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Evolution of nef variants in gut associated lymphoid tissue of rhesus macaques during primary simian immunodeficiency virus infection

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Abstract

We utilized the simian immunodeficiency virus model of AIDS to examine evolution of *nef* gene in gut-associated lymphoid tissue (GALT) during primary and early asymptomatic stages of infection. Macaques were infected with a cloned virus, SIVmac239/nef-stop harboring a premature stop codon in the *nef* gene. Restoration of the *nef* open reading frame occurred in GALT early at 3 days post-infection. Analysis of *nef* sequences by phylogenetic tools showed that evolution of *nef* was neutral thereafter, as evidenced by the ratio of synonymous to nonsynonymous substitutions, a star pattern in unrooted trees and distribution of amino acid replacements fitting a simple Poisson process. Two regions encoding for a nuclear localization signal and a CTL epitope were conserved. Thus, GALT was a site for strong positive selection of functional *nef* during initial stages of infection. However, evolution of the *nef* gene thereafter was neutral during early asymptomatic stage of infection.
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Keywords: SIV-Nef; Evolution; Positive selection; Neutral model; Adaptive; GALT; Synonymous and nonsynonymous

Introduction

Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections are characterized by a high degree of viral genomic diversity which could arise from a combination of transcription errors by the error-prone reverse transcription and immune-mediated positive selection for viral variants (Bebenek et al., 1989; Bonhoeffer et al., 1995; Coffin, 1996; McMichael and Phillips, 1997; Roberts et al., 1988; Temin, 1993). HIV appears to evolve differentially in various tissues including lymph nodes, brain and lung (Ait-Khaled et al., 1995; Delassus et al., 1992; Haggerty and Stevenson, 1991; Itescu et al., 1994; Kodama et al., 1993; Korber et al., 1994; Wong et al., 1997). Molecular evolutionary studies have examined the role of viral evolution in disease progression by performing longitudinal analysis of genetic diversity and divergence in vivo (Ganeshan et al., 1997; Shankarappa et al., 1999; Wolinsky et al., 1996; Zanotto et al., 1999). These studies showed a positive relationship between the genetic diversity and disease progression. However, others have reported a lack of relationship between emergence of viral variants and disease outcomes (Markham et al., 1998; Ross and Rodrigo, 2002; Strunnikova et al., 1995, 1998; Zanotto et al., 1999).

Gut associated lymphoid tissue (GALT) harbors more than 80% of the total lymphoid tissue in the body. It is an early site for active HIV and SIV replication, and severe depletion of $CD4^+$ T cells accompanied by CD8 T cell lymphocytosis and virus specific cytotoxic T cell (CTL) responses (Heise et al., 1993; Mattapallil et al., 1999; Shacklett et al., 2000; Smit-McBride et al., 1998; Veazey et al., 1998). Since GALT provides a microenvironment conducive for active viral replication, it may serve as an ideal site for viral evolution and a source for the emergence of viral variants that could be attributed to the disease outcome (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995; Kodama et al., 1993; Mariani et al., 1996). Data on the evolution of viral variants in GALT during primary SIV or HIV infection are either limited or

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nonexistent. During primary SIV and HIV infections, high viral replication coincides with severe CD4$^+$ T cell depletion in the GALT and this is followed by a clinically asymptomatic stage with decreased viral loads. Both CD4$^+$ T cells and macrophages are important targets of HIV and SIV in the GALT. However, due to changes in mucosal cell populations following severe loss of CD4$^+$ T cells, macrophages may serve as an important viral reservoir. It is unclear whether divergence of viral variants would reflect the changes in GALT microenvironment and consequently disease stages.

The *nef* gene of HIV and SIV encodes for a 25–27 kDa multifunctional protein that has been shown to play a key role in SIV and HIV pathogenesis (Piguet and Trono, 1999; Trono, 1995). While Nef is not required for viral replication in tissue culture, it is necessary for the development of high viral loads and disease progression in vivo (Kestler et al., 1991). Experimental infection of adult rhesus macaques with SIVmac239 virus with nef deletions showed low viral loads, near normal CD4$^+$ T cell counts and absence of disease symptoms (Deacon et al., 1995). The importance of *nef* gene in HIV-1 infection is suggested by the findings that a few HIV-1-infected long-term nonprogressors with low viral loads harbor HIV variants containing deletions in the *nef* gene (Deacon et al., 1995; Kirchhoff et al., 1995). These studies and others suggested that mutations in the *nef* gene could impact disease outcome in SIV and HIV infections (Huang et al., 1995; Shugars et al., 1993). In contrast, some studies did not find any correlation between *nef* gene variation and disease progression (Mourich et al., 1999; Zhu et al., 1996).

Evolutionary theories have been used to gain insights into the correlation between viral population dynamics, host–pathogen interactions, disease progression and clinical outcome (Domingo et al., 1996; Nowak et al., 1991; Tersmette et al., 1989; Williamson, 2003; Wolinsky et al., 1996). An application of evolutionary theories will be valuable to interpret viral sequences derived from the GALT of SIV-infected macaques or HIV-infected patients with diverse disease progression rate. Two models can be considered in the study of evolution of *nef* variants. The adaptive model postulates that new quasi-species in the viral population may have a selective advantage over the ancestral strain or possibly may survive better in the later stages of infection. Under the adaptive model, selective forces include host immune responses, replication fitness, cell tropism and co-receptor usage which may greatly influence viral genetic variation. Positive selection results in the distribution of genetic variants about the best-adapted genotype. The frequency of these variants in the quasi-species reflects the competitive advantage of some variants to replicate over others. Positive selection ensures efficient outgrowth of variants with enhanced replication fitness while negative selection eliminates low fitness mutants. On the other hand, the neutral model postulates that emergence of new variants is based on random events and these variants have no selective advantage. As a result, variants in the viral quasi-species may not show preferentially mutated codons.

In the present study, we tested the hypothesis that the selection and emergence of *nef* variants occur in the GALT during the early stages of infection and that these dominant variants may partly account for the variable disease outcomes observed in HIV-1-infected patients and SIV-infected macaques. To study the evolution of the *nef* gene in the GALT, 11 rhesus macaques were inoculated with SIVmac239/nef-stop virus and examined for genetic diversity of the *nef* gene at 3 days to 23 weeks post-infection (wk pi), a time period spanning the development of primary SIV infection and early asymptomatic period following the resolution of primary acute stage of infection. SIVmac239/nef-stop virus is a molecular clone that results in a single viral species with a single *nef* gene sequence variant in the early stages of infection. This molecular clone has a TAA premature stop codon at position 93 of Nef that abrogated the synthesis of a full-length functional Nef in vivo (Kestler et al., 1990). Evolution of the *nef* gene in vivo involved the reversion of this stop codon to synthesize a full-length functional Nef protein. The use of this molecular clone has allowed us to examine the viral genetic diversity during the course of infection in the absence of confounding factors such as the complexity of infecting quasi-species, selection of variants at the time of viral transmission and the biologic phenotype of the infecting virus. We sought to examine the correlation between genetic variations in the *nef* gene in GALT during primary SIV infection in the context of viral loads and disease progression. Our results demonstrated that GALT is an early site for positive selection of full-length functional Nef with a reversion of the stop codon to an open reading frame immediately following infection. However, thereafter, the evolution of the *nef* gene was neutral as confirmed by the comparison of synonymous and non-synonymous substitution rate and monophylogenetic star topology that supports the neutral model of evolution.

Results

Viral burden in GALT during primary and asymptomatic stages of SIV infection

The presence of SIV infection was detected in jejunal tissue samples of 11 rhesus macaques at different time points following SIVmac239/nef-stop infection (3 days to 23 wk pi). This time period spanned the development of primary acute stages of infection with high viral loads and subsequent asymptomatic period with suppressed viral loads in response to the host anti-viral immune responses. The viral RNA levels were quantified by bDNA assay (Table 1) and virally infected cells were localized by in situ hybridization (data not shown). The peak viral loads were detected at 2 wk pi (1×10^7 RNA copies/mg of tissue). However, one of the four animals at 2 wk pi had relatively lower SIV

Table 1
Animal numbers, necropsy time points, viral load and percentage of stop codon reversions

Animal #	Necropsy time point (weeks post-infection)	Viral load $\log_{10}$ RNA copies/ml of plasma[a]	% stop codon revertants[b]
25541	3 days[c]	3.95	77
25546	1	3.95	11
25605	2	7.06	71
25618	2	6.55	64
23894	2	3.00	90
23930	2	7.32	45
24231	8	5.3	100
24255	8	4.22	91
24219	13	4.11	93
24242	23	5.67	100
24263	23	3.90	66

[a] Plasma viral loads were determined by bDNA assay.
[b] Percentage of revertants represents the % of clones that were found to have reversed the early stop codon at position 93 of the parental inoculum.
[c] Reversion of stop codon to generate a full-length Nef occurred early in infection (3 days) as seen in rhesus macaque 25541.

RNA load (1×10^3 RNA copies/mg of tissue). The viral loads declined (1×10^4 and 1×10^5 RNA copies/mg tissue) by 2 to 3 logs in animals during the asymptomatic stage of infection (8 to 23 wk pi). One of the two animals at 8 wk pi did not have detectable levels of anti-SIV antibodies and was considered to be a rapid progressor. This particular macaque had a high viral load (1×10^6 RNA copies/mg tissue). There was a 1.5 log difference in viral loads between the rapid progressor and animal with a regular disease course at 8 wk pi. At 23 wk pi, one of two rhesus macaques displayed high viral loads. The variation in jejunal tissue viral loads between the two macaques at 23 wk pi may be explained by the presence of viral targets and reservoirs that sustain viral replication in the macaque with high viral loads.

Early selection of a full-length Nef reading frame in GALT by reversion of premature stop codon in SIVmac239/nef-stop virus

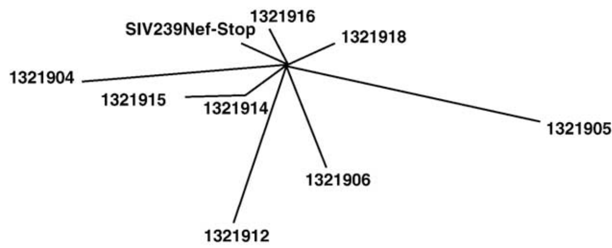
We examined the status of the premature stop codon at position 93 of the Nef protein among the deduced amino acid sequences from intestinal tissues at different time points following SIV239/nef-stop infection. In 10 of 11 animals in the study, a majority of the nef variants had reversions of the premature stop codon (TAA) to CAA (Q), GAA (E) or in-frame deletion of the entire stop codon. At day 3 post-infection, all 10 nef variants derived from GALT of rhesus macaque 25541 had a reversion of the premature stop to a sense codon (Table 1, see Supplementary Figs. A and B). In the remaining 10 of 11 animals, the majority of the clones showed a reversion of the premature stop codon to either a glutamine or glutamic acid residue with an exception of animal 24255 analyzed at 8 wk pi with a rapid disease course. In three of the 10 nef variants derived from this animal, the premature stop codon was substituted with a tyrosine residue. In rhesus macaque 25546 sacrificed at 1 wk pi, six of seven clones retained the premature stop codon suggesting that reversion of the stop codon occurred in vivo (Fig. A1). This strongly supports for an essential role of a functional Nef protein during the early stages of infection in lymphoid tissue as well as its importance in disease progression and development of high viral loads as previously reported (Kirchhoff et al., 1999).

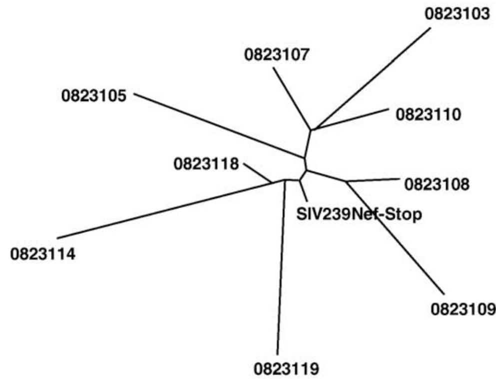
Phylogenetic analyses of nef sequences

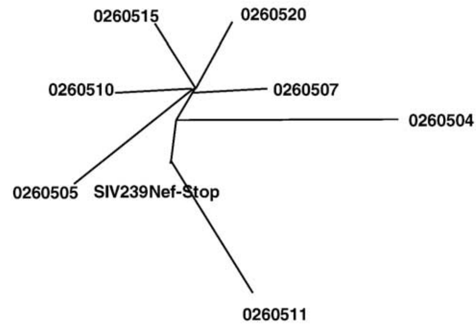
To construct evolutionary relationships of the SIV *nef* gene sequences among all 11 SIV-infected rhesus macaques during the course of SIV infection with different viral loads, phylogenetic analyses were performed using the PHYLIP program package (Felsenstein, 1989). Pairwise comparisons and distance matrices were generated using Dnadist program (Distance matrix program). Phylogenetic trees were constructed from the distance matrices using the Neighbor TreeMaker Program. Final tree topologies were visualized with TreeView.

We have used unrooted trees to display this information. The radial tree topologies consistently showed that the sequences from each animal clustered together and resembled a star burst topology with branches radiating from a point of divergence. In most cases, the center of the star was one step from the parental SIVmac239 *nef*-stop variant as shown by the phylogenetic trees of *nef* gene sequences from three of 11 macaques (Figs. 1A–C). We have interpreted this star pattern to mean that, beyond the reversion of the premature stop codon, other amino acid replacements are neutral. Expectations of the adaptive model would show an emergence of an adaptive variant out of one of the branches upon which new variants would emerge (i.e. appearance of a star out on the branch). Within the phylogenetic trees, all sequences radiated from the putative parental node (SIVmac239/nef-stop) indicating increasing divergence with time but with no evidence that any of the variants were favored over ancestral forms, other than the selective pressure for the stop codon (position 93) to change in order to produce an open reading frame. These results emphasize the importance of the functional *nef* gene expression in lymphoid tissue during the early stages of viral infection.

To further investigate the phylogenetic relationship between sequences from different animals in primary and asymptomatic stages of SIV infection, phylogenetic analyses were performed on variants pooled from all 11 animals (Fig. 2). As was seen in Fig. 1, no unique clustering of variants derived from either early or asymptomatic stages of infection was observed. Likewise, there were no clustering similarities between rapid and slow disease progressors. These data show that different variants evolved in different animals independent of the phenotype or lineage of cellular reservoirs, disease stage or outcome.







0.01

Fig. 1. Phylogenetic analysis of nef sequences from rhesus macaques at different stages of SIV infection. Unrooted phylogenetic tree of variants from rhesus macaques (A) 24219; (B) 24231; (C) 25605. *Nef* sequences where used to construct a neighbor-joining consensus tree as described in Materials and methods. The scale bar represents 0.01 substitutions per nucleotide position (s/nt). Sequence identities are coded as follows: the first two digits refer to the disease stage post-infection; the third, four and fifth digits stand for the last three digits of the animal number, while the last two digits stand for the clone number. Thus, sequence "0823109" can be decoded as "08" week post-infection; rhesus macaque number (24231) and clone # 09.

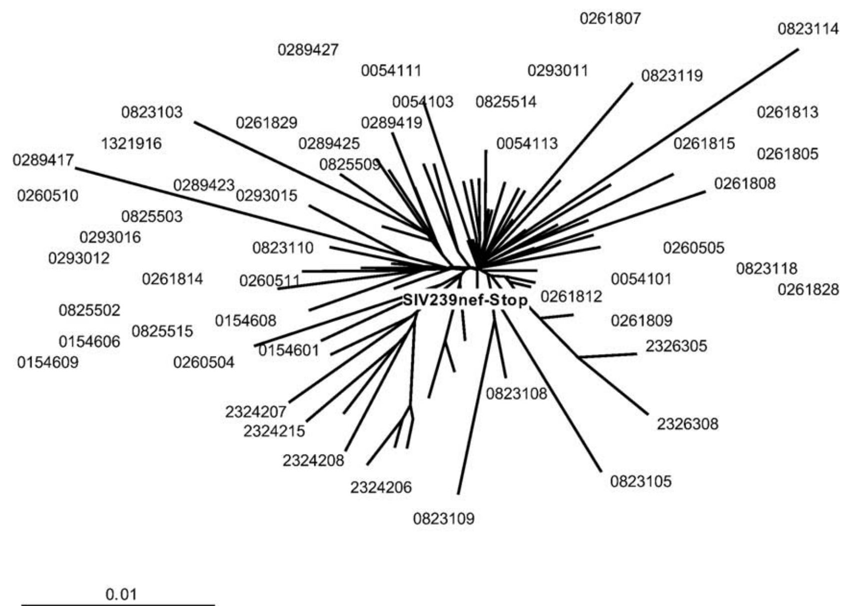


Fig. 2. Unrooted tree of SIV nef sequences from all 11 SIV-infected rhesus macaques. The trees show a distribution of a group of 60 sequences pooled from the 11 SIV-infected rhesus macaques shown in Table 1. Other tress reconstructed from sequence data sets from individual animals display a similar star phylogeny. Sequence identities are coded as describe in the Fig. 1 legend. Half of the sequence numbers have been omitted from the figure for presentation purposes.

Synonymous vs. nonsynonymous substitutions support neutral evolution

Nef variants have been suspected to vary with disease progression. This has led to the speculation that particular variants may be associated with different stages of disease, cellular targets and reservoirs or clinical outcome (Shpaer and Mullins, 1993; Zanotto et al., 1999; Zhu et al., 1996). The phylogenetic pattern of amino acid substitutions observed in the different animals (as shown in Figs. 1 and 2) appeared unrelated to disease outcome and hence the occurrence of these substitution may be random. A common analytical approach to address whether or not amino acid replacements are selected over parental genotypes is to compute the rate of synonymous (silent or *ds*) and nonsynonymous (amino acid altering or *dn*) nucleotide substitutions. The ratio of synonymous to nonsynonymous, *ds/dn*, is indicative of whether base substitution at a degenerate site is positively selected, negatively selected or neutral (Nei and Gojobori, 1986). The idea behind this approach is that if mutations were selected, we would expect *dn* to have a higher value than the one that would be expected from simple mutation pressure (which is the factor that determines the value of *ds*). Thus, the *ds/dn* ratio (where *ds* is the number of silent or synonymous mutations per synonymous site and *dn* is the number of amino acid replacements per nonsynonymous site) can be regarded to reflect the relative rate of *nef* evolution and the selection for or against amino acid changes. The values for the *ds/dn* of the *nef* gene in 11 rhesus macaques are shown in Table 2. The ratios varied from 0.73 to 3.65 with a mean ratio of 1.84. If all codon positions observed were equally free to vary without selection either for or against mutations in *nef*, then a ratio greater than 1.0 would be expected. If transition and transversion mutations were equally likely, the ratio would be expected to be exactly 1.0; however, since transition rates are greater than transversion and third position transitions favor synonymous changes, the ratio of *ds/dn* would be expected to be >1.0. A value of 1.84 is just slightly greater than would be expected, so we reasoned that the relatively moderate *ds/dn* ratio is suggestive of *nef* being under extremely weak functional constraint against amino acid replacements. If there was a positive selection for amino acid replacements, a *ds/dn* <1 would be expected; hence, we saw no evidence for positive selection. Based on the comparison of *ds/dn* (Table 2) with viral load and infection course (Table 1), no obvious difference in *ds/dn* ratios could be discerned between animals at different stages of infection or with divergent disease outcome. Indeed, the differences observed in the *ds/dn* ratios in Table 2 are not statistically significant. So far, our phylogenetic analysis is consistent with the null hypothesis that all of the amino acid changes observed in *nef* are neutral. Perhaps more surprisingly, there is only the smallest indication of any functional constraint acting on *nef* with the exception of maintaining an open reading frame. As we show below, there are two short regions that seem to display functional constraint and are highly conserved.

Table 2
Synonymous and nonsynonymous substitutions

Animal #	Sd	Sn	*ds/dn*
25541	6	19	2.15
25546	2	7	2.46
25605	4	14	1.97
25618	15	16	1.77
23894	7	9	1.74
23930	4	11	1.23
23231	10	48	1.29
24255	7	14	2.88
24219	6	12	3.65
24242	6	19	.73
24263	1	18	1.43
Weighted average			1.84

ds and *dn* are substitution rates in terms of the observed number of synonymous and nonsynonymous changes divided by the total number of synonymous and nonsynonymous changes possible. The numbers are computed from the comparison of SIVmac239 Nef stop sequence to each of the other sequence variants from the same animal. *ds/dn* is a weighted average from those comparisons. Sd is the total number of observed synonymous changes and Sn is the total number of observed nonsynonymous changes within each sequence data set. The variance of *ds/dn* values among the different animals is expected for a stochastic process acting under a mean of 1.84.

Linear distribution of amino acid replacements in Nef are Poisson distributed or nearly indistinguishable from random

If amino acid replacements in Nef protein were completely neutral, we would expect to see these changes across the protein to be randomly distributed or to state this in statistical terms, to be Poisson distributed. The probability that any region of length (d) across the gene has zero mutations would be given by the zero term of the Poisson distribution, e^{-md}, where m is the mutation frequency (Syvanen, 1984). This will give the probability that the codons are contained within a run (defined as a cluster of adjacent amino acids with zero replacements or no changes) that is greater than or equal to d. Thus, the probability of having a run between i and j codons in length is:

$$e^{-mi} - e^{-mj}.$$

This term multiplied by the total number of mutations +1 will give the expected number of runs of that size within the gene under study. A correction for multiple hits that produce only a single (or zero) observed change is not needed in the current study because the total number of mutations is relatively low.

The actual distribution of amino acid changes observed in this study and a display of each codon position and the numbers of replacements encountered are shown in Fig. 3. This distribution of amino acid changes can be compared with the expectation of a purely random process. A

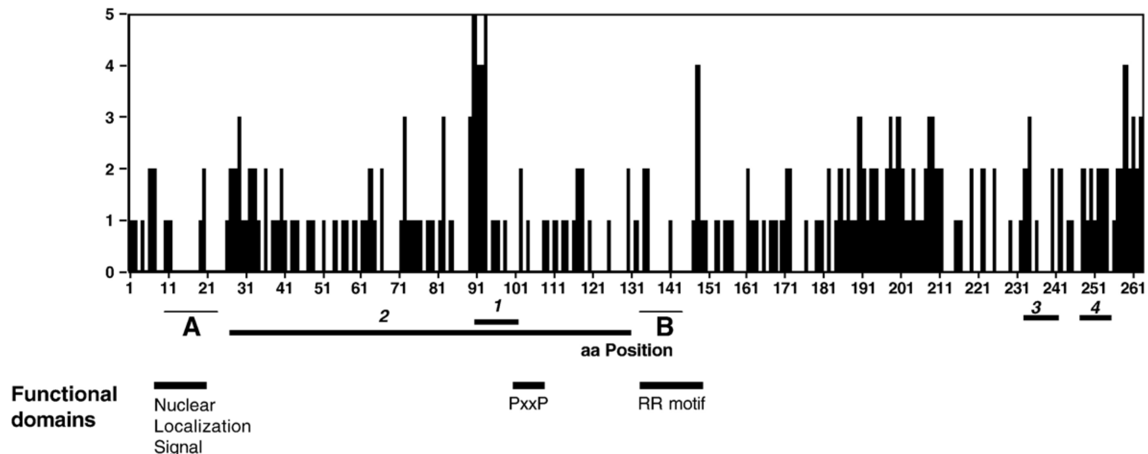


Fig. 3. Linear distribution of deduced amino acid replacements across the SIVmac239 Nef protein. The number of substitutions per amino acid position is indicated on the *y* axis while the amino acid position is shown on the *x* axis. Observed deletions 1, 2, 3 and 4 are indicated by thick bold lines while gap regions A and B are shown with a thin line below the amino acid positions. Known functional domains e.g. nuclear localization signal, PxxP and RR motifs are indicated with thick bold dotted lines. The distribution of amino acid replacements was compiled from all of the variants encountered in the study from all 11 rhesus macaques.

total of 233 codon replacements are distributed over 263 positions. If each position was as free to vary as any other, we would conclude that the probability for any codon to experience a change is 233/263, which is the mutation frequency for this study. We have calculated the expected distribution from this number using the equation derived in the previous paragraph. The results of the observed and expected number of amino acid replacements per codon position are shown in Table 3. It is evident that there is no significant difference between the observed and expected distributions (the chi square value is 3.5 over 5 degrees of freedom). Thus, overall replacements in Nef protein are Poisson distributed or indistinguishable from random.

There is one feature of the linear distribution amino acid substitutions depicted in Fig. 3 that does appear to vary from the expectations of a purely random process. This is the presence of a cluster of adjacent amino acids with zero replacements that can be called runs of zero hits. There are two regions that seem to have fewer mutations than expected. One region consists of a 15 amino acid cluster from position 11 to 25 and the other run is made of an 11 amino acid cluster from position 135 to 145. These two runs could represent domains of Nef that are functionally constrained. The first run, residues 11 to 25, encompasses a region postulated to be a putative nuclear localization signal. The second run (residues 135 to 145) includes the di-arginine motif (137–138) and a CTL epitope (Mortara et al., 1998, 2000; Sawai et al., 1996). Based on our data, we conclude that the *nef* gene has two noncontiguous regions that appear to be functionally constrained on the basis of amino acid replacement rates. The remaining regions of Nef are free to vary randomly.

Table 3
Number of adjacent codons with zero mutations

N[a]	Observed[b]	Expected[c]
0	114	108
1	86	97
2	48	43
3	12	12.8
4	4	3
5	1	0.5

[a] Size of run of codons with zero mutations.
[b] Number of runs of size N that are encountered at each codon and derived from F).
[c] Expected number of runs from a Poisson process (see text). Where m = 233/263.

Amino acid substitutions in Nef functional domains

Our analysis shows that the overall pattern of changes can be accounted for by neutral evolution theory. However, this is not to say that some of the changes did not influence disease outcome. We identified specific amino acid substitutions in known functional domains of SIV Nef protein or at regions proximal to these domains. These amino acid substitutions occurred randomly and at very low frequencies in any particular animals. No commonly shared substitutions were observed among the asymptomatic animals except for random, low frequency, nonunique amino acid changes. A Q41K substitution was observed in five of nine clones in macaque 23242 at 23 wk pi. This substitution lies within the SQS motif involved in AP-1 recruitment and endocytosis. We also observed a V102M substitution in six of the nine clones at the PxxP domain in the same animal (Supplementary Fig. B). This proline rich motif is involved in SH3 binding and MHC-1 down-regulation. No relationship was established between specific amino acid substitution at functional domains of Nef protein and disease stage.

Evaluation Nef variants and functional domains in an SIV-infected animal with a rapid disease course

To investigate whether any specific mutations in Nef were associated with a rapid disease course, we analyzed SIV-Nef variants from two rhesus macaques 24231 and 24255 which showed diverse infection course (regular vs. rapid) at 8 wk pi. With regard to the occurrence of unique and animal specific substitutions, an E188G substitution was observed in four of 10 clones derived from an animal 24231 with a regular disease course (Supplementary Fig. B). This site has been shown to be important for COP-1 recruitment, that is involved in Nef-induced CD4 down-regulation (Janvier et al., 2001). Whereas, a unique reversion of the premature stop at position 93 to tyrosine (Q93Y) was observed in three of the 10 clones derived from the rapid progressor animal (24255). This substitution falls within the central acidic cluster shown to be involved in MHC-1 down-regulation (Schwartz et al., 1996). Thus, the main difference observed between these two animals was the substitution of premature stop with a tyrosine residue at position 93 observed in the rapid progressor (24255), and the presence of E188G in variants derived from rhesus macaque with a regular disease course.

Analysis of amino acid substitutions at Nef CTL epitopes

HIV-1-specific CTL responses are an important component of host immune responses in suppressing HIV-1 infection. Knowledge of CTL epitopes is critical for the development of effective HIV vaccines. We examined the occurrence of amino acid substitutions at SIV Nef CTL epitopes listed in the Los Alamos HIV molecular Immunology database. In addition, Nef CTL epitope (128–137) and (159–167) regions were previously reported to be sites for the emergence of CTL escape variants in SIV-infected rhesus macaques immunized with Nef peptides. We defined Nef CTL escape variants as those clones in which 50% or more of the clones harbored amino acid substitution at the CTL epitopes. Interestingly, the CTL epitope region (159–167) displayed a Y167F variability at position 167 in six of nine clones derived from rhesus macaque 24242 with high viral load at 23 wk pi. All the other CTL epitopes including epitope cluster 128–137 were highly conserved. Our results show a Y167F substitution at Nef CTL epitope cluster (159–167) in one of 11 animals. However, whether this substitution facilitates immune evasion was not determined in our study.

Discussion

The genetic variability in HIV and SIV is due to high mutation rates generated during reverse transcription by the error-prone viral encoded reverse transcriptase (Goodenow et al., 1989; Hahn et al., 1986; Meyerhans et al., 1989; Zhang et al., 1997). One of the major questions is how selective pressure exerted by the host immune system or other pathogenic factors contribute to these complex mixture of genetically diverse but related viral quasi-species (Kimata et al., 1999; McNearney et al., 1992; Pang et al., 1992; Zhu et al., 1993). Besides intrinsic host factors, viral genetic diversity has been shown to contribute to the variable disease outcome observed in HIV-1-infected patients and macaques experimentally infected with SIV. Nef, an auxiliary gene of HIV and SIV, is not required for viral replication in cell culture, but is an important viral determinant for the development of high levels of viremia and disease progression in vivo (Deacon et al., 1995; Kestler et al., 1991; Sawai et al., 2000). This is further supported by the observation that infection of rhesus macaques with molecularly cloned SIV bearing defined mutations at various domains of Nef shows variations in disease course suggesting that Nef variants may impact on disease course and outcome in SIV and HIV infection (Hahn et al., 2003; Kestler et al., 1991; Kirchhoff et al., 1999; Mortara et al., 1998; Mortara et al., 2000; Sawai et al., 2000; Stephens et al., 1996).

Our study on the evolution of the *nef* gene sequences in GALT of rhesus macaques at 3 days to 23 wk pi demonstrated that there is a strong selective pressure for restoring the nef open reading frame in vivo in GALT immediately following infection. All but one animal (at 1 wk pi) restored the nef open reading frame. However, beyond this change, all subsequent changes appear to be completely random. Neutrality of *nef* evolution is supported from an analysis of variants in *nef* using three different molecular evolutionary procedures. First, the star phylogenies in Figs. 1A–C and Fig. 2 provide evidence that is consistent with a model that the evolution of the *nef* gene is neutral during the early stages of infection.

Second, the ratio of substitutions in *nef* that cause amino acid replacements to those that are silent (the *ds/dn* ratio) is very close to the value expected for random variations of the genetic code when translational stop mutations are lethal (Table 2). This means that the rate of amino acid replacements is very close or slightly less than the rate expected from the mutation rate itself. Thirdly, the distributions of changes across the gene are predictable by a Poisson process. Even though casual examination of the distribution of variants (Fig. 3) appears to reveal hyper-variable regions, these are expected by chance (Table 3).

However, one deviation from a purely random process of amino acid replacements is seen in two regions that contain a run of nonmutated amino acids that are adjacent to one another. These regions include residues 11 to 25 at the N-terminus nuclear localization signal. The second region (residues 135 to 145) encompasses a CTL epitope and the RR (137–138) motif shown to interact with cellular serine kinases and implicated in pathogenesis. Previous studies have reported the emergence of CTL escape variants in SIV-infected rhesus macaques immunized with SIV Nef peptides

containing Nef CTL epitopes (Nef 125–147 and 128–137) prior to the challenge with pathogenic SIV (Evans et al., 1999; Friedrich et al., 2004). These studies suggested that immune responses against Nef CTL epitopes might have exerted selection pressures facilitating the adaptive evolution and emergence of Nef variants within Nef CTL epitope regions (O'Connor et al., 2002; Peyerl et al., 2004). In our study (experimental SIV infection in absence of prior immunizations), the second run (defined as a cluster of adjacent amino acids with zero replacements) of amino acid residues harboring a Nef CTL epitope with zero substitution rates indicated that the evolution of this particular Nef CTL epitope region was constrained. In support of our findings, the evolutionary constrained region of Nef observed in our study, residues 135–145 correspond to the highly hydrophobic crevices between the two anti-parallel alpha helices in the highly conserved core of Nef protein (Bauer et al., 1997; da Silva and Hughes, 1999; Peyerl et al., 2004; Zhang et al., 1997). It has been suggested that Nef CTL epitopes tend to coincide with structural and functional domains and thus not free to vary randomly (Hahn et al., 2003; Padua et al., 2003; Plikat et al., 1997; Yang et al., 2003; Zhu et al., 1996). This would be consistent with the lower rates of evolution at these regions.

Previous studies have attempted to delineate the evolutionary properties of *nef* in viral replication and pathogenesis (Plikat et al., 1997). Plikat et al. monitored HIV-1 *nef* quasi-species that appeared in a single patient infected with HIV following blood transfusion and showed that short-term evolution of HIV-1 *nef* quasi-species was consistent with the neutral model. More recently, evaluation of the evolutionary dynamics and conservation of functional domains of HIV-1 Nef during maternal–fetal transmission indicated that both maternal and infant nef sequences were under positive selection pressure and that amino acid substitutions at most of the sites including functional domains essential for Nef activity were either conserved or neutral (Hahn et al., 2003). Our study, for the first time has examined nef sequences in GALT during primary and early asymptomatic stages of viral infection. Our results are consistent with Plikat et al. (1997) study but do not support the work of Hahn et al. (2003).

In summary, GALT is an early site for strong positive selection for functional full-length Nef during primary SIV infection. This was evidenced by the reversion of the premature stop codon in SIVmac239/nef stop. Using molecular evolutionary tools, we show that SIVmac239 *nef* variants in GALT emerge by neutral evolution. Through the *nef* sequence analysis, we have identified for the first time, two functionally constrained regions in Nef, a putative nuclear localization signal and a previously characterized CTL epitope that are highly conserved and may be important for *nef* function. Detection of conserved regions of Nef through phylogenetic analyses may identify potential targets for development of HIV vaccines and new therapeutics.

Materials and methods

Animals, virus infection and tissue collection

Eleven rhesus macaques (*Macaca mulata*) from the California Regional Primate Research Center (CRPRC) were used in this study. Animals were sero-negative for simian T cell leukemia virus-1 (STLV-1) and simian retrovirus-1 (SRV-1). All animals were housed in accordance with American Association for Accreditation of Laboratory Animal care guidelines. Rhesus macaques were intravenously inoculated with SIVmac239/nef stop, a molecularly cloned virus. This clone has a premature TAA stop signal at codon 93 of the *nef* gene (Kestler et al., 1990, 1991). Animals were euthanized during primary infection and in clinically asymptomatic infection. Animals in primary SIV infection were sequentially sacrificed at 3 days post-infection (pi) (n = 1), 1 wk pi (n = 1) and 2 wk pi (n = 4). Whereas, animals in asymptomatic stage of infection were euthanized at 8 wk pi (n = 2), 13 wk pi (n = 1) and 23 wk pi (n = 2) (Table. 1). Intestinal tissues were collected at necropsy and immediately frozen in liquid nitrogen for the determination of viral loads and for the isolation of genomic DNA.

Quantitation of SIV RNA loads

SIV RNA loads in intestinal tissue samples were determined by branched DNA (bDNA) assay (Bayer Diagnostics, Emeryville, CA) as previously described (Pachl et al., 1995).

DNA isolation and amplification of SIV nef gene

Genomic DNA was extracted from frozen jejunal tissues obtained at necropsy by proteinase K treatment, phenol/chloroform purification and ethanol precipitation. SIV nef sequences were amplified by nested PCR using outer primers [sense, 5′-CAGGACTGAACTGACCTACCTACA-ATATGG-3′ (nt 9051–9080); antisense, 5′-ACATCCCCTT-GTGGAAAGTCCCTGCTGTTT-3′ (nt 9868–9898)] and inner nef specific primers [sense 5′-TGGGTGGAGCTATT-TCCATGAGGCGGTCC-3′ (nt 9077–9106); antisense 5′-TCAGCGAGTTTCCTTCTTGTCAAGCCAT-3′ (nt 9842–9868)], numbering based on SIVmac239 genome (Gene accession no. M33262) (Regier and Desrosiers, 1990).

The PCR reactions were performed in 1× PCR reaction buffer containing1.5 mM $MgCl_2$, 200 μM each of the four dNTPs (dATP, dCTP, dGTP and dTTP), 1.5 pmol of each primer pair, 1 μg of template DNA and 2.5 U of Expand High Fidelity Polymerase (Boehringer Mannheim). The thermal cycling conditions for the first round PCR amplification were as follows: first, the template DNA was denatured at 93 °C for 3 min, followed by 35 cycles using the following profile: denaturation at 93 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 3

min. For the nested PCR amplification, 1 μl of first round product was used and the same thermal cycling conditions were applied except for an additional extension at 72 °C for 20 min.

Cloning and sequencing of nef variants

PCR products from three different PCR reactions per animal sample were pooled together and cloned into TOPO TA cloning vector (Invitrogen Corp., San Diego, CA). Ligated DNA was transfected into competent DH5α cells. Ten clones or more per animal were randomly selected and plasmid DNA purified using Qiagen Miniprep Kit (Qiagen). Clones were sequenced bi-directionally using a Taq Dye-Deoxy Terminator cycle sequencing kit (Amersham Life Sciences, Piscataway, NJ). Sequence editing was performed using Sequencher 3.0 software (Gene Codes, Ann Arbor, MI). Nucleic acid and deduced amino acid sequence alignments were performed with Vector NTI Suite software (InforMax Inc., Frederick, MD) and Clustal X (Thompson et al., 1997).

Phylogenetic sequence analyses

Multiple *nef* gene sequence alignments were performed using Clustal X program (Thompson et al., 1997). Final alignments were manually edited to eliminate gaps. Phylogenetic analyses were performed using the neighbor-joining method available in the PHYLIP program package (Phylogeny Inference Package, written by J Felsenstein) (Felsenstein, 1989). Dnadist program (Distance matrix program) was used to perform pairwise nucleotide comparisons and generation of distances matrices. Phylogenetic trees were constructed from the distance matrices by the neighbor-joining method using the Neighbor TreeMaker Program. Final tree topologies were visualized with TreeView.

Synonymous Nonsynonymous Analysis program (SNAP) written by B Korber (http://hiv.lanal.gov) was used to estimate rate of synonymous (*ds*) and nonsynonymous (*dn*) substitutions as computed by method of Nei and Gojobori (Korber, 2000; Nei and Gojobori, 1986). This method defines *ds* as the number of observed synonymous substitutions per possible synonymous substitution and *dn* as the number of observed nonsynonymous substitutions per possible nonsynonymous substitutions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2005.08.013.

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