Protection against Lethal Simian Immunodeficiency Virus SIVsmmPBj14 Disease by a Recombinant Semliki Forest Virus gp160 Vaccine and by a gp120 Subunit Vaccine

SALLY P. MOSSMAN,^{1†} FRANCOISE BEX,² PETER BERGLUND,³ JAMES ARTHOS,^{4‡} SHAWN P. O'NEIL,¹ DAVID RILEY,^{4§} DONALD H. MAUL,¹ CLAUDINE BRUCK,⁵ PATRICIA MOMIN,⁵ ARSENE BURNY,⁶ PATRICIA N. FULTZ,⁷ JAMES I. MULLINS,^{4§} PETER LILJESTRÖM,³
AND EDWARD A. HOOVER^{1*}

Department of Pathology, Colorado State University, Fort Collins, Colorado 80523¹; Department of Internal Medicine, University of Texas, Southwestern Medical Center at Dallas, Dallas, Texas 75235-8594²; Department of Biosciences, Karolinska Institute, Novum, S-141 57 Huddinge, Sweden³; Department of Microbiology & Immunology, Stanford University School of Medicine, Stanford University, Stanford, California 94305-5402⁴; Department of Molecular Biology, SmithKline Beecham Biologicals, B-1330 Rixensart,⁵ and Departement de Biologie Moleculaire, Laboratoire de Chimie Biologique, Université Libre de Bruxelles, 1640 Rhode-Saint-Genese,⁶ Belgium; and Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294-2170⁷

Received 7 August 1995/Accepted 7 December 1995

Infection of pigtail macaques with SIVsmmPBj14, biological clone 3 (SIV-PBj14-bcl3), produces an acute and usually fatal shock-like syndrome 7 to 14 days after infection. We used this simian immunodeficiency virus (SIV) model as a rapid and rigorous challenge to evaluate the efficacy of two SIV Env vaccine strategies. Groups of four pigtail macaques were immunized four times over a 25-week span with either a recombinant Semliki Forest virus expressing the SIV-PBj14 Env gp160 (SFV-SIVgp160) or purified recombinant SIV-PBj14 gp120 (rgp120) in SBN-1 adjuvant. Antibody titers to SIV Env developed in all immunized animals (mean peak titers prior to challenge, 1:1,700 for SFV-SIVgp160 and 1:10,500 for rgp120), but neither neutralizing antibodies nor SIV-specific T-cell proliferative responses were detectable in any of the vaccinees. All macaques were challenged with a 100% infectious, 75% fatal dose of SIV-PBj14-bcl3 at week 26. Three of four control animals died of acute SIV-PBj14 syndrome on days 12 and 13. By contrast, all four SFV-SIVgp160-immunized animals and three of the four rgp120-immunized animals were protected from lethal disease. While all virus-challenged animals became infected, symptoms of the SIV-PBj14 syndrome were more severe in controls than in vaccinees. Mean virus titers in plasma at 13 days postchallenge were approximately 10-fold lower in vaccinated than control animals. However, there was no apparent correlation between survival and levels of peripheral blood mononuclear cell-associated culturable virus, provirus load, or any antiviral immunologic parameter examined. The results indicate that while immunization with SFV-SIVgp160 and rgp120 did not protect against virus infection, these Env vaccines did lower the virus load in plasma and protect against the lethal SIV-PBj14 challenge.

Envelope glycoproteins have been the primary focus for the development of vaccines to control the AIDS epidemic. By using primate models of infection of chimpanzees with human immunodeficiency virus (HIV) and macaques with simian immunodeficiency virus (SIV), it has been shown that Env vaccines can induce neutralizing antibodies and cell-mediated immune responses and can in some cases protect against homologous challenge (6, 17, 19, 30, 33). Moreover, the passive transfer of Env-specific antibodies has been shown to confer protection to recipients (12, 24). However, the relationship between indicators of immunogenicity measured in vitro and protective immunity assessed in vivo is far from clear (1, 6, 19, 37). While the most potent vaccination efficacy demonstrated to date was associated with an attenuated SIV strain which

replicated to very low levels in macaques but protected against a high-dose challenge of pathogenic virus (8), the safety of such vaccines in neonates and, by analogy, immunocompromised individuals remains in question (3). Vaccination with infectious recombinant vectors containing genes encoding HIV or SIV proteins offers the possibility of expressing antigens in multiple host cells in the same context as in the cognate infection but without the safety concerns associated with a live attenuated retrovirus vaccine. In this respect, recombinant vaccinia viruses expressing SIVmne Env have been successful in protecting macaques against a homologous virus challenge (19, 20), but these results have not been achieved with more virulent viruses in other laboratories (2, 9). Complete suppression of HIV replication or "sterilizing immunity," however, may not be feasible or be a reasonable absolute criterion by which candidate SIV and HIV vaccines should be judged (2, 7, 18, 22, 23, 28, 34). Rather, immunization with the goal of sufficiently suppressing early virus replication to prevent or delay the onset of disease may be a more realistic prospect.

In this study, we evaluated two SIV Env vaccine strategies: (i) an infectious, suicidal recombinant Semliki Forest virus (SFV) expressing the SIV-PBj14 *env* gene product (SFV-SIVgp160), and (ii) a recombinant Env subunit vaccine com-

* Corresponding author. Mailing address: Department of Pathology, Colorado State University, Fort Collins, CO 80523. Phone: (970) 491-7861. Fax: (970) 491-0523.

† Present address: Regional Primate Research Center, University of Washington, Seattle, WA 98195.

‡ Present address: NIH/NIAID/LIR, Bethesda, MD 20892.

§ Present address: Departments of Microbiology and Medicine, University of Washington, Seattle, WA 98195.

posed of the glycosylated SIV-PBj14 surface glycoprotein, gp120, formulated in adjuvant (rgp120). Immunized animals were challenged with the acutely fatal SIV-PBj14, clone bcl3 (14, 15, 21). An advantage of using this SIV variant is that the *in vivo* protective effects elicited by vaccines can be assessed rapidly. Here, we present evidence that both the SFV-SIVgp160 and SIVrgp120 vaccines provided macaques with protection from lethal SIV-PBj14 challenge but not from infection.

MATERIALS AND METHODS

Animals. Twelve female juvenile pigtail macaques (*Macaca nemestrina*), 1 to 2 years of age and weighing 3 to 4 kg, were obtained from Charles River Primates Corp. All animals were negative for SIV, simian type D retrovirus, and SFV.

Vaccine preparations. (i) **SFV-SIVgp160.** The *env* gene of the molecular clone SIV-PBj14-mcl1.9 (11) was amplified by PCR with the following oligonucleotides as 5' and 3' primers, respectively: 5' GATCGGATCCTCTTGGGAATCAGCTGCTTATCG 3' and 5' GATCGGATCCTCACAAGAGAGTGAGTCAAGC 3'. The fragment was then cloned into the pSFV3 expression vector (27) by using the *Bam*HI restriction sites incorporated into the PCR primers. This strategy places the SIV *env* coding sequence in frame with the initiation codon of the SFV capsid gene, downstream of the SFV subgenomic promoter, and modifies the first two amino acids of the full-length envelope glycoprotein (Asp-Pro instead of Gly-Cys). The plasmid was then used as a template for *in vitro* synthesis of recombinant RNA. This RNA was cotransfected with pSV-Helper 2 RNA, carrying the SFV structural genes, into BHK-21 cells for production of recombinant SFV-SIVgp160 virus stocks, as described in references 4 and 27. Production of SFV-*lacZ* recombinant virus has been described previously (27).

BHK-21 cells were infected with SFV-SIVgp160 at a multiplicity of infection of 10 for 24 h as previously described (27). The cells were then scraped into sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and the cell lysates were separated on 10% acrylamide gels and transferred to nitrocellulose paper. Blots were probed with a pool of anti-SIV Env monoclonal antibodies or with serum from a macaque infected with SIV-PBj14. Detection was performed with enhanced chemiluminescence Western blot (immunoblot) reagents (Amersham, Arlington Heights, Ill.).

BHK-21 cells infected with SFV-SIVgp160 for 9 h were washed and incubated in L-methionine-free minimal essential medium for 30 min, labelled for 15 min with L-[³⁵S]methionine (1 mCi/ml), and chased for 15, 30, and 90 min in medium containing unlabelled L-methionine. The cells were scraped, lysed, and immunoprecipitated with a pool of anti-SIV Env monoclonal antibodies. Immunoprecipitated proteins were separated on 10% acrylamide gels and detected by autoradiography.

On the day of the immunizations, SFV-SIVgp160 and SFV-*lacZ* particle preparations were activated by incubation with 200 μ g of α -chymotrypsin (Sigma, St. Louis, Mo.) per ml for 30 min at room temperature. The chymotrypsin was then inactivated by the addition of 700 μ g of aprotinin (Sigma) per ml. The activated SFV particles were stored on ice until used for inoculation into animals.

Dilutions of activated SFV-SIVgp160 and SFV-*lacZ* stocks were plated onto BHK-21 monolayers, incubated at 37°C overnight, and then fixed in ice-cold methanol for 5 min. Infectious-unit counts were derived for SFV-SIVgp160 stocks by an immunofluorescence assay with serum from a pigtail macaque chronically infected with SIV-PBj14-mcl1.9 as the primary antibody. The secondary antibody was goat anti-monkey immunoglobulin G (IgG) (whole molecule) conjugated to fluorescein isothiocyanate (Cappel, Durham, N.C.). Infected cells were detected by green fluorescence under a UV microscope. SFV-*lacZ*-infected cells were detected by incubation of monolayers with the β -galactosidase substrate, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Sigma). Positive cells were stained with a dark-blue precipitate.

(ii) **rgp120.** Recombinant soluble gp120 was obtained by expression of the envelope gene of SIV-PBj14-mcl1.9 (11) in Chinese hamster ovary cells (36). Construction of the gp120 expression system followed the strategy of Planelles et al. (32). Briefly, a fragment of the PBj envelope comprising residues +2 to +523 was PCR amplified from PBj14-mcl1.9 by using the error-correcting polymerase Pfu (Stratagene, La Jolla, Calif.). Amplification was limited to five cycles to minimize base misincorporation by Pfu. This fragment was inserted into the mammalian expression vector JW8282 (38a) such that it was under the control of a cytomegalovirus promoter and flanked by a bovine growth hormone polyadenylation signal. Plasmid was transfected into the dihydrofolate-deficient Chinese hamster ovary cell line DXB11 by calcium phosphate transfection (38). Cell clones expressing high levels of gp120 were identified and amplified as described by Deen et al. (10). Concentrated culture supernatants were passed over a GNA lectin column, eluted with α -mannopyranoside (16), and then passed over an anti- α_2 -macroglobulin column. Recombinant gp120 purity was estimated at greater than 85% by silver stain gel analysis and found to be coprecipitable with soluble CD4. The rgp120 was emulsified in SBN-1 adjuvant (SmithKline Beecham, Rixensart, Belgium) prior to inoculation into animals. SBN-1 adjuvant is an oil-water emulsion believed both to exert a depot function to retaining

antigen and to stimulate antigen-presenting cells. As with most adjuvants, its true mode of action is unknown.

Vaccinations. Twelve pigtail macaques were apportioned into two groups of four vaccinees and one group of four controls. All animals were immunized at weeks 0, 5, 16, and 25. One group of four animals (53-423, 53-430, 53-429, and 53-459) received the SFV-SIVgp160 construct. The first three immunizations of recombinant SFV were divided between intramuscular and intravenous routes. The final boost was administered by the intramuscular route only. At the time of immunization, titers of activated SFV stocks were measured *in vitro* to determine the exact dose inoculated. Doses were similar for each vaccination and averaged 2.7×10^8 infectious units per immunization per animal. The second group of four macaques (53-425, 53-426, 22-144, and 72-14) was inoculated intramuscularly with 100 μ g of rgp120 formulated in SBN-1 adjuvant per animal. Two control animals (53-437 and 53-442) were inoculated with SFV-*lacZ*, and two controls (53-456 and 53-458) received SBN-1 adjuvant in phosphate-buffered saline (PBS).

Production of primary macaque cell cultures. Primary fibroblast and epithelial cell cultures were established from rhesus macaque lung and kidney tissues. Pieces of tissue 3 cm² were removed from a naive animal at necropsy and stored on ice in Eagle minimum essential medium (Irvine Scientific, Santa Ana, Calif.) containing 20% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 100 U of penicillin per ml, 100 μ g of streptomycin per ml, 1% nystatin (Gibco-BRL, Gaithersburg, Md.) and 2 mM L-glutamine (JRH Biosciences, Lenexa, Kans.). The tissues were then minced into 2- to 3-mm³ fragments and were digested for 2 h at 37°C in serum-free medium containing 2 mg of collagenase per ml and 1 mg of hyaluronidase per ml. Cells and tissue fragments were then washed twice in medium and seeded into 25-cm² flasks containing complete Eagle minimal essential medium with 20% fetal bovine serum. Once confluent, the monolayers were washed with PBS to remove nonadherent debris and were trypsinized and replated at a 1:2 dilution. Dominant cell types in these cultures were either epithelial or fibroblastic in morphology.

Challenge virus. A stock of SIV-PBj14-bcl3 was prepared in pigtail macaque peripheral blood mononuclear cells (PBMC) previously stimulated for 3 days in 10 μ g of phytohemagglutinin (PHA; Sigma) per ml. Female juvenile pigtail macaques, age matched to the vaccinees, were inoculated intravenously with 10-fold dilutions of virus stock to determine the minimum dose inducing clinical disease. The end point was reached at a 10^{-4} dilution of the virus stock, which was equal to 10 animal infectious doses and one 50% tissue culture infectious dose (TCID₅₀) in PHA-stimulated human PBMC. Of four macaques inoculated with the 10^{-4} dose, three died of acute SIV-PBj14 disease and the final animal developed severe clinical signs but survived the acute disease phase. This dose was used to challenge vaccinated and control macaques at week 26, 1 week after the final boost.

Mouse inoculations. Suckling mice, 1 to 3 days old, were inoculated intracerebrally through the cranium via a 27-gauge needle with 10^7 PFU of wild-type SFV or with 10^7 infectious units of activated or inactivated SFV-SIVgp160 vaccine stock in 30- μ l volumes. Death of inoculated animals was used as an indicator of the presence of wild-type SFV.

Virus isolation. Macaques were bled at necropsy and/or at 10, 13, 17, 24, and 67 days postchallenge (p.c.). PBMC were separated from EDTA-treated (final concentration, 0.15%) blood by Ficoll density gradient centrifugation (LSM; Organon Teknica, Durham, N.C.), and virus titers were determined in 24-well plates by coculture with 2×10^6 PHA-stimulated human PBMC per well in RPMI 1640 medium (Irvine Scientific) containing 100 U of interleukin-2 (Cetus Corp., Emeryville, Calif.) per ml, 2 μ g of polybrene (Sigma) per ml, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. Medium was replaced twice a week, and supernatants were frozen at -20°C once a week for SIV antigen capture enzyme-linked immunosorbent assay (ELISA) (22). At 10 and 20 days after initiation of cultures, 2×10^6 fresh human PHA-stimulated PBMC were added to each well. Fresh cell-free plasma was subjected to titer determination and cultured in human PBMC in the same way.

Quantitative provirus PCR assay. (i) **Construction of reporter templates.** PBMC DNA was extracted with Isoquick nucleic acid extraction kits (Micro-Probe Corp.) and quantitated by spectrophotometry at A_{260} , assuming that 1 μ g of DNA represented 1.5×10^5 cells. An SIV *gag* reporter template plasmid (pDRSC109) containing a 178-bp DNA fragment in pCRII (Invitrogen Corp., San Diego, Calif.) was generated. This plasmid contains binding sites for DR9 (GACAGATTGGATTAGCAGAAAGCCTGTGGA) and DR10 (GCTTCCTCAGTATGTTTCACTTCTCTCTGCGTG) that amplify a highly conserved region of SIV and HIV-2 *gag* (bases 156 to 264 of SIV-PBj14-mcl1.9). The internal sequence of the reporter template is nonhomologous and corresponds to bases 7486 to 7594 of HIV-1 HXB2 but matches the corresponding SIV/HIV-2 PCR products in length, G+C content, and calculated melting temperature. Another plasmid, pDRSIV109, containing the 178-bp SIV *gag* sequence in the pCRII vector was also constructed.

Each plasmid stock was purified on CsCl gradients and then quantified by multiple parallel end point dilution followed by nested PCR to determine the precise concentration of amplification targets in the DNA.

(ii) **Proximal DNA quantitation.** All PCRs were performed in duplicate in 10 mM Tris (pH 8.3)-2 mM MgCl₂-50 mM KCl-300 μ M deoxynucleoside triphosphates-200 nM each primer-1% glycerol-1.25 U of Ampli-Taq polymerase per

μ l. Amplification was carried out in a Perkin-Elmer 9600 thermocycler for four cycles of 94°C for 10 s, 53°C for 10 s, and 72°C for 10 s followed by 28 cycles of 90°C for 10 s, 55°C for 10 s, and 72°C for 10 s, with a final extension at 72°C for 5 min.

PCR products were assayed in microwell plates with reagents from the HIV-1 detection kit (Roche Molecular Systems, Branchburg, N.J.). Briefly, biotin-labeled PCR product was denatured and hybridized for 2 h at 37°C in 96-well plates coated with an oligonucleotide probe. Hybridized PCR product was detected with a streptavidin-horseradish peroxidase conjugate followed by tetramethyl-benzene substrate. The reaction was stopped with 1.0 N sulfuric acid after 10 min, and the A_{410} was read.

Specific sequences within PCR-amplified DNA were detected with the following probes, designed to have similar melting temperatures. The SIV *gag* reporter product was hybridized to DR78 (GTATGCCCCCTCCCATCAGAGGA CAAATTAGATG), and the SIV *gag* PCR product was hybridized to DR109 (GTTTATGCTCCATTAGTTCCGACAGGTTCAAGTTTCAGAAA).

SIV Env antibody ELISA. Macaque sera were tested for SIV Env antibodies by using capture monoclonal antibody 101.1, which detects SIV gp120 and SIV gp160 (22).

SIV neutralizing antibody. Twofold dilutions of heat-inactivated macaque sera were incubated in round-bottom 96-well plates with 10 TCID₅₀ of SIV-PBj14-bcl3 per well for 1 h at 37°C. CEMx174 cells (5×10^5) were added to each well, and the plate was incubated overnight at 37°C. The next day, the cells were washed three times, and subsequently the medium was changed twice weekly. Supernatants were screened for SIV antigen once a week by SIV p27 ELISA, as described previously (22). The neutralizing titer was defined as the highest serum dilution that completely inhibited SIV replication. Serum from a pigtail macaque chronically infected with SIV-PBj14-mcl1.9 was included in each assay as a positive control and consistently gave a titer of 1/160 in this assay.

SFV neutralizing antibody. Twofold dilutions of heat-inactivated pre- and postimmunization macaque sera were incubated in 96-well plates at 37°C for 1 h with 50 PFU of wild-type SFV per well. BHK-21 cells were added at 7×10^4 cells per well, and the plates were incubated for 4 days until there was 100% cytopathic effect in negative control wells. The neutralizing titer for each serum sample was the highest dilution that completely inhibited SFV cytopathic effect.

Clinical evaluations. Animals were examined daily, and the following symptoms associated with SIV-PBj14 infection were scored on a numeric scale from 0 to 18: stool consistency, hydration status, abdominal discomfort, skin rash, grooming condition, attitude, and weakness. In addition, when animals were anesthetized for blood collection on days 10, 13, 17, 24, and 67, temperatures and weights were recorded. Clinical scores were determined independently by two people, and the final daily index was the mean score for all parameters in both evaluations.

Lymphocyte subset analysis. Lymphocyte subset analysis was performed as described previously (21). A 50- μ l sample of whole blood collected in EDTA was mixed with fluorochrome-conjugated monoclonal antibody and incubated for 20 min in the dark at room temperature. Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated irrelevant mouse antibodies (MsIgG-FITC and MsIgG-PE, including isotypes IgG1, IgG2a, IgG2b, and IgG3 [Coulter Corp.]) were used as controls. CD4 T lymphocytes were enumerated with OKT4-FITC (Ortho Diagnostics, Raritan, N.J.), CD8 T lymphocytes were enumerated with Leu-2a-PE (Becton Dickinson, Mountain View, Calif.), and monocytes were enumerated with MY4-FITC (Coulter Corp., Hialeah, Fla.). Following labeling, samples were processed with a Coulter Q-Prep workstation before analysis on a Coulter Epics II flow cytometer.

Lymphocyte proliferation assays. Cryopreserved, Ficoll-separated PBMC from postimmunization, prechallenge blood samples were cultured in round-bottom 96-well plates at 5×10^5 cells per well in RPMI 1640 medium containing 15% heat-inactivated AB-positive human serum, 2% L-glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. Cells were incubated with either 5 μ g of heat-inactivated SIV-PBj14-bcl3 virions per ml, 10 μ g of concanavalin A per ml as a positive control, or medium alone. The plate was incubated for 3 days at 37°C and then pulsed overnight with 0.5 μ Ci of aqueous [*methyl*-³H]thymidine (Amersham). Cells were harvested the next day, and the stimulation index was calculated as the ratio of radioactivity incorporated by PBMC in the presence of antigen to that in medium alone. The assay was optimized and controlled by using postchallenge PBMC from the same animals. At 2 months postchallenge, stimulation indices ranged from 4 to 6, whereas PBMC from naive animals did not proliferate. The mean stimulation index for PBMC exposed to mitogen was 19.

RESULTS

Expression of Env by SFV-SIVgp160. To characterize the Env glycoproteins produced by the recombinant SFV expression system, BHK-21 cells infected with the SFV-SIVgp160 recombinant were analyzed by Western blotting (Fig. 1A). Env-specific monoclonal antibodies bound to the full-length precursor glycoprotein, gp160 (molecular mass, 185 kDa for SIV-PBj14) (Fig. 1A, lane 1). Polyclonal serum from an SIV-

FIG. 1. (A) Western blots of cell extracts from BHK-21 cells infected with SFV-SIVgp160 (lanes 1 and 3) or uninfected BHK-21 cells (lanes 2 and 4). Blots were probed with a pool of SIV Env-specific monoclonal antibodies (lanes 1 and 2) or with a polyclonal SIV-specific macaque serum (lanes 3 and 4). (B) BHK-21 cells infected with SFV-SIVgp160, labeled with [³⁵S]methionine, and chased for 15 (lane 1), 30 (lane 2), and 90 (lane 3) min before immunoprecipitation with a pool of SIV Env-specific monoclonal antibodies.

PBj14-infected macaque also recognized the surface (130-kDa) and transmembrane (41-kDa) subunits generated by cleavage of the envelope precursor (Fig. 1A, lane 3). Pulse-chase labeling demonstrated that a detectable portion of the precursor was processed into its subunits within the 90-min chase period (Fig. 1B, lane 3). This suggests that the envelope glycoproteins produced by the SFV expression system are glycosylated and correctly folded and processed, as has been shown for HIV-1 gp160 and gp120 molecules produced in the SFV expression system (31).

SFV biosafety. Recombinant suicidal SFV particles were designed to undergo a single round of replication *in vivo*, and since the helper virus is not packaged, no progeny virions should be produced. As a further biocontainment precaution, the vaccine particles contain a defective structural p62 protein, which requires cleavage by chymotrypsin treatment *in vitro* to render the virus infectious. Therefore, any second-round virions produced *in vivo* would be noninfectious in the absence of chymotrypsin (5, 13, 26, 27, 40).

Intracerebral inoculation of suckling mice is the most sensitive method of detecting wild-type SFV and was therefore used to test vaccine particle stocks for wild-type replicating virus arising through recombination *in vivo*. Whereas five of five mice inoculated with wild-type SFV died at 1 to 2 days postinoculation, 11 of 11 animals inoculated with activated (6 mice) or inactivated (5 mice) SFV-SIVgp160 survived the inoculations and were symptom free. An additional nine mice were inoculated with activated SFV-SIVgp160 and then sacrificed at 3 days postinoculation. The brains from these animals were minced and cocultured with BHK-21 monolayers. These cultures were negative for SFV as determined by lack of cytopathic effect in the BHK-21 indicator cells. It was concluded that production of wild-type replicating SFV from vaccine preparations did not occur (<1 infectious unit/ 10^8 particles) and that the vector was therefore safe for use in macaques. Inoculation of $>10^8$ particles into macaques was also found to be safe in that none of the six animals receiving recombinant SFV vaccines developed signs of wild-type SFV infection.

SFV infectivity for macaque cells *in vitro*. Additional preliminary *in vitro* experiments were performed to ascertain which macaque cells might be susceptible to SFV infection.

FIG. 2. Mean SIV gp160 ELISA antibody titers in sera from animals immunized with SFV-SIVgp160 (□) or rgp120 (◇). The times at which immunizations were performed are denoted by open arrows, and the time of challenge is denoted by a solid arrow.

Infection was detected by immunofluorescence assay for expression of SIV Env. Both primary macaque kidney fibroblasts and lung epithelial cells were infected by SFV-SIVgp160, and the titers were equal to those on BHK-21 cells. We were unable to detect expression of Env in SFV-SIVgp160-inoculated pigtail PBMC, suggesting that these cells may be restricted for SFV replication. The SFV-SIVgp160 vaccine and SFV-lacZ control were inoculated into macaques by two routes, intramuscular and intravenous. The intramuscular route was used since it was established that SFV could infect macaque fibroblasts. Although PBMC were apparently not infected in vitro, the recombinant SFV was inoculated intravenously to maximize the potential for distribution of virus throughout the body.

Humoral immune responses to SIV and SFV. Serum antibody to SIV gp160 was produced in the rgp120 group after the first immunization, while that in the SFV-SIVgp160 group was undetectable until after the second immunization (Fig. 2). Antibody titers in both groups reached maximum levels after the third vaccination and then declined. Mean peak titers were 1:10,500 in rgp120-immunized animals and 1:1,700 in SFV-SIVgp160-immunized animals. Env antibody titers did not reach peak values in either vaccine group after the fourth immunization, 1 week before challenge. At challenge, mean anti-SIV titers were 1:2,400 in rgp120-inoculated animals and 1:600 in SFV-SIVgp160 vaccinees. Throughout the vaccination schedule, animals inoculated with the recombinant gp120 protein maintained higher Env antibody titers than those exposed to SFV-SIVgp160. This finding probably reflects the fact that the Env glycoprotein produced by the SFV vector represents only 1% of that present in the rgp120 inoculations. No SIV-neutralizing activity was detected in sera screened at the time of challenge and at 9 weeks p.c.

Neutralizing antibodies against SFV were induced in all animals inoculated with SFV constructs and had reached a mean titer of 1:480 by the time of challenge (Fig. 3).

Cell-mediated immunity. Cell-mediated immunity was analyzed by evaluating T-cell proliferative responses to SIV antigen. PBMC were tested at 1 month after the third immunization and at the time of challenge. Stimulation indices were ≤ 1 for all animals at both time points, indicating the absence of detectable SIV-specific T-cell proliferation.

Survival and clinical disease. All macaques were challenged

FIG. 3. Neutralizing-antibody titers to SFV for the four animals immunized with SFV-SIVgp160, 53-429 (□), 53-429 (◇), 53-423 (○) and 53-430 (△), and for the two control animals inoculated with SFV-lacZ, 53-437 (■) and 53-442 (■). Times of immunization are denoted by open arrows, and the time of challenge is denoted by a solid arrow.

at 26 weeks, 1 week after the final immunization. Three of the four control animals, one infected with SFV-lacZ (53-442) and two adjuvant-only controls (53-456 and 53-458), died on day 12 or 13 p.c. (Fig. 4). None of the 4 SFV-SIVgp160-vaccinated animals died. One of the four animals vaccinated with rgp120 (22-144) died of the SIV-PBj14 syndrome at 8 days p.c. All animals that survived beyond day 14 p.c. remained asymptomatic at 12 months p.c. Whereas one of four control macaques survived the 75% lethal dose of SIV-PBj14-bcl3, all SFV-SIVgp160-immunized and three of four rgp120-immunized animals survived challenge.

The severity of disease symptoms was ranked by a clinical scoring system for the following: diarrhea, skin rash, behavioral depression, degree of dehydration, weakness, abdominal discomfort, and coat quality. All challenged animals developed symptoms consistent with SIV-PBj14 infection. The mean score for the naive controls was greater than that for either group of vaccinees (Fig. 5), including the sole survivor (53-437), although this was not assigned statistical significance. All signs of disease in all surviving macaques had resolved by day 17 p.c.

Hematologic responses. Declines in absolute numbers of circulating CD4 and CD8 lymphocytes occurred in all groups

FIG. 4. Survival of animals vaccinated with SFV-SIVgp160 (—) or rgp120 (---) and for unimmunized control animals (— · — · —) after challenge with SIV-PBj14.

FIG. 5. Clinical scores associated with SIV-PBj14-induced disease for animals vaccinated with SFV-SIVgp160 ($\square$) or rgp120 ($\diamond$) and for unvaccinated control animals ($\circ$). Each point represents the mean of combined scores for all surviving animals in each group.

during the acute SIV-PBj14 infection. Recovery to normal cell numbers correlated with resolution of symptoms, by 17 days p.c. (Fig. 6A and B). Similar declines were also seen in total leukocyte counts, neutrophils, B cells, erythrocytes, and hemoglobin concentration (data not shown). Conversely, monocyte numbers increased, reaching a peak at 13 to 17 days before returning to normal values by 24 days p.c. (Fig. 6C). None of the above parameters differed significantly among the challenged vaccinated and control groups.

Virus burden. (i) Plasma. Virus titers in plasma in the two control macaques surviving at 13 days p.c. (53-458 and 53-437) were 10,000 TCID₅₀/ml; this was ≥ 10 -fold higher than those in vaccinees (Fig. 7). Terminal virus titers were not determined for the control animals that died on day 12 p.c., 53-456 and 53-442. These results suggest a direct association between virus load in plasma and severity of disease. However, animal 22-144 died early in the acute phase of infection, at day 8 p.c., with a relatively low virus titer in plasma of 1,000 TCID₅₀/ml. Thus, other factors in addition to virus load in plasma may play a role in lethality, including individual animal susceptibility to SIV-PBj14 infection.

(ii) PBMC. The frequency of PBMC-associated virus was highest in two animals vaccinated with rgp120 (22-144 and 53-426) (Fig. 8). One of these animals (22-144) died at 8 days p.c., whereas the other macaque (53-426) survived the acute phase of infection. Overall, the mean PBMC proviral burden determined by quantitative PCR did not differ between vaccinated and control animals (Fig. 9). The single highest burden, 98 copies per 10,000 cells, was detected in control animal 53-458 at 10 days postinoculation, although at the time this animal died (13 days p.c.), the proviral burden had declined to 47 copies. Taken together, the PBMC virus isolation and PCR results demonstrated little or no association between cell-associated virus titers and prognosis regarding survival from acute SIV-PBj14 virus infection.

DISCUSSION

The SIV-PBj14 challenge model represents a rapid and rigorous system for evaluating vaccine efficacy. Here we show that recombinant Env vaccines can protect against lethal SIV-PBj14-bcl3 challenge but not against virus infection. Suppression of cell-free but not cell-associated virus in the blood of vaccinated macaques was associated with survival past the critical acute phase of SIV-PBj14 infection. These results confirm

FIG. 6. Hematologic data for animals vaccinated with SFV-SIVgp160 ($\diamond$) or rgp120 ($\circ$) and for unvaccinated control animals ($\square$). (A) Mean CD4 cell numbers; (B) mean CD8 cell numbers; (C) mean monocyte numbers.

previous findings demonstrating that Env subunit (2, 22) or whole inactivated (18) SIV vaccines can suppress virus replication and prolong survival in SIV-infected macaques.

SFV, an alphavirus with a single-stranded positive-sense RNA genome, is an excellent candidate as an expression vector (4, 5, 26, 27, 31, 40, 41). It replicates efficiently in a wide range of eukaryotic cells and shuts off host protein synthesis to redirect cell metabolism to expression of virus-encoded genes. The efficacy of the SFV system in inducing immune responses to foreign antigens has been previously tested by infection of mice with a recombinant SFV expressing the nucleoprotein (NP) of influenza virus. Both humoral immune responses and anti-NP specific major histocompatibility complex class I-restricted CD8⁺ cytotoxic T lymphocytes were detected (40, 41). We have shown that the SIV envelope glycoprotein produced by the SFV expression system is matured and processed in the native form as shown by its molecular mass, its recognition by

FIG. 7. Plasma viremia, measured by coculture assay, following challenge with SIV-PBj14 for unvaccinated controls (A), SFV-SIVgp160 vaccinees (B), and rgp120 vaccinees (C). Solid symbols represent animals that died during the acute infection.

FIG. 8. PBMC-associated virus titers, measured by coculture assay, following challenge with SIV-PBj14 for unvaccinated controls (A), SFV-SIVgp160 vaccinees (B), and rgp120 vaccinees (C). Solid symbols represent animals that died during the acute infection.

SIV Env-specific immunological reagents, and the fact that it is cleaved appropriately into two subunits.

Measurement of SIV neutralizing antibodies and helper T-cell proliferative responses in the macaques failed to identify an immune correlate for the protection from fatal disease. Other SIV vaccine studies have also failed to demonstrate prechallenge neutralizing activity in macaque sera but have induced protective immunity (2, 29). These results may reflect the inadequacy of *in vitro* assays in measuring neutralizing activity that is relevant *in vivo*. CD8⁺ cytotoxic T-lymphocyte activity has been shown to be induced by a recombinant SFV vector expressing HIV-1 Env (4), by other live vector vaccines (25, 35), and by subunit vaccines formulated in SBN-1 adjuvant. However, the correlation between cytotoxic T-lymphocyte induction and protective immunity to challenge also remains ambiguous (39). In the present study, SIV Env-specific antibody titers were the only indication of immune status in the

vaccinated macaques. That anti-Env titers had declined from peak levels by the time of challenge raises the possibility that a greater degree of protection against clinical disease would have been observed if the animals were challenged earlier in the course of immunization.

The results presented here suggest that the SFV expression vector is a safe and promising system which warrants further experimentation. A more robust immune response may be elicited by combining SFV priming with rgp120 boosting. It is possible that the ability of the SFV-SIVgp160 to boost the SIV-specific immune response was compromised by the induction of neutralizing activity against SFV. Direct inoculation of viral RNA, instead of particles, could circumvent problems associated with development of immunity to the vector (40). Moreover, the ability of SFV to replicate in epithelial cells suggests a potential for vaccination at mucosal surfaces.

Should complete eradication of infecting virus from an im-

FIG. 9. Provirus copy numbers in PBMC measured by quantitative PCR assay following challenge with SIV-PBj14. The copy number is plotted for unvaccinated controls (A), SFV-SIVgp160 vaccinees (B), and rgp120 vaccinees (C). Solid symbols represent animals that died during the acute infection.

munized individual (sterilizing immunity) prove an unrealistic prospect for lentivirus infections, a vaccine that affects disease may be a feasible alternative for preventing or delaying AIDS in HIV-infected humans. The two recombinant envelope vaccine strategies presented here are promising in that they elicited significant, albeit partial, protection in a rapidly lethal SIV challenge system.

ACKNOWLEDGMENTS

This study was supported by grant UO1 AI27136 from DAIDS, NIAID, NIH, USPHS, and by the Swedish Medical Research Council, Swedish Research Council for Engineering Sciences and the EVA Programme.

REFERENCES

- Abimiku, A. G., G. Franchini, J. Tartaglia, K. Aldrich, M. Myagkikh, P. D. Markham, P. Chong, M. Klein, M.-P. Kieny, R. C. Gallo, and M. Robert-Guroff. 1995. HIV-1 recombinant poxvirus vaccine induces cross-protection against HIV-2 challenge in rhesus macaques. *Nat. Med.* 1:321-329.
- Ahmad, S., B. Lohman, M. Marthas, L. Giavedoni, Z. El-Amad, N. L. Haigwood, C. J. Scandella, M. B. Gardner, P. A. Luciw, and T. Yilma. 1994. Reduced virus load in rhesus macaques immunized with recombinant gp160 and challenged with simian immunodeficiency virus. *AIDS Res. Hum. Retroviruses* 10:195-204.
- Baba, T. W., Y. S. Jeong, D. Penninck, R. Bronson, M. F. Greene, and R. M. Ruprecht. Pathogenicity of live attenuated SIV after mucosal infection of neonatal macaques. *Science*, in press.
- Berglund, P., M. Quesada-Rolander, R. Thorstensson, P. Putkonen, G. Bihelfeld, and P. Liljestrom. 1995. Unpublished data.
- Berglund, P., M. Sjoberg, H. Garoff, G. J. Atkins, B. J. Sheahan, and P. Liljestrom. 1993. Semliki Forest virus expression system: production of conditionally infectious recombinant particles. *BioTechnology* 11:916-920.
- Berman, P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershsberg, E. K. Cobb, and J. W. Eichberg. 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature (London)* 345:622-625.
- Cohen, J. 1993. A new goal: preventing disease, not infection. *Science* 262:1820-1821.
- Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 258:1938-1941.
- Daniel, M. D., G. P. Mazzara, M. A. Simon, P. K. Sehgal, T. Kodama, D. L. Panicali, and R. C. Desrosiers. 1994. High-titer immune responses elicited by recombinant vaccinia virus particle boosting are ineffective in preventing virulent SIV infection. *AIDS Res. Hum. Retroviruses* 10:839-851.
- Deen, K. C., J. S. McDougal, R. Inacker, G. Folena-Wasserman, J. Arthos, J. Rosenberg, P. J. Maddon, R. Axel, and R. W. Sweet. 1988. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. *Nature (London)* 331:82-84.
- Dewhurst, S., J. E. Embretson, D. C. Anderson, J. I. Mullins, and P. N. Fultz. 1990. Sequence analysis and acute pathogenicity of molecularly cloned SIVsmm-PBj14. *Nature (London)* 345:636-640.
- Emini, E. A., W. A. Schleif, J. H. Nunberg, A. J. Conley, Y. Eda, S. Tokiyoshi, S. D. Putney, S. Matsushita, K. E. Cobb, C. M. Jett, J. W. Eichberg, and K. K. Murthy. 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature (London)* 355:728-730.
- Federal Register. 1993. Addition of Appendix D-LX to the NIH Guidelines Regarding Semliki Forest Virus. *Hum. Gene Ther.* 5:401.
- Fultz, P. N., H. M. McClure, D. C. Anderson, and W. M. Switzer. 1989. Identification and biologic characterization of an acutely lethal variant of simian immunodeficiency virus from sooty mangabeys (SIV/SMM). *AIDS Res. Hum. Retroviruses* 5:397-409.
- Fultz, P. N., and P. M. Zack. 1994. Unique lentivirus-host interactions: SIVsmmPBj14 infection of macaques. *Virus Res.* 32:205-225.
- Gilljam, G. 1993. Envelope glycoproteins of HIV-1, HIV-2, and SIV purified with Galanthus nivalis agglutinin induce strong immune responses. *AIDS Res. Hum. Retroviruses* 9:431-438.
- Hart, M. K., T. J. Palker, T. J. Matthews, A. J. Langlois, N. W. Lerche, M. E. Martin, R. M. Searce, C. McDaniel, D. P. Bolognesi, and B. F. Haynes. 1990. Synthetic peptides containing T and B cell epitopes from human immunodeficiency virus envelope gp120 induce anti-HIV proliferative responses and high titers of neutralizing antibodies in rhesus monkeys. *J. Immunol.* 145:2677-2685.
- Hirsch, V. M., S. Goldstein, N. A. Hynes, W. R. Elkins, W. T. London, P. M. Zack, D. Montefiori, and P. R. Johnson. 1994. Prolonged clinical latency and survival of macaques given a whole inactivated simian immunodeficiency virus vaccine. *J. Infect. Dis.* 170:51-59.
- Hu, S.-L., K. Abrams, G. N. Barber, P. Moran, J. M. Zarlino, A. J. Langlois, L. Kuller, W. R. Morton, and R. E. Benveniste. 1992. Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. *Science* 255:456-459.
- Hu, S.-L., K. Abrams, L. Misher, V. Stallard, P. Moran, J. M. Zarlino, A. J. Langlois, L. Kuller, W. R. Morton, and R. E. Benveniste. 1992. Evaluation of protective efficacy of recombinant subunit vaccines against simian immunodeficiency virus infection of macaques. *J. Med. Primatol.* 21:119-125.
- Israel, Z. R., G. A. Dean, D. H. Maul, S. P. O'Neil, M. J. Dreitz, J. I. Mullins, P. N. Fultz, and E. A. Hoover. 1993. Early pathogenesis of disease caused by SIVsmmPBj14 molecular clone 1.9 in macaques. *AIDS Res. Hum. Retroviruses* 9:277-285.
- Israel, Z. R., P. F. Edmonson, D. H. Maul, S. P. O'Neil, S. P. Mossman, C. Thiriart, L. Fabry, O. Van Opstal, C. Bruck, F. Bex, A. Burny, P. N. Fultz, J. I. Mullins, and E. A. Hoover. 1994. Incomplete protection, but suppression of virus burden, elicited by subunit simian immunodeficiency virus vaccines. *J. Virol.* 68:1843-1853.
- Johnston, M. I., J. S. Noe, and J. Y. Killen. 1994. Recent advances in AIDS

- vaccine research and development. *AIDS Res. Hum. Retroviruses* **10**:S317–S323.
24. Lewis, M. G., W. R. Elkins, F. E. McCutchan, R. E. Benveniste, C. Y. Lai, D. C. Montefiori, D. S. Burke, G. A. Eddy, and A. Shaffer. 1993. Passively transferred antibodies directed against conserved regions of SIV envelope protect macaques from SIV infection. *Vaccine* **11**:1347–1355.
  25. Li, S., V. Polonis, H. Isobe, H. Zaghouni, R. Guinea, T. Moran, C. Bona, and P. Palese. 1993. Chimeric influenza virus induces neutralizing antibodies and cytotoxic T cells against human immunodeficiency virus type 1. *J. Virol.* **67**:6659–6666.
  26. Liljeström, P. 1994. Alphavirus expression systems. *Curr. Opin. Biotechnol.* **5**:495–500.
  27. Liljeström, P., and H. Garoff. 1991. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Bio/Technology* **9**:1356–1361.
  28. Marthas, M. L., S. Sujipto, J. Higgins, B. Lohman, J. Torten, P. A. Luciw, P. A. Marx, and N. C. Pedersen. 1990. Immunization with a live, attenuated simian immunodeficiency virus (SIV) prevents early disease but not infection in rhesus macaques challenged with pathogenic SIV. *J. Virol.* **64**:3694–3700.
  29. Murphy-Corb, M., L. N. Martin, B. Davison-Fairburn, R. C. Montelaro, M. Miller, M. West, S. Ohkawa, G. B. Baskin, J.-Y. Zhang, S. D. Putney, A. C. Allison, and D. A. Eppstein. 1989. A formalin-inactivated whole SIV vaccine confers protection in macaques. *Science* **246**:1293–1297.
  30. Murphy-Corb, M., R. C. Montelaro, M. A. Miller, M. West, L. N. Martin, B. Davison-Fairburn, S. Ohkawa, G. B. Baskin, J.-Y. Zhang, S. D. Putney, A. C. Allison, and D. A. Eppstein. 1991. Efficacy of SIV/DeltaB670 glycoprotein-enriched and glycoprotein-depleted subunit vaccines in protecting against infection and disease in rhesus monkeys. *AIDS* **5**:655–662.
  31. Paul, N. L., M. Marsh, J. A. McKeating, T. F. Schulz, P. Liljeström, H. Garoff, and R. A. Weiss. 1993. Expression of HIV-1 envelope glycoprotein by Semliki Forest virus vectors. *AIDS Res. Hum. Retroviruses* **9**:963–970.
  32. Planelles, V., N. L. Haigwood, M. L. Marthas, K. A. Mann, C. Scandella, W. D. Lidster, J. R. Schuster, R. Van Kuyk, P. A. Marx, M. B. Gardner, et al. 1991. Functional and immunological characterization of SIV envelope protein produced in genetically engineered mammalian cells. *AIDS Res. Hum. Retroviruses* **7**:889–898.
  33. Shaffer, A., P. B. Jahrling, R. E. Benveniste, M. G. Lewis, T. J. Phipps, F. Eden-McCutchan, J. Sadoff, G. A. Eddy, and D. S. Burke. 1991. Protection of macaques with a simian immunodeficiency virus envelope peptide based on conserved human immunodeficiency virus type 1 sequences. *Proc. Natl. Acad. Sci. USA* **88**:7126–7130.
  34. Shaffer, A., M. G. Lewis, F. E. McCutchan, R. E. Benveniste, P. B. Jahrling, R. L. Hickman, C. Y. Lai, D. S. Burke, and G. A. Eddy. 1992. Vaccination of macaques with SIV conserved peptides suppressed infection and prevented disease progression and transmission. *AIDS Res. Hum. Retroviruses* **8**:1483–1487.
  35. Shen, L., Z. W. Chen, M. D. Miller, V. Stallard, G. P. Mazzara, D. L. Panicali, and N. L. Letvin. 1991. Recombinant virus vaccine-induced SIV-specific CD8⁺ cytotoxic T lymphocytes. *Science* **252**:440–443.
  36. Urlaub, G., and L. A. Chasin. 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc. Natl. Acad. Sci. USA* **77**:4216–4220.
  37. Vaslin, B., R. Le Grand, G. Vogt, O. Benveniste, G. Gras, P. Roques, P. Stoessel, P. L. Salk, J. Salk, and D. Dormont. 1994. Induction of humoral and cellular immunity to simian immunodeficiency virus: what are the requirements for protection? *Vaccine* **12**:1132–1140.
  38. Wigler, M., M. Perucho, D. Kurtz, A. Pellicer, R. Axel, and S. Siverstein. 1980. Transformation of mammalian cells with an amplifiable dominant-acting gene. *Proc. Natl. Acad. Sci. USA* **77**:3567–3570.
  - 38a. Wissink, J., and J. I. Mullins. Unpublished data.
  39. Yasutomi, Y., S. Koenig, R. M. Woods, J. Madsen, N. M. Wassef, C. R. Alving, H. J. Klein, T. E. Nolan, L. J. Boots, J. A. Kessler, E. A. Emini, A. J. Conley, and N. L. Letvin. 1995. A vaccine-elicited, single viral epitope-specific cytotoxic T-lymphocyte response does not protect against intravenous, cell-free simian immunodeficiency virus challenge. *J. Virol.* **69**:2279–2284.
  40. Zhou, X., P. Berglund, G. Rhodes, S. E. Parker, M. Jondal, and P. Liljeström. 1994. Self-replicating Semliki Forest virus RNA as recombinant vaccine. *Vaccine* **12**:1510–1514.
  41. Zhou, X., P. Berglund, H. Zhao, P. Liljeström, and M. Jondal. 1995. Generation of cytotoxic and humoral immune responses using non-replicative recombinant Semliki Forest virus. *Proc. Natl. Acad. Sci. USA* **92**:3009–3013.