunized macaques in this study. Regardless, these observations of both higher than expected humoral responses and sustained SIV-specific T cell proliferative responses suggested either persistent expression of this vaccine provirus plasmid or SIVΔvif virus replication of very low magnitude.

Effects of immunization with SIV/CMVΔvif proviral DNA on peripheral blood virus loads and CD4 T cell concentrations after vaginal challenge with SIVmac251

The second objective of this study was to test the efficacy of vif-deleted SIV proviral DNA vaccine against vaginal challenge with the highly pathogenic isolate SIVmac251. All vaccinated macaques became viremic after vaginal challenge, whereas one unvaccinated control remained negative for plasma viral RNA for all time points tested after challenge (Fig. 4A and B). Although mean plasma virus loads, measured as viral RNA copies per ml, were lower for vaccinated macaques compared to unvaccinated controls over the first sixteen weeks after vaginal challenge, differences in loads between the two groups were not statistically significant ($P=1.2–1.5$) during the first eight weeks post-challenge with analyses including unvaccinated control macaque (30037) that remained uninfected after challenge. However, statistical analysis revealed significant differences between virus loads for the animal groups at two ($P=0.026$), four ($P=0.004$), and eight ($P=0.041$) weeks after challenge when this uninfected control animal was excluded from the analysis (Fig. 4B). By twenty weeks after challenge, mean virus loads were similar between vaccinated macaques and unvaccinated controls. Importantly, three of the five infected animals within the unvaccinated control group were rapid progressors and euthanized due to SIV-associated illnesses, including diarrhea and weight loss, by sixteen weeks after challenge (Fig. 4B). In contrast, only one out of six vaccinated and infected animals was euthanized within this same time period (Fig. 4A). Study animals were maintained up to 12–14 months after challenge to determine effects on long-term survival imposed by vaccination. A Kaplan–Meier survival analysis revealed a difference in median survival times for vaccinees (fifty one weeks) and unvaccinated controls (sixteen weeks) (Fig. 4D). Therefore an enhanced survival rate during the acute phase of infection and a greater median survival time was observed for vaccinated animals. However, long-term survival of animals from both groups were comparable, with two vaccinated and two unvaccinated animals controlling virus loads to approximately $5 \times 10^5$ RNA copies per ml of plasma at the termination of the study.

All six vaccinated animals demonstrated a precipitous decline in the peripheral blood CD4 T cell population within two weeks after challenge followed by a stabilization of values for the percentages of this T cell subset (Fig. 5A). The CD4 T cell decline at two weeks post-challenge produced a significant difference between mean values for peripheral blood CD4 T cell percentages in SIV-infected vaccinees and unvaccinated controls ($P=0.015$) (Fig. 5B). One possible cause for the accelerated CD4 T cell depletion upon challenge would be a vaccine-induced expansion of SIV-specific CD4 T cell population as indicated by the strong T cell proliferative responses detected after immunization. This potential mechanism is supported by findings from other studies showing preferential infection by HIV-1 for virus-specific CD4 T cell populations (Douek et al., 2002; Harari et al., 2002). In contrast, unvaccinated viremic control animals experienced a slower progressive reduction in CD4 T cell percentages that occurred over an eight week time period after challenge. Nevertheless, the abrupt reduction in the peripheral blood CD4 T cell population in vaccinated macaques was associated with lower peripheral blood virus loads at early time points post-challenge, when compared to unvaccinated controls (Fig. 4A). Two of the three unvaccinated animals were euthanized by sixteen weeks after challenge due to SIV-related
clinical disease; however, these animals did not exhibit significant changes in CD4 T cell counts during the acute phase of challenge infection. Percentages of CD4 T cells were comparable for both vaccinated and unvaccinated macaques by four weeks after challenge and remained relatively stable and similar for those animals in both groups that survived until termination of the study. The role of major histocompatibility complex class I haplotypes in control of virus load for macaques involved in this vaccine study was unclear as all animals were negative for the rhesus macaque Mamu-A\(^*\)01 allele. Two of the five vaccinated animals (26349 and 27513) that survived past the acute phase of SIVmac251 infection, were positive for the rhesus macaque Mamu-B\(^*\)17 haplotype previously considered to be predictive of control of SIVmac239 virus load and disease progression (O’Connor et al., 2003; Yant et al., 2006). However, findings from a more recent report indicated that inheritance of this haplotype was not predictive for control of SIVmac239 virus load and disease progression (Wojcechowskyj et al., 2007). Taken as a whole, these observations indicated that a single modality vaccine composed of a vif-deleted SIVmac239 proviral plasmid was capable of reducing virus loads and mortality during the acute phase of SIVmac251 infection. However, vaccination was not effective for inducing sustained control of challenge virus replication or virus-associated peripheral blood CD4 T cell depletion after high dose vaginal challenge with pathogenic SIVmac251.

**Cellular immune responses after challenge with SIVmac251**

For vaccinated macaques, the SIVmac251 challenge induced a rapid ablation of SIV-specific proliferative responses that had been moderate to robust just prior to challenge (Fig. 3A). SIV-specific proliferative responses were entirely suppressed in all vaccinated animals and also absent in unvaccinated controls at two weeks after challenge (Fig. 5C). By four weeks after challenge, low to moderate proliferative responses were observed in two vaccinated and two unvaccinated macaques; this most likely reflected responses related to virus replication rather than recall responses associated with vaccination, with the exception of responses demonstrated by one unvaccinated macaque (30037). Interestingly, proliferative responses were observed for this particular control animal that appeared to resist infection with SIVmac251 based on the absence of a detectable plasma viremia, antiviral antibody, and SIV-associated disease throughout the 12 month period of assessment after challenge. Proliferative responses observed for this unvaccinated macaque substantiated exposure to challenge virus and suggested transient...
virus replication that was subsequently controlled sufficiently to prevent detection of infection by standard assays used in this study. Extended follow-up assessment of this animal beyond 12 months after challenge and assay of multiple lymphoid tissues for the presence of SIV proviral DNA at necropsy would be necessary to determine if this animal suffered an occult infection as previously described by McChesney et al. (1998) or completely resisted SIVmac251 infection.

Virus-specific IFN-γ ELISPOT responses were variable but mostly low to absent after challenge for all vaccinated macaques (Fig. 6A). The one vaccinated animal (27101) that exhibited moderate to low ELISPOT responses consistently at time points both before and after challenge, exhibited declining responses by twelve weeks after challenge. These declining responses were coincident with a progressive reduction in peripheral blood CD4 T cells over the sixteen week time period after challenge (Fig. 5A), at which time the animal was euthanized. Measurable IFN-γ ELISPOT responses to SIV were not detected in unvaccinated animals after challenge (Fig. 6B). In general, IFN-γ ELISPOT responses observed for vaccinated animals were reduced, compared to responses prior to challenge, and were comparable to those measured for unvaccinated control animals, with the exception of two vaccinated animals (27101 and 27674).

Weak SIV-specific IFN-γ ELISPOT responses in vaccinated animals after vaccination and challenge suggested a poor virus-specific CD8 T cell response was induced by this plasmid vaccine. The absence of a protective SIV-specific CD8 T cell response may have augmented the susceptibility of virus-specific CD4 T cells during the acute phase of infection. Accordingly, an enhanced susceptibility of virus-specific CD4 T cells to infection as previously described (Brenchley et al., 2004), combined with insufficient virus-specific CD8 activity could possibly explain both the abrupt but transient depletion of peripheral blood CD4 T cells and absolute ablation of previously strong proliferative responses observed for vaccinated animals during the very early acute phase of stage of infection. Furthermore, a more recent study suggested that the preponderance of HIV-1-specific CD4 T cells in infected patients are of a less mature phenotype and may be more susceptible to infection and depletion by HIV-1 when compared to terminally differentiated virus-specific CD4 T cells (Brenchley et al., 2006). The possibility of induction of incompletely differentiated virus-specific CD4 T cells by SIV/CMVΔvif plasmid vaccine may also directly relate to the disparity observed between SIV-specific T cell proliferative and IFN-γ ELISPOT responses noted in vaccinated macaques, as well as the rapid drop in CD4 T cell percentages after challenge. Future studies characterizing T cell phenotypes represented by these IFN-γ ELISPOT responses and proliferative responses, as well as testing for additional cellular immune response functions including antigen-specific induction of expression of different cytokines and degranulation markers, will be necessary to address these issues and to fully characterize SIV/CMVΔvif DNA-induced cellular responses.

SIV Env antibody responses after challenge with SIVmac251

SIV envelope-specific antibody titers increased by ten-fold or more over base-line titers for five of six vaccinated animals by
two weeks after SIVmac251 challenge. In contrast, measurable antibody titers were not detected until four weeks after challenge in unvaccinated animals, with only three of the six animals demonstrating SIV Env antibody (Fig. 7A). One of the three antibody-negative unvaccinated controls was also negative for plasma viremia and appeared resistant to SIVmac251 challenge (Fig. 4B). The absence of SIV antibodies for the remaining two unvaccinated animals was expected because these two animals (24078 and 30839) proved to be rapid progressors that were euthanized at sixteen weeks after challenge due to SIV-associated clinical illness. These findings suggested that the rise in antibody titers detected for vaccinated animals within two weeks of challenge most likely reflected vaccine-related anamnestic responses. By eight weeks after challenge, Env-specific antibody titers were comparable between vaccinated and unvaccinated animals and did not correlate with suppression of virus loads.

Increasing Env-specific antibody avidity was observed over the eight week time period after challenge for four of the six vaccinated macaques and for two of three unvaccinated animals that were antibody-positive (Fig. 7B). In contrast, the unvaccinated animal (24759) that exhibited a decrease in antibody avidity between four and eight weeks after challenge, was also a rapid progressor that was euthanized at sixteen weeks after challenge. Otherwise, antibody avidity responses were comparable between vaccinated macaques and unvaccinated controls by eight weeks after challenge with the exception of one vaccinated animal (27513) that exhibited a significantly higher avidity compared to all other animals. Overall, the magnitude of Env antibody titer and avidity did not correlate with suppression of virus load after challenge with pathogenic SIVmac251, although the absence of antibody was associated with rapid disease progression. Importantly, high SIV-specific antibody responses
were not predicted given that this vaccine approach consisted of a single modality extremely attenuated proviral DNA vaccine that lacked booster immunizations with viral antigens expressed from a viral vector or as protein subunits. However, future examination for neutralizing antibody responses will be necessary to provide a more complete analysis of humoral responses induced by this single modality proviral DNA vaccine.

The question remains as to whether this vif-deleted SIV provirus plasmid functioned as a highly attenuated virus or as a defective proviral DNA vaccine. Regardless, the results of this study supported the “threshold hypothesis” which states that immunization efficacy is directly related to the extent of vaccine virus replication in vivo (Johnson et al., 1999; Ruprecht et al., 1996). Despite the fact that vaccine-induced suppression of challenge virus loads was insufficient to provide sustained control of virus infection after a highly pathogenic SIVmac251 challenge, SIV/CMVΔvif plasmid immunization generated cellular and humoral responses that were consistent with, or actually improved over those reported for other vif-deleted lentivirus infections and for defective SIV or SHIV proviral DNA vaccines (Akahata et al., 2000; Gorelick et al., 2000; Liu et al., 2006; Singh et al., 2005; Wang et al., 2000). Correlates for vaccine-induced suppression of challenge virus loads during acute infection were not readily apparent. However, strong SIV-specific T cell proliferative responses, and increasing antiviral antibody titers associated with booster immunizations, suggested either persistent vaccine plasmid expression or low level SIVΔvif replication in the host. Additionally, studies examining SIV/CMVΔvif proviral DNA-inoculated macaques for tissue reservoirs of either replicating SIVΔvif virus, or persistent SIV/CMVΔvif plasmid, will be required to determine the origin for the enhanced proliferative and antibody responses observed for this vif-deleted proviral DNA vaccine. The immunogenicity, limited suppression of challenge virus loads, and improved median survival time associated with this vif-deleted SIVmac239 proviral DNA vaccine, supports further examination of this proviral DNA vaccine as a component of multiple modality vaccine approaches that include immunogens expressed by viral vectors, as well as co-immunization with vectors expressing immunomodulators.

Materials and methods

Plasmid vaccine preparation

The proviral DNA genome of a previously described SIV vif-deletion mutant, SIVmac239Δvif (Zou and Luciw, 1996), was modified by the replacement of the 5′ LTR with a hybrid LTR encoding the enhancer region of the human CMV IE gene promoter. Briefly, a hybrid LTR (CMV-LTR) encoding a 514 nt fragment of the CMV IE promoter with enhancer elements, nucleotides-1 to -53 of the U3 domain, and the R and U5 domains of SIVmac239 LTR, was constructed by standard molecular cloning strategies. Next, reporter plasmids encoding the luciferase gene directed by either the hybrid CMV-LTR (pCMV-LTR/Luc) or the wild type SIVmac239 LTR (SIV-LTR/Luc) were also prepared. Finally, the hybrid CMV-LTR was substituted for the 5′LTR of vif-deletion mutant SIVmac239Δvif for construction of a SIVmac239-based provirus directed by a potentially stronger promoter. The SIVmac239Δvif plasmid encoding the 5′ hybrid LTR was further modified by replacement of the truncated nef gene encoded by the original pMA239 provirus plasmid used to construct SIVmac239Δvif, by insertion of a nef-containing subgenomic fragment from SIVmac239/nef-open to produce the vaccine provirus plasmid SIV/CMVΔvif (Fig. 1A). The structure of this vaccine provirus was confirmed by nucleotide sequencing. Vaccine plasmid DNA (SIV/CMVΔvif) was purified using a commercial Endofree kit (DNA Maxi prep; Qiagen, Valencia, CA) and tested for endotoxin concentrations with a commercial kit (E-Toxate, Sigma, St. Louis, MO).

Replication assays for vaccine provirus plasmid

Replication of SIV/CMVΔvif was tested by transfection of the SupT1 cell line, and by infection of rhesus macaque PBMC and the macaque MM221 T cell line (provided by Ronald Desrosiers, New England Primate Research Center). SupT1 cells, human embryonic kidney 293T cells, and MM221 T cells were cultured using protocols previously described (Himathongkham et al., 2002; Zou and Luciw, 1996). Rhesus macaque PBMC were prepared by Ficoll–Hypaque density centrifugation and cultivated as previously described (Himathongkham et al., 2002). For assay of replication in the SupT1 cell line, cells were transfected with provirus plasmids (5 μg) including SIV/CMVΔvif and SIVmac239/nef-open using electroporation protocols previously reported (Zou and Luciw, 1996). To assess SIV/CMVΔvif replication in MM221 cells and macaque PBMC, plasmids were first transfected into 293T cells using Eugene 6 lipofection reagent (Roche Molecular Biochemicals, RMB, Indianapolis, IN) according to previously described protocols (Gememiano et al., 2004). Supernatants were harvested from transfected 293T cells two days later and filtered through a 0.45 μm filter. MM221 cells and PBMC (1.5 × 10⁶ cells) were washed in RPMI media and re-suspended in 0.5 ml of filtered transfected cell supernatant for a 3 h incubation at 37 °C. Cells were next washed twice with RPMI media and then cultivated using previously described protocols (Himathongkham et al., 2002). Cell culture supernatants from provirus plasmid-transfected SupT1 cells and infected cultures of MM221 cells and macaque PBMC were collected every three to four days and assayed by a commercial ELISA (Coulter, Hialeah, FL) for SIV p27Gag concentrations.

DNA immunization and virus challenge

Animals used in this study included colony-bred, multi-parous female rhesus macaques (Macaca mulatta) housed at the California National Primate Research Center and previously determined to be negative for antibodies to simian type D retrovirus, SIV, HIV-2, and simian T cell leukemia virus type 1. Tissue typing demonstrated that all animals were negative for the major histocompatibility complex (MHC) class I allele Manu-1A*01 and two animals within the vaccine group (26349 and 27513) were positive for MHC class I allele Manu-B*17.
Animals were housed and maintained according to regulations and guidelines of the Institutional Animal Care and Use committee. Physical examinations were performed at regular time intervals for body weight, lymphadenopathy, splenomegaly, and opportunistic infections. Clinical criteria for euthanasia included three or more of the following: a greater than 10% loss of body weight, chronic diarrhea unresponsive to therapy, persistent infections unresponsive to therapy, persistent dehydration without fluid supplementation, and persistent and marked hematologic abnormalities.

For this vaccine study, six female rhesus macaques were inoculated with 600 μg of SIV/CMVΔvif plasmid DNA by the intramuscular route and 400 μg of vaccine DNA by the intradermal route to constitute a total vaccine DNA inoculum of 1 mg. At six weeks and twenty two weeks after priming immunization, animals received booster inoculations of SIV/CMVΔvif plasmid DNA 1 mg; these inoculations were divided between intramuscular and intradermal routes as described for the initial immunization. At twenty six weeks after the priming immunization (four weeks after the second booster inoculation), vaccinated animals and six unvaccinated control female rhesus macaques were challenged by the vaginal route using protocols and a SIVmac251 virus stock previously described (Marthas et al., 2001). Briefly, animals received two intravaginal inoculations of 1 ml undiluted SIVmac251 stock containing 1×10⁵ 50% tissue culture infectious doses (TCID50) with these two inoculations separated by a 4 h time period over one day. Blood samples were collected at two to four week intervals after immunization and challenge for assessment of virus-specific immune responses and monitoring for hematologic alterations by complete blood cell count and CD4/CD8 subset analysis.

Virus load determination

Viral RNA concentrations in plasma were measured using a previously described real-time reverse transcriptase (RT) TaqMan PCR assay with a sensitivity limit of 50–100 copies of SIV RNA per ml of plasma (Leutenegger et al., 2001). Briefly, viral RNA extracted from plasma with a commercial kit (QIAamp Viral RNA Mini Kit; Qiagen, Valencia CA) was amplified in a single step RT-PCR reaction using previously described conditions and primers (SIV510f and SIV592r), TaqMan probe (SIV535p) specific for SIVmac 251 gag, MMLV-RT (Applied Biosystems), and AmpliTaq Gold DNA polymerase. For assay of PBMC for proviral DNA, genomic DNA was extracted with a commercial kit (DNeasy Blood and Tissue Kit; Qiagen) and processed using the Applied Biosystems 6100 Nucleic Acid Prep Station. Real-time PCR conditions for detection of cell-associated viral DNA were previously described (Leutenegger et al., 2001) and included assay for rhesus IL-2 as a housekeeping gene for all reactions. All reactions were carried out in an ABI PRISM Sequence Detector (Applied Biosystems).

T cell proliferation assay

SIV-specific T cell proliferation responses were assayed in freshly isolated peripheral blood mononuclear cells (PBMC) using a traditional assay based on ³H-labeled thymidine incorporation as previously described (Abel et al., 2003; McChesney et al., 1998). AT-2-inactivated SIV (kindly provided by Jeff Lifson, Laboratory of Retroviral Pathogenesis, SAIC Frederick, Bethesda, MD) in concentrations of 0.1, 1, and 10 ng per well in a 96-well plate, served as viral antigen.

IFN-γ ELISPOT assay

Cryopreserved PBMC were tested in duplicate using a previously described recombinant vaccinia virus (r-VV)-based ELISPOT assay for detection of antigen-specific IFN-γ expression (Larsson et al., 1999; Shacklett et al., 2002). Recombinant vaccinia viruses expressing SIVmac251 Gag (vAbT252), Env (vAbT2531), Pol (vAbt258), and Nef or control r-VV (Therion Biological, Cambridge, MA) were used as antigens. After an overnight incubation with r-VV, PBMC were transferred to a 96-well ELISPOT plate (U-Cytech, Utrecht, The Netherlands) coated with monoclonal antibody specific for macaque IFN-γ. Spot-forming cells (SFC) were counted using a digital ELISPOT reader (AID, Automation Diagnostika, Cell Technology Inc., Columbia MD). Results were presented as SFC per 10⁶ cells after subtraction of background SFC measured for control vaccinia virus. Values greater than 50 SFC per 10⁶ cells were considered positive.

Antibody assays

Plasma antibodies specific to SIVmac were assayed after vaccination and challenged using a commercial HIV-1/HIV-2 ELISA (Genetics Systems Corporation, Redmond, Washington). Plasma antibodies reactive to native SIV envelope proteins were measured with a previously described ConA ELISA (Cole et al., 1997). However, a four-fold increase in concentration of SIVmac239 virus was used to coat ELISA plates to enhance the sensitivity of this ELISA. Avidity of plasma antibodies to native envelope proteins was determined by measuring resistance of antibody-envelope glycoprotein complexes to 8 M urea in a ConA ELISA (Cole et al., 1997). The avidity index calculation was based on the following equation: (OD₄₅₀ of phosphate buffered saline-washed wells/OD₄₅₀ of urea-washed wells)×100%.

Statistics

Graphs and statistical analyses were performed using GraphPad Prism using the Mann–Whitney–U test for comparison of virus loads and CD4 T cell percentages between vaccinated and unvaccinated control animals after challenge. P values of <0.05 were considered significant. A survival curve and analysis was performed using the Kaplan–Meier method (GraphPad Prism).

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References


