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The surface glycoprotein of a natural feline leukemia virus subgroup A variant, FeLV-945, as a determinant of disease outcome

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Abstract

Feline leukemia virus (FeLV) is a natural retrovirus of domestic cats associated with degenerative, proliferative and malignant diseases. Studies of FeLV infection in a cohort of naturally infected cats were undertaken to examine FeLV variation, the selective pressures operative in FeLV infection that lead to predominance of natural variants, and the consequences for infection and disease progression. A unique variant, designated FeLV-945, was identified as the predominant isolate in the cohort and was associated with non-T-cell diseases including multicentric lymphoma. FeLV-945 was assigned to the FeLV-A subgroup based on sequence analysis and receptor utilization, but was shown to differ in sequence from a prototype member of FeLV-A, designated FeLV-A/61E, in the long terminal repeat (LTR) and the surface glycoprotein gene (SU). A unique sequence motif in the FeLV-945 LTR was shown to function as a transcriptional enhancer and to confer a replicative advantage. The FeLV-945 SU protein was observed to differ in sequence as compared to FeLV-A/61E within functional domains known to determine receptor selection and binding. Experimental infection of newborn cats was performed using wild type FeLV-A/61E or recombinant FeLV-A/61E in which the LTR (61E/945L) or LTR and SU (61E/945SL) were exchanged for that of FeLV-945. Infection with either FeLV-A/61E or 61E/945L resulted in T-cell lymphoma of the thymus, although 61E/945L caused disease significantly more rapidly. In contrast, infection with 61E/945SL resulted in the rapid induction of a multicentric lymphoma of B-cell origin, thus recapitulating the outcome of natural infection and implicating FeLV-945 SU as a determinant of disease outcome. Recombinant FeLV-B was detected infrequently and at low levels in multicentric lymphomas, and was thereby not implicated in disease induction. Preliminary studies of receptor interaction indicated that virus particles bearing FeLV-945 SU bind to the FeLV-A receptor more efficiently than do particles bearing FeLV-A/61E SU, and that soluble SU proteins expressed from the viruses demonstrate the same differential binding phenotype. Preliminary mutational analysis of FeLV-945 was performed by exchanging regions containing either the primary receptor binding determinant, VRA, the secondary determinant, VRB, or a proline-rich region, PRR, with that of FeLV-A/61E. Results implicated a region containing VRA as a minor contributor, while a region containing VRB largely conferred increased binding efficiency.

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Conflicts of Interest Statement

The authors declare no conflicts of interest related to the performance of this work.

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Keywords

feline leukemia virus; lymphoma; surface glycoprotein; receptor binding domain

Feline leukemia virus (FeLV) is a naturally occurring gammaretrovirus endemic in an outbreeding mammalian species, the domestic cat. As a simple retrovirus, the FeLV genome encodes only those gene products required for its structure and replication. Despite this simplicity, the outcome of FeLV infection in the natural host is complex and unpredictable, including malignant, proliferative and degenerative diseases of lymphoid, myeloid or erythroid origin (Rezanka et al., 1992). This complex pathogenic spectrum is thought to reflect the heterogeneity of the domestic cat population that serves as host together with natural genetic variation among FeLV isolates. Indeed, FeLV occurs in nature not as a single genomic species but as a genetically complex family of closely related viruses. Genetic variability in natural FeLV is a consequence of replication mediated by the viral reverse transcriptase, a relatively low fidelity polymerase, and by recombination with endogenous FeLV-related sequences integrated in 9 – 16 copies throughout the feline genome (Levy, 2008; Overbaugh and Bangham, 2001; Pontius et al., 2007). The consequence of this genetic variation is a complex virus population that is continuously shaped by selective pressures *in vivo* and from which variants arise as predominant species. The selective pressures operative in natural FeLV infection are not well understood, but are thought to include such factors as replicative fitness and alternative receptor utilization, and may have significant consequences for infection and disease progression in the cat (Overbaugh and Bangham, 2001).

Natural isolates of FeLV most commonly exhibit sequence variation within the viral long terminal repeat (LTR) or the surface glycoprotein (SU) gene, both of which are implicated as determinants of disease outcome (Neil et al., 1991; Overbaugh and Bangham, 2001). The LTR encodes a major determinant of tumorigenic potential and disease specificity of FeLV, particularly within the repeat elements that function as transcriptional enhancers. The LTR of FeLV, like other gammaretroviruses, functions in the malignant process by directing high levels of virus expression in relevant target tissues and also by insertionally activating oncogenes at the sites of proviral integration. The capacity for LTR-driven transcriptional activation, and thus its impact on disease outcome, may be tissue-specific based on binding sites in the LTR for transcription factors and co-activators expressed in tissue-specific patterns (Fan, 1997; Finstad et al., 2004; Levy, 2008; Prabhu et al., 1999). The surface-exposed envelope glycoprotein, SU, governs entry into target cells by interaction with a specific host cell receptor. Thus, SU impacts disease progression and outcome through its direct impact on target cell selection and entry kinetics (Kristal et al., 1993; Lauring et al., 2001; Neil et al., 1991; Rigby et al., 1992; Tailor and Kabat, 1997).

FeLV SU as a determinant of receptor utilization and disease outcome

Differential receptor utilization among natural isolates has defined four FeLV subgroups, designated A, B, C and T, that are associated with distinct pathogenic mechanisms and disease outcome. FeLV-A is a weakly pathogenic, ecotropic, horizontally transmissible virus thought to represent the predominant agent spread cat-to-cat in nature. The known isolates of FeLV-A share ~97% amino acid sequence identity in SU, particularly remarkable since they were isolated over more than a decade from distant geographic locations across the world (Donahue et al., 1988; Neil et al., 1991; Overbaugh and Bangham, 2001). FeLV-B, -C, and -T subgroups are thought to arise from FeLV-A *de novo* through point mutation, insertion and/or recombination with endogenous FeLV-related sequences during virus replication in the infected animal. While FeLV-A infection is typically associated with the

induction of thymic lymphoma of T-cell origin (Neil et al., 1991; Rezanka et al., 1992), the FeLV-B, -C and -T subgroups are specifically associated with lymphoma, anemia or immunodeficiency disease, respectively (Donahue et al., 1991; Neil et al., 1991; Rohn et al., 1998). Distinct cellular receptors have been identified for each of the FeLV subgroups, all of which are multiple membrane-spanning receptors that function in transport of small molecules. FeLV-A, -B, and -C utilize their respective receptor for both binding and entry. The recently identified receptor for FeLV-A is a thiamine transporter termed FeTHTR1 (Mendoza et al., 2006). FeLV-A displays a fairly strict ecotropic host range, but has been shown to replicate at low levels in a few non-feline cell types including human 293T cells and canine D-17 cells (Mendoza et al., 2006; Nakata et al., 2003). FeLV-B exhibits a broad host range that reflects the patterns of expression of its receptors, one of two phosphate transporters designated FePit1 and FePit2 (Anderson et al., 2001; Boomer et al., 1997; Takeuchi et al., 1992). FeLV-C enters target cells via a heme transporter, designated FLVRC, and also exhibits a broad host range (Quigley et al., 2000; Quigley et al., 2004). FeLV-T binds to FePit1 as receptor, but is unique among the FeLV subgroups in that a soluble co-factor, FeLIX, is required for FeLV-T entry. FeLIX is expressed from endogenous FeLV sequences as a truncated SU protein and contains the N-terminal receptor binding domain (Anderson et al., 2000). To the extent that the surface receptor proteins are variably expressed on different types of host cells, the various FeLV subgroup SU proteins act as preliminary determinants of disease outcome by determining tissue tropism. FeLV-C, for instance, is associated with a rapidly degenerative anemia, a reflection of its entry into erythroid progenitor cells through FLVRC (Quigley et al., 2000; Quigley et al., 2004). In the case of FeLV-T, while the FePit1 receptor is abundantly expressed on many cell types, the required co-factor for entry, FeLIX, is expressed predominantly in T cells (Lauring et al., 2001).

FeLV-945, the predominant isolate in a cohort of naturally infected animals with non-T-cell diseases

The unique FeLV variant designated FeLV-945 was isolated from a temporal and geographic cohort of naturally infected cats from essentially a single veterinary practice in Pasadena, California over a period of six years (the gift of Dr. Murray Gardner). The cohort included twelve cases of multicentric lymphoma, four cases of thymic lymphoma, one case of mast cell leukemia, and two cases each of myeloproliferative disorder and anemia. Neither fresh nor fixed tissues from the cohort were available for analysis; thus, the cell type of origin of the multicentric lymphomas could not be determined with certainty. Southern blot analysis of the T-cell receptor beta locus in genomic DNA isolated from the tumors demonstrated a non-T-cell origin. FeLV-945, the predominant isolate identified in non-T-cell diseases in the cohort, was shown to differ in sequence from all previously known FeLV isolates in the LTR and the SU gene (Chandhasin et al., 2005a; Chandhasin et al., 2005b; Chandhasin et al., 2004; Levesque et al., 1990).

The FeLV-945 LTR contains in the U3 region a unique sequence motif, specifically, a tandem triplication of a 21-base pair (bp) sequence positioned 25-bp downstream from the canonical transcriptional enhancer (Chandhasin et al., 2004; Prabhu et al., 1999). Functional studies showed that the 21-bp triplication contributes transcriptional enhancer function to the LTR, that it functions preferentially in a cell type-specific manner and that it confers a replicative advantage to the virus in those cells (Athas et al., 1995b; Chandhasin et al., 2004; Prabhu et al., 1999). The mechanism by which the 21-bp triplication acts is dependent on two binding sites for the transcription factor, c-Myb, which cross the repeat junctions formed by the triplication itself; thus, the binding sites would not occur were the tandem triplication not precisely conserved. The c-Myb protein was shown to bind to the triplication, to recruit the transcriptional co-activator, CBP, and thereby to positively

regulate FeLV-945 replication *in vitro* (Finstad et al., 2004). LTRs were isolated from diseased tissues in the cohort that contained two, three or four tandemly repeated copies of the 21-bp sequence, but triplication of the 21-bp repeat was found to confer the optimal replicative advantage to the virus in feline cells (Chandhasin et al., 2004). These findings are consistent with the predominance of the triplicated form of the repeat as represented in FeLV-945, and suggest that predominance in the cohort may reflect replicative fitness mediated by a requirement for c-Myb binding.

Sequence analysis of FeLV-945 SU demonstrated unique sequence elements, particularly in the amino-terminal domain demonstrated in naturally occurring and recombinant FeLVs to be responsible for receptor selection and binding (Chandhasin et al., 2005a; Chandhasin et al., 2005b). As is typical of gammaretroviruses, the amino-terminal region of the FeLV SU protein comprises a receptor-binding domain (RBD) followed by a proline-rich region (PRR) that mediates conformational changes required for entry. Two variable regions within RBD, designated VRA and VRB, define the specificity for receptor binding (Battini et al., 1995; Boomer et al., 1997; Rigby et al., 1992; Sugai et al., 2001; Taylor and Kabat, 1997). Analysis of FeLV-945 SU demonstrated its relatedness to exogenous FeLV-A, but showed that it differs in amino acid sequence from an FeLV-A prototype to a greater extent than the known FeLV-A isolates differ among themselves (Athas et al., 1995a; Prabhu et al., 1999), and that the sequence differences are concentrated in the functional domains VRA, VRB, and PRR (Boomer et al., 1997; Sugai et al., 2001; Taylor and Kabat, 1997). Specifically, the FeLV-945 SU protein was shown to be 89%, 73%, and 85% identical in predicted amino acid sequence to prototype FeLV-A/61E SU across VRA, VRB and PRR, respectively. In contrast, the segments of FeLV-945 SU between variable regions were 94% identical to FeLV-A/61E, and the 197 residues of the predicted TM product were 97% identical to FeLV-A/61E (Athas et al., 1995a; Prabhu et al., 1999). By comparison, the known natural isolates of FeLV subgroup A are nearly identical (>97%) in amino acid sequence across the entire SU protein, although they were isolated from distant geographic locations across the world over a period of many years (Donahue et al., 1988; Neil et al., 1991; Overbaugh and Bangham, 2001). Multiple proviruses isolated from cases of multicentric lymphoma and myeloproliferative disease in the cohort were observed to share most of the unique sequence features of FeLV-945 SU (Chandhasin et al., 2005a). Thus, FeLV-945 SU, while closely related to FeLV-A, was shown to contain sequence differences localized to functional domains identified as determinants of receptor interactions and entry. Despite these sequence differences, FeLV-945 was shown by measurements of host range and superinfection interference to utilize the FeLV-A receptor, and thus to represent a member of the FeLV-A subgroup (Chandhasin et al., 2005a). This observation was noteworthy in that FeLV-945 occurred in malignant and proliferative diseases of non-T-cell origin, specifically multicentric lymphoma and myeloproliferative disease, while natural and experimental FeLV-A infection is typically associated with thymic lymphoma of T-cell origin (Athas et al., 1995a; Levesque et al., 1990; Neil et al., 1991; Rezanka et al., 1992).

Functional significance of the unique SU protein encoded by FeLV-945

The possible impact of the mutational changes in FeLV-945 SU on its biologic and pathogenic properties was examined through the use of a set of recombinant virus constructs in which the unique sequence elements of FeLV-945 were substituted into FeLV-A/61E, a prototype member of FeLV-A. Specifically, two recombinant viruses were constructed: **61E/945L**, in which the LTR of FeLV-945 was substituted into FeLV-A/61E, and **61E/945SL**, in which both the SU gene and LTR of FeLV-945 were substituted into FeLV-A/61E (Chandhasin et al., 2005a; Chandhasin et al., 2005b). Experiments were first performed to determine whether the mutational changes in FeLV-945 SU influenced the rate of virus replication *in vitro*, thereby contributing to predominance of the isolate in the cohort from

which it was identified. Plasmid DNAs encoding infectious viruses FeLV-A/61E, 61E/945SL or 61E/945L were introduced into FEA cells (feline embryonic fibroblasts), FC6.BM (adherent feline bone marrow-derived cells) and 3201 (feline T- lymphoid cells), and virus replication was quantified at regular intervals thereafter by measuring reverse transcriptase activity in culture supernatants. The results demonstrated significantly more efficient replication of the 61E/945L recombinant as compared to FeLV-A/61E in all cell lines, thus recapitulating the previous finding that the triplication-containing FeLV-945 LTR confers a replicative advantage. The additional substitution of FeLV-945 SU as represented in 61E/945SL conferred a small but statistically significant replicative advantage in FEA cells (Chandhasin et al., 2005a).

To examine the impact of the unique sequence elements of FeLV-945 on disease outcome, neonatal cats were inoculated intraperitoneally with one of three viruses: (1) prototype FeLV-A/61E, infection with which induces T-cell lymphoma of the thymus after long latency (Chandhasin et al., 2005b; Rezanka et al., 1992), (2) 61E/945L or (3) 61E/945SL. The results implicated both the FeLV-945 LTR and SU as determinants of disease outcome. First, the triplication-containing LTR of FeLV-945 was found to direct a significantly more rapid progression to disease. Kaplan-Meier survival estimate and analysis of the data by the log-rank test showed that virus containing the FeLV-945 LTR (i.e., either 61E/945L or 61E/945SL) caused disease significantly more rapidly than did FeLV-A/61E (avg. 47 weeks vs. avg. 78 weeks). Substitution of the FeLV-945 LTR into FeLV-A/61E did not alter the tumorigenic spectrum, however, as both FeLV-A/61E and 61E/945L induced thymic tumors of T-cell origin (Chandhasin et al., 2005b). In contrast, substitution of both the FeLV-945 LTR and SU gene redirected the disease outcome entirely, in that FeLV-A/61E induced a thymic lymphoma of T-cell origin but 61E/945SL induced a multicentric lymphoma of B-cell origin. These findings identify the FeLV-945 LTR and SU gene as determinants of disease, indicating that the LTR determines the kinetics of disease induction and SU determines the tumorigenic spectrum (Chandhasin et al., 2005b).

Recombinant FeLV-B in diseased tissues in the cohort

As described above, FeLV-A is thought to represent the naturally horizontally transmissible FeLV subtype that spreads cat-to-cat in nature. During infection *in vivo*, however, viruses with a novel envelope gene arise by recombination between the exogenous FeLV-A and FeLV-related genetic elements endogenous to the domestic cat genome. Of these novel viruses, the group designated FeLV-B are of uncertain disease association but have been linked to the induction of lymphoma. FeLV-B can be identified in 30% – 50% of natural infections, always in conjunction with FeLV-A, but is overrepresented in the diseased tissues of animals with lymphoma as compared to asymptomatic, FeLV-infected cats. FeLV-B has been identified in the majority of thymic lymphomas in particular (Coelho et al., 2008; Jarrett, 1980, 1992; Neil et al., 1991; Roy-Burman, 1995; Sheets et al., 1993; Tsatsanis et al., 1994). Having identified FeLV-945 SU as a determinant of non-T-cell disease in infected cats, the question arose as to whether FeLV-945 SU acts together with recombinant FeLV-B in pathogenesis. Thus, the frequency of occurrence and nature of FeLV-B was examined in diseased tissues from the cohort in which FeLV-945 was originally identified, and from twenty experimentally infected animals from previous studies. Southern blot and PCR amplification demonstrated FeLV-B in the genomic DNA of 13 of 17 natural or experimental cases of thymic lymphoma. In contrast, FeLV-B was detectable by Southern blot in only 3 of 15 natural or experimental cases of multicentric lymphoma, and was detectable only by PCR amplification in 3 additional cases. As measured by Fisher Exact Test, the incidence of FeLV-B at levels high enough to be detected by Southern blot was significantly greater in thymic lymphoma than in multicentric lymphoma ($p = 0.015$) (Ahmad and Levy, 2010). These results confirmed previous demonstrations of the frequent

association of FeLV-B recombinants with thymic lymphoma (Jarrett, 1980, 1992; Roy-Burman, 1995; Sheets et al., 1993; Tsatsanis et al., 1994), but demonstrate occurrence of FeLV-B in multicentric lymphoma at low frequency and low levels.

Further analysis was performed to establish the recombination junctions and infectivity of FeLV-B from proviruses in genomic DNA of multicentric and thymic lymphomas. The results of recombination site analysis demonstrated that FeLV-B proviruses in multicentric lymphomas were generated using a recombination junction near the 3' end of the envelope gene, and contain more endogenous FeLV sequence than do recombinants in thymic lymphomas. As endogenous FeLV sequences are defective and do not encode infectious particles (McDougall et al., 1994), these observations suggested that the inclusion of increasing amounts of endogenous FeLV-derived sequence in the recombinants in multicentric lymphomas may have reduced their infectivity. To test this possibility, genomic DNA of thymic and multicentric lymphomas was introduced into canine D-17 cells in culture, and the culture supernatant was examined at regular intervals thereafter for evidence of productive FeLV-B infection. The results demonstrated that FeLV-B proviruses in the genomic DNA of thymic lymphomas were generally infectious, but those in the DNA of multicentric lymphomas were not. Taken together, the infrequent and low level detection of FeLV-B recombinants, and their lack of infectivity in multicentric lymphomas, do not support a significant role for FeLV-B in disease induction (Ahmad and Levy, 2010).

Distinctive receptor-binding properties of FeLV-945 SU

Despite the ecotropic host range, receptor utilization and common assignment to FeLV subgroup A, the mutational changes in FeLV-945 SU have been shown to confer at least two distinctive biologic and pathogenic properties. First, FeLV-945 SU was shown to confer a small but statistically significant replicative advantage over FeLV-A/61E in some feline cells (Chandhasin et al., 2005a). Second, FeLV-945 SU was shown to act as a determinant of pathogenesis *in vivo* by directing a shift in disease outcome from thymic to multicentric lymphoma when substituted into FeLV-A/61E (Chandhasin et al., 2005b). Recent preliminary studies indicate a third functional difference conferred by the unique sequence of FeLV-945 SU in its interaction with receptor. Specifically, virus binding assays were performed using infectious FeLV-A/61E or 61E/945SL, or using pseudotypes bearing the SU proteins of those viruses, in which virus particles were incubated with feline 3201 T-lymphoid cells. Cell-bound virus was then detected by incubation with an anti-SU monoclonal antibody and analyzed by flow cytometry. The results indicated that particles bearing FeLV-945 SU bind to feline cells more efficiently than do particles bearing FeLV-A/61E SU. Soluble SU proteins expressed from FeLV-945 SU similarly bound to feline cells, or to murine cells expressing the FeLV-A receptor, with greater efficiency than did soluble SU expressed from FeLV-A/61E. Increased efficiency of receptor binding by FeLV-945 may influence pathogenesis by increasing the kinetics of virus spread *in vivo*, and/or by allowing the virus to access and enter a novel target cell with a lower receptor density. Preliminary studies have been conducted to map the domain of FeLV-945 SU responsible for increased receptor binding by using mutagenesis and reciprocal exchange substitutions between regions containing the VRA, VRB and PRR domains of FeLV-945 and FeLV-A/61E. Binding assays performed with the resulting SU proteins implicate a region containing the VRB domain as largely responsible for the increased receptor binding of FeLV-945 SU, although a region of the protein containing VRA appears to make a contribution as well (L. Bolin, C. Chandhasin, P. A. Lobelle-Rich, L. M. Albritton, and L. S. Levy, preliminary observations). Taken together, these studies implicate increased efficiency of receptor binding in the distinctive pathogenesis associated with FeLV-945 infection.

Summary and Conclusions

FeLV is a simple gammaretrovirus, but is associated with the induction of a complex spectrum of diseases. Genetic variation induced by the error-prone reverse transcriptase and/or by recombination with endogenous FeLV-related elements, leads to a natural population of virus that is genetically complex and from which variants are continuously selected. FeLV-945 is the predominant isolate in a cohort of naturally infected animals with an unusual disease spectrum in which non-T-cell multicentric lymphoma predominates. The SU protein encoded by FeLV-945 bears a unique amino acid signature in the functional domains of the protein that is apparently responsible for its unusual properties, including the ability to direct the induction of multicentric lymphoma upon experimental infection. While the mechanism by which FeLV-945 SU acts in disease induction is not known, it has been shown to mediate unusual biological properties including a replicative advantage over prototype FeLV-A and a more efficient binding to host cell receptor. The enhanced SU binding to receptor may influence the outcome of infection by at least two mechanisms: (1) by increasing kinetics of virus spread *in vivo*, and/or (2) by permitting efficient entry into a novel population of target cells, perhaps one with low receptor density. Future studies are designed to define the residues within FeLV-945 SU responsible for its distinctive properties and to explore the mechanisms of their influence on disease outcome *in vivo*.

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