SIV envelope evolution and virus virulence
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Chapter 6

Increased heterogeneity of SIVsm env Associated with Low Viral Load in Macaca mulatta
Increased Heterogeneity of SIVsm *env* Associated with Low Viral Load in *Macaca mulatta*

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Abstract: Lentiviruses in primates susceptible to AIDS swarm as a quasispecies of genetic and biological variants which evolve as the infection persists. The development of AIDS is associated with the evolution of highly fit and highly virulent end-stage variants. To determine when virulent end-stage variants emerge in a susceptible host we correlated the virus load (prognostic for disease progression) with the degree of variation of the C1 to CD4 binding regions of the env. This was studied during the sequential in vivo passage of SIVsm DeltaB670 in a uniform group of age and sex matched rhesus monkeys (Macaca mulatta). Interestingly, the most marked sequence variation was observed in animals participating in earlier passages with low viral loads. Certain genotypic motifs found in the highly fit end-stage variants could be traced back to animals with low virus loads and a long survival history. These data suggest that the evolution of highly-fit end-stage variants begins during periods of low viral loads. They may be in part be driven by host immune responses early in infection until they become highly fit and capable of immune escape.

Introduction: The use of SIV in macaques allows for the analysis of virus of known sequence, and parameters or determinants of disease progression. The various strains of SIV differ in their infective and disease causing capacities (1,24) and in their various natural hosts as well as in experimental infections in other primate species (25,38). Patterns of disease have been widely investigated with studies on prior immunization and analysis of the progressors and slow progressors after infection to show that early replication patterns correlate with disease outcome (20,42). The route of transmission has also been shown to influence early viral selection and diversity (6,40). Virus load is a measure of disease progression in susceptible hosts (9,20,22,31,38,42). In disease resistant hosts viral loads as high as $10^5$ to $10^7$ copies per millilitre have been reported (38,42).

The ability of lentiviruses to absorb remarkable variations in their genomes and remain replication competent or fit is due to their quasispecies nature (17). The various copies of the SIV genome present during infection are all slightly different from one another due to the infidelity of their own reproductive machinery, and thus the virus may be more or less fit because of these variations. The size of the infectious inoculum is crucial as the variability of the virus can be detrimental if the inoculum is too small and only incompetent or less fit variants are transmitted alone (8,15). Increases in the size of the infectious inoculum have been shown to exponentially increase the virulence or pathogenicity of the virus during infection (8,35). Whereas dilute passages of virus, or lowered inoculum size promotes the expression of genetic and phenotypic variants (39) there is a finite limit to the extent to which the transmitted quasispecies, or inoculum can be reduced (8), limited reduction in the size of the transmitted quasispecies gives less prevalent or less replication competent variants a chance at the limited number of target cells. Experimental infection with a pathogenic clone can result in rapid progression as the initial burst of replication of a fit variant can overcome the effect of dose (23). Although quasispecies support the replication of the most fit variants by virtue of their quicker replication capacity (better able to compete to be the founder of the next round of replication or infective party to a new target cell) the sequence space within which they operate (17) is prone to disruption by the stochastic action of newly derived mutants of already fit variants (7). Although the derivation of new more fit variants is the basis for genetic variation during adaptation and infection the antigenic variation is not necessarily dependent upon immune selection as has long been proposed (13), but also to the action of the stochastic behaviour of large fit quasispecies (7). The combination of viral variation and immune response leads to a change in the biological properties of the during
infection and frequently a change in the tropism of the target cells (10), and the evolution of more virulent variants during progression to AIDS (19,28). The stabilization levels of viremia are predictive of the outcome of the infection (19,43), and increased viral load is a marker for a more rapid progression to AIDS.

To study the molecular evolution of a pathogen during cross species transfer the sooty mangabey strain of SIVsm was sequentially passed six times (23) in Asian rhesus macaques. Pathogenesis was studied by following the fixation of env sequences, plasma viral load, disease characteristics, and length of the asymptomatic period during adaptation to a new host. The highest rates of evolution as measured by intrasample nonsynonymous variation were found in the initial passages and correlated with low virus load. These infections demonstrated that large amounts of nonsynonymous variation, or antigenic diversity are related to decreased replication kinetics, or less fit early passage variants of SIVsm. Eventually the selection and fixation of these nonsynonymous variations led to increased loads and high virulence.

Materials and Methods:

**Virus.** The SIV strain used in the multiple passage is of the SIVsm family of lentiviruses (SIVmac, SIVsmmH4 and SIVPBj14) originally discovered in sooty mangabeyes or in captive Asian macaques infected accidentally with SIVsm (11,12,21). The SIVsm DeltaB670 strain used is well characterised and has been used in previous experimental infections of rhesus macaques because it accurately reproduces the clinical manifestations of AIDS seen in humans (2-4,18,26,32,34,37,41,49). The virus stock has been thoroughly analysed as to genotypic and phenotype variation during experimental infection of non-human primates and during in vitro infection of rhesus macaque and human PBMCs (2,30).

**Passage.** As reported elsewhere (23), the passage was carried out using 16 age matched Asian rhesus macaques (Macaca mulatta) of two years of age. The passage with SIVsmDeltaB670 was carried out using intravenous inoculation, with the first rhesus macaque receiving 5x10^2 infectious doses of cell free virus. The following rhesus macaques (for P2 to P6A and P6B, there were two infections at the P6 level) were inoculated with 2x10^6 uncultured cryopreserved peripheral blood mononuclear cells (PBMC) taken at the symptomatic stage of disease from the preceding rhesus macaque. Routine clinical biochemistry and haematological analysis was performed at two week intervals, and following two months, at four week intervals. The CD4+ T cell changes in concentration were monitored by FACSscan (Becton Dickinson) using double labelling with Leu3a and 4B4, as described earlier (33). The concentrations of SIV p27 antigen were measured with an SIV p27 antigen capture assay (Coulter Corporation).

**Clinical Findings During Serial Passage:** The levels of plasma antigen (p27) and CD4+ T cells were measured at all available time points. Shortening of the asymptomatic period coincided with decreased viral diversity, greater plasma antigen levels, and a more rapid CD4+ T cell loss. The concentrations of SIVp27 and relative percentages of CD4+ T cells were monitored throughout the passage, no data was available for passage one (P1), except for the last serum sample, as used for RT-PCR amplification, cloning, and load determination. The P5 resulted in unexpectedly quick progression to AIDS, and no samples were available for haematological assay or for sequence, or load determination. The passage of SIVsm
DeltaB670 in juvenile rhesus monkeys resulted in the decrease of the asymptomatic period from eighteen months to one and two months, in the fifth and sixth passage respectively. Disease development was characterised by rapid loss of CD4+ T cells persistent and persistently high level plasma viremia (23). The pathological and clinical manifestations of disease did not change during the decrease in the clinical latency. The rapid reduction of the asymptomatic period occurs within three consecutive passages, dropping to weeks after P3 (23).

**Viral load determination:** Determination of SIV RNA levels in plasma of infected macaques was carried out using a highly sensitive and reproducible quantitative competitive (QC) RT-PCR assay (43). Briefly, 200 pl of plasma was added to 600 pl of guanidine-isothiocyanate-based lysis solution containing 300 copies of internal standard RNA. The RNA was precipitated by propanol-2 and was reversed transcribed and amplified with rTth DNA polymerase. The amplification products were hybridised in six five-fold dilutions to a capture probe that was that was covalently bound to microwell plates. The amplification products were detected by a streptavidin-horseradish-peroxidase-mediated calorimetric reaction. The amplified internal standard was hybridised to a rearranged 26-bp capture probe in separate microwells. The number of RNA copies in the plasma sample was calculated from the optical density of the sample wells compared to that of the corresponding internal standard. The number of RNA copies in the plasma sample was calculated from the optical density of the sample wells compared to that of the corresponding internal standard well. Data was plotted for each individual animal involved in the sequential *in vivo* passage as the concentration of RNA per ml of plasma (23).

**Env RT-PCR, Cloning Strategy, and Sequencing:** Genomic viral RNA was extracted from macaque serum, amplified using RT-PCR and 60 env genes were sequenced form the V1 to V4, and analysed using phylogenetic analysis (44), and Ks/Ka variation (46). Briefly, viral RNA was extracted using silica beads and a chaotropic agent according to the Boom method (5), and used as template in a reverse transcriptase reaction followed by PCR amplification. The 3' PCR primer was also used to prime the reverse transcriptase reaction, followed by a single PCR reaction and cloning (44). The time points for sampling by sequencing of serum viral RNA were seroconversion and death. The sequenced clones were named by passage number (P1 to P6A and B) and start (S) or death (D). The sequences were aligned using CLUSTAL W(18) and adjusted by eye. Synonymous and nonsynonymous nucleotide variations (P-Distances) were calculated by using the MEGA program (29,46). Intrasample variation calculations are the result of comparisons of clones from the same time-point (within a sample).

**Results:** Increasingly rapid progression to AIDS caused by the large inoculum size occurred in the first three passages. Macaques infected with only 50 MID₅₀ of another nonpassaged SIVsm strain died between twelve and thirty weeks with loads of up to 10⁹ per millilitre of plasma (20). During the first passage the virus at time of death showed a large amount of heterogeneity (46). During the second (and later) passages there was a very rapid progression to AIDS and death. As has been shown (23) the monkeys with extended TDPI (time of death post infection) showed a lower load and some containment of viremia before death, whereas the fast progressing monkeys showed no containment of viremia reaching a plateau at a high early peak load. The data were analysed according to TDPI, synonymous and nonsynonymous intrasample genetic variation, and virus load at peak, start (first available sample), and death.
Figure 1: Nonsynonymous (A), and Synonymous (B) P-Distances within the V1 to V4 sequenced env clones versus the virus viral load in the serum of the serially passaged SIVsm. P-Distances are the numbers of nucleotide substitutions between two strands of nucleic acid divided by the number of nucleotides they comprise in length, and gives an estimate of the average genetic heterogeneity.
The nonsynonymous and synonymous genetic variation versus viral load (Figure 1A & B) follow different patterns, with the nonsynonymous variation being biphasic with a steep decline from the initially heterogenous primary inoculum at the lowest viral load. The synonymous variation follows no particular pattern when compared to either viral load (Figure 1A), or TDPI (similar pattern, not shown). The nonsynonymous genetic heterogeneity decreased almost three fold in the first passage and then remained at a low level thereafter when compared to virus load (Figure 1B); this biphasic pattern is similar to the relationship of TDPI and viral load (23). The increasing peak viral load follows a consistent decline in the TDPI and also in nonsynonymous genetic variation (Figure 2).

Figure 2: Time of death post infection versus nonsynonymous genetic variation.

**Discussion:** Lentiviral evolution during sequential passage is under the influence of population passage (8,35), purifying selection and the steady state variability or quasispecies of an adapted virus population (7,14,17), and antigenic variation which is not dependent upon immune selection (13,47). Sequential passage of a sooty mangabey derived SIVsm in rhesus macaques resulted in marked increases in the virulence of the virus. A genetic quasispecies with a wide range around the master sequence was found during adaptation and was initially associated with lower viral loads. The peak loads increased from $10^5$ in the first passage to $10^9$ in the end of the fifth passage. The genetic variation of the evolving virus decreased from the first passage to the third passage and remained at a basal level with a nominal amount of nonsynonymous variation (Figure 1A). Heterogeneity of the passaged virus was always present and displayed a quasispecies nature. The TDPI of the passages decreased in a similar pattern as the genetic variation and remained at an exceedingly short average after the fourth passage (Figure 2).
Rapid progression to AIDS occurred in the absence of an effective immune response (viremia was controlled to some extent only in one monkey in passage two and three). Antigenic variation is decreased by the rapid adaptation and the selection for the most rapidly replicating viral variant. Rapid replication after the initial selection and dissemination preclude the immune system of producing even a weak or shortened suppression of viral replication.

A large number of seedling viruses used as the inoculum increases the chances that a better adapted or more rapidly replicating virus will exist in the inoculum population. Progeny will be selected as founders by the preceding rounds of replication and infection thereby raising the ability to replicate of the entire virus population. During decreasing TDPI and genetic variation of the quasispecies its phenotype narrows (15,16,36). The large number of genomes present overcomes the stochastic events of reverse transcriptase errors, sampling errors during fluctuating viral loads and immune or tissue specific selection as the quasi-species swarm evolves through sequence space towards elevated terrain (17). Nonsynonymous genetic variation is the hallmark of lentiviral infection and over time the heterogeneity or breadth of the quasispecies will alter according to selection and escape event, as will the master or consensus sequence encoding the aggregate of the quasispecies existing at any time. A virus is in constant competition due to the competitive exclusion principle (7). The complex mixture of unique antigenically distinct viral genomes compete as a most fit variant is selected over its nearest relatives (derived by errors of the reverse transcriptase) which may have some advantage, be neutral, or have some disadvantage, as compared to the other viruses present. Selection of fitness occurs by several processes of replication kinetics, cell tropisms, and immune escape ability. The sequence most fit (replicating to the highest levels, tropically advantaged, or more immune resistant) in a given host is at a peak of efficiency, and variation is of a limited value, as the virus would then shift to a lower terrain in the fitness landscape or lesser ability to replicate (17). The life cycle of SIV is dependent upon acclimatization to produce increased virulence (27) as is seen by the rapid and directed direction of the nonsynonymous variation.

The events that take place early in HIV/SIV infection have a distinct role in the outcome of the infection. Diverse profiles of plasma viremia occur during infection and progression and the early containment is a prognostic indicator of a long asymptomatic period, and early viremia is predictive of a more rapid disease progression (20,31,48). The emergence of cytopathic and antigenic variants influences the progression to disease (19,28). The selection early in the infection of antigenically distinct env fixations (nonsynonymous variations) may also be an indicator of progression (45). The viruses containing advantageous mutations early in infection are selected during the heterogeneous phase of the infection and fixed in a sequential fashion leading to a more fit quickly replicating variant (Figure 1A). The consensus sequence or intrasample nonsynonymous variation is seen to follow a biphasic pattern after the needed variations present in the primary inoculum are found, selected and fixed (45). These variants correlate directly with the viral load, whereas the synonymous or antigenically indistinct variations have no correlation (Figure 1B). This implies that the low virus load correlated env variations explored by the virus and fixed could be the cause of the increased load and virulence. The existence of the variations early in infection would lead one to believe that the development of pathogenesis is caused by the rapid replication (even in the earliest infection the load is $10^6$ copies per ml of plasma), and continuous adaptation of the virus in selecting the virulence inducing substitutions in the env of the early passages until
they are all present in one genome. The study of these early infection selections could lead to the finding of env variations that could be used as a prognostic indicator. As demonstrated in this study the ability to replicate efficiently with decreasing genetic heterogeneity are key predictors of a rapid progression to AIDS.

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