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NOTES

A Neutralizing Antibody-Inducing Peptide of the V3 Domain of Feline Immunodeficiency Virus Envelope Glycoprotein Does Not Induce Protective Immunity

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Specific-pathogen-free cats, immunized with a 22-amino-acid synthetic peptide designated V3.3 and derived from the third variable region of the envelope glycoprotein of the Petaluma isolate of feline immunodeficiency virus (FIV), developed high antibody titers to the V3.3 peptide and to purified virus, as assayed by enzyme-linked immunoassays, as well as neutralizing antibodies, as assayed by the inhibition of syncytium formation in Crandell feline kidney cells. V3.3-immunized animals and control cats were challenged with FIV and then monitored for 12 months; V3.3 immunization failed to prevent FIV infection, as shown by virus isolation, anti-whole virus and anti-p24 immunoglobulin G antibody responses, and positive PCRs for *gag* and *env* gene fragments. Sequence analysis of the V3 region showed no evidence for the emergence of escape mutants that might have contributed to the lack of protection. The sera of the V3.3-hyperimmunized cats and two anti-V3.3 monoclonal antibodies neutralized FIV infectivity for Crandell feline kidney cells at high antibody dilutions but paradoxically failed to completely neutralize FIV infectivity at low dilutions. Moreover, following FIV challenge, V3.3-immunized animals developed a faster and higher antiviral antibody response than control cats. This was probably due to enhanced virus replication, as also suggested by quantitative PCR data.

Feline immunodeficiency virus (FIV) is a widespread lentivirus of domestic cats. As it shares numerous biological and pathogenetic features with human immunodeficiency virus, it is considered a valuable model for studies of antilentiviral vaccination strategy, drug evaluation, and immunodeficiency lentivirus pathogenesis (23).

FIV-neutralizing antibodies (NA) have been described only recently and are poorly characterized (7, 34). Assays based on the inhibition of FIV infectivity for the Crandell feline kidney (CrFK) fibroblastoid cell line have shown that infected cats develop NA which are broadly reactive, as shown by their presence in infected cats from different geographical areas and their ability to neutralize different FIV isolates (34, 35). Active production of these NA is evident at 5 to 6 weeks of infection and plateaus at high titers in 3 to 5 months. FIV also induces type-specific NA that are demonstrable by using lymphoid cells as substrates (2).

The viral antigen(s) and epitope(s) involved in FIV neutralization are also poorly understood. The *env* gene product seems to constitute the major target for NA. The gene encodes the surface envelope (SU) glycoprotein of about 95 kDa in its glycosylated form (6) and the transmembrane (TM) glycoprotein of 40 kDa. As in human immunodeficiency virus and other lentiviruses, *env* gene diversities among different isolates cluster in discrete segments known as variable regions. Eight or possibly nine variable regions have been identified in FIV (20, 22, 25, 29). By the use of synthetic peptides, we recently established the presence of several conserved immunoreactive domains in the V3 region (Cys-373 to Cys-424, according to Phillips et al. [25], or Cys-366 to Cys-417, according to Talbott et al. [33]; the latter numeration is used in the present paper). Analysis of FIV-infected cat sera by four partially overlapping peptides spanning the entire V3 region evidenced three linear antibody-binding domains, two of which are in the half-terminal part of V3. Other linear immunoreactive domains were identified in regions V4 (Gly-467 to Ans-481), V5 (Cys-534 to Gly-568), and V6 (Pro-593 to Lys-610) (13). Studies from other laboratories employing synthetic peptides or polypeptides expressed as fusion proteins defined nine antibody-binding domains, of which five are in the SU glycoprotein and four are in the TM glycoprotein (1, 8, 21).

Three possible sites directly or indirectly involved in FIV neutralization have been described so far: two map in regions V4 and V5 of gp95, as shown by the fact that a single amino acid substitution in V4 and V5 (amino acid positions 483 and 560, respectively) conferred resistance to virus neutralization (28, 30), while the third neutralization site is located in the V3 region. The last site was evidenced by findings showing that a 22-amino-acid synthetic peptide, designated V3.3 (GSWFRAI SSWKQRNRWEWRPDF) and spanning the sequence Gly-385 to Phe-406 of the Petaluma isolate (FIV-P), inhibited the FIV-neutralizing activity of pooled immune cat sera and, when

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TABLE 1. Serological findings in SPF cats hyperimmunized with V3.3 synthetic peptide

Cat	Immunization	Antibody titer[a]		
		Anti-V3.3	Anti-FIV	Neutralizing
784	V3.3	>25,600	800	256
199	V3.3	>25,600	400	256
192	V3.3	>25,600	400	64
3368	None	<50	<50	<8
338	None	<50	<50	<8
2906	None	<50	<50	<8

[a] Anti-V3.3 and anti-FIV antibodies were assessed by ELISA (13); NA were assayed by inhibition of syncytium formation in CrFK cells (34).

injected into specific-pathogen-free (SPF) cats, elicited NA that effectively neutralized the infectivity of the homologous FIV isolate and a heterologous FIV isolate for CrFK cells (13). The V3.3 peptide could therefore be of great interest as a component of candidate vaccines against FIV.

In the present study, we investigated whether V3.3 immunization of cats would confer protection against experimental FIV infection. For this purpose, 8-month-old SPF cats were immunized subcutaneously with 1 mg of V3.3 peptide, synthesized as previously reported (13), in Freund's complete adjuvant (1:1) (total volume, 2 ml); animals were then given boosters of 500 μg of peptide in Freund's incomplete adjuvant on days 15, 30, 45, 60, and 75. Two weeks later, the three immunized cats and an equal number of uninjected control SPF cats were challenged intravenously with 20 50% cat infectious doses of FIV-P grown in FL4 cells (38) (kind gift of Janet Yamamoto), which had been passaged 196 times in vitro and then titrated in vivo in groups of four 8-month-old SPF cats.

As shown in Table 1, prior to challenge, the V3.3-immunized animals showed high immunoglobulin G antibody titers to the V3.3 peptide and to purified FIV-P, as well as FIV NA at titers ranging from 1:64 to 1:256. The animals were then monitored for 12 months for viremia by virus isolation from peripheral blood mononuclear cells (PBMC), essentially as previously described (16), for the presence of proviral DNA in PBMC by PCR for *gag* (16) and *env* genes (see Table 2, footnote *a*) and for immunoglobulin G antibody to purified whole FIV-P by a standard enzyme-linked immunosorbent assay (ELISA) and to the p24 core antigen by a capture ELISA based on anti-p24 monoclonal antibodies (14). Moreover, at 4, 24, and 50 weeks postchallenge (p.c.), the viral load in PBMC was estimated by quantitative PCR with an internal standard, essentially as described by Pistello et al. (26).

As shown in Table 2, FIV was isolated at 4 weeks p.c. from pooled PBMC from both animal groups; thereafter, it was isolated only occasionally and only from V3.3-immunized cats until week 50 p.c., when it was isolated from all the V3.3-immunized animals and from two control cats. This was in keeping with previous observations that the virus can be isolated only sporadically from the PBMC from cats infected with low doses of FIV-P adapted to grow in tissue culture, at least during the first months of infection (3, 15). On the other hand, PCR for *gag* and *env* detected one or both of the proviral DNA segments in cats from both groups at all times tested, with the exception of one animal from the control group whose result was negative for both proviral segments at 4 weeks p.c. Quantitative PCR showed no major differences in the viral loads of V3.3-immunized and control animals at 4 weeks p.c.; however, at 6 and 12 months p.c., significantly higher viral loads were detected in V3.3-immunized animals ($P < 0.05$, according to the two-tailed Student t test).

With whole FIV as the antigen, the antibody response to infection (Fig. 1) was first evident at 4 to 8 weeks p.c., without major differences between the groups; however, at 12 to 26 weeks p.c., two cats immunized with V3.3 exhibited significantly higher levels of anti-FIV antibodies than control animals ($P < 0.05$ at week 16). Moreover, in all the cats immunized with V3.3, the antibody response to p24 core antigen developed considerably faster, peaked earlier, and reached significantly higher levels than did the response in control cats ($P < 0.05$ from week 12 through week 26); especially striking was the fact that the V3.3-immunized cats reached the highest anti-p24 antibody titers as early as 4 to 16 weeks p.c. By comparison, control cats reached lower levels of anti-p24 antibody as late as 24 to 26 weeks p.c. The counts of circulating $CD4^+$ and $CD8^+$ T lymphocytes remained in the normal range throughout the follow-up period in all animals (39).

Taken together, the above data clearly demonstrate that V3.3 immunization of cats does not prevent development of FIV infection in spite of the induction of high NA levels. This is in keeping with previous results showing that subunit vaccines are generally poorly effective against lentiviruses (10, 11), but the reasons for this are not understood. In vitro and in vivo

TABLE 2. Results of virus isolation and PCR for proviral DNA from the PBMC of V3.3-immunized or control cats challenged with FIV-P

Cat	V3.3 immunization	Result[a] at FIV postchallenge week:						
		0	4[b]	8	12	16	24	50
784	+	−−	+− (130)	+++	−+−	++−	−++ (420)	+++ (610)
199	+	−−	−+ (30)	−++	−++	−++	−+− (380)	+++ (430)
192	+	−−	++ (80)	−++	−++	+++	−++ (320)	+++ (520)
3368	−	−−	+− (110)	−+−	−++	−++	−++ (180)	+++ (400)
338	−	−−	−− (<10)	−+−	−+−	−++	−++ (ND[c])	+++ (280)
2906	−	−−	+− (60)	−+−	−++	−++	−++ (260)	−++ (350)

[a] Virus isolation and PCR analysis to detect *gag* gene fragments were performed as reported previously (16); PCR for the *env* gene fragment was carried out by the same procedure as for the *gag* genes but with the following primers and probes: TTAAGCTATTGTACAGACCC (positions 7212 to 7231) for the *env* sense strand, TTCTACAGCTTTTGTCATAT (positions 7816 to 7835) for the *env* antisense strand, and AGAAGTTCAGGAGATTATGG (positions 7545 to 7564) for the *env* probe. For weeks 0 and 4, results of PCR detection of *gag* sequences and PCR detection of *env* sequences are presented, in that order; for weeks 8 to 50, results of virus isolation, PCR detection of *gag* sequences, and PCR detection of *env* sequences are presented, in that order. The numbers in parentheses at 4, 24, and 50 weeks p.c. indicate the estimated number of proviral genomes per microgram of genomic DNA determined by quantitative PCR (26).

[b] At week 4, virus isolation was performed with the pooled PBMC from each group of three cats, and results were positive for both groups.

[c] ND, not determined.

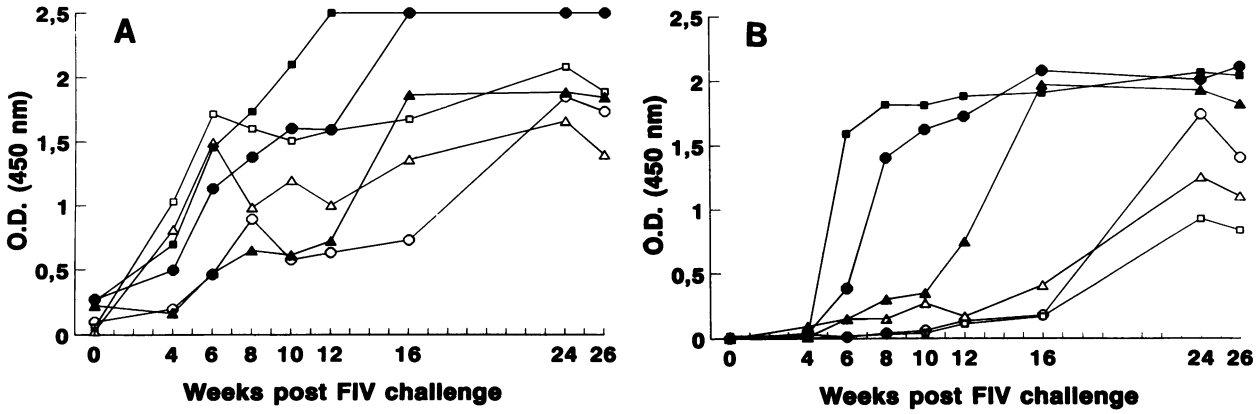

FIG. 1. Anti-FIV antibodies (A) and anti-p24 antibodies (B) in V3.3-immunized (solid symbols) and control (open symbols) cats challenged with 20 50% cat infectious doses of FIV, as measured by optical density (O.D.) at 450 nm. Each curve represents an individual animal.

growth in the presence of NA has been shown to select for lentivirus mutants that resist antibody-mediated neutralization (5, 30, 37). Thus, the failure of V3.3 immunization to protect against FIV might be due to the rapid emergence of similar mutants also in vivo under the pressure of anti-V3.3 immunity. In the first approach to evaluate this possibility, we sequenced the V3 *env* region detected by nested PCR of the PBMC obtained from two cats from each group at 6 months p.c. Briefly, PBMC DNA was first amplified (35 cycles; 94°C for 30 s, 60°C for 30 s, and 72°C for 180 s) with primers 237S (CC AGGAATAAACCCATTTAGGGTACCTG, positions 6372 to 6399) and 238AS (CTCATGCCAGTCCACCCTTTTTTC, positions 9103 to 9126) (kind gift of Antony de Ronde); 4 μl of the product was then amplified (35 cycles; 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s) with the internal primers S (ACA GACCCGTTACAAATCCCAC, positions 7224 to 7245) and AS (TTCTACTGCTTTTGTCATATTG, positions 7814 to 7835). Single-strand amplifications were then performed with the S or AS primer alone (45 cycles; 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s). The single strands were then freed of deoxynucleoside triphosphates, excess primers, and salts by ultrafiltration; verified by electrophoresis in 2% agarose; and sequenced with an automated apparatus (ALF DNA sequencer; Pharmacia, Uppsala, Sweden). As shown in Fig. 2, the sequence of the virus used for challenge exhibited two amino acid substitutions (F→L at position 388 and P→Q at position 416) compared with the published sequence of FIV-P (33), most probably as a consequence of its prolonged propagation in tissue culture prior to use as a challenge. Three substitutions were observed in the V3 sequence of the virus from the challenged animals, but they appeared to be independent of the immune status of the animals prior to challenge. Substitution at position 403 (R→G) was seen in cat 338 of the control group only. Substitution K→G at position 409 was seen in all infected cats. At position 407, the substitution K→E was complete only for cat 3368 while in the others both residues were detected, thus suggesting the coexistence of viral variants in a single infected cat, which is similar to what has been reported for human immunodeficiency virus (31). These limited sequence data do not entirely rule out the development of escape mutants in the V3.3-immunized cats, as the pathways that lead to immune escape in lentiviruses appear to be

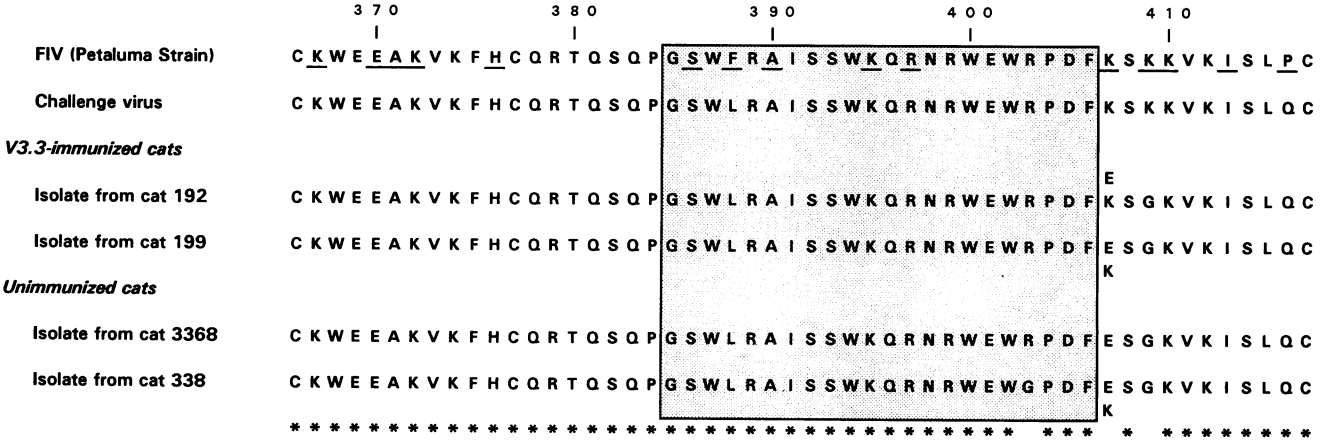

FIG. 2. Amino acid sequence of the V3 region deduced by direct sequencing of PCR-amplified fragments from the PBMC from two V3.3-immunized and two naive cats at 6 months p.c. The V3.3 domain is bounded by the rectangle, the underlined letters in the Petaluma strain indicate highly variable amino acid positions, and the asterisks indicate conserved amino acids compared with those of the virus used for challenge. The coexistence of K and E at position 407 was due to the contemporary detection of both A and G as the first nucleotide of the codon. These amino acids have been separately reported in previously published sequences (32, 33).

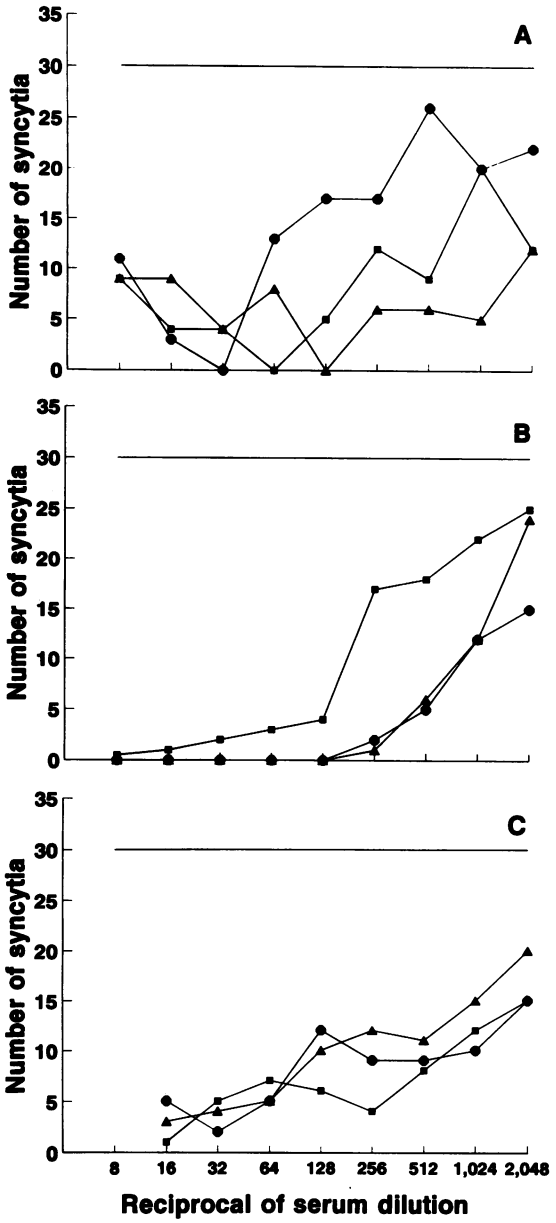

FIG. 3. FIV neutralization by V3.3-immunized cat sera (A), V3.3-immunized, FIV-challenged cat sera (B), and FIV-challenged cat sera (C), as assessed by inhibition of FIV-induced syncytium formation in CrFK cells. Twofold dilutions of heat-inactivated sera in phosphate-buffered saline were mixed with 50 syncytium-forming units of FIV-P in 24-well plates. After 1 h at room temperature, 10^4 CrFK cells that had been adapted to grow in 0.5% fetal bovine serum in 0.5 ml of medium were added to each well. Six days later, the cultures were stained and the syncytia were counted under a microscope. Each curve represents an individual animal; the horizontal lines indicate the number of FIV-induced syncytia in the absence of cat serum.

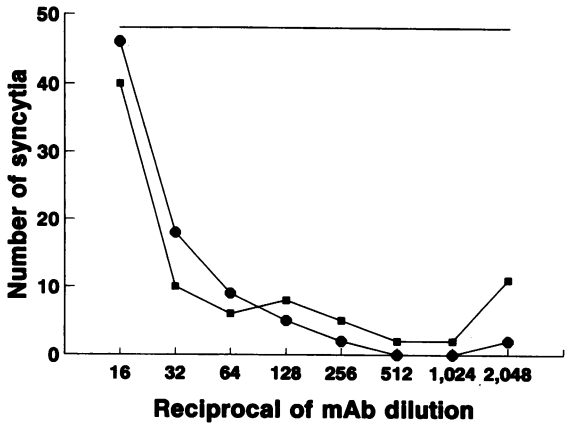

FIG. 4. FIV neutralization by anti-V3.3 monoclonal antibodies (mAb), as assessed by inhibition of FIV-induced syncytium formation in CrFK cells. Twofold dilutions of protein G-purified anti-V3.3 monoclonal antibodies, diluted in phosphate-buffered saline at a concentration of 1 mg/ml, were mixed with 50 syncytium-forming units of FIV-P in 24-well plates. After 1 h at room temperature, 10^4 CrFK cells that had been adapted to grow in 0.5% fetal bovine serum in 0.5 ml of medium were added to each well. Six days later, the cultures were stained and the syncytia were counted under a microscope. Each curve represents an individual monoclonal antibody; the horizontal line indicates the number of FIV-induced syncytia in the absence of monoclonal antibody.

multiple and include single amino acid substitutions that may affect V3 although they are located outside this region (28, 30, 37). However, the development of neutralization-resistant mutants is unlikely to play a major role in the failure of V3.3 immunization to protect against subsequent FIV challenge, especially in view of the fact that antibodies raised in cats against V3.3 peptide neutralized various divergent FIV isolates in vitro with similar efficiencies (12, 13).

In a previous study, sera from infected cats exhibited high titers of antibodies that neutralized the infectivity of FIV for CrFK cells but not the infectivity for lymphoid cells and cats (2). As anti-V3.3 sera also failed to neutralize FIV on lymphoid cells (data not shown), a clear possibility is that the V3.3 region contains a neutralization epitope(s) that is active solely on specific cell substrates, such as the CrFK cell line, and therefore cannot elicit significant levels of protection in vivo.

The situation is compounded by the observation of paradoxical behavior of hyperimmune anti-V3.3 cat sera in the neutralization assay on CrFK cells. While anti-V3.3 sera obtained from cats early during the immunization process showed normal dilution-dependent neutralization curves (13), the sera from the hyperimmunized cats used in this study neutralized FIV infectivity completely at high dilutions, but at low dilutions, the sera neutralized FIV infectivity only partially (Fig. 3A). This paradoxical partial neutralization at a high serum concentration was no longer evident in serum samples obtained from V3.3-immunized animals 1 month after challenge with FIV-P (Fig. 3B) and was not observed in sera from naive FIV-P-infected SPF cats (Fig. 3C). V3.3 hyperimmunization may have induced high levels of anti-idiotypic antibodies which mimic the antigenic epitope(s) and compete with NA, but we have observed the same paradoxical phenomenon with two of six anti-V3.3 immunoglobulin G monoclonal antibodies raised in mice by immunization with the V3.3 peptide (12). As shown in Fig. 4, these monoclonal antibodies induce 100% and more than 90% inhibition of FIV-induced syncytium formation at dilutions of 1:512 and 1:1,024, respectively, but have little if any neutralizing activity at high antibody concentrations. Although the basis of this phenomenon is not understood, it recalls antibody-dependent enhancement, which, however, is known to occur at subneutralizing concentrations of immune serum. Antibody-dependent enhancement has been shown for a num-

ber of viruses, including human immunodeficiency virus type 1, simian immunodeficiency virus, and, more recently, FIV (2), but the mechanisms involved are not clear (19, 27); antibody-dependent enhancement results in a paradoxical increase in the number of infected cells and represents a concern because of its suspected role in producing enhanced viremias, shorter incubation periods, increased disease severity, and vaccine failure (for a review, see reference 18).

Our findings also suggest that V3.3 immunization might have led to an exacerbation of FIV infection. Compared with naive cats, V3.3-immunized animals showed (i) prompter and higher antiviral antibody responses, (ii) a more frequent isolation of the virus, and (iii) higher viral loads in PBMC as detected by PCR. A similar enhancement of infection after immunization of cats with FIV antigens in immune stimulating complexes (9) has been reported, as has enhancement in animals infected with other retroviruses, such as feline leukemia virus (24), equine infectious anemia virus (36), and caprine arthritis-encephalitis virus (17). In the last lentivirus model, a correlation between an increased severity of disease and the presence of enhancing antibodies has been noted (17). It may be worth mentioning that NA can reportedly modulate antivirus cell-mediated immunity in a protective or harmful way and that, paradoxically, a preexisting NA may also increase susceptibility to virus-induced disease (4).

In conclusion, the key question for anti-FIV vaccination strategy as well as for anti-human immunodeficiency virus strategy—i.e., that of what types of immune responses it would be preferable to elicit (humoral versus cell mediated, T_H1 cells versus T_H2 cells)—is essentially unanswered; anti-FIV humoral immunity might protect against the systemic spread of the virus but might have little influence on virus-infected cells and on cell-to-cell virus spread in lymphoid organs. Both NA immunity and cell-mediated immunity are probably required for protection against FIV infection.

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