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The induction of in vivo superinfection and recombination using feline immunodeficiency virus as the model

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Summary. This study investigated the hypothesis that under certain conditions, superinfection of cats with feline immunodeficiency virus (FIV), may occur. One FIV isolate (T91) was used to inoculate three FIV and FeLV-free cats. Blood from an FIV-infected cat (N), which contained two variants and differed from T91 by at least 5% in nucleotide sequence in the *env* gene, was inoculated into a fourth cat. Both T91 and blood from N were inoculated simultaneously into a fifth cat. After 22 weeks, two of the three cats initially infected with T91 were challenged with blood from N. At 30 weeks following initial infection, peripheral blood mononuclear cells were obtained from all cats, DNA was extracted, and a segment of the *env* gene was PCR amplified, cloned and sequenced. Nucleotide sequence analysis of the cloned PCR product showed that virus strains used in initial infection were recovered from cats not challenged with a second variant. Challenge of cats with the blood of N following initial infection with T91 resulted in superinfection occurring in one cat and recombination occurring in the other. Furthermore, the use of blood as a source of challenge, in cats where superinfection and simultaneous infections were attempted, may have induced the appearance of variants which more closely resembled the most heterologous strain present in the infectious source.

Introduction

Feline immunodeficiency virus (FIV) is a lentivirus which shares many characteristics with human immunodeficiency virus-1 (HIV) and is associated with the development of an acquired immune deficiency syndrome (AIDS)-like disease in cats. Because of the close similarities between FIV and HIV, particularly in terms of replication strategy, cell tropism, and the clinical stages of disease, FIV is considered to be an appropriate model for the study of HIV infection and AIDS [7, 13, 8, 26].

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Since the advent of the AIDS epidemic, and with as many as 40 million people predicted to become infected with HIV by the year 2000 [32], the development of a vaccine against the infection has become a major challenge for researchers. Although many promising results have been obtained regarding HIV vaccine development [3, 8, 23, 28], most have used challenge with cell-free homologous virus as the index of protection. It has been suggested that the use of cell-free virus as the challenge may be an inadequate test for the effectiveness of an HIV vaccine [31]. The major reasons cited for this is that the mode of natural infection in human beings, semen and blood, contain large numbers of virus-infected cells and relatively little cell-free virus [31]. A further issue to be addressed is the existence of a wide variety of virus variants that have little or no cross-neutralisation to each other [19]. Indeed, the extensive genomic variability among different HIV-1 strains as well as within a given individual has been well established [1, 9, 16, 25, 30].

Furthermore, dual infections with HIV-1 and HIV-2 have been reported in patients from West Africa [4, 29] and Brazil [27]. By restriction enzyme analysis, superinfection of a chimpanzee with a second distinct strain of HIV has been demonstrated [6]. However, there is compelling evidence that persons infected with one variant of HIV are refractory to infection with a second variant [11]. Furthermore, it has been shown at the cell level that once cells become infected with an initial variant of HIV, virus infected cells are resistant to superinfection with a different virus variant [33]. Elucidation of this protective mechanism at the level of both the host and cell may lead to the development of an effective vaccine against HIV. It has also been demonstrated that seropositive donors who carry multiple variants of HIV-1 in their peripheral blood transmit only one variant and probably always the same variant to the recipients of the infection [17, 35].

Although the demonstration of superinfection and recombination has been well established both in vitro and in vivo for oncoviruses [2, 5, 6, 15, 34], little is known of either superinfection or recombination of lentiviruses in vivo. In this study we investigated the possibility that under certain conditions, superinfection with FIV may occur in vivo. By comparing nucleotide sequences of clones derived from the first and second variable domains within the *env* region of FIV proviral DNA obtained from experimentally infected cats following challenge with a second strain of FIV, our findings show that the superinfection does occur in vivo, and superinfection as well as simultaneous infection may have resulted in the appearance of variants which more closely resembled the most heterologous strain present in the infectious source.

Materials and methods

Source of FIV

The experimental infections were accomplished using T91, a cell culture maintained isolate originating from an adult male cat naturally infected with FIV. T91 was recovered from this cat by co-cultivation of its peripheral blood mononuclear cells (PBMCs) with an IL-2

Table 1. Schedule of inoculation of cats with FIV, their age at initial infection and when proviral DNA obtained for PCR, cloning and sequencing

Cat No.	Age at initial infection (weeks)	Initial infecting virus	Challenge virus (at 22 weeks)	Proviral DNA obtained (weeks post-initial infection)
1	14	N	None	8
2	36	T91	None	60
3	32	T91	N	30
4	32	T91	N	30
5	14	T91 and N simultaneously	None	8

sensitive T-lymphoblastoid cell line (MYA-1) of feline origin [20]. The sequence of a 568 base pair segment of the *env* region of T91 is known [10]. The second source of virus was blood from an adult female cat (N). The sequence of an isolate from this cat (N91) from the same *env* region as that described for T91 is also known [10]. A second sequence from N (N93) was obtained from PCR of salivary gland DNA. The salivary gland was collected post-mortem and was stored at −70 °C until DNA was extracted.

Experimental design

Five FIV, FeLV-free cats between $3\frac{1}{2}$ and 8 months of age at the time of initial infection were used. The schedule of initial infection and subsequent challenge (if any) is shown in Table 1. If infected with T91, the particular cat was inoculated subcutaneously with 1 ml of cell culture supernatant containing $10^4 TCID_{50}$/ml of FIV. If infected initially or challenged with N, the cat was intravenously injected with 0.5 ml of heparinised blood obtained from N immediately prior to injection.

Cat 1 was inoculated with N, cats 2, 3, 4 with T91, and cat 5 simultaneously with T91 and N (Table 1). The initial infection of each cat was confirmed by ELISA for the presence of FIV antibody. Following the initial infection, two of these (cats 3 and 4) were inoculated intravenously with N. Cats were then bled at varying times post-initial infection. PBMCs from the blood samples were isolated as described previously by Meers and colleagues [20].

Preparation of cellular DNA

Briefly, PBMCs (approx. 1×10^6) were lysed in 500 µl lysis buffer (10 mM Tris-Cl, pH 8; 2 mM EDTA, pH 8; 400 mM NaCl; 5% SDS); and digested with 100 µl of proteinase K (100 µg/ml) at 65 °C for 1 h and then overnight at 37 °C. Proteins were then precipitated with 100 µl of saturated NaCl solution [22]. DNA containing supernatant was precipitated in ethanol, and the pellet obtained was washed with 70% ethanol, and resuspended in 20 µl of sterile distilled water.

PCR

PCR primer pairs which encompassed the region between L6299 and R6866 were used to amplify a fragment of 568 bases. L6299 is located at positions 6299–6319 of *env* (gp 120) and

has the sequence 5′ AGGACCAGAAGAAGCTGAAGA 3′, while R6866 is located at positions 6866–6846 of *env* (gp 120) and has the sequence 5′ TTCTGGTGCCCAACAATCCCA 3′. Amplification of FIV proviral DNA sequences was achieved using the PCR as marketed by Perkin Elmer Cetus (Norwalk, CT). Purified double-stranded genomic DNA (1 to 5 μg) was added to 50 μl of total reaction mix. The reaction mix contained a final concentration of 100 pmole of each primer, 200 μM of each dNTP, and reaction buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$, and 0.1 mg/ml gelatin). The reaction was overlayed with paraffin oil, and cycled on a Corbett thermocycler (Corbett Research, Australia). After heat denaturation at 94 °C for 5 min, 2.5 units Taq polymerase (Perkin Elmer Cetus, U.S.A.) was added and the primers annealed at 55 °C for 1 min, extension was carried out at 72 °C for 2 min and denaturation at 94 °C for 30 sec. At the end of the 35th cycle, extension was carried out at 72 °C for 8 min. PCR products were resolved on 4% NuSieve agarose (FMC BioProducts, U.S.A.) gels stained with 0.5 μg/ml ethidium bromide.

Cloning of PCR-products

Bands corresponding to 568 basepairs were exised and gel purified with β-agarase (New England BioLabs, U.S.A.) and cloned into pGEM-T vector (Promega, U.S.A.). Following transformation into *Escherichia coli* (Sure cells; Stratagene), recombinants were selected on plates containing IPTG and X-gal (Boehringer Mannheim). The identity of positive clones were confirmed by restriction endonuclease digestion of plasmid miniscreen DNA.

DNA sequencing

Sequencing reactions were performed by the dideoxynucleotide chain termination method using a M13 universal sequencing primer and DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturers' instructions. The sequence was resolved on an Applied Biosystems model 373A automated DNA sequencer. Sequences obtained were screened and aligned via the Australian National Genomic Information Services (ANGIS) for homology.

Results

In a previous study [10], a 5′ segment of the *env* gene coding for an N-terminal region of gp 120 spanning the first and second variable domains of FIV [24] was analysed because of its high variability relative to other regions of the genome. In the present study, we analysed the same region to allow direct comparison with previous results. DNA from each of the five cats was subjected to PCR amplification, the products of which were cloned, and individual clones isolated and sequenced. The results are shown in Tables 2 and 3.

From cat 1, which was inoculated with N only, the clones recovered were virtually those of N93, with nucleotide sequence identity of 99.4%. Also, the nucleotide sequences of three randomly selected clones from this cat were identical to each other.

Similarly, the sequences from cat 2, which was inoculated with T91 only, were virtually those of T91, with nucleotide sequence identity of 99.4%. Other clones from this cat were also identical to each other in nucleotide sequence.

Differing results were obtained from the two cats (cats 3 and 4), which were initially infected with T91 and then later challenged with N at 22 weeks following

Table 2. Percent identity in a 516-nucleotide domain of an N-terminal region of *env* gp 120 from FIV isolates obtained from five experimental cats at 30 weeks post-initial infection

	T91	N93	N91	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5
T91								
N93	94.8							
N91	88.0	91.3						
Cat 1	94.1	99.4		100				
Cat 2	99.4	95.0		94.3	100			
Cat 3	98.8	95.6		94.8	99.4	100		
Cat 4	89.4	93.4	97.5	94.2	89.6	90.0	100	
Car 5	89.7–90.7	92–94	95.9–96.9	93.3–94.4	89.9–90.6	90.2	98.3–99.4	97–99

Table 3. Percent identity in a 172-amino acid sequences an N-terminal region of *env* gp 120 from FIV isolates obtained from five experimental cats at 30 weeks post-initial challenge

	T91	N93	N91	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5
T91								
N93	95.1							
N91	87.7	89.8						
Cat 1	92.4	99.4		100				
Cat 2	97.7	91.9		91.6	100			
Cat 3	96.0	94.8		93.9	96.4	100		
Cat 4	89.1	91.8	92.1	92.4	89.5	91.5	100	
Car 5	88.8–89.4	91.8–92.7	91.2–92.4	91.5–92.4	87.7–88.3	90.9–91.5	95.8–97.6	97.3–99.7

initial infection. The nucleotide sequence (106 base pairs) at the 5′ end obtained from cat 3, consisted of a short segment which corresponded with N93 sequence followed by a longer segment corresponding to T91. The clones were identical, with nucleotide sequence identities being 98.8% when compared with T91 and 95.6% with N93.

Although it was apparent that the nucleotide sequences of four randomly selected clones from cat 4 were derivatives of N93, they were significantly different from N93, with up to 6.6% variation in nucleotide sequence. The sequences of clones derived from this cat differ from T91 by approximately 11%. Of 516 basepairs, 33 were different from either T91 or N93. These nucleotide changes correspond to those of N91, an isolate obtained from N, 2-years previously. Overall nucleotide sequences of all clones derived from this cat more closely resembled N91, with sequence identity of 97.5%, and all other randomly selected clones from this cat were identical.

In contrast, sequences of clones recovered from cat 5 that was simultaneously infected with both T91 and N, were not identical, with up to 3% nucleotide

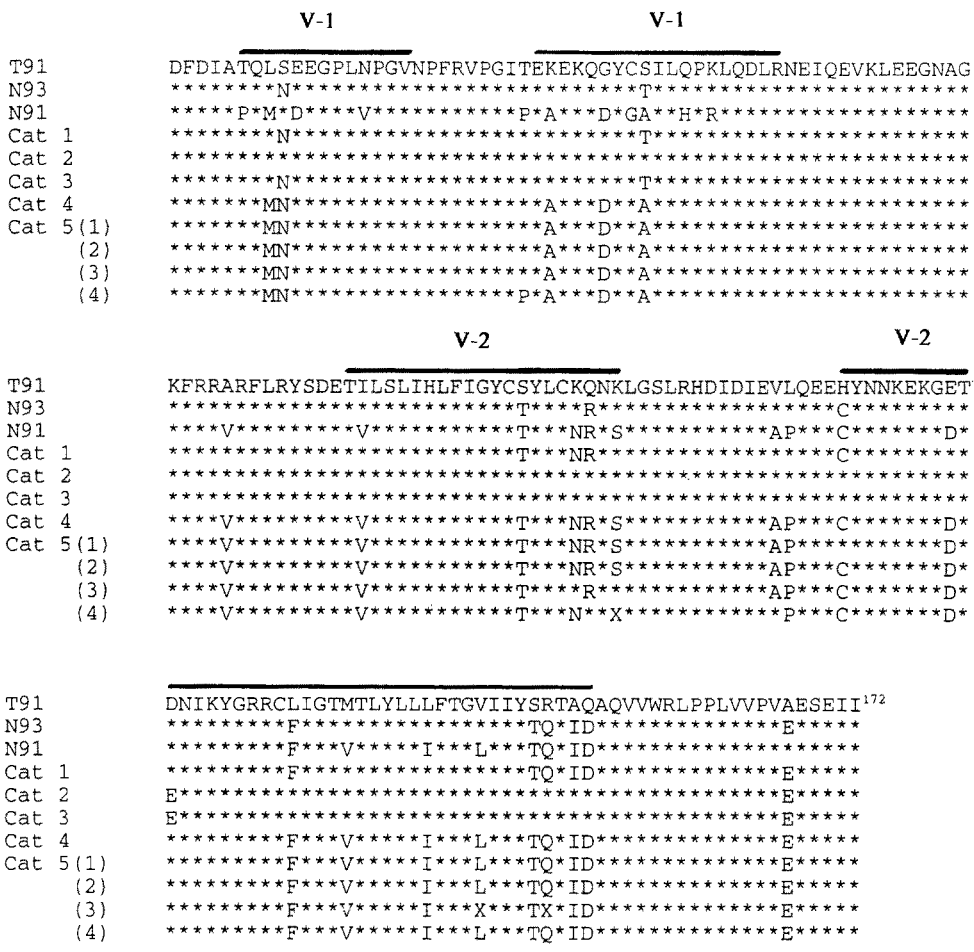

Fig. 1. Comparison of deduced amino acid sequences of an N-terminal region of *env* gp120 from clones isolated from five experimental cats. Asterisks indicate sequence identity with T91. Bars denote variable domains according to [24]

variation between clones, but as found with clones obtained from cat 4, sequences appeared to be most closely related to N91 with the order of 95.9–96.9% identity. Of the 516 nucleotides, 31 (6%) did not correspond to either T91 or N93. Nucleotide sequences obtained from four randomly selected clones of cat 5 were similar to those derived from cat 4, with nucleotide sequence identities as high as 99.4%, and were different from those of T91 which have identities of 89.7–90.7% and to a lesser extent (identities of 92–94%) from N93.

The nucleotide sequence variation also resulted in predicted amino acid change in cats 3, 4 and 5. These tended to cluster within the variable regions (Fig. 1). Significantly, of 29 nucleotide changes observed in clones derived from cats 4 and 5 which did not correspond to either T91 or to N93, eighteen (60%) were non-synonymous. As was found with the nucleotide sequence, the amino acid identity between FIV sequences from cats 4 and 5 was high being in the order of 95.8–97.6%. In contrast, the amino acid sequence of clones derived

from both cats 4 and 5 appeared to be significantly different from T91, with identities of 89.1% for cat 4 and 88.8–89.4% for cat 5. Similarly, amino acid sequences of clones derived from both of these cats were also substantially different from N93, but to a lesser extent than T91, with identities of 91.8% for cat 4 and 91.8–92.7% for cat 5. The predicted amino acid sequence of clones isolated from cats 1 and 2 were virtually identical with N93 and T91 respectively, each with a identity of 99.4%.

Discussion

This study demonstrates that both superinfection of cats and recombination of different FIV variants can occur in vivo under experimental conditions. The phenomena of superinfection and recombination suggests the response by the host to the initial infection with a pathogenic lentivirus is insufficient to resist challenge with a second, heterologous virus. The experiments were designed to test the hypothesis that the host response to primary FIV infection would offer protection against heterologous challenge. Protection may still be provided under natural conditions as the magnitude of the challenge in this experiment, in terms of the amount of virus and the route of administration (intravenously), was substantial. Proof that in vivo superinfection for a member of the lentivirus group has been established, and this opens the way to more closely define the conditions under which superinfection occurs. The use of FIV infection as a model for such studies would seem ideal. The establishment of the criteria for withstanding challenge with heterologous virus has particular significance with respect to the vaccine development.

While superinfection and recombination occurred in the experiments described, there were a number of additional findings which were not considered likely at the start of the experiment. It would appear that the use of FIV-infected blood, which contained at least two variants of FIV, as the challenge, showed that the most heterologous variant became the predominant superinfecting variant. It also introduced the question of the success of in vitro growth of variants compared to the success of in vivo replication. It appears that the state of the host has a profound influence on which particular variants predominate. The FIV variant recovered from the FIV-free cat inoculated with blood from N only was identical to the variant recovered from the salivary gland of cat N, which suggests that this sequence (N93) was the predominant variant present in N.

Given that the infections of cats 1 and 2 reflect what occurs in a single infection where the clones were readily identifiable as N93 and T91 respectively, and the sequences of all clones were identical, it is evident that superinfection has occurred in one cat (cat 4). However, not only has superinfection occurred, but the recovered sequences of FIV variant (N91) from this cat was significantly different from N93 (the predominant strain), indicating challenge with at least two isolates in the presence of the first strain (T91), and the host's accompanying immune response, may have induced selection away from the predominant

variant. Considering the occurrence of directional nucleotide changes amongst FIV variants within a cat over two years [10], and therefore taking N93 as the ancestor of N91, it is likely that the blood given to the cats contained N91 as well as N93. Indeed, this has been shown in hepatitis C (HCV)-human carriers who were superinfected with a second HCV strain [14]). It was reported that following recovery, the follow-up samples showed significant genetic variation compared with the original HCV strain. Furthermore the rapid generation of HIV sequence variation within an individual over a very short period of time has also been reported [25].

This selection of variants has also occurred in cat 5 in which simultaneous infection was attempted. However, in this case clones were not identical and were substantially different from both T91 and N93, and closest to N91 (homology of 95.9–96.9%). The finding of 3% variation between clones from the same cat was at variance with the clones recovered from the other 4 cats, where all clones were identical, suggesting that simultaneous infection may have induced the appearance of distinct but highly related "quasi-species" [9, 21], resulting in four randomly selected clones not identical to each other.

We have shown that superinfection of cats with a heterologous FIV can occur in vivo, and that superinfection as well as simultaneous infection has induced selection of variants which are distinguishable from the predominant strain and resemble N91 (the original strain).

As the nucleotide sequences obtained from cats 4 and 5 were those of N91, and as N93 and T91 have a very high sequence homology (95%), FIV-specific cytotoxic T-lymphocytes which recognized epitopes common to N93 and T91 may have selectively killed T91- as well as N93-infected cells, thus allowing expression of N91.

In contrast, nucleotide sequences derived from randomly selected clones from cat 3 were not of N91 origin. Sequences of FIV variants derived from this cat, while substantially of T91 origin, contained a nucleotide sequence (106 base pairs) corresponding to the 5′ end of N93. This strongly suggests that recombination occurred. As there is a significant homology between T91 and N93 (95%), it was possible for crossover to have taken place between two viral variants following challenge with N. It has been well established that retroviruses undergo genetic recombination in vitro with a frequency of 40% per cycle during reverse transcription [12].

Amongst other factors, superinfection may have occurred because the challenge dose was too great for all cats to resist superinfection. Thus, when considering the development of candidate vaccines, several important factors must be taken into account including: the likely dose of infectious virus; the route of infection; and the age of the host during natural infection, and most importantly the health of the animal including whether it has clinical AIDS. The ability to superinfect cats may depend on the age of the cat at the time of initial infection, and the subsequent development of the immune response. Furthermore, local immune responses may provide a protective mechanism against superinfection following mucosal challenge which may be overcome by

intravenous challenge. The mechanism, which allows recombination to take place in one cat, and superinfection in the other despite the identical experimental protocols to which both cats were subjected to, remains unclear. Regardless of this, the findings reported here need to be taken into account when considering the development of effective HIV vaccines.

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