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Neutralization of Feline Immunodeficiency Virus by Polyclonal Feline Antibody: Simultaneous Involvement of Hypervariable Regions 4 and 5 of the Surface Glycoprotein

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Sites involved in antibody-mediated neutralization of feline immunodeficiency virus were mapped by reciprocal exchange of envelope fragments or amino acids between molecular clones of feline immunodeficiency virus with different susceptibilities to neutralization by a polyclonal cat serum. Combinations of mutations within HV-4 or within HV-4 and HV-5 changed the susceptibility of the viruses to neutralizing antibody.

Feline immunodeficiency virus (FIV) is a lentivirus that causes a severe impairment of immune function in experimentally and naturally infected cats, which eventually results in the development of a syndrome quite similar to AIDS in humans (1, 4, 8, 11, 12, 14, 18, 19, 22, 25, 28). For both human immunodeficiency virus type 1 and FIV it has been shown that single amino acid substitution mutations in the envelope glycoprotein, within or outside the presently identified VN epitopes, may confer resistance to virus-neutralizing (VN) monoclonal or polyclonal antibody (13, 16, 20, 23, 27). It has also been postulated that glycosylation may play an important role in the proper expression or masking of VN epitopes (2, 5, 6, 9, 10).

We have recently described two molecular clones of FIV (FIV19k1 and FIV19k32), simultaneously derived from a naturally infected cat (21). These clones differed in their susceptibilities to virus neutralization in feline thymocytes by serum S1422, obtained from a specific-pathogen-free cat, 22 weeks after infection with FIV19k1 (23). The surface glycoproteins (SU) of the two molecular clones were shown to differ in only five amino acids (21). We have demonstrated that a single substitution mutation at amino acid position 560 (HV-5) or at position 483 of FIV19k1 conferred resistance to virus neutralization by S1422 (20, 23). In the present study we have further evaluated the involvement of different sites of the SU protein in antibody-mediated neutralization by reciprocal exchange of FIV19k1 and FIV19k32 envelope gene fragments and by site-directed mutagenesis. This included a highly conserved potential N-linked glycosylation site at amino acid position 481 (24).

Combinations of point mutations were made in the SU protein of FIV19k32, on basis of the FIV19k1 sequence, in order to obtain a virus neutralization-sensitive mutant of FIV19k32. To this end, site-directed mutagenesis and gene fragment exchange were carried out as previously described (20, 23). Replication competence of the molecular and chimeric clones was confirmed by showing that the production of FIV antigen in independent duplicate thymocyte cultures was of the same order of magnitude as FIV antigen production of cultures infected with FIV19k1 or FIV19k32 (less than $10^{0.5}$ times difference), under previously described conditions (20, 23). Two virus neutralization-sensitive chimeric viruses, FIV19k32^{mutA} (Asp-454 to Asn and Ser-483 to Leu) and FIV19k32^{mutB} (Ser-483 to Leu and HV-5 region of 19k1), were thus constructed. The VN indices of S1422 for the respective viruses were measured as previously described (20, 23) and were 14 and 25 on day 8 and 5 and 9 on day 12 (Fig. 1A). FIV19k1 and FIV19k32 and the chimeric viruses FIV19k1^{mutA}, FIV19k1^{mutB}, FIV19k32^{mutC}, FIV19k32^{mutD}, and FIV19k32^{mutE}, which had been generated previously and contained only one or two point mutations compared with their parent viruses, were also tested in the same experiment. Essentially the same results were obtained as in previous studies (20, 23) (Fig. 1A). These data show that a point mutation reciprocal to the Leu-to-Ser mutation at amino acid position 483, which has been shown to render FIV19k1 insensitive to neutralization by S1422 (20), renders FIV19k32 sensitive to this neutralization only if at least an additional mutation at amino acid position 454 is created or additional exchange of HV-5 is achieved (Fig. 1A).

The Leu-to-Ser substitution at amino acid position 483 of FIV19k1 introduces a potential N-linked glycosylation site at position 481. To investigate the role of this potential N-linked glycosylation in the mechanism of neutralization and escape, 12 chimeric clones were constructed by site-directed mutagenesis (Fig. 1B). To test whether the Ser at amino acid position 483 itself was important, we substituted in the SU protein of FIV19k1, FIV19k32, FIV19k32^{mutA}, and FIV19k32^{mutB} the Leu by a Thr at this position. This resulted in chimeric clones FIV19k1^{mutD}, FIV19k32^{mutF}, FIV19k32^{mutA1}, and FIV19k32^{mutB1}, respectively, with a potential N-linked glycosylation site in the absence of a Ser at this position. Replication of FIV19k1^{mutD} in the presence of S1422 proved to be inhibited (Fig. 1B). Thus, independently from the potential N-linked glycosylation site, the actual amino acid present at amino acid position 483 does influence the S1422 virus neutralization phenotype of the virus. The VN phenotypes of chimeric clones FIV19k32^{mutF}, FIV19k32^{mutA1}, and FIV19k32^{mutB1} were similar to that of the parent virus FIV19k32: none of these chimeric viruses were neutralized by S1422 (Fig. 1B).

To disrupt the potential N-linked glycosylation site at position 481 in the SU protein of FIV19k1^{mutB}, FIV19k32, FIV19k32^{mutC}, and FIV19k32^{mutE}, the Asn at amino acid po-

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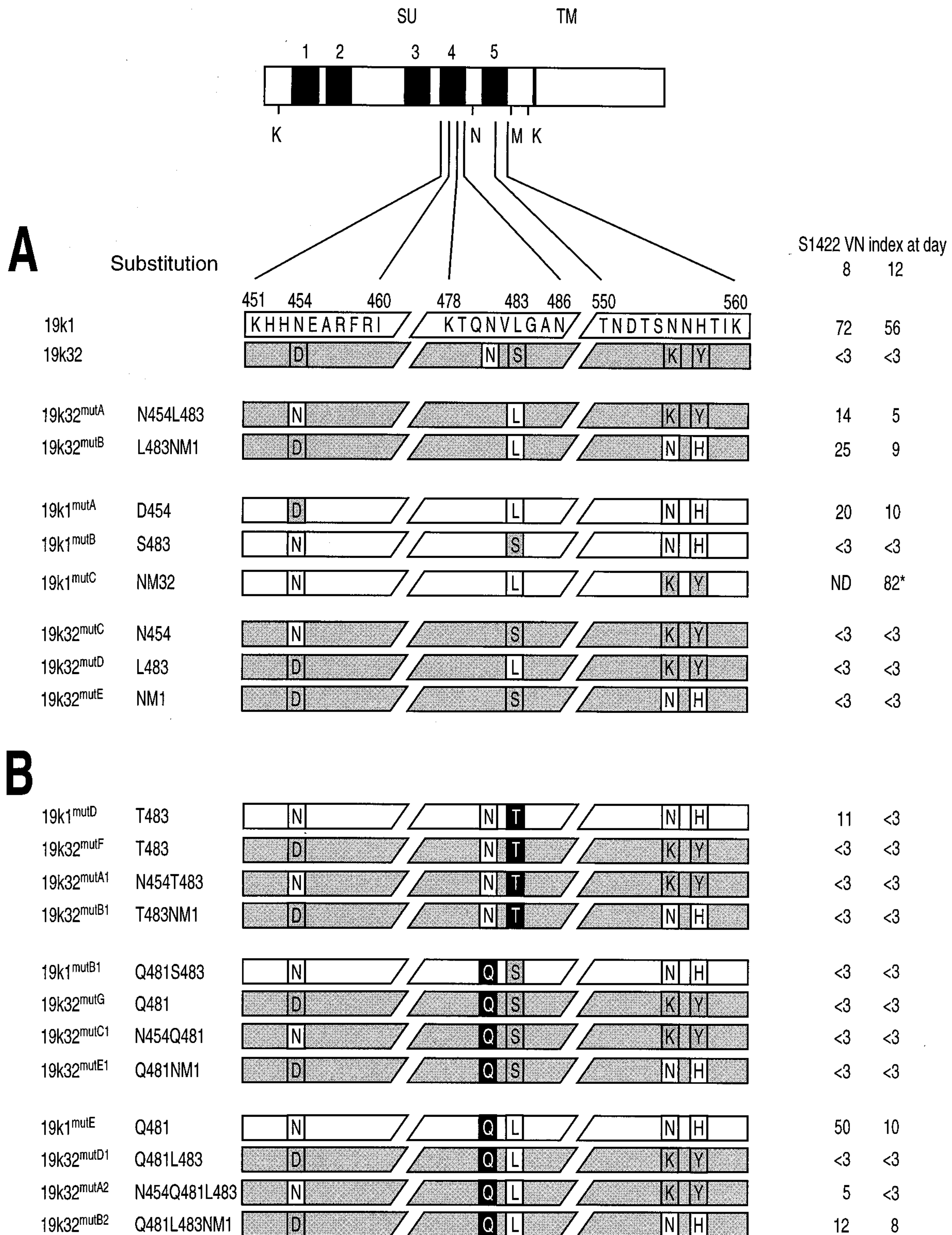

FIG. 1. Schematic representation of parts of the HV-4 and HV-5 regions of FIV19k1 and FIV19k32 and chimeric viruses constructed by exchange of single or multiple amino acids in the HV-4 and HV-5 regions of FIV19k1 and FIV19k32 (A) and by altering the potential N-linked glycosylation site at amino acid position 481 (B). S1422 VN indices measured on days 8 and 12 against the respective viruses are also shown (*, data from reference 20). The envelope glycoprotein is diagrammed at the top. The hypervariable regions are numbered 1 through 5. Restriction enzyme cleavage sites: K, *Kpn*I; M, *Mst*II; N, *Nsi*I. Open and shaded bars, sequences of FIV19k1 and FIV19k32, respectively. The amino acid sequence of clone FIV19k1 is shown. The individual mutated (and relevant nonmutated) amino acids are indicated, with shading and lack of shading indicating the virus of origin (see above). Amino acids which alter the potential N-linked glycosylation site and which are not present in FIV19k1 or FIV19k32 are highlighted in black.

sition 481 was substituted by a Gln, another uncharged polar amino acid. This resulted in chimeric viruses FIV19k1^{mutB1}, FIV19k32^{mutG}, FIV19k32^{mutC1}, and FIV19k32^{mutE1}, respectively. None of these viruses were neutralized by S1422 (Fig. 1B).

Finally, to control for a potential virus neutralization site disruption by the presence of a Gln at amino acid position 481, the Asn at amino acid position 481 of clones FIV19k1, FIV19k32^{mutD}, FIV19k32^{mutA}, and FIV19k32^{mutB} was also substituted by a Gln, resulting in chimeric clones FIV19k1^{mutE}, FIV19k32^{mutD1}, FIV19k32^{mutA2}, and FIV19k32^{mutB2}, respectively. With the exception of FIV19k32^{mutA2}, the introduction of Gln at this position did not change the S1422 virus neutralization phenotype of these viruses significantly. The replication of FIV19k32^{mutA2} proved to be slightly inhibited. The neutralization of the parent virus of FIV19k32^{mutB2} was not significantly different from neutralization of the mutated virus (Fig. 1B).

In the experiments presented in this article we have used a thymocyte virus neutralization assay, since viral progeny of molecular clones 19k1 and 19k32 replicates only in feline T cells, a primary target for FIV infection. It has been shown that sera from FIV-infected cats that neutralize FIV infection of CrFK cells with CrFK-adapted FIV strains fail to neutralize FIV infection of thymocytes, peripheral blood mononuclear cells, or a T-lymphoid cell line (3, 23, 26). The FIV-neutralizing activity demonstrated in the CrFK virus neutralization assay proved to be mediated predominantly by antibodies directed to an immunodominant epitope in HV-3 (7, 15). This suggests that HV-3-specific antibodies largely fail to neutralize FIV in the thymocyte virus neutralization assay and that the HV-3 domain, if at all, is only indirectly involved in the mechanisms of virus neutralization in feline thymocytes.

Be it as it may, in the present article we have shown that both the HV-4 and the HV-5 domains of two closely related FIV clones are directly involved in FIV neutralization in feline thymocytes. With bacterially expressed envelope fragments of FIV it was shown that the HV-4 region contains a highly type-specific antigenic site (7, 17). However, in Pepscan analysis of both regions using 12-mers we could not obtain evidence for true linear epitopes in these regions (20, 23) (unpublished data), indicating that the epitopes involved are to a certain extent conformation dependent. It is interesting to note that the location of the regions involved in FIV neutralization identified here is reminiscent of the CD4 binding site of human immunodeficiency virus type 1, which is a discontinuous site with contributions mostly from the carboxy-terminal half of the SU protein. Antibodies to the CD4 binding site have VN activity, just like the antibodies present in serum S1422. On the basis of these similarities it may be hypothesized that the HV-4 and HV-5 regions of the SU protein contribute to the definition of the binding site for the cellular receptor of FIV.

Together with previously reported results (20, 23) the data presented show that mutations in the HV-4 and HV-5 regions influence the susceptibility of FIV to antibody-mediated neutralization.

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