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Molecular and Biological Analyses of Quasispecies during Evolution of a Virulent Simian Immunodeficiency Virus, SIVsmmPBj14

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A prototypic simian immunodeficiency virus (SIVsmm9), isolated from a naturally infected sooty mangabey (*Cercocebus atys*), was passaged in vivo in a pig-tailed macaque (*Macaca nemestrina*) having the identifier PBj. When PBj died of a typical AIDS-like syndrome 14 months after infection, the virus isolated from its tissues was subsequently shown to differ from SIVsmm9 genetically and biologically. Most notably, this isolate, SIVsmmPBj14 (SIV-PBj14), is the most virulent primate lentivirus known: it induces acute disease and death within 6 to 10 days after intravenous inoculation into pig-tailed macaques. Between the time of infection with SIVsmm9 and isolation of SIV-PBj14, isolates were obtained periodically from peripheral blood mononuclear cells of PBj. To establish the temporal relationship between evolution of new biologic properties and fixation of specific mutations in the virus population, these sequential SIV-PBj isolates were characterized for unique properties of SIV-PBj14 that appeared to correlate with acute lethal disease. These properties included the ability to replicate in quiescent macaque peripheral blood mononuclear cells, to activate and induce proliferation of $CD4^+$ and $CD8^+$ cells, and to exhibit cytopathicity for mangabey $CD4^+$ lymphocytes. Consistent with earlier studies, a major change in biologic properties occurred between 6 (SIV-PBj6) and 10 (SIV-PBj10) months, with the SIV-PBj8 quasispecies exhibiting properties of both earlier and later isolates. Multiple biologic clones derived from the 6-, 8-, and 10-month isolates also exhibited diverse phenotypes. For example, one SIV-PBj10 biologic clone resembled SIVsmm9 phenotypically, whereas three other biologic clones resembled SIV-PBj14. To evaluate genetic changes, proviral DNA of the biologic clones generated from SIV-PBj6, -PBj8, and -PBj10 was amplified by PCR in the U3 enhancer portion of the long terminal repeats (LTR) and the V1 region of *env*, where the greatest nucleotide diversity between SIVsmm9 and SIV-PBj14 resided. Nucleotide sequence data indicated that all biologically cloned viruses are distinct and that insertions/duplications of 3 to 27 nucleotides (in multiples of three) had accumulated stepwise in the *env* V1 region, beginning with SIV-PBj8. In addition, one of four SIV-PBj8 biologic clones had a 22-bp duplication in the LTR which is characteristic of SIV-PBj14. When virus mixtures containing different proportions of two SIV-PBj10 biologic clones with opposite phenotypes were tested, the SIV-PBj14 phenotype was clearly dominant, since mixtures with as few as 10% of the viruses being SIV-PBj14-like exhibited all the properties of the lethal isolate. The results suggest that neither the duplication of the NF-κB binding site in the LTR nor the duplications/insertions in *env* V1 (nor a combination of both mutations) were sufficient to confer the SIV-PBj14 biologic phenotype. However, because some of the unique SIV-PBj14 properties segregate, further analysis of biologically and molecularly cloned viruses derived from these sequential isolates should lead to the identification of viral determinants for specific traits.

Because of the quasispecies nature of the primate lentiviruses (24, 40), the biologic properties of any one isolate are a composite of phenotypes, each determined by a unique genotype or subset of genotypes. This concept was demonstrated for the human immunodeficiency virus type 1 (HIV-1) by generation and evaluation of properties of biologically or molecularly cloned viruses isolated from peripheral blood mononuclear cells (PBMC) from individuals in different stages of disease (10, 25, 41). A priori one might assume that differences in phenotypes of isolates from one individual directly reflect the most prevalent members of the quasispecies at a given time. However, it is possible that some biologic properties are dominant, in which case a phenotype might be determined by a minor species in the population, or alternatively that a specific phenotype results from interactions between two or more viruses with properties different from those observed (16, 30, 45).

Transition of the phenotypically dominant members of a quasispecies from less pathogenic to more pathogenic viruses is one of several hypotheses to explain HIV-related disease and its variable progression to AIDS (7, 8, 47). On the basis of the characterization of multiple isolates obtained both cross-sectionally and longitudinally from individuals in various cohorts, transition of HIV-1 from non-syncytium-inducing (NSI) to syncytium-inducing (SI) phenotypes appears to be correlated with and to predict loss of $CD4^+$ lymphocytes (9, 18, 38, 39, 42, 46). This shift from an NSI to SI phenotype is also associated with a broadening of cellular tropism from macrophage-tropic to macrophage/T-cell-tropic isolates, which is manifest by an acquired ability to replicate in continuous T-cell lines (7, 25, 42). While one can speculate about mechanisms for disease progression in HIV-infected individuals and the possible role of HIV-1 variants with different biologic properties, these assumptions cannot be tested directly because animal models of HIV-1 infection that result in disease are not available. Because of the molecular and biologic similarities between HIV

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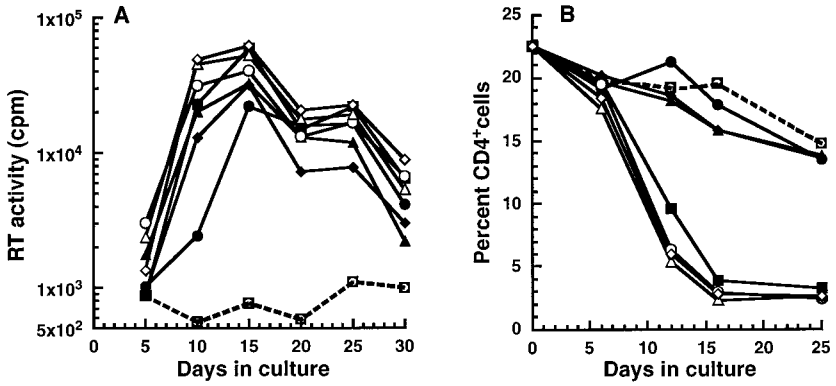

FIG. 1. Comparison of SIVsmm9 and the sequential SIV-PBj isolates for replication in mitogen-stimulated PBMC from sooty mangabeys (A) and CPE on mangabey CD4$^+$ lymphocytes (B). The data are from a representative experiment in which cultures of mangabey PBMC were infected with equivalent amounts of virus and were monitored in parallel. ◘, uninfected; ●, SIVsmm9; ▲, SIV-PBj3; ◆, SIV-PBj6; ■, SIV-PBj8; ○, SIV-PBj10; △, SIV-PBj12; ◇, SIV-PBj14.

and simian immunodeficiency virus (SIV), however, SIV infection of macaques provides an excellent model with which to test various hypotheses.

One particular isolate of SIV, SIVsmmPBj14 (SIV-PBj14), is especially useful not only for studying mechanisms of pathogenicity but also for evaluating strategies for prophylactic and therapeutic intervention. SIVsmmPBj14 was isolated from pig-tailed macaque (*Macaca nemestrina*) PBj at the time of its death, 14 months after inoculation with SIVsmm9 (22). This latter strain was originally isolated from a naturally infected sooty mangabey monkey (*Cercocebus atys*) and induces a slowly progressive AIDS-like disease after experimental infection of rhesus (*Macaca mulatta*) and pig-tailed (20, 21, 23) macaques, but it is not pathogenic for mangabeys. In contrast, SIV-PBj14 induces acute disease and death within 6 to 10 days not only in pig-tailed and cynomolgus (*Macaca fascicularis*) macaques but also in sooty mangabeys (22, 29). Generation of biologic clones from SIV-PBj14 demonstrated the quasispecies nature of this isolate, including the identification of viruses phenotypically similar to the parental SIVsmm9 (22). Thus, derivation of acutely lethal molecularly cloned viruses required the use of a biologically cloned isolate, SIV-PBj14-bcl3, that reproducibly induced acute disease and death in pig-tailed macaques (12, 34). Nucleotide sequence comparisons between SIVsmm9 and SIV-PBj14-derived infectious molecular clones (which also induce acute disease and death) revealed that a maximum of 57 point mutations plus two duplications/insertions were sufficient to encode the acutely lethal phenotype on the SIVsmm9 genetic background (11–13). These two insertions included a 22-bp duplication encompassing an NF-κB enhancer element in the long terminal repeat (LTR) and a 15-bp duplication in the V1 region of the *env* gene. Evaluation of infectious chimeric viruses generated by recombination between defined regions of SIV-PBj14 and the unrelated SIVsmmH4 molecular clones indicated that more than one region of the SIV-PBj14 genome contributed to, but that the 22-bp duplication of NF-κB was not required for, full manifestation of the unique lethal phenotype (33, 34).

Between the time of inoculation of macaque PBj with SIVsmm9 and its death, viruses were periodically isolated from this animal's PBMC. These isolates were designated SIV-PBj3, -PBj4.5, and -PBj6, etc. (for isolates obtained, respectively, 3, 4.5, and 6, etc., months after infection), and were cryopreserved as P0 and P1 stocks, which represent viruses from the initial coculture of PBj's PBMC with human PBMC and first-passage virus stocks generated on human PBMC, respectively. The availability of these sequential isolates provides a unique opportunity to study the genetic and biologic evolution of a virulent lentivirus. Furthermore, since we have identified eight in vitro properties that differ between SIVsmm9 and SIV-PBj14, it may be possible to localize determinants of individual biologic phenotypes to specific genes or regions of the SIV genome. Moreover, because some of the properties segregate, the contribution of individual properties to the acute disease syndrome can be evaluated in vivo. This report describes the generation and characterization of biologically cloned viruses from three SIV-PBj sequential isolates (SIV-PBj6, -PBj8, and -PBj10) that were obtained before and after major transitions in phenotype occurred.

MATERIALS AND METHODS

Viruses and cells. Virus stocks of SIVsmm9 and the SIV-PBj sequential isolates were generated on phytohemagglutinin P (PHA)-stimulated normal human PBMC by a single passage of SIVsmm primary isolates (P0). P0 isolates were obtained by cocultivation of normal human PBMC with PBMC from mangabey FEd (for SIVsmm9) or macaque PBj at different times after the inoculation of SIVsmm9. Biologically cloned viruses derived from SIV-PBj6, -PBj8, and -PBj10 were generated by two cycles of serial limiting dilution and infection of human PBMC, as described previously (22). Titers of virus stocks, defined as 50% tissue culture infectious doses ($TCID_{50}$), were determined by limiting dilution and infection in triplicate of PHA-stimulated human PBMC in 12-well culture plates; cultures were monitored every 4 to 5 days for reverse transcriptase (RT) activity. All cultures of human and macaque PBMC were maintained in complete medium consisting of RPMI 1640 medium supplemented with glutamine, antibiotics, 10% fetal bovine serum, and recombinant human interleukin-2 (IL-2; 8 U/ml), unless otherwise specified. All analyses of replicative properties of viruses were done with primary PBMC obtained from normal SIV-seronegative pig-tailed macaques, sooty mangabeys, or humans; PBMC were obtained from heparinized whole blood processed on Ficoll-sodium diatrizoate gradients (LSM; Organon Teknika, Durham, N.C.).

Replicative properties of viruses. After separation from blood, PBMC were washed with phosphate-buffered saline, and 10^7 cells were placed in culture in individual 25-cm^2 flasks in a total volume of 10 ml where they were infected immediately with virus (resting PBMC) or stimulated 3 days with PHA (human PBMC) or concanavalin A (sooty mangabey PBMC) before infection. Complete medium was used, except in experiments to evaluate replication in resting macaque PBMC, in which case the medium lacked IL-2 (RPMI–10% fetal bovine serum). As a measure of virus replication, aliquots of cell-free supernatants were removed periodically to determine RT activity; culture volumes were maintained by the addition of fresh medium. To determine percentages of CD4$^+$ and CD8$^+$ lymphocytes in uninfected and infected cultures, aliquots of 2×10^5 cells were removed, washed, and incubated with equal amounts of phycoerythrin-labeled Leu3a (CD4) and fluorescein isothiocyanate (FITC)-labeled Leu2a (CD8) (Becton-Dickinson, Mountain View, Calif.). Lymphocytes were gated according to forward-scatter versus side-scatter characteristics, and negative cells were identified with FITC-labeled immunoglobulin G1 (IgG1) and phycoerythrin-labeled immunoglobulin-G2a control monoclonal antibodies. A minimum of 10^4 cells was analyzed with a FACS-STAR flow cytometer (Becton-Dickinson).

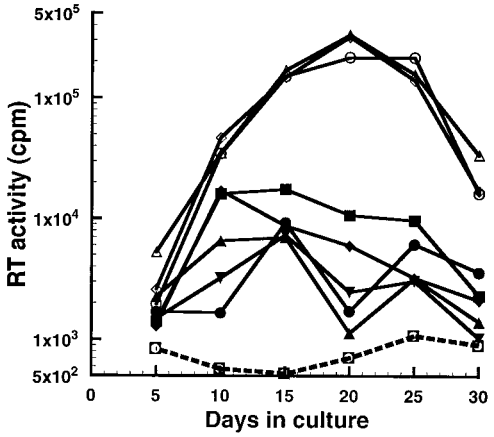

FIG. 2. Replication of SIVsmm9 and SIVsmmPBj sequential isolates in quiescent pig-tailed macaque PBMC. Equal amounts of virus ($TCID_{50}$) were used to infect PBMC immediately after separation from whole blood. No mitogens or exogenous IL-2 was added to the cultures. ◘, uninfected; ●, SIVsmm9; ▲, SIV-PBj3; ▼, SIV-PBj4.5; ◆, SIV-PBj6; ■, SIV-PBj8; ○, SIV-PBj10; △, SIV-PBj12; ◇, SIV -PBj14.

Activation and proliferation of PBMC by virus. After infection of resting pig-tailed macaque PBMC, activation was defined as increases in percentages of lymphocytes expressing the CD25 antigen (IL-2 receptor alpha subunit), determined by FACScan analysis with FITC-labeled anti-TAC monoclonal antibodies (Becton-Dickinson). The total number of cells and cell viabilities were determined by trypan blue dye exclusion. The ability of viruses to induce proliferation of resting macaque PBMC was evaluated by incorporation of [^{3}H]thymidine 6 days after incubation of 10^5 cells with virus containing 10 ng of $p27^{gag}$, as previously described (34). Levels of p27 in virus stocks were determined with an SIV-p27 enzyme-linked immunosorbent assay kit (Coulter Electronics, Inc., Hialeah, Fla.). Stimulation indices were calculated by dividing the counts per minute incorporated in the presence of virus by counts per minute incorporated by cells in medium only.

PCR amplification, cloning, and sequencing. Nucleotide sequences were determined with proviral DNA from PBMC in which each biologic clone was propagated; infected cells were harvested at the same time supernatants of biological clones were collected for viral stocks, and genomic DNA was isolated. This genomic DNA served as a template for PCR amplification of approximately 500 bp spanning the NF-κB site in the U3 enhancer region of the LTR. The oligonucleotide primers were PBj-LTR-A, 5′-AGAAGAGTTTGGTAGTCAGT CAGG-3′, and PBj-LTR-D, 5′-ACCAGGCGGCGGCTAGGAGAGATG-3′. Fragments of approximately 460 bp spanning the V1 region of *env* were also amplified and sequenced with the following primers: PBj-*env*-I, 5′-CAATGCT TGCCAGATAATGGTGATTACTC-3′, and PBj-*env*-II, 3′-GTCACAGGATT CTTGAATAACACTGGT-3′. After purification with the Geneclean Kit (Bio 101, Vista, Calif.), the PCR-amplified products were cloned into the PCR vector, which was then transfected into competent *Escherichia coli* cells (TA Cloning kit; Invitrogen, San Diego, Calif.). DNA from multiple positive clones representing each SIV-PBj biologic clone was prepared and sequenced by the dideoxynucleotide chain termination method (Sequenase version 2 Kit; U.S. Biochemicals, Cleveland, Ohio).

RESULTS

Biologic properties of SIV-PBj sequential isolates. In previous studies (22) we showed that SIVsmm9 and some of the SIV-PBj sequential isolates differed in their abilities to replicate in human T-cell lines and the abilities to be neutralized by serum from macaque PBj. The intermediate isolates, however, were not tested for the unique properties of SIV-PBj14 that were identified subsequently and that, in analyses of biologically and molecularly cloned viruses derived from SIV-PBj14, appeared to correlate with acute disease (12, 20, 34). One such property exhibited by SIV-PBj14, but not its parent virus SIVsmm9, was cytopathicity for mangabey $CD4^+$ cells (12, 20). Infection of concanavalin A-stimulated PBMC from SIV-seronegative sooty mangabeys with equivalent amounts of the sequential isolates resulted in comparable production of progeny virus, although in some experiments the kinetics of SIVsmm9 replication appeared to lag behind that of the other isolates (Fig. 1A). Distinct differences were noted, however, in the loss of $CD4^+$ lymphocytes from the cultures as virus accumulated in the medium (Fig. 1B). Only those isolates obtained at 8 months and later times after infection of PBj were cytopathic, whereas all isolates were cytopathic for macaque $CD4^+$ lymphocytes (data not shown).

In contrast to the requirement of other primate lentiviruses that $CD4^+$ lymphocytes be activated before efficient replication and production of virus (31, 37, 44, 48). SIV-PBj14 replicated efficiently in quiescent (or resting) lymphocytes (19). When the sequential isolates were evaluated for this property, only SIV-PBj10 and -PBj12 achieved levels similar to that of SIV-PBj14 (Fig. 2). Since basal levels of activated lymphocytes in peripheral blood vary among individual animals at any one time, in some experiments other isolates produced low levels of virus, but these amounts (determined by RT activity) were always 10% or less than that produced by the last three SIV-PBj isolates. This ability to replicate efficiently in quiescent lymphocytes might be linked to the abilities of SIV-PBj14 not only to activate lymphocytes but also to induce proliferation (19, 34, 43). Activation of lymphocytes was monitored by increases in CD25 expression after infection of resting PBMC, which was shown previously to occur after maximum levels of virus were reached (19). Consistent with previous results, effi-

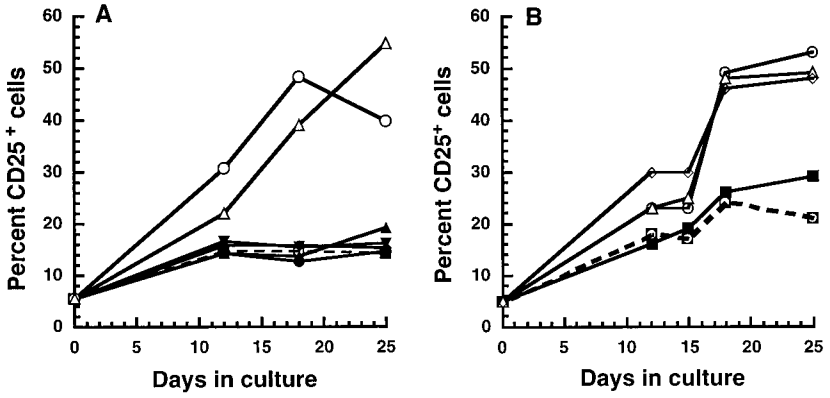

FIG. 3. Activation of quiescent pig-tailed macaque PBMC in cultures infected with the SIVsmmPBj sequential isolates. Aliquots of cells were removed from cultures periodically and tested for the presence of CD25 by FACScan. ◘, uninfected; ●, SIVsmm9; ▲, SIV-PBj3; ▼, SIV-PBj4.5; ◆, SIV-PBj6; ■, SIV-PBj8; ○, SIV-PBj10; △, SIV-PBj12; ◇, SIV-PBj14. Data from two experiments are shown.

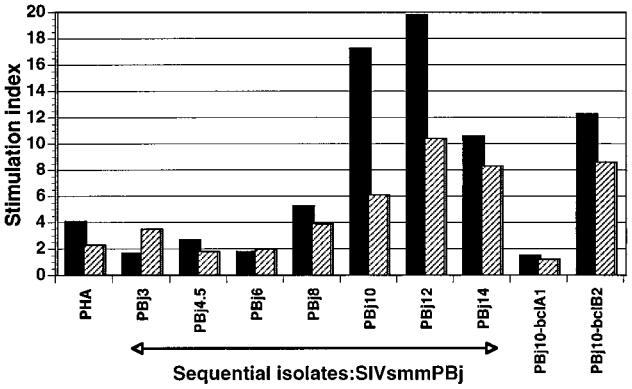

FIG. 4. Proliferation of quiescent pig-tailed macaque PBMC induced after 6 days of incubation with the SIV-PBj sequential isolates and two biologic clones derived from SIV-PBj10. The solid and striped bars represent separate experiments with PBMC from two different macaques. Stimulation indices were obtained by dividing counts per minute incorporated by cells incubated with PHA or virus (10 ng of p27) by counts per minute of cells in medium alone.

cient replication in resting pig-tailed macaque PBMC by SIV-PBj10, -PBj12, and -PBj14, but not earlier isolates, induced expression of CD25 (Fig. 3). Furthermore, since activation of lymphocytes does not always lead to proliferation, incorporation of [^{3}H]thymidine into cellular DNA was measured. This assay showed that infection of resting macaque PBMC with these same three isolates and, to a lesser extent, SIV-PBj8, also induced significant levels of proliferation (Fig. 4). Thus, these results are consistent with our earlier conclusion that during persistent infection of macaque PBj, a major change in the phenotype of the SIVsmm quasispecies occurred between 6 and 10 months after inoculation of SIVsmm9 (Table 1).

Generation and characterization of biologically cloned viruses. Because SIV exists as a quasispecies in vivo; there were several possibilities to explain the observed phenotypic changes of the sequential SIV-PBj viruses: (i) there was a turnover in the major virus form(s) present in the peripheral blood of macaque PBj; (ii) a variant that remained a minor virus form, but that was dominant over other members of the quasispecies, evolved; or (iii) two or more distinct variants that complemented one another arose, resulting in a new phenotype. To address these possibilities, biologically cloned viruses were obtained by serial limiting dilutions of SIV-PBj6, -PBj8, and -PBj10, the isolates that spanned the transition period;

TABLE 1. Biologic properties of SIVsmm9 and sequential isolates of SIVsmmPBj

Isolate	Presence of the following property[a]:			
	Replication	Activation	Induction proliferation	CPE
smm9	−	−	−	−
PBj3	−	−	−	−
PBj4.5	−	−	−	−
PBj6	−	−	−	−
PBj8	−	−	−/+	+
PBj10	+	+	+	+
PBj12	+	+	+	+
PBj14	+	+	+	+

[a] Biologic properties tested included replication in resting PBMC from pig-tailed macaques, activation and induction of proliferation, of macaque PBMC, and CPE on mangabey CD4 lymphocytes (see text). +, isolate exhibits property; −/+, partial activity; −, no or minimal activity.

TABLE 2. Biologic and molecular properties of SIVsmm9 and biologically derived clones from SIV-PBj

Isolate and clone	Presence of the following property:				
	Replication[a]	Activation[a]	CPE[a,b]	LTR[c]	*env* V1[d]
PBj6-bcl					
A-1	−	−	−	−	−
A-2	−	−	−	−	−
A-3	−	−	−	−	−
PBj8-bcl					
A-1	−	−	−/+	+	+ (3)
B-1	−	−	−/+	−	+ (6)
C-1	−	−	−/+	−	+ (9)
D-1	−	−	−/+	−	+ (9)
PBj10-bcl					
A-1	−	−	−	+	+ (27)
B-2	+	+	+	−	+ (15)
C-1	+	+	+	−	+ (27)
C-4	+	+	+	−	+ (27)
smm9	−	−	−	−	−
PBj14-bcl[e]					
1	−/+	−	−	−	+ (15)
3	+	+	+	+	+ (15)

[a] For biologic properties, see text and footnote *a* to Table 1.
[b] −, not cytopathic; −/+, intermediate levels of CPE; +, complete loss of $CD4^+$ cells.
[c] The presence of the 22-bp duplication in the LTR is denoted by +. −, no duplication.
[d] −, V1 regions having the same number of base pairs as the V1 region of SIVsmm9; +, V1 regions containing duplications, with the number of bases duplicated shown in parentheses.
[e] SIV-PBj14 biologic clones are described in reference 22.

multiple biologic clones (bcl) were then characterized for phenotypes (Table 2). Of the biologic clones evaluated, all exhibited phenotypes similar or identical to that of SIVsmm9, with the exception of clones derived from SIV-PBj10; three of four clones derived from SIV-PBj10 reproduced the phenotypes of SIV-PBj14, whereas one clone (PBj10-bclA-1) had phenotypic properties identical to those of SIVsmm9. Since SIV-PBj8 P0 and P1 virus stocks exhibited cytopathic effects (CPE) for mangabey $CD4^+$ lymphocytes equivalent to that of SIV-PBj14, it was unexpected that only intermediate levels of $CD4^+$ cell depletion were seen in mangabey cultures infected with each of 15 biologic clones derived from SIV-PBj8. It was possible, however, that members of the SIV-PBj8 quasispecies synergized to effectively eliminate $CD4^+$ cells from the cultures. When all 15 SIV-PBj8 biologic clones were mixed, this possibility appeared unlikely, since levels of cytopathicity did not differ from those seen with individual clones. Thus, it appeared that CPE for mangabey $CD4^+$ lymphocytes resulted from a variant(s) that comprised a small fraction of the population but was dominant.

Molecular analysis of biologically cloned viruses. In addition to distinct phenotypes, SIVsmm9 and SIV-PBj14 also differed with respect to genotypes. Differences in the nucleotide sequences of proviral DNA of these two viruses were identified previously; in particular, SIV-PBj14 had acquired a 22-bp duplication in the U3 enhancer region of the LTR, that resulted in two NF-κB binding sites and a 15-bp duplication in the V1 region of the *env* gene (11–13). Therefore, to define the evolution of these mutations, 10 to 35 cloned fragments generated by PCR amplification of cellular DNA from PBMC infected

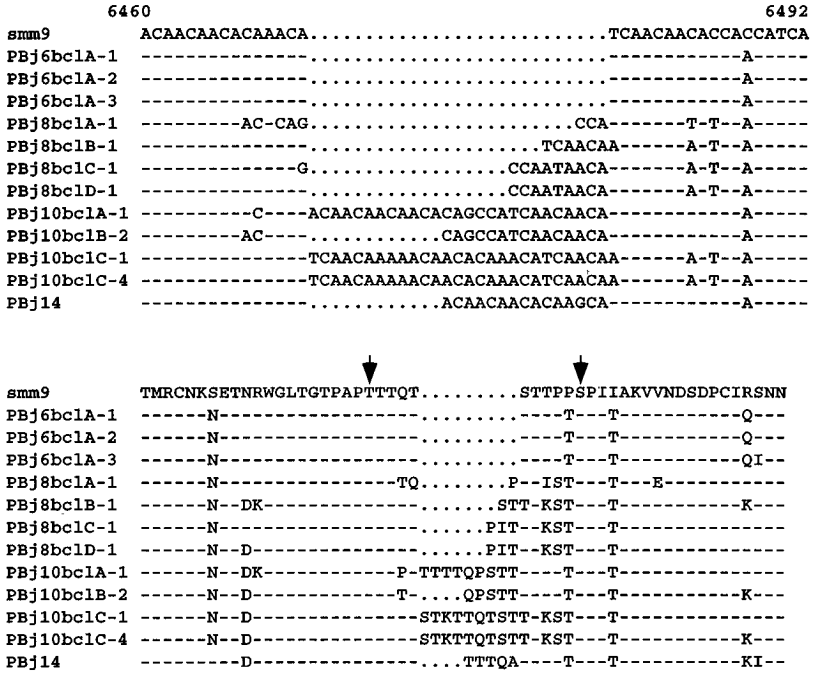

FIG. 5. Evolution of variation in the *env* V1 region of SIVsmm9 following infection of macaque PBj. (Upper panel) Extent of nucleotide sequence duplication/insertion (nucleotides 6460 and 6492 in SIVsmm9 [32]) in the biologically cloned viruses from SIV-PBj6, -PBj8, and -PBj10. (Lower panel) Amino acid sequences of the entire V1 regions of these clones. Arrows mark the amino acids encoded by the sequences shown above. Dashes indicate identity, and dots indicate gaps introduced to optimize alignments.

with the SIV-PBj6, -PBj8, and -PBj10 biologically cloned viruses were sequenced in these two regions of the viral genome. Only two biologic clones, one clone derived from SIV-PBj8 (bclA-1) and one derived from SIV-PBj10 (bclA-1), had the 22-bp duplication in the LTR; however, both of these biologically cloned viruses displayed the SIVsmm9 phenotype (Table 2). Although all PCR-generated clones derived from the SIV-PBj6 biologic clones lacked duplications or insertions in these two genomic regions, all were unique because of several distinct point mutations (data not shown).

The composition of the V1 regions of the various biological clones derived from SIV-PBj8 and -PBj10 was more complex (Table 2). In addition to point mutations, duplications/insertions in V1 occurred in multiples of 3 bases (3 to 27 bases), which primarily involved C's and A's and generated codons for threonine (Fig. 5). That V1 regions from SIV-PBj8 viruses had acquired three- to nine-base duplications, whereas those derived from SIV-PBj10 had 15- to 27-base duplications/insertions, indicated that these mutations accumulated incrementally as disease progressed. Furthermore, of the 45 point mutations identified in all biologic clones in the region encoding the entire 49 amino acids in the SIVsmm9 genome, 100% resulted in amino acid changes, strongly indicating nonrandom selection. This high degree of amino acid changes and the observation that the preponderance of nucleotide substitutions were G→A (40%) or C→A (38%) are consistent with the results of earlier studies (2, 4, 27, 28, 36).

Identification of a dominant phenotype. Although a majority of the SIV-PBj10 biologic clones that were tested reproduced the SIV-PBj14 phenotype, mixing experiments were performed to determine whether the most prevalent virus in a population determined the phenotype of the quasispecies. Since SIV-PBj10-bclA-1 and -bclB-2 were generated from the same pool or swarm of viruses but had distinct properties, different proportions of these two viruses were tested for the ability to replicate in resting pig-tailed macaque PBMC, to induce expression of CD25 on macaque lymphocytes, and to exhibit cytopathicity for mangabey $CD4^+$ lymphocytes (Table 3). Infection of the various cell populations with a total of 100 $TCID_{50}$ of the two biologically cloned viruses demonstrated that all mixtures containing at least 10% SIV-PBj10-bclB-2 exhibited the phenotype of this virus, whereas only those mixtures with as little as 5% or less of this virus (and 95% or more of bclA-1) exhibited properties indicative of SIVsmm9. These results indicated, therefore, that biologic properties associated with the SIV-PBj14 acutely virulent phenotype are dominant over those of its parent virus.

TABLE 3. Biologic properties of mixtures with different proportions of SIV-PBj10-bclA-1 and SIV-PBj10-bclB-2

$TCID_{50}$s of indicated PBj10-bcl clone in mixed infections[a]		Presence of the following property[b]:		
A-1	B-2	Replication	Activation	CPE
100	0	−	−	−
99	1	−	−	−
95	5	−	−	−
90	10	+	+	+
75	25	+	+	+
50	50	+	+	+
25	75	+	+	+
0	100	+	+	+

[a] Numbers represent actual $TCID_{50}$s of the respective viruses used to infect PBMC cultures.

[b] For biologic properties, see text and footnote *a* to Table 1.

DISCUSSION

In this study we demonstrated that some of the unique properties associated with the acutely lethal disease induced by SIV-PBj14 evolved independently in the SIVsmm9-related quasispecies generated in macaque PBj. Furthermore, biologic properties previously shown to correlate with high-level virus burdens and rapid death, including induction of proliferation of quiescent lymphocytes, are dominant in a mixed virus population containing as little as 10% SIV-PBj14-like viruses. Although transition in viral phenotypes occurred at approximately 8 months after infection, macaque PBj had decreased percentages and absolute numbers of $CD4^+$ cells in peripheral blood at 6 weeks after infection (which continued to decline) and developed persistent thrombocytopenia at 3 months (23). Thus, progression to AIDS preceded by several months the presence of the SIV-PBj14-like phenotype—a phenotype consistent with a switch from NSI (SIVsmm9) to SI (22). This is somewhat different from HIV infection, in which progression to AIDS and rapid loss of $CD4^+$ lymphocytes generally occur in association with transition from NSI to SI viruses (9, 47). However, it is possible that variants resembling SIV-PBj14 evolved earlier than 6 to 8 months but comprised less than 10% of the quasispecies.

We previously speculated that cytopathicity for mangabey $CD4^+$ lymphocytes might explain induction of acute disease and death in mangabeys by SIV-PBj14 (12). It is possible that acquisition of mutations that increase cytopathicity and virulence in the natural host also might result in increased virulence in other species. Because the SIV-PBj8 quasispecies was cytopathic for mangabey $CD4^+$ lymphocytes but only marginally induced proliferation of resting macaque PBMC, evaluation of the pathogenesis of this isolate in pig-tailed macaques might provide information on the contribution of this property to acute pathogenicity. Since members of the quasispecies with a replicative advantage might be amplified preferentially in vivo, infection of macaques with SIV-PBj8 and molecular and biologic characterization of viruses recovered early after inoculation might also provide insight into transmission of complex viral mixtures.

Do the observed genetic changes contribute to the various SIV-PBj14 phenotypic properties? Although substantial evidence has been generated by us and others to indicate that the 22-bp duplication in the LTR is not required (33, 34), it is possible that, in conjunction with other strain-specific sequences or genes, the second NF-κB site might contribute to the SIV-PBj14 phenotype. Dollard et al. (15) demonstrated that molecular constructs and recombinant viruses containing this duplication exhibit enhanced transcription and kinetics of replication, respectively. Transfer of the SIV-PBj14 U3 enhancer region into an apathogenic SIVagm molecular clone rendered the virus able to replicate in resting macaque and African green monkey PBMC (14). Since SIVagm already has two NF-κB sites that are identical to those in SIV-PBj14, this latter result suggests that other enhancer elements, such as the SP1 or NFAT binding sites, all of which differ in nucleotide sequence between SIVagm and SIV-PBj14, may be important. Moreover, these results imply that the SIV-PBj14 properties being evaluated require the interaction of two or more viral determinants, which may differ on different viral genetic backgrounds.

An additional determinant important for induction of the lethal disease by SIVsmm recombinant clones maps to *env*. The mutation(s) in the V1 region of *env* probably has little influence on the pathogenicity of SIV-PBj14; in studies by Novembre et al. (34), one virulent and two nonvirulent SIV-PBj14-derived molecular clones were conserved in this domain. Comparable mutations that result in the accumulation of serine and threonine residues also occur in the V1 region of SIVmac239, SIVmac251-32H, SIVsmF236, and SIVMne during disease progression in macaques (1, 4–6, 27, 35, 36). Overbaugh and Rudensey (35) proposed that increased O-linked glycosylation on these serine/threonine-rich regions may increase cell-cell recognition and play a role in syncytium formation by infected lymphocytes. Alterations in potential sites for N-linked glycosylation in the V1 region, as in other SIV isolates, were also noted (1, 5, 6, 35). However, none of these altered sites in the SIV-PBj biologic clones was in the two small hypervariable regions identified in the SIVMne V1 region, where numerous new N-linked glycosylation sites were created as the disease progressed (35).

Because of the complex interaction of the primate lentiviruses with the immune system, immune-mediated selection for more-pathogenic variants may be important. It is of interest, therefore, that the V1 region of SIVmac239 contains an epitope recognized by $CD8^+$ cytotoxic T lymphocytes, suggesting that escape from cellular immunity contributes to SIV pathogenesis (17). Although we do not know whether macaque PBj had cytotoxic T lymphocytes against this epitope, escape from neutralizing antibodies was documented for viruses isolated from PBj at 8 months and later (22). An SIV immunodominant neutralization epitope(s) has not been identified. In fact, SIV neutralization epitopes appear to be conformational (26) and therefore discontinuous. Thus, the identification of the epitope involved in the evolution of SIV-PBj14 and its relevance to pathogenesis will require substantial work. Sequence analysis of neutralization-resistant variants of SIVmac239 failed to localize relevant mutations, since amino acid changes were present in all five variable regions of the *env* gene (3). In addition to possible immune selection, the potential contribution of 1 or more of the 57 point mutations that appear specific for SIV-PBj14 compared with SIVsmm9 (11) cannot be overlooked. The biologic clones derived from the sequential PBj isolates, however, will be and indeed have already been proven valuable in our attempts to map viral determinants for specific properties in the SIV genome.

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