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Role of Immune Responses against the Envelope and the Core Antigens of Simian Immunodeficiency Virus SIVmne in Protection against Homologous Cloned and Uncloned Virus Challenge in Macaques

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We previously showed that envelope (gp160)-based vaccines, used in a live recombinant virus priming and subunit protein boosting regimen, protected macaques against intravenous and intrarectal challenges with the homologous simian immunodeficiency virus SIVmne clone E11S. However, the breadth of protection appears to be limited, since the vaccines were only partially effective against intravenous challenge by the uncloned SIVmne. To examine factors that could affect the breadth and the efficacy of this immunization approach, we studied (i) the effect of priming by recombinant vaccinia virus; (ii) the role of surface antigen gp130; and (iii) the role of core antigens (Gag and Pol) in eliciting protective immunity. Results indicate that (i) priming with recombinant vaccinia virus was more effective than subunit antigen in eliciting protective responses; (ii) while both gp130 and gp160 elicited similar levels of SIV-specific antibodies, gp130 was not as effective as gp160 in protection, indicating a possible role for the transmembrane protein in presenting functionally important epitopes; and (iii) although animals immunized with core antigens failed to generate any neutralizing antibody and were infected upon challenge, their virus load was 50- to 100-fold lower than that of the controls, suggesting the importance of cellular immunity or other core-specific immune responses in controlling acute infection. Complete protection against intravenous infection by the pathogenic uncloned SIVmne was achieved by immunization with both the envelope and the core antigens. These results indicate that immune responses to both antigens may contribute to protection and thus argue for the inclusion of multiple antigens in recombinant vaccine designs.

The envelope antigen of human immunodeficiency virus type 1 (HIV-1) is a major determinant for virus infectivity, cellular tropism, cytopathicity, and in vivo pathogenicity and is the primary target for virus neutralizing antibodies (14, 98). Most of the early efforts in AIDS vaccine development have therefore focused on the envelope glycoproteins as the target antigen, especially the surface antigen gp120 (22, 36, 56). The efficacy of this approach has been demonstrated primarily in chimpanzees (8, 9, 12, 13, 24, 30, 58) and more recently in macaques, using chimeric simian-human immunodeficiency viruses (11, 46, 55, 64, 91). Protection has been attributed to neutralizing antibodies, particularly those directed to the V3 hypervariable region of gp120 (8, 26, 89). However, antisera from gp120-immunized vaccinees failed to neutralize primary HIV-1 isolates (35, 59), and the potential efficacy of such vaccines in humans has been debated (10, 19).

We and others have used simian immunodeficiency viruses (SIV) as a model to gain insights into the correlates of protection and the requirements for an efficacious vaccine against primate lentiviruses. Recombinant subunit envelope vaccines alone have shown little or no efficacy (61, 67). Various degrees of success have been reported by investigators using a combination immunization strategy in which a poxvirus-vectored vaccine was used for priming and subunit immunogens for boosting (1, 3, 21, 29, 38, 42, 45, 70). We recently reported that while such a combination immunization regimen resulted in complete protection against a pathogenic cloned virus, E11S, only partial protection was achieved against the uncloned virus, SIVmne (76). The breadth of protection by recombinant gp160 vaccines thus appears restricted. Given the hypervariable nature of the envelope antigen, it remains important to explore approaches that will broaden the protective immunity of envelope-based vaccines.

The core antigens of HIV-1 are attractive targets for vaccine development not only because they are relatively conserved but also because they contain major determinants for recognition by cytotoxic T lymphocytes (CTL) (15, 16, 60, 71), which are believed to play an important role in controlling virus infection (52, 83, 84). However, the protective role of immune responses to the core antigens has not been directly demonstrated. Emini et al. (25) failed to protect chimpanzees against HIV-1 IIIB infection with a Gag p55 antigen produced in yeast cells. Similarly, a peptide vaccine containing a conserved CTL epitope of SIV failed to protect macaques against SIVmne E11S, despite the presence of a robust CTL response (100). Core antigens have also been used in combination with enve-

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lope antigens in vaccine studies. However, results have been variable, ranging from a complete lack of protection (21) to variable degrees of partial protection with reduction of viral load and prolongation of survival (1, 3, 38). The potential role of immune responses to the core antigens are difficult to discern from these studies in part because of a lack of direct comparisons and in part because of the divergent nature of the challenge models and immunization regimens used.

In the present study, we sought to determine the role of the envelope and the core antigens in eliciting protective responses by the combination immunization approach. Specifically, we examined the effect of priming by recombinant vaccinia virus, the role of the surface antigen gp130 versus whole envelope protein gp160, and the role of core antigens, alone or in combination with envelope antigens, in eliciting protective immunity against SIV infection. Our results indicate that immune responses to both antigens may contribute to protection and that protection is feasible with recombinant vaccines against a primate lentivirus of moderate complexity and pathogenicity.

MATERIALS AND METHODS

Immunogens and immunization regimen. The following recombinant viruses were used for primary immunizations: v-SE5, which expresses the native full-length gp160 antigen of SIVmne cl 8 from the vaccinia virus 7.5K promoter (44); v-SE6, which expresses only the surface antigen gp130 from the 7.5K promoter; v-SG11, which contains the entire *gag-pol* coding region of SIVmne under the control of vaccinia virus H6 (40K) promoter; and v-SGE14, which contains both the full-length *env* and the *gag-pol* constructs in v-SE5 and v-SG11 under the control of their respective promoters. All recombinant viruses were constructed by using a plaque-purified New York City Board of Health strain of vaccinia virus (v-NY) (43) as the vector. Immunizations with recombinant viruses were performed by skin scarification at approximately 10^8 PFU per dose. All subunit and particle vaccines were produced by African green monkey kidney (BSC-40) cells infected with recombinant vaccinia virus expressing the corresponding antigens (gp160, gp130, Gag-Pol, or Gag-Pol-Env) under the control of the late vaccinia virus 11K promoter. Full-length SIVmne envelope glycoprotein rgp160 molecules produced by this system are glycosylated and are capable of binding to recombinant human CD4. They have an apparent molecular mass of 160 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and are oligomeric (mostly trimeric or tetrameric) by size exclusion chromatography (data not shown). The rgp130 molecules are CD4-binding monomeric glycoproteins secreted from infected cells. Each dose contained approximately 100 μg of gp160 or gp130, corresponding to 200 and 110 μg of total proteins, respectively (51). The Gag-Pol and Gag-Pol-Env antigens expressed by v-SG11 and v-SGE14, respectively, are processed and assembled in particle forms similar to those described for HIV-1 antigens (33, 34, 41). Each dose of Gag-Pol particles contained 500-μg equivalents of p27 antigens. Each dose of Gag-Pol-Env particles contained 500-μg equivalents of p27 antigens, plus approximately 30 μg of envelope proteins copurified with the core particles (pseudovirions). All subunit and particle immunogens were formulated in incomplete Freund's adjuvant and were administered intramuscularly. Methods for the construction, preparation, and characterization of SIVmne recombinant viruses and subunit immunogens are similar to those previously described for the HIV-1 counterparts (34, 39, 42, 51) and will be described in further detail elsewhere (47). Cynomolgus macaques (*Macaca fascicularis*; $n = 4$/group) were immunized at 0, 3, 18, and 24 months as described in Table 1.

Challenge virus and conditions. SIVmne was isolated from a pigtailed macaque (*M. nemestrina*) with lymphoma and was propagated on HuT78 cells (4). E11S is a single-cell clone of SIVmne-infected HuT78 cells that produced large amounts of envelope glycoproteins (6). Animals were challenged by intravenous injection at 4 weeks after the last immunization. Challenge with SIVmne clone E11S was performed with 10 to 100 animal infectious doses (AID) of virus grown on macaque (*M. fascicularis*) peripheral blood mononuclear cells (PBMC). The animals in groups B, E, and F (Table 1) that were completely protected against E11S challenge were boosted again at 8 to 9 months after the challenge and were rechallenged 4 weeks later with uncloned SIVmne grown on HuT78 cells at 2 to 20 AID. Blood samples were collected on the day of challenge, at 2, 4, 6, and 8 weeks after each challenge, and monthly thereafter. Plasma and serum samples were collected and stored until used at −70 and −20°C, respectively. Lymph node biopsy specimens were obtained at indicated times after challenge and were frozen at −70°C for DNA analysis or fixed for histological examinations. Animals were housed in the Washington Regional Primate Research Center and were under the care of licensed veterinarians. Euthanasia was performed on the basis of the following criteria: (i) AIDS; (ii) termination of experiment; or (iii) unrelated cause. Euthanasia is considered to be AIDS related if the animal exhibits $CD4^+$ cell depletion (≤200 cells/mm^3) in the peripheral blood and two or more of the following conditions: wasting, unsupportable diarrhea, opportunistic infections, proliferative diseases (e.g., lymphoma), and abnormal hematology (e.g., anemia or thrombocytopenia).

TABLE 1. Immunization regimen

Group	Immunization		Macaque
	Primary	Booster(s)[a]	
A	rgp160	rgp160	91286 91292 91268 91262
B	v-SE5 (gp160)	rgp160	J90304 91272 91250 91263
C	v-SE6 (gp130)	rgp130	91255 91254 91244 91293
D	v-SG11 (Gag-Pol)	Gag-Pol particles	91245 91278 91288 91253
E	v-SE5 + v-SG11[b]	Gag-Pol particles + rgp160[c]	91251 91281 91248 91283
F	v-SGE14 (Gag-Pol-gp160)[d]	Gag-Pol-Env particles[e]	91287 91291 91282 91247
Control			91077 91078 M92387 93057

[a] Booster immunizations were performed by intramuscular injections at 3, 18, and 24 months after the primary immunization.

[b] Animals received equal doses (10^8 PFU) of each recombinant virus administered separately by skin scarification at opposite sites along the midline of the back.

[c] Inoculum was formulated as a mixture of core particles (containing 500-μg equivalents of p27 antigen) and rgp160 (100 μg).

[d] Inoculum contained a single recombinant virus (10^8 PFU) expressing the *gag-pol* genes under the control of vaccinia 40K promoter and the full-length *env* gene under the 7.5K promoter.

[e] Immunogen was produced from recombinant vaccinia virus-infected cells and was composed of both the core (Gag-Pol) and the envelope antigens in a mature virus-like particle structure (pseudovirions). Each inoculum contained a 500-μg equivalent of p27 antigens and approximately 30 μg of envelope proteins.

Virus isolation. PBMC were isolated over Histopaque-1077 (Sigma Chemical Co., St. Louis, Mo.) as described previously (5, 7). Briefly, 4×10^6 PBMC were cocultivated with 5×10^6 AA-2CL5 cells, and cultures were maintained for 8 to 9 weeks. Virus was detected by reverse transcriptase assays performed as described previously (7). A positive value means positive results in reverse transcriptase assays, and a negative value means no reverse transcriptase activity detected after 8 to 9 weeks of cocultivation.

Serum neutralization assays. Neutralizing antibodies against uncloned SIVmne and SIVmne clone E11S were measured in CEM-x174 cells by methods similar to those described by Montefiori et al. (62). The uncloned SIVmne used for neutralization studies was grown on HuT78 cells and was identical to the challenge stock but prepared at different times. The E11S clone used for neutralization assays was grown on macaque PBMC after the initial isolation and propagation in HuT78 cells (4). It was derived from the same stock as the challenge virus but grown on PBMC from a different *M. fascicularis*. Neutralization of a related but heterologous virus, SIVmac251, was measured in HuT78 cells as described by Langlois et al. (54). Twofold serum dilutions (heat inactivated at 56°C for 30 min) were done in 96-well plates. The neutralization titer is expressed as the reciprocal serum dilution that inhibits 50% of SIVmne-induced cytopathic effect in CEMx174 cells or 90% syncytium formation by SIVmac251-infected HuT78 cells.

ELISA. SIV-specific antibodies were measured by enzyme-linked immunoabsorbent assay (ELISA) as described previously (40) except that sucrose gradient-purified and disrupted whole SIVmne clone E11S virion was used as antigen in ELISA. Endpoint titers were determined as the reciprocal of the highest serum dilution that resulted in an optical density reading threefold greater than that obtained with negative control sera.

Nested PCR analysis. PBMC were isolated from EDTA-treated blood by Hypaque-Ficoll gradient centrifugation, and nucleic acid was extracted by means of standard techniques. One microgram of total nucleic acid was used as a template for a two-step amplification by PCR using a nested set of oligonucleotides primers specific for the envelope regions. The conditions for the first and second rounds of amplification were as described elsewhere (44). The final amplified fragment was approximately 642 bp. Amplified products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Results for a subset of samples were also confirmed by PCR with primers from long terminal repeat (LTR)-*gag* regions.

Semiquantitative PCR analysis of proviral DNA load. Proviral DNA in PBMC was measured by PCR with the use of radiolabeled primer incorporation for quantification (75) and was expressed as copies of proviral genome detected per million PBMC. Briefly, 1 μg of DNA from each sample was amplified in a PCR mixture that contained 0.2 μM each primer, 200 μM each of four deoxynucleoside triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1.0 U of *Taq* polymerase (Perkin-Elmer Cetus, Branchburg, N.J.) in a volume of 50 μl. The reaction mixture was subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 60°C, and elongation for 3 min at 70°C. The oligonucleotide primers used were derived from the nucleotide sequence of SIVmne (GenBank accession no. M32741 [37]). They consist of a primer pair specific for the envelope region, env10 (nucleotide 7191 to 7211 [sense]) and env12 (7541 to 7561 [antisense]), and a pair of primers specific for the LTR-*gag* region, S1 (228 to 251 [sense]) and S8 (536 to 559 [antisense]). The amplified products for the *env* and LTR-*gag* sequences are 370 and 330 bp, respectively. One oligonucleotide of each complementary pair was 5′ end labeled with [^{32}P]ATP by the use of polynucleotide kinase (New England Biolabs, Inc., Beverly, Mass.). The ^{32}P-labeled PCR products obtained by amplification were analyzed by electrophoresis on 8% nondenaturing polyacrylamide gels and quantified by autoradiography with phosphorimager analysis (75). Quantification of SIV DNA was determined with a standard curve generated by known quantities of a plasmid clone of E11S.

RT-QC (quantitative competitive)-PCR determination of plasma viral RNA. Plasma viral RNA was prepared as described by Watson et al. (97). The viral RNA samples were serially diluted in a 96-well PCR microplate into a reaction buffer containing a fixed copy number of a competitor RNA containing an internal deletion. The template and the competitor were subjected to reverse transcription (RT) followed by PCR. The primers used are from the SIVmne *gag* sequence: 5′ primer (5G) from nucleotides 675 to 698 (AAAGCCTGTTGGA GAACAAAGAAG); 3′ primer (3Diii) from nucleotides 993 to 1011 (AATTT TACCCAGGCATTTA). The internal RNA control contains a deletion of 82 bp which enables the discrimination between products amplified from the viral (336-bp) and the control (254-bp) templates. The conditions for the RT reaction and PCR were as described by Watson et al. (97).

Lymphocyte subset analysis. Cell surface immunofluorescence was quantified by use of a FACScan flow cytometer and Lysis II software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Lymphocyte subsets (CD4, CD8, CD2, and CD20) of whole heparinized blood samples were evaluated by conventional methods.

Statistical analysis. Differences in proportions were tested by Fisher's exact test. Differences in the mean peak proviral load for control animals and immunized animals were tested by using the *t* test. Repeated-measures analysis of variance (ANOVA) models with weeks as a fixed within-subject factor and group as a fixed between-subject factor were used to test for differences in viral load between the control group and the immunized groups in the first 20 weeks of infection. To determine if protection correlated with SIV-specific antibody responses, bivariate ANOVA models with protection status and group as fixed effects were used (the interaction between group and protection status was tested but was not significant in any of the models). If the global hypothesis test testing whether all groups were the same was rejected, all pairwise differences were tested by the Bonferroni method to adjust for multiple comparisons. The median time to AIDS for immunized and control animals was compared by using the Mann-Whitney *U* test.

RESULTS

Immunization regimen and outcome of challenge with clone E11S. Twenty-four juvenile cynomolgus macaques (*M. fascicularis*) were divided into six groups and were immunized at 0, 3, 18, and 24 months as described in Table 1. Except for animals in group A, which were immunized with subunit recombinant gp160 (rgp160) only, all animals received primary immunizations of recombinant vaccinia virus(es) followed by booster immunizations with recombinant subunit or particle vaccines. Most animals developed low levels of SIV-specific antibody responses after the primary immunization (Fig. 1). After the first booster immunization, all animals showed a significant increase in SIV-specific antibodies (Fig. 1). Although antibody titers declined after the booster immunization, they generally did not fall below the baseline (1:100). The exceptions, however, were animals immunized with subunit rgp160 only (group A). SIV antibody titers in these animals decreased to baseline level by the time of the second booster immunization (Fig. 1A). Even after the second boost, two of four animals in this group failed to generate a sustained antibody response. In contrast, all animals primed with recombinant vaccinia virus(es) showed recall responses to boosts and retained low to moderate levels of antibody titer between boosts (Fig. 1).

Four weeks after the third boost, all immunized animals, together with four naive controls, were challenged intravenously with the homologous pathogenic virus clone E11S grown on macaque PBMC. Infection was monitored by virus isolation by coculture, nested PCR analysis, and measurements of anamnestic response and seroconversion to nonvaccine antigens. Results of virus isolation and PCR analysis are summarized in Table 2.

Among animals that received just the envelope immunogens, the only group that showed significant protection were those that received rgp160 in a recombinant vaccinia virus priming and subunit protein boosts (group B). Three of the four animals in this group were completely protected (Table 2). One animal (macaque 91272) was transiently virus positive by PCR analysis and PBMC coculture, respectively, at weeks 2 and 4 after challenge. Compared with the cumulative data for 15 control animals from three separate experiments (76), immunized animals in group B showed significant protection (3 of 4 immunized animals versus 1 of 15 controls; $P = 0.02$) (Table 3).

In contrast, immunization with subunit rgp160 alone (group A) or with the surface antigen rgp130 in a prime-and-boost regimen (group C) was much less effective. Three of four animals in each group became infected after challenge (Table 2). The proportion of uninfected animals in each group failed to reach statistical significance (1 of 4 in the experimental groups versus 1 of 15 in the cumulative controls; $P = 0.4$) (Table 3). However, some degree of protection was apparent in these animals since the quantity and the duration of detectable proviral DNA in their PBMC were considerably less than those of naive controls (Fig. 2). The mean peak proviral loads ($\log_{10}$ copies/10^6 PBMC) at 2 weeks after challenge were 4.04 for the control animals and 2.97 and 2.33 for the immunized animals in groups A and C, respectively (Table 3). This difference was highly significant for animals in group C ($P = 0.004$) but marginally so for those in group A ($P = 0.07$). To test whether animals in groups A and C had lower viral loads in primary infection (through week 20), we performed repeated-measures ANOVA on proviral loads as described in Materials and Methods. Animals in both group A and group C showed significant reduction of viral load during primary infection compared to the controls (P for groups A and C are 0.005 and 0.001, respectively). Thus, immunization with gp130 in a prime-and-boost regimen, or even with subunit rgp160 alone, resulted in some degree of protection.

All four animals in group D that received only the core antigens (Gag-Pol) became infected after challenge (Table 2). However, they all showed significantly reduced viral load compared to the controls as early as 2 weeks after challenge (mean viral load of $\log_{10}$ 2.42 copies/10^6 PBMC for group D versus 4.04 for the controls; $P = 0.001$) (Fig. 2). The duration of detectable proviral DNA in their PBMC was also reduced. One animal (macaque 91278) was virus positive by PCR analysis

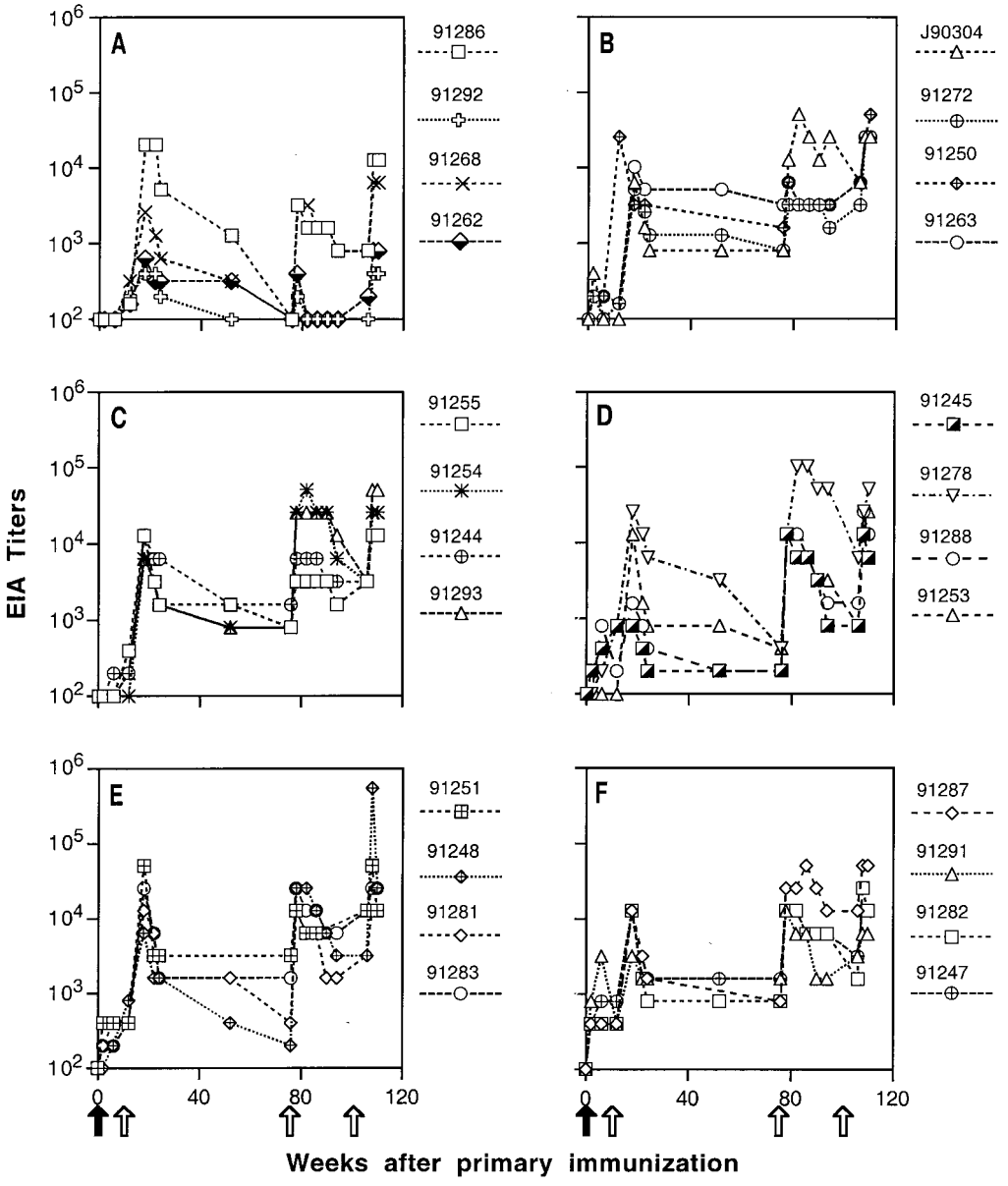

FIG. 1. ELISA analysis of SIV-specific antibody responses in immunized macaques prior to challenge. Dilutions of macaque sera collected at the indicated times were incubated with disrupted, sucrose gradient-purified SIVmne virions proteins on microtiter plates. Endpoint ELISA (EIA) titers were defined as the reciprocal of the highest dilution that gave an optical absorbance value at least threefold higher than the average values obtained with SIV-negative macaque sera. Panels A through F correspond to experimental groups A through F (Table 1). Positions of the arrows indicate the times of primary (solid arrows) and booster (open arrows) immunizations.

only at week 2 after challenge. Proviral loads in primary infection (through week 20) were also significantly lower for group D animals than for the control animals ($P < 0.001$). These results indicate that although immune responses to the core antigens were not protective, they facilitated the clearance of virus-infected cells and the control of infection.

Animals immunized with both envelope and core antigens (groups E and F) showed high degrees of protection. Three of the four animals in group E and all four animals in group F were completely protected against E11S challenge (Tables 2 and 3; Fig. 2E and F). Compared to the data for the cumulative controls, the *P* values were 0.02 and 0.001 for groups E and F respectively. This result, together with those from animals immunized with Gag-Pol particles only (group D), reaffirms the important role of immune responses against the envelope antigens in protection against the homologous virus.

SIV-specific antibody responses in animals challenged with clone E11S. To determine if protection correlated with SIV-specific antibody responses, we analyzed by ELISA and virus neutralization assays the sera collected from immunized animals on the day of challenge. With the exception of two animals in group A (macaques 91292 and 91262), all immunized animals generated moderate levels of SIV-specific antibody response as measured by whole-virus ELISA (Fig. 3C). Among animals immunized with envelope antigens, the mean ELISA titer for animals in group A ($\log_{10}$ 3.39) was significantly lower than that for animals in groups B ($\log_{10}$ 4.45) and C ($\log_{10}$ 4.37) ($P = 0.05$ in both cases), indicating the potential advantage of

TABLE 2. Virus isolation and nested PCR analysis of PBMC and lymph node cells from macaques after intravenous challenge with clone E11S

Group[a]	Macaque	Results of analysis[b] at indicated wk postchallenge														
		2	4	8	12	16	20	28	36	44	60	99	148		183	200
A	91286	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−		−/−	
	91292	+/+	+/+	−/+	+/+	−/−	+/+/+	−/+	−/+	−/−	−/−	−/+	−/−		−/+	E
	91268	+/+	−/+	−/−	−/−	+/+	−/+/+	−/+	−/−	−/+	−/−	−/−	−/−		−/−	E
	91262	+/+	+/+	+/+	−/−	−/−	+/+/+	−/+	+/+	+/+	+/+	+/+	A			
B	J90304	−/−	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	R						
	91272	−/+	+/−	−/−	−/−	−/−/+	−/−/+	−/−	+/−	−/+	+/+	A				
	91250	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	R						
	91263	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	R						
C	91255	+/+	+/+	−/+	+/−	−/−	−/−/+	−/−	−/+	−/−	−/+	−/+	+/+		+/+	E
	91254	+/+	+/+	−/−	+/−	−/−	−/−/+	−/−	−/−	−/−	−/−	−/+	−/−		−/−	E
	91244	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−		−/−	
	91293	+/+	+/+	−/+	−/+	NT/−/+	−/+	−/−	−/+	−/−	−/−	−/−	−/−		−/−	E
	2	4	8	12	16	20	28	36	45	66	99	150	173	183	220	
D	91245	+/+	+/+	−/+	+/+	+/+	−/+/+	−/+	−/−	−/+	−/−	−/+	−/−	−/−	−/−	E
	91278	−/?	−/−	−/−	−/−/−	−/−	−/−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	
	91288	+/+	−/+	−/−	−/−/+	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	
	91253	+/+	−/+	−/+	−/+	−/+/+	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	E
E	91251	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	R						
	91281	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	R						
	91248	+/+	+/+	+/+	−/+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	A	
	91283	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	R						
F	91287	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	−/−	R						
	91291	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	R						
	91282	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	−/−	R						
	91247	−/−	−/−	−/−	−/−	−/−/−	−/−	−/−	−/−	R						
Control	91077	+/+	+/+	+/+	−/+	−/+/+	−/+	−/+	−/+	−/−	−/+	−/−	−/−	−/−	−/+	E
	91078	+/+	+/+	+/+	−/+	−/+	−/+/+	−/+	−/+	+/+	−/+	−/+	−/−	−/−	−/−	E
	M92387	+/+	+/+	+/+	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	A		
	93057	+/+	+/+	+/+	+/+	+/+/+	−/+	−/+	−/+	−/+	−/+	−/+	−/−	−/−	NT/−	E

[a] See Table 1 and Materials and Methods for details of the immunization regimen.
[b] Presented as positive (+) or negative (−) in the following assays: virus isolation from PBMC/PCR analysis of PBMC DNA/PCR analysis of lymph node DNA. When only two results are indicated, they refer to those from the first two assays. The cause of death is denoted as AIDS-related euthanasia (A) or elective euthanasia (E). R, reassigned to be rechallenged with uncloned SIVmne; nt, not tested; ?, results inconclusive.

primary immunization with recombinant vaccinia virus. However, there is no apparent correlation between the titer of SIV-specific antibodies on the day of challenge and protection.

Serum neutralization activities were measured by two different assays. Only a minority of animals had detectable levels of neutralizing antibody against the homologous virus on the day of challenge (Fig. 3B). However, all animals immunized with envelope antigens alone, or with both envelope and core antigens, showed low to moderate levels of neutralizing activities against a related but heterologous virus, SIVmac251 (Fig. 3A). As expected, no neutralizing antibody was detected in animals immunized with core antigens alone. Again, there was no correlation between protection and serum neutralizing activity as measured by either assay.

Protection against uncloned SIVmne. To examine the breadth of protective immunity, we boosted 10 animals in groups B, E, and F that were completely protected against E11S and rechallenged them with uncloned SIVmne. These animals were virus negative at all times for 8 to 9 months after the E11S challenge by all criteria tested (virus isolation, nested PCR analysis of PBMC and lymph node cells, anamnestic response, and, for group B animals, seroconversion to nonvaccine antigens). As expected from our previous studies (76), only partial protection against the uncloned SIVmne was achieved by immunization with rgp160 alone with the prime-and-boost regimen (group B). One animal was protected (macaque 91263), one was transiently virus positive (macaque 91250), and one was

TABLE 3. Summary of results after E11S challenge

Group[a]	Protection against E11S challenge			
	No. protected/ total	P^b	Mean peak proviral load[c] ($\log_{10}$ copies/ 10^6 PBMC)	P
A	1/4	0.4	2.97	0.07
B	3/4[d]	0.02		
C	1/4	0.4	2.33	0.004
D	0/4[e]	1.0	2.42	0.001
E	3/4	0.02		
F	4/4	0.001		
Control	0/4		4.04	
Cumulative controls[f]	1/15			

[a] See Table 1 and Materials and Methods for details of the immunization regimen.
[b] Calculated by using data from cumulative controls. If data from concurrent controls are used, only results from group F after E11S challenge are statistically significant ($P = 0.03$).
[c] At 2 weeks after challenge.
[d] The infected animal in this group (macaque 91272) was virus positive by nested PCR and virus isolation from PBMC only at weeks 2 and 4, respectively, after challenge. However, its virus load increased significantly about 1 year after infection, and the animal eventually developed AIDS (76).
[e] One of the infected animals (macaque 91278) was virus positive only by semiquantitative PCR analysis of PBMC collected at weeks 2 after challenge.
[f] Including 15 animals infected intravenously with E11S in three separate experiments (76).

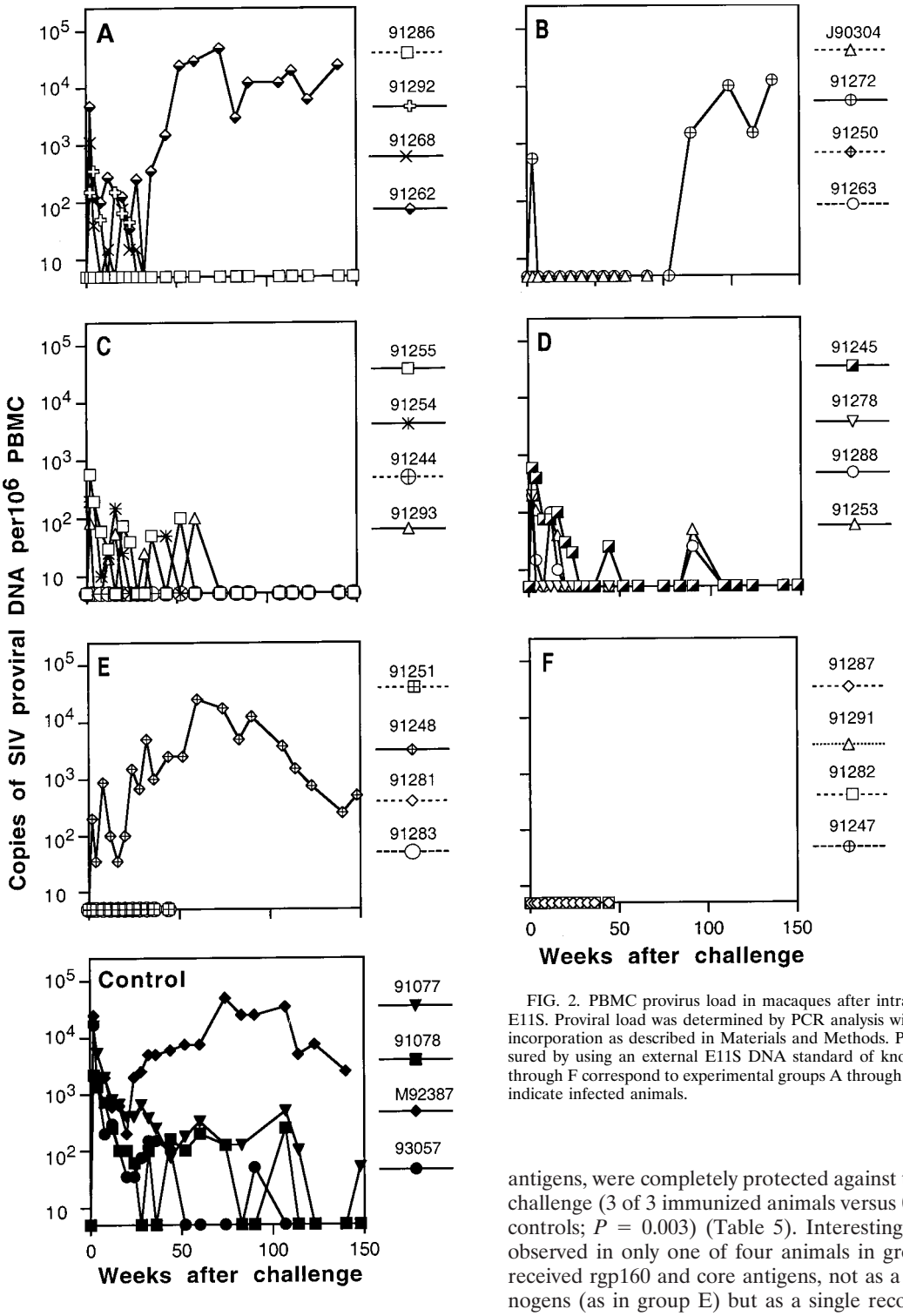

FIG. 2. PBMC provirus load in macaques after intravenous challenge with E11S. Proviral load was determined by PCR analysis with radiolabeled primer incorporation as described in Materials and Methods. Proviral DNA was measured by using an external E11S DNA standard of known quantity. Panels A through F correspond to experimental groups A through F (Table 1). Solid lines indicate infected animals.

persistently infected (macaque J90304) (Table 4). Concordant results were obtained by virus isolation and nested PCR analyses of PBMC and lymph node cells (Table 4), by QC-PCR analyses of viral load in PBMC (data not shown) and plasma (Fig. 4), and by ELISA of SIV-specific serum antibody responses after challenge (Fig. 5). In contrast, all three animals in group E, which were immunized by both gp160 and core antigens, were completely protected against the uncloned virus challenge (3 of 3 immunized animals versus 0 of 10 cumulative controls; $P = 0.003$) (Table 5). Interestingly, protection was observed in only one of four animals in group F, which also received rgp160 and core antigens, not as a mixture of immunogens (as in group E) but as a single recombinant virus for priming and pseudovirion for boosting. Again, concordant results were obtained from all analyses performed to determine protection or infection in these animals (Table 4; Fig. 4 and 5).

SIV-specific antibody responses in animals challenged with uncloned SIVmne. After the first challenge by E11S virus, titers of SIV-specific antibodies in all the protected (i.e., uninfected) animals declined approximately 10-fold over 6 to 7 months and then stabilized (data not shown). A final booster immunization was given 4 weeks prior to the uncloned virus challenge, re-

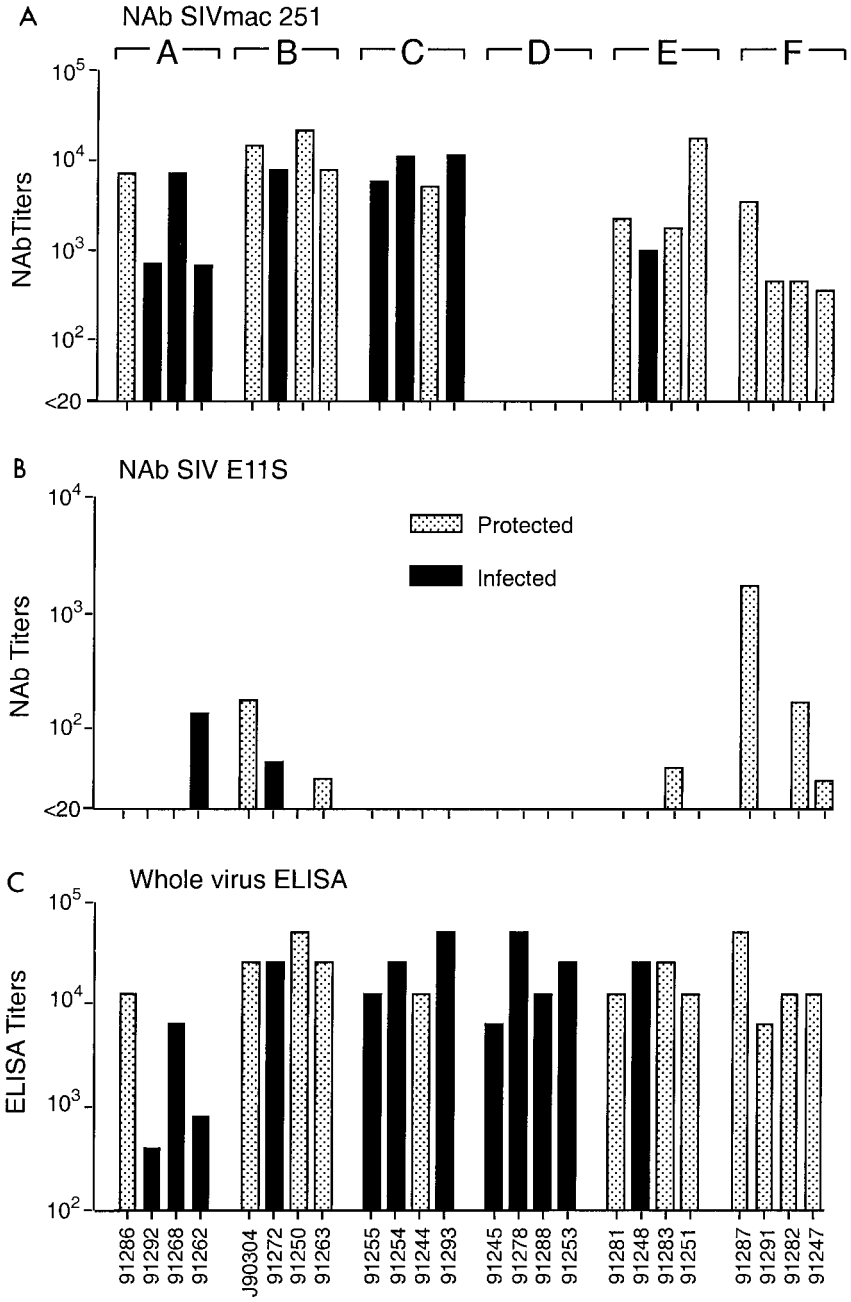

FIG. 3. SIV-specific antibody responses in immunized macaques on day of challenge with E11S. Sera collected on the day of challenge were analyzed for neutralizing antibody (NAb) activities against the homologous challenge virus E11S or a heterologous virus, SIVmac251. Neutralizing titers are expressed as the reciprocal serum dilutions that results in >90% reduction of syncytium formation by SIVmac251 on HuT78 cells (A) or >50% reduction of cytopathicity of E11S-infected CEMx174 cells (B). Serum reactivity with disrupted SIVmne E11S virion proteins was analyzed by ELISA, and the results are expressed as endpoint titers (C). Designations of experimental groups (A through F) are indicated at the top.

sulting in recall responses in most of the animals. On the day of challenge, all but one animal (macaque 91282) had SIV-specific antibody titers comparable to those prior to the E11S challenge (compare Fig. 6C and 3C). With the exception of the same macaque 91282, comparable titers of neutralizing antibodies against SIVmac251 were also detected (Fig. 6A and 3A). All animals also showed low to moderate levels of serum neutralizing antibodies against the challenge virus, uncloned SIVmne (Fig. 6B). However, we failed to observe any correlation between protection and the titer of SIV-specific antibodies as determined by any of these three assays. After uncloned SIVmne challenge, SIV-specific antibody titers declined in all protected animals and one transiently virus-positive animal (macaque 91250), whereas titers increased in all persistently infected animals, as in the infected control animals (Fig. 5).

Clinical outcome of infection. Infected animals were maintained for more than 3 years after challenge to determine the clinical outcome of infection and the effects of immunization. Animals were monitored periodically for lymphocyte subsets, hematology, blood chemistry, body weight, opportunistic infections, and proliferative diseases. Figures 7 and 8 summarize

TABLE 4. Virus isolation and nested PCR analysis of PBMC and lymph node cells from macaques after intravenous challenge with uncloned SIVmne

Group[a]	Macaque	Results of analysis[b] at indicated wk postchallenge														
		2	6	8	12	16	20	32	48	56	64	87	126	136	162	220
B	J90304	+/+	+/+/+	+/+	−/+	+/+	−/+	+/+	+/+	+/+	+/+	+/+	−/+	+/+	−/+	A
	91250	+/−	−/+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	
	91263	−/−	−/−/−	−/−	−/−	−/−	−/?	−/−	−/−	?	−/−	−/−	−/−	−/−	−/−	
E	91251	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	
	91281	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	
	91283	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	
F	91287	+/+	−/+	−/−	−/+	−/+	−/−	+/+	−/+	+/+	+/+	+/+	A			
	91291	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	
	91282	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	A				
	91247	−/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	A					
Control	91064	+/+	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	A		
	91070	+/+	+/+	NT/+/+	+/+	+/+	+/+	−/+	+/+	+/+	+/+	+/+	+/NT	+/+	A	
	92168	+/+	+/+	+/+/+	−/+	−/+	−/+	+/+	−/+	−/+	−/+	−/−	−/−	+/+	−/−	E

[a] See Table 1 and Materials and Methods for details of the immunization regimen.
[b] Presented as in Table 2. The cause of death is indicated as AIDS-related euthanasia (A) or elective euthanasia (E). NT, not tested; ?, results inconclusive.

their peripheral blood $CD4^+$ cell levels and survival time after challenge.

To help conserve animals, we only used a small number of control animals in this study. We therefore included in our analysis cumulative data from three studies (76) in which control animals were similarly infected with E11S or uncloned SIVmne. As reported previously, 50 to 60% of naive control *M. fascicularis* infected with either E11S or uncloned SIVmne developed AIDS-like diseases within 3 years after infection (Fig. 7 and 8, Control). However, $CD4^+$ cell depletion occurs more rapidly in some of the animals infected with the uncloned virus (Fig. 8) than in those infected with E11S (Fig. 7).

Prior immunization did not appear to affect significantly the clinical course of infection. There was no statistically significant difference between controls and each group of immunized and infected animals in their rate of $CD4^+$ cell decline and progression to AIDS. Among 12 immunized animals infected with E11S, 3 were euthanatized due to AIDS between 1.9 to

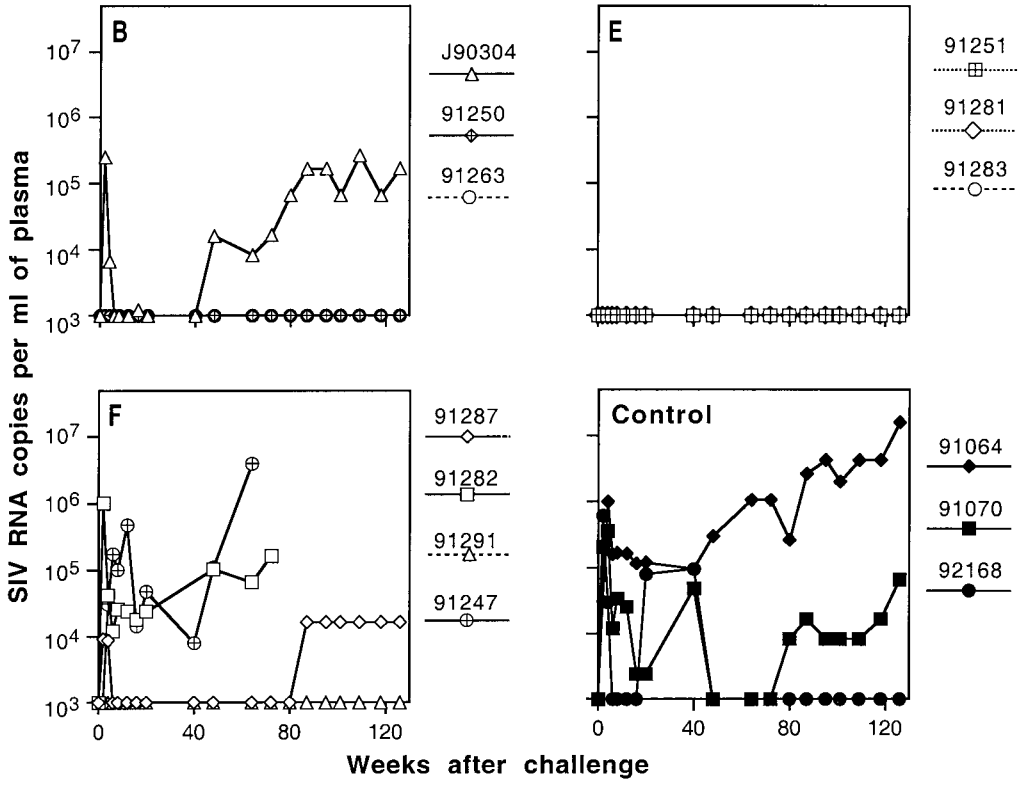

FIG. 4. Plasma viral load in macaques after uncloned SIVmne challenge. Plasma viral load was determined by RT-QC-PCR using an internally controlled template as described in Materials and Methods. Panels B, E, and F correspond to experimental groups B, E, and F. Solid lines indicate infected animals.

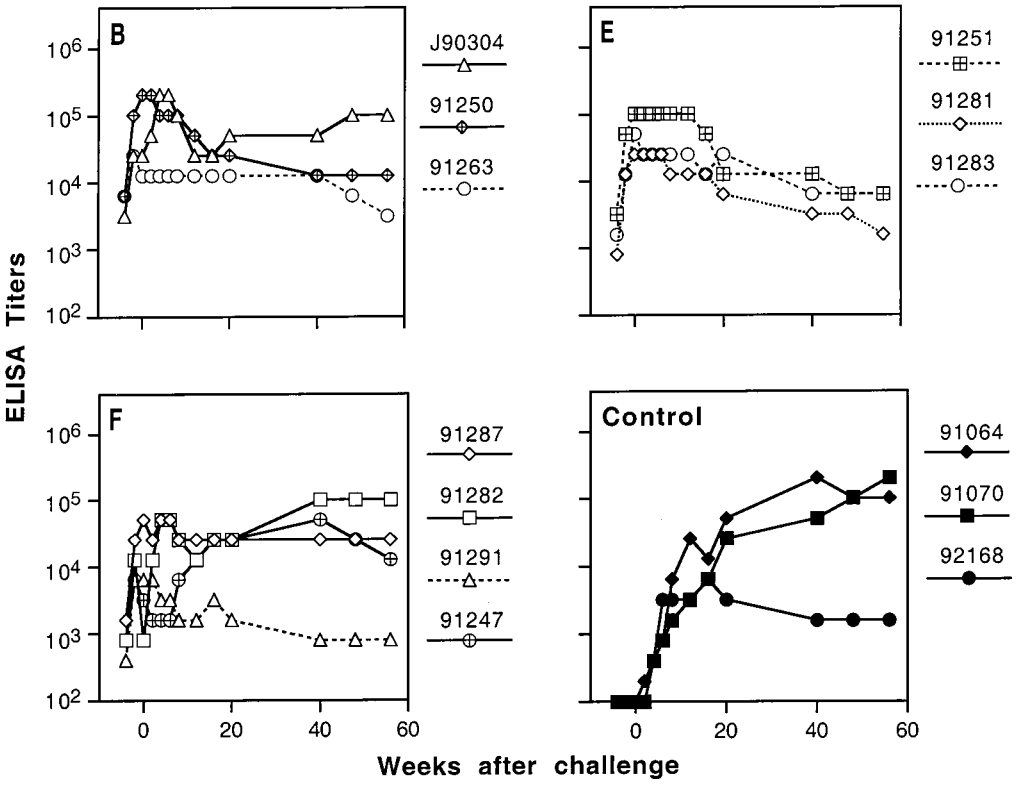

FIG. 5. ELISA analysis of SIV-specific antibodies in macaques after uncloned SIVmne challenge. Animals in experimental groups B, E, and F (represented by panels B, E, and F) that were protected against E11S challenge were held for 8 to 9 months to confirm their virus-negative status. They were then boosted again and challenged 4 weeks later with uncloned SIVmne (week 0 on the abscissa). Endpoint titers were defined as the reciprocal of the highest dilution that gave an optical absorbance value at least threefold higher than the average values obtained with SIV-negative macaque sera. Solid lines indicate infected animals.

3.5 years after challenge (Fig. 7A to F). In comparison, 5 of 10 naive controls infected with the same virus were euthanatized within the same period ($P = 0.4$). Among animals infected with the uncloned virus, 4 of 5 of the immunized animals developed AIDS (Fig. 8B, E, and F), compared with 6 of 10 of the naive controls ($P = 0.6$). The median survival times for immunized and control macaques that developed AIDS after infection with uncloned SIVmne were 64 and 90.5 weeks, respectively (Fig. 8, $P = 0.7$).

All immunized animals that were completely protected against virus challenge showed no sign of infection throughout the study period (8 to 9 months for animals used for rechallenge and up to 3.5 years for the rest) (Tables 2 and 4). Their clinical status and $CD4^+$ cell levels remained normal during this time (Fig. 7 and 8).

DISCUSSION

We have shown that vaccine efficacy is dependent on the immunization regimen as well as the nature of the immunogens. To date, immunization with recombinant subunit vaccines containing either the envelope or the core antigens has not resulted in complete protection against pathogenic primate lentiviruses (3, 21, 29, 38, 70, 76). By incorporating both immunogens in a prime-and-boost immunization strategy, we achieved complete protection against intravenous infection by an uncloned virus of moderate pathogenicity, SIVmne.

We previously reported that immunization with the envelope antigen rgp160 in a prime-and-boost regimen resulted in complete protection against intravenous and intrarectal challenge by a homologous cloned virus SIVmne E11S (44, 76, 77). In this study, we examined factors that potentially affect the breadth and the efficacy of this immunization approach. Our findings indicate that priming with recombinant vaccinia virus played a key role in potentiating the protective efficacy of envelope-based vaccines. The mechanism by which this was achieved remains unclear. However, one or more known properties of vaccinia virus infection may have contributed. These include endogenous expression of antigens resulting in presentation by the major histocompatibility complex class I molecules and the generation of CTL responses (20, 88, 101), in situ presentation of native antigenic structures (33, 39, 41), prolonged and low-level expression of antigens, and possible adjuvant effects due to the cumulative local and systemic re-

TABLE 5. Summary of results after uncloned SIVmne challenge

Group[a]	Protection against uncloned SIVmne challenge	
	No. protected/total	P^b
B	1/3[c]	0.2
E	3/3	0.003
F	1/4	0.3
Control	0/3	
Cumulative controls[d]	1/10	

[a] See Table 1 and Materials and Methods for details of the immunization regimen.

[b] Calculated by using cumulative controls.

[c] One of the two infected animals, macaque 91250, was virus positive only by virus isolation at week 2 after challenge and by nested PCR at week 6.

[d] Including 10 animals infected intravenously with uncloned SIVmne in three separate experiments (76).

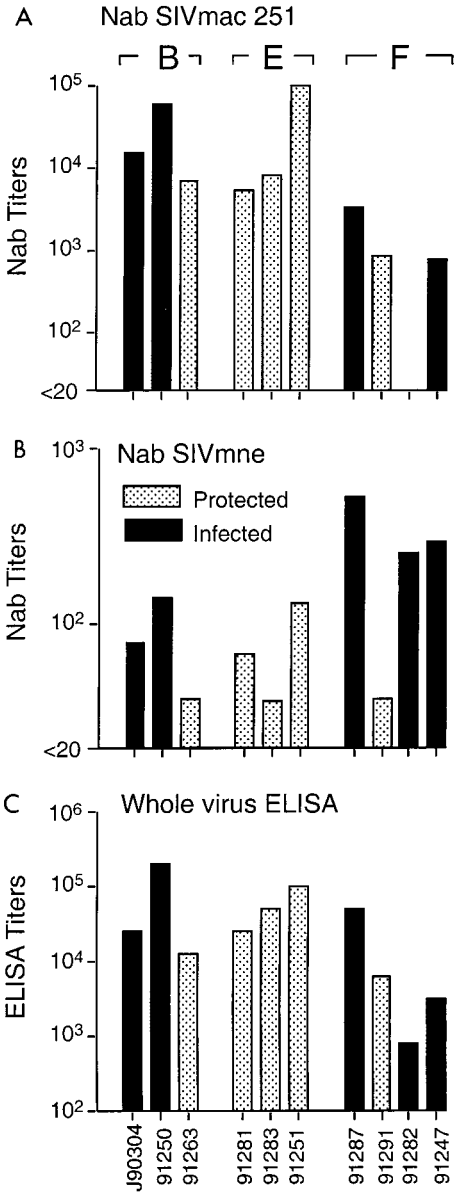

FIG. 6. SIV-specific antibody responses in immunized macaques on day of challenge with uncloned SIVmne. Sera collected on the day of rechallenge were analyzed for neutralizing antibody (NAb) activities against the homologous challenge virus uncloned SIVmne or a heterologous virus, SIVmac251. Neutralizing titers are expressed as the reciprocal serum dilutions that results in >90% reduction of syncytium formation by SIVmac251 on HuT78 cells (A) or >50% reduction of cytopathicity of uncloned SIVmne-infected CEMx174 cells (B). Serum reactivity with disrupted SIVmne E11S virion proteins was analyzed by ELISA, and the results are expressed as endpoint titers (C). Designations of experimental groups (B, E, and F) are indicated at the top.

sponses to vaccinia virus infection (66). Since the initial success with the prime-and-boost strategy, a number of investigators have used other viral vectors as well as naked DNA for priming immune responses (1, 3, 38, 55, 70, 80). It would be of interest to compare these approaches to gain further insight on the requirements for efficient priming. It also remains to be shown whether subunit envelope vaccines alone, perhaps with improved adjuvants and immunization regimen, could prime protective responses as well as live viruses.

Our results also showed that immunization with the full-length envelope antigen rgp160 was superior to the surface antigen rgp130, thus indicating a potentially important role for the transmembrane protein (TM). It is possible that immune responses directed to TM itself may contribute to protection. Multiple antigenic sites, including immunodominant epitopes, have been identified in the TM of HIV-1 (27, 31, 72, 99) and SIV (90, 92). They serve as targets for antibody-dependent cellular cytoxicity (57, 94), virus neutralization (68, 69, 93), complement-binding antibody (63, 81, 82), lymphoproliferative (87), and CTL (48, 49, 84, 85, 95) responses. Although their roles in vivo have yet to be defined, one or more of these responses may participate in protection. Alternatively, TM may help present functionally important epitopes in the surface antigen by maintaining proper oligomeric structure (17, 23, 78). Recent studies revealed important functional and antigenic differences between oligomeric and monomeric forms of HIV-1 envelope proteins (14, 65, 79, 86). It is possible that such differences contribute in part to the apparent disparity in the protective efficacy of rgp130 and rgp160. It is of interest that partial protection was observed in macaques immunized with virion-derived oligomeric, but not monomeric, forms of gp130 (73). In that study, better clinical outcome was correlated with high neutralizing antibodies and long-lasting reactivity to the TM after challenge (74). Finally, the full-length and functionally competent envelope glycoprotein expressed in the context of recombinant vaccinia virus priming may expose epitopes important for cell fusion and virus infectivity. LaCasse et al. recently described a "fusion-competent" vaccine that was capable of eliciting broadly neutralizing antibodies against primary HIV-1 isolates (53). It is of interest to determine if one or more of these responses may contribute to the protective immunity elicited by the native full-length envelope vaccines in the prime-and-boost regimen described here.

Immunization with core antigens alone failed to protect against the homologous cloned virus E11S. However, even in the absence of any neutralizing (or *env*-specific) antibodies, immunized macaques showed significantly reduced viral load compared to controls as early as 2 weeks after infection. The duration of detectable proviral DNA in their PBMC was also reduced, with one animal (macaque 91278) showing only transient infection. These results indicate that immune responses to the core antigens may not protect against infection but may help to control infection, perhaps by facilitating the clearance of virus-infected cells. The mechanism by which this is achieved has yet to be determined. Kent et al. (50) examined a subset of animals in this study and found SIV-specific lymphoproliferative responses in eight of eight animals prior to challenge but no CTL response. CTL response was detected after challenge in a number of animals, including one, macaque 91278, that was immunized with core antigens alone and showed only transient viremia after infection. Thus, the rapid onset and persistent CTL responses induced after infection may play an important role in controlling infection after challenge. It should be noted, however, that CTL responses to a single Gag epitope failed to protect against the same E11S challenge virus (100). Viral load was not determined in that study. It is possible that effective CTL responses to multiple targets, primed by immunization and expanded after exposure, reduce viral load sufficiently, resulting in transient or inapparent infection and thus partial if not complete protection.

Immunization with both envelope and core antigens was highly protective. This was most apparent in animals immunized with these antigens as separate immunogens (group E). Significant protection was observed in these animals against not only the homologous virus E11S but also the uncloned virus upon rechallenge. We reported earlier that immunization with rgp160 alone by the prime-and-boost regimen has only

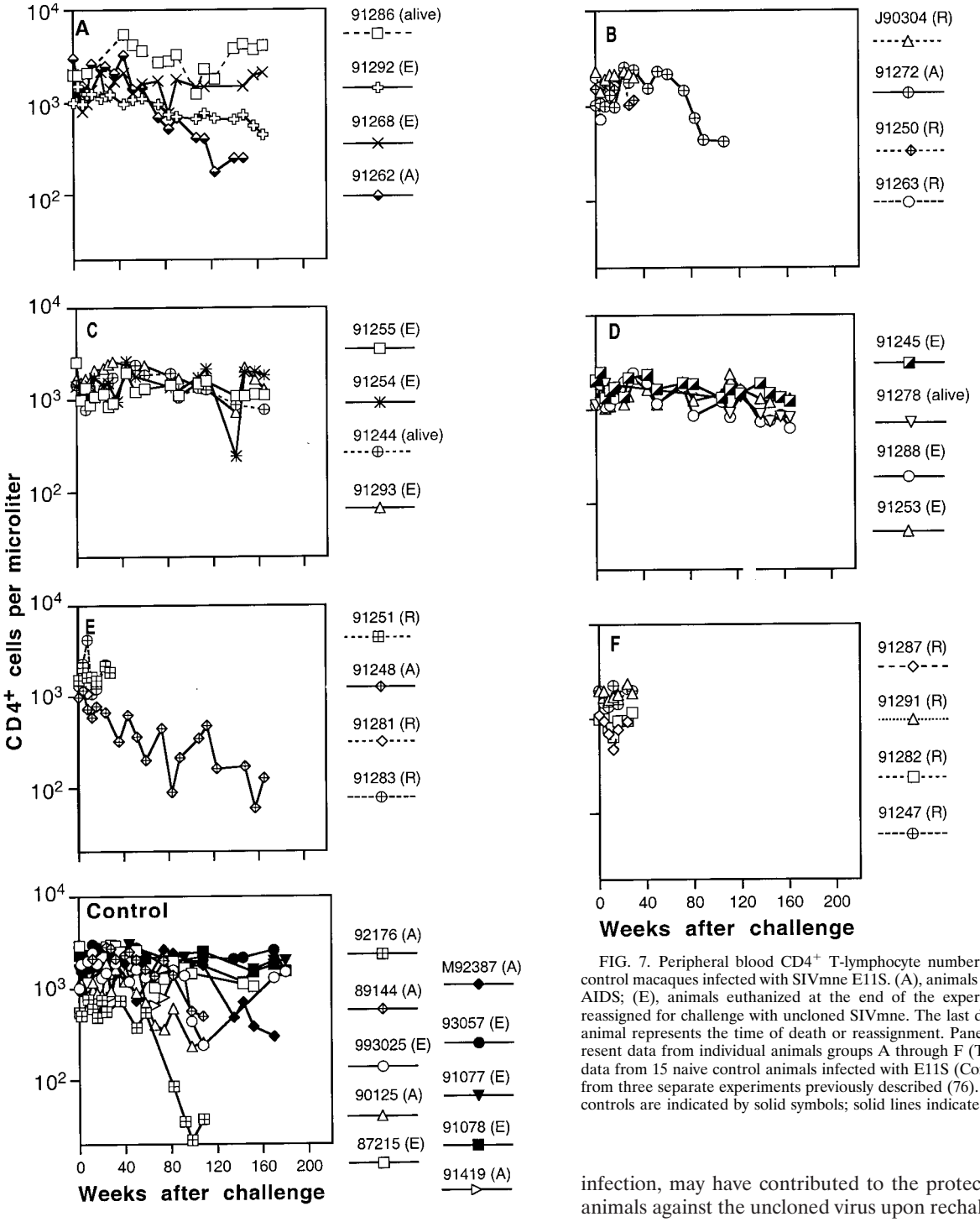

FIG. 7. Peripheral blood $CD4^+$ T-lymphocyte numbers in immunized and control macaques infected with SIVmne E11S. (A), animals sacrificed because of AIDS; (E), animals euthanized at the end of the experiment; (R), animals reassigned for challenge with uncloned SIVmne. The last datum point for each animal represents the time of death or reassignment. Panels A through F represent data from individual animals groups A through F (Table 1). Cumulative data from 15 naive control animals infected with E11S (Control) were obtained from three separate experiments previously described (76). Data for concurrent controls are indicated by solid symbols; solid lines indicate infected animals.

limited efficacy against an intravenous infection by the uncloned virus, especially the non-E11S-type (or variant-type) viruses present in the challenge stock (76). Therefore, it is likely that immune responses to the core antigens may have contributed to controlling infection by both the E11S- and variant-type viruses. This is consistent with the highly conserved nature of the core antigens. Alternatively, prior exposure to the E11S virus, even without resulting in an apparent infection, may have contributed to the protection in group E animals against the uncloned virus upon rechallenge. This possibility cannot be excluded without a direct comparative study. However, it should be noted that we found no sign of infection in these animals by multiple and stringent assays over a period of 8 to 9 months after E11S challenge. Furthermore, complete protection was not achieved against intravenous infection by the uncloned SIVmne in animals immunized only with rgp160, including those previously challenged by and protected from E11S (76). Similarly, three of four animals in group F of the present study became infected by the uncloned virus, even though they were previously protected against E11S. Therefore, complete protection against the uncloned virus observed

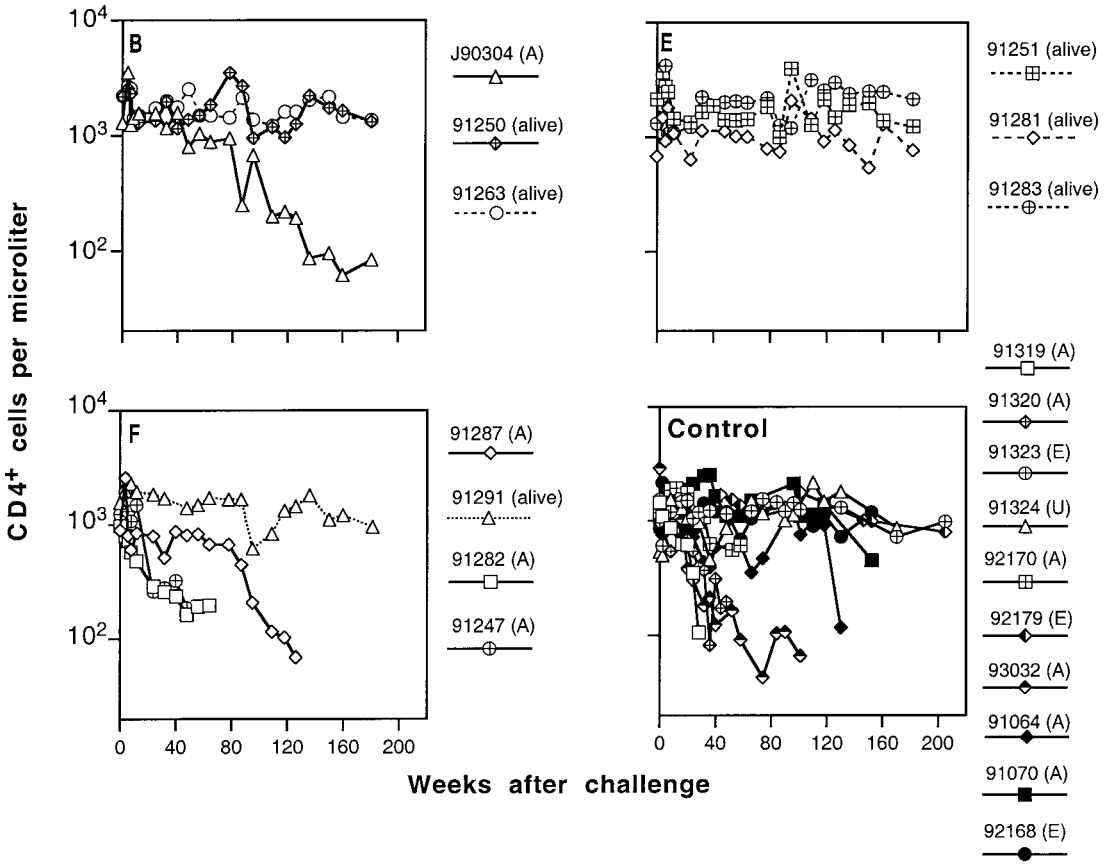

FIG. 8. Peripheral blood CD4+ T-lymphocyte numbers in immunized and control macaques infected with uncloned SIVmne. Designations (A) and (E) are as described in the legend to Fig. 7; the animal that died of causes unrelated to AIDS is labeled (U). The last datum point for each animal represents the time of death or termination of the experiment. Panels B, E, and F represent data from individual animals in groups B, E, and F (Table 1). Cumulative data from 10 naive control animals infected with uncloned SIVmne (Control) are obtained from three separate experiments previously described (76). Data for concurrent controls are indicated by solid symbols; solid lines indicate infected animals.

in animals in group E could not be attributed solely to the potential effects of prior exposure to E11S. Nevertheless, a challenge study with a newly immunized cohort will be needed to confirm the findings of the present study.

It is not clear why protection against the uncloned virus was much less efficient in group F animals than in those in group E, since they were all immunized with both envelope and core antigens, albeit presented in different forms. Several factors could have contributed. First, the quantity of envelope antigens present in the Gag-Pol-Env (pseudovirion) preparations was considerably less (~3-fold) than that in the mixed immunogens used in group E. Second, the envelope antigens used for groups E and F may have very different compositions and conformations. The surface protein could have shed from the pseudovirion preparations, or its conformation could have been altered, during formulation. The suboptimal responses to the envelope antigens in the group F animals, together with responses to the core antigens, may have been sufficient to protect against E11S infection but not against the uncloned virus. Finally, the lack of protection may also be due to poor responses in individual animals in group F. Recall responses to the last booster immunization in macaques 91282 and 91247 were so short-lived that their antibody titers as determined by one or more assays were significantly lower at the time of rechallenge compared to the E11S challenge. Our results therefore demonstrate that although multicomponent particle vaccines, such as the psuedovirions described here, may have advantages over subunit immunogens, significant problems need to be resolved before such potentials can be realized.

Results from this study have several important implications for the current efforts in HIV-1 vaccine development. First, findings from our present and previous (44, 76, 77) studies demonstrate the feasibility of immunization with recombinant vaccines against a primate lentivirus of moderate complexity and pathogenicity. This is significant in view of the recent findings concerning the safety of live attenuated virus approaches (2, 18, 32). Second, results from the present study (i.e., the comparison between immunization with subunit rgp160 alone versus priming with live recombinant virus and boosting with gp160) point to the advantages of a combination approach such as the prime-and-boost regimen described here. Large-scale clinical trials were recently initiated to evaluate the potential efficacy of subunit vaccines based solely on HIV-1 surface antigens (28, 96). While the efficacy of any vaccine can be demonstrated only by properly designed clinical trials, our studies indicate that immunization with subunit immunogens alone, especially with monomeric surface antigens, may not be the most effective approach. Finally, our results indicate that complex immune mechanisms targeted to multiple antigens may contribute to protection. These results indicate the potential advantage for the inclusion of multiple antigens in a combination immunization strategy for the design of recombinant vaccines against AIDS.

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