Systemic Immunization with an ALVAC-HIV-1/Protein Boost Vaccine Strategy Protects Rhesus Macaques from CD4+ T-Cell Loss and Reduces both Systemic and Mucosal Simian-Human Immunodeficiency Virus SHIV$_{KU2}$ RNA Levels

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Transmission of human immunodeficiency virus type 1 (HIV-1) occurs primarily via the mucosal route, suggesting that HIV-1 vaccines may need to elicit mucosal immune responses. Here, we investigated the immunogenicity and relative efficacy of systemic immunization with two human ALVAC-HIV-1 recombinant vaccines expressing Gag, Pol, and gp120 (vCP250) or Gag, Pol, and gp160 (vCP1420) in a prime-boost protocol with their homologous vaccine native Env proteins. The relative efficacy was measured against a high-dose mucosal exposure to the pathogenic neutralization-resistant variant SHIV$_{KU2}$ (simian-human immunodeficiency virus). Systemic immunization with both vaccine regimens decreased viral load levels not only in blood but unexpectedly also in mucosal sites and protected macaques from peripheral CD4+ T-cell loss. This protective effect was stronger when the gp120 antigen was included in the vaccine. Inclusion of recombinant Tat protein in the boosting phase along with the Env protein did not contribute further to the preservation of CD4+ T cells. Thus, systemic immunization with ALVAC-HIV-1 vaccine candidates elicits anti-HIV-1 immune responses able to contain virus replication also at mucosal sites in macaques.

Due to the alarming spread of human immunodeficiency virus type 1 (HIV-1) infection worldwide, the development of a prophylactic vaccine is critical. Vaccine strategies for AIDS have included the use of structural and nonstructural HIV subunit proteins or peptides, naked DNA, bacterial and viral live vectors, or combinations of the above. Most of the vaccines developed thus far induce T-cell responses but are unable to induce neutralizing antibodies to the Env of primary HIV-1 isolates. The fact that a decrease in plasma viremia (9, 41) is concomitant with the appearance of virus-specific cytotoxic T lymphocytes (CTLs) and depletion of CD8+ T cells in simian immunodeficiency virus (SIV)-infected macaques or HIV-infected chimpanzees suggests that CD8+ T cells are able to partially control HIV-1/SIV replication (15, 36, 43, 52). Indeed, several "T-cell vaccines" based on DNA and live vectors confer protection from high-level replication of challenge viruses in rhesus macaques (2, 4, 8, 30, 31, 48). The extent of the decrease in viral replication induced by these vaccines is variable in nonhuman primates, and protection from disease appears to be dependent on the virulence of the virus used in the challenge experiments (28).

The relative efficacy of poxvirus-based vaccine candidates with various degrees of attenuation has been extensively studied in rhesus macaques after challenge with SIV, SHIV, and HIV-2 isolates (1, 3, 8, 24, 25, 29–31, 48). These vaccine modalities elicit variable levels of cell-mediated immune response and prevent infection after challenge exposure to viruses with low virulence, such as HIV-2 (3, 25), and to other somewhat-attenuated SIV isolates (34, 35). Importantly, these vaccine modalities were also able to significantly reduce virus load after challenge with highly pathogenic SIV isolates (8, 29, 32,

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TABLE 1. Nomenclature of ALVAC recombinant vaccines used for immunization

Immunogen	Vaccine
ALVAC-SIV$_{mac251}$-gag-pol	vCP172
ALVAC-HIV-1$_{IIIB}$-gag-pol-gp120-env	vCP250
ALVAC-HIV-1$_{IIIB}$-gag-pol-gp160-env	vCP1420

48). An ALVAC-SIV vaccine encoding the *gag*, *pol*, and *env* genes of the SIV$_{mac251}$ isolate was able to reduce plasma virus load during primary infection and conferred protection from CD4$^+$ loss during both acute and chronic phases of infection after rectal exposure to a highly pathogenic SIV$_{mac251}$ isolate (50).

Among the pox vector-based vaccines, several ALVAC-based HIV-1 vaccines have been tested in phase I and II clinical trials and have been shown to be safe and immunogenic in humans (10, 14, 18, 21, 27). Whether the immunogenicity of these vaccine candidates will be sufficient to protect humans from HIV-1 remains unknown (5, 11, 44). The ongoing human phase III trial in Thailand will provide key information in this regard. Here, we designed a study to assess whether systemic immunization with recombinant canarypox expressing either HIV-1 gp120 or gp160 followed by a boost with either purified gp120 or gp140 proteins would confer protection after mucosal challenge with the pathogenic SHIV$_{KU2}$. We also investigated whether the addition of the Tat protein of HIV-1 as an immunogen could provide better protection, since a few studies have demonstrated that immunization of macaques with Tat protein either alone or as a Nef-Tat fusion protein in a multicomponent subunit vaccine confers an advantage in a SHIV$_{89.6P}$ model (12, 13, 56) but not in other studies (53). Here, we found that immunization of macaques with this type of ALVAC-based vaccine formulation elicited both antibody and cellular responses and significantly decreased plasma viremia and CD4$^+$ T-cell loss after rectal exposure to the SHIV$_{KU2}$ isolate. Tat protein immunization had no additive effect on the reduction of virus load in vaccinated rhesus macaques, as also observed by others (42, 46, 53).

MATERIALS AND METHODS

Vaccines and immunization protocol. Thirty-three rhesus macaques of Indian origin were used in the present study. The care and use of the animals were in compliance with all relevant institutional guidelines. The ALVAC-based SIV and HIV-1 vaccines used in the present study are presented in Table 1. vCP250 expressing the HIV-1$_{IIIB}$ gag-pro-gp120 TM *nef-pol* genes epitope, vCP1420 expressing the HIV-1$_{IIIB}$ gag-pro-gp160 *nef-pol* genes epitope, and vCP172 expressing the SIV$_{mac251 (K6W)}$ *gag* and *pol* genes (22) were generated as previously described (50). The HIV-1 gp120 and gp140 used as protein boost were purified from the serum-free culture supernatant of HUT78 cells chronically infected with HIV-1$_{IIIB}$ by immunoaffinity chromatography using anti-gp120 and anti-gp41 monoclonal antibodies as described previously (40). Recombinant Tat protein from the HIV-1$_{IIIB}$ isolate was purified from bacterial culture as described elsewhere (16). Both ALVAC-derived vaccines and the subunit glycoprotein and Tat protein boost were delivered via the intramuscular route. Env and Tat proteins were administered in QS-21 adjuvant (provided by Antigenics, Inc., Framingham, MA). Animals in group A (six macaques) received 10^8 PFU of ALVAC-gp120 (vCP250) and ALVAC-SIV-gag-pol (vCP172) at weeks 0, 4, 26, 225, and 233 and gp120 protein boost on weeks 52, 78, 225, and 233; group B animals (six macaques) were inoculated with 10^8 PFU of ALVAC-gp160 (vCP1420) and vCP172 at weeks 0, 4, 26, 225, and 233 and oligomeric gp140 protein boost on weeks 52, 78, 225, and 233. Animals in group C (six macaques) were inoculated with 10^8 PFU of vCP1420 and vCP172 at weeks 0, 4, 26, 52, 78, 225, and 233 and simultaneously received oligomeric gp140 protein boost on

weeks 4, 26, 52, 78, 225, and 233. Three animals per group from groups A, B, and C also received Tat protein (50 μg) by the intramuscular route on weeks 221, 225, and 233 adjuvanted in QS-21. Group D animals (six macaques) received 10^8 PFU of vCP172 at weeks 0, 4, 26, 52, 78, 225, and 233, whereas animals from group E (nine macaques) were kept as naive controls. All animals from groups A, B, C, and E, and three animals from group D were challenged intrarectally with SHIV$_{KU2}$ 4 weeks after the last immunization.

Immunoprecipitation of gp120 from chicken embryo fibroblasts infected with ALVAC-HIV-1-gag-pol-env (gpe). Freshly isolated chicken embryo fibroblast cells (Charles River SPAFAS, Wilmington, MA) were cultured in complete RPMI medium containing 10% fetal calf serum overnight. The monolayer was washed with RPMI medium and infected with 5 ml of either vCP250 or vCP1420 (multiplicity of infection = 5) at 37°C for 2 h. Residual virus inoculum was removed by washing the monolayer with 10 ml of complete medium, and the cells were cultured with 20 ml of complete RPMI medium for 72 h. After the culture revealed a cytopathic effect, cells were washed with phosphate-buffered saline (PBS) and labeled with [^{35}S]methionine (50 μCi/ml) in methionine-free RPMI medium containing 2% dialyzed fetal calf serum for 18 h. A flask of uninfected fibroblasts was also labeled with [^{35}S]methionine (50 μCi/ml) as a negative control. Both cells and supernatant were harvested, solubilized, and immunoprecipitated with serum from an HIV-1-infected individual as described elsewhere (49).

Protein immunogens. HIV-1 envelope glycoproteins gp120 and gp140 were purified from the culture supernatant of HUT78 cells lines by immunoaffinity chromatography as described elsewhere (39, 40). Briefly, a clone of a chronically infected HUT78 cell line was adapted to grow in serum-free conditioned medium. The culture supernatant was harvested when the cells were cultured to the log phase, and the glycoproteins were purified by immunoaffinity chromatography using anti-gp120 and anti-gp41 murine monoclonal antibodies. Recombinant Tat protein was expressed in bacteria and purified as described before (16).

Challenge virus stock. The SHIV$_{KU2}$ challenge stock was prepared by culturing phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) from an infected macaque that was inoculated intravenously with SHIV$_{KU2}$. SHIV$_{KU2}$ was derived from in vivo animal passage of SHIV$_{HXB2}$ (38), and the Env proteins of both viruses had significant sequence homology. However, SHIV$_{KU2}$ was shown to have characteristics similar to primary isolates and behaved as a biological variant of SHIV$_{HXB2}$ in terms of neutralization properties (19). Moreover, our preliminary titration experiments clearly demonstrated SHIV$_{KU2}$ transmits efficiently via the mucosal route (data not shown). The titer of the SHIV$_{KU2}$ challenge stock was determined in vivo in rhesus macaques by inoculating rectally six animals with different dilutions of virus stock (data not shown). Since six of six animals inoculated with 1 ml of undiluted virus stock became infected, as evidenced by high plasma viremia, this dose of virus was selected for all challenge studies.

Binding and neutralizing antibody assays. Serum samples were tested for HIV- and SIV-specific antibody responses by using an enzyme-linked immunosorbent assay (ELISA) described elsewhere (50). Briefly, polystyrene plates (Greiner Bio-One, Longwood, FL) were coated with HIV-1$_{IIIB}$ gp140 protein (50 ng/well) in 100 μl of phosphate buffer (pH 7.2) at 4°C overnight. The solution was aspirated, the wells were washed with wash buffer (PBS containing 0.5% Tween 20) four times, and each well was treated with 200 μl of blocking buffer (water containing 2.5% bovine serum albumin and 1.25% dry milk) for 1 h at 37°C. After a wash with wash buffer, 100 μl of serum serially diluted in Dilsim II (bioMerieux, Durham, NC) was added to each well, followed by incubation for 1 h at 37°C. Plates were again washed with wash buffer, and each well was incubated for 1 h at 37°C with 100 μl of 1:5,000 dilution of goat anti-human horseradish peroxidase-labeled immunoglobulin G (KPL Labs, Gaithersburg, MD) in conjugate buffer (PBS containing 10% normal goat serum, 0.1% Tween 20, and 0.2% bovine serum albumin). Plates were then thoroughly washed with wash buffer, and each well was reacted with 100 μl of Enhanced K-Blue Substrate (Neogen, Lexington, KY) at room temperature for 30 min. Reaction was terminated by adding 100 μl of 2N H$_2$SO$_4$ to each well, and the optical density was read in an ELISA plate reader at 450 nm. Antibody titers were defined as the highest dilution of immune serum yielding an optical density greater than two times the optical density detected with a corresponding dilution of preimmune serum. To assess SHIV-specific serum-neutralizing activity, two types of assays were conducted with sera from the vaccinated animals. In the first assay, sera were tested for their ability to neutralize a T-cell-line-adapted stock of SHIV$_{HXB2}$ assayed in MT2 cells as described previously (45). In the second assay, neutralization of the challenge stock of SHIV$_{KU2}$ was examined in PHA-activated human PBMC by measuring a reduction in viral p27 Gag antigen synthesis (45).

CTL assay using recombinant vaccinia virus. PBMC (10^7) from the monkeys were cultivated in vitro with paraformaldehyde-fixed, autologous B-LCL infected with either vaccinia-HIV-1_{BH10} Env or vaccinia-SIV$_{mac251}$ Gag. On day 3 of culture, 20 U of recombinant human interleukin-2/ml was added to the cultures. On day 12 of culture, the lymphocytes were centrifuged over a Ficoll-diatrizoate gradient and assessed as effector cells in a ^{51}Cr release cytotoxicity assay. Target cells were autologous B-LCL (10^6) cultured overnight with vaccinia virus-SIV$_{mac251}$ Gag, HIV-1_{BH10} Env, or vaccinia virus expressing herpes equine virus (control) at a multiplicity of infection of 10 PFU/cell. Target cells were then washed and labeled with 100 μCi of sodium ^{51}Cr for 1.5 h. After the washing step, 10^4 target cells were added per well in 96-well U-bottom plates in 100-μl volumes. Effector cells were added in another 100-μl volume at various concentrations to give effector/target ratios of 20:1, 10:1, 5:1, and 2.5:1. Plates were incubated at 37°C for 4 h. Then, 50 μl of supernatant was transferred to counting plates, and 200 μl of scintillation fluid was added and analyzed in a 1450 Microbeta liquid scintillation counter. Specific release was calculated as follows: [(experimental release − spontaneous release)/(100% release − spontaneous release)] × 100. Only cytotoxic activities above 10% were considered positive.

Virological assays. Animals were bled periodically after challenge, and the viral load in plasma was assessed by using a nucleic acid sequence-based amplification assay (NASBA) to quantify SIV RNA (51). In addition, PBMC collected from animals 21 days after virus challenge were subjected to quantitative virus isolation by coculture with PHA-activated human PBMC to confirm virus transmission. The CD4$^+$ T-cell count in the PBMC of challenged animals was determined by standard flow cytometric analysis.

For viral load quantification in tissues, total RNA was extracted from homogenized tissues by a method described previously (17). Briefly, a small portion of the tissue was excised and lysed by homogenization in a tube homogenizer with sodium citrate lysis buffer (pH 7.0) containing guanidine thiocyanate, sarcosyl, and β-mercaptoethanol. The lysed sample was treated with sodium acetate, extracted with phenol-chloroform (5:1), and RNA was precipitated with isopropanol. Extracted RNA was further purified by dissolving the pellet in sodium citrate lysis buffer and by reprecipitation with isopropanol. After a wash with 70% ethanol, the RNA pellet was air dried and dissolved in water, and the RNA content was quantitated by measuring the optical density at 260 nm. Quantitation of viral RNA was performed in a small aliquot (0.5 to 1 μg) of total RNA extract by NASBA as described elsewhere (51). This assay has a lower limit sensitivity of 500 copies of RNA, and the SIV RNA load was expressed as viral RNA copies per μg of total tissue RNA.

Statistical analysis. Analyses of the area under the curve (AUC) and the peak of plasma viral RNA loads were performed by using the Wilcoxon rank sum test and Wilcoxon-Gehan tests, respectively. CD4$^+$ T-cell counts, adjusted for baseline counts, were square-root transformed before analysis and were compared by using the Kruskal-Wallis test and repeated measures analysis of variance. The statistical analysis included CD4$^+$ T-cell values at days 28, 42, 58, 83, and 119. Viral RNA levels were tested by the Wilcoxon-Gehan test in three tissues where sufficient detectable levels were found and by the Fisher exact test in two tissues where vaccinated animals had undetectable levels.

RESULTS

Vaccines and study design. The aim of the present study was to assess the immunogenicity and relative efficacy of multiple immunizations of two ALVAC-based HIV-1 vaccines expressing either the uncleavable Env precursor gp160 (vCP1420) or the gp120-TM fusion protein (vCP250). Expression of Env proteins by these two constructs were assessed in chicken embryo fibroblasts. vCP1420 expressed the Env precursor that remained cell associated, whereas in the case of the vCP250 construct the gp120 was shed in the supernatant (Fig. 1a, lanes 1 and 3 and lanes 4 and 6, respectively). The Env proteins were neither detected by normal human serum (Fig. 1a, lanes 2 and 5) nor by the immune sera in mock-infected cells (Fig. 1a, lanes 7 and 9). All three proteins used as subunit boosts were homogeneous preparations with a high level of purity as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles for HIV-1_{IIIB} gp140 (Fig. 1b, lane 1),
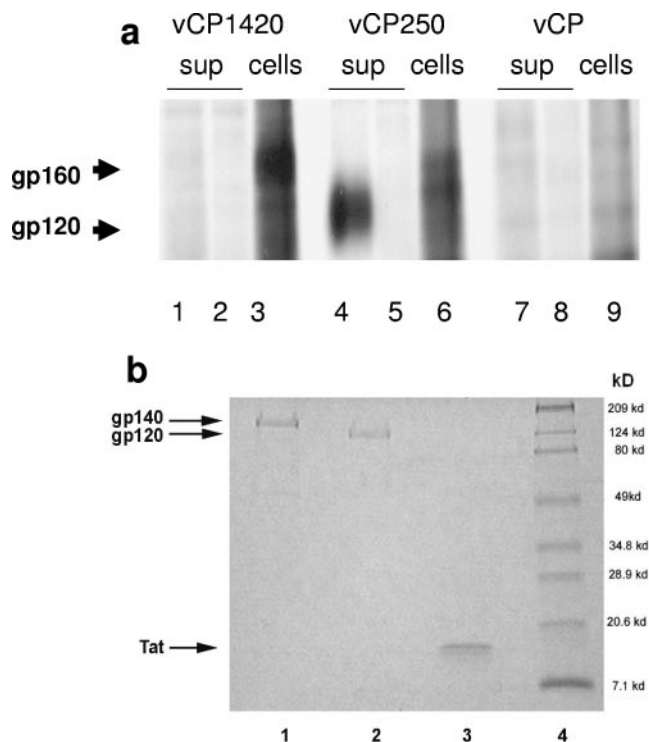

FIG. 1. (a) Expression of HIV-1_{IIIB} Env proteins in chicken embryo fibroblasts infected with vCP 1420 and vCP250 ALVAC-HIV-1 vaccines. Chicken embryo fibroblasts were infected with vCP1420 (lanes 1 to 3), vCP250 (lanes 4 to 6), and mock virus (lanes 7 to 9) and labeled with [^{35}S]methionine overnight. Cells (lanes 3, 6, and 9) and supernatants (lanes 1, 2, 4, 5, 7, and 8) were lysed and immunoprecipitated with normal human serum (lanes 2, 5, and 8) or serum from an HIV-1-infected individual (lanes 1, 3, 4, 6, 7, and 9) and analyzed by SDS-PAGE, as described in Materials and Methods. (b) PAGE profiles of Env and Tat proteins. Proteins were heated at 95°C for 5 min with SDS sample buffer under reducing conditions, electrophoresed on 10 to 20% gradient gel, and stained with Coomassie blue. Lane 1, gp140; lane 2, gp120; lane 3, Tat; lane 4, molecular weight marker. (c) Schematic representation of the study design. The asterisks designate the time of Tat administration in half of the animals in groups A, B, and C.

gp120 (Fig. 1b, lane 2), and recombinant Tat protein (Fig. 1b, lane 3).

The experimental vaccination regimen included five groups of animals (A to E) (Fig. 1c). Group A was immunized with vCP250 and boosted with the homologous cleaved gp120 protein. Animals in group B were immunized with vCP1420 and boosted with purified uncleaved gp140. Group C animals received simultaneously both vCP1420 and gp140. Group D in addition to animals from groups A to C were immunized with an ALVAC construct expressing only Gag and Pol of the SIV$_{mac251}$ isolate (vCP172), whereas group E animals were left naive. A subgroup of macaques from groups A, B, and C was also immunized with Tat protein (see Materials and Methods). Macaques from all groups were challenged with SHIV$_{KU2}$ rectally 4 weeks after the last immunization. We chose to use multiple doses of recombinant ALVAC because ALVAC-based vaccines have been used up to 12 times with minimal interference of vector-directed immunogenicity in cancer patients (33).
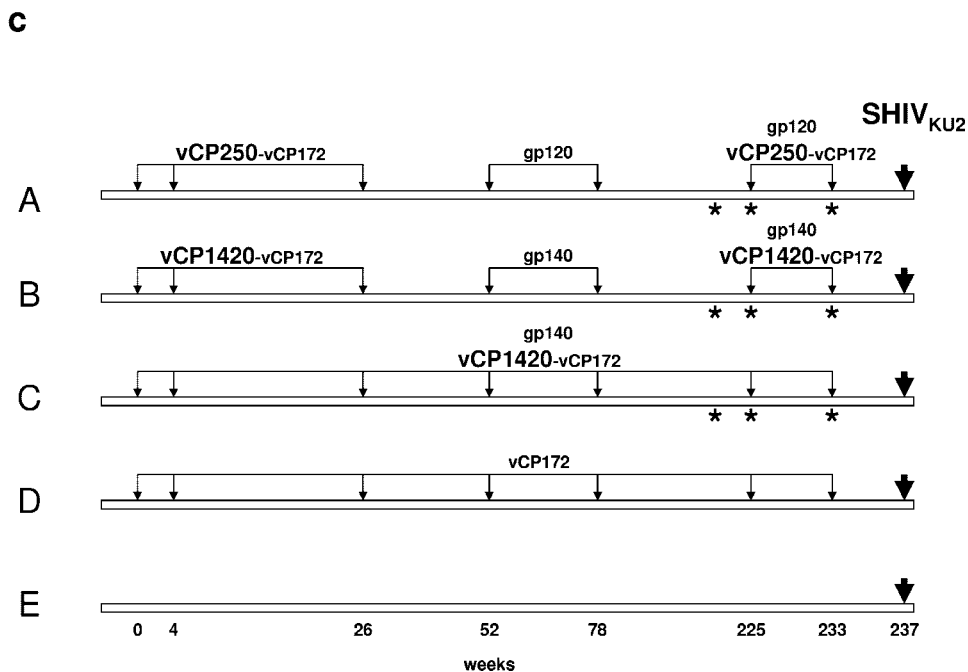
FIG. 1—*Continued.*

HIV-1-specific immune responses elicited by vaccination. Endpoint serum antibody titers to HIV-1 antigens measured by ELISA demonstrated that, during the ALVAC priming phase until week 52, group A animals receiving ALVAC-gp120 had higher anti-Env antibody titers compared to group B animals primed with ALVAC-gp160 (Fig. 2A). As expected, animals in group C that received both ALVAC-gp160 and gp140 proteins simultaneously during this phase had the highest anti-Env titers, especially after immunization on week 26. Antibody titers to Env were markedly enhanced in all groups of animals after boosting with the Env protein on week 52, with comparable titers in groups A and C and a slightly lower titer in group B. Similar increases in anti-Env titers were noted after Env protein boosts on weeks 78 and 225, and at the end of the immunization regimen the antibody titers were equivalent in all groups (Fig. 2a). Since antibodies to Tat protein have been correlated to protection in some studies (12) but not in others (53), we investigated their possible contribution by immunizing a subgroup of ALVAC-env-immunized animals with Tat protein. Immunization of animals with Tat protein elicited ELISA antibody titers to Tat (Fig. 2b), their titers increased progressively after each inoculation and, at the end of the immunization regimen, all immunized groups had equivalent titers of anti-Tat antibodies.

Neutralizing antibody titers in sera from all ALVAC-vaccinated animals (groups A to C) collected at weeks 28, 54, 80, and 234 were measured against both the laboratory-adapted SHIV$_{HXB2}$ in MT2 cells and the primary challenge stock SHIV$_{KU2}$ in human PBMC. Since binding antibody titers were weak before week 28 (Fig. 2a), no neutralization assay was conducted with sera from earlier time points. As shown in Fig. 2c, neutralizing antibodies to the laboratory-adapted SHIV$_{HXB2}$ were detected in most animals of groups A and C on week 28 with neutralization titers increasing significantly

after subsequent protein boost. Interestingly, sera from group A had slightly higher neutralizing titers compared to the other groups. None of the sera neutralized the SHIV$_{KU2}$ challenge stock in a human PBMC-based assay (titer < 1:5 as based on 80% reduction in p27 synthesis to be considered positive) (data not shown).

Cell-mediated immune responses induced by vaccination. Cellular responses induced by vaccination were measured by cytotoxic assays specific for the SIV Gag and the HIV-1 Env in blood of all animals twice during the immunization regimen (weeks 28 to 54). CTL responses to Env were low in all groups and did not differ in animals immunized with the two constructs (group A versus groups B and C) (Table 2). Five Mamu-A*01-positive animals were also included in the study: four in group B and one in group D. Memory response to the dominant Gag p11C epitope was demonstrated in all of these immunized macaques (52).

ALVAC-HIV-1-gpe vaccines decrease plasma viremia and protect from CD4$^+$ T-cell loss. After challenge exposure, the contribution of the various immunization regimens to the containment of viral replication and CD4$^+$ T-cell loss was assessed. Plasma viral RNA loads of all groups are shown in Fig. 3. Eight of nine naive control macaques became viremic after intrarectal challenge exposure to SHIV$_{KU2}$. Plasma viral RNA levels during primary viremia in the ALVAC-SIV-gag-pol-immunized (group D) animals were lower than in the naive animals from group E, but the difference was not significant ($P = 0.73$) (Fig. 3a and Table 3), indicating the limitation of a vaccine constituted of only the Gag and Pol product. Similarly, set point viremia did not differ significantly in these two animal groups. Thus, these animal groups were combined in a single control group to increase the statistical power of the analysis.

Among vaccinated macaques, two animals each from groups A and B and one animal from group C (a total of 5 of 18) had
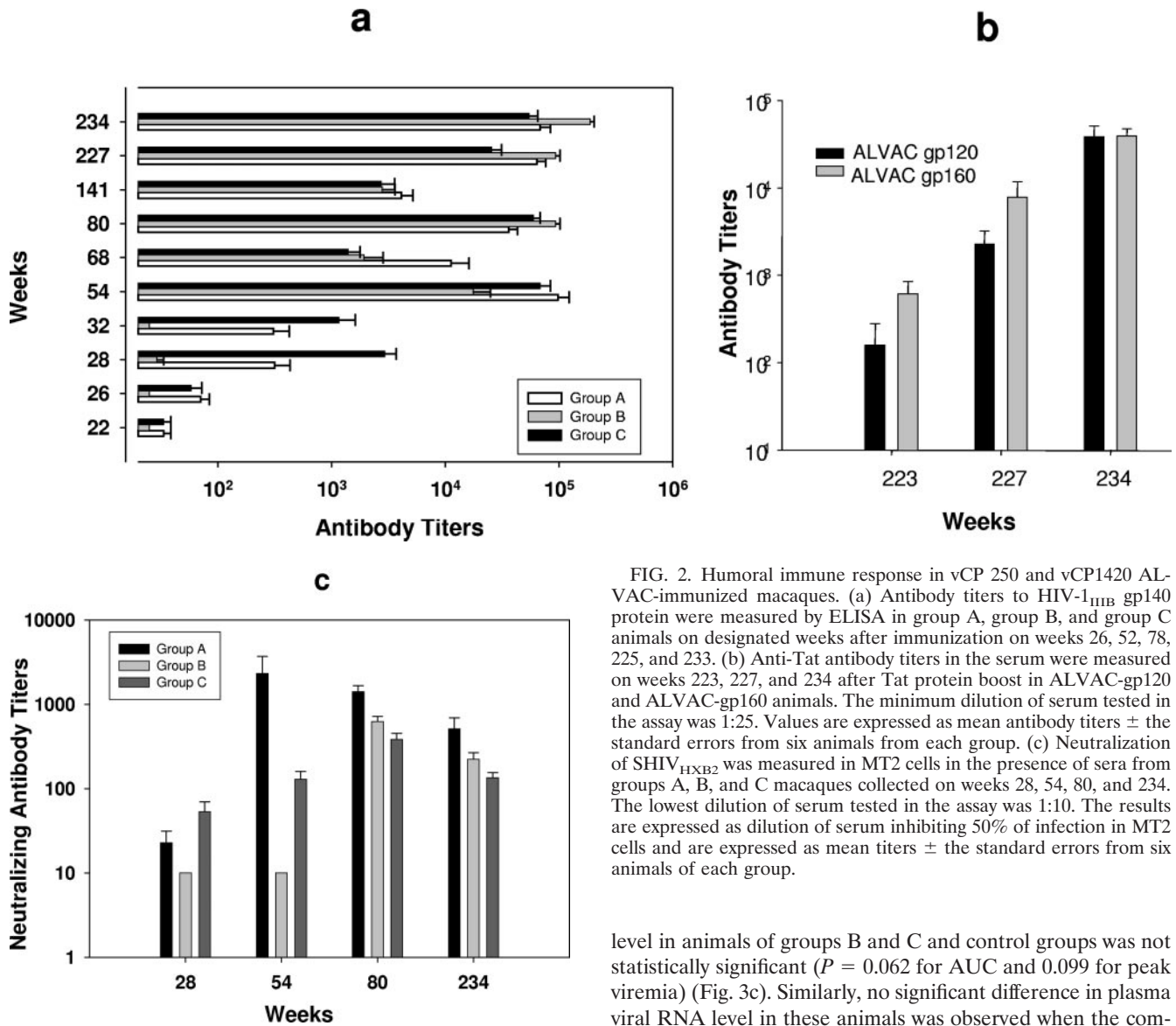
a

b

c

FIG. 2. Humoral immune response in vCP 250 and vCP1420 AL-VAC-immunized macaques. (a) Antibody titers to HIV-1$_{IIIB}$ gp140 protein were measured by ELISA in group A, group B, and group C animals on designated weeks after immunization on weeks 26, 52, 78, 225, and 233. (b) Anti-Tat antibody titers in the serum were measured on weeks 223, 227, and 234 after Tat protein boost in ALVAC-gp120 and ALVAC-gp160 animals. The minimum dilution of serum tested in the assay was 1:25. Values are expressed as mean antibody titers ± the standard errors from six animals from each group. (c) Neutralization of SHIV$_{HXB2}$ was measured in MT2 cells in the presence of sera from groups A, B, and C macaques collected on weeks 28, 54, 80, and 234. The lowest dilution of serum tested in the assay was 1:10. The results are expressed as dilution of serum inhibiting 50% of infection in MT2 cells and are expressed as mean titers ± the standard errors from six animals of each group.

consistently undetectable levels of plasma viremia (<2,000 RNA copies/100 μl input). However, these animals mounted weak titers of anti-p27 antibodies, suggesting occult infection (data not shown).

Plasma viremia in animals from group A differed significantly from the combined control group (D and E) ($P = 0.041$ within the first 2 weeks from challenge measured as the total AUC and $P = 0.013$ for peak viremia) (Fig. 3b and Table 3). This difference also remained statistically significant when plasma viremia of group A was compared only with naive animals in group E ($P = 0.049$ for AUC and 0.026 for peak viremia) (Table 3). When plasma viremia in groups B and C was compared, no significant difference was noted during primary viremia between the two groups ($P = 0.73$). These two groups were subsequently combined for assessing the vaccine effect of the vCP1420 construct. Interestingly, the plasma virus

level in animals of groups B and C and control groups was not statistically significant ($P = 0.062$ for AUC and 0.099 for peak viremia) (Fig. 3c). Similarly, no significant difference in plasma viral RNA level in these animals was observed when the comparison included naive animals only (group E) ($P = 0.072$ for AUC and 0.089 for peak viremia). Comparison of groups A, B, and C combined with the control group (groups D and E)

TABLE 2. Cytotoxic activity[a] in response to Gag and Env in immunized macaques

Group	Vaccine regimen	No of positive animals/total no. tested			
		Wk 28		Wk 54	
		Env (HIV-1$_{BH10}$)	Gag (SIV$_{mac251}$)	Env (HIV-1$_{BH10}$)	Gag (SIV$_{mac251}$)
A	vCP250 + vCP172 + gp120	1/6	0/6	0/6	0/6
B	vCP1420 + vCP172 + gp140	2/6	0/6	2/6	0/6
C	vCP1420 + vCP172 + gp140 simultaneously	1/6	0/6	0/6	2/6
D	vCP172	0/6	1/6	0/6	1/6

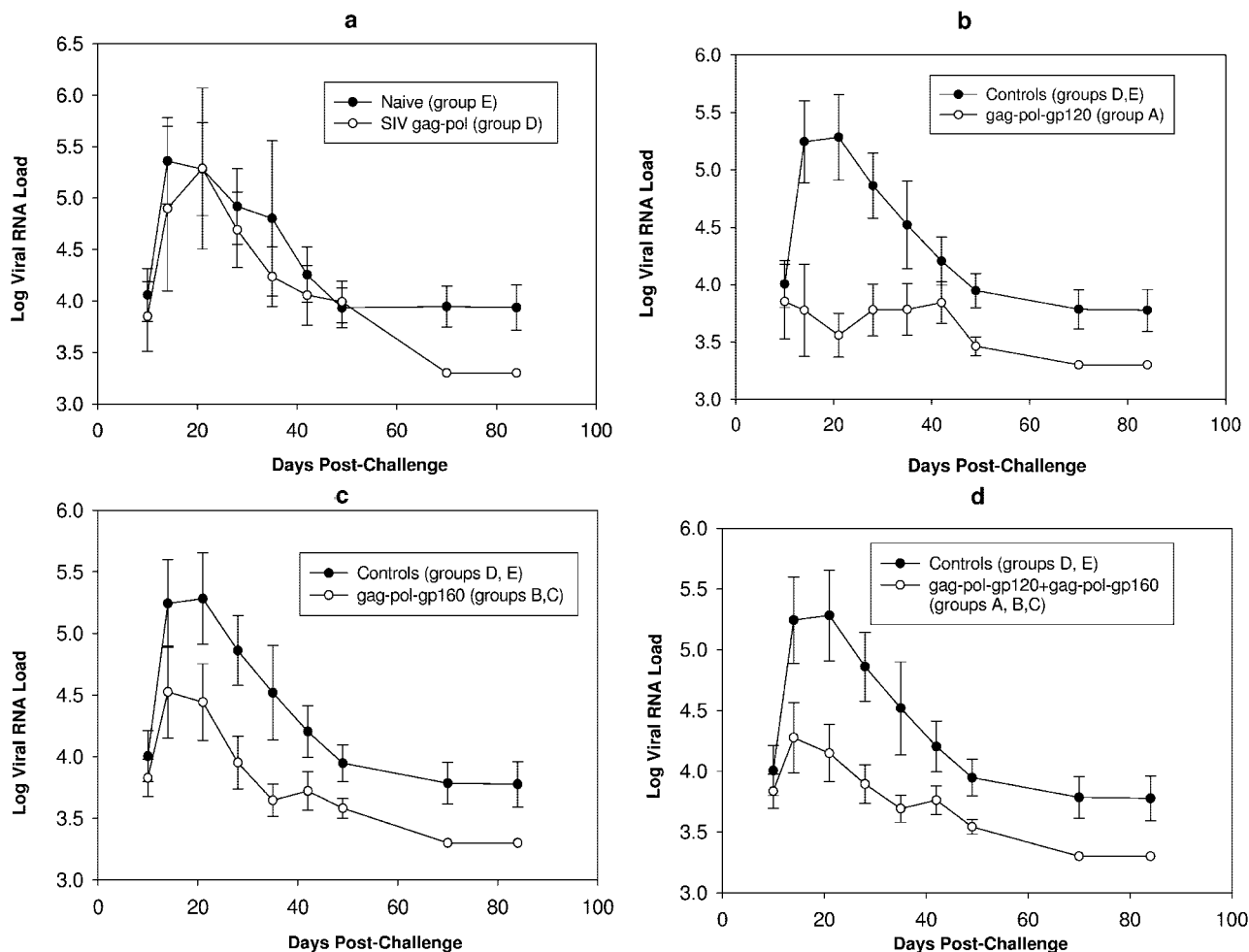[a] Only cytotoxic activity 10% above background was scored positive.

FIG. 3. Plasma viral RNA load in ALVAC-immunized macaques challenged with SHIV$_{KU2}$ by the rectal route. Plasma virus load was compared between naive group E and SIV-gag-pol group D (a), control (groups D and E) and vCP250-immunized/gp120-boosted group A animals (b), control (groups D and E) and vCP1420-immunized/gp140-boosted groups B and C animals (c), and control (groups D and E) and vCP250-immunized/gp120-boosted group A animals plus vCP1420-immunized/gp140-boosted groups B and C animals (d). Values are expressed as log-transformed mean RNA loads ± the standard errors from animals from each group.

again revealed a significant difference in plasma viremia (P values of 0.019 for both AUC and peak viremia) (Fig. 3d), and this difference remained significant when only naive animals were included in the analysis ($P = 0.027$ for AUC and 0.025 for peak viremia). Although vCP250 vaccination resulted in a more effective control of plasma virus level than vaccination with vCP1420, the difference in plasma viremia between group A and groups B and C was not statistically significant ($P = 0.29$ for AUC and 0.26 for peak viremia) (Table 3). Taken together, these results demonstrate that Env protein contributed to the protection against pathogenic SHIV challenge. Further, the form of Env protein presented to the immune system determined the protective efficacy of the vaccine.

We also assessed the effect of vaccination in protection from CD4$^+$ T-cell loss. For this, CD4$^+$ T-cell counts before virus challenge and at different time points were measured (Fig. 4 and Table 4). Differences in CD4$^+$ T-cell loss among group D and E animals were not significant ($P = 0.40$), as expected (Table 4). In contrast, there was a significant preservation of CD4$^+$ T cells in macaques from group A compared to naive

TABLE 3. Statistical analysis of plasma viral RNA load measured in different vaccine groups after SHIV$_{KU2}$ challenge

Group comparison[a]	P	
	AUC	Peak viremia
vCP250/gp120 (group A) vs naive (group E)	0.049	0.026
vCP1420/gp160 (groups B and C) vs naive (group E)	0.072	0.089
vCP172 (group D) vs naive (group E)	0.73	0.73
vCP250/gp120 (group A) and vCP1420/gp160 (groups B and C) vs naive (group E)	0.027	0.025
vCP250/gp120 (group A) vs vCP172 (group D) and naive (group E)	0.041	0.013
vCP1420/gp160 (groups B and C) vs vCP172 (group D) and naive (group E)	0.062	0.099
vCP250/gp120 (group A) and vCP1420/gp160 (groups B and C) vs vCP172 (group D) and naive (group E)	0.019	0.019
vCP250/gp120 (group A) vs vCP1420/gp160 (groups B and C)	0.29	0.26

[a] vCP250, ALVAC-HIV-1$_{IIIB}$-gpe (gp120 Env); vCP1420, ALVAC-HIV-1$_{IIIB}$-gpe (gp160 Env); vCP172, ALVAC-SIV$_{mac251}$-gp.
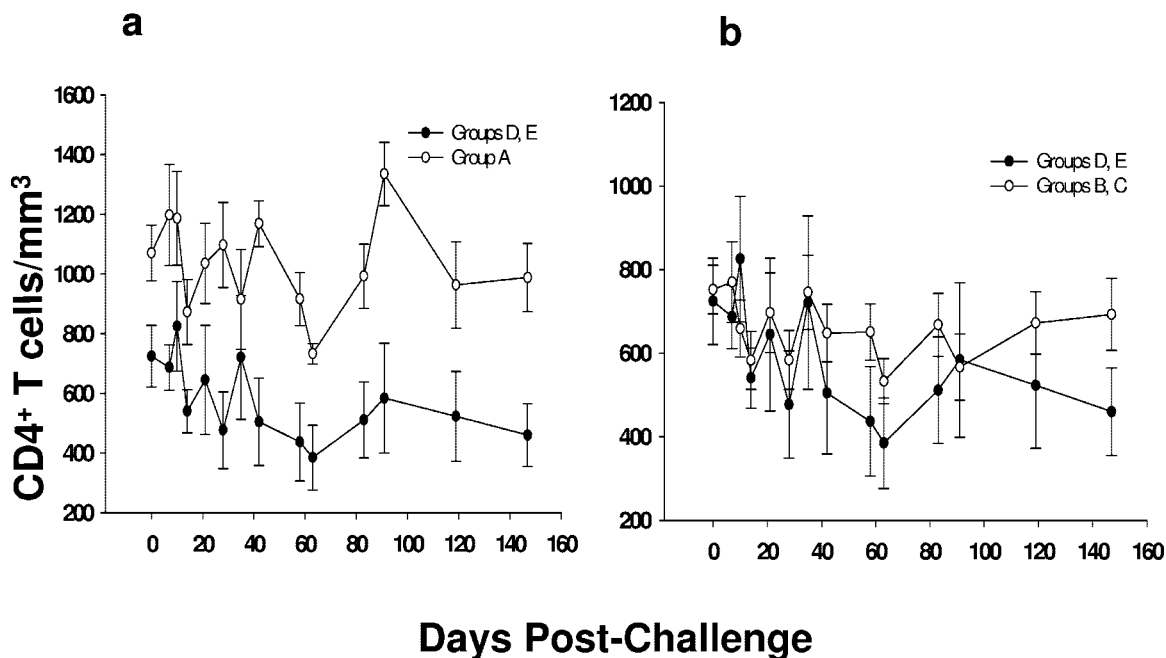
a

b

Days Post-Challenge

FIG. 4. CD4$^+$ T-cell counts in ALVAC-immunized and control macaques after challenge exposure to SHIV$_{KU2}$. Mean CD4$^+$ T-cell counts were compared between control groups (groups D and E) and vCP250 group A (a) and vCP1420 groups B and C (b), respectively. Values on day 0 represent average CD4$^+$ T-cell counts from two time points measured before challenge.

and ALVAC-SIV-gag-pol-vaccinated macaques (groups D and E) with a P value of 0.014 (Fig. 4A and Table 4). Preservation of CD4$^+$ T cells in group A was also significant compared to naive animals in group E ($P = 0.020$). CD4$^+$ T cells were also preserved in group B and C animals compared to the control group (groups D and E) ($P = 0.039$) (Fig. 4b). However, this difference was not significant when a comparison was made among groups B, C, and E ($P = 0.081$).

Tat protein did not confer further virological benefit. Since half of the animals from groups A, B, and C were immunized with Tat protein three times before virus challenge, we sought to determine whether the immunization with Tat would further contribute to containment of viral replication and preservation of CD4$^+$ T cells. The statistical analysis on the plasma virus level in macaques immunized with Tat protein and with those animals that did not receive Tat demonstrated that Tat did not contribute further to protection ($P = 0.32$ for AUC) (Table 5). Furthermore, the addition of Tat to animals in groups A, B, and C demonstrated no difference compared to non-Tat-immunized animals from the same three groups ($P = 0.10$ for AUC for group A; $P = 0.39$ for groups B and C combined). Similarly, Tat protein did not affect virus levels when Tat-

immunized animals from groups A, B, and C together were compared to the non-Tat-immunized group ($P = 0.67$ for AUC).

SIV RNA load in tissues of ALVAC-HIV-1-gpe-vaccinated animals after challenge with SHIV$_{KU2}$. We previously observed using a peptide-based vaccine that mucosal delivery of HIV/SIV antigens resulted in a better control of virus RNA levels in tissue than systemic immunization after mucosal challenge with the same viral strain (SHIV$_{KU2}$) (7). However, as we have demonstrated that systemic delivery of a recombinant poxvirus vaccine such as NYVAC-SIV results in generation of memory T cells present in mucosal sites as well as systemic sites (54), we sought to determine whether systemic immunization of animals with the ALVAC-SIV/HIV vaccine candidate would affect virus RNA levels in mucosal tissues. To this end, total RNA from lymph nodes and gut-associated tissues of both ALVAC-HIV-1 (groups A to C) and ALVAC-SIV-gag-pol (group D) vaccinated and naive animals (group E) was harvested and SIV RNA was quantitated by NASBA. As shown in Fig. 5, viral RNA levels in several tissues of the vaccinated animals were consistently lower than the level in the tissues of the control animals. Statistical analysis of these re-

TABLE 4. Preservation of CD4$^+$ T cells in vaccinated and control macaques

Group comparison	P
vCP250 (group A) vs naive (group E)	0.020
vCP1420 (groups B and C) vs naive (group E)	0.081
vCP172 (group D) vs naive (group E)	0.40
vCP250 vs vCP172 + naive	0.014
vCP1420 vs vCP172 + naive	0.039

TABLE 5. Statistical analysis of the effect of Tat protein on viral RNA load in different vaccine groups

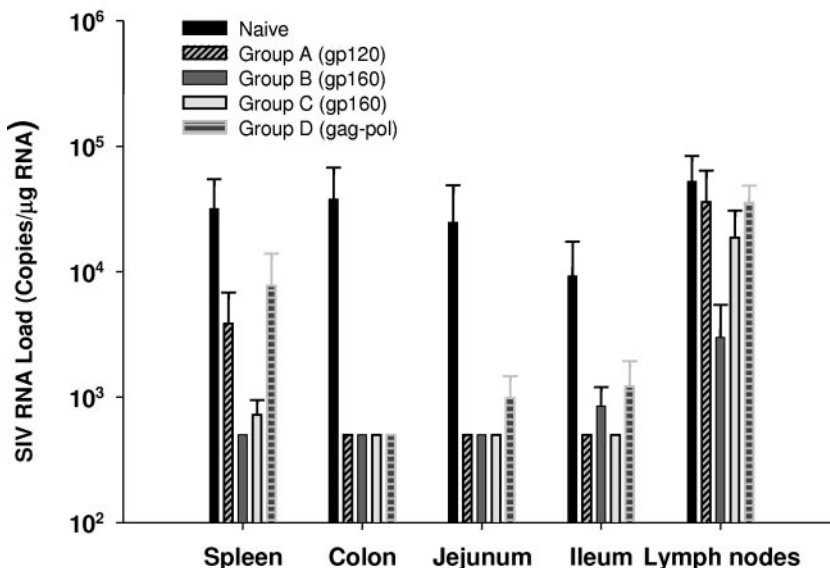| Group comparison	AUC
Tat-immunized vs all non-Tat-immunized	0.32
Tat-immunized vs non-Tat-immunized vCP1420/gp160	0.39
Tat-immunized vs non-Tat-immunized vCP250/gp120	0.10
Tat-immunized vs non-Tat-immunized vCP250/gp120 and vCP1420/gp160	0.67

FIG. 5. RNA virus levels in the tissues of macaques in groups A (vCP250), B and C (vCP 1420), and E (naive) at euthanasia. SIV RNA virus levels in tissues were compared between naive animals, vCP250 group A, vCP1420 group B, vCP1420 group C, and vCP172 group D separately.

sults indicated that the RNA load in spleens, colons, and ilea of vaccinated animals was significantly lower than for naive animals ($P = 0.0016$ for spleen, 0.0041 for colon, and 0.015 for ileum). However, the difference in lymph nodes did not reach statistical significance. Interestingly, the RNA load in colons, jejuna, and ilea of SIV-gag-pol-vaccinated animals (group D) was lower than for the naive animals (group E), although this difference was not noted for plasma viremia. However, no statistical analysis was performed because of the low number of animals enrolled in group D.

Postchallenge neutralizing antibody response. Neutralization of challenge virus as well as SHIV$_{HXB2}$ was examined with sera collected 2, 4, and 8 weeks postchallenge. None of the serum from the challenged animals neutralized SHIV$_{KU2}$ (data not shown). However, sera from groups A, B, and C neutralized SHIV$_{HXB2}$ almost to a similar level, whereas the sera from ALVAC-SIV-gag-pol-immunized animals (group D) and naive group E had no neutralizing activity on weeks 2 and 4 but developed a weak response compared to the immunized groups on week 8 (Fig. 6).

DISCUSSION

Results from several clinical trials have demonstrated that canarypox-based HIV-1 vaccines are safe and immunogenic in humans (6, 14, 18, 20, 37, 47). We previously evaluated the relative efficacy of this vaccine modality in rhesus macaques immunized with a recombinant SIV$_{mac}$ canarypox virus expressing SIV *gag*, *pol*, and *env* genes (ALVAC-SIV-gpe) and exposed mucosally to the pathogenic SIV$_{mac251}$ strain. The ALVAC-SIV recombinant vaccine elicited both antibodies and CTL responses against SIV Gag and Env antigens in immunized animals. After mucosal challenge exposure, a decrease in SIV$_{mac251}$ plasma RNA levels was observed during primary infection. In addition, the immunized macaques were protected from CD4 loss during both acute and chronic phases of

infection (50) and survived longer than nonimmunized macaques. In that study, however, boosting of ALVAC-SIV-gpe-immunized animals with SIV gp120 did not further contribute to protection (50). Because the Env protein of SIV differs substantially from the Env of HIV, we wanted to assess more directly the contribution of two Env proteins using HIV-1 antigens. Thus, the present study was designed to assess the relative efficacy of an ALVAC-based HIV-1 vaccine con-
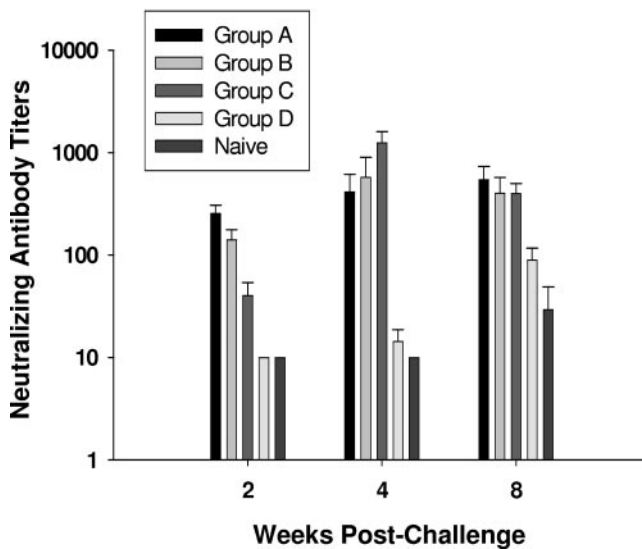

FIG. 6. SHIV$_{HXB2}$ neutralizing antibody titers in the sera of infected macaques. Neutralization of SHIV$_{HXB2}$ was measured in MT2 cells in the presence of sera from groups A, B, C, D, and naive macaques collected on 2, 4, and 8 weeks after rectal inoculation with SHIV$_{KU2}$. The lowest dilution of serum tested was 1:10. The results are expressed as dilutions of serum inhibiting 50% of infection in MT2 cells and are expressed as mean titers $\pm$ the standard errors from animals of each group.

structed in the same way as the vaccine used in the phase III trial in Thailand and compare it with another construct whereby both expression of the canarypox Env and the protein boost differed. The SHIV$_{KU2}$ used as a challenge virus is a neutralization-resistant phenotype of SHIV$_{HXB2}$ and has biological characteristics similar to primary virus. The present study clearly demonstrates that immunization of macaques with a prime-boost including ALVAC-HIV-1-gpe encoding the gp120 gene (vCP250) and ALVAC-SIV-gag-pol (vCP174), followed by a gp120 protein boost, was effective in controlling plasma viremia, as well as the loss of CD4 cells in macaques following intrarectal challenge with a pathogenic X4-tropic SHIV$_{KU2}$ isolate. In contrast, immunization of macaques with ALVAC-HIV-1-gpe encoding the uncleavable gp160 gene (vCP1420) followed by gp140 boost did not significantly affect viral replication or CD4$^+$ T-cell count.

The protective immunological responses induced by these vaccine regimens in this animal model, however, remain unclear. Both vaccines combined with the Env protein boost elicited strong binding antibodies in ALVAC-HIV-1-gpe-primed animals, and these antibodies were able to neutralize the T cell-line-adapted SHIV$_{HXB2}$ isolate. Similar induction of neutralizing antibodies by protein boost against homologous HIV-1 isolate was reported in ALVAC-HIV-1-primed human volunteers (47). However, we were unable to detect neutralizing antibodies against the challenge virus SHIV$_{KU2}$. Hence, neutralizing antibodies may not play a major role in the containment of SHIV$_{KU2}$ in ALVAC-HIV-1-gpe-immunized animals. However, other antibody-dependent immune mechanisms such as antibody-dependent cellular cytotoxicity in sera or other body fluids may play a role in the efficacy of these vaccine candidates, as demonstrated in the use of a replication-competent adenovirus-based vaccine candidate (26).

These ALVAC vaccine candidates elicit durable memory responses directed to both dominant and subdominant CTL epitopes (52), and such immune response may account for virus containment after challenge exposure (52). In phase I trials with an ALVAC prime/gp120 boost combination (6, 47), these vaccines induced proliferative responses in most of the immunized people. Interestingly, in the SIV$_{mac251}$ macaque model, poxvirus-based HIV vaccine candidates induce proliferative response and inversely correlate with the virus level (30, 31, 55).

The results presented here demonstrate that ALVAC-gp120-immunized animals had a significantly lower virus load compared to ALVAC-gp160-immunized animals, suggesting that the form of Env presented during the primary immunization phase may influence the protective immune response. This hypothesis is supported by the finding that priming of macaques with ALVAC expressing gp120 elicited more durable and higher titers of binding antibodies than the ALVAC-gp160-expressing vaccine, leading to a better priming effect (see Fig. 2A). However, boosting of ALVAC-primed animals with either gp120 or gp140 (groups A and B) or simultaneous immunization with ALVAC and gp140 protein (group C) decreased these differences in antibody titers and, at the time of SHIV$_{KU2}$ challenge, no differences were observed between these groups. Interestingly, the neutralizing antibody response to SHIV$_{HXB2}$ appeared to be consistently higher in the ALVAC-gp120-immunized group (group A) compared to AL-

VAC-gp160-immunized animals (groups B and C), suggesting that a gp120-based ALVAC vaccine may elicit a more functionally active antibody response compared to immunization with gp140. This again may be due to a better priming effect observed with the ALVAC-gp120-immunized group than with the ALVAC-gp160-immunized group.

As described here, the SIV RNA load associated with the gut-associated tissues of ALVAC-HIV-1-immunized animals was contained compared to naive animals. Although the RNA load in lymph nodes was lower in immunized animals, such reduction was not statistically significant. This containment of virus replication in tissues following ALVAC-HIV-1 vaccination suggests that tissue-specific cellular responses may contribute to this type of virus containment.

Recent studies have demonstrated that a subunit vaccine composed of Tat protein of HIV-1 may contain viral replication following challenge with pathogenic SHIV isolates (12, 56). However, such a protective effect of Tat protein against SHIV challenge was not reproduced in an independent challenge study (53). We examined here whether the immunization of ALVAC-primed animals with Tat and Env proteins could confer additional protective advantage over boosting with Env protein alone. The results presented here demonstrate that, although immunization of ALVAC-primed animals with purified Tat protein clearly elicited strong anti-Tat antibody response, such antibodies did not enhance the efficacy of the ALVAC vaccine against SHIV challenge. It is not clear why the Tat protein did not exhibit any efficacy against SHIV$_{KU2}$ challenge. Several factors, such as the functional state of the Tat protein, the animal species, the timing of immunization, and the quality of anti-Tat antibodies elicited by the vaccine, may account for this difference.

A number of clinical studies performed in human volunteers have clearly demonstrated that ALVAC vaccines administered either as a single immunogen or in combination with a subunit protein boost elicit both humoral and cellular immune responses (23). The relevance to humans of our findings in macaques will be assessed in the ongoing phase III trail in Thailand, designed to evaluate the protective efficacy of an ALVAC-based vaccine against HIV infection.

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