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Intrahost Human Immunodeficiency Virus Type 1 Evolution Is Related to Length of the Immunocompetent Period

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The antigenic diversity threshold theory predicts that antigenic sites of human immunodeficiency virus type 1, such as the V3 region of the external glycoprotein gp120, evolve more rapidly during the symptom-free period in individuals progressing to AIDS than in those who remain asymptomatic for a long time. To test this hypothesis, genomic RNA sequences were obtained from the sera of 44 individuals at seroconversion and 5 years later. The mean number of nonsynonymous nucleotide substitutions in the V3 region of the viruses circulating in 31 nonprogressors ($1.1 \times 10^{-2} \pm 0.1 \times 10^{-2}$ per site per year) was higher than the corresponding value for 13 progressors ($0.66 \times 10^{-2} \pm 0.1 \times 10^{-2}$ per site per year) ($P < 0.01$), while no difference between the mean numbers of synonymous substitutions in the two groups was seen ($0.37 \times 10^{-2} \pm 0.1 \times 10^{-2}$ and $0.51 \times 10^{-2} \pm 0.2 \times 10^{-2}$ per site per year for nonprogressors and progressors, respectively; $P > 0.1$). The mean ratios of synonymous nucleotide *p* distance to nonsynonymous *p* distance were 0.35 for nonprogressors and 0.62 for progressors. The number of nonsynonymous substitutions was not associated with virus load or virus phenotype, which are established predictors of disease progression, but correlated strongly with the duration of the immunocompetent period ($r^2 = 0.41$; $P = 0.001$). This indicates that there is no causative relationship between intrahost evolution and $CD4^+$ cell decline. Our data suggest that intrahost evolution in human immunodeficiency virus type 1 infection is driven by selective forces, the strength of which is related to the duration of the immunocompetent period.

High levels of genetic variation among human immunodeficiency virus type 1 (HIV-1) isolates are observed within infected individuals (12, 20, 23, 34, 35, 44). Genetic variation of the region encoding the HIV-1 external glycoprotein gp120 has been shown to directly influence the HIV-1 phenotype (7, 19). In particular, the third variable domain, V3, is implicated in a number of biological properties of the virus, including cell tropism, infectivity, and cytopathicity (8–11, 30, 43). Certain amino acid changes in the V3 domain were associated with the appearance of syncytium-inducing (SI) virus strains (10, 13, 17), which correlate with rapid $CD4^+$ cell decline and disease progression (28, 48). The V3 domain includes recognition sites for both humoral and T-cell immune responses (18, 38, 41, 42, 45–47). Early in infection, antibodies are induced that bind efficiently in vitro to synthetic peptides mimicking the deduced V3 amino acid sequence of the genomic RNA in virions abundant at seroconversion (50, 53). These antibodies can block infection by HIV-1 strains with closely related V3 domains in cell cultures (51). Binding to continuous V3 epitopes and neutralization of viral infectivity require exposure of the V3 epitope on the surface of the virion. The production of virus stocks in T-cell lines may lead to the exposure of V3 loops, which can, however, be hidden on the virus surface in vivo (6). Zwart et al. concluded from their work that the role of V3-directed neutralizing antibodies in preventing the development of immunodeficiency appears to be nondecisive in natural HIV-1 infection (52). Still, nonsynonymous nucleotide substitutions (which result in a change of an amino acid) are accumulated more rapidly than the synonymous ones (which do not lead to a change of the amino acid) within the V3 domain in the early period of infection, indicating the selection pressure on this region. Some of these nonsynonymous substitutions are related to resistance to neutralization (50), some are related to antibody enhancement (27), and some are related to the biological phenotype of the virus (30). Each of these characteristics has been postulated to be involved in HIV-1 pathogenesis.

Disease progression following HIV-1 infection is directly related to the level of virus replication (24, 39). The greater and the more persistent HIV-1 production is, the faster HIV-1 causes immunodeficiency. Independently of virus production, the biological phenotype is also a predictor of disease progression (28, 48). On the basis of these observations, one may hypothesize that higher levels of replication and a shift in the biological phenotype of HIV-1 may increase variation due to the high misincorporation rate of reverse transcriptase and negative selection pressure against genomes less fit for replication and infection of particular cell types. This hypothesis predicts that V3 variation is positively correlated with disease progression, the level of virus replication, and the shift from the non-SI (NSI) to the SI phenotype but negatively correlated with the rate of $CD4^+$ cell decline. To test this hypothesis, the predominant genomic RNA sequences encoding the HIV-1 gp120 V3 region in virus populations present at seroconversion and 5 years later in sera of 13 progressors and 31 nonprogressors were studied. The relationship between the rate of evolution of the V3 region and disease progression, as well as markers of disease progression, such as $CD4^+$ cell decline, HIV-1 p24 production, the HIV-1 RNA level in serum, and the biological phenotype of the virus, was studied.

MATERIALS AND METHODS

Patients. Serum samples were obtained at seroconversion and 5 years later from 44 participants in the Amsterdam prospective cohort study of homosexual men who seroconverted in 1985 to 1988. At the time of the second sampling, 13 persons had developed AIDS, while the other 31 remained asymptomatic. In 15

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patients (11 progressors and 4 nonprogressors), the $CD4^+$ cell count dropped below 200/μl during the 5-year period, while in 29 individuals (2 progressors and 27 nonprogressors), the $CD4^+$ cell count was above 200/μl. During the total follow-up period (mean of 6 years [range, 5 to 7 years] after seroconversion for progressors; mean of 8 years [range, 6 to 9 years] for nonprogressors), 38 of 44 individuals (11 of 13 progressors and 27 of 31 nonprogressors) developed a $CD4^+$ cell decline to below 400/μl; in 21 of 44 individuals, (7 of 13 progressors and 14 of 31 nonprogressors) the $CD4^+$ cell count dropped to below 200/μl. Virus phenotype and p24 production were determined as described earlier (29).

Sequence analysis. The procedures for viral isolation, reverse transcription, amplification, and sequencing were described in detail earlier (13, 33). Briefly, RNA was isolated from 50 or 100 μl of serum by the method of Boom et al. (4). Viral RNA was transcribed into cDNA by using the 3′-V3 NOT primer. Part of each sample on which no reverse transcription step was performed and an extraction control to which no serum was added were used as negative controls. The cDNA obtained was subjected to a nested PCR. The outer primers used for the first PCR were 5′-V3-NOT and 3′-V3-NOT; the inner primers used for the second PCR were SP6-5′-ksi and T7-3′-ksi. Nested PCR resulted in the amplification of a sequence of approximately 290 bp. Double-stranded sequencing was performed on an automatic sequencer (model 373A; Applied Biosystems, Foster City, Calif.) with the *Taq* polymerase dye primer sequencing kit (Applied Biosystems). When directly obtained sequences included illegible positions, nested PCR products were cloned by using the TA cloning system (Invitrogen, San Diego, Calif.), and a consensus sequence of six clones was used for analysis (4 of 88 samples had to be cloned).

Nucleotide sequences were aligned manually. All positions with an alignment gap in at least one sequence were excluded from any pairwise sequence comparison. Synonymous and nonsynonymous nucleotide p distances (K_s and K_a, respectively) were calculated by using the MEGA program (32). The K_s/K_a ratio for the group of patients was calculated according to the formula $K_s/K_a = (\Sigma M_{si}/\Sigma S_{si})/(\Sigma M_{ai}/\Sigma S_{ai})$, where ΣM_{si} and ΣM_{ai} are the sums of intrapatient (weighted) mutation events at coding silent and nonsilent sites, respectively, and ΣS_{si} and ΣS_{ai} are the sums of intrapatient (weighted) coding silent and nonsilent sites, respectively. The phylogenetic analysis was performed with the PHYLIP package by using the neighbor-joining algorithm (16); the distance matrix was generated with Kimura's two-parameter model (25). Principal coordinate analysis was done with the PCOORD software (21). All statistical calculations were done with the SPSS/PC+ software (version 5.0; SPSS Inc., Chicago, Ill.). The nonparametric Mann-Whitney test was used to compare group means.

Viral RNA quantitation. The determination of viral RNA copy number in patient sera or plasma samples was performed with the commercial NASBA HIV-1 RNA QT kit (Organon Teknika, Turnhout, Belgium), which includes all reagents for nucleic acid isolation, amplification, and quantitation (49). To determine the amount of HIV-1 RNA in a sample, three distinguishable Q-RNAs in different concentrations are mixed, coisolated, and coamplified with the wild-type sample. The initial input of wild-type RNA can be calculated from the ratio of its signal to the signals of internal standards. All details of this method were described previously (49). A well-characterized virus stock dilution, containing 2.9×10^4 viral particles per ml, was used as a positive control in all quantifications.

RESULTS

Phylogenetic clustering of V3 sequences is not related to disease progression. Phylogenetic analysis of the sequence set revealed that 5 years of viral evolution did not separate the sequences obtained at seroconversion from those obtained 5 years later (Fig. 1). Neither early nor late sequences clustered according to disease progression or immune status. Attempts to separate the sequence set according to disease progression or immune status by signature pattern or principal coordinate analyses failed (data not shown). A stable His or a change to His at the 5-year point at position 308 (IHIGPGRAF) at the crown of the V3 loop was seen in 9 of 13 progressors (69%) and in 19 of 31 nonprogressors (61%) ($P > 0.1$). A stable Pro or a change to Pro at the 5-year point was seen in 4 of 13 progressors (31%) and in 8 of 31 nonprogressors (26%) ($P > 0.1$). Nonsynonymous substitutions at codon 308 occurred in 6 of 13 progressors (46%) and in 15 of 31 nonprogressors (48%) ($P > 0.1$). Similarly, amino acid changes at this position occurred in 8 of 15 individuals (53%) with $CD4^+$ cell counts below 200/μl and in 13 of 29 individuals (45%) with $CD4^+$ cell counts above 200/μl at the 5-year time point ($P > 0.1$).

V3 evolution is unrelated to prognostic markers for disease progression. The mean HIV-1 RNA copy number in serum at seroconversion was higher in progressors than in nonprogressors ($10^{5.3}$ versus $10^{4.8}$); however, this difference was not significant in this study population ($P > 0.1$). At the 5-year point, the mean HIV-1 RNA copy number was $10^{4.8}$ in progressors and $10^{4.5}$ in nonprogressors ($P > 0.1$), and the HIV-1 RNA load in persons with $CD4^+$ cell counts below 200/μl was significantly higher than in those who had $CD4^+$ cell counts above 200/μl ($10^{4.9}$ and $10^{4.4}$, respectively; $P = 0.01$), while no difference was seen at seroconversion ($10^{5.1}$ and $10^{4.9}$, respectively; $P > 0.1$). No association was found between RNA copy number, p24 production (either at seroconversion or at the 5-year point), and numbers of synonymous or nonsynonymous substitutions accumulated (data not shown). Virus isolates obtained from all persons during the first year after seroconversion were determined to be NSI. During the course of infection, a switch of virus phenotype from NSI to SI was observed in seven patients, without a relation to disease progression. No difference in evolution rate was detected between patients with stable NSI viruses and those with switch NSI-SI viruses isolated over the period studied (data not shown).

V3 evolution is inversely related to disease progression and immune suppression. The comparison of genetic distances between early and late samples revealed a difference in virus evolution between progressors and nonprogressors (Fig. 2). While the mean number of synonymous nucleotide substitutions was similar in progressors and nonprogressors ($0.51 \times 10^{-2} \pm 0.2 \times 10^{-2}$ and $0.37 \times 10^{-2} \pm 0.1 \times 10^{-2}$ per site per year, respectively; $P > 0.1$), a significantly higher number of nonsynonymous substitutions was detected in nonprogressors compared with progressors ($1.1 \times 10^{-2} \pm 0.1 \times 10^{-2}$ versus $0.66 \times 10^{-2} \pm 0.1 \times 10^{-2}$ per site per year; $P < 0.01$). This difference was observed for the whole V3 region as well as for its central part, the V3 loop, between two cysteine residues (synonymous substitutions $0.16 \times 10^{-2} \pm 0.1 \times 10^{-2}$ and $0.38 \times 10^{-2} \pm 0.1 \times 10^{-2}$ per site per year [$P > 0.1$], and nonsynonymous substitutions, $1.1 \times 10^{-2} \pm 0.1 \times 10^{-2}$ and $0.74 \times 10^{-2} \pm 0.1 \times 10^{-2}$ per site per year [$P < 0.01$], for nonprogressors and progressors, respectively). The mean K_s/K_a ratio was 0.35 in nonprogressors and 0.62 in progressors. Nonsynonymous substitutions were more frequent in viral genomes isolated from individuals with $CD4^+$ cell counts above 200/μl at the 5-year time point than in viral genomes from individuals with $CD4^+$ cell counts below 200/μl ($1.04 \times 10^{-2} \pm 0.1 \times 10^{-2}$ and $0.74 \times 10^{-2} \pm 0.1 \times 10^{-2}$ per site per year, respectively; $P = 0.04$). The numbers of synonymous substitutions did not differ ($0.42 \times 10^{-2} \pm 0.1 \times 10^{-2}$ and $0.4 \times 10^{-2} \pm 0.1 \times 10^{-2}$ per site per year, respectively; $P > 0.1$).

A low level of $CD4^+$ cells is associated with strong inhibition of immune system functions. Analysis of virus evolution over 5 years as a function of the time between seroconversion and the decline of the $CD4^+$ count to below 200/μl revealed a highly significant association of these two parameters for nonsynonymous substitutions ($P = 0.001$) but not for synonymous ones ($P > 0.1$) (Fig. 3). The same analysis for the time that the $CD4^+$ cell count fell below 400/μl showed no significant correlation (data not shown).

DISCUSSION

The sequences of the V3 domain did not cluster according to disease progression or immune status. This observation indicates that genetic polymorphism of the V3 region is not directly related to AIDS pathogenesis. Wolfs et al. (50) have shown that in the course of HIV-1 infection, a fixed nonsynonymous substitution may occur at position 308 (IHIGPGRAF), directly influencing V3 peptide binding, and Kliks et al. (27) have shown that changes at position 308 may directly

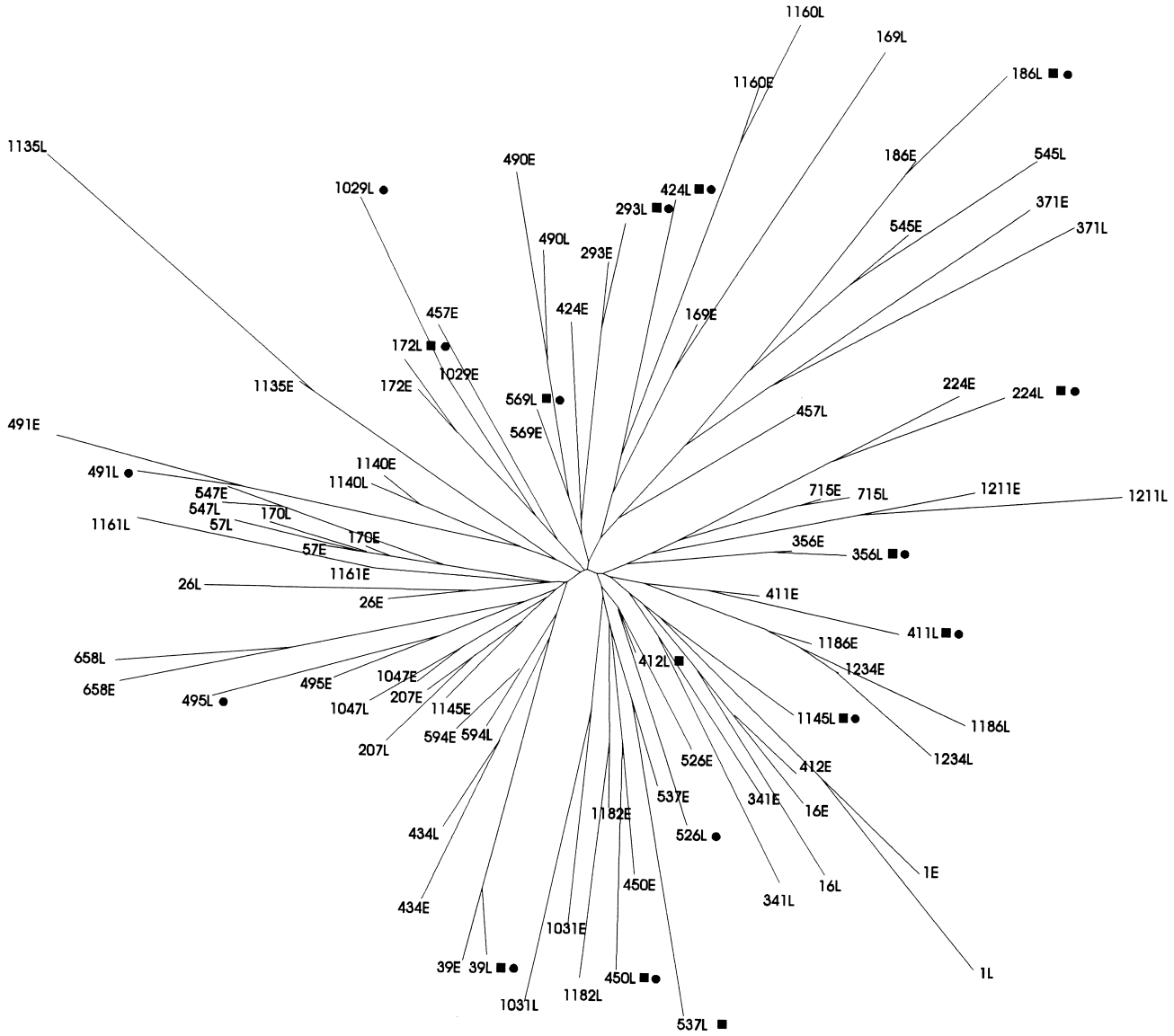

FIG. 1. Phylogenetic tree of V3 sequences derived at seroconversion and 5 years later. Numbers indicate patient codes. E, early sequence; L, late (5-year) sequence; ■, progressors, ●, individuals with $CD4^+$ cell counts below 200/μl at the 5-year time point.

impact upon the biological effect of antibodies, leading to either sensitivity, enhancement, or resistance to neutralization (27). Indeed, we were able to confirm the observation of Wolfs et al. that a change at position 308 occurs in about half of infected individuals. Although such a change may very well lead to resistance to neutralization or even enhancement, this apparently has no biological significance, since progressors showed a change at position 308 equally as frequently as nonprogressors. No preference was seen for a particular amino acid residue at position 308 (i.e., His versus Pro) that was related to health or to AIDS. In accordance with the observations in the present paper, we also did not find such a correlation previously when we specifically studied AIDS-related dementia (31), as has been suggested by Power et al. (40).

Similar to the findings of our previous study (24), the HIV-1 genomic RNA copy number at seroconversion was not significantly different for progressors and nonprogressors. Slightly higher levels of HIV-1 RNA were found at the 5-year point in progressors. This difference reached statistical significance when immune status was used as a criterion for patient selection. These results confirm accumulating evidence that high and persistent levels of virus production are directly related to disease progression (22, 24, 39).

The nonsynonymous evolution rate clearly showed an inverse relationship with the development of immunodeficiency, while the synonymous evolution rate was not associated with immune status and disease progression. The observation that the numbers of synonymous substitutions in progressors and nonprogressors did not differ indicates a similar misincorporation rate of the replication machinery of HIV-1 strains circulating in both groups of patients. The absence of a relationship between the nonsynonymous evolution rate and the level of virus production indicates that the replication and production of HIV is only a minor factor contributing to the final evolution rate of the V3 region. The inverse relationship between the number of nonsynonymous substitutions and disease progres-

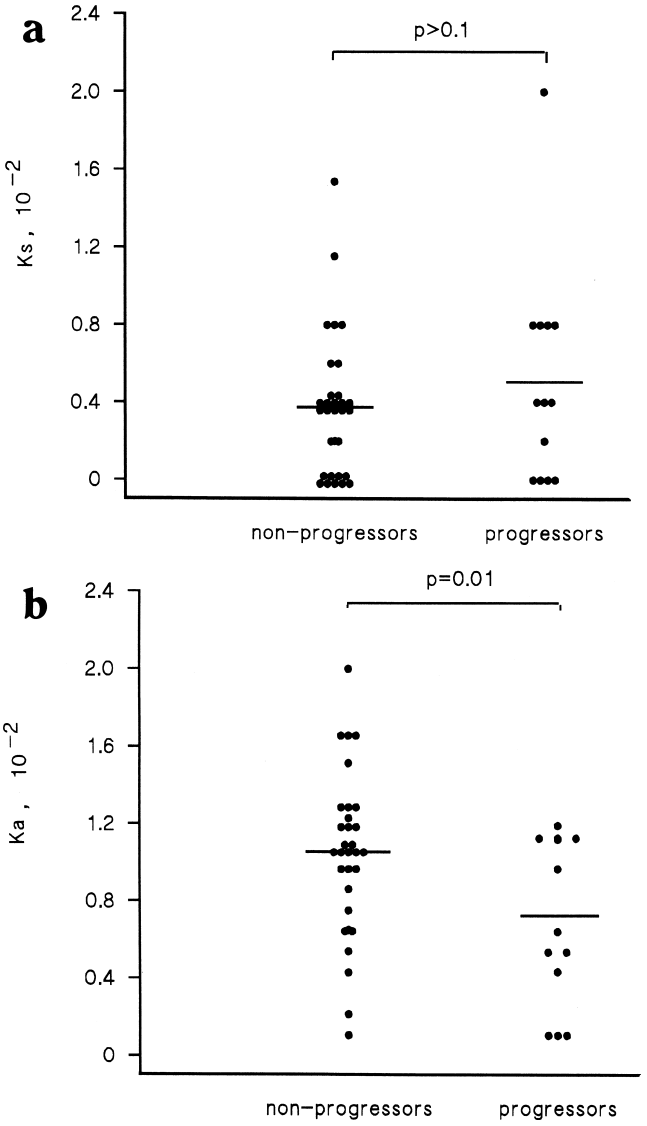

FIG. 2. Numbers of synonymous (a) and nonsynonymous (b) nucleotide substitutions per site per year in the V3 region in progressors and nonprogressors. Horizontal lines, means.

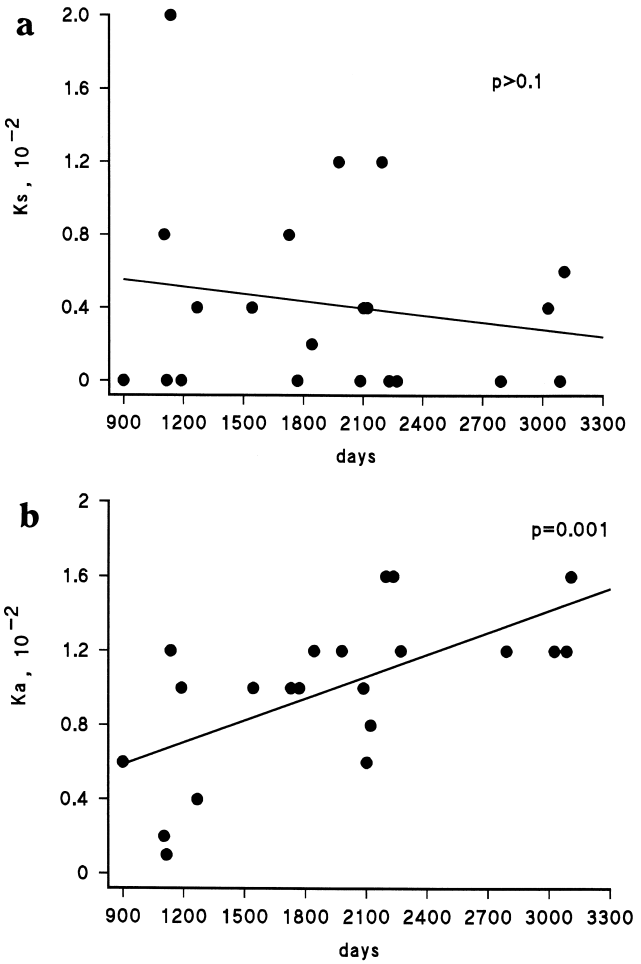

FIG. 3. Numbers of synonymous (a) and nonsynonymous (b) substitutions per site per year over the 5-year period in relation to the duration of the immunocompetent period in HIV-1 infection. For panel b, $r = 0.64$, and $r^2 = 0.41$.

sion and immunodeficiency indicates either that intact immunity drives V3 evolution (i.e., positive selective forces are operational in nonprogressors) or that virulence suppresses V3 evolution (i.e., negative selective forces are operational in progressors). The lower K_s/K_a ratio in nonprogressors compared with progressors indicates that positive selection for nonsynonymous substitutions in nonprogressors overrides relative negative selection in progressors.

Positive selection for nonsynonymous substitutions in V3 was shown to be related to the duration of the immunocompetent period. This observation is in accord with the findings of Delwart et al. (12) of greater HIV-1 quasispecies complexity being associated with a slow decline of $CD4^+$ cell numbers. The findings of Delwart et al. on quasispecies complexity and our own on the evolution of the consensus or dominant sequence do not support the model of Nowak et al. (36, 37), which predicts that a highly diverse HIV-1 population, rather than a specific depletion of immune cells, causes escape from the immune system and disease progression.

Delwart et al. (12) explained their findings as an effect of the immune response against the virus driving intrahost evolution. Similarly, our present findings might be explained by assuming that the immune system of progressors is less efficient in controlling the virus, simultaneously leading to more rapid disease progression and to slower viral evolution. It seems clear that selective forces are involved, in particular because the K_s/K_a ratio for the V3 region is low. An explanation involving pressure from the immune system is attractive because of the high levels of V3-directed antibodies and their neutralizing capacity in vitro. This explanation presupposes that the immune response to the V3 region is strong and effective in postponing immunodeficiency and AIDS. Recent evidence, however, convincingly shows that V3-specific antibodies are relatively ineffective in neutralizing HIV-1 primary isolates because the native V3 domain may be hidden on the surface of macrophage-tropic isolates (6). However, Bonhoeffer et al. (3) found a significantly lower K_s/K_a ratio in the T-lymphocyte-tropic HIV-1 sequences than in the macrophage-tropic variants, suggesting that the immune pressure may act more strongly against T-cell-tropic HIV-1 strains. On the other hand, V3-specific antibody titers appear not to be related to immunocompetence or the absence of HIV-related disorders (22, 52).

These observations suggest that V3-specific antibodies may not provide a strong selective force, and they make it difficult to explain the observed difference in evolution rate between progressors and nonprogressors as a result of a humoral immune response directed against the V3 region. Klein et al. (26) recently demonstrated a persistent Gag-specific cytotoxic T-lymphocyte response in nonprogressors and its loss during the progression to AIDS, which coincided with the $CD4^+$ cell decline and severe deterioration of T-cell function. Taking into account these observations and the fact that the V3 region contains the antigenic sites for cellular immunity (42), it is attractive to hypothesize that the stronger (or broader) cytotoxic T-lymphocyte response in nonprogressors drives HIV-1 evolution.

An alternative explanation may be based on the model of Domingo and coworkers, who have demonstrated antigenic variation in the absence of immune selection for the foot-and-mouth disease virus (5, 14, 15). This model states that an evolution of the consensus sequence, like we observed, of an RNA region encoding an antigenic site can be driven by positive selection without involvement of the immune system. Positive selection can be mediated either by fixation of replacements arising from their beneficial effects on biological functionality or by beneficial replacements or compensatory changes elsewhere in the genome. Both are seen with HIV-1. V3 contains determinants for cytopathicity, infectivity, and cell tropism (8, 10, 11, 17, 28, 43), and a virus may escape V3 antibodies by a mutation outside the antigenic epitope but in the same external envelope protein (1) or even in a separate but structurally linked protein, gp41 (2). The model of Domingo et al. explains fixations of replacements at any genomic site where replacements are tolerated to more than the average degree in the viral genome.

Having argued that the immune pressure on the V3 region is not necessarily the only selective force determining the V3 evolution rate, several alternative explanations can be suggested. One possibility is that there is an inherent difference between the virus variants in progressors and nonprogressors, for example, differences in the cell type in which the virus replicates most efficiently or virus dissemination differences due to greater efficiency of cell-to-cell spread of virus than of cell-free virus infection. In general, functional differences between the viruses in progressors and nonprogressors can be assumed, in which one virus variant does not evolve rapidly because it is already optimally adapted to its host environment and, by its very nature, is more detrimental to the host than a less well adapted virus.

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