Journal of General Virology (2010), **91**, 242–249 **DOI** 10.1099/vir.0.015404-0

Neutralization of feline immunodeficiency virus by antibodies targeting the V5 loop of Env

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Neutralizing antibodies (NAbs) play a vital role in vaccine-induced protection against infection with feline immunodeficiency virus (FIV). However, little is known about the appropriate presentation of neutralization epitopes in order to induce NAbs effectively; the majority of the antibodies that are induced are directed against non-neutralizing epitopes. Here, we demonstrate that a subtype B strain of FIV, designated NG4, escapes autologous NAbs, but may be rendered neutralization-sensitive following the insertion of two amino acids, KT, at positions 556–557 in the fifth hypervariable (V5) loop of the envelope glycoprotein. Consistent with the contribution of this motif to virus neutralization, an additional three subtype B strains retaining both residues at the same position were also neutralized by the NG4 serum, and serum from an unrelated cat (TOT1) targeted the same sequence in V5. Moreover, when the V5 loop of subtype B isolate KNG2, an isolate that was moderately resistant to neutralization by NG4 serum, was mutated to incorporate the KT motif, the virus was rendered sensitive to neutralization. These data suggest that, even in a polyclonal serum derived from FIV-infected cats following natural infection, the primary determinant of virus-neutralizing activity may be represented by a single, dominant epitope in V5.

Received 27 July 2009
Accepted 17 September 2009

INTRODUCTION

Feline immunodeficiency virus (FIV) was first isolated in 1986 in Petaluma, CA, USA, from a cat with an immunodeficiency-like syndrome (Pedersen *et al.*, 1987) and is an important pathogen of domestic cats worldwide. FIV is the feline homologue of human immunodeficiency virus (HIV) and these viruses share many biological and pathogenic features (Bendinelli *et al.*, 1995; English *et al.*, 1993; Johnson *et al.*, 1994), typified by a selective targeting of $CD4^+$ T lymphocytes and the induction of a progressive immunosuppression (Ackley *et al.*, 1990).

The induction of an effective humoral response against lentivirus infection is a key element in the immunological control of the disease, and the primary target for neutralizing antibodies (NAbs) is the virus envelope glycoprotein (Env) (Binley *et al.*, 2004; Frost *et al.*, 2005; Wei *et al.*, 2003). Env varies by up to 30 % amongst FIV subtypes (Hosie & Beatty, 2007) and, thus, the preparation of an immunogen capable of inducing broadly neutralizing antibody responses against such diverse isolates of FIV would be of great value to the development of an FIV vaccine. Further, the development of a vaccine against FIV would have implications extending beyond veterinary medicine; FIV is the only non-primate lentivirus that induces AIDS-like symptoms in its natural host and, as such, is a valuable animal model for both prophylactic and therapeutic studies for HIV (Bendinelli *et al.*, 1995; Elder *et al.*, 1998; Okada *et al.*, 1994). Moreover, cats have the advantage of being easier to breed and have shorter life cycles than other animal models currently used for HIV research (Miller *et al.*, 2000).

Previous studies showed that vaccination with whole inactivated vaccines or DNA vaccines can protect cats against challenge with either low-virulence/homologous strains of FIV, but not against virulent/heterologous primary strains (reviewed by Hosie & Beatty, 2007). A vaccine capable of protecting cats against infection with a wide range of virulent primary isolates remains a challenge, primarily because of the limited number of conserved neutralization epitopes that have been identified and the failure to present such epitopes appropriately in vaccines. Steric factors may render neutralization epitopes inaccessible to NAbs (Chiarantini *et al.*, 1998; Labrijn *et al.*, 2003), whilst most of the antibodies generated by vaccination may

The GenBank/EMBL/DDBJ accession numbers for the novel FIV *env* sequences generated in this study are GU066862–GU066866.

Supplementary tables showing oligonucleotides used for site-directed mutagenesis of V5 and GenBank numbers for new and reference sequences are available with the online version of this paper.

be directed against non-neutralizing epitopes (Dhillon *et al.*, 2007; Richman *et al.*, 2003). Accordingly, the identification of conserved determinants for neutralization will be an important advance in the design of the next generation of FIV vaccines.

Understanding the biological basis for the induction of antibodies with broad neutralizing activity is pivotal to the development of efficacious lentivirus vaccines and to the generation of vaccines that will protect against a broad range of primary isolates. Encouraging results have indicated that it is possible to protect cats against challenge with primary FIV isolates, depending on their regional distribution. By vaccinating cats with a whole inactivated vaccine based on the subtype B Pisa M2 strain, cats were protected from natural challenge with subtype B isolates of FIV (Pistello *et al.*, 1997). It has been suggested that subtype B isolates may be more ancient and, accordingly, more host-adapted (Bachmann *et al.*, 1997), hence they may be more readily neutralizable by the host humoral response. In contrast, whilst subtype A viruses display similar levels of non-synonymous mutations, such viruses display half as many synonymous mutations, suggesting a more recent spread and a lower level of host–virus adaptation (Bachmann *et al.*, 1997). As the major FIV subtypes are restricted in their global distribution, it has been proposed that it may be necessary to design several regional vaccines rather than a single, worldwide-protective vaccine (Steinrigl & Klein, 2003).

In this study, we investigate the initial observation that a subtype B strain resisted neutralization by its homologous serum. By applying targeted mutagenesis across the *env* gene, we identified the V5 loop as the primary determinant of escape from neutralization and localized the primary determinant to a lysine–threonine (KT) motif. Additional subtype B strains bearing the same motif were also sensitive to neutralization. Furthermore, incorporation of these residues into a neutralization-resistant subtype B strain rendered the virus more sensitive to neutralization, confirming the monospecificity of the neutralizing serum. These data indicate that the functional neutralizing response in natural infection with FIV may be highly specific and that immune evasion *in vivo* may stem from the mutation of as few as two amino acids in the V5 loop.

RESULTS

Phylogenetic analysis

An unrooted neighbour-joining phylogenetic tree was generated based on 651 nt spanning the *env* V3–V5 sequence from the five isolates included in the study, as well as reference sequences from subtypes A, B, C, D and E. The phylogenetic tree (Fig. 1) demonstrates that the five Japanese isolates were genetically related and clustered within subtype B with bootstrapping support of 100 %. KNG1 and TOT1 were highly similar at the nucleotide level and indeed had identical V3–V5 amino acid sequences and clustered together on a single tree branch (Fig. 1).

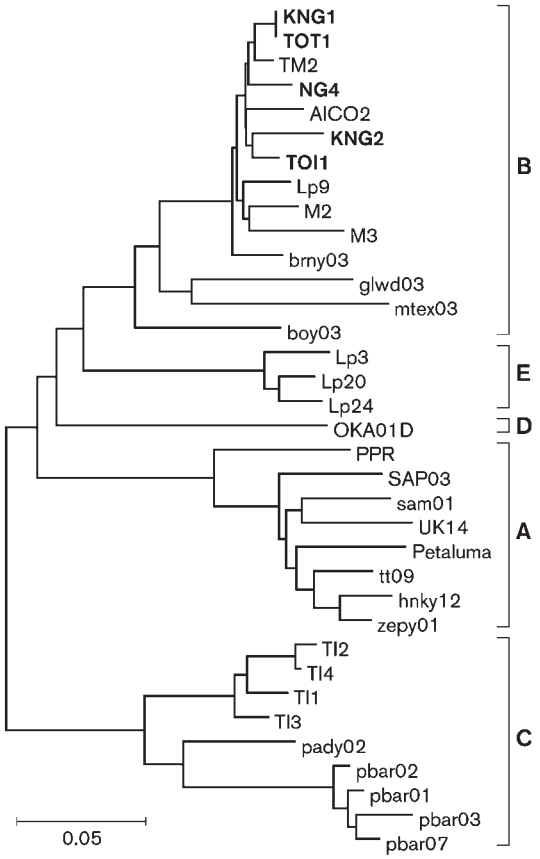

Fig. 1. Midpoint-rooted neighbour-joining tree of the FIV V3–V5 *env* gene sequence. The tree shows the five Japanese sequences included in the study (in bold type), as well as reference sequences from subtypes A, B, C, D and E.

Cats show different NAb responses to homologous and heterologous strains

Sera from cats KNG1 and TOI1 showed no evidence of neutralizing activity against any of the pseudotypes tested, including those bearing their homologous Env proteins, and serum from cat KNG2 showed no neutralizing activity against the KNG1, NG4 or TOT1 pseudotypes, moderate neutralizing activity against the KNG2 pseudotype and weak neutralizing activity against the TOI1 pseudotype (Table 1). In contrast, sera from cats NG4 and TOT1 showed strong neutralizing activity against pseudotypes expressing Env proteins of the heterologous KNG1, TOT1 and TOI1 strains, and moderate responses against pseudotypes expressing the Env of strain KNG2. The pseudotype expressing the NG4 Env resisted neutralization by four heterologous sera and showed only weak neutralizing activity against its homologous serum (Table 1). Although KNG1 and TOT1 shared similar *env*

Table 1. Percentage neutralization values (at a 1 : 10 serum dilution) for five HIV(FIV) pseudotyped viruses tested against homologous and heterologous sera for neutralization

Neutralizing activity is categorized as weak (40–59 %), moderate (60–79 %) or strong (80–100 %). Negative values indicate *in vitro* enhancement of infection and are not significant.

Env	Serum				
	KNG1	KNG2	NG4	TOT1	TOI1
KNG1	−7	−12	86	99	−78
KNG2	67	71	73	64	61
NG4	4	25	46	−31	−15
TOT1	−31	−48	80	99	−57
TOI1	15	41	98	96	−8

sequences (97 % similarity), they did not induce similar humoral immune responses in their respective hosts.

Escape from homologous neutralization by NG4

NG4 was neutralized only weakly or resisted neutralization by the two cross-neutralizing sera NG4 and TOT1, respectively. The finding that NG4 was neutralized only weakly by its homologous serum, a serum that cross-neutralized the other four viruses, may indicate the evolution *in vivo* of a neutralization-escape mutant that, at the time of sampling, was the dominant species in the periphery, as five independent Env clones for each virus were sequenced, all of which gave rise to identical V5 amino acid sequences (data not shown). However, as blood samples from cat NG4 at earlier time points in infection were not available, it was not possible to identify earlier strains that were most likely to have contributed to the generation of the NAbs. However, given the similarity between the five subtype B isolates, sites of amino acid divergence from the consensus sequence that were identified in NG4 would be likely to contribute to the relative resistance of this strain to neutralization by its homologous serum. Accordingly, these non-synonymous residues were targeted by site-directed mutagenesis in an attempt to render NG4 more sensitive to neutralization by its homologous serum. Initially, Env was mutated at the V3 loop, a known target for NAbs (Lombardi *et al.*, 1993; Osborne *et al.*, 1994). A glutamine residue (377) was substituted with a histidine (Q377H), resulting in a net increase in the charge of the V3 loop. However, the Q377H mutation did not render NG4 more sensitive to neutralization (Fig. 2). Next, the NG4 Env was mutated at the crown of the predicted V5 loop (Fig. 3), targeting serine residue 550 (S550N), creating a potential site for *N*-linked glycosylation. When tested against its homologous serum, the S550N mutant did not show enhanced sensitivity to neutralization (Fig. 2; wild type, 46 % neutralization at a 1 : 10 dilution; S550N, 38 % at a 1 : 10 dilution), indicating that the absence of asparagine 550 alone and ablation of the predicted site for *N*-linked glycosylation did not contribute to the escape from neutralization by homologous serum. The NG4 Env was then mutated within the V5 loop by the insertion of two amino acids, lysine and threonine, at positions 556 and 557 [S556(KT)557K]. Interestingly, this mutation rendered NG4 sensitive to neutralization, with strong neutralizing activity (98 %) observed at a 1 : 10 dilution (Fig. 2a, b). Moreover, the S556(KT)557K insertion also rendered NG4 sensitive to the broadly neutralizing TOT1 serum (Fig. 2c, d), indicating that, despite the independent origins of the two viruses, both cats responded to infection by targeting V5. Next, we asked whether the increase in polarity afforded by the inserted lysine residue contributed to the formation of the neutralizing determinant. An S556(K)557K mutant was prepared in which only a lysine residue was inserted. The NG4 S556(K)557K mutant was tested against NG4 serum and showed an intermediate pattern of neutralization (Fig. 2), indicating that, whilst the lysine residue was clearly important, the additional threonine residue enhanced the formation of the neutralizing determinant. These data emphasize the dominance of V5 in the neutralization of FIV and are consistent with a hypothesis whereby the broadly neutralizing NG4 serum selected for mutant viruses bearing a deletion in V5 in the host animal.

Neutralization sensitivity of KNG2

Of the five isolates studied, KNG2 was the most divergent at V5. In comparison with the other strains, KNG2 was moderately sensitive to neutralization by all sera tested, including the NG4 and TOT1 sera that showed strong neutralizing activity against KNG1, TOT1 and TOI1. Based on our finding that NG4 could be rendered sensitive to neutralization by a single change in the amino acid sequence of the V5 loop, we asked whether the partial resistance of KNG2 was conferred by its divergent V5 loop. Firstly, a KNG2 mutant was prepared bearing a DN556KT mutation, targeting the same residues shown earlier to confer neutralization sensitivity upon NG4. The DN556KT mutation did not display improved sensitivity to neutralization relative to the wild type. As the sequence of KNG2 diverged from the consensus at 7 aa, we considered that the context of the mutation may have been important for formation of the epitope. Accordingly, the V5 loop was mutated in stages towards identity with the (highly sensitive) KNG1 and TOT1 sequences; initially a 5 aa change was created (553NGNDN558→553DNSDT558), followed by the full mutation (551STNGNDNKMT558→551GTDNSKTKMA558). Both KNG2 mutants were then tested for sensitivity to neutralization by the TOT1 serum. The 551STNGNDNKMT558→551GTDNSKTKMA558 variant was neutralized efficiently by the TOT1 serum, whilst the 553NGNDN558→553DNSDT558 mutant showed an intermediate sensitivity (Fig. 4). The conversion of KNG2 from neutralization-resistant to neutralization-sensitive by changing the sequence of V5 to a sequence similar to those of the neutralization-sensitive KNG1 and TOT1 strains is

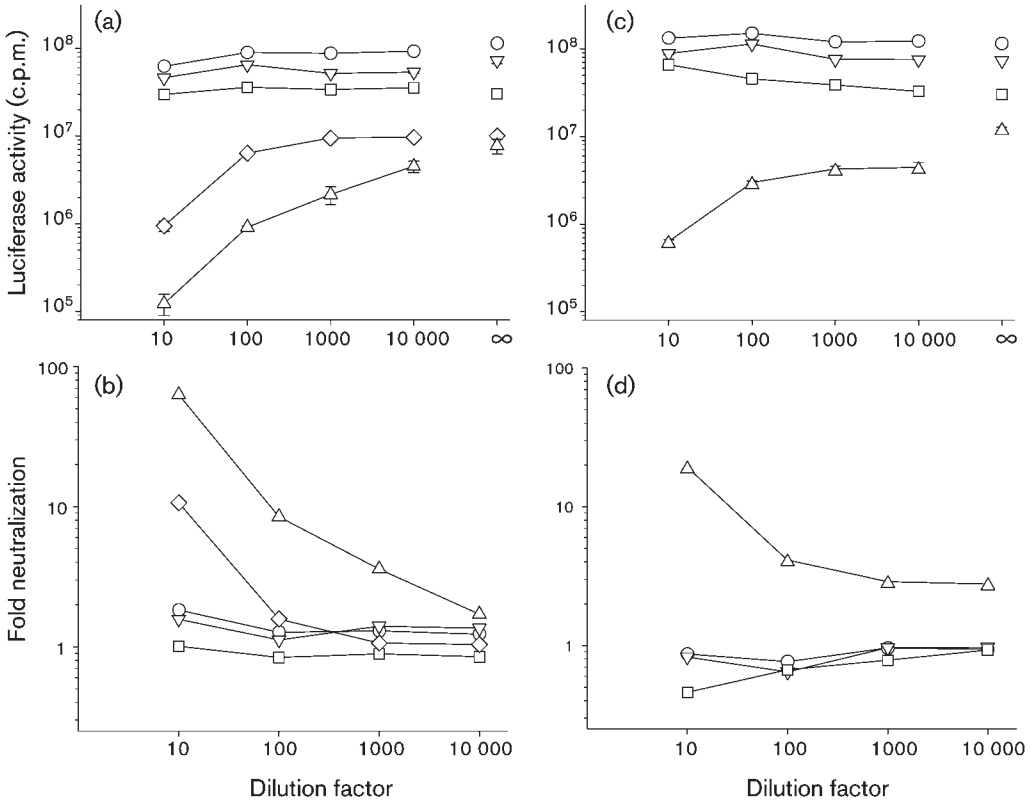

Fig. 2. Neutralization of wild-type NG4 (○) and NG4 mutants [▽, NG4 S550N; □, NG4 Q377H; ◇, NG4 S556(K)557K; △, NG4 S556(KT)557K] by homologous serum (a, b) and by TOT1 serum (c, d). (a, c) Luciferase activity (c.p.m.) with successive dilutions of NG4 serum (dilution factors 1 : 10, 1 : 100, 1 : 1000 and 1 : 10 000; no serum, ∞). (b, d) Fold neutralization relative to no-serum control. Each point represents the mean ± SEM (n=3).

consistent with V5 being a primary determinant of neutralization sensitivity and the specificity of the neutralizing sera.

The results of this study indicate the importance of V5 to antibody-mediated neutralization of FIV. Whilst anti-V5 antibodies can neutralize primary isolates of FIV very efficiently, the virus appears able to escape neutralization readily by mutating V5. What, then, are the prospects for designing an FIV vaccine that induces broadly neutralizing antibodies targeting V5? A comparison of published V5 sequences (Fig. 5) would suggest that the V5 region has highly conserved structural features, such as the cysteines at positions 533, 546, 548 and 561 (numbering as in KNG1 and 2, TOI1 and TOT1; Fig. 5). However, the region between cysteine residues 548 and 561 appears to be remarkably flexible in its ability to tolerate amino acid substitutions, glycosylation (potentially *O*- and *N*-linked) and length polymorphisms, suggesting that it is under pressure to vary from the host immune response. The relative conservation of the region between cysteines 533 and 548 may indicate that this region is either inaccessible or structurally constrained, perhaps through interactions with adjacent regions of Env. If possible, targeting the humoral response to this region may induce more broadly neutralizing antibodies.

DISCUSSION

In this study, we demonstrate that the V5 loop of FIV Env is a primary target for virus-neutralizing antibodies. Although previous studies *in vitro* indicated that the V3 loop was a major determinant for virus neutralization, subsequent studies revealed that the significance of antibodies targeting this region to the neutralizing response had been overemphasized by the cellular substrate used to quantify neutralization. Formerly, assays were based on inhibiting infection of Crandell feline kidney (CrFK) cells with 'CrFK-adapted' strains of virus. Prior to measuring neutralization in this system, the challenge virus had to be selected for growth in CrFK cells, thereby selecting for viruses that were capable of CD134-independent infection through a direct interaction with CXCR4 (analogous to CD4-independent infection with HIV). The V3 loop plays a critical role in the FIV Env–CXCR4 interaction; accordingly, CrFK-based assays exaggerated the importance of NAbs binding this region. Subsequent studies

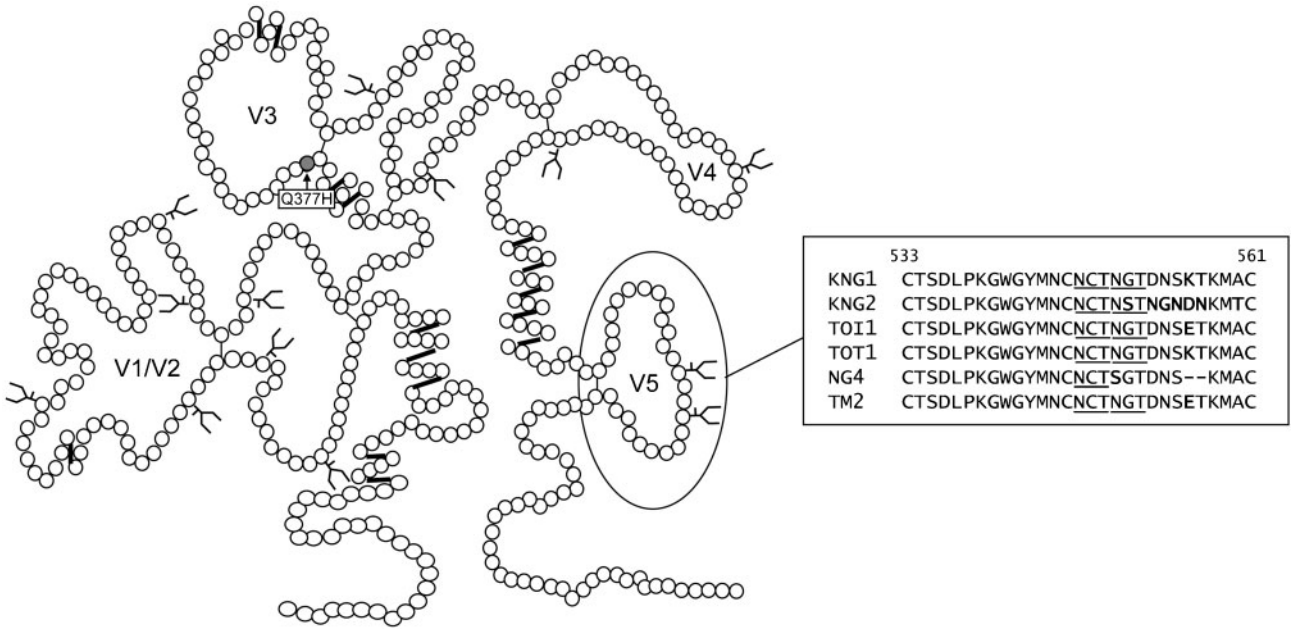

Fig. 3. Schematic structural model of the FIV SU protein, illustrating the locations of the Q377H mutation in V3 and of the V5 loop. The inset displays a comparison of the sequences from the five study strains, KNG1, KNG2, TOI1, TOT1 and NG4, and the reference strain TM2 (GenBank accession no. M59418).

revealed that, when primary (non-CrFK-adapted) isolates were assayed on interleukin-2-dependent T cells, the correlation between the efficiency of virus neutralization and the response to V3 was diminished. Here, we extend previous studies that indicated that, in biologically relevant *in vitro* systems, the variable loops V4 and V5 appear to be the major targets for effective NAbs. Moreover, we found that, in polyclonal sera from infected cats, deletion of as few as two amino acids from V5 converts a virus from a strongly neutralized to a weakly neutralized or neutralization-resistant phenotype. Each of the isolates described in the current study was confirmed as being CD134-dependent by plating the virus pseudotypes on target cells expressing CD134. No evidence of CD134-independent infection via CXCR4 alone was detected (data not shown), confirming that the isolates were primary and not cell-culture-adapted.

Amongst the variable regions in FIV Env, V5 appears to be highly polymorphic between strains, with variations in amino acid sequence, glycosylation and extended stretches of serine and threonine residues being common. These data suggest that there is a selective pressure within the host for variation in V5, consistent with escape from an effective immune response. The fact that minimal variation in V5 is sufficient to either reduce substantially or ablate completely the neutralizing capacity of the host humoral response is of concern for the design of effective FIV vaccines. How, then, are we to elicit broadly neutralizing antibodies *in vivo*? Clearly, the V4 and V5 loops are exposed and immunogenic, and antibodies targeting these regions neutralize virus. However, it would appear that these regions may represent little more than a decoy for the virus, presenting the immune system with an easy target for NAbs. By the time functional NAbs develop, virus variants that can escape the monospecific serum may have already evolved *in vivo*. The solution to this dilemma would appear to lie in the identification of framework determinants in Env that induce antibodies that are broadly active, refocusing the immune response either by silencing the immunodominant (but ultimately ineffectual) determinants in V4 and V5, or by presenting them in a such a way as to induce antibodies that recognize structures or motifs that are difficult or impossible for the virus to vary.

Previous studies have demonstrated the importance of V5 in virus neutralization (Giannecchini *et al.*, 2001; Pistello *et al.*, 2003; Siebelink *et al.*, 1995). V5 was mapped by using synthetic peptides for a possible linear epitope based on the sequence of the 19k1-560 strain (Siebelink *et al.*, 1995), and it was demonstrated that a single mutation at position 560 may be involved concurrently with another mutation at 483 (V4 loop) in one or more conformational epitopes, but no linear epitope was mapped in V5. Further, two independent mutations have been reported that resulted in the creation of potential *N*-linked glycosylation sites in V4 (K481N) and V5 (S557N) and which contributed to the conversion from a neutralization-sensitive to a neutralization-resistant phenotype (Giannecchini *et al.*, 2001; Pistello *et al.*, 2003). In comparison, the conversion from a weakly neutralized or neutralization-resistant to a strongly neutralized phenotype described herein was mediated by

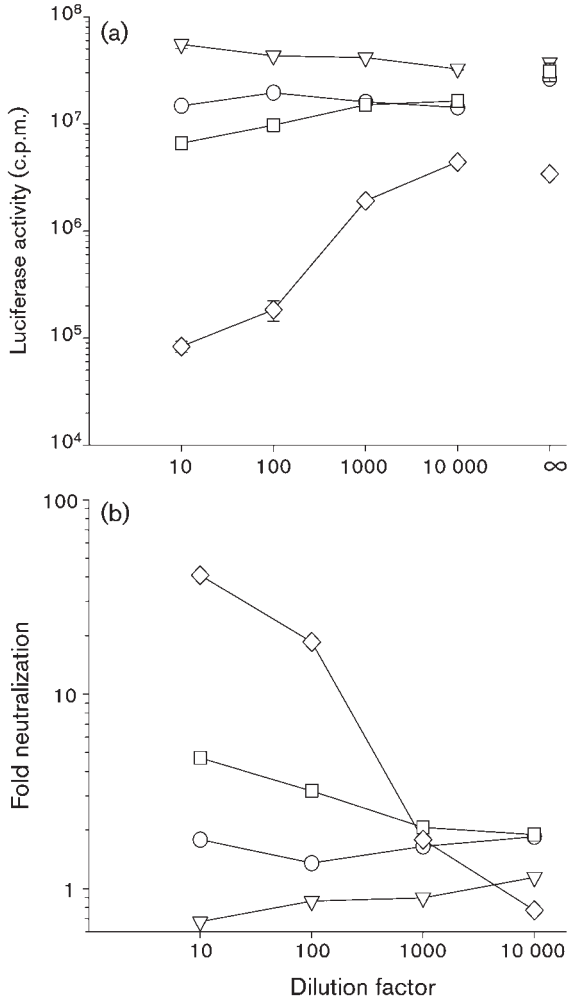

Fig. 4. Conferral of neutralization sensitivity to KNG2 by V5-loop transfer. (a) Luciferase activity (c.p.m.) with successive dilutions of TOT1 serum (dilution factors 1 : 10, 1 : 100, 1 : 1000 and 1 : 10 000; no serum, ∞). (b) Fold neutralization relative to no-serum control. Each point represents the mean ± SEM (n=3). ○, KNG2 WT; ▽, KNG2 556KT557; □, KNG2 553DNSDT558; ◇, KNG2 551GTDNSKTKMA558.

mutation of V5 alone. The V5 loop of FIV Env is highly variable among isolates. Variation occurs not only within the amino acid sequence, but also in the length of the central region (up to 14 residues) towards the end of the loop formed by disulfide-bond linkage, indicating that this particular region may play a significant role in virus evolution and escape from neutralization. It is notable that the length polymorphisms in this region are attributable to a reiteration of codons encoding serine and threonine residues. Threonine and serine have hydroxyl side chains and may, potentially, undergo *O*-linked glycosylation. It is generally believed that, due to their small molecular size, *O*-linked oligosaccharides have only minor effects on the formation of the glycan shield on HIV Env compared with *N*-linked oligosaccharides. However, these oligosaccharides usually have diverse structures with no common carbohydrate core and it is difficult to predict how efficient they may be in shielding neutralization epitopes, especially when there are several adjacent molecules (Bernstein *et al.*, 1994). Moreover, X-ray crystallography studies on HIV-1 gp120 showed that glycosylation may have significant effects on the conformational stability of Env, as well as an indirect effect on more distant sequences along the secondary structure. This, in turn, would affect the interaction between the epitope and NAbs, rendering epitopes less accessible for antibody binding (Huang *et al.*, 1997).

In conclusion, we identified a neutralization determinant in the FIV V5 loop that is associated with the conversion of strongly neutralized to weakly neutralized or neutralization-resistant strains. The determinant is apparently linear and involves at least 10 aa (GTDNSKTKMA) within the V5 region, a highly variable region amongst FIV strains. The identification of this neutralization determinant will inform vaccine design in the future in order to induce more potent NAb responses.

METHODS

Blood samples and collection of peripheral blood mononuclear cells (PBMCs) and sera. All blood samples were collected from five naturally infected cats from three different regions of Japan (isolate NG4 originated in Isikawa, KNG1 and KNG2 from Kanagawa and TOI1 and TOT1 from Tokyo). Four cats (KNG1, NG4, TOT1 and TOI1) were apparently healthy and displayed no clinical signs (analogous to the asymptomatic stage of HIV infection), whereas one cat, KNG2, presented with severe gingivostomatitis (analogous to the symptomatic stage of HIV infection). PBMCs were fractionated from 5 ml heparinized whole blood by centrifugation over Ficoll-Paque density separation medium (GE Healthcare). Aliquots of sera were stored at −80 °C prior to use in neutralization assays.

Cloning and pseudotype virus production. DNA was extracted from PBMCs by using a DNA Whole Blood extraction kit (Qiagen). The FIV *env* gene was amplified by PCR, using forward and reverse primers containing recognition sites for the restriction enzymes *Sal*I and *Not*I, and then cloned into a low-copy-number eukaryotic expression vector, VR1012 (VICAL Inc.). To prepare HIV(FIV) pseudotypes, 3.3×10^5 HEK-293T cells were transfected with 5 μg VR1012 expressing the FIV *env* clone and 5 μg pNL-Luc-E-R+ (Connor *et al.*, 1995) (an Env-deleted HIV provirus that incorporates a luciferase reporter gene) by using SuperFect reagent (Qiagen), following the manufacturer's instructions. Culture supernatants were harvested at 48–72 h post-transfection, filtered at 0.45 μm and frozen at −80 °C until required.

DNA sequencing. The FIV *env* genes were sequenced by using a BigDye Terminator v1.1 kit (Applied Biosystems). The reaction mixture consisted of 100 ng purified DNA μl^{-1}, 3.2 pmol each primer, 4 μl sequencing buffer and 2 μl sequencing enzyme in 20 μl reactions. Cycling conditions were one cycle at 96 °C for 1 min, followed by 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Sequencing was performed by using an ABI3700 automated capillary array sequencer (Applied Biosystems). Raw chromatographic data were analysed by using 'Contig Express' sequence-analysis software within the Vector NTI suite of programs (Invitrogen).

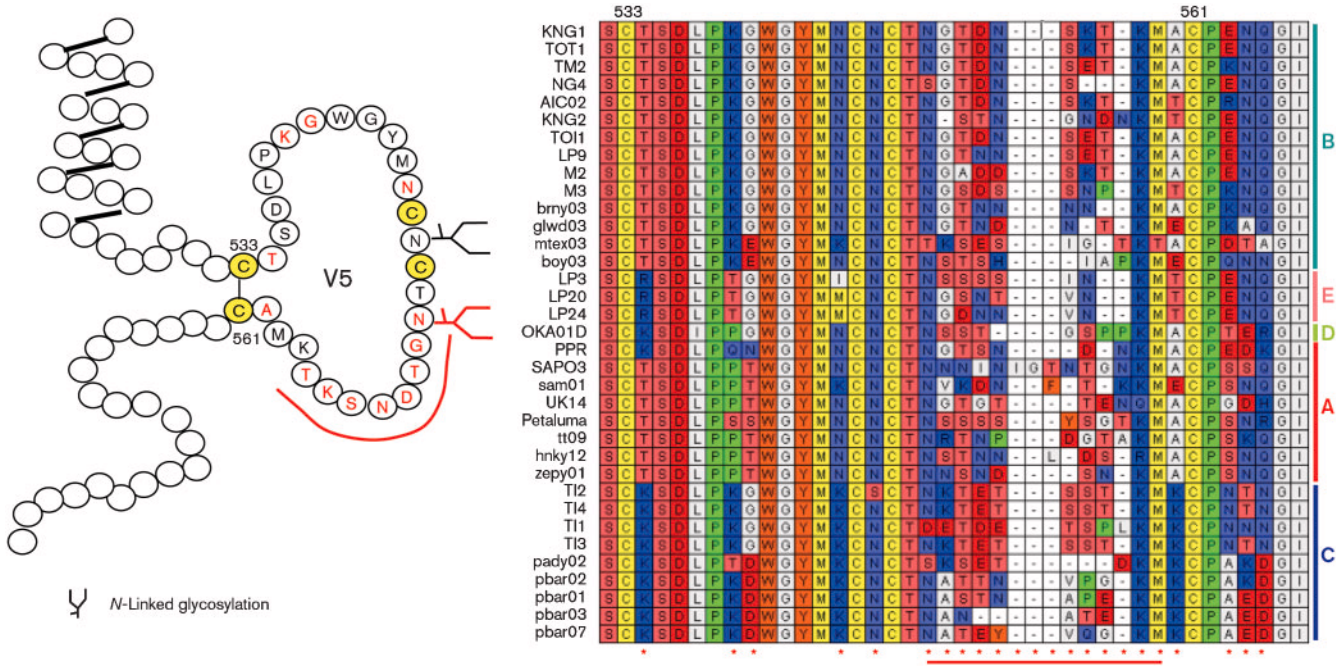

Fig. 5. Amino acid alignment of the V5 region from diverse FIVs. Within the framework of the predicted loop bounded by cysteines 533 and 561 (numbering as in KNG1 and 2, TOI1 and TOT1), extensive variation is evident, the majority of which resides between cysteines 548 and 561. Hypervariable residues are marked (red asterisks). Colours indicate side chains: red, acidic; white, hydrophobic; light blue, amido; orange, aromatic; dark blue, basic; pink, hydroxyl; green, proline; yellow, thiol.

Multiple alignments and phylogenetic analysis. Nucleotide sequence analysis was performed on a 651 nt fragment spanning the V3–V5 region of the FIV *env* gene. The generated consensus sequence comprised sequences of the five isolates included in the study, as well as reference sequences. Multiple alignments were performed by using the CLUSTAL_X (version 2.0.1) (Larkin *et al.*, 2007) and BioEdit (version 7.0.9.0) (Hall, 1999) applications, followed by manual adjustment to maximize similarities. Alignments were translated and the resulting amino acid-based alignments were used as an exact guide for repositioning of improper gapping, particularly where sequences differed in length. DNA distance matrices were calculated with DNADIST from the PHYLIP software package (version 3.68, 2008) (Felsenstein, 1989) using the F84 model and an empirical transition/transversion ratio of 2. Phylogenetic trees were constructed by the neighbour-joining method. Robustness of the tree was evaluated by bootstrap analysis on 1000 replicates to assess the support at each of the internal nodes of the neighbour-joining tree. The phylogenetic tree was committed for final editing and graphical representation using MEGA4 (Tamura *et al.*, 2007).

Virus-neutralization assay. Twenty-five microlitres of heat-inactivated serum (four tenfold serial dilutions starting at a 1 : 10 dilution) and 25 µl luciferase HIV(FIV) pseudotypes were co-incubated in triplicate wells of 96-well flat-bottom CulturPlate-96 assay plates (Perkin Elmer) for 1 h at 37 °C before 50 µl CLL-CD134 cells (5×10^5 cells ml^{-1}) (Willett *et al.*, 2006) were added. At 72 h post-infection, 100 µl Steadylite HTS (Perkin Elmer) luciferase substrate was added to each well and luciferase activity was quantified by single photon counting on a MicroBeta luminometer (Perkin Elmer). The percentage neutralization was calculated as follows: [(no-serum luciferase activity−serum luciferase activity)/no-serum luciferase activity] × 100. Empirical values of ⩾80 and 60–79 % were considered as strong and moderate neutralization, respectively.

Site-directed mutagenesis. Mutations were introduced into the FIV *env* gene by using a QuikChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. Briefly, each 50 µl reaction contained 50 ng DNA (VR1012 plasmid carrying the FIV *env* gene), 125 ng each primer, 1 × *Pfu* reaction buffer, 1 µl dNTP mix and 2.5 U *Pfu*Ultra high-fidelity DNA polymerase (included in the QuikChange kit). Cycling temperatures were as follows: initial denaturation at 95 °C for 30 s, followed by 12–18 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min and extension at 68 °C for 7 min with no final extension hold. The product was then treated with *Dpn*I endonuclease in order to digest the methylated parental DNA template. *Dpn*I-treated DNA was then transformed into XL1-Blue Supercompetent cells (included in the QuikChange kit) and the *env* gene was sequenced to confirm the presence of the desired mutation. Oligonucleotide primers for mutagenesis are detailed in Supplementary Table S1, available in JGV Online.

Nucleotide sequence accession numbers. GenBank accession numbers for control reference sequences used in the phylogenetic analysis and new sequences reported in this study are listed in Supplementary Table S2 (available in JGV Online).

ACKNOWLEDGEMENTS

We would like to thank the Kyoritsu–Seiyaku Corporation for financial support, the Faculty of Veterinary Medicine and Pfizer Corporation for scholarship funding for A. S. and the veterinary practitioners who collected samples from naturally infected cats for this study. N. L. and E. L. M. are funded by a grant awarded to B. J. W. and M. J. H. from the Wellcome Trust.

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