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Comparison of Whole Gene and Whole Virus Scrambled Antigen Approaches for DNA Prime and Fowlpox Virus Boost HIV Type 1 Vaccine Regimens in Macaques

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ABSTRACT

T cell immunity plays a critical role in controlling HIV-1 viremia, and encoding a limited set of HIV-1 genes within DNA and poxvirus vectors can, when used sequentially, induce high levels of T cell immunity in primates. However, a limited breadth of T cell immunity exposes the host to potential infection with either genetically diverse HIV-1 strains or T cell escape variants of HIV-1. In an attempt to induce maximally broad immunity, we examined DNA and recombinant fowlpox virus (rFPV) vaccines encoding all HIV-1 genes derived from a global HIV-1 consensus sequence, but expressed as multiple overlapping scrambled 30-amino acid segments (scrambled antigen vaccines, or SAVINEs). Three groups of seven pigtail macaques were immunized with sets of DNA and rFPV expressing Gag/Pol antigens only, the whole genome SAVINE antigens, or no HIV-1 antigens and T cell immunity was monitored by ELISpot and intracellular cytokine staining. High levels of cross-subtype HIV-specific T cell immunity to Gag were consistently induced in the seven macaques primed with DNA and rFPV vaccines expressing Gag/Pol as intact proteins. It was, however, difficult to repeatedly boost immunity with further rFPV immunizations, presumably reflecting high levels of anti-FPV immunity. Unfortunately, this vaccine study did not consistently achieve a broadened level of T cell immunity to multiple HIV genes utilizing the novel whole-virus SAVINE approach, with only one of seven immunized animals generating broad T cell immunity to multiple HIV-1 proteins. Further refinements are planned with alternative vector strategies to evaluate the potential of the SAVINE technology.

INTRODUCTION

HIV IS A FORMIDABLE PATHOGEN and a great challenge to vaccine development. Although tremendous successes have been achieved in the development of antiretroviral drug therapy, the progress of vaccine development has been slow. These difficulties are in part the nature of HIV replication, which can evade host immune responses, both T cell and humoral,[1–4] as a result of its high replication rate, frequent recombination events, and low fidelity of replication.[5,6]

Although desirable, preventive vaccines based on the generation of neutralizing antibodies are still in the early stages of development. The most advanced current HIV vaccine efforts are directed toward the generation of effective $CD8^+$ cytotoxic T lymphocyte (CTL) responses that aim to control viral replication and prevent the onset of disease that is associated with progressive $CD4^+$ T cell depletion.[7] The generation of HIV-specific $CD4^+$ T cell immunity, often lacking in HIV-infected individuals, is also highly desirable.[8,9] Recombinant viral carrier vaccine vectors, such as vaccinia, avian poxviruses (e.g., canarypox and fowlpox viruses [rFPV]), and adenoviruses, either alone or in combination with DNA vaccination, have been most effective in inducing T cell immunity.[10–14] Although live, attenuated SIV vaccines are highly effective in macaques,[15] and

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induce broadly targeted $CD4^+$ and $CD8^+$ T cell responses and neutralizing antibodies,[16] such vaccines pose an unacceptable safety risk.[17,18]

Vaccines that promote cellular immunity, in particular CTLs, must generally express HIV proteins (from HIV-1 genes) intracellularly for presentation by MHC class I. Expression of all native viral genes within cells in their native form poses an unacceptable safety risk and most viral vector vaccines express a single or limited set of HIV genes, generally reflecting only a modest portion of all available viral antigenic material.[10,11,14,19] This focuses the T cell response to a limited set of viral antigens, restricting the impact that the immune responses can have on viral replication, particularly on genetically divergent HIV-1 strains. A limited breadth of T cell immunity may promote the eventual emergence of vaccine escape mutants.[20–22] Ideally, CTL-inducing vaccines would induce strong responses across a multitude of epitopes presented by an individual's MHC haplotype in order to have maximum impact on viral replication in the first few weeks of infection and limit CTL escape.

The *s*crambled *a*ntigen *vacc*ine (SAVINE) concept achieves this objective in principle by expressing all 9 HIV-1 proteins as a reassortment of 189 thirty-amino acid segments. These multiple 30-amino acid segments making up the SAVINE open reading frames are long enough to encode all HIV-1 $CD4^+$ and $CD8^+$ epitopes yet short enough to ensure that few if any functional viral components are expressed. The SAVINE concept utilizes segments of HIV-1 encoding 30 amino acids with 15-amino acid overlap between adjacent segments randomly scrambled to express three large (~5700 bp), nonfunctional polypeptides [S. Thomson *et al.* (submitted for publication)] that were inserted into DNA and rFPV vectors. Thus, the SAVINE concept potentially offers many of the advantages afforded by live, attenuated vaccines (expressing virtually all HIV-1 or SIV proteins for T cell recognition) without the inherent safety risk.

Another aspect of HIV vaccines is their limited global reach. Because of the enormous sequence diversity of HIV-1, vaccines usually encode a representative subtype sequence, preferably a strain with relatively high identity with current circulating strains of at least one particular subtype.[20,23] However, the cross-recognition of vaccine-induced immunity may be limited across divergent HIV-1 subtypes. Because the SAVINE vaccine is designed synthetically, a global consensus sequence,[23] formulated by a hierarchical system that is based on the global prevalence of HIV subtypes, can be used.

The immunogenicity of SAVINE DNA and rFPV combination vaccines has been demonstrated in murine studies.[22a] In this report, the immunogenicity of whole-HIV-1 SAVINE DNA and rFPV vaccines was tested for the first time in a nonhuman primate model and compared with a more conventional HIV DNA prime–rFPV vaccine combination that expressed only Gag and Pol proteins.

MATERIALS AND METHODS

Vaccines

The SAVINE vaccines are described in greater detail elsewhere.[22a] The starting HIV-1 sequence used for the SAVINE vaccine was a consensus sequence designed on the basis of a biased ranking of subtype consensus sequences (A/E > C > B > D > others).[23,24] Subtype E and subtype A differ mainly in their envelope sequences and so were considered virtually equal in the ranking. Two isolate sequences were utilized, GenBank accession U51189 and U46016, respectively, where consensus sequences were unavailable for some E and C subtype HIV proteins. The hypervariable regions of the HIV envelope protein were also excluded from the vaccine design because of extreme sequence variability. A number of degeneracies were also incorporated into the consensus sequence to cater to heterologous HIV viruses according to the following: (1) subtype ranking, (2) no more than eight combinations possible in any nine-amino acid stretch, and (3) their amino acid similarity. The specific sequences used have been submitted to GenBank (accession numbers AY787839–AY787841).

Three HIV-1 SAVINE open reading frames (ORFs) were constructed synthetically in a stepwise fashion. Twenty-two subcassettes were constructed using 189 purified 100-mer oligonucleotides covering all HIV-1 ORFs (Invitrogen, Carlsbad, CA). Construction of each subcassette used multiple steps of asymmetric polymerase chain reaction (PCR) extension of each 90-bp (30-amino acid) segment to progressively add on the long oligonucleotides.[25] ORF1 and ORF2 sequences were each separated into seven subcassettes of approximately 840 bp each and ORF3 was separated into eight subcassettes, the last subcassette being approximately 340 bp. Each of the 22 subcassettes was constructed using in most cases 10 polyacrylamide gel (6% urea–TBE [Tris–borate–EDTA])-purified 100-mer oligonucleotides and 2 short 20-mer oligonucleotides (Invitrogen). Construction of each subcassette was begun at both ends simultaneously, using multiple steps of asymmetric PCR extension to progressively add on the long oligonucleotides, and standard PCR reagents and conditions. The two halves of each subcassette were then joined using standard splicing by overlap extension, using both short primers and standard PCR reagents and conditions. Each subcassette fragment was digested with *Bam*HI and *Eco*RI restriction enzymes and ligated into the general cloning plasmid pBluescript II KS^- cut with the same enzymes (Stratagene, La Jolla, CA) and checked by sequencing. Because of the degenerate design of the HIV-1 SAVINE we selected at least two clones for each subcassette in order to subsequently make a second version of the HIV-1 SAVINE. The appropriate subcassettes were joined again in multiple steps of restriction enzyme digestion, ligation, and PCR amplification, using Elongase (Invitrogen). To facilitate subcassette joining each subcassette had one of the following cohesive restriction enzymes sites at each end, *Spe*I, *Avr*II, *Nhe*I, or *Xba*I, in such an arrangement that when joined to another subcassette alternative sites remained at the ends to allow for the next joining step. For example, the subcassettes in ORF1 (SC1–SC7) were flanked with the following restriction enzyme sites: -SC1-*Spe*I, *Xba*I-SC2-*Nhe*I, *Spe*I-SC3-*Avr*II, *Nhe*I-SC4-*Xba*I, *Spe*I-SC5-*Avr*II, *Nhe*I-SC6-*Xba*I, *Nhe*I-SC7, respectively. Once each full-length ORF fragment was obtained it was digested with restriction enzymes *Bam*HI and *Eco*RI and ligated into pBluescript II KS^- cut with the same enzymes (Stratagene) and checked again by sequencing. Each ORF was then subcloned into the plasmids pHIS-64 (using enzymes *Xba*I and *Xho*I or *Xba*I and *Sal*I where appropriate) and pAF09[26] (using

enzymes *Bam*HI and *Sal*II). The DNA vaccine plasmid pHIS-64 (H. Davis, Coley Pharmaceutical Canada, Ottawa, ON, Canada) carries kanamycin selection, a cytomegalovirus (CMV) promoter, the bovine growth hormone poly(A) signal and 14 primate-optimized CpG immunostimulatory sequences.[27] The pAF09-derived plasmids were used to generate rFPV by marker rescue recombination as described previously.[26] The ability of each final synthetic SAVINE ORF to be expressed was demonstrated by subcloning each separately in frame with the enhanced green fluorescent protein (EGFP). When each SAVINE-EGFP plasmid was transfected into 293 cells fluorescence was detected in each case (data not shown; and Thomson *et al.*[22a]).

The B-subtype DNA (pHIS-HIV-B) and rFPV (rFPV-HIV-B) vaccines are described elsewhere.[27] Briefly, the DNA vaccine (pHIS-HIV-B) contained approximately 65% of the B subtype pNL(AD8) provirus, with sequence expressing modified Gag, modified RT, protease, Rev, Tat, Vpu, truncated Nef (expressing the first 31 amino acids only), and truncated Env (expressing the first 275 amino acids only). This modified HIV-1 genome was inserted into the plasmid DNA vaccine vector pHIS-64. rFPV-HIV-B expressed only the Gag and Pol (RT protease) regions of pNL(AD8).[27]

Animals

Twenty-one pigtail macaques (*Macaca nemestrina*) were obtained and held at the simian retrovirus (SRV)-free captive breeding facilities of the Primate Research Center at Bogor Agricultural University (Bogor, Indonesia). Offspring from this breeding colony were rescreened for the presence of SRV by PCR enzyme immunoassay (EIA), and Western blot assay. Only confirmed-negative animals were used in the study. Animals were randomly assigned into three groups of seven animals. The macaques received three DNA vaccinations (SAVINE vaccine for group A, and B-subtype DNA vaccine for group B) at weeks 0, 4, and 8, and were boosted with rFPV-SAVINE vaccine (group A) or the rFPV-HIV-B vaccine (group B) twice at weeks 12 and 16. Group C received the empty DNA and FPV vectors at the same time points. At the completion of the standard vaccination protocol, a cross-over design was studied whereby the SAVINE-immunized animals received the rFPV-HIV-B standard vaccine and the B subtype-immunized animals received the rFPV-SAVINE at week 20 (Table 1). All experiments were performed at the Primate Research Center in Bogor in accordance with the *Guide for the Care and Use of Laboratory Animals* issued by the Institute of Laboratory Animal Resources (ILAR), and were approved by the Animal Care and Use Committee of the Primate Research Center, Bogor Agricultural University.

Antigens

Peptides used in the study for restimulation of peripheral blood mononuclear cells (PBMCs) from immunized animals were obtained through the NIH AIDS Research and Reference Reagent Program, (Division of AIDS, NIAID, NIH, Bethesda, MD) (15-mer sets of HIV subtype B consensus Gag, Pol, Rev, Tat, Vpu, Vif, Nef, Vpr, HIV-1 subtypes A and C consensus Gag, and HIV-1_{MN} Env). All peptides were dissolved in dimethyl sulfoxide (DMSO) at high concentration (>200 μg/ml per peptide) and used in culture at 1 μg/ml per peptide. Rev, Tat, and Vpu were combined into a single pool, as were Vif, Nef, and Vpr peptides. Aldrithiol-2-inactivated whole HIV-1_{MN} particles and equivalent amounts of control microvesicles were kindly provided by J. Lifson (AIDS Vaccine Program, National Cancer Institute, Bethesda, MD) and used at a final concentration of 5 μg/ml. Staphylococcal B enterotoxin (SEB; Sigma, St. Louis, MO) was used as a positive control superantigen at 10 μg/ml (intracellular cytokine staining), 10 mg/ml (ELISpot).

ELISpot assay for IFN-γ-producing lymphocytes

Fresh PBMCs from multiple time points were stimulated with sets of peptide pools of Gag, Pol, Env, Rev/Tat/Vpu, and Vif/Nef/Vpr as well as whole intact inactivated HIV-1 and SEB proteins as controls. The samples were assayed with a monkey interferon γ (IFN-γ) enzyme-linked immunospot (ELISpot) kit (U-CyTech, Utrecht, The Netherlands) according to the manufacturer's instruction manual and as described elsewhere.[27,28] Briefly, PBMCs were stimulated with pools of overlapping 15-mer HIV-1 peptides or whole inactivated HIV-1 for 18 hr, washed, and then transferred to anti-IFN-γ monoclonal antibody-coated plates and incubated for 5 hr. Cells were lysed and wells were incubated with biotinylated anti-IFN-γ polyclonal rabbit antibody for 1 hr, followed by incubation with a gold-labeled anti-biotin IgG antibody for 1 hr. IFN-γ spots were developed, shipped to Australia for counting on an automated reader (AID, Strassberg, Germany), and results were normalized to antigen-specific IFN-γ-secreting precursor frequency per 10^6 PBMCs.

TABLE 1. VACCINE REGIMEN

Vaccine regimen	*No. of animals/ group*	*Immunization time point (week)*					
		0	*4*	*8*	*12*	*16*	*20 (cross-over)*[a]
SAVINE	7	DNA (SAVINE)	DNA (SAVINE)	DNA (SAVINE)	FPV (SAVINE)	FPV (SAVINE)	FPV (HIV-B)
HIV-B	7	DNA (HIV-B)	DNA (HIV-B)	DNA (HIV-B)	FPV (HIV-B)	FPV (HIV-B)	FPV (SAVINE)
Control	7	Control[b]	Control	Control	Control	Control	Control

[a]SAVINE and HIV-B FPV vaccines were administered to HIV-B and SAVINE groups, respectively, at week 20.
[b]Control vaccines consisted of empty DNA and FPV vectors.

Intracellular cytokine staining

Induction of HIV-specific intracellular IFN-γ expression in $CD4^+$ and $CD8^+$ lymphocytes was assessed by flow cytometry as previously described.[27,28] Briefly, 200 μl of whole blood was incubated with peptide pools or control DMSO and the costimulatory antibodies anti-CD49d and CD28 (BD Biosciences Pharmingen, San Diego, CA) for 7 hr. Brefeldin A (10 μg/ml; Sigma) was included during the last 5 hr of incubation. Anti-CD4–PE and anti-CD8–PerCP (BD Biosciences Pharmingen) were added to each well and incubated for 30 min. Red blood cells were lysed (FACS lysing solution; BD Biosciences Pharmingen) washed with phosphate-buffered saline (PBS) and the remaining cells were permeabilized (Cytofix/Cytoperm; BD Biosciences Pharmingen). Permeabilized cells were then incubated with anti-IFN-γ-FITC antibody (Mabtech, Stockholm, Sweden) before paraformaldehyde fixation. Fixed cells were frozen at -70°C and shipped on dry ice to the University of Melbourne (Parkville, VIC, Australia) for acquisition (FACScan; BD Biosciences Immunocytometry Systems, San Jose, CA). The percentage of antigen-specific gated lymphocytes expressing IFN-γ was assessed for both $CD4^+$ and $CD8^+$ lymphocytes.

p24 Gag antibody EIA

p24 protein of HIV-1 virus was obtained from Protein Sciences (Meriden, CT). Briefly, 96-well Nunc immunoplates (NUNC; Nalge Nunc International, Roskilde, Denmark) were coated with 50 μl of p24 protein at 2 μg/ml in borate buffer (pH 9.6). Plasma samples from each animal from multiple time points were incubated in the wells for 1 hr at 37°C. Horseradish peroxidase-conjugated anti-monkey IgG (Sigma) was then incubated for 1 hr and 30 min at 37°C. Tetramethyl benzidine (TMB) substrate (Sigma) was added for 15 min at room temperature for color development. The reaction was stopped by

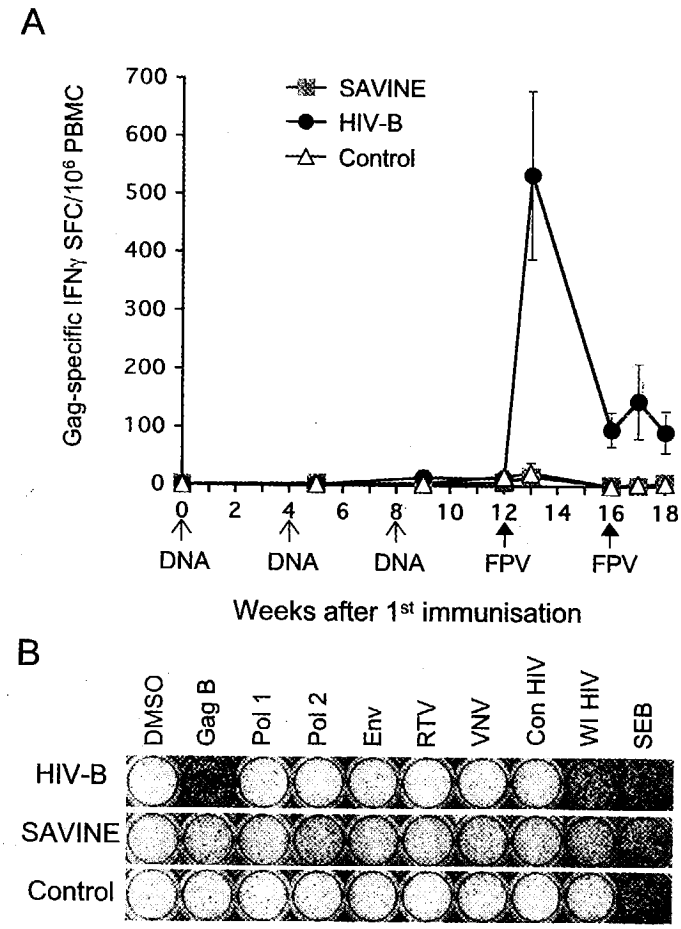

FIG. 1. Subtype B Gag-specific cellular immune response by IFN-γ ELISpot assay. (**A**) Time course of mean responses and SE in groups of seven macaques immunized with HIV-B DNA and rFPV vaccines, SAVINE DNA and rFPV vaccines, and control vaccines (empty DNA and FPV vectors). (**B**) Visual representation of responses to a panel of HIV-1 subtype B peptide antigens and whole, inactivated HIV-1 in the ELISpot assay at week 13, when responses peaked. Antigens studied were Gag B (consensus B HIV-1 Gag 15-mer peptide pool), Pol 1 and 2 (peptide pools comprising the first and second half of HIV-1 consensus B Pol), Env (Env MN peptide pool), RTV (a pool comprising consensus B Rev, Tat, and Vpu peptides), VNV (pooled consensus B Vif, Nef, and Vpr peptides), WI HIV (adrithiol-2-inactivated HIV particles and its control antigen, Con HIV), and the mitogen SEB (*Staphylococcus* enterotoxin B). SFC, spot-forming cells.

adding 2 M H_2SO_4 and optical density was measured at 450 nm with an enzyme-linked immunosorbent assay (ELISA) reader.

RESULTS

T cell immunity by IFN-γ ELISpot

To assess T cell immunity induced by the vaccines, HIV-specific IFN-γ-expressing T cells were quantitated at multiple time points by ELISpot in all 21 animals, using pools of 15-mer peptides spanning the Gag and Pol proteins from HIV-1 B consensus subtype, Env proteins from HIV-1_{MN}, Rev/Tat/Vpu and Vif/Nef/Vpr pooled peptides from HIV-1 consensus B subtype, and whole intact inactivated HIV-1 virions. The animals in the group receiving HIV-B subtype DNA and rFPV vaccines showed a dramatic elevation in the number of Gag and whole inactivated HIV-specific T cells at week 13, 1 week after the first rFPV boosting immunization (Fig. 1A and B). This specific T cell immunity, however, was directed only against Gag proteins of HIV-1, with no responses to Pol, Env, or regulatory proteins (Fig. 1B). This is consistent with only Gag and Pol being shared by the DNA and rFPV vaccines, and Pol being expressed at much lower levels than Gag.[27] There was no further elevation of IFN-γ-producing lymphocytes after the second boost of rFPV at week 16, likely reflecting anti-vector immunity to the FPV.

In general, there was no significant induction of T cell immunity among animals in the group receiving SAVINE DNA and rFPV vaccine regimen (Fig. 1A). One SAVINE-immunized animal (5721), however, showed low-level but broad induction of T cell immunity to Gag, the second half of Pol (Pol-2), and pooled Vif/Nef/Vpr proteins, as well as whole inactivated HIV-1, likely reflecting the recognition of Gag epitopes (Fig. 1B). No control animals had HIV-specific immunity present to HIV-1 peptides and whole inactivated HIV-1.

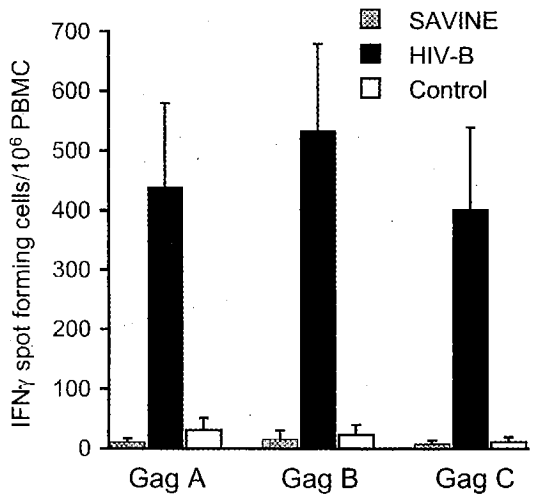

FIG. 2. Cross-subtype T cell responses. Vaccine-induced cross-subtype responses. Mean (+SE) group T cell responses by IFN-γ ELISpot to consensus Gag peptide pools from subtypes A, B, and C at the peak of the immune response at week 13.

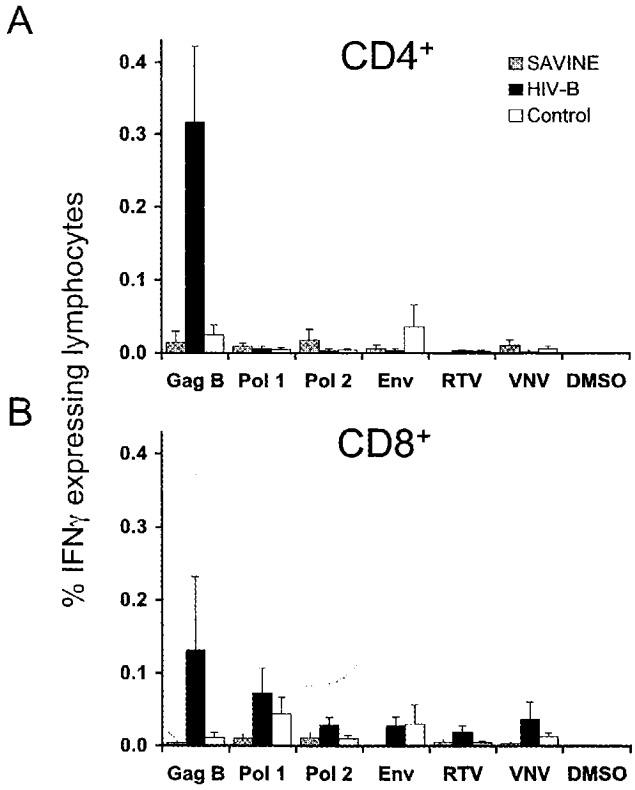

FIG. 3. HIV-specific $CD4^+$ and $CD8^+$ T cell immunity by intracellular cytokine staining (ICS). Flow cytometric analysis of whole blood intracellular IFN-γ staining 2 weeks after the first rFPV booster (week 14). Group mean and SE from seven macaques for (**A**) HIV-specific $CD4^+$ lymphocytes and (**B**) HIV-specific $CD8^+$ lymphocytes expressing intracellular IFN-γ in response to HIV peptide restimulation. Antigens: HIV-1 Consensus subtype B peptide pools for Gag (Gag B), first and second half of Pol (Pol 1 and Pol 2, respectively), Rev/Tat/Vpu (RTV), Vif/Nef/Vpr (VNV), and HIV-1 MN Env (Env).

Cross-subtype immune responses

To assess the capacity of the vaccines to induce immunity to various HIV-1 subtypes, PBMCs were evaluated for the specific production of IFN-γ by ELISpot to overlapping Gag peptides derived from consensus sequences of subtypes A, B, and C (Fig. 2). The HIV-B DNA and rFPV vaccine combination, expressing HIV-1 sequences from strain NL4.3 (a subtype B strain), induced strong responses to consensus Gag B peptide sequences as well as to consensus Gag A and C peptides in all animals. Subtype A Gag responses were 82% and subtype C Gag responses were 75% the magnitude of the response to the more closely related consensus B Gag pool. The potential for induction of cross-subtype immunity could not be examined for the SAVINE vaccines because the induced responses were low. The one SAVINE-immunized animal that did respond weakly to HIV-1 Gag (5721, shown in Fig. 1B), however, also had significant recognition of HIV-1 subtype B, A, and C Gag (data not shown).

$CD4^+$ and $CD8^+$ T cell immunity by intracellular cytokine staining

To confirm the ELISpot data on the T cell immunogenicity of the vaccines and to phenotype the responding T cells, intracellular cytokine staining was also performed at week 14, 2 weeks after the first rFPV boost. Previous observations showed that the maximal induction of T cell immunity occurs 1 to 3 weeks after the first booster vaccine.[28a] A mean of 0.3% of all $CD4^+$ lymphocytes and 0.1% of all $CD8^+$ lymphocytes were specific for HIV-1 Gag in animals that received the B-subtype vaccine regimen (Fig. 3A and B). These data correlate with the high level of Gag-specific T cell immunity detected by ELISpot. No significant $CD4^+$ or $CD8^+$ T cell responses were detected in the SAVINE-immunized or control animals. No significant responses to other HIV-1 proteins (Pol, Env, and pools of regulatory protein peptides) were detected in either $CD8^+$ or $CD4^+$ lymphocytes from any of the immunized animals (Fig. 3B).

HIV-1 Gag p24-specific antibody detection

Although most prime–boost vaccine regimens target the induction of T cell immunity, humoral immunity was also assessed by the detection of antibodies to p24 protein of HIV-1 Gag, the most immunogenic of the HIV proteins in the HIV-B-immunized animals. The antibody capture ELISA showed an increase in p24 antibodies in animals immunized with B-subtype vaccines, as shown by the optical density reading 2 weeks after the second boost of rFPV-HIV-B (Fig. 4). As expected, animals in the SAVINE and control groups did not show significant antibody responses. The priming immunization with the DNA vaccines expressing either SAVINE or HIV-B antigens did not induce humoral immunity in the animals.

Effect of rFPV vaccine on total lymphocyte numbers

After administration of the first rFPV vaccine at week 12, we detected a dramatic 4-fold increase in PBMC recovery 1 week later (Fig. 5). This effect was common to all three vaccine groups (receiving recombinant or nonrecombinant FPVs) and therefore not related to the vaccine inserts. This phenomenon was transient and numbers of PBMCs purified from blood returned to baseline levels 3 weeks later, before administration of a second rFPV booster vaccine. No such phenomenon was observed after each of the DNA vaccinations (data not shown). The sharp increase in PBMC yield coincided with the peak immune response (Fig. 1). However, a similar effect on PBMC numbers was not observed after a second rFPV booster vaccination, with only a small increase in cell number (Fig. 5). This was associated with a negligible boost in HIV-specific T cell immune response at the same time point (week 17; Fig. 1A).

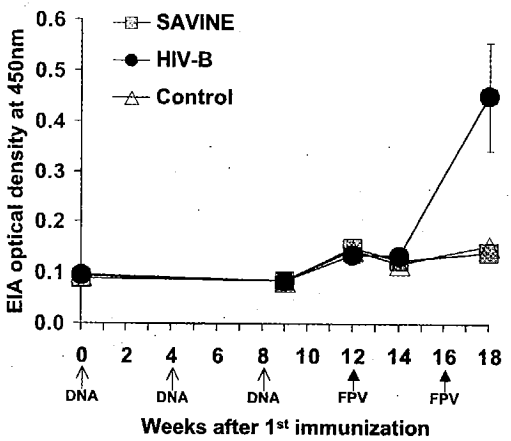

FIG. 4. p24 antibody responses. Plasma samples from five time points that cover the priming DNA and boosting rFPV immunizations were assayed for the presence of antibodies to p24 protein of Gag. Antibody responses were detected 2 weeks after the second rFPV booster in samples from the group receiving the B-subtype vaccine regimens, but not in animals from the group receiving SAVINE regimens.

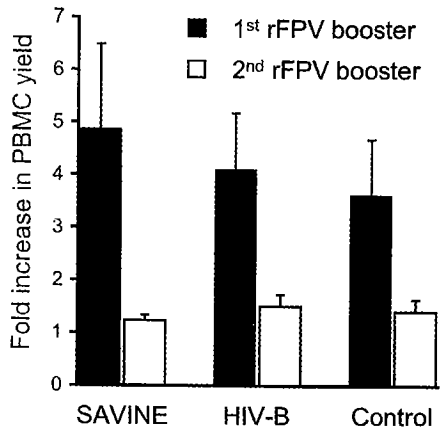

FIG. 5. FPV immunization on PBMC yield. Influence of rFPV vaccination on PBMCs recovered from 9 ml of heparinized blood. Results are expressed as the fold increase in PBMC number 1 week after vaccination over the number of PBMCs isolated at the time of vaccination.

Cross-over immunization

There was no significant HIV-specific immunity in the SAVINE-vaccinated animals after three DNA primings and two rFPV boostings. In an attempt to determine whether either the SAVINE DNA or SAVINE rFPV vaccine was primarily responsible, we performed a cross-over immunization. The SAVINE-vaccinated animals were vaccinated with rFPV-HIV-B (to determine whether they had been primed effectively by the SAVINE DNA) and the HIV-B DNA/rFPV-immunized animals were vaccinated with rFPV-SAVINE (to determine whether rFPV-SAVINE could boost preprimed HIV-specific cellular and humoral immunity). However, there was no induction of T cell or humoral immunity in the SAVINE-vaccinated animals 2 weeks after the rFPV-HIV-B boost, and no boosting of HIV-specific immunity in the HIV-B-immunized animals occurred after the rFPV-SAVINE boost (Fig. 6A and B).

DISCUSSION

This study characterized the safety and cellular and humoral immunogenicity of DNA and fowlpox virus HIV-1 prime–boost

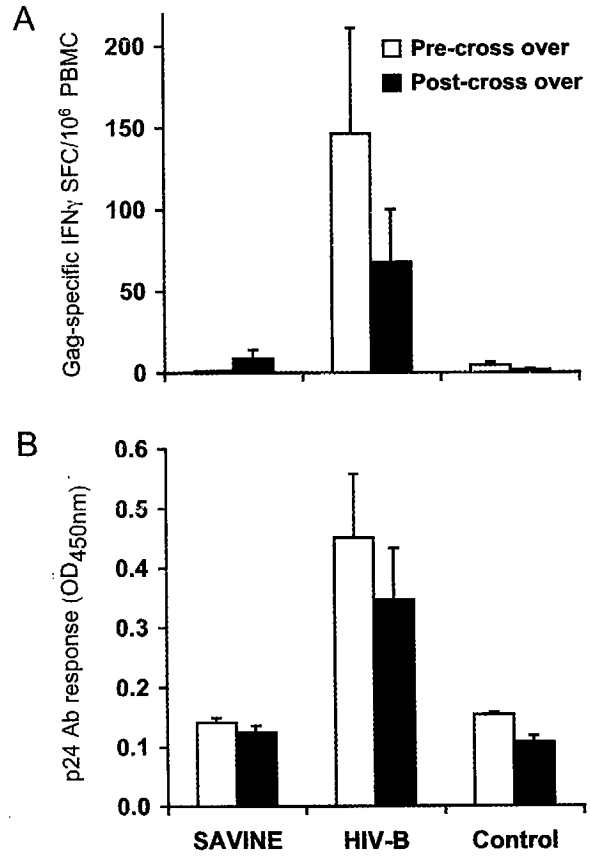

FIG. 6. Cross-over immunization. Cellular and humoral immune responses after cross-over immunization. The SAVINE- and HIV-B-immunized macaques were twice given a final immunization with rFPV-HIV-B and rFPV-HIV-SAVINE, respectively, at week 20 and (**A**) IFN-γ ELISpot responses to HIV-1 B subtype peptides and (**B**) p24 antibody responses were studied 2 weeks later. SFC, spot-forming cells.

regimens in macaques, using a standard whole gene vaccine approach, and a novel scrambled antigen vaccine approach with the potential to induce immune responses across all HIV-1 proteins. DNA and viral vector prime–boost vaccine regimens have shown great promise for the induction of high levels of T cell immunity, but have to date, for safety and technical reasons, primarily expressed a limited number of shared HIV or SIV proteins (up to three).[11,14,29]

The standard whole gene DNA/rFPV prime–boost approach (HIV-B) expressing shared Gag and Pol antigens induced high-level T cell immunity by ELISpot to Gag antigens. There was encouraging (75–82%) cross-reactivity to nonsubtype B HIV-1 strain A and C gag peptides, similar to previous studies in HIV-infected subjects.[30] Both $CD4^+$ and $CD8^+$ T cell immunity was detected to Gag by ICS in the standard HIV-B-immunized animals, with greater proportions of HIV-specific T cells in the $CD4^+$ lymphocyte compartment. Although both $CD4^+$ and $CD8^+$ T cell immunity to HIV is likely to be helpful in controlling HIV, the destruction of HIV-specific $CD4^+$ T cells in HIV-1 infection of humans is recognized as a critical defect and vaccine-induced $CD4^+$ T cell immunity may be crucial in generating effective immunity.[8,9,31] The T cell immunity induced was limited to Gag antigens, presumably reflecting the low levels of Pol antigens expressed, by an infrequent frameshift read-through in the Gag–Pol overlap, from these vaccines (our unpublished data). Additional studies with one or more rFPVs expressing multiple HIV-1 proteins are currently underway.

Anti-Gag antibodies were also readily detected after rFPV boosting in the HIV-B whole gene regimen, although, interestingly, this was primarily detected after the second (rather than the first) rFPV booster. The T cell immunity induced by the DNA/rFPV was, however, difficult to boost further by additional rFPV vaccinations, presumably reflecting immunity against the FPV vector. We have previously shown that strong antibody responses are induced to FPV after one or more rFPV immunizations.[27] Interestingly, we observed a marked enhancement of PBMC numbers (reflective of high lymphocyte counts) 1 week after the first rFPV boost, equal across all three groups, and presumably stimulated by the FPV vector. This immune-stimulating capacity may, as an adjuvant effect, have assisted in the production of high levels of HIV-specific immunity. We have subsequently confirmed this effect of increased total lymphocyte counts in other rFPV vaccine studies in macaques (our unpublished data). This boost in lymphocyte numbers was not observed after the second rFPV boost, again presumably reflecting significant antivector immunity. If continuous high levels of effector immunity are required for protection from HIV-1 in humans, the inability to repeatedly boost immune responses with rFPV vaccines may limit their long-term utility, as with other live vector approaches.[32]

Although safe in macaques, the novel SAVINE approach induced only low-level T cell immunity by ELISpot in only one of the seven immunized animals, although reactivity across multiple HIV-1 antigens was detected in this one responding animal. Our previous murine data had showed promising immunogenicity of SAVINE DNA and FPV regimens (our unpublished data) and it was not clear why these regimens failed to induce similar immunity in macaques, although translation of promising murine results to outbred nonhuman primates can be difficult.[28] In an effort to elucidate whether the DNA or rFPV-SAVINE vaccines were responsible for the lack of immunogenicity of this prime–boost combination, we performed a cross-over immunization experiment; however, this also did not result in boosted immunity. In retrospect, however, anti-FPV immunity is likely to have limited this response.

The limited response generated by the SAVINE vaccine compared with the whole gene approach may be linked to the key differences between these approaches. The whole gene vaccine used here expressed Gag and Pol proteins that are likely to be relatively stable for a period of time and produce pseudoviral particles that can escape the cell. The longer, unstructured synthetic scrambled SAVINE vaccine polypeptides are likely to be relatively short lived within the cells in which they are expressed. The poorer stability and increased containment at the site of immunization therefore may affect the SAVINE strategy to a greater extent than the whole gene vaccine strategy if a suboptimal vector is used. In support of this interpretation we have subsequently observed that priming mice with a recombinant vaccinia virus expressing HIV SAVINE inserts instead of a DNA vaccine and then boosting with rFPV significantly in-

creases the strength of the HIV T cell immunity (our unpublished data). Additional macaque experiments utilizing a vaccinia and FPV prime–boost regimen are now planned to confirm these data in nonhuman primates.

In summary, we demonstrated high levels of cross-subtype HIV-specific T cell immunity in macaques primed with whole gene Gag-expressing DNA and rFPV vaccines. It was, however, difficult to repeatedly boost immunity with further rFPV immunizations. Although this study did not consistently achieve a broadened level of T cell immunity to multiple HIV genes utilizing a novel whole virus scrambled antigen SAVINE approach, additional experiments are planned with alternative vector strategies to further evaluate the potential of this technology.

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