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Vaccine Protection by a Triple Deletion Mutant of Simian Immunodeficiency Virus

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Twelve rhesus monkeys were vaccinated with SIVmac316Δnef (lacking nef sequences), and 12 were vaccinated with SIVmac239Δ3 (lacking nef, vpr, and upstream sequences in U3). SIVmac316 and SIVmac239 differ by only eight amino acids in the envelope; these changes render SIVmac316 highly competent for replication in macrophages. Seventeen of the animals developed persistent infections with the vaccine viruses. Seven of the 24 vaccinated animals, however, developed infections that were apparently transient in nature. Six of these seven yielded virus from peripheral blood when tested at weeks 2 and/or 3, three of the seven had transient antibody responses, but none of the seven had persisting antibody responses. The 24 monkeys were challenged in groups of four with 10 rhesus monkey infectious doses of wild-type, pathogenic SIVmac251 at weeks 8, 20, and 79 following receipt of vaccine. None of the seven with apparently transient infections with vaccine virus were protected upon subsequent challenge. Analysis of cell-associated viral loads, $CD4^+$ cell counts, and viral gene sequences present in peripheral blood in the remainder of the monkeys following challenge allowed a number of conclusions. (i) There was a trend toward increased protection with length of time of vaccination. (ii) Solid vaccine protection was achieved by 79 weeks with the highly attenuated SIV239Δ3. (iii) Solid long-term protection was achieved in at least two animals in the absence of complete sterilizing immunity. (iv) Genetic backbone appeared to influence protective capacity; animals vaccinated with SIV239Δ3 were better protected than animals receiving SIV316Δnef. This better protection correlated with increased levels of the replicating vaccine strain. (v) The titer of virus-neutralizing activity in serum on the day of challenge correlated with protection when measured against a primary stock of SIVmac251 but not when measured against a laboratory-passaged stock. The level of binding antibodies to whole virus by enzyme-linked immunosorbent assay also correlated with protection.

Infection of rhesus monkeys with simian immunodeficiency virus (SIV) is a useful model for assessing novel vaccine strategies for AIDS. SIV is a primate lentivirus that produces persistent, fatal infections of rhesus monkeys that are characterized by progressive deterioration in immune function and opportunistic infections similar to those associated with human immunodeficiency virus infection of humans. Both SIV and HIV use the CD4 molecule as the initial receptor for entry into cells, and they contain a similar complement of auxiliary genes that likely play similar roles in regulating viral replication (for reviews, see references 12, 18, and 42).

Studies with rhesus monkeys have shown that vaccine protection against intravenous challenge with SIV can be achieved. While some strains of SIV have proven relatively easy to protect against, others have been quite resistant (9, 14, 17, 24, 33, 38–40). Meaningful levels of protection against the virulent SIVmac251 have been achieved only through the use of cellular antigens under stringently proscribed conditions (3, 6, 41) and by live attenuated nef deletion mutants (2, 7). Because of the somewhat discouraging results with traditional and recombinant strategies and because of the historical success of attenuated viral vaccines, we have been pursuing a live attenuated vaccine strategy that depends upon deletion of viral genes (7, 11, 13, 15).

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Previous studies have shown that molecularly cloned SIVmac239 with *nef* sequences deleted (SIVmac239Δnef) is nonpathogenic for juvenile and adult rhesus monkeys (7, 28). SIVmac239Δnef produces a persistent infection that generates whole-virus enzyme-linked immunosorbent assay (ELISA) and neutralizing antibody responses that increase over months to years (7, 28). Challenge of these animals with pathogenic SIVmac251 or SIVmac239 at 2 1/4 years after infection with the deletion mutant resulted in 100% protection (7).

The present study was designed to investigate the effects of vaccine strain and time of vaccination on protection. Neither of the deletion mutant vaccine strains used for the present study has been tested previously. The results of our study indicate that protective capacity appears to increase with length of time of vaccination, that solid protection can be achieved with the triply deleted SIVmac239 by 79 weeks postvaccination, and that solid long-term vaccine protection is achieved with this approach in the absence of complete sterilizing immunity.

MATERIALS AND METHODS

Study design. Twenty-four rhesus monkeys (*Macaca mulatta*) were inoculated intravenously with either SIV239Δ3 (12 animals) or SIV316Δnef (12 animals). Four monkeys vaccinated with SIV239Δ3 and four monkeys vaccinated with SIV316Δnef were challenged with pathogenic SIVmac251 at 8 weeks (group A), 20 weeks (group B), or 79 weeks (group C). Animals were monitored for cell-associated virus loads by limiting dilution culture, for plasma p27 antigen by antigen capture, lymphocyte subset composition by flow cytometry, and antibody responses by whole-virus ELISA and neutralization assay. PCR was also used to assay for nef-deleted and nef-open sequences in peripheral blood mononuclear cells (PBMC) at selected time points. Six naive animals, two at each challenge time point, were sham vaccinated and challenged with wild-type SIVmac251 to

TABLE 1. Experimental design

Group (wk of challenge) and vaccine	Animals
A (8)	
SIVmac316Δnef	92B, 8QO, R49, R03
SIVmac239Δ3	92A, R24, R54, R08
No vaccine	16156, H103
B (20)	
SIVmac316Δnef	H093, 92E, H048, R30
SIVmac239Δ3	99S, 91Q, 99R, R15
No vaccine	92C, R11
C (79)	
SIVmac316Δnef	H17, 91M, R19, R18
SIVmac239Δ3[a]	R21, H061, R17, 8R0, 280-91, 296-91
No vaccine	16125, R05

[a] Because four of the animals in group C did not have typical persistent infections, 280-91 and 296-91 were added to increase the number of vaccinated animals in this group.

serve as controls for challenge virus infectivity. An additional two animals were enrolled in the 79-week challenge group (280-91 and 296-91) in order to increase the number of test animals at the late time point. Animals remained in the study until they were euthanized because of the onset of the immunodeficiency disease. Animals still alive remain in the study at the time of manuscript preparation.

Animals. Rhesus monkeys were received from Laboratory Animal Breeders and Services, Yemassee, S.C.; Caribbean Primate Research Center, Sabana Seca, P.R.; or Oregon Regional Primate Research Center, Beaverton, Oreg. Upon receipt, the monkeys underwent a 6-week quarantine, undergoing three intradermal tuberculin tests, sampling for hematology and serum chemistry profile, analysis of a rectal swab for bacterial culture and feces for occult blood, and ovum and parasite determinations. In addition, each animal was screened for antibody status with respect to SIV, simian type D retrovirus, simian T-cell lymphotropic virus type 1, herpes B, and measles viruses. All animals were antibody negative for SIV, simian type D retrovirus, and simian T-cell lymphotropic virus type 1. All animal care and use procedures conformed to the revised Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Vaccine and challenge viruses. SIVmac239Δ3 and SIVmac316Δnef stocks were prepared following transfection of cloned DNA into CEMx174 cells as described by Gibbs et al. (20). The SIVmac316 strain differs from the SIVmac239 strain by only eight amino acids in the envelope (34, 35). The clone used had a full-length transmembrane protein. Supernatants were harvested at 15 days (SIVmac239Δ3) and 11 days (SIVmac316Δnef) following transfection, clarified by low-speed centrifugation, and filtered through a 0.45-μm-pore-size filter. Aliquots were stored in the vapor phase of liquid nitrogen. The preparation and titration of the SIVmac251 challenge stock have been described elsewhere (7, 31).

Vaccination and challenge virus inoculation. Monkeys were inoculated intravenously with 1 ml containing 5 ng of p27 of SIVmac316Δnef or 5 ng of p27 of SIVmac239Δ3 diluted in RPMI 1640 without fetal bovine serum. Control monkeys received 1 ml of RPMI 1640. SIVmac316Δnef stock containing 174 ng of p27 antigen per ml was diluted by adding 1 ml of stock to 33.8 ml of medium. SIVmac239Δ3 stock containing 334 ng of p27 antigen per ml was diluted by adding 1 ml of stock to 65.8 ml of medium. For wild-type challenge, each animal received a 1-ml intravenous inoculation containing 10 animal infectious doses of uncloned SIVmac251 prepared in RPMI 1640 with 10% fetal bovine serum, penicillin-streptomycin, and L-glutamine.

Flow cytometry. Whole-blood collected in EDTA was analyzed for lymphocyte subsets CD4 (OKT4a [Ortho] and Anti-Leu-3a [Becton Dickinson, Franklin Lakes, N.J.]), CD8 (Anti-Leu-2a [Becton Dickinson]), and CDw29 (4B4 [Coulter Immunology, Hialeah, Fla.]) by a whole-blood lysis technique. Antibody (5 to 20 μl, depending on the antibody) was added to 100 μl of EDTA whole blood and incubated in the dark for 10 min. Lysing solution (2 ml; Becton Dickinson) was added, and samples were incubated for 10 min at room temperature. Stained cells were washed once in minimum essential medium with 5% fetal calf serum and then fixed in 0.5% paraformaldehyde. Samples were analyzed on a Becton Dickinson FACScan cytometer.

Cell-associated virus loads by limiting-dilution coculture. PBMC were separated over Ficoll-Paque from heparinized whole blood. Twelve serial 1:3 dilutions of isolated PBMC, beginning with 10^6 cells, were cocultured in duplicate with 10^5 CEMx174 cells per well in 24-well plates (total volume, 1 ml). After 3 to 4 days of culture, 1 ml of medium (RPMI 1640 containing 10% fetal bovine serum, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), L-glutamine, penicillin-streptomycin, 2-mercaptoethanol, and amphotericin B [Fungizone]) was added to each well. The cultures were split every 3 to 4 days at a 1:1 dilution. Supernatant samples were collected after 21 days of culture and stored frozen at ≤−70°C until being assayed for p27 antigen with the Coulter p27 antigen assay kit.

Assay of neutralizing antibodies to laboratory-passaged SIVmac251. Serum was heat inactivated for 30 min at 56°C. Twofold serial dilutions of heat-inactivated serum, 25 μl per well, were incubated for 1 h at 37°C in 5% CO_2 with 25 μl of diluted SIVmac251 per well in 96-well plates. The SIVmac251 for these assays had been extensively passaged and is sensitive to neutralization. Following the 1-h incubation, 3×10^4 MT4 cells were added at 50 μl per well, and the cultures were incubated at 37°C in 5% CO_2 for 13 or 14 days with additional media (RPMI 1640 containing 10% fetal bovine serum, HEPES, L-glutamine, penicillin-streptomycin, 2-mercaptoethanol, and amphotericin B) added twice a week. Neutralizing antibodies were determined by the presence of viable cells as determined by a standard MTT assay (4, 8).

Assay of neutralizing antibodies to a primary stock of SIVmac251. A primary stock of SIVmac251 was prepared by a single expansion of the animal challenge stock in rhesus PBMC. Neutralization of the rhesus PBMC-grown, animal challenge stock of SIVmac251 was measured in CEMx174 cells by a reduction in SIV $p27^{gag}$ antigen synthesis. Cell-free virus (20 μl containing 11 ng of p27 per ml) was incubated with an equal volume of 1:2-diluted, heat-inactivated test serum (final dilution, 1:4) for 1 h at 37°C in duplicate wells of 96-well U-bottom plates. An additional six wells containing no test serum (control wells) were included to determine the kinetics of virus replication in the absence of neutralizing antibodies. CEMx174 cells (10^5 cells in 100 μl) were added to each well and incubated at 37°C for 4 h. Cells were then washed three times with 200 μl of growth medium, resuspended in 250 μl of growth medium, and incubated at 37°C in fresh 96-well flat-bottom culture plates. Cell suspensions (25 μl) were collected every day beginning on day 3 and mixed with 225 μl of 0.5% Triton X-100, and virus production was quantified by p27 immunoassay as suggested by the supplier (Coulter Immunology). Fresh growth medium was added in place of the cell suspensions that were removed each day. SIV p27 production in control wells increased linearly from 0.5 to 12.5 ng/ml on days 3 to 7. SIV p27 production in the presence of test sera was measured on day 6, at which time control wells contained an average of 7.2 ng of p27 per ml.

Whole-virus ELISA. Immulon-2 flat-bottom 96-well plates (Dynatech Laboratories, Chantilly, Va.) were coated for 2 h at 37°C with 100 μl per well of Sepharose-purified, detergent-disrupted SIVmac251 diluted in a carbonate buffer (8). The plates were washed three times with phosphate-buffered saline (PBS) containing 1% Tween 20 (D-PBS). Serum was assayed in duplicate 1:100 dilutions prepared in D-PBS with 1% bovine serum albumin. The plates were incubated at 37°C for 2 h, washed three times, and then incubated for 1 h at 37°C with 100 μl of the alkaline phosphatase-conjugated goat anti-human immunoglobulin G (heavy and light chains) per well. Following a 1-h incubation at 37°C, the plates were washed three times and the color was developed with 200 μl of *p*-nitrophenyl phosphate (Sigma, St. Louis, Mo.) per well. The color reaction was stopped after 30 min at room temperature by adding 50 μl of 3 N NaOH per well. Absorbance was analyzed at 405 nm on a Perkin-Elmer microtiter plate reader. To minimize plate-to-plate variability, the results were normalized by multiplying the average absorbance for each sample by a normalization factor (known absorbance of the positive serum divided by the measured absorbance per plate for the positive serum).

PCR methodology. Chromosomal DNA was extracted from viably frozen PBMC by using an IsoQuick kit (Microprobe, Garden Grove, Calif.) according to the manufacturer's instructions. The cells were quickly thawed and pelleted in an Eppendorf tube by centrifugation. The supernatant was decanted, and the pellet was resuspended in 100 μl of sample buffer. After a 5-min incubation, 100 μl of lysis buffer was added and the sample was gently mixed. Extraction matrix, 700 μl, and extraction buffer, 400 μl, were added. The extract was mixed and centrifuged for 5 min. DNA was precipitated by mixing 400 μl of the upper layer with 40 μl of sodium acetate and 440 μl of isopropanol. The sample was centrifuged for 15 min, and the pellet was washed once with 70% ethanol, dried, and resuspended in 75 μl of RNase-free water. Five microliters of each DNA preparation was diluted 1:200, and the absorbance was measured at 260 nm. Turboboosted and nested PCR was performed with 2.5 μg of total genomic DNA subjected to 10 amplification cycles (1 min at 94°C, 1 min at 55°C, and 1 min 45 s at 72°C; 2.5 mM $MgCl_2$) with 5 pmol of primer 9065-to-9087 (5′-CCTACCTA CAATATGGGTGGAGC-3′) and primer 10005-to-9983 (5′-TCTGCCAGC CTCTCCGCAGAGCG-3′). The total reaction volume was 95 μl. After 10 cycles were completed, a 5-μl solution containing 45 pmol of each primer and 2.5 U of *Taq* polymerase was added and 35 cycles were performed as before. Five microliters of this PCR product was used as template for the nested PCRs as follows: 50 pmol each of primers 9110-to-9135 (5′-CCGTCTGGAGATCTGCGACA GAGACT-3′) and 9984-to-9962 (5′-CGACTGAATACAGAGCGAAATGC-3′) were used in a 100 μl reaction mixture. The parameters were 35 cycles for 1 min at 94°C, 1 min at 55°C, and 1 min 45 s at 72°C with 3.5 mM $MgCl_2$. Master mixes containing buffer, $MgCl_2$, nucleotides, and primers were prepared, aliquoted, and stored frozen at −20°C. The 2.5 μg of chromosomal DNA for the turboboosted reactions was denatured for 10 min at 95°C and immediately placed at 0°C. The master mixes were preincubated at 55°C. Chromosomal DNA and *Taq* polymerase were added, and the mix was then topped with mineral oil. After the first 10 cycles were completed, additional primers and enzyme were added and the PCR continued for another 35 cycles. After the turboboosted PCR was

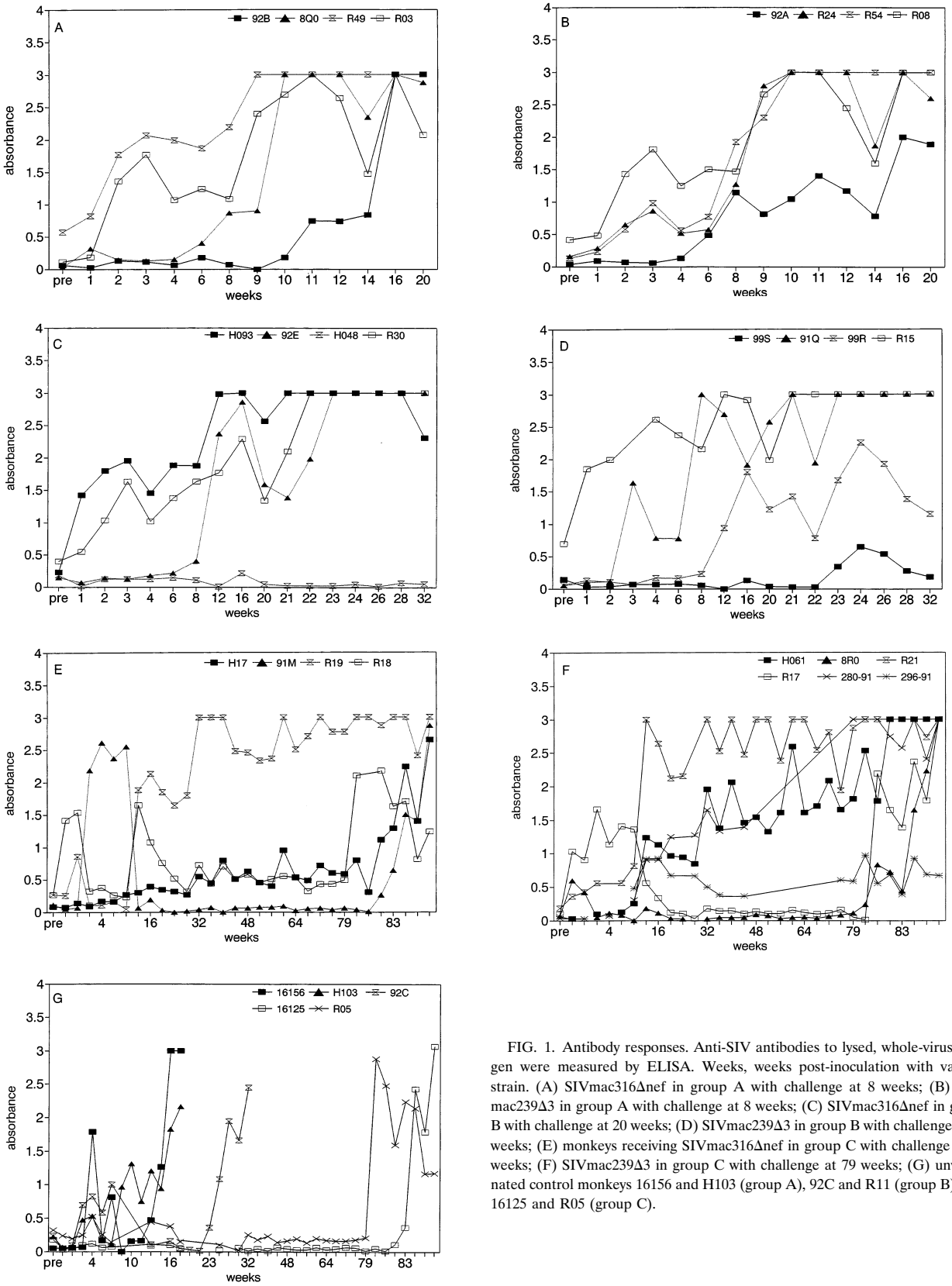

FIG. 1. Antibody responses. Anti-SIV antibodies to lysed, whole-virus antigen were measured by ELISA. Weeks, weeks post-inoculation with vaccine strain. (A) SIVmac316Δnef in group A with challenge at 8 weeks; (B) SIVmac239Δ3 in group A with challenge at 8 weeks; (C) SIVmac316Δnef in group B with challenge at 20 weeks; (D) SIVmac239Δ3 in group B with challenge at 20 weeks; (E) monkeys receiving SIVmac316Δnef in group C with challenge at 79 weeks; (F) SIVmac239Δ3 in group C with challenge at 79 weeks; (G) unvaccinated control monkeys 16156 and H103 (group A), 92C and R11 (group B), and 16125 and R05 (group C).

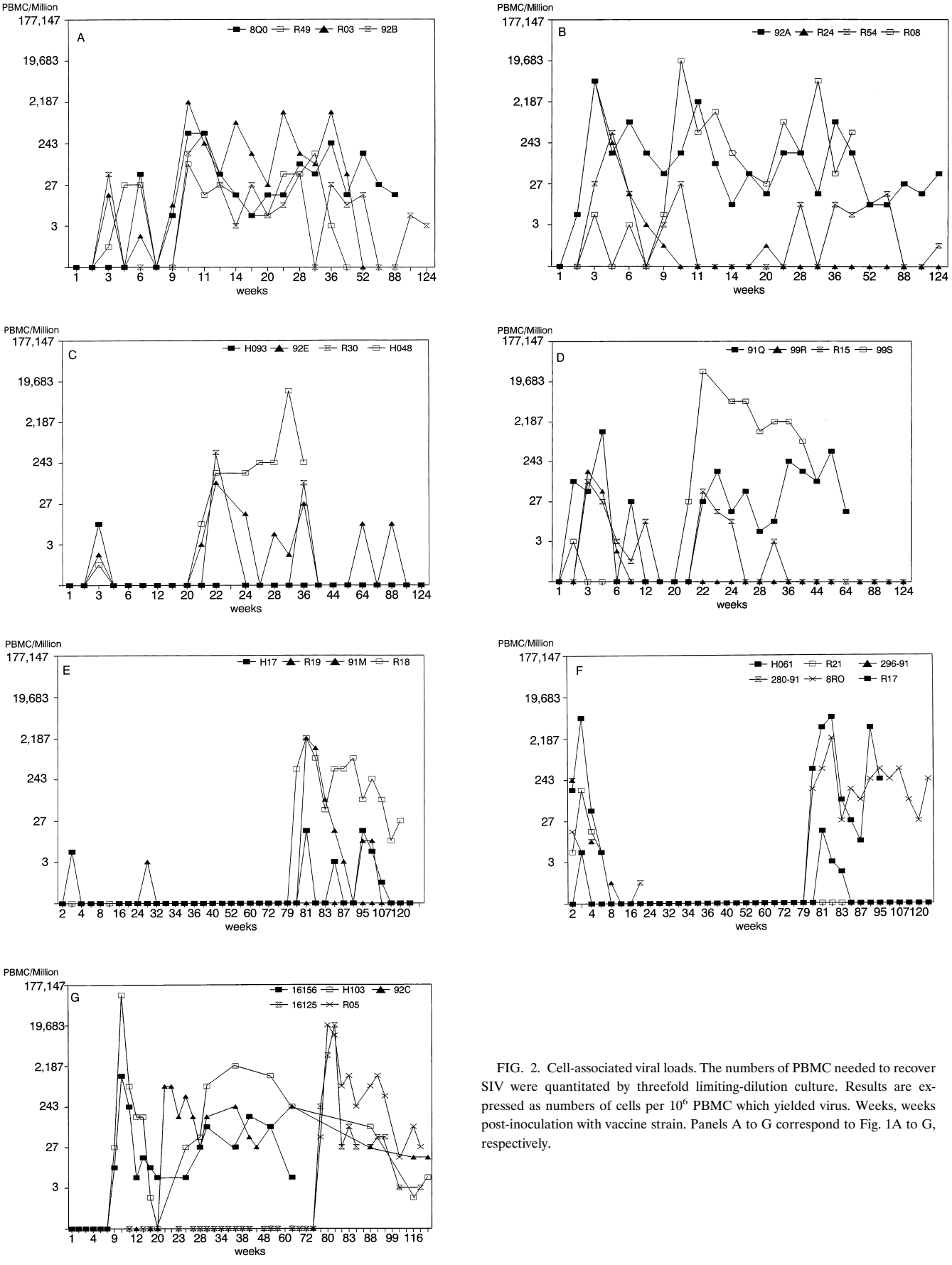

FIG. 2. Cell-associated viral loads. The numbers of PBMC needed to recover SIV were quantitated by threefold limiting-dilution culture. Results are expressed as numbers of cells per 10^6 PBMC which yielded virus. Weeks, weeks post-inoculation with vaccine strain. Panels A to G correspond to Fig. 1A to G, respectively.

TABLE 2. Effect of vaccine strain on peak virus load[a]

Vaccine and group	Peak virus load[b]
SIVmac316Δnef	
A	27
B	6
C	6
SIVmac239Δ3	
A	610
B	243
C	545

[a] The 17 monkeys with persistent vaccine virus infections and monkeys 280-91 and 296-91 were used for these calculations.
[b] Mean number of cells per 10^6 PBMC yielding vaccine virus recovery 2 to 6 weeks postvaccination.

completed, 5 μl of the product was used as template for the nested reactions. The master mix was preincubated at 55°C, the 5-μl template and 2.5 U of *Taq* polymerase were added, and the amplification was carried out. Each sample was tested in triplicate. For analysis of the PCR product 10 μl of each nested PCR product was electrophoresed through 1.5% agarose gels containing ethidium bromide. Gels were run at 45 V for 4 h and photographed.

RESULTS

Vaccine phase. Twelve rhesus monkeys were vaccinated with SIVmac239Δ3, and 12 were vaccinated with SIVmac316Δnef (Table 1). Each monkey received vaccine virus containing 5 ng of p27. Seventeen of the 24 inoculated monkeys exhibited an infection course that was in general similar to what has been described previously for SIVmac239Δnef (7, 28). These seventeen developed anti-SIV antibodies detected by whole-virus ELISA (Fig. 1). Antibodies first became detectable 2 to 12 weeks following inoculation, and they persisted at moderate or high levels until the time of challenge (Fig. 1). Furthermore, vaccine virus was recovered on one or more occasions from all 17 animals in the weeks immediately following vaccine virus inoculation (Fig. 2). We conclude that these 17 had typical, low-level, persistent infections with the mutant virus strains.

Among these 17 with low-level persistent infections with mutant virus, peak levels of recoverable virus and, most prominently, stabilized levels of recoverable virus were typically much lower than levels previously observed in monkeys receiving parental SIVmac239 or SIVmac251 (7, 9, 19, 25, 28). Furthermore, plasma antigenemia was not observed in any of these 17 in the weeks immediately following vaccination. The levels of recoverable virus from PBMC of monkeys that received SIVmac239Δ3 were on average considerably higher than those in monkeys receiving SIVmac316Δnef (Fig. 2 and Table 2). Peak levels of recoverable virus from monkeys that were persistently infected with SIVmac239Δ3 were on average 54 times higher than those in monkeys infected with SIVmac316Δnef (Table 2).

Seven of the 24 inoculated rhesus monkeys, however, showed a transient course of infection that we have not seen previously (Table 3). Six of these seven had SIV vaccine strain recovered from their peripheral blood at week 2, week 3, or weeks 2 and 3. In all but one of these cases, virus was recovered at more than one dilution of PBMC. Three of the seven (91M, R18, and R17) had transient antibody responses that subsequently subsided to background levels (Fig. 1 and Table 3). The other four never mounted detectable anti-SIV antibody responses (Fig. 1 and Table 3). These apparently transient infections occurred with both vaccine strains: four occurred with SIVmac316Δnef and three occurred with SIVmac239Δ3. The nature of these infections appears to be similar to previous descriptions of similar occurrences following mucosal exposure of macaque monkeys to SIV (5, 32, 36).

Challenge phase. (i) Monkeys with apparently transient infections. None of the monkeys with apparently transient vaccine virus infections were protected against subsequent challenge. All seven had a rapid, dramatic increase in anti-SIV antibody levels immediately after intravenous challenge with uncloned, pathogenic SIVmac251 (Table 3 and Fig. 1). Cell-associated virus loads quickly increased and were maintained at high levels subsequent to challenge (Fig. 2). PCR readily detected wild-type virus subsequent to challenge (Table 4). Four of these seven (HO48, 99S, R18 and R17) died or had to be sacrificed as a result of their SIV infections 18 to 44 weeks after challenge. The remaining three showed declines in $CD4^+$ lymphocyte concentrations after challenge (Fig. 3).

Since none of these seven monkeys exhibited a typical course of persistent infection with vaccine virus and since none resisted subsequent challenge, these seven monkeys are not included in the subsequent analyses.

(ii) Group A. Only two of the seven animals remaining in group A were protected when challenged with wild-type, pathogenic SIVmac251 at 8 weeks postvaccination. Both of these protected animals (R24 and 92A) were vaccinated with SIVmac239Δ3. R24 maintained very low cell-associated virus burdens after challenge (Fig. 2B), and PCR either detected no viral sequences or detected Δ3 virus only (Table 4). $CD4^+$ lymphocyte concentrations have remained stable in R24 (Fig. 3B). While 92A has maintained cell-associated virus loads at a measurable level postchallenge (Fig. 2B), PCR detected only Δ3 sequences postchallenge (Table 4) and $CD4^+$ lymphocyte concentrations have remained above 30% for more than 2 years after challenge (Fig. 3B).

The remaining five animals in group A maintained high cell-associated virus burdens postchallenge (Fig. 2A and B) and had wild-type DNA sequences readily detected by PCR postchallenge (Table 4). Four of these five (8QO, R49, RO3, and RO8) died or had to be sacrificed 22 to 80 weeks following challenge. The fifth of these, R54, remains alive with declining $CD4^+$ cell numbers (Fig. 3B).

(iii) Group B. Three of the remaining six monkeys in group B were protected when challenged with wild-type pathogenic SIVmac251 at 20 weeks postvaccination. One of the protected monkeys (HO93) was vaccinated with SIVmac316Δnef, and two (99R and R15) were vaccinated with SIVmac239Δ3. While R15 clearly exhibited the presence of wild-type virus postchallenge, this animal was nonetheless strongly protected over the

TABLE 3. Rhesus monkeys with apparently transient infections with vaccine virus

Animal	Vaccine	Wk of recovery[a]	PBMC load[b]	Antibody	Wk of challenge[c]
92B	SIVmac316Δnef	3	54	−[d]	8
H048	SIVmac316Δnef	3	1	−	20
99S	SIVmac239Δ3	2	3	−	20
8RO	SIVmac239Δ3	2	18	−	79
		3	6		
91M	SIVmac316Δnef	3	6	Transient	79
R18	SIVmac316Δnef	−	−	Transient	79
R17	SIVmac239Δ3	3	6	Transient	79

[a] Week postvaccination at which the SIV vaccine strain was recovered.
[b] Number of cells per 10^6 PBMC that harbored a recoverable SIV vaccine strain.
[c] None of the animals were protected.
[d] −, negative.

TABLE 4. PCR results

Group and animal	Sequences detected at wk[a]:											
	2	3	8	10	20	22	38	40	48	81	88	99–100
ΔnefA												
92B			−, −, −	wt, wt, wt					wt, −, wt			wt, wt, wt
8QO			−, −, −	wt, wt, wt					Δn, wt, wt			
R49			Δn, Δn, Δn	−, wt, wt								
R03			Δn, Δn, Δn	wt, wt, wt								
Δ3A												
92A			Δ3, Δ3, Δ3	Δ3, Δ3, Δ3						Δ3, Δ3, Δ3		Δ3, Δ3, Δ3
R24			Δ3, Δ3, −	−, Δ3, Δ3						−, Δ3, Δ3		−, −, −
R54			Δ3, Δ3, Δ3	−, Δ3, Δ3					−, −, −			wt, wt, wt
RO8			−, −, −									
ΔnefB												
HO93		Δn, Δn, Δn			Δn, Δn, Δn	Δn, Δn, Δn		Δn, Δn, −	−, −, −		Δn, Δn, Δn	
92E		Δn, Δn, Δn			Δn, −, Δn	wt, wt, wt[b]		wt, wt, wt[b]	−, wt, −		wt, wt, wt	
HO48		−, −, −			−, −, −	wt, wt, wt	wt, wt, wt					
R30		Δn, Δn, Δn			Δn, Δn, Δn	wt, wt, wt		wt, wt, wt[b]	−, −, wt		−, wt, −	
Δ3B												
99S	−, −, −				−, −, −	wt, wt, wt		wt, wt, wt				
91Q	Δ3, Δ3, Δ3				Δ3, Δ3, Δ3	Δ3, Δ3, Δ3		wt, wt, wt	wt, wt, wt			
99R	Δ3, −, Δ3				Δ3, −, −	Δ3, Δ3, −		−, −, −	−, −, −		Δ3, Δ3, −	
R15	Δ3, −, Δ3				Δ3, Δ3, Δ3	wt, wt, wt[c]		Δ3, Δ3, wt[d]	−, −, Δ3		Δ3, −, −	
ΔnefC												
H17		−, −, −					−, −, −			−, wt, −		
91M		−, −, −					−, −, −			wt, wt, wt		
R19		Δn, Δn, Δn					−, −, −			−, −, −		
R18		−, −, −					−, −, −			wt, wt, wt		
Δ3C												
HO61		Δ3, Δ3, Δ3					Δ3, Δ3, Δ3			wt, Δ3, Δ3		
8R0		−, −, −					−, −, −			wt, wt, wt		
R21		Δ3, Δ3, Δ3					Δ3, −, −			−, −, −		
R17		−, −, −					−, −, −			wt, wt, wt		
280-91										Δ3, Δ3, Δ3		
296-91										Δ3, −, −		

[a] Results of three independent analyses for each PBMC sample. Δn, Δ3, and wt, Δnef, Δ3, and wild-type sequences, respectively. −, no sequence detected.
[b] Lesser amounts of Δnef were also detected.
[c] Lesser amounts of Δ3 were also detected.
[d] The wild-type signal was very weak.

long term. After challenge, virus loads became transiently high in R15 but subsequently subsided to undetected levels (Fig. 2D). Wild-type DNA sequences were strongly detected by PCR at week 22, i.e., 2 weeks after challenge, but not at later times (Table 4). This animal has now survived more than 2 years following challenge, maintaining low virus burdens and stable $CD4^+$ lymphocyte counts (Fig. 3D). SIV was not recovered from the other two protected animals postchallenge (Fig. 2C and D), and PCR detected either no viral DNA or vaccine strain DNA only (Table 4). Their $CD4^+$ lymphocyte counts have also remained stable for more than 2 years following challenge (Fig. 3C and D).

Three of the remaining animals in group B (91Q, 92E, and R30) did not appear to be protected following challenge. Cell-associated virus loads increased after challenge (Fig. 2C and D), and wild-type virus sequences were detected by PCR long after challenge (Table 4). 91Q died 30 weeks after challenge.

(iv) Group C. Because four animals randomized to group C at the initiation of the study did not exhibit persistent vaccine virus infections, two monkeys (296-91 and 280-91) vaccinated with SIVmac239Δ3 at the New England Regional Primate Research Center around the same time were added to group C. Solid protection was achieved in four of four monkeys vaccinated with SIVmac239Δ3 (HO61, R21, 296-91, and 280-91) and in one of two monkeys vaccinated with SIVmac316Δnef (R19). HO61 appeared to be similar to R15 in group B in that solid long-term protection was achieved in the absence of sterilizing immunity.

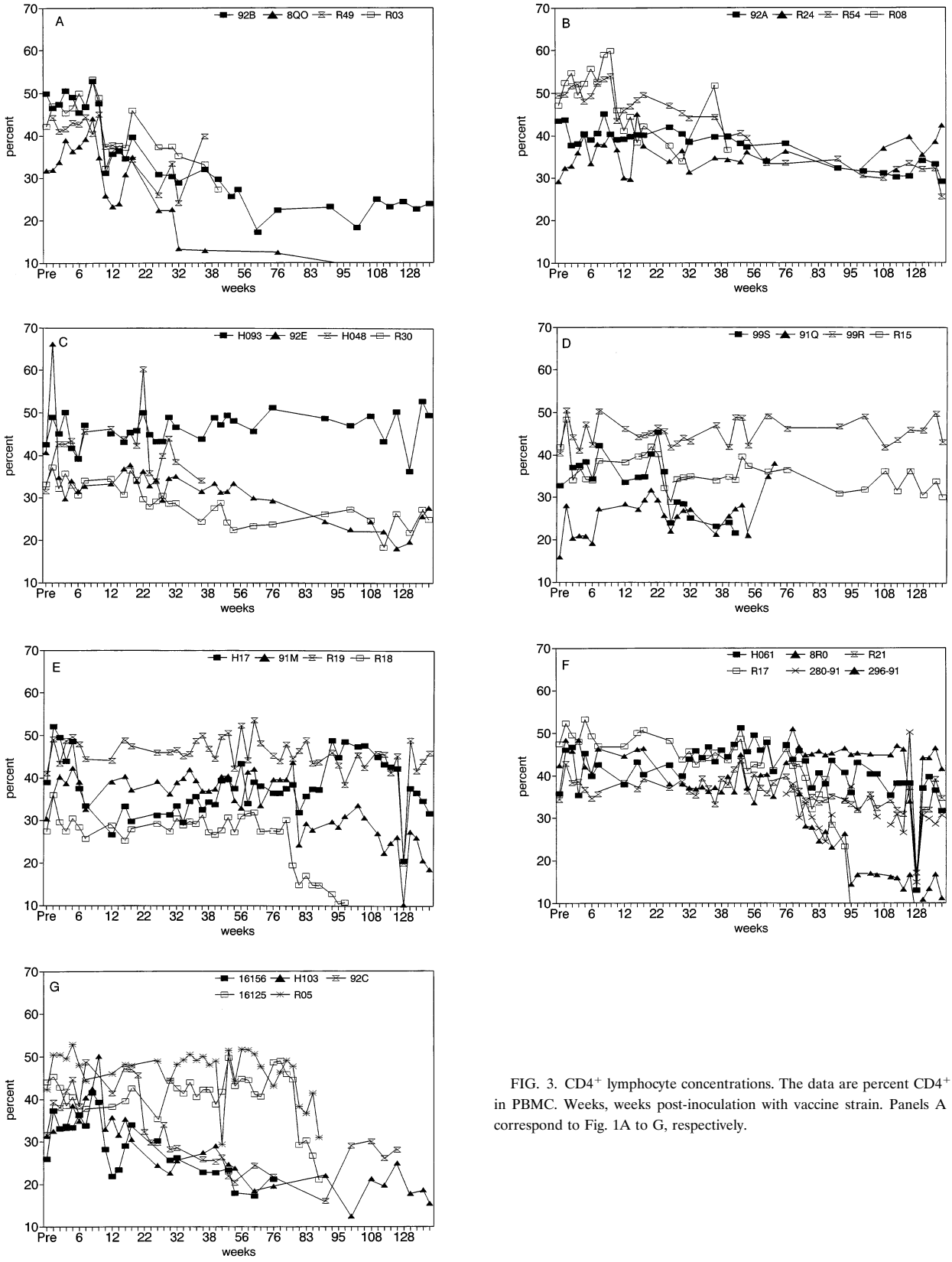

FIG. 3. $CD4^+$ lymphocyte concentrations. The data are percent $CD4^+$ cells in PBMC. Weeks, weeks post-inoculation with vaccine strain. Panels A to G correspond to Fig. 1A to G, respectively.

TABLE 5. Protection

Vaccine	No. of animals protected in group:		
	A	B	C
SIVmac316Δnef	0/3	1/3[a]	1/2[b]
SIVmac239Δ3	2/4[c]	2/3[d]	4/4[e]

[a] HO93 was protected.
[b] R19 was protected.
[c] 92A and R24 were protected.
[d] 99R and R15 were protected. Evidence of challenge virus was detected in R15.
[e] HO61, R21, 296-91, and 280-91 were protected. Evidence of challenge virus was detected in HO61.

(v) Summary. A summary of the protective outcomes is given in Table 5.

Correlates of protection. Stored sera taken from all test animals on the day of, just prior to, challenge were used to measure the level of neutralizing activity against a laboratory-passaged stock of SIVmac251. The neutralization assay was run independently in two different laboratories (at TSI Mason and the New England Regional Primate Research Center), and all results agreed within a factor of 4, i.e., 2 twofold dilutions. No correlation was observed between the level of neutralizing activity and protection in this assay (Fig. 4A). However, neutralizing activity did correlate with protection when measured against a primary stock of SIVmac251 derived from the challenge virus stock (Fig. 4B). Antibodies that bound to whole virus by ELISA also showed considerably higher levels in protected animals (Fig. 4C). At the 1:400 dilution of serum used, protected animals showed a mean absorbance (0.493) which was 2.5-fold higher than that of unprotected animals (0.197). The median absorbance in protected animals was 2.1-fold higher than that of unprotected animals. This correlation was significant with a P value of 0.033 by Student's two-tailed t test.

We next analyzed peak levels of cell-associated virus loads 2 to 6 weeks after vaccination for a possible correlation with protection. In this case, protected animals showed 9-fold (mean) or 33-fold (median) greater virus loads (243 versus 27 and 202 versus 6 cells, respectively, per 10^6 PBMC yielding the SIV vaccine strain at peak) (Fig. 4D) ($P = 0.048$). Consistent with this observation and the fact that SIVmac239Δ3 overall gave somewhat greater protection than SIVmac316Δnef (Table 5), higher cell-associated virus loads were observed with SIVmac239Δ3 than with SIVmac316Δnef (Fig. 5).

DISCUSSION

Unusual, apparently transient infections have been observed previously by others following mucosal exposure of some macaque monkeys to SIV (5, 32, 36). While we have not observed such a pattern of infection previously in more than 30 monkeys inoculated intravenously with attenuated SIV mutants (19, 25, 26, 28), we clearly observed it with reasonably high frequency in the current study. The factors which influence the variable occurrence of this phenomenon remain undefined. In a recent titration of a stock of SIVmac239Δ3 in 20 rhesus monkeys, only one exhibited this atypical pattern, and this occurred with the lowest dilution containing infectious virus (unpublished results). Thus, dose may be one factor which influences the occurrence of such atypical infections. The proportion of defective viruses in individual virus stocks and genetic variation of the individual monkey hosts could also conceivably be contributing factors.

Clerici et al. (5) found that monkeys with such transient infections were protected against subsequent challenge. We observed no protective effects in any of the seven monkeys with apparently transient infections when they were subsequently challenged in the present study. Several key differences in the designs of the studies likely contributed to the different outcomes of challenge. Clerici et al. used initial mucosal exposure and subsequent mucosal challenge; we used intravenous vaccination and intravenous challenge. Also, the different strains of SIV used for challenge in the two studies may differ with respect to the inherent ease with which they can be protected against.

Among the animals with persistent infection by vaccine virus, there was a clear trend toward increased protection with time of vaccination. Vaccination was mostly ineffective when duration was only 8 weeks and was almost completely protective by 79 weeks. This time dependence seems more consistent with immune-mediated protection than with simple interference with viral replication since virus burdens do not increase noticeably after the resolution of the initial burst in virus replication. The time dependence of protection would be expected to vary with the individual virus strains used. In the current study, a slightly heterologous virus strain was used for challenge. More or less time to achieve solid immunity would be expected with greatly divergent or homologous challenge strains, respectively.

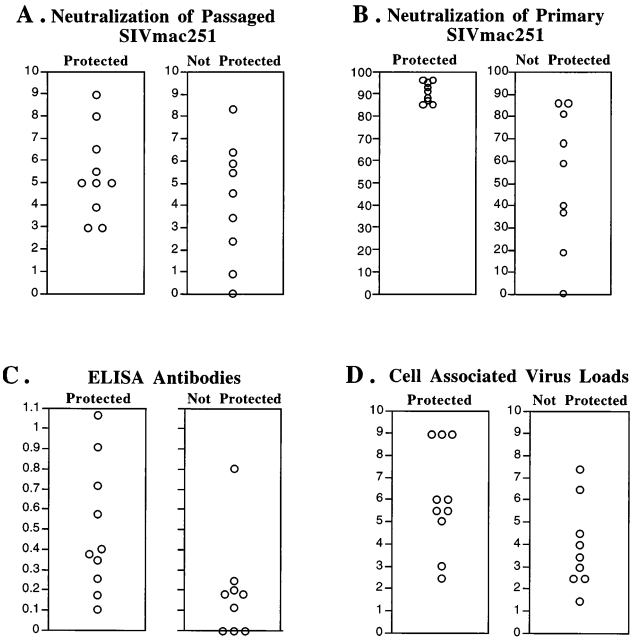

FIG. 4. Correlations with protection. (A) Neutralizing antibodies to laboratory-passaged SIVmac251 do not correlate with protection. Each point represents the reciprocal dilution of serum that yielded 50% neutralization with serum taken on the day of, just prior to, challenge. 0, <1:20; 1, 1:20; 2, 1:40; 3, 1:80; 4, 1:160; 5, 1:320; 6, 1:640; 7, 1:1,280; 8, 1:2,560; 9, 1:5,120; 10, 10,240. (B) Neutralizing antibodies to a primary stock of SIVmac251 correlate with protection. Each point represents the reduction in virus yield (percent) in the presence of test serum (1:4) taken on the day of, just prior to, challenge. (C) Binding antibodies to whole-virus by ELISA correlate with protection. Each point represents the A_{410} obtained with a 1:400 dilution of serum taken on the day of, just prior to, challenge. This dilution of serum was chosen so that most samples yielded absorbance in the linear range of the spectrophotometer. All samples were run in parallel at the same time. (D) Cell-associated virus loads appear to correlate with protection. Each point represents the peak of virus load during the first 6 weeks after vaccination (Fig. 1) measured as the number of cells per 10^6 PBMC which yielded recoverable virus. 0, <1; 1, 1; 2, 3; 3, 9; 4, 27; 5, 81; 6, 243; 7, 729; 8, 2,187; 9, 6,561.

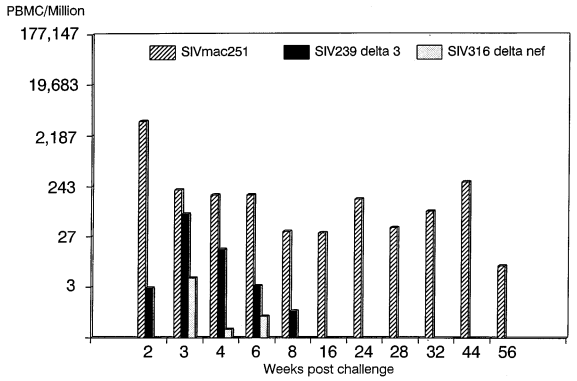

FIG. 5. Cell-associated virus loads are different with different SIV strains. The numbers of cells per 10^6 PBMC needed for virus recovery are the averages for all animals used in this study.

Genetic backbone also appears to influence protective capacity. We observed greater protection with SIVmac239Δ3 (8 of 11) than with SIVmac316Δnef (2 of 8). We feel that the decreased level of protection observed with SIVmac316Δnef is likely to be due to the eight amino acid changes in the envelope compared with the envelope of SIVmac239. Previous studies have indicated that vpr contributes little to the ability of SIVmac239 to replicate in rhesus monkeys (19, 23, 30) and that upstream sequences in the long terminal repeat function primarily or exclusively as nef coding sequences (25, 29). Thus, the level of attenuation of SIVmac239Δ3 is expected to be similar to, or only slightly greater than, SIVmac239Δnef, in agreement with the in vivo measurements. The eight amino acid changes in SIVmac316 compared with SIVmac239 impart on the virus a high replicative capacity for macrophages (34, 35). However, early cell-associated virus loads with SIVmac316Δnef appear to be considerably less than those with SIVmac239Δnef. This is consistent with other experiments which indicate lower cell-associated virus burdens with SIVmac316 than with SIVmac239 (37a). Thus, these eight amino acid changes in the envelope could alter the ability of virus variants to serve as vaccines by altering the replicative capacity of virus and/or by altering the immunogenic properties of the envelope protein.

Long-term vaccine protection was observed in at least two animals (HO61 and R15) in the absence of sterilizing immunity. Thus, despite the clear establishment and replication of the pathogenic challenge virus, it was subsequently controlled and long-term protection was achieved. In the previous report that used SIVmac239Δnef as a vaccine, one of the four protected animals had a spike in cell-associated virus loads 4 weeks after challenge (7). While samples were not saved for genetic analysis, it is likely that this animal also had a take of the challenge virus. It has now been more than 4 years since these animals described by Daniel et al. (7) were challenged; they are all still alive and healthy with very low virus loads and stable $CD4^+$ lymphocyte counts. It should be noted, however, that solid long-term protection against SIVmac251 was not observed with other vaccine approaches in the absence of sterilizing immunity (9, 10, 16). At least three of the nonprotected animals in the current study (R30 and 92E in group B Δnef and H17 in group C Δnef) appeared to show a significant beneficial effect of prior vaccination on the basis of reduced virus loads and disease progression. Evidence for a delay in disease progression in the absence of sterilizing immunity has also been obtained in other settings (1, 10, 22, 27).

One advantageous feature of the design of our vaccine study is that the similar numbers of protected and unprotected animals allow detailed investigation of the correlates of protection. Virus-neutralizing activity in sera taken on the day of, just prior to, challenge did not correlate with protection when measured against laboratory-passaged SIVmac251. This does not necessarily mean, however, that neutralizing antibodies have nothing to do with the protection that was observed. It is possible that the nature, specificity, or affinity of particular neutralizing activities could correlate with protection. Indeed, a correlation with protection was observed when neutralization was measured with a primary stock of SIVmac251. The level of binding antibodies to whole virus by ELISA also appeared to correlate with protection. Evidence for an in vivo protective capacity of nonneutralizing antibody has been presented for other viral systems (21, 37).

The peak levels of the replicating vaccine strain early after administration also appeared to correlate with protection. Our ability to make this correlation is constrained by the limited sampling times at weeks 1, 2, 3, 4, and 6 after vaccination. We do not feel that this correlation is indicative of viral interference, since the time dependence of protection and the absence of sterilizing immunity in some of the protected animals are more consistent with immune-mediated protection. Furthermore, $CD4^+$ cells from SIVmac239Δnef- and SIVmac239Δ3-vaccinated animals support replication of wild-type SIVmac239 at levels similar to those for $CD4^+$ cells from uninfected monkeys (27a). Thus, the protection is likely to be immune mediated.

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