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FOREWORD

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Induction of Type 1 Immune Responses to SIV by IFN- γ

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Principal Investigator: T. Yilma

INTRODUCTION

Our long term goal is the development of a safe and efficacious vaccine against infection with the human immunodeficiency virus (HIV). The immediate objective of this study is to develop vaccinia virus (VV) vector vaccines against acquired immunodeficiency syndrome (AIDS) using simian immunodeficiency virus (SIV) as a model in rhesus macaques. On the basis of both biologic and genetic features, SIV infection of macaques is the best animal model for HIV infection and AIDS (Desrosiers, 1990; Gardner, 1990; Hirsch *et al.*, 1989). The development of the live VV vaccines is based on results obtained from previous studies in our laboratory and others demonstrating the adjuvant and attenuating effects of lymphokine interferon-gamma (IFN- γ) and recombinant VV (rVV) expressing the IFN- γ alone, coexpressed with immunogen, or fused to immunogen (Anderson, Fennie, and Yilma, 1988; Giavedoni *et al.*, 1992). Based on the above research, we have developed a vaccine based on a recombinant VV that expresses the IFN- γ , SIV virus like particles (VLPs) containing SIVgag and env, and the SIV nef protein, that will induce Type 1 immune responses to these antigens and hence protection against SIV infection. Such a vaccine combines the effectiveness of a live attenuated vaccine with the safety of a subunit immunogen and will have these advantages:

- 1. Antigens expressed by rVV are effective in inducing both cytotoxic T-lymphocyte (CTL) and humoral immune responses;
- 2. IFN-γ attenuates VV virulence;
- 3. IFN- γ will favor a Type 1 immune response to SIV antigens;
- 4. VLPs retain the antigenic integrity of the whole SIV virus;
- 5. Inclusion of nef, a non-structural protein which is expressed early in SIV infection will induce the CTLs that act on infected cells soon after exposure to SIV;
- 6. Should lack of sterilizing immunity occurs, an immune response to nef will select against SIVnef⁺ variants so that attenuated SIVnef⁺ viruses will be favored.

There is a strong evidence that supports the critical role of the cell-mediated arm of the immune system in the control of HIV infection (Buseyne *et al.*, 1993; Ramsay, Ruby, and Ramshaw, 1993; Salk *et al.*, 1993; Shearer and Clerici, 1992). Also, the preferential development of one T helper cell subset is often apparent at the early stages of an infection, suggesting that the mechanism that drives the immune response in one direction or the other operates soon after exposure to antigen (Reed and Scott, 1993). Therefore, we believe that the inclusion of IFN γ in our vaccine will generate a safe, attenuated VV vector, and will prompt the immune system to develop a protective immune response to SIV antigens, mediated by T_H1 cells. Since, anti-SIV antigens in their natural conformation. The incorporation of SIV envelop into VLPs will improve its presentation to the immune system, in a way that can not be achieved by subunit preparations. Additionally, the co-expression of SIV VLPs by rVV, and subsequent booster immunizations with SIV VLPs produced in a baculovirus vector, will induce long lasting cell-mediated as well as

humoral immunity. There are two reasons for including nef in the vaccine preparation. First, VVexpressed nef will generate nef specific CTLs in immunized macaques; these CTLs will recognize a gene product that is expressed early after infection, and therefore will kill infected cells before the release of infectious virus. Second, the immune response to this protein may prevent disease by creating an immune environment in which nef SIV variants have a selective advantage. These variants are viruses in which the nef gene product has been eliminated as an immune target, and will likely be early non-sense mutations of deletions of the nef gene. Under the selective pressure, the SIV population in an immunized host could evolve to a nef phenotype. Once the nef SIV population takes over, it may prevent disease progression in two ways: 1) the virus population is less pathogenic by virtue of being nef (Kestler *et al.*, 1991); 2) the population could mimic the behavior of the nef-deletion mutant described above (whose manner of protection is unknown) and induce protective immunity (Daniel *et al.*, 1992).

During the first phase of the project, we completed the construction, characterization and preparation of high-titer stocks of a number of rVVs that express the IFN- γ gene and/or SIVgag, env, and nef genes. Immunological blots, PCR and protein gel electrophoresis were used to confirm that each of the proteins expressed by these rVVs retain biological and antigenic activities. We have also demonstrated that intravenous (I/V) inoculation of rVVs did not induce generalized VV infection in SIV-infected macaques. On the contrary, it was self limiting since we were not able to recover live rVV from plasma or PBMCs. The concurrent SIV infection of the intravenously vaccinated macaques did not alter, and there was even a functional recovery of the lymph nodes of 3 out 4 inoculated macaques.

In June 1998 progress report, we reported the immune responses of three groups of rhesus macaques vaccinated with rVVs. The first group of five animals that was vaccinated with vHu γ /SIVgen, a Wyeth recombinant that expresses the human interferon gamma, and the SIV gag, env, and nef genes. The second group of five macaques received a similar dose of vSIVgen, a Wyeth rVV that expresses the SIV gag, env, and nef genes. The third group has two animals that served as controls and were inoculated with wild type VV (Wyeth strain). All animals have been inoculated with one ml of sterile DMEM containing 10⁷ plaque forming units (pfu) of the corresponding VV intramuscularly (I/M) thrice on week 0 for priming and week 8, and 26 for boosting the immune responses. The resolution of VV infection, immune responses to SIV antigens, and lymphokine (IFN γ) activity in plasma were investigated. Finally, these macaques were boosted with 50 ug of gag/env virus like particles produced in insect cells. All macaques plus two control animals were inoculated with SIV_{Anef} two weeks after the final boost. Fifty weeks after the inoculation with SIV_{Anef}, all of the macaques plus two naive controls were challenged with SIV_{mec251}. We report the outcome of challenge studies and the type of immune responses generated to the SIV antigens in the presence or absence of co-expressed IFN- γ .

MATERIALS AND METHODS

1. Preparation of rVV Inoculum: Large-scale preparation of rVV was conducted as following: HeLa cells were infected with each rVV at a multiplicity of infection (moi) of 3.0 in spinner cultures. After 48-hour incubation in Jokliks modified Eagles medium (JME) with 5% FBS at 37 °C, the cell pellets were harvested and resuspended in JME. The concentration of infectious virus was titrated by a VV plaque assay. Purification of rVV was performed by sucrose cushion ultracentrifugation. Briefly, the virus was well homogenized and sonicated in JME. The homogenate was layered over an equal-volume of 36% sucrose cushion (prepared in 10 mM Tris, pH9.0) in SW28 rotor tubes. After ultracentrifugation (40,000 x g for 60 minutes), each virus pellet was washed with JME to remove any residual sucrose and resuspended in JME. The purified rVVs were titrated by VV plaque assay.

2. Preparation of Virus-like Particles: Upon infection of a permissible cell, Simian Immunodeficiency Virus (SIV) genome encodes for gag, polymerase, gp160 and accessory proteins. The gag protein of SIV and other lentiviruses (HIV, BIV) assembles into virus-like particles (VLPs) on the surface of eukaryotic cells (Delchambre et al., (1989) EMBO J., 8(9):2653-60; ?; ?). We investigated the incorporation of surface glycoprotein (gp160) into the envelope of VLPs during the co-expression of gp160 and gag of SIVmac239 in insect cells (Sf9). The gp160 and gag genes were inserted separately or together into the genome of Autographa californica nuclear polyhedrosis virus (AcNPV) under polyhedrin promoter by homologous recombination. AcNPV expressing gp160 or gag and AcNPV expressing gp160 and gag were used to infect the insect cells for characterization of recombinant gp160, gag, and VLPs from Sf9 cells by biochemical and immunological methods. Electron microscopic (EM) examination of the infected Sf9 cells showed the assembly and release of VLPs from the plasma membrane surfaces. The expression of VLPs by baculovirus is so high that there is particle formation on the membranes even inside the cytoplasm. The extracellular VLPs were further purified by 20-60% sucrose step gradient. These VLPs contained both gag and gp160 as demonstrated by westernblot analysis and also their ability to exhibit an increase in the anti-gp160 and anti-gag antibodies in primed mice. Also, the negative staining and transmission electron microscopy of VLPs labeled with anti-gp130 antibodies and immunogold was a direct evidence of presence of gp160 on the surface of purified VLPs. We demonstrated the incorporation of surface glycoprotein gp160 into envelope of VLPs assembled from gag in insect cells. This incorporation of gp160 into the envelope of VLPs in insect cells does not require any SIV factor. Milligram quantities of these VLPs were produced in insect cell culture in flasks and suspension culture in small scale bioreactor.

3. Vaccination of Rhesus Macaques with Recombinant VV and gag/env VLPs: Twelve colony-bred, juvenile rhesus macaques (*Macaca mulatta*) seronegative for simian type D retroviruses, simian T-cell leukemia virus, and SIV, were employed in this experiment. They are housed at the California Regional Primate Research Center (CRPRC) in accordance with the American Association for Accreditation of Laboratory Animal Care Guidelines and University of California Animal Care Policies. Two groups of five macaques were vaccinated I/M with 10⁷ pfu

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of rVV and the third control group consisting of two macaques was inoculated with a similar dose of wild type VV (Wyeth strain). After 8 and 26 weeks, the macaques were boosted by vaccinating with the same viruses, dose and route. Behavior and clinical condition of the vaccinated animals were routinely recorded by the staff of the CRPRC.

After 35 weeks of last VV inoculations, the animals in first two groups were inoculated with 50 ug of gag/env VLPs intramuscularly as a final boost.

4. Collection of tissue samples from vaccinated macaques: Animals were sedated, and 5 ml of blood was withdrawn in EDTA tubes. Additionally, EDTA tubes were filled with blood for CBC and surface immunofluorescence (Delchambre *et al.*, 1989). Using sterile surgical techniques, peripheral lymph nodes (axillary) biopsies were performed on macaques by the certified veterinarian of CRPRC. Lymph node tissues were immersed in RPMI-fungizone and shipped along with the blood to the processing laboratory. The lymph node were cut in two pieces, one half was processed to obtain cells and nucleic acids, and the other half was immersed in formaldehyde (for immuno-histochemistry and in-situ hybridization). The blood samples and lymph node biopsies were taken at 2 week intervals for first 8 weeks and then monthly basis until the boost with VLPs.

5. Co-culture of PBMCs and LNCs with BSC 40 for VV Isolation: The presence of infectious VV in PBMCs and LNCs of vaccinated macaques was determined by co-cultivation of rhesus PBMCs and LNCs with BSC 40 cells. One million PBMCs or LNCs were added to BSC 40 cells in 6 well plates and incubated for 48 hours. Non-adherent cells were removed and the remaining cells were observed for VV CPE. When CPE was not evident, adherent cells were removed from the plate with trypsin and cell lysates were prepared by cycles of freeze/thaw and sonication. These cell lysates were used to infect fresh 6 well-plates of BSC 40 cells that were maintained in 37°C incubator for another 48 hours for recording VV CPE.

6. PCR Amplification of gag and B13R Sequences in PBMCs and LNCs of Vaccinated Macaques: The rVV sequences in the tissues from vaccinated macaques were identified by amplifying the B13R and the recombinant gag regions by polymerase chain reaction. DNA from 1 x 10^6 PBMCs and LNCs was isolated by DNA extraction column (QIAGEN, Inc., Santa Clarita, CA). One tenth of each DNA sample was used in a polymerase chain reaction with forward and reverse oligonucleotide primers complimentary to B13R and gag genes. Negative and positive controls were the DNA from PBMCs/LNCs from unvaccinated macaques and rVV-infected BSC 40 cells respectively. Each PCR was performed with the 2.5 units of Taq DNA polymerase enzyme from Promega Corporation. After 37 cycles of PCR, one third of the PCR product was loaded on 1% agarose gel that was stained with ethidium bromide.

7. Lymphokine Activity in Plasma of Rhesus Macaques: The antiviral activity induced by interferon present in macaques plasma samples was determined by prevention of cytopathic effects (CPE) of EMCV (encephalomyocarditis virus) on human A549 cell lines (Giavedoni *et al.*, 1992).

Plasma samples were diluted in duplicate in a 96 well plate, A549 cells (human lung cells) were added to each well, and the plate was incubated at 37°C. After 24 hour incubation, 1×10^4 plaque forming units (pfu) of EMCV was added to each well, and the plates incubated for 24 hours at 37°C. The plates were read under the microscope and stained. The units of interferon were expressed as the reciprocal of the last dilution that provided 50% or more protection against virus infection (CPE).

8. Immune Responses to VV, gp160, gag, and nef Antigens:

(a) Anti-VV Antibody Titers by Plaque Reduction Assay: Antibodies induced by the vaccine vector were measured by VV plaque reduction assay on the BSC 40 cells (Andrew, Coupar, and Boyle, 1989). Four-fold serial dilutions of plasma, in duplicate, were mixed with a predetermined amount of VV. The virus antibody mixture was incubated for 1 hour at 37°C and then used to infect BSC 40 cells to detect virus infectivity. Antibody titers are expressed as the inverse of the highest sample dilution where the number of plaques is reduced at least 50%

(b) Anti-gp160, -gag, and -nef by Western Blot Analysis: Two week post-primary vaccination plasma samples usually have very low levels of anti-SIV antibodies. To detect these low level antibodies in plasma of 2 WPV, we transferred the capture antigens (gp160, gag, nef) to Immobilon-P (PVDF) membranes for western blots (Ahmad *et al.*, 1994). One blot was prepared and adsorbed with a 1:100 dilution of each serum sample in 3% blotto (non-fat dry milk in Tris.HCl, NaCl buffer). Anti-monkey Ig conjugated to horseradish peroxidase (Cappel Inc., Alexandria, VA) was used as the second antibody (diluted to 1:2,000 in 3% blotto). The color was developed by adding 1% AEC in acetate buffer (pH 5.5) and hydrogen peroxide.

© Antibody to gp160, -gag, and -nef measured by ELISA: Before the last boost with baculovirus expressed VLPs, these ELISAs contained the following capture antigens expressed in baculovirus vectors: (1) SIVmac p55gag, (2) SIVmac gp160 env, and (3) SIVmac nef (Ahmad *et al.*, 1994; Giavedoni *et al.*, 1993). After boost with VLPs, the immune responses to antigens were quantitated with the ELISA using whole SIVmac251 antigen. Anti-monkey Ig conjugated to horseradish peroxidase (Cappel Inc., Alexandria, VA) was used as second antibody for these ELISAs. Two-fold serial dilutions of plasma were assayed in duplicate and mean values were calculated. Positive controls were plasma samples from macaques infected with uncloned SIVmac251. Negative controls were plasma samples from macaques never exposed to SIV. Antibody titers were expressed as the inverse of the highest sample dilution where optical density (OD) was at least double the negative control OD.

9. Lymphocyte phenotyping. PBMCs from macaques were stained with anti-human monoclonal antibodies to CD4 (phycoerithrin-conjugated OKT4, Ortho Diagnostic Systems Inc., Raritan, NJ) or to CD8 (Leu 2a-FITC, Becton Dickinson Immunocytometry Systems, San Jose, CA)) as instructed by the manufacturers, and immunofluorescence was measured with a dual-

laser flow cytometer (FACSCAN, Becton Dickinson).

10. Proliferation of Lymphocytes to Viral Antigens. PBMCs or LNCs from macaques were plated at 10⁵ cells/well in quadruplicate in 96 well plates. Each sample was stimulated with baculovirus-expressed gag, gp160, a control antigen (vesicular stomatitis virus glycoprotein), SIVmac251, ConA or DME and the incorporation of ³H thymidine measured. Samples were collected using a semi-automated cell harvester, washed and then counted on a beta scintillation counter. Samples were considered positive if the amount of radioisotope incorporated in the cells was two or more times that of the negative control antigen.

11. Inoculation of Immunized Macaques with SIV Δ nef: Vaccinated macaques were inoculated with SIV $_{\Delta nef}$ 2 weeks after vaccination with VLPs; two naive controls were included at the time of challenge. The viral inoculum, 100 tissue culture infectious dose 50 (TCID₅₀), was given intravenously in a single 1 ml dose.

12. Challenge of Immunized Macaques with SIV_{mac251}: Vaccinated macaques were challenged with SIV_{Anef} 2 weeks after vaccination with VLPs; two naive controls were included at the time of challenge. The viral inoculum, 10 TCID₅₀ (approximately 60AID50), was given intravenously in a single 1 ml dose. Animals will be euthanized if they show three or more of the following clinical observations: a) weight loss greater than 10% in two weeks or 30% in two months, b) chronic diarrhea unresponsive to treatment, c) infections unresponsive to antibiotic treatment, d) inability to maintain body heat or fluids without supplementation, e) persistent, marked hematologic abnormalities including lymphopenia, anemia, thrombocytopenia, or neutropenia, and f) persistent, marked splenomegaly or hepatomegaly.

13. Cell-associated viral load. Cell-associated virus, latent or productive, was measured by limiting-dilution assay (4 replicates per dilution) of PBMCs and LNCs, with CEM-x-174 cells in 24-well plates (30). Twice weekly, aliquots of the culture medium were assayed for the presence of the SIV major core protein (p27) by ELISA (23). When p27 antigen was detected at 2 consecutive time points, cultures were recorded as virus positive. Endpoint cultures were maintained and tested for four weeks before being scored as negative. Virus levels were calculated according to the method of Reed and Muench (29) and expressed as TCID₅₀ per 10⁶ cells.

14. Detection of proviral SIV DNA. DNA was isolated from 2x10⁵ cells (PBMCs, LNCs or CEM-x-174 cells) using a DNA isolation kit (Quiagen, Chatsworth, CA). The presence of SIV proviral sequences was confirmed by PCR amplification of the env-3'LTR region, employing primers A (⁵GTACCATGGCCAAATGCAAG³, sense primer, nt. 8720) and E (⁵AAATCCCTTCCAGTCCCCCC³, antisense, nt. 9710). The proviral DNA was hot-started with Mg⁺⁺

beads (Invitrogen Corp., La Jolla, CA) at 94°C for 5 min, annealed at 65°C for 1 min, and extended at 72°C for 2 min; then the denaturation time was reduced to 1 min, and the cycle was repeated 35 times.

15. Analysis of the lymphokine pattern released by T4 cells in immunized macaques.

In order to determine whether the presence of IFN- γ modified the induction of T_H cell subsets, we started to evaluate the pattern of lymphokine released by T cells after antigen-specific stimulation. PBMCs from immunized macaques were used to seed 96-well plates (10⁵ cells/well) and 100 µl of various preparations was added by quadruplicate. Baculovirus-expressed SIV gag and env were used as antigenic stimulus; uninfected insect cell preparations and culture medium served as negative controls. IFN- γ , IL-2, IL-4, and IL-10 were measured. Detection of IFN- γ and IL-2 indicate a pattern characteristic of T_H1 cells, whereas IL-4, IL-5 and IL-10 are indicative of T_H2 cells.

Detection of lymphokine-specific mRNA: PBMC are cultured with RPMI 1640 with 10% FCS, 1% penicillin streptomyacin, .5ug of antigen (gag or gp160), for 5 days. T4 cells are then isolated from the media using magenetic beads, (Dynal cat. 111.15) attaining a purity of 97%, and then detached from the magnetic beads by Detachabead (Dynal cat. 125.03). The mRNA is isolated from the cells by Trizol Reagent according to the manufacturer's instructions (Gibco cat. 15596-018). RT-PCR was preformed using digoxygenin labled dNTPs and primer pairs specific for IL-2, IL-4, IL-10, and INF- γ (Table 2). Quantitation of the PCR products was determined by using a PCR-ELISA kit (Borrehinger Mannheim cat. 1636111) and biotin labeled internal primers for each lymphokine.

RESULTS AND DISCUSSION

1. Intramuscular Vaccination of Juvenile Rhesus Macaques with rVV is Safe: This study used a total of 16 juvenile rhesus macaques placed in five groups as outlined in Table I. The first group consisted of five animals which were vaccinated with vHu γ /SIVgen, a Wyeth recombinant that expresses the human interferon gamma, and the SIV gag, env, and nef genes. The second group had five macaques that received a similar dose of vSIVgen, a Wyeth recombinant that expresses the SIV gag, env, and nef genes. The third group had two control animals that received wild type Wyeth VV (vWY). The fourth group was inoculated with SIV_{Aner} and the fifth group was added at the time of challenge with SIV_{mac251} as naive controls.

We decided to vaccinate the rhesus macaques with rVV by I/M inoculations rather than I/V. The intramuscular inoculations were performed for the following reasons: 1) There is a slight probability of systemic VV infection complication of I/V inoculation of rVV especially in individuals with a compromised immune system, although I/V inoculation of VV in SIV-infected macaques did cause any complications as reported last year. 2) In a comparative study in cattle, I/M inoculations of rVV expressing the surface glycoproteins of rinderpest virus were superior to intradermal (I/D) vaccinations in inducing immune responses to the antigens and providing

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sterilizing immunity to 1,000X lethal challenge with rinderpest virus (**unpublished data**). 3) Moreover, I/M vaccination of VV was used in some human vaccinee populations during the smallpox eradication program.

Eating patterns, elimination, and behavior of all macaques were noted to be normal on a daily basis. No local or systemic reactions to the vaccine were recorded. Once or twice a month, a physical examination was performed to measure body weight. No indication of lymphadenopathy and splenomegaly was noticed by palpitation. Peripheral blood was collected immediately prior to vaccination; differential blood counts and chemistries (CBC) were found in normal range except a slight rise in the lukocytes in response to VV infection. None of the animal exhibited thrombocytopenia and anemia consequent to the vaccination. All of these procedures and diagnostic tests were performed by veterinary staff at the CRPRC at U. C. Davis.

2. Resolution of VV infection in PBMCs and LNCs Rhesus Macaques: One of the major concerns in the use of live VV as a vaccine vector is the safety to vaccinees and contact populations of humans and animals. In this study, we followed the co-culture assays and polymerase chain reaction to determine the fate of rVV in PBMCs and lymph node cells (LNCs) of vaccinated macaques. PBMCs from all twelve vaccinated macaques were co-cultured with BSC 40 cells, a cell line highly susceptible to VV infection. No extensive CPE or individual virus plaques were observed on BSC 40 cell monolayer. Similar studies with LNCs are in progress. We could not isolate the rVV or vWY from PBMCs collected as early as one week post-primary and two weeks post-secondary vaccination in all twelve macaque.

We resorted to a more sensitive technique, polymerase chain reaction (PCR) to identify the presence of VV in PBMCs and LNCs from vaccinated macaque since VV is a highly cell associated virus. We were able to identify the presence of rVV nucleotide sequences one week after primary vaccination but not the infective capability of rVV.

The gag and B13R regions of the rVV genome were amplified from total DNA extracted from PBMCs and LNCs. In our laboratory, the gag region is commonly used to identify SIV in virus, cell, and tissue preparations from cultured cells and animal tissues. The B13R open reading frame is present in the VV *Hind* IIIB genomic region close to the right inverted terminal repeat. This immune-modulating gene codes for a serine protease inhibitor (serpin) homolog (Kotwal and Moss, 1989). Our laboratory has recently cloned and characterized the B13R gene of the VV. As part of NIH-sponsored project, we have developed a number of oligonucleotide primers to amplify the B13R region of the VV genome present in various tissues. The oligonucleotide B13R primers have been used successfully yielding DNA bands of the expected size (1.1 kbp), whose identity was confirmed by cloning, restriction endonuclease analysis, and/or sequencing. Consequently, these primers were chosen to amplify the B13R region of the VV genome present in vertices.

In the PBMC samples from 1 WPV, one animal (MMU 28067) was positive for the presence of the gag gene; all others were negative. None of the samples were positive for the presence of B13R sequences. This indicates that rVV or VV infection in PBMCs was resolved within one week of inoculation in rhesus macaques. Moreover, the gag oligonucleotide primers

are more sensitive than B13R primers in identifying the rVV sequences in the DNA from tissues. When the LNCs were assayed for the presence of these two gene sequences, 4 of 5 animals vaccinated with the vSIVgen and none of the animals vaccinated with the vHu γ /SIVgen were positive for gag sequences 1 WPV. These results suggest that VV infection is resolved more rapidly in PBMCs than in LNCs, possibly by different mechanisms. Moreover, the expression of IFN γ by the rVV appeared to aid in earlier resolution of the infection in lymph nodes of macaques.

Two weeks after the second vaccination (first boost) with rVVs, PBMCs from all 10 macaques were negative for the virus as determined by amplification of gag region. These samples are being processed for B13R region amplifications by PCR. From limited results available, the rVV infection was completely resolved in these primed macaques within two weeks after first boost.

3. Immune responses of Rhesus Macaques to VV: All vaccinated animals were positive for anti-VV antibodies as determined by the plaque reduction assay (Figure 1). The anti-VV antibody titers ranged <4-64 after the primary immunization. At 8 WPV, the mean anti-VV titer was 18.4 \pm 26 in vHu γ /SIVgen-vaccinated macaques compared to 35.2 \pm 28 in vSIVgen-vaccinated macaques. This may be due to the lower replication of vHu γ /SIVgen and an indication of attenuation of VV by the expression of IFN γ during primary infection. However, after the second vaccination with rVV, the mean anti-VV titer in vHu γ /SIVgen-vaccinated macaques was approximately twice that in vSIVgen-vaccinated macaques. This increase may be due to the immune enhancing activity of the IFN γ . In our laboratory, we have seen immune enhancement by IFN γ in cattle and mice (Anderson, Fennie, andYilma, 1988). Upon revaccination with rVV, there appears to have been a replication of the rVV in the macaques because a 5-20 fold increase in anti-VV responses was observed 2-8 weeks after the second inoculation. There was an anamnestic response to the second boost with VV, however this was not as large as the first one.

4. Immune responses of Rhesus Macaques to SIV Antigens (gp160, gag, nef): All macaques in first and second group responded to primary vaccination by producing the antibodies to SIV antigens. We expected very low titers of anti-gag, -gp160, and -nef antibodies during the first two weeks after the primary vaccination with rVV, thus western blots were used to detect antibodies against the respective antigens. However, the average titers to all antigens were high by 1 WPV. On the 8th WPV, the macaque received the first boost with the same dose of respective rVV and route of inoculation. All the animals vaccinated with vSIV γ gen and vSIVgen had anamnestic antibody responses to the first boost and had high titers of antibodies to gp160, gag, and nef antigens of the SIV (Figures 2,3,4). The monkeys were given a second boost with the rVVs on week 26 post-vaccination, however there was only an anamnestic response to gp160.

5. Lymphokine Responses of Rhesus Macaques Vaccinated with the rVV: Lymphokines were measured by a PCR assay for several time points (Table 3). These results suggest that the

immune response is a Th2, not the predicted Th1 response. However the data is incomplete at this time so no conclusions can be drawn as yet.

6. Proliferation of PBMCs After Challenge with SIV_{mac251}: As a measure of immune function, the proliferation of PMBCs was measured to various SIV antigens (Table 4). These antigens included baculovirus expressed SIV gag and gp160 and SIV_{mac251}. There was no clear correlation with proliferation and protection against high virus loads after challenge although there was some indication that there was more proliferation in the group given vSIVγgen as a primary vaccine.

7. Virus Loads After Inoculation with SIV_{Anef}: All animals were inoculated with 100TCID50 of SIV_{Anef} 63 weeks post-vaccination and all of the monkeys became infected with this virus. All animals had low to undetectable titers of virus in PBMCs and LNCs by 9 weeks post-inoculation. There was no difference in the average titers of virus and no discernable difference in the animals vaccinated with rVVs when compared to the controls (Figure 5).

8. Virus Loads After Challenge with SIVmac251: All macaques were challenged with 10 TCID50 of virulent SIVmac251 50 weeks post-inoculation with SIVAnef. One week post-challenge, 6/10 of the rVV vaccinated animals were negative for the challenge virus, 1/4 of the vaccinated controls had undetectable SIVmac251, and both of the naive controls had high titers of the challenge virus. By 2 weeks post-challenge, 3/10 of the rVV vaccinated animals were still negative for SIVmac251, and 1/4 of the vaccinated controls still remained negative. By 4 weeks post-challenge, SIVmac251 was detected in all of the animals although a few had a small amount of detectable SIVAner proviral DNA. Currently (12 weeks post-challenge) 3/5 of the animals given vSIVygen have no detectable virus by co-culture or PCR in their PBMCs. In the vSIVgen-vaccinated group, 2/5 are virus negative and 1/4 of the control vaccinated monkeys have no detectable virus. The two control animals have higher average virus loads and lower average CD4 counts and one of them has a rash, often an early sign of SAIDS. The CD4 counts have dropped on average, but only 6/14 vaccinated animals have dropped more than 50% (Figure 6). The average titers of the different vaccinated groups of animals does not suggest any increase in efficacy that can be measured at this time point although the naive controls do have a significantly higher virus load (Figure 7). These observations suggest that the vaccinated animals are controlling the infection with the challenge virus better than either the vaccine or naive controls and will probably survive the infection much longer than the naive controls. In a previous experiment, we found that 5/20 monkeys given a similar vaccination regime survived more than 3 years after challenge (unpublished data). No SIVmac251 was detected in these animals and 3/5 had no detectable virus. A ll of the survivors were vaccinated with rVVs expressing SIVgp130 or gp160 and then SIVAner. None of the VV or SIVAner controls survived this length of time suggesting that vaccination with rVVs and then an attenuated live SIV did enhance the efficacy of the vaccination protocol.

CONCLUSIONS

We utilized the research project (contract #DAMD17-95-C5054) funded by US Department of Army for expanding our studies to improve upon our preliminary observations that prior immunization with recombinant subunit vaccines against simian immunodeficiency virus with a deletion in the nef gene (SIV_{Aner}) may enhance both safety and efficacy of live attenuated vaccines for AIDS. The most effective vaccines for prevention of infection and/or disease from viruses have been live attenuated vaccines. In some instances, live attenuated viruses may be the safest and most effective vaccines available. For example, inactivated morbillivirus vaccines resulted in high rates of atypical and sometimes dangerous responses to infection with the wild type virus (Craighead, 1975; Norrby, 1975), perhaps due to problems in MHC class I presentation of antigens caused by UV or formalin inactivation. (Cardoso *et al.*, 1995). This problem can be overcome by expression of antigens in live recombinant vectors, subunit, or DNA vaccines with the proper adjuvants. However, in those cases where viruses are difficult to propagate *in vitro* or are extremely dangerous; inactivated, subunit or recombinant vaccines may be the only reasonable method of vaccine development.

In the search for a safe and effective vaccine for AIDS, all of these approaches have been taken. Using the SIV macaque model, currently several conclusions have been found: 1. Immune responses to foreign antigens in the vaccine and in the challenge virus are protective in inactivated whole virus vaccines (Stott, 1991; Stott et al., 1990). 2. To protect against intravenous infection with virulent SIV_{mac251}, only two vaccines have been successful, SIV_{Δner} and SIV_{Δ3} (Daniel *et al.*, 1992). Other vaccines, recombinant live vaccines, SHIV, and subunit vaccines have been successful in delaying onset of disease, but not infection with virulent virus (Joag et al., 1998; Kent et al., 1996; Lu et al., 1998; Mossman et al., 1996; Putkonen et al., 1998; Putkonen et al., 1995) However, SIVAner and SIVA3 are not highly effective against infection until 12-15 months postvaccination and both have been reported to cause disease in some adult and neonatal macaques (Baba et al., 1995; Baba et al., 1999; Baba et al., 1996; Wyand et al., 1997). In addition, it has been reported that nef-truncated HIV-1 and HIV-2 can cause AIDS in infected humans.(Learmont et al., 1999; Switzer et al., 1998) We have been exploring the possibility of combining current models of vaccines to improve the safety and efficacy of live attenuated vaccines. We hypothesized that by vaccinating with a recombinant vaccine first, chances of the attenuated live vaccine causing disease by itself might be decreased. This approach may also decrease the time required for induction of sterilizing immunity and protection against infection with virulent virus.

Twelve rhesus macaques were inoculated I/M with one ml of sterile, cold DMEM containing 10⁷ pfu of rVV. We decided to vaccinate the rhesus macaques with the rVV by I/M inoculations rather than I/V since, in a comparative study in cattle, I/M inoculations of rVV expressing the surface glycoproteins of rinderpest virus outperformed the traditional I/D route in eliciting immune responses to the antigens and provided sterilizing immunity to higher challenge doses of virulent rinderpest virus. Moreover, the I/M route vaccination of VV was used safely and effectively in some human vaccinees during the smallpox eradication program. Additionally, there is a slight probability of systemic VV infection complication of I/V inoculation of VV in

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SIV-infected macaques did not cause any complications.

Although the primary antibody response to rVVs expressing IFN- γ was lower than that to rVVs not expressing this lymphokine, the anamnestic response to VV after the second and third vaccinations suggests that: 1) The vaccination did not induce a sterilizing immunity to VV and 2) Expression of IFN- γ increased the antibody response to VV and gp160, suggesting that IFN- γ has a detectable immune enhancing activity on the anamnestic antibody response to some antigens. All macaques in first and second group responded to primary vaccination by producing the antibodies to VV and SIV antigens (Figures 1-4). By 8 WPV, all animals except controls had high titers of antibodies to the gag, gp160, and nef antigens of the SIV. After the second vaccination, animals had higher anamnestic responses to gp160 than to nef and gag antigens. These results indicate that the rVVs and the I/M route used in this study have induced high anti-SIV responses in macaques.

No infectious VV was detected by coculture of PBMCs and LNCs with the highly susceptible cell line BSC 40. This suggests that the amount of virus circulating in the animal by one week after primary and two weeks after secondary vaccination is very low. This result implies that the chance of accidental infection by contact is very low. The PCR results suggest that there is a very low level of virus still present in the animals after vaccination and that: 1) The virus expressing IFN- γ is cleared more rapidly than virus not expressing the lymphokine, increasing the attenuation and safety of the vaccine. 2) The virus is cleared more slowly in the LNCs than in PBMCs. This suggests that the theories about virus retention in lymph nodes as a method of maintaining the immune response to antigens may have some validity and perhaps the mechanisms of virus clearance differ in these two parts of the body.

All of the monkeys vaccinated with vSIV γ gen and vSIVgen were given a final boost with baculovirus-expressed gp160 and gag on week 61 post-vaccination and these 10 animal plus the two VV controls and two naive animals were inoculated with SIVAner 63 weeks post-vaccination. A ll of the animals became infected with a high dose of $SIV_{\Delta nef}$ (100 TCID₅₀), indicating that at this dose there was no protection, even with high levels of antibody, against infection with an attenuated virus. All animals quickly controlled the infection and by the time of challenge (50 WPI) virus titers in PBMCs and LNCs were low to undetectable by co-culture or PCR. None of the monkeys had any sign of immunodeficiency or illness during this time. The animals were challenged intravenously with 10 TCID50 (approximately 60 AID50) of SIVmac251 and were able to control but not resist infection with the challenge virus. All of the macaques were positive for SIVmac251 by four weeks post-challenge but most of the animals were able to control the infection quickly and the virus load returned to prechallenge levels by four to eight weeks postchallenge in 80% of the rVV-vaccinated animals. The virus in the co-culture plates were mainly SIVmac251 although the vaccine virus was also detectable at lower levels in some of the animals. There appears to be a slightly lower virus load in the animals vaccinated with vSIVygen compared to vSIVgen and SIVAner, however this is not statistically significant. By twelve weeks post-challenge, three of the five animals given vSIVygen had undetectable virus loads in the PBMCs compared to two of five animals given vSIVgen and the average titers are lower in the vSIV γ gen-vaccinated group (76 TCID₅₀ versus 315 TCID₅₀). Since money is lacking for the

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maintanence of these animals, they will be euthanized in the next month and a long term study is not possible for financial reasons.

There was no correlation with proliferation or antibody titer and resistance to high virus loads after challenge. The most proliferation was observed in the vSIV γ gen vaccinated group on week 4 post-challenge. This observation does suggest that the cell mediated response has been increased in this group but it is not statistically significant.

Thus, vaccination with a recombinant vaccinia virus vaccine for SIV followed by inoculation with an attenuated SIV_{Anef} appears to improve the efficacy of the vaccine since vaccinated monkeys are controlling the infection with SIV_{mac251} better than the naive or SIV_{Anef} controls based on relative virus loads in PBMCs and LNCs. However, it did not improve the efficacy of the attenuated live vaccine against intravenous infection with virulent SIV_{mac251}. These results indicate that, although infection with SIV_{mac251} was not prevented after prevaccination with rVVs expressing SIV antigens and interferon- γ and 34 - 50 weeks of infection with SIV_{Anef}, this vaccination scheme was effective in decreasing the virus load in these animals for at least the first 12 weeks.

The conclusions to be drawn from these experiments are 1. Recombinant subunit vaccines do not provide sterilizing immunity against SIV; however, they significantly reduce virus load and increase survival time. 2. Prior immunization with recombinant subunit vaccines against SIV_{Aner} may enhance both safety and efficacy 3. Inclusion of SIV gag and nef and the human IFN- γ proteins in the recombinant vaccines did not increase the resistance to infection with SIV_{Aner} or SIV_{mac251}. 4. The inclusion of human IFN- γ in the recombinant vaccine does appear to decrease the virus load after challenge, although not significantly.

Induction of Type 1 Immune Responses to SIV by IFN- γ

Table 1: Immunization and challenge of macaques.

N	Vaccination (10 ⁷ PFU, im) (0 week)	1 st boost (10 ⁷ PFU, im) (8 weeks)	2nd boost (10 ⁷ PFU, im) (26 weeks)	3 rd boost (61 weeks)	Inoculation (100 TCID ₅₀ , iv) (63 weeks) SIV Δnef	Challenge* (10 TCID ₅₀ , iv) (113 weeks) SIVmac251
5	vSIVygen	vSIVygen	vSIVygen	bSIVge	$\mathrm{SIV}_{\Delta \mathrm{nef}}$	SIV _{mac251}
5	vSIVgen	vSIVgp120	vSIVgen	bSIVge	$\mathrm{SIV}_{\Delta \mathrm{nef}}$	SIV _{mac251}
2	vv	vv	vv	bWT	$\mathrm{SIV}_{\Delta nef}$	SIV_{mac251}
2	SIVAnef				$\mathrm{SIV}_{\Delta\mathrm{nef}}$	SIV _{mac251}
2	Naive Control					$\mathrm{SIV}_{\mathrm{mac251}}$

vSIVγgen- rVV expressing SIV gp160, gag, nef and human IFN-γ vSIVgen- rVV expressing SIV gp160, gag, and nef bSIVge- baculovirus expressed SIV gp160 and gag, bWT - wild type baculovirus VV- vaccinia virus

Principal Investigator: T. Yilma

TABLE 2: Sequence of oligonucleotide primers for PCR and internal probes

PCR-primer pairs for various lymphokines (Bost et al., 1997)

Lymphokine	5' oligo-primers	3' oligo- primers
IL-2	attacaagaatcccaaactcacag	gtcaaagcatcatctcaacac tgacct
IL-4	cggcaactttgcccacggacacaa	ctgaaacggctcgacaggaa cctc
I L -10	atgccccaagctgagaaccacgaccca ggatagctgacccagccccttgaga	
INF-y	gttttgggttctcttggctgtta	caagtgatggctg aactgtcgc

Biotin labeled internal probes for various lymphokines (Benveniste et al. 1996)

Lymphokine	Internal probe
IL-2	agggatctgaaacaacactgatgtgtga
IL-4	cagcagtttcacaggcacaagcagc
正-10	ggccgtggagcaggtgaagaatgccttta
INF-y	tgaccagaggatccaaaagagtgtg

Principal Investigator: T. Yilma

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TABLE 3: Level of lymphokine expression in 1 x 10⁶ PBMCs stimulated with SIV-gp160 at 9 wpi in monkeys vaccinated with vHu γ /SIVgen+VLPs or vSIVgen+VLPs and challenged with 100 TCID₅₀ of SIV_{Auf}.

Groun #	Animal	Vaccination	Challenge	11,-2	11,-4	11-10	
a dwarp)		Pico-gram of m	RNA per 10 ⁶ PBN	ИC
1	MMU 27436	vHuy/SIVgen + VLPs	$SIV_{\Delta nef}$			8.0	
	MMU 27672	vHuy/SIVgen + VLPs	$SIV_{\Delta nef}$			80.0	
	MMU 27991	vHuy/SIVgen + VLPs	$SIV_{\Delta nef}$			36.0	
	MMU 28067	vHuy/SIVgen + VLPs	$SIV_{\Delta nef}$			56.0	
	MMU 28243	$vHu\gamma/SIVgen + VLPs$	$\mathrm{SIV}_{\mathrm{\Delta nef}}$		12.0	100.0	
ç	MMATT 28281	vSIVgen + VI Ps	SIV		24.4	132.0	
4	NAME 78258	wSIV and + VI Pa	SIV.			ċ	
	NANTI 28401	vSIVgen + VLPs	SIV	12.0	14.8	136.0	
	MMU 28472	vSIVgen + VLPs	SIV	5.2	10.0	108.0	
	MMU 28703	vSIVgen + VLPs	$\mathrm{SIV}_{\mathrm{Anef}}$				116.0
~	MMU 29116	νWY	$SIV_{\Delta nef}$				26.0
I	MMU 29117	۲WV	$SIV_{\Delta nef}$	-			¢. ;
	MMU30992	ı	$SIV_{\Delta nef}$	÷			92.0
	MMU30994	I	$\mathrm{SIV}_{\Delta \mathrm{nef}}$				36.0

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TABLE 4: PROLIFERATION RESULTS AFTER SIVmac251 CHALLENGE (% POSITIVE)

WEEK	VACCINE	SIVmac251	gag (2.5 ul)	gag (5 ul)	gp 160
0	vSIVygen	0	80%		0
-	vSIVgen	40%	40%		20%
	SIVAnef	0	40%		20%
	NONE	0	0		0
1	vSIVvgen	0	0		20%
•	vSIVgen	0	20%		0
	STVAnef	25%	0		0
	None	0	0		0
2	vSIVvgen	20%	0		20%
2	vSIVgen	0	20%		20%
	SIVAnef	25%	50%		0
	None	0	0		0
4	vSIVvgen	40%	80%	100%	40%
•	vSIVgen	0%	20%	40%	40%
	SIVAnef	0	50%	50%	0
	None	0	0	0	0
8	vSIVvgen	0	60%	80%	20%
Ŭ	vSIVgen	0	20%	20%	20%
	SIVAnef	0	50%	25%	0
	None	0	0	0	0
12	vSIVvgen	0	0	0	0
	vSIVgen	20%	60%	20%	0
	SIVAnef	0	50%	25%	0
	None	0	0	0	0

Induction of Type 1 Immune Responses to SIV by IFN- γ

FIGURE 1



FIGURE 1: Antibody to VV was measured by a plaque reduction assay. The animals were vaccinated with VV on weeks 0, 8, and 26. They were boosted with baculovirus-expressed SIV gag and gp160 on week 61 (vlps) and then given SIV_{Aner} two weeks later. vSIV_γgen is a rVV that expresses SIV gag, gp160, nef and human IFN- γ . vSIVgen expresses SIV gp160, gag and nef. vWY is the parental Wyeth strain of VV used to construct the recombinants.

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Induction of Type 1 Immune Responses to SIV by IFN-y

FIGURE 2



FIGURE 2: Antibody to SIV gp160 was measured by an ELISA using plates coated with baculovirus-expressed SIV gp160. The animals were vaccinated with VV on weeks 0, 8, and 26. They were boosted with baculovirus-expressed SIV gag and gp160 on week 61 (vlps). Since the monkeys have antibody to the baculovirus antigens after this inoculation, this assay cannot be used to measure antibody accurately after this time point. vSIVygen is a rVV that expresses SIV gag, gp160, nef and human IFN- γ . vSIVgen expresses SIV gp160, gag and nef. vWY is the parental Wyeth strain of VV used to construct the recombinants.

Induction of Type 1 Immune Responses to SIV by IFN- γ

FIGURE 3



FIGURE 3: Antibody to SIV gag was measured by an ELISA using plates coated with baculovirus-expressed SIV gag. The animals were vaccinated with VV on weeks 0, 8, and 26. They were boosted with baculovirus-expressed SIV gag and gp160 on week 61 (vlps). Since the monkeys have antibody to the baculovirus antigens after this inoculation, this assay cannot be used to measure antibody accurately after this time point. vSIVygen is a rVV that expresses SIV gag, gp160, nef and human IFN- γ . vSIVgen expresses SIV gp160, gag and nef. vWY is the parental Wyeth strain of VV used to construct the recombinants.

Induction of Type 1 Immune Responses to SIV by IFN- γ

FIGURE 4



FIGURE 4: Antibody to SIV nef was measured by an ELISA using plates coated with baculovirus-expressed SIV nef. The animals were vaccinated with VV on weeks 0, 8, and 26. They were boosted with baculovirus-expressed SIV gag and gp160 on week 61 (vlps). Since the monkeys have antibody to the baculovirus antigens after this inoculation, this assay cannot be used to measure antibody accurately after this time point. vSIVygen is a rVV that expresses SIV gag, gp160, nef and human IFN- γ . vSIVgen expresses SIV gp160, gag and nef. vWY is the parental Wyeth strain of VV used to construct the recombinants.

Average Antibody Responses to the SIV nef Protein

Principal Investigator: T. Yilma

FIGURE 5



FIGURE 5. Virus loads in LNCs were measured by co-culture of LNCs with CEM-X-63 cells and the virus was detected by an ELISA that detects production of SIV p27. Animals were inoculated with 100 AID50 of SIV_{Aner} 63 weeks after the first vaccination with rVVs. Fifty weeks after the inoculation with the attenuated SIV, the monkeys were challenged with 10 AID50 of SIV_{mac251}. vSIV_γgen is a rVV that expresses SIV gag, gp160, nef and human IFN- γ . vSIVgen expresses SIV gp160, gag and nef.

Induction of Type 1 Immune Responses to SIV by IFN- γ

FIGURE 6



FIGURE 6. Virus loads in PBMCs were measured by co-culture of PBMCs with CEM-X-63 cells and the virus was detected by an ELISA that detects production of SIV p27. Animals were inoculated with 100 AID50 of SIV_{Δnef} 63 weeks after the first vaccination with rVVs. Fifty weeks after the inoculation with the attenuated SIV, the monkeys were challenged with 10 AID50 of SIV_{mac251}. vSIV_γgen is a rVV that expresses SIV gag, gp160, nef and human IFN- γ . vSIVgen expresses SIV gp160, gag and nef.

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Induction of Type 1 Immune Responses to SIV by IFN- γ

FIGURE 7

Average CD4 + Counts



FIGURE 7. The number of CD4+ cells in the blood samples were estimated by staining the cells with anti-human monoclonal antibodies to CD4 (phycoerithrin-conjugated OKT4) and immunofluorescence was measured with a dual-laser flow cytometer. Animals were inoculated with 100 AID50 of SIV_{Aner} 63 weeks after the first vaccination with rVVs. Fifty weeks after the inoculation with the attenuated SIV, the monkeys were challenged with 10 AID50 of SIV_{mac251}. vSIV_γgen is a rVV that expresses SIV gag, gp160, nef and human IFN- γ . vSIVgen expresses SIV gp160, gag and nef.

Induction of Type 1 Immune Responses to SIV by IFN- γ

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