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Potent Antibody-Mediated Neutralization and Evolution of Antigenic Escape Variants of Simian Immunodeficiency Virus Strain SIVmac239 In Vivo▽

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Here, we describe the evolution of antigenic escape variants in a rhesus macaque that developed unusually high neutralizing antibody titers to SIVmac239. By 42 weeks postinfection, 50% neutralization of SIVmac239 was achieved with plasma dilutions of 1:1,000. Testing of purified immunoglobulin confirmed that the neutralizing activity was antibody mediated. Despite the potency of the neutralizing antibody response, the animal displayed a typical viral load profile and progressed to terminal AIDS with a normal time course. Viral envelope sequences from week 16 and week 42 plasma contained an excess of nonsynonymous substitutions, predominantly in V1 and V4, including individual sites with ratios of nonsynonymous to synonymous substitution rates (*dN*/*dS*) highly suggestive of strong positive selection. Recombinant viruses encoding envelope sequences isolated from these time points remained resistant to neutralization by all longitudinal plasma samples, revealing the failure of the animal to mount secondary responses to the escaped variants. Substitutions at two sites with significant *dN*/*dS* values, one in V1 and one in V4, were independently sufficient to confer nearly complete resistance to neutralization. Substitutions at three additional sites, one in V4 and two in gp41, conferred moderate to high levels of resistance when tested individually. All the amino acid changes leading to escape resulted from single nucleotide substitutions. The observation that antigenic escape resulted from individual, single amino acid replacements at sites well separated in current structural models of Env indicates that the virus can utilize multiple independent pathways to rapidly achieve similar levels of resistance.

The primate lentiviruses, including human immunodeficiency virus type 1 (HIV-1), HIV-2, and the various simian immunodeficiency viruses (SIVs) establish persistent infections characterized by months or even years of continuous, uninterrupted cycles of viral replication in most infected individuals. The capacity of the primate lentiviruses to replicate at high levels even in the face of adaptive, virus-specific immune responses results from a variety of complementary immune evasion strategies (13). The viral envelope proteins form a large, heterotrimeric complex that is expressed on the surfaces of both virions and infected, virus-producing cells and therefore constitute the major target for antibody-mediated immune responses. Viral mechanisms for circumventing humoral immunity include strategies that reduce immunogenicity and the induction of neutralizing antibodies, such as the use of a two-receptor entry mechanism, entropic masking, extensive glycosylation, and the destruction of CD4 T helper cells (12, 15, 23, 29, 50, 51). Nonetheless, virus-specific antibodies are still elicited in the infected host, but the high degree of viral diversity generated in vivo provides a ready substrate for the rapid selection of antigenic escape variants (9, 10). In the case of major histocompatibility complex class I-restricted epitopes, the best-documented examples of escape all involve alterations in the processing, presentation, or recognition of individual, linear peptides (reviewed in reference 14). While antigenic escape from antibody responses is also well documented, because B-cell epitopes can involve discontinuous or conformational determinants, the exact physical mechanism(s) by which amino acid variation results in escape is difficult to discern. To complicate matters, several groups have described changes resulting in escape from neutralizing serum/plasma that do not alter residues directly involved in antibody binding but which may act indirectly by altering the accessibility or conformation of regions targeted by neutralizing antibodies (5, 45, 47, 49).

Experimental infection of rhesus macaques with SIV results in a disease course that mirrors the development of AIDS in HIV-infected patients. Like HIV-infected patients, SIV-infected macaques develop robust but ineffective anti-SIV envelope antibody responses. In contrast to HIV infection, where the details of the initial event including the exact sequence of the original infecting virus may be unknown, in experimental infections of macaques with defined molecular clones of SIV, the exact sequence of the inoculating virus is known, and the precise dose, timing, and route of infection can be controlled. The ability to compare sequences that evolved in the host with the inoculum facilitates the unambiguous reconstruction of the direction and timing of sequence evolution and the identification of specific changes related to antigenic escape.

SIVmac239 is a molecular clone of a pathogenic, T-cell-

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tropic biological isolate and reproducibly causes AIDS in rhesus macaques (19, 39). Similar to primary isolates of HIV-1, SIVmac239 preferentially targets CCR5-positive memory T cells (38) and is relatively resistant to antibody-mediated neutralization (18). Animals infected with this virus typically mount antibody responses that can only weakly neutralize SIVmac239 (17, 18). These characteristics of the virus, combined with the existence of a robust and well-characterized animal infection model, make SIVmac239 infection a useful system for studying antibody-mediated neutralization and viral mechanisms of immune evasion.

Previously, studies of in vivo evolution of the envelope sequence of SIV in rhesus macaques chronically infected with SIVmac239 (4) and pig-tailed macaques infected with SIVMne-CL8 (37) revealed strikingly similar patterns of viral envelope sequence evolution in these heterologous viruses during infection of their respective hosts. The emergence of neutralization-escape variants has been well-documented in SIVmac239-infected rhesus macaques (3, 4, 20). However, it is still not clear which among the many reported amino acid changes are responsible for antigenic escape, to what degree different changes contribute to escape, and whether some of these changes have a combined effect. Here, we report the identification of an SIVmac239-infected rhesus macaque that developed an atypical, highly potent neutralizing antibody response to SIVmac239. Although this animal eventually succumbed to AIDS, it provided a unique opportunity to study the effects of potent antibody-mediated neutralization on SIVmac239 infectivity and the in vivo evolution of antibody-resistant variants.

MATERIALS AND METHODS

Viruses and plasma samples. All samples described in this study were obtained from archived material at the New England Primate Research Center (NEPRC). Rhesus macaques Mm333 and Mm322 were unvaccinated, control animals challenged with SIVmac239 as part of an unrelated vaccine study (27); in the same study, Mm139, Mm526, and Mm536 were vaccinated with an attenuated SIVmac239 variant (ΔV1ΔV2). All four animals were challenged with 20 intravenous infectious doses of SIVmac239. The SIVmac239 viral stock for inoculation was produced by transient transfection of rhesus macaque peripheral blood lymphocytes with cloned SIVmac239 proviral DNA, and day 11 supernatants from these fresh cultures were passed to fresh peripheral blood lymphocyte cultures from the same animals; then cell-free day 8 supernatants were harvested, titers were determined, and samples were cryo-preserved (25).

SIVmac239-infected rhesus macaques (*Macaca mulatta*) were housed at the NEPRC in a biosafety level 3 animal containment facility in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. Research, including plasma procurement, was conducted according to the principles described in the Guide for the Care and Use of Laboratory Animals and was approved by the Harvard Medical School Animal Care and Use Committee (33). The AE637 pool used for the neutralization assay and Pepscan analysis was a mixture of plasma collected at week 20 postinoculation from eight rhesus macaques infected with SIVmac239 variants expressing wild-type envelope.

Plasma SIV RNA measurements. Plasma SIV RNA loads were measured by quantitative real-time reverse transcription-PCR (RT-PCR) using previously described methods (8). This method has a threshold sensitivity of 20 SIV *gag* RNA copies/ml of plasma.

Cloning and sequencing of *env* from plasma. SIV RNA was isolated by affinity column purification using a High Pure Viral RNA kit (Roche) following the manufacturer's protocol. Ten nanograms of viral RNA was amplified by a one-step, combined RT-PCR using Superscript III One-step RT-PCR (Invitrogen) for 40 rounds with oli149 (forward, 5′-ACCGCCCTCTAGAAGCATGCTATA ACACATGCTATTG-3′) and oli150 (reverse, 5′-CCTCACAAGAGAGTGAG CTCAAGCC-3′). The 2.8-kbp RT-PCR product was gel purified and used for cloning into the pCR-TOPO TA cloning vector in the TOPO TA cloning kit (Invitrogen) following the manufacturer's protocol. After transformation of Stbl3 *Escherichia coli* cells (Invitrogen), single ampicillin-resistant colonies were grown overnight at 30°C for plasmid preps, which were completely sequenced on both strands by primer extension using 10 oligonucleotide primers (five forward and five reverse) by Retrogen Inc. (San Diego, CA).

Sequence analysis. Viral *env* sequences were aligned using the CLUSTAL W algorithm and, where necessary, adjusted manually. To identify sites of potentially strong positive selection, aligned sequences were used to generate a phylogenetic tree using the neighbor-joining method, and the alignment and tree files were then used as input for the CODEML program in the PAML suite of molecular evolutionary analysis software (53). Sites with ratios of nonsynonymous to synonymous substitution rates (*dN*/*dS*) indicative of positive selection were identified using CODEML, essentially as described previously (34, 52).

Plasmids, transfections, and virus production. The full-length SIVmac239-expressing plasmid has been described previously (56). Mutant envelope clones from week 16 and week 42 were subcloned into the full-length SIV proviral DNA expression vector by digesting the TOPO *env* clones with SacI and SphI and then purifying and ligating the 2,780-bp fragment into the same sites of the full-length vector. The single-substitution mutant virus vectors were constructed by subcloning of appropriate DNA fragments generated by restriction digests of the TOPO envelope clones or by PCR-based site-directed mutagenesis. All single-substitution clones generated using PCR were verified by sequencing to ensure the absence of unintended mutations. SIVsmmPGm5.3 was produced from an expression vector that has been previously described (35). Three days posttransfection, the supernatant was harvested and subjected to centrifugation to eliminate cell debris, and the p27 concentration of the supernatant containing infectious virus was determined using an SIV p27 antigen capture assay kit (Advanced BioScience Laboratories, Kensington, MD), following the manufacturer's protocol.

Virus infectivity and neutralization assays. Viruses for infectivity and neutralization assays were produced by transient transfection of HEK293T/17 cells with full-length, virus-expressing vector using previously described methods (56). To determine the infectivity of each virus, virus-containing supernatant was serially diluted in 100 μl of tissue culture medium and added to 100 μl of 5×10^3 C8166-SEAP cells, which contain a stably integrated, Tat-inducible secreted alkaline phosphatase (SEAP) reporter gene (described in reference 28), and incubated in 5% CO_2 at 37°C. Three days postinfection, SEAP activity was measured using a chemiluminescence assay, as previously described (28). The chemiluminescence signal was plotted against p27 concentration for each virus (in duplicate samples). Plasma samples for neutralization assays were heat inactivated at 56°C for 30 min and then briefly spun at 4,000 × *g* to pellet out any insoluble aggregates. Neutralization assays were carried out in duplicate for each virus and each plasma sample using procedures previously described (57).

Purification of total IgG from plasma. Total immunoglobulin G (IgG) was purified from 250 μl of heat-inactivated (56°C for 30 min) Mm333 week 82 plasma and plasma from an uninfected, specific-pathogen-free rhesus macaque using protein A/G-agarose beads (Pierce) following the manufacturer's protocol. After elution, the purified IgG was dialyzed against 1× phosphate-buffered saline (PBS), pH 7.4, and then the final volume was adjusted to 250 μl with the buffer. Purity and yield of IgG were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie staining and Western blotting. Contaminating protein in the elution and loss of IgG during purification, dialysis, and concentration were both minimal.

Env peptide ELISA. SIVmac239 Env 15-mer peptides were obtained from the NIH AIDS Research and Reference Reagent Program. Single wells of 96-well half-area, high-binding plates (Costar) were coated with 50 μl of each peptide diluted to 40 μg/ml with PBS and incubated at 37°C for 1 h. The wells were blocked with 5% nonfat powdered milk in PBS at 37°C for 1 h. A total of 50 μl of heat-inactivated plasma diluted 1:20 with 5% milk in PBS was added to each well and incubated at 37°C for 2 h. After five washes with PBS containing 0.05% Tween 20, 50 μl of horseradish peroxidase-conjugated goat anti-human IgG antibody (Santa Cruz Biotechnology), diluted 1:1,000 in 5% milk in PBS, was added to each well, and the plates were incubated at 37°C for 1 h. The plates were then washed 10 times with PBS-Tween, and 50 μl of TMB (3,3′,5,5′-tetramethylbenzidine) reagent (Calbiochem) was added to each well. Thirty minutes later, 50 μl of 250 mM hydrochloric acid was added to each well, and the optical density at 450 nm was measured using a Wallac Victor plate reader (Perkin Elmer).

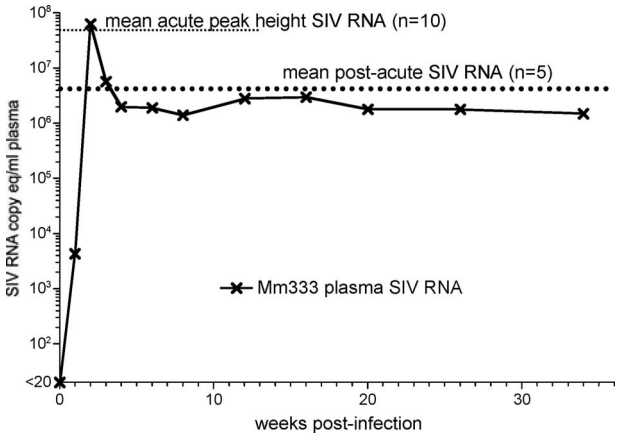

FIG. 1. Viral replication in animal Mm333. Viral RNA loads were measured from longitudinal plasma samples (at 0, 1, 2, 3, 4, 6, 12, 16, 20, 26, and 34 weeks postinoculation) taken from animal Mm333 using quantitative real-time RT-PCR. Mean acute peak height and mean post-acute SIV RNA load values (indicated by dotted lines) for SIVmac239-infected rhesus macaques were obtained from a previous study (16) and are included for the purpose of comparison.

RESULTS

Identification of an SIVmac239-infected rhesus macaque with a high-titer neutralizing antibody response against SIVmac239. In a screen of archived plasma samples collected during past vaccine studies conducted at the NEPRC, we identified samples taken from an Indian-origin female rhesus macaque that exhibited an unusually high-titer neutralizing antibody response against SIVmac239. Records indicated that this animal served as an unvaccinated control that had been inoculated intravenously with SIVmac239 (27). At 82 weeks postinoculation, animal Mm333 developed AIDS and was euthanized according to the principles described in the Guide for the Care and Use of Laboratory Animals (33). SIV viral RNA loads measured from longitudinal plasma samples showed acute and post-acute levels typical of SIVmac239-infected rhesus macaques (Fig. 1). Moreover, postinfection survival time for Mm333 (82 weeks) was similar to survival times of other SIVmac239-infected animals (16).

Longitudinal plasma samples harvested on the day of inoculation as well as at weeks 4, 16, 20, 42, and 58 postinoculation were tested in vitro for neutralizing activity against SIVmac239 produced by transient transfection of HEK293T/17 cells (Fig. 2). Plasma samples taken at 42 weeks postinfection from four other rhesus macaques that were tested in parallel yielded 50% neutralization titers against SIVmac239 at a plasma dilution between 1:32 and 1:128 (Fig. 2, panel A). A pool of plasma samples (20 weeks postinfection) from multiple rhesus macaques chronically infected with SIVmac239 yielded 50% neutralization titers near a 1:32 plasma dilution. In contrast, Mm333 developed detectable neutralizing activity by week 16 (50% neutralization at a plasma dilution of 1:100 to 1:300), and neutralizing potency peaked around week 42 with 50% neutralizing titers against SIVmac239 in excess of a 1:1,000 plasma dilution (Fig. 2B). Thus, the neutralizing titer of Mm333 plasma against SIVmac239 was nearly 10-fold more effective than other SIVmac239-infected macaques. To our knowledge, this degree of neutralizing activity against SIVmac239 has never previously been described.

To confirm that the in vitro neutralizing activity in plasma samples from Mm333 was antibody mediated, we purified total IgG from the terminal plasma sample (taken at 82 weeks postinfection) using a protein A/G-agarose column. IgG was purified from 250 μl of plasma, and after elution, dialysis, and concentration, the IgG fraction was adjusted with PBS to a final volume of 250 μl. In this way, the neutralizing activities of plasma and purified IgG against SIVmac239 could be directly compared as a function of reciprocal dilution (Fig. 3). For comparison, IgG was also purified in parallel from plasma collected from an uninfected rhesus macaque. Neither the plasma nor the purified IgG from the uninfected macaque neutralized SIVmac239. The neutralizing activities of the IgG fraction and of the plasma of Mm333 were very similar (50% neutralization titer of ≈1:500). Thus, the SIVmac239-neutralizing activity detected in plasma of Mm333 was antibody mediated.

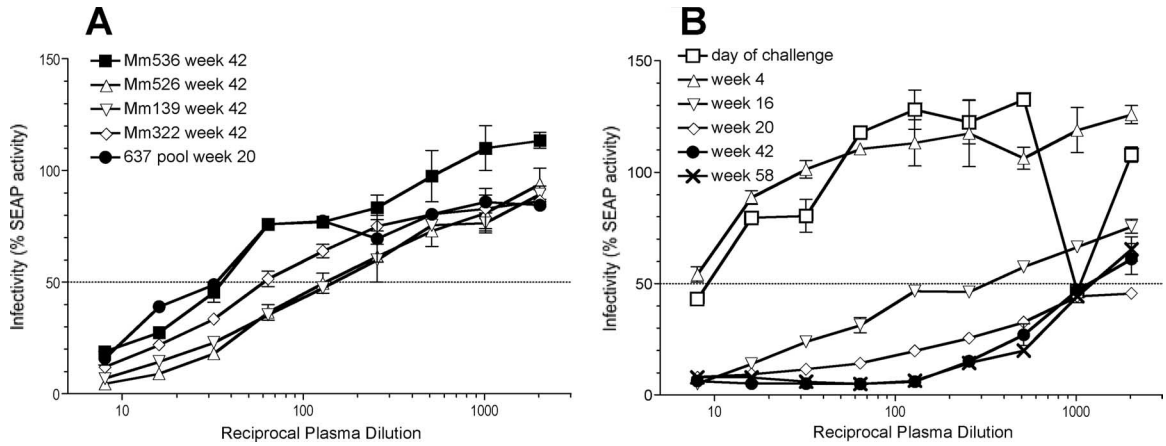

FIG. 2. Development of neutralizing plasma titers against SIVmac239 in Mm333. Plasma samples were tested for their ability to neutralize SIVmac239 using C8166-SEAP indicator cells. (A) Neutralization profile of week 42 plasma from four SIVmac239-positive rhesus macaques and a pool of plasma (week 20) from multiple SIVmac239-positive animals. (B) Neutralization profile of Mm333 for longitudinal plasma samples harvested at day of inoculation and at weeks 4, 16, 20, 42, and 58 postinfection, as indicated. All samples were tested in duplicate.

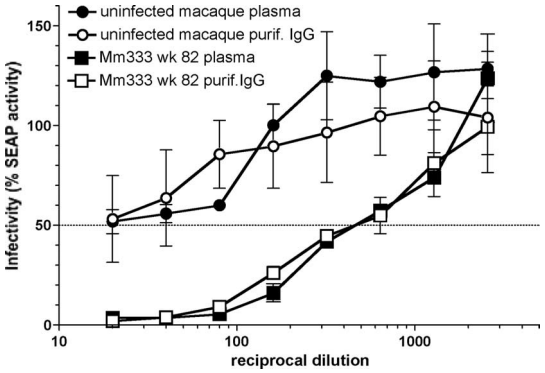

FIG. 3. Neutralization of SIVmac239 by Mm333 plasma is antibody mediated. The neutralization profiles of plasma and purified (purif) total IgG fractions are compared for Mm333 (week 82 postinoculation) and an uninfected macaque. Total IgG was purified from plasma using protein A/G beads. The purified IgG fraction volume was reconstituted with 1× PBS to be equivalent to the starting plasma sample volume so that the neutralizing titers of plasma and purified IgG could be directly compared. All samples were tested in duplicate. wk, week.

Reactivity profile of Mm333 plasma to SIVmac239 Env peptides. We next examined the specific reactivity profile of Mm333 plasma to peptides within the SIVmac239 envelope protein sequence. Ninety-six-well microtiter plates were coated with a collection of 218 15-mer peptides, overlapping by 10 amino acids and spanning the entire SIVmac239 gp160 envelope protein. Plasma pooled from multiple SIVmac239-infected rhesus macaques (AE637 pool, 20 weeks postinfection) with only weak neutralizing activity and week 26 plasma from Mm333 were tested by enzyme-linked immunosorbent assay (ELISA) for Env peptide-specific binding of IgG (Fig. 4). As a control, we were able to observe specific binding of the rhesus anti-SIV Env monoclonal antibody 3.11H to two overlapping peptides in the V3 loop (peptides 6606 and 6607) (data not shown), both of which include sequence corresponding to the linear epitope of the monoclonal antibody (11).

The Env peptide reactivity profile of the pooled plasma with weak neutralizing activity (Fig. 4A) can be categorized mainly

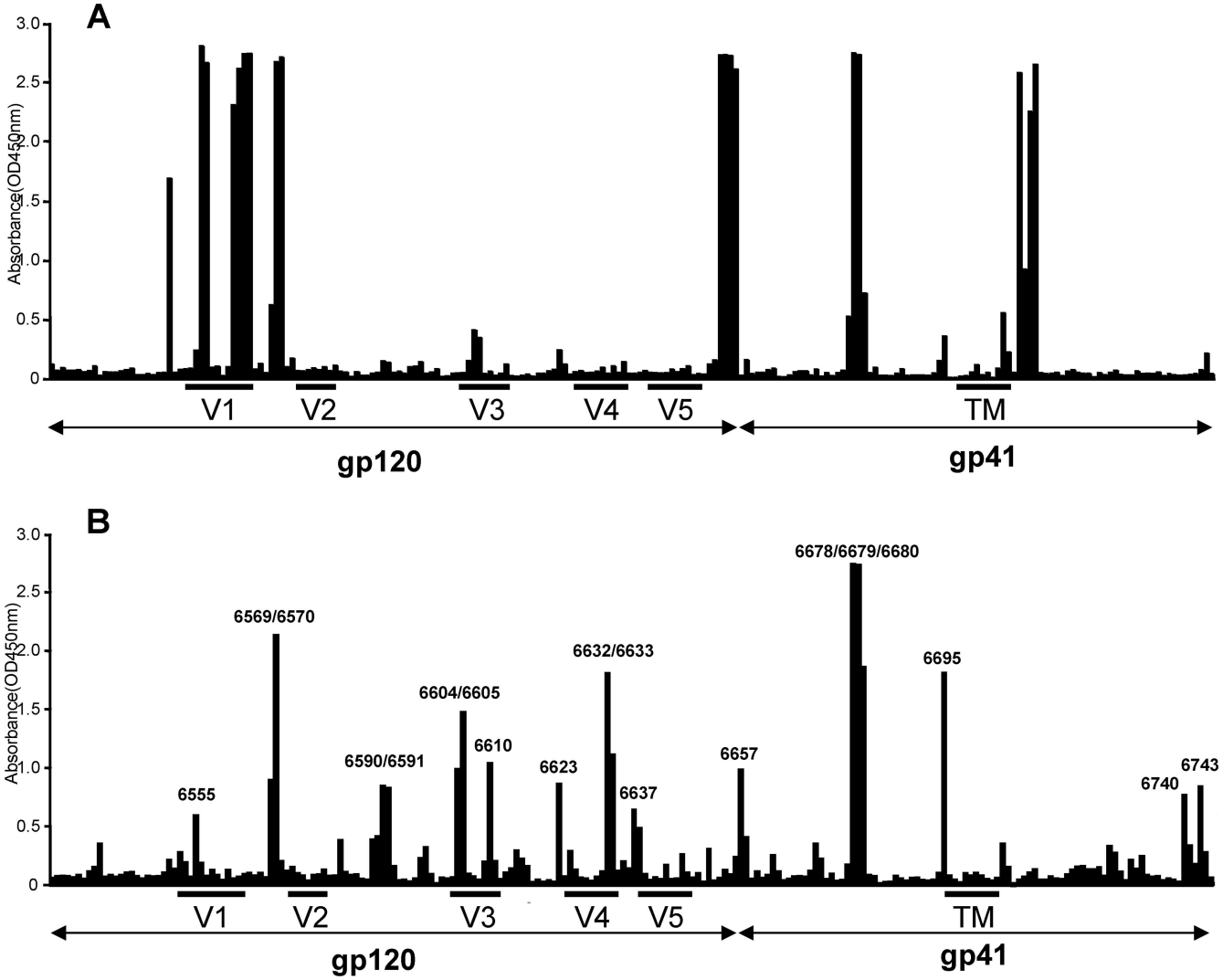

FIG. 4. Profile of Mm333 plasma binding to overlapping 15-mer SIVmac239 Env peptides by ELISA. (A) Binding profile of the week 20 plasma pool from multiple SIVmac239-positive, nonneutralizing animals. (B) Binding profile of week 26 Mm333 plasma. The numbers above the bars indicate peptides to which binding of fivefold above background was observed. OD, optical density; TM, transmembrane.

TABLE 1. SIVmac239 Env peptides strongly bound by IgG in Mm333 plasma

Peptide[a]	Sequence[b]	Region (aa position)[c]
6555	ITMRCNK**S**ETDRWGL	V1 (109–123)
6569	MISCKFNMTGLKRDK	V1/V2 (165–179)
6570	KFNMTGLKRDKKKEY	V1/V2 (169–183)
6590	SGFMPKCSKVVVSSC	β7 (249–263)
6591	PKCSKVVVSSCTRMM	β7/α1a (253–267)
6604	YNLTMKCRRPGNKTV	V3 (305–319)
6605	MKCRRPGNKTVLPVT	V3 (309–323)
6610	VFHSQPINDRPKQAW	V3 (329–343)
6623	PGGG**D**PEVTFMWTNC	CD4bl (381–395)
6632	**A**NQK**P**KEQHKRNYVP	V4 (417–431)
6633	**P**KEQHKRNYVPCHIR	V4 (421–435)
6637	IINTWHKVGKNVYLP	C4 β20/β21 (437–451)
6657	TGGTSRNKRGVFVLG	Cleavage site (517–531)
6678	KDQAQLN**A**WGCAFRQ	gp41 HR1 (601–615)
6679	QLN**A**WGCAFRQVCHT	gp41 HR1/loop (605–619)
6680	WGCAFRQVCHTTVPW	gp41 HR1/loop (609–623)
6695	L QKLNSWDVFGNWFD	gp41 MPER (669–683)
6740	WGDLWETLRRGGRWI	gp41 CT (849–863)
6743	RWILAIPRRIRQGLE	gp41 CT (861–875)

[a] Peptides to which Mm333 plasma IgG exhibited high binding (arbitrarily set at fivefold above background) in the peptide ELISA assay are listed in order from the N to C terminus of SIVmac239 Env.

[b] Amino acids shown in boldface and underlined indicate where substitutions occurred in sequences from at least two *env* clones isolated from Mm333 (Fig. 5). Substitutions that resulted in premature stop codons are excluded.

[c] The region of Env in which each peptide lies or structural motifs previously described in crystallographic studies are identified (6, 26, 55), followed by the corresponding amino acid (aa) position of the peptide within SIVmac239 Env. CD4bl, CD4 binding loop; HR, heptad repeat; HR1/loop, loop between HR1 and HR2; MPER, membrane-proximal external region; CT, cytoplasmic tail.

into four regions: in and around V1 (peptides 6550, 6556 and 6557, 6562 to 6565, and 6569 to 6571), the C terminus of gp120 (peptides 6653 to 6656), the ectodomain of gp41 (peptides 6677 to 6680), and the cytoplasmic tail (peptides 6709 to 6712). The pattern of reactivity to these four regions has been observed with individual plasma samples from three SIVmac239-infected rhesus macaques as well as two plasma pools from multiple SIVmac239-infected animals (E. Yuste, J. Bixby, J. Lifson, S. Sato, W. E. Johnson, and R. C. Desrosiers, unpublished data). In contrast, the plasma from Mm333 exhibited a distinct reactivity profile (Fig. 4B); the peptides to which Mm333 plasma antibodies bound at least fivefold above background are listed in Table 1. Reactivity of Mm333 plasma to peptides in and around V1, in the C terminus of gp120, and in the cytoplasmic tail of gp41 was either much weaker or undetectable. Moreover, an unusual reactivity pattern for Mm333 plasma was detected and included multiple peptides in and around the V3-V4 region of gp120 (a region of the envelope that had almost no reactivity with the pooled plasma from the other SIVmac239-infected animals). Two peptides in this core region of gp120 raise interest: peptide 6623, which contains the GGDPE motif that makes up the CD4-binding loop (31, 36), and peptide 6637, which corresponds to the β20/β21 ribbon (6, 24). The latter makes up part of the bridging sheet, a structure in the HIV-1 envelope that contains residues that are essential for interaction with CD4 (24) and the coreceptor (42). Additionally, Mm333 plasma displayed unique reactivity to a single peptide (peptide 6695) in the membrane-proximal external region of gp41. This peptide lies in a region within the SIVmac239 gp41 that is homologous to the region of the HIV-1 gp41 containing the epitope of the broadly neutralizing monoclonal antibody 2F5 (32).

Sequence evolution of SIVmac239 *env* during replication in rhesus macaque Mm333. Despite the development of a potent neutralizing antibody response, Mm333 failed to clear or control SIVmac239 infection, as evidenced by post-acute viral loads in excess of 10^6 SIV RNA copies/ml (Fig. 1). Moreover, SIVmac239 replication kinetics in Mm333 did not differ significantly from those observed in animals with typical, low-titer neutralizing antibody responses (Fig. 1). To investigate the hypothesis that the rapid emergence of antigenic escape variants permitted typical levels of viral replication in Mm333, despite the development of an antibody response capable of neutralizing the inoculating SIVmac239 virus, the complete SIVmac239 envelope gene was amplified by RT-PCR using SIV RNA isolated from Mm333 plasma as a template. *env* sequences were amplified from two time-points: week 16, when the neutralizing activity was first detected, and week 42, when the observed neutralizing activity reached its peak.

Ten envelope clones from week 16 and seven clones from week 42 were sequenced and aligned with the parental SIVmac239 envelope sequence (Fig. 5). An average of 9.4 (ranging from 6 to 13) and 19 (ranging from 12 to 24) nucleotide substitutions were present in the week 16 and week 42 clones, respectively. For both time points, a majority of substitutions resulted in amino acid replacements. Out of a total of 222 substitutions, 167 (75.2%) resulted in a change of amino acid. Nonsynonymous changes clearly predominated in V1 (22 of 26 substitutions, or 84.6%) and in V4 (26 of 28 changes, or 92.9%). We also identified specific amino acid positions as probable sites of positive selection using the nonsynonymous/synonymous rate ratio (*dN*/*dS*) with a method previously applied to longitudinal HIV-1 Env sequences isolated from a single HIV-1 patient by Nielsen and Yang (34). The analysis identified residues S116, P421, and D511 (posterior probability of >99%) and residues A138 and K349 ($P > 95\%$) as having undergone strong positive selection. For the week 42 samples, additional hotspots could be observed just downstream of the V3 loop, in the C terminus of gp120, and in the ectodomain of gp41. Very few changes accumulated in V2, V3, or V5, suggesting that substitutions in these regions did not confer a selective advantage during in vivo replication.

The valine at amino acid position 67 was replaced with a methionine in 16 of 17 clones and with a leucine in 1 clone. The arginine at amino acid position 751 was replaced with a glycine in all but one clone. These two substitutions are universally observed in virus isolated from SIVmac239-infected macaques (1, 4, 22, 30). However, these also appear frequently when SIVmac239 is passaged in cultured cells and cell lines (data not shown), strongly arguing that such changes affect viral replication and not escape from host immune pressure. A previous report describes V67M as one of three substitutions within gp120 that impart macrophage tropism (30). R751G is the result of a single A-to-G nucleotide substitution; however, this change also alters a codon in the second exon of *rev* (1). Neither change significantly altered viral infectivity in single-cycle infectivity assays (Fig. 6B), but this does not rule out subtle improvements in viral fitness that may manifest only over the course of multiple, complete cycles of viral replication

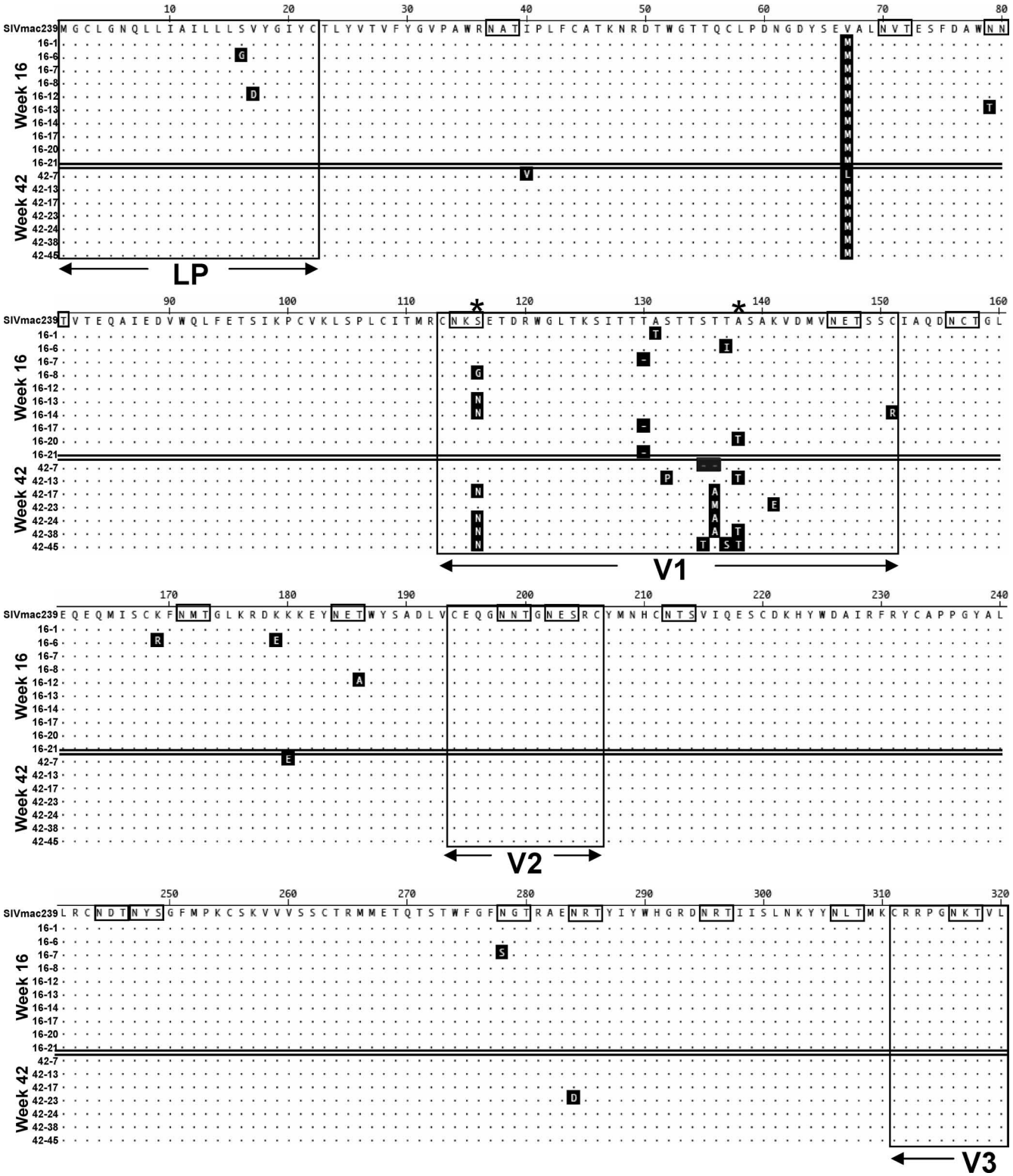

FIG. 5. Amino acid sequence alignment of envelope clones isolated from Mm333 week 16 and week 42 plasma. The full-length *env* coding region was amplified and cloned from plasma viral RNA by RT-PCR, followed by TOPO-TA cloning. Ten individual envelope clones from week 16 plasma and seven from week 42 plasma were sequenced, and variations from the parental SIVmac239 amino acid sequence are shown as white letters within black boxes. The vertical dotted line indicates the cleavage site that separates gp120 and gp41. The leader peptide (LP), variable domains V1 through V5, heptad repeats 1 and 2 (HR1 and HR2), and the transmembrane domain (TM) are outlined and indicated under each region. N-linked glycosylation motifs [NX(S/T)] in the parental sequence are boxed. Positively selected residues identified by *dN*/*dS* analysis are indicated by asterisks.

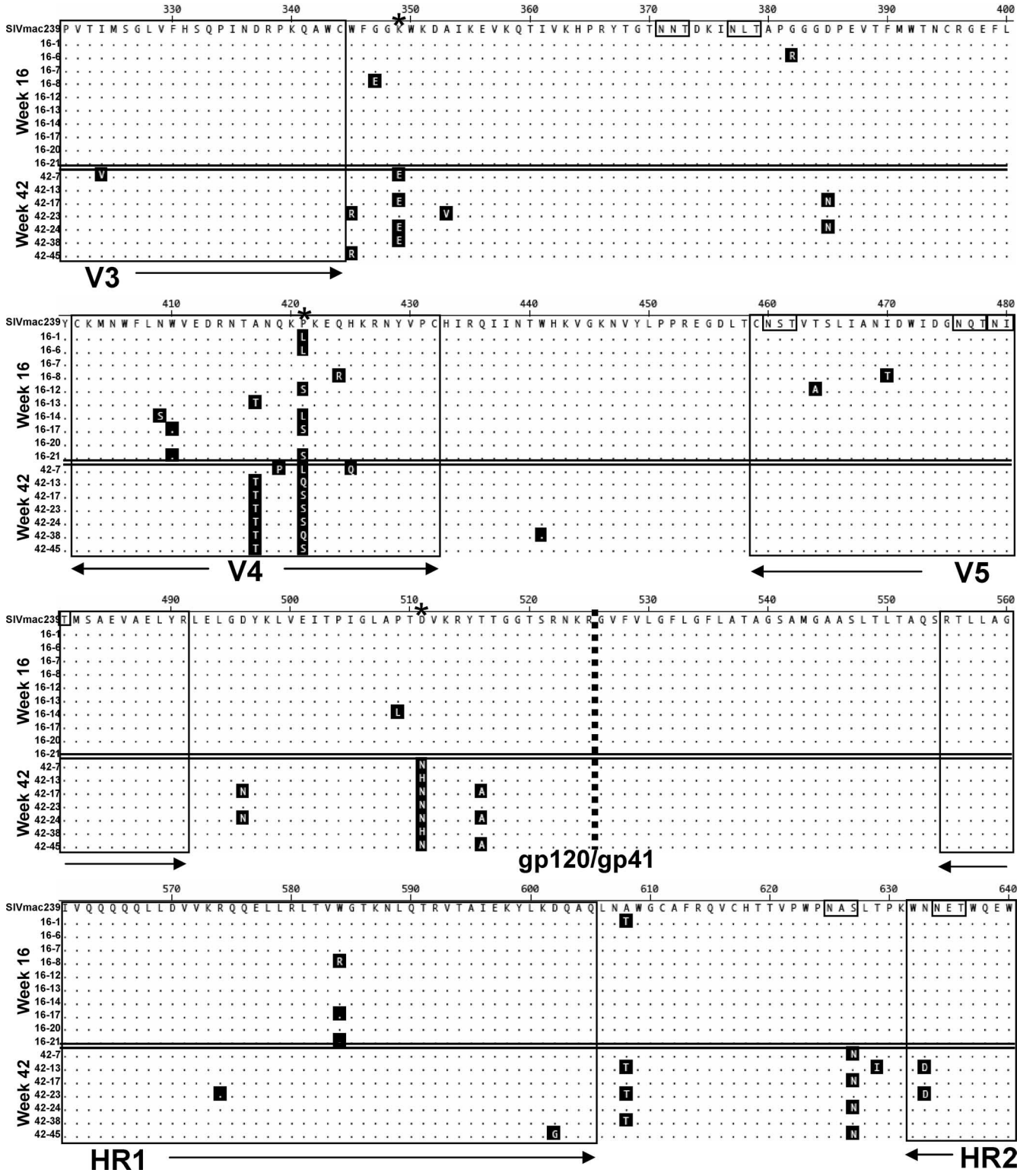

FIG. 5—*Continued.*

(the infectivity assay only assesses entry and early post-entry stages of the viral replication cycle).

The envelope gene of SIVmac239 encodes 26 possible *N*-glycan attachment sites [NX(S/T)], where X represents any amino acid except proline). Additionally, there is a serine/threonine-rich stretch within V1 (^{126}TKSITTTASTTSTTAS^{139}) that creates potential O-linked glycan attachment sites (5). Both losses and gains of N- and potential O-linked glycan

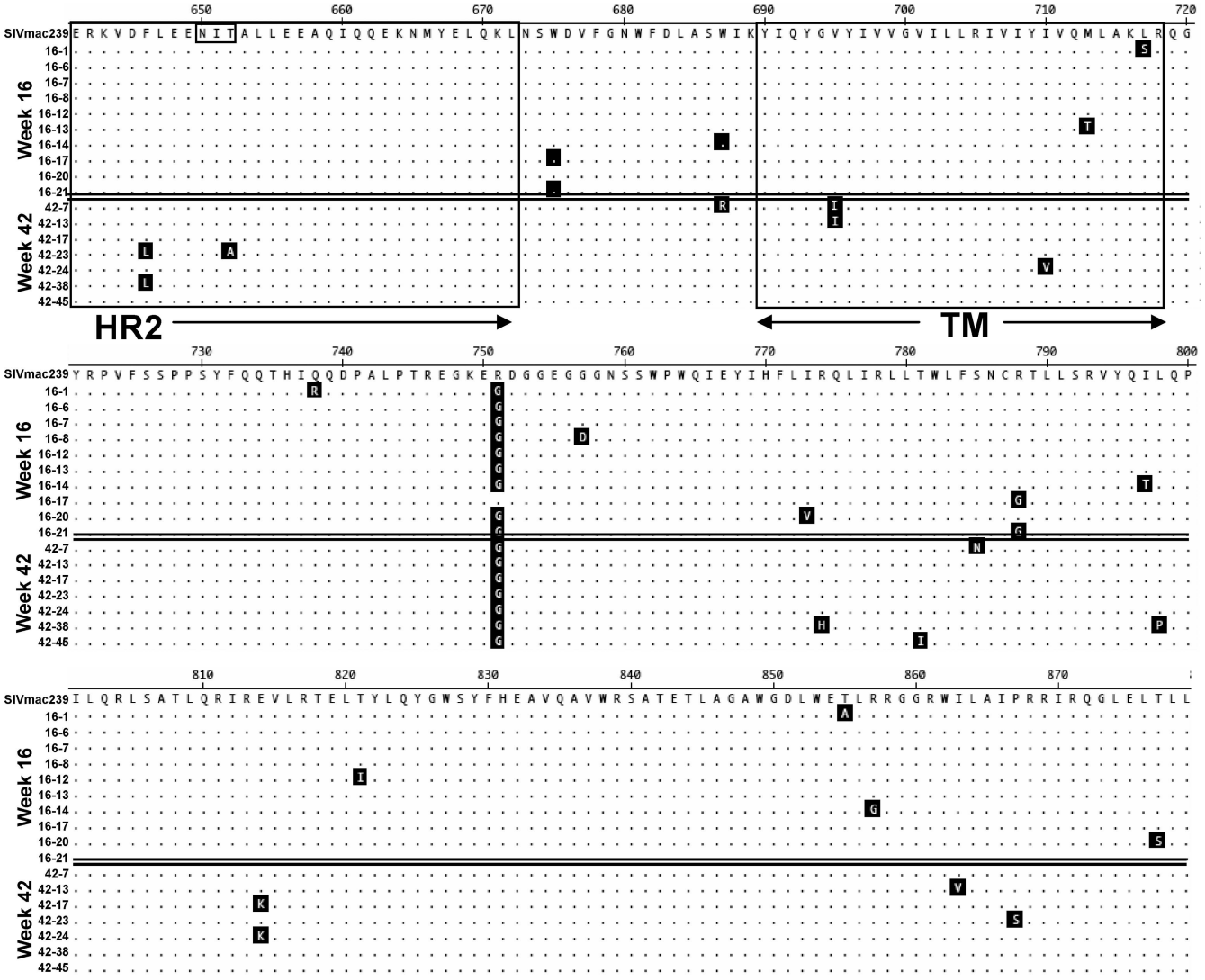

FIG. 5—*Continued.*

attachment sites were observed in a number of clones: loss of N-linked glycan attachment sites due to substitutions at amino acid positions 79, 116, 186, 278, 284, and 652 were seen, as were deletions or substitutions of S and T residues within the V1 S/T stretch. A new *N*-glycan attachment site was created within V4 due to a change of an alanine to threonine at amino acid position 417 in one week 16 clone and in all but one of the week 42 clones. In some clones, substitutions eliminating *N*-glycan attachment sites (S116N and S627N) resulted in the simultaneous creation of new attachment sites. Thus, two N-linked glycan sites were shifted by two residues at two positions, 114 to 116 in V1 and 625 to 627 in the gp41 ectodomain. Changes in V1, A131T and A138T, each adding a threonine within the S/T stretch (and thus creating potential O-linked glycan attachment sites), were also observed.

With the exception of V67M and R751G, the most commonly altered residue was the proline at position 421 in V4 (P421Q, P421L, and P421S), which was replaced in 6 of 10 week 16 clones and in all of the week 42 clones. In addition, two mutations just downstream of the V3 loop, W345R and K349E, were observed in multiple week 42 clones. One week 16 clone and two week 42 clones contained either a G382R or a D385N substitution in the GGDPE CD4-binding site motif (residues 383 to 387) (31, 36). In the C terminus of gp120 immediately downstream of V5, D511 was replaced with either N or H in all of the week 42 clones, and a T516A change was observed in three. In gp41, A608T and S627N changes had occurred in week 16 and week 42 clones.

In order to test the effects of specific amino acid substitutions on neutralization resistance, we first tested the neutralization sensitivity/resistance of each full-length Env clone to Mm333 plasma. To do this, the 12 envelope sequences with intact open reading frames were used to replace the parental *env* in an expression vector encoding full-length SIVmac239 proviral DNA, and virus was produced by transient transfection of HEK293T cells (56). The infectivities of virion stocks with substitutions in the envelope sequences were compared with the infectivity of the parental SIVmac239. Four clones

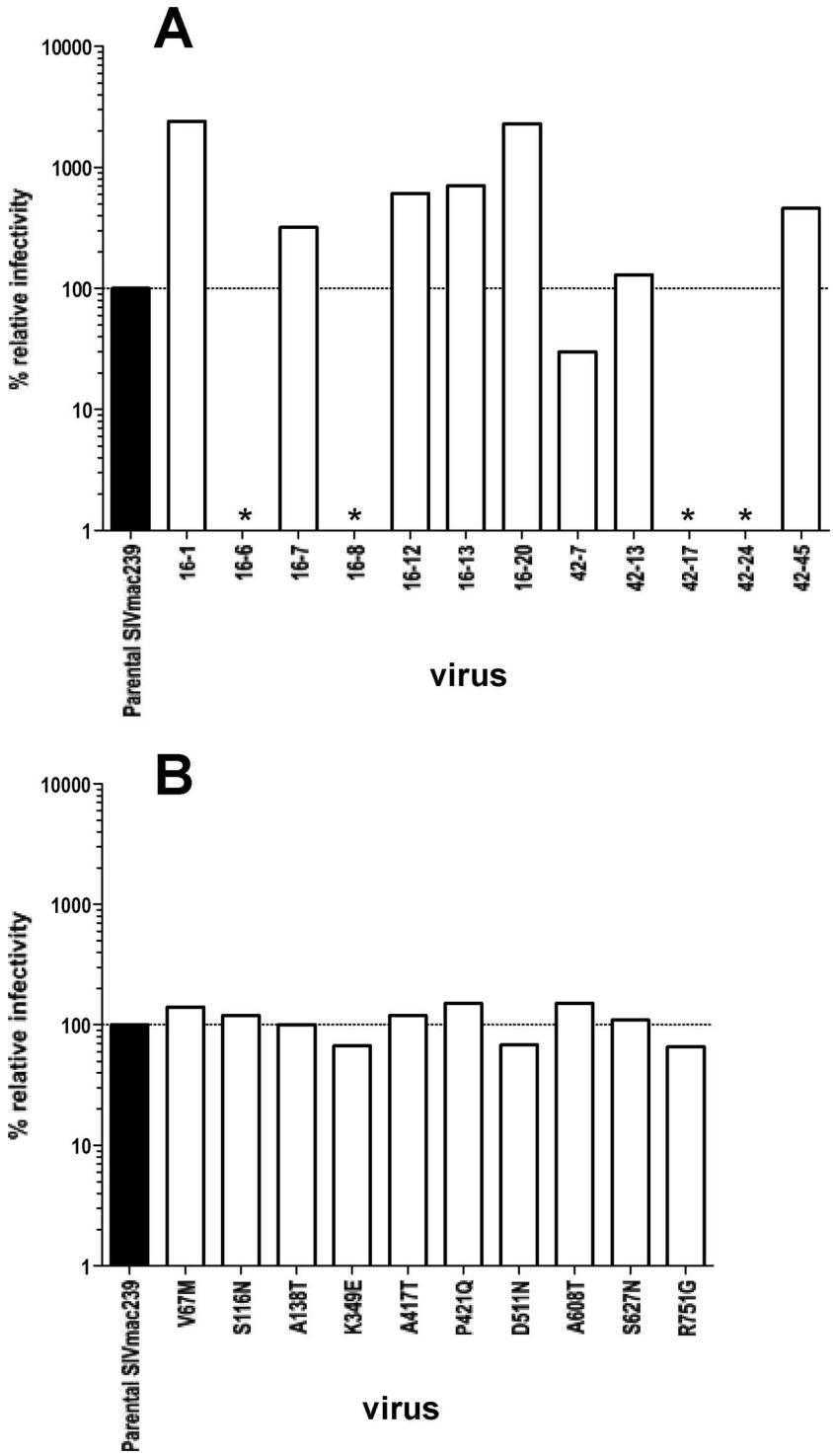

FIG. 6. Relative infectivity of SIVmac239 *env* variants. Infectivity of viruses with week 16 and week 42 full-length envelope clones (sequences are shown in Fig. 5) (A) and single amino acid substitutions (B) were assessed in C8166-SEAP cells. Clones containing stop codons in the *env* open reading frame were excluded. The percent relative infectivity was calculated as the percentage of the reciprocal of the amount of p27 (in nanograms) of variant virus that gives the same SEAP activity as 1 ng of parental SIVmac239 (100%), determined by infecting the reporter cell line with serially diluted stocks of each virus. An asterisk indicates that SEAP activity above background (uninfected cells) was not detected at the highest concentration of the particular virus tested.

were noninfectious (three of these contained alterations of the CD4-binding GGDPE motif described above), one clone (clone 7 of the week 42 clones, designated 42-7) exhibited threefold lower infectivity than parental SIVmac239, and the remaining clones (clone 1 of week 16 [16-1] and clones 16-7, 16-12, 16-13, 16-20, 42-13, and 42-45) displayed infectivity greater than the parental virus by 3- to >20-fold (Fig. 6A). Clone 42-7, the only infectious clone with decreased infectivity

in the reporter cell line, was the only variant that contained the change V67L instead of V67M. There was no obvious correlation between the level of infectivity in C8166-SEAP indicator cells and the time point from which each envelope clone was isolated.

Neutralization of *env* variants by Mm333 plasma. Neutralization of the eight infectious viral clones by archived, longitudinal plasma samples from Mm333 was assessed. The plasma samples tested were from week 16 (Fig. 7A), when neutralizing activity first became detectable; week 42 (Fig. 7B), when the neutralizing activity reached its peak; and a terminal sample taken at week 82 (Fig. 7C). Week 16 plasma exhibited mild neutralizing activity against SIVmac239, and none of the *env*-variant viruses were more sensitive to neutralization than the SIVmac239 parent. When tested against week 42 plasma, only three clones (16-7, 16-12, and 42-7) were as sensitive to neutralization as the parental virus, and all the other variants were 20- to 50-fold less sensitive. Testing against week 82 plasma revealed a pattern similar to that seen with week 42 plasma, with the exception of clone 42-7, which was resistant to the week 82 plasma. Thus, substitutions conferring escape from antibody-mediated neutralization were already frequent in Mm333 by week 16 postinfection. Moreover, the neutralization-resistant envelope sequences present by week 16 postinfection were also resistant to neutralization by plasma from all later time points tested, including terminal samples taken at week 82 (66 weeks later). Two clones (16-7 and 16-12), which lost N-linked glycan attachment sites and did not gain any additional glycan attachment sites, were neutralization sensitive. In contrast, clone 16-13, which lost one N-linked glycan site but gained a new N-linked site and one potential O-linked site, was highly resistant.

To identify specific envelope sequence changes responsible for the neutralization-resistant phenotype, single amino acid substitutions that were common to more than one neutralization-resistant clone but were absent from the neutralization-sensitive variants were introduced into the parental SIVmac239 proviral sequence, either by subcloning or by site-directed mutagenesis. Substitutions that were observed in only one clone were excluded from the analysis as these could not be distinguished from errors that might have occurred during RT-PCR amplification. Ten SIVmac239 variants bearing single substitutions were expressed by transient transfection of HEK293T cells. The infectivity of each single-substitution variant in C8166-SEAP cells was similar to that of parental SIVmac239 and did not deviate by more than 50% (Fig. 6B). The effects of the single residue changes on neutralization resistance were tested against the terminal plasma sample (week 82) of Mm333 (Fig. 8). The A138T substitution in V1 and the A417T and P421Q substitutions in V4 each imparted a dramatic increase in resistance to the Mm333 plasma of between 30- to 50-fold relative to the parental virus (Fig. 8A). Thus, single residue changes in V1 (A138T) or in V4 (A417T or P421Q) were each sufficient to confer a high level of resistance to the potently neutralizing plasma when tested individually. In addition, the two changes in the gp41 ectodomain resulted in moderate increases in neutralization resistance: about threefold for A608T and sevenfold for S627N (Fig. 8A). None of the other substitutions tested altered sensitivity or resistance to neutralization by Mm333 plasma by more than twofold (Fig.

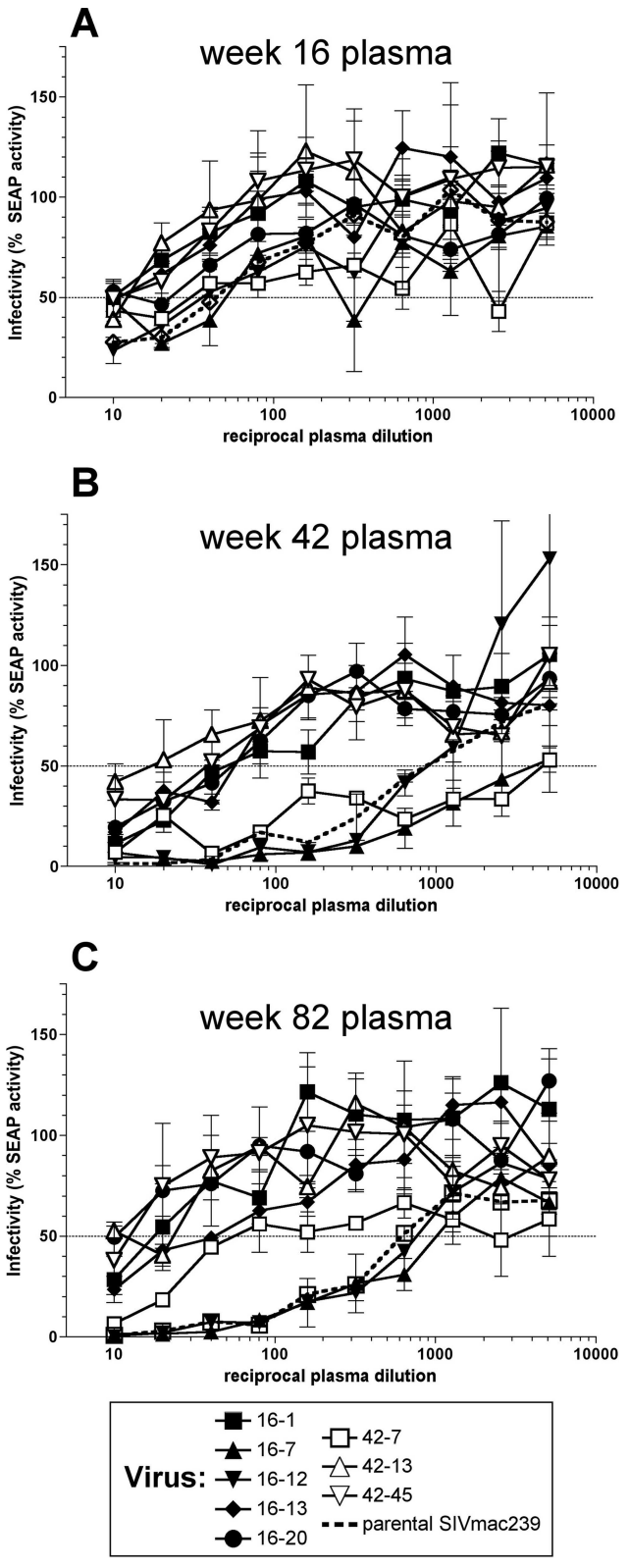

FIG. 7. Neutralization resistance of week 16 and week 42 *env* clones to longitudinal Mm333 plasma samples. SIVmac239 variants encoding *env* sequences isolated from week 16 (clones 16-1, 16-7, 16-12, 16-13, and 16-20) and week 42 (clones 42-7, 42-13, and 42-45) were tested for neutralization resistance to Mm333 plasma from week 16, week 42, and week 82 using C8166-SEAP cells. All samples were tested in duplicate.

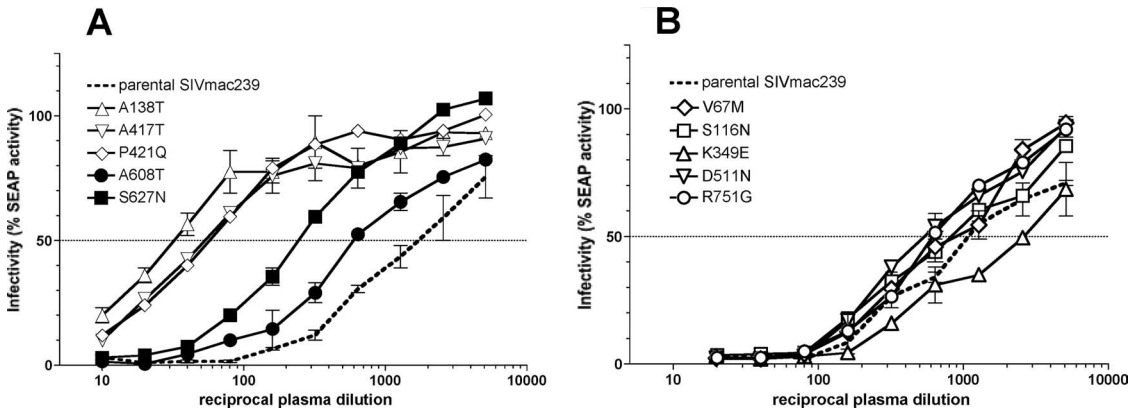

FIG. 8. Neutralization escape imparted by single substitutions in Env. Single residue substitutions in Env identified in neutralization escape clones isolated from Mm333 week 16 and week 42 plasma were introduced in parental SIVmac239 and tested for neutralization resistance to week 82 plasma. (A) Substitutions that increased neutralization resistance to Mm333 plasma. (B) Substitutions that did not affect neutralization resistance. All samples were tested in duplicate.

8B). Two changes that conferred increased resistance to neutralization (A138T and P421Q) were also identified by *dN*/*dS* analysis as having a high probability ($P > 99\%$) of evolving under strong positive selection.

Neutralizing activity of Mm333 plasma was also tested against a heterologous SIV molecular clone, SIVsmmPGm5.3 (35). The envelope of this SIV strain shares 83% amino acid identity with SIVmac239 Env, with most of the divergence mapping to the V1 and V4 loops and the variable region immediately downstream of the V3 loop. SIVsmmPGm5.3 was neutralized by Mm333 week 82 plasma at only very high concentrations (50% neutralization at a plasma dilution of 1:16 to 1:32) (data not shown).

DISCUSSION

The SIV strain SIVmac239 is typically resistant to antibody-mediated neutralization by autologous, virus-specific antibody responses, a property it shares with many primary isolates of HIV-1. Archived samples from rhesus macaque Mm333, which produced high-titer neutralizing antibody responses to SIVmac239, provided a unique opportunity to investigate neutralization of SIVmac239 and to map the evolution of antigenic escape variants. Analysis of neutralizing activity in longitudinal plasma samples revealed that by week 42 postinoculation, the 50% neutralizing antibody titer against the inoculum virus had reached 1:1,000 (plasma dilution), at least an order of magnitude greater than we have previously found in animals experimentally infected with SIVmac239 (16–18, 57).

Despite the presence of a vigorous, virus-specific neutralizing antibody response, we did not observe any detectable signs of control of SIV infection by animal Mm333 compared to other SIVmac239-infected macaques, suggesting possible rapid escape of the virus from neutralizing antibody responses in this animal. Indeed, viral escape variants resistant to multiple longitudinal plasma samples were already circulating at high frequency in the animal by week 16 postinfection (the earliest time point analyzed). Furthermore, these escape variants were also resistant to the terminal plasma sample harvested 66 weeks later (week 82 postinfection). In other words, de novo antibody responses with the capacity to neutralize the escape variants did not subsequently develop in this animal. This observation differs from previous studies of HIV-1, such as those described by Richman and colleagues (41) and by Wei et al. (48), in which the development of neutralizing antibody responses and the emergence of escape variants were analyzed in HIV-1-positive patients. In those studies, 50% neutralizing titers against autologous HIV-1 reached 1:100 or greater as early as 8 to 10 weeks after infection in two patients (one from each study) and by 3 to 6 months in others. In each patient, viral escape variants emerged, but after a lag of several months secondary neutralizing antibody responses to the resistant variants also appeared. In contrast, we found that SIVmac239 escape variants that were already present by week 16 postinfection in Mm333 were also resistant to plasma harvested 42 weeks (6 months) and even 82 weeks (16 months) later. The present study did not address the inability of the infected animal to mount an effective neutralizing antibody response to escape variants. We speculate that rapid emergence of resistant virus permitted uninterrupted, high levels of viral replication (Fig. 1), and the consequent destruction of CD4-positive T helper cells may have prevented further evolution and maturation of the virus-specific neutralizing antibody response.

By introducing selected single amino acid substitutions observed in the envelope sequences of escape variants into the parental SIVmac239 sequence, we determined that neutralization resistance was conferred independently by specific, single amino acid changes in either V1 or V4. When tested separately, substitutions A138T (V1 loop), A417T (V4 loop), or P421Q (V4 loop) each individually conferred nearly complete resistance to neutralization by Mm333 plasma. Two additional changes in gp41, A608T and S627N, resulted in intermediate levels of resistance (Fig. 8A). Thus, single amino acid replacements at positions widely separated in the primary Env sequence independently conferred moderate to high levels of resistance.

Intuitively, one might expect that these positions are in close proximity in the folded protein or, alternatively, that they are spatially juxtaposed in the fully assembled heterotrimeric complex, where they form either a single epitope or closely over-

lapping, conformational epitopes. However, in the unliganded SIV gp120 structure reported by Chen and colleagues, the V1-V2 stem and the V4 loop are clearly located at opposite ends of the protein (6), and it is unlikely that the loops would be in close proximity within the monomer. Cryoelectron tomography models published by two independent groups indicate that the V1-V2 and the V4 domains of different subunits are probably well separated within the trimeric envelope spikes of intact virions (58, 59); similarly, a hypothetical model of the SIV trimer proposed by Chen et al. also indicates that the V1-V2 and V4 domains of different gp120 subunits are not juxtaposed (6). If the structural models are correct, then changes in the two spatially distinct domains (V1 and V4) must influence resistance to neutralization by independent mechanisms although how this might be achieved is not clear. One possibility is that such changes indirectly affect the packing of *N*-glycans across the trimer (P. Kwong, personal communication), thus altering much of the antigenic surface. In the case of V1, a theoretical trimer model based on the unliganded SIVgp120 crystal structure indicates a potential intermolecular interaction between V1-V2 and V3 of adjacent monomers (6). If true, perhaps the adaptive changes in V1 influence the trimeric arrangement of protomers in such a way as to reduce or obscure access to the primary targets of neutralization. Another possibility is that both V1 and V4 independently influence a third domain or region more directly involved in antibody recognition. Whatever the explanation, for any such model residues such as A138T, A417T, or P421Q somehow serve as molecular "switches," whereby a single amino acid replacement at any one of these sites allows the virus to respond rapidly to selection by polyclonal neutralizing antibody responses. Watkins et al. proposed a similar model based on the observation that single amino acid changes (one in gp120 and one in gp41) selected by passage of HIV-1 in the presence of neutralizing patient serum could independently confer resistance to antibody-mediated neutralization (47). Such hypothetical models do not preclude additional adaptations that facilitate escape directly through alteration of individual antibody binding sites, but a global mechanism of escape may be critical in the context of polyclonal antibody responses directed at distinct epitopes or in situations where multiple B-cell receptors converge on similar or overlapping targets.

We also analyzed sequences for evidence of strong positive selection operating on individual residues in the Env proteins by calculating the ratio of the rates of nonsynonymous and synonymous change for each site across the entire *env* open reading frame (34, 52). Five sites stood out with high posterior probabilities of positive selection; these were at positions 116, 138, 349, 421, and 511. These included two of the sites harboring changes conferring a high level of resistance to neutralization (A138T in V1 and P421Q in V4), consistent with the hypothesis that these changes conferred a strong selective advantage in vivo. Notably, there were in fact three variants at position 421 (P421Q/L/S) (Fig. 5), suggesting that the elimination of proline, more than the incorporation of a new amino acid, was responsible for the observed effect. An earlier study also described the emergence of changes in the V4 domain of SIVmac239 in vivo, including a four-amino-acid deletion encompassing P421 (residues 420 to 423) (4). A follow-up study reported that this deletion, as well as several of the other changes in V4, resulted in loss of binding to recombinant gp120 by multiple anti-gp120 murine monoclonal antibodies (7). While the positively selected changes at positions 116, 349, and 511 did not appear to alter resistance/sensitivity to neutralization, these may represent adaptations to other selective pressures, such as cytotoxic T lymphocyte or T helper responses. Importantly, while *dN*/*dS* analysis is useful for identifying sites under strong positive selection, it depends on sufficient signal being present in the sequence data (2, 54), and not all positively selected adaptations will necessarily be detected by this approach. For example, amino acid changes at 417 (V4), 608 (gp41), and 627 (gp41) also clearly contributed to neutralization resistance and are likely to have been selected in vivo although *dN*/*dS* analysis alone was not suggestive of positive selection. A broader analysis involving more sampling both within and between individual SIV-infected macaques, coupled with functional assays, could ultimately help identify sites most frequently subject to positive selection and perhaps reveal common themes among the observed patterns of escape from neutralizing antibody responses.

Amino acid substitutions that did not result in an observable decrease in sensitivity to Mm333 plasma (Fig. 8B) or an increase in infectivity in our reporter cell line (Fig. 6B) may still have been selected in vivo for a variety of reasons (52). As mentioned above, some changes may reflect escape from other immunological pressures, including virus-specific cellular immune responses, whereas others may reflect compensatory adjustments that served to accommodate those changes directly involved in escape. Very small selective advantages can translate into large relative increases in viral fitness in the infected host, such that even those changes with effects on viral replication too subtle to be detected in a single round of infection will nonetheless be strongly selected over the course of multiple rounds of viral replication in vivo (9, 10). The reporter cell line assays were performed by shuttling *env* sequences into the parental SIVmac239 and testing a single round of infectivity; thus, it is also possible that the phenotypic consequences of some changes may only manifest in the context of other changes not present in the parental SIVmac239 backbone. If any differences in infectivity were unique to primary macaque cells or dependent on conditions present in the infected animal, these would also not be readily detected by in vitro infectivity assays.

The patterns of sequence evolution described here are not unique to animal Mm333 or to the SIVmac239 strain of SIV. Several of the changes we found to be involved in escape were also noted previously (3, 4, 20) although in the previous studies individual substitutions were not assessed for their contributions to antigenic escape. In a study that analyzed evolution of the V1-V2 region of a primary biological isolate of SIVsmm in both sooty mangabeys and rhesus macaques, Vanderford et al. noted multiple sites under strong positive selection in V1 in both species, including the same *N*-glycan attachment site altered by substitution S116N in SIVmac239 (Fig. 5) (46). Rudensey et al. analyzed the evolution of sequence changes in *env* in a different species of macaque (*Macaca nemestrina*) infected with a different SIV isolate (SIVmne) and specifically found that changes in N-linked and O-linked glycosylation of V1 conferred resistance to neutralization by macaque serum (44). These were very similar to changes we found in the corre-

sponding region of SIVmac239, including potential changes in both N-linked and O-linked glycosylation. Moreover, the reported changes in V1 sequence of SIVmne-CL8, like the changes we identified in V1, V4, and gp41 of SIVmac239, did not define the neutralizing epitope(s) themselves but, rather, influenced neutralization by altering or masking epitopes involving other regions of the Env spike. Two of the independent changes conferring escape in Mm333 mapped to the V4 loop, including the replacement of a proline (P421Q) and the creation of a new *N*-glycan attachment site (A417T) (Fig. 5). Similarly, Wei et al. found that changes in glycosylation pattern in V4 were among the key substitutions associated with antigenic escape in HIV-1-infected patients (48). We also identified two substitutions in gp41, each of which individually conferred moderate levels of resistance to neutralization by longitudinal plasma samples from Mm333. A similar result was reported several years ago for HIV-1 selected by passage in cultured cells in the presence of neutralizing patient serum (40, 43). In those studies, a single alanine-to-threonine replacement in the viral gp41 protein was sufficient to confer escape from antibodies directed at the gp120 subunit, indicating that changes in one subunit can affect neutralization-sensitive epitopes in the other (21, 45, 49). Thus, while it is likely that antigenic escape can also occur through direct alteration of specific antibody binding sites, it is clear from this study and the work of other groups that primate lentiviruses can rapidly and efficiently generate resistant variants through single amino acid changes in different regions of Env. This is distinct from known or suspected modes of lentiviral escape from virus-specific cytotoxic T lymphocytes, where resistance typically involves alterations that directly affect the processing, presentation, or recognition of individual epitopes (14).

The observation that amino acid changes scattered throughout the viral envelope proteins can facilitate antigenic escape from high-titer neutralizing plasma independently of one another highlights another challenge in the design of immunogens to elicit potent neutralizing antibody responses. In addition to the poor immunogenicity of the viral Env spike, the nature of the single residue changes that lead to escape suggests that the relevant target(s) can be rapidly and efficiently concealed via several, independent pathways. The identification of animals such as Mm333 is a first step toward isolating monoclonal antibodies that can neutralize SIVmac239. Cloning and characterization of neutralizing monoclonal antibodies from such animals and experimental definition of their cognate binding sites will complement studies on antigenic escape and hopefully advance our understanding of the molecular mechanisms of neutralization and viral immune evasion.

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