Antibodies specific for hypervariable regions 3 to 5 of the feline immunodeficiency virus envelope glycoprotein are not solely responsible for vaccine-induced acceleration of challenge infection in cats

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In a previous vaccination study in cats, the authors reported on accelerated feline immunodeficiency virus (FIV) replication upon challenge in animals vaccinated with a candidate envelope subunit vaccine. Plasma transfer studies as well as antibody profiles in vaccinated cats indicated a causative role for antibodies directed against the hypervariable regions HV3, HV4 and HV5 (HV3–5) of the envelope glycoprotein. The present study was designed to investigate further the contribution of antibodies in envelope vaccine-induced acceleration of FIV infection. To this end, regions HV3–5 of the envelope glycoprotein were deleted from the original vaccine, thus addressing the contributing role of antibodies directed against these hypervariable regions. Interestingly, this approach did not prevent acceleration of challenge infection. Analysis of the antibody responses in the respective groups suggested that removal of HV3–5 redirected the humoral immune response towards other regions of the envelope glycoprotein, indicating that these regions can also induce antibodies that accelerate virus replication.

INTRODUCTION

Since the identification of feline immunodeficiency virus (FIV) in 1986 (Pedersen *et al.*, 1987), several candidate FIV vaccines have been developed and evaluated (Elyar *et al.*, 1997; Uhl *et al.*, 2002). The development of a successful FIV vaccine would not only be of veterinary interest, but could also provide insight into correlates of protective immunity in lentivirus infections. It has been demonstrated that infections with the lentiviruses FIV and human immunodeficiency virus type 1 (HIV-1) share biological and clinical characteristics and exhibit a similar immunopathogenesis, which makes FIV infection of cats a valuable animal model for AIDS in a natural host (Bendinelli *et al.*, 1995; Willett *et al.*, 1997a).

The most promising results with candidate vaccines have been obtained using classical approaches such as inactivated whole virus and infected cell vaccines (Yamamoto *et al.*, 1991, 1993; Matteucci *et al.*, 1999, 2000; Pu *et al.*, 2001; Finerty *et al.*, 2001). However, from these studies it was not clear what the immune correlates of protection were. It has been reported that protection correlated with the presence of virus-neutralizing antibodies on the day of challenge (Yamamoto *et al.*, 1991, 1993), but vaccine-induced protection has also been observed in the absence of virus-neutralizing antibodies (Matteucci *et al.*, 1996). Candidate FIV subunit vaccines tested to date have failed to induce protective immunity. The majority of these vaccines were based on the use of the FIV envelope glycoprotein (gp130). Vaccines consisted of recombinant bacterial proteins (Siebelink *et al.*, 1995c; Lutz *et al.*, 1995; Verschoor *et al.*, 1996; Leutenegger *et al.*, 1998), recombinant baculovirus proteins (Lutz *et al.*, 1995; Leutenegger *et al.*, 1998), recombinant vaccinia virus protein (Siebelink *et al.*, 1995c; Huisman *et al.*, 1998), peptides representing parts of the envelope protein (Lombardi *et al.*, 1994; Finerty *et al.*, 2000, 2001; Flynn *et al.*, 1995) or recombinant vectors expressing the envelope protein or parts thereof (Gonin *et al.*, 1995; Tijhaar *et al.*, 1997). Recently, partial protection was reported using an envelope-encoding DNA vaccine co-injected with an IL12 expression plasmid (Boretti *et al.*, 2000; Leutenegger *et al.*, 2000).

In contrast, acceleration rather than reduction of FIV replication was observed upon challenge of vaccinated cats in a number of studies (Hosie *et al.*, 1992; Lombardi *et al.*, 1994; Siebelink *et al.*, 1995c; Richardson *et al.*, 1997, 2002; Karlas *et al.*, 1999; Giannecchini *et al.*, 2002). This phenomenon is characterized by an accelerated viraemia and sometimes increased virus loads in FIV-vaccinated animals compared with mock-vaccinated animals. The accelerated virus replication correlated with the presence of envelope-specific antibodies at the time of challenge in some studies

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(Lombardi *et al.*, 1994; Siebelink *et al.*, 1995c; Karlas *et al.*, 1999; Giannecchini *et al.*, 2002), but not in others (Hosie *et al.*, 1992; Richardson *et al.*, 1997, 2002). Hence, the mechanism underlying this enhancement phenomenon has not been fully elucidated. Transfer studies using plasma of vaccinated animals strongly suggested the involvement of plasma-associated factors, like virus-specific antibodies or cytokines (Siebelink *et al.*, 1995c). Moreover, the accelerated viraemia also correlated with the presence of antibodies specific for the hypervariable regions HV3, HV4 and HV5 (Siebelink *et al.*, 1995c). We showed that cat antisera raised against FIV molecular clone 19k1 neutralized this virus but enhanced the replication of a closely related molecular FIV clone, 19k32, *in vitro* (Siebelink *et al.*, 1992; unpublished results). Since these two clones differ in their envelope amino acid sequence at only four positions, all located in regions HV4 and HV5, we hypothesized that antibody responses against these regions are involved in antibody-mediated acceleration of infection.

Here, we report on the *in vivo* evaluation of a recombinant FIV envelope glycoprotein candidate vaccine from which the regions HV3–5 were deleted. The results show that antibodies directed against HV3–5 are not solely responsible for vaccine-induced acceleration of infection.

METHODS

Cells and viruses. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using Lymphoprep (Axis-Shield), aliquoted and stored at −135 °C. Assays were performed retrospectively to guarantee identical experimental conditions for all samples. After thawing, PBMCs were allowed to recover overnight in RPMI (Cambrex Bioscience) supplemented with penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), L-glutamine (2 mM), β-mercaptoethanol (2×10^{-5} M) and 10 % foetal bovine serum (Greiner Bio-One) (R10F medium). The following day, the cells were washed and subsequently cultured in R10F medium supplemented with concanavalin A (4 μg ml^{-1}; Sigma-Aldrich) and recombinant human interleukin 2 (rhuIL2, 200 IU ml^{-1}; Red Swan Pharma Logistics). After 3 days, cells were washed and cultured in R10F medium plus rhuIL2 (200 IU ml^{-1}).

Crandel feline kidney (CrFK) cells originated from an FIV-susceptible clone, CrFK 1D10, originally provided by N. Pedersen (Yamamoto *et al.*, 1988) and were cultured in Dulbecco's modified Eagle's medium (Cambrex Bioscience) supplemented with penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), L-glutamine (2 mM), β-mercaptoethanol (2×10^{-5} M) and 10 % foetal bovine serum (D10F medium).

FIV AM19 was used as the challenge virus, as described previously (Siebelink *et al.*, 1995c). This virus was originally isolated from PBMCs of a naturally infected cat, and an *in vitro*-grown virus stock was subsequently titrated *in vivo* (Siebelink *et al.*, 1995c).

FIV AM6c was used in the CrFK-based virus neutralization assay (see below). This virus was also isolated from a naturally infected cat and subsequently adapted to replicate on CrFK 1D10 cells (Siebelink *et al.*, 1995b). The *env* sequence homology of FIV AM6c and the molecular clone FIV 19k1 is 94·8 %.

Vaccines: recombinant vaccinia virus (rVV) constructs. The candidate vaccines used in this study were either identical to or derivatives of the vGR657x15 vaccine described previously (Rimmelzwaan *et al.*, 1994), which has been shown to be responsible for vaccine-induced enhancement of challenge infection (Siebelink *et al.*, 1995c). vGR657x15 consists of a VV-expressed, lentil lectin–Sepharose column-purified FIV envelope protein. The cleavage site between the surface and transmembrane protein has been deleted through site-directed mutagenesis to facilitate incorporation into immune-stimulating complexes (iscoms) (Rimmelzwaan *et al.*, 1994). For clarity, this vaccine is referred to as FIV-Env-iscom.

A schematic representation of the regions deleted from the ΔEnv construct is provided in Fig. 1. To generate the FIV-ΔEnv vaccines, the envelope of FIV 19k1 was amplified using two different primer sets [ΔENV1 (5′-GGGTACCTGGAATAACAC-3′) with ΔENV2 (5′-GGTCGACCCACCATCCACATTTTGG-3′, *Sal*I), and ΔENV3 (5′-GGTCGACGGCATCTTAAGAAATTGG-3′) with ΔENV4 (5′-CCTACCCAATCTTCCCAC-3′, *Sal*I) (restriction sites underlined)] generating two fragments, 5′ΔEnv and 3′ΔEnv. These fragments were linked by the introduced *Sal*I site. This resulted in an envelope sequence missing aa 358–567 (based on the envelope sequence of FIV 19k1; GenBank accession no. M73964), which constitute HV3–5. Internal *Kpn*I sites were used to exchange ΔEnv with the original envelope sequence in pGR657x15, the plasmid originally used to generate vGR657x15 (Rimmelzwaan *et al.*, 1994). Subsequent generation of rVV-GR657x15-ΔEnv, production, isolation, purification and incorporation into iscoms was done as described for rVV-GR657x15 (Rimmelzwaan *et al.*, 1994). The obtained vaccine is referred to as FIV-ΔEnv-iscom.

Polyclonal rabbit sera raised against bacterial fusion proteins were used to characterize and identify the produced envelope proteins; serum Ra2194 recognized HV4 and serum Ra2279 recognized epitopes located between HV2 and HV3 (de Ronde *et al.*, 1994).

Animals, vaccination and challenge. The specific-pathogen-free (SPF) cats used in this study were obtained from Harlan (Horst). An external ethical committee approved the protocol used in this study. Twelve animals were randomly distributed into groups: group 1 ($n=3$) was vaccinated with the original FIV-Env-iscom preparation; group 2 ($n=4$) was vaccinated with the FIV-ΔEnv-iscom preparation; group 3 ($n=5$) received PBS (non-vaccinated controls). Originally, group 1 consisted of four animals; however, one animal died during the vaccination scheme due to reasons not related to the experimental set-up of this study. Cats were inoculated with the respective vaccines at weeks 0, 6 and 10, identical to the previous study (Siebelink *et al.*, 1995c). Each vaccine dose contained 1·5 μg envelope protein. Animals were challenged intramuscularly with 20 CID_{50} FIV AM19 2 weeks after the last vaccination. PBMCs and plasma samples were obtained before and 1, 2, 3, 4, 6 and 8 weeks post-challenge.

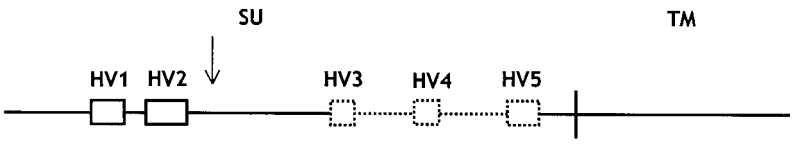

Fig. 1. Full-length FIV precursor envelope glycoprotein. The regions that were deleted to generate the ΔEnv glycoprotein are dashed. SU, surface subunit; TM, transmembrane subunit; HV, hypervariable region. The arrow indicates the highly hydrophobic leader sequence.

Virus neutralization assay. A virus neutralization assay based on the inhibition of FIV AM6c infection of CrFK cells was performed as described previously (Siebelink *et al.*, 1995c).

Env ELISA

Cloning into prokaryotic expression vectors. A modified version of the pThio plasmid (Invitrogen) was used to generate expression vectors containing FIV 19k1 (Siebelink *et al.*, 1992) *env* sequences. The modifications were introduced for detection and purification purposes. Using primers ThioHisfor (5′-GCGTCTAGAATCCCTAACCCTCTCC-3′, *Xba*I) and ThioHisback (5′-CGCGCTGCAGTCAATGGTGATGGTGATGATG-3′, *Pst*I) and pMT/V5-His (Invitrogen) as a template, the V5 epitope and the hexahistidine (6 × His)-tag were amplified, after which the PCR product was cloned into *Xba*I/*Pst*I-digested pThio. The obtained expression vector was named pThio/V5-His. Expression products from this expression vector were thioredoxin fusion proteins, containing a C-terminal V5 epitope for detection purposes and a 6 × His-tag for purification.

Using Pwo *Taq* polymerase (Stratagene), *env* sequences were directly amplified from a 3′-19k1 pUC19 subclone (Siebelink *et al.*, 1992). For the Env-SU (surface subunit region) construct, the primers 5′-CGGGATCCGACCCATTACAAATCCCACTG-3′ (*Bam*HI) and 5′-CGTCTAGATCTTTTTCTTCTAGGTTTATATTC-3′ (*Xba*I) were used. For the Env-TM (transmembrane region) construct, the primers 5′-CGGGATCCGGCATCTTAAGAAATTGGTAT-3′ (*Bam*HI) and 5′-CGTCTAGATTGATTACATCCTAATTCTTGC-3′ (*Xba*I) were used. The obtained amplicons were subsequently digested with *Bam*HI and *Xba*I and cloned into *Bam*HI/*Xba*I-digested pThio/V5-His.

The Env-SU construct incorporated Env 19k1 aa 322–611 (Env19k1, NCBI nucleotide accession no. M73964), containing HV3, HV4 and HV5 and flanking regions that were not deleted from the ΔEnv constructs. The Env-TM construct incorporated Env 19k1 aa 567–701, containing the principal immunodominant domain.

The generated plasmids were checked by restriction enzyme analysis and sequencing.

Expression and purification of bacterial recombinant fusion proteins. For expression of the fusion proteins, plasmid DNA was transfected into BL21pLysS bacteria (Stratagene). After culturing a single colony in 10 ml SOB medium [20 g Tryptone Peptone l^{-1} (Difco), 5 g yeast extract l^{-1} (Difco), 0·5 g NaCl l^{-1}, 2·5 mM KCl, 10 mM $MgCl_2$, pH 7·0] with 100 μg ampicillin ml^{-1} for 2 h, the bacteria were subsequently cultured in 500 ml SOB/ampicillin until an OD_{600} of 0·5–0·6 was reached. Expression was induced by adding IPTG (Roche) to a final concentration of 1 mM. After 4 h of induction at 20–37 °C, depending on the construct, the bacteria were pelleted by centrifugation at 900 ***g*** for 15 min and immediately used for purification or frozen overnight at −20 °C.

Subsequently, the pelleted bacteria were solubilized overnight in 5 ml lysis buffer (6 M guanidine/HCl, 0·1 M NaH_2PO_4, 0·01 M Tris/HCl pH 8·0) per 500 ml bacterial culture. The resulting lysate was centrifuged for 15 min at 10 000 ***g*** at 4 °C. The supernatant was then incubated with ProBond resin (Invitrogen) for 1 h at room temperature. After stacking and washing of the column material, guanidine/HCl was replaced with urea by washing the column with 10 ml wash buffer (8 M urea, 0·1 M NaH_2PO_4, 0·01 M Tris/HCl pH 6·3). After an additional washing step with wash buffer (pH 5·9), elution was carried out with elution buffer (8 M urea, 0·1 M NaH_2PO_4, 0·01 M Tris/HCl pH 4·5). Washing and elution steps were carried out at 4 °C. Purification was checked by SDS-PAGE on 15 % polyacrylamide gels and Western blot analysis using an anti-V5 monoclonal antibody (Invitrogen). Elution fractions containing the highest concentration of fusion protein were pooled and used in the ELISA.

ELISA. ELISA plates (96-well; Corning) were coated with 100 ng of recombinant protein in PBS (Gibco) per well. As a control antigen, an FIV Orf A–thioredoxin fusion protein was used, expressed and purified as for the envelope fusion proteins. After blocking with ELISA buffer consisting of Meddens reagent (Meddens, Woerden, The Netherlands) supplemented with 0·5 % BSA and 0·05 % Tween 20, the plasma samples were incubated in a 1 : 100 dilution in ELISA buffer for 1 h at 37 °C. Mouse anti-cat IgG (1 : 300; Serotec) was used as a first conjugate, followed by HRP-conjugated rabbit anti-mouse IgG (1 : 1000; DAKO). 3,3′,5,5′-Tetramethylbenzidine (TMB) in TMB diluent (Meddens) was used as substrate. The reaction was stopped after 5 min by adding an equal volume of 2 M H_2SO_4. Background values against the Orf A fusion protein were subtracted from the Env-SU and Env-TM values to obtain specific OD_{450} values.

Virus loads

Plasma virus load. Plasma virus loads were determined with a real-time PCR (*Taq*Man) assay according to a protocol described previously (Klein *et al.*, 1999; Leutenegger *et al.*, 1999) on an ABI prism 7700 Sequence Detection System (Applied Biosystems). Primers and probe sequences (Klein *et al.*, 1999; Leutenegger *et al.*, 1999) were adapted to the FIV 19k1 sequence when different. Primer concentrations of 15 pmol (300 μM) and 30 pmol (600 μM) were found to be optimal for the forward and reverse primers, respectively. The optimal probe concentration was 10 pmol (200 μM). The real-time PCR was performed using the EZ-core kit (Applied Biosystems). The RT-PCR cycling programme was initiated with a 2 min 50 °C uracyl amperase step, followed by a 30 min 60 °C RT step, 5 min denaturation at 95 °C and 45 cycles of a two-step PCR consisting of 20 s at 95 °C and 1 min at 62 °C. Data were collected during the annealing step (62 °C). A diluted 19k1 virus stock was used in each run to obtain a standard curve. Feline plasma (190 μl) was spiked with a known phocine distemper virus stock that was used as an internal control to control for efficiency of RNA isolation (unpublished data). RNA was extracted from plasma (total 200 μl input) and concentrated fourfold (50 μl output) with a Magnapure LC Isolation Station (Roche), using the Magna Pure LC Total Nucleic Acid Isolation kit (Roche).

Cell-associated virus load. Provirus loads or cell-associated virus loads were determined using a slightly modified infectious centre test, described previously (Siebelink *et al.*, 1995c). Briefly, threefold serially diluted PBMC samples obtained after challenge infection were co-cultured in 96-well U-bottom plates (Greiner Bio-One) with a concanavalin A- and rhuIL2-stimulated mixture of PBMCs from two SPF cats in 10 wells. Culture medium containing rhuIL2 was added weekly to maintain the cultures. After 4 weeks, the culture supernatants were analysed for the presence of FIV antigen by ELISA (Siebelink *et al.*, 1990). The number of infected cells in the PBMCs was calculated from the results of the ELISA by assuming that one infected cell gave rise to FIV antigen production after co-cultivation with the stimulated PBMCs, when one or more cultures tested in the 10 wells were negative for FIV antigen.

Statistical analyses. For statistical analysis of the data between two groups, the Mann–Whitney test was used.

RESULTS

Generation of FIV-ΔEnv-iscom

BHK cells were infected in parallel with rVV-GR657x15-Env or rVV-GR657x15-ΔEnv. Two days post-infection, crude cell-lysates were prepared and after purification of

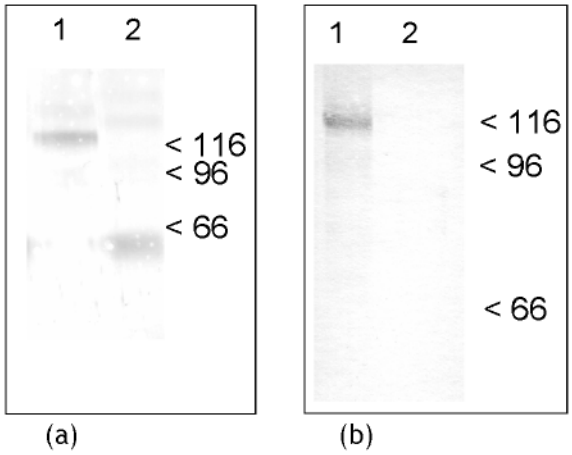

Fig. 2. Western blot analysis of candidate vaccine preparations using polyclonal rabbit serum directed against the region between HV2 and HV3 (a) and against HV4 (b) as the primary antibody. Lane 1, FIV-Env-iscom; lane 2, FIV-ΔEnv-iscom. Molecular mass markers are indicated on the right of each panel.

the FIV glycoproteins, iscoms were prepared and quality controlled as described previously (Rimmelzwaan *et al.*, 1994). In addition, iscom preparations were analysed by SDS-PAGE followed by Western blotting. As shown in Fig. 2(a), the FIV-Env-iscom preparation contained an envelope protein with a molecular mass of 130 kDa, confirming the expression of the non-cleaved 688 aa FIV envelope glycoprotein by rVV-vGR657x15, as reported previously (Rimmelzwaan *et al.*, 1994). Deletion of HV3–5 resulted in expression of a protein with a molecular mass of approximately 60 kDa. Western blot analysis using polyclonal antisera against HV2–3- and HV4-specific envelope regions confirmed the successful deletion of HV3–5. As can be seen in Fig. 2(a), the α-HV2–3 serum clearly identified the expression of both the intact envelope and the envelope glycoprotein from which HV3–5 were deleted, while with the α-HV4 serum (Fig. 2b), the intact envelope glycoprotein was detected but not the envelope glycoprotein from which HV3–5 had been deleted.

Vaccine-induced immune responses

Virus-neutralizing antibodies. Cats vaccinated with FIV-Env-iscoms developed virus-neutralizing antibody (VNA) titres against FIV infection of CrFK cells with titres ranging from 160 to 5120 on the day of challenge (Table 1). The titres were comparable to those measured on the day of challenge in the study by Siebelink *et al.* (1995c), in which the same candidate vaccine was used. In plasma samples of control cats and those vaccinated with FIV-ΔEnv-iscoms, VNAs (titres $\geqslant$20) were not demonstrated on the day of challenge.

Envelope-specific antibodies. To study the immunogenicity of the FIV-ΔEnv-iscoms, plasma samples of vaccinated cats were tested in an ELISA for the detection of envelope protein-specific antibodies. As shown in Fig. 3(a), all cats vaccinated with the FIV-Env-iscoms had developed significant antibody responses against Env-SU by 2 weeks after the last vaccination ($P=0{\cdot}025$, compared with control animals). Two cats vaccinated with FIV-ΔEnv-iscoms also mounted an antibody response against Env-SU.

The same two animals in group 2 also mounted a significant Env-TM-specific antibody response. In contrast, cats from the control group 3 and group 1 failed to develop an antibody response against the TM part of the envelope glycoprotein.

Outcome of challenge infection

Plasma virus loads. Plasma virus load kinetics in the respective groups of vaccinated cats were similar, irrespective

Table 1. Virus neutralizing antibody titres

Group	Vaccine	Cat no.	Virus neutralization titre (1/*x*) (weeks post-challenge)						
			0	1	2	3	4	6	8
1	FIV-Env	1	160	160	640	5 120	10 240	2 560	640
		2	5 120	2 560	1 280	5 120	>40 960	10 240	5 120
		3	320	80	160	80	640	640	1 280
2	FIV-ΔEnv	4	<20	<20	20	80	2 560	1 280	5 120
		5	<20	<20	20	40	40	10 240	5 120
		6	<20	<20	<20	40	1 280	2 560	1 280
		7	<20	<20	<20	40	160	10 240	10 240
3	PBS	8	<20	<20	<20	<20	<20	160	2 560
		9	<20	<20	<20	<20	40	2 560	10 240
		10	<20	<20	<20	<20	<20	80	40 960
		11	<20	<20	<20	<20	<20	320	5 120
		12	<20	<20	<20	<20	<20	2 560	ND

ND, Not determined.

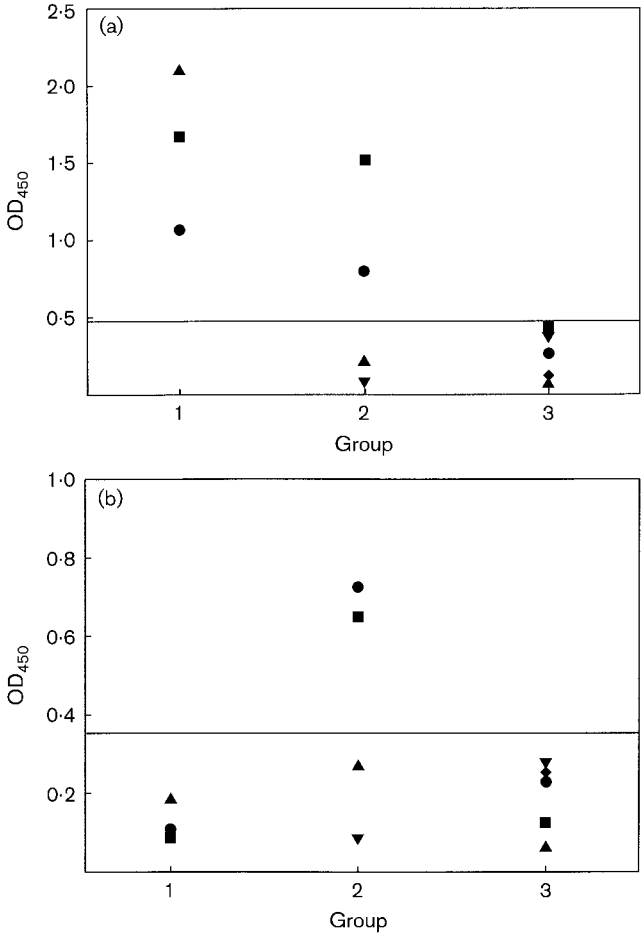

Fig. 3. Env-ELISA. Plasma samples obtained on the day of challenge were diluted 1 : 100 and tested by ELISA against 100 ng coated recombinant bacterial fusion protein encoding either the surface (a) or the transmembrane (b) subunit of the envelope protein. Group 1, FIV-Env-iscom; group 2, FIV-ΔEnv-iscom; group 3, PBS. Symbols represent individual cats.

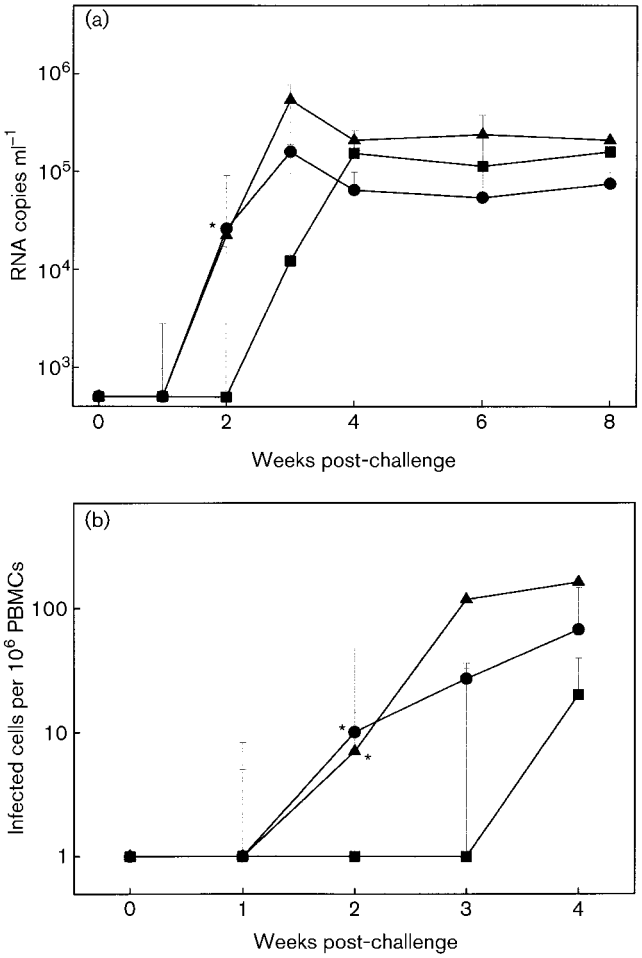

Fig. 4. Virus loads. Plasma and PBMCs isolated after challenge infection were quantitatively analysed for the presence of FIV particles (a) and provirus (b). ●, Group 1, ▲, group 2, ■, group 3. An asterisk denotes a significant difference compared with control group, $P<0{\cdot}05$.

of the vaccine used (Env vs ΔEnv, Fig. 4a). At two weeks post-challenge, plasma virus loads were significantly higher in group 2 (median 22 400 copies ml^{-1}) compared with control group 3 (median 0, $P=0{\cdot}041$). Group 1 (FIV-Env-iscom, median 26 000 copies ml^{-1}) also exhibited a trend towards higher virus loads in circulation ($P=0{\cdot}167$). Peak virus loads ranged from 60 000 to 4 600 000 copies ml^{-1}, with no significant differences between groups. However, envelope protein-vaccinated animals reached peak plasma virus loads 1–3 weeks earlier than animals in the control group. These plasma virus load kinetics suggested vaccine-induced acceleration of infection in envelope-vaccinated animals.

Cell-associated virus loads. Accelerated virus replication upon challenge infection was more pronounced when cell-associated virus loads were analysed in the respective groups (Fig. 4b): animals vaccinated with the respective candidate envelope vaccines exhibited significantly higher numbers of FIV-infected cells at 2 weeks post-challenge [median 30 and 42·5 infected cells $(10^6$ PBMCs$)^{-1}$, respectively], compared with control animals in which no FIV-infected cells were found at this time ($P<0{\cdot}05$). Peak provirus loads ranged from 25 to >1000 infected cells $(10^6$ PBMCs$)^{-1}$, with no significant differences between groups. FIV envelope-vaccinated animals showed peak provirus levels 2–4 weeks earlier than animals in the control group.

Post-challenge virus-neutralizing antibody kinetics

Upon infection, all cats vaccinated with FIV-Env-iscoms developed an anamnestic antibody response. Virus-neutralizing antibody titres $\geqslant 5120$ were measured in individual cats from 3 weeks post-challenge onward (Table 1). The FIV-ΔEnv vaccinated cats (group 2) exhibited faster VNA titre kinetics compared with the

mock-vaccinated cats (group 3), as two out of four cats developed VNA titres from 2 weeks post-challenge onward (all cats were positive at 3 weeks post-challenge), while most mock-vaccinated cats did not develop VNA titres until week 6 post-challenge.

DISCUSSION

In the present study it was shown that antibodies directed against FIV envelope regions HV3–5 are not solely responsible for vaccine-induced acceleration of challenge infection. We have previously shown that vaccination with an envelope protein iscom preparation predisposed cats to accelerated viraemia upon challenge infection with FIV (Siebelink *et al.*, 1995c). Antibodies directed against the envelope protein were considered to be involved in this phenomenon, since naïve cats exhibited an accelerated viraemia upon passive transfer with plasma obtained from the vaccinated animals and subsequent virus challenge. The accelerated virus replication correlated with the presence of virus-neutralizing and HV3–5-specific serum antibodies on the day of challenge (Siebelink *et al.*, 1995c). In addition, vaccination of cats with a peptide containing the V3 region of the FIV envelope protein induced enhanced virus replication, which correlated with accelerated VNA titre kinetics (Lombardi *et al.*, 1994). To address the role of HV3–5-specific antibodies in vaccine-induced acceleration of infection, we vaccinated cats with iscom candidate vaccines containing either the entire FIV envelope glycoprotein (Siebelink *et al.*, 1995c) or the envelope glycoprotein from which regions HV3–5 were deleted (FIV-ΔEnv-iscom). It was shown that plasma virus and provirus load kinetics were similar for both the FIV-Env-iscom and the FIV-ΔEnv-iscom vaccinated cats, indicating that antibody responses against HV3–5 were not solely responsible for FIV envelope vaccine-induced acceleration of infection. Passive transfer of post-vaccination plasma and subsequent infection of naïve cats could provide supportive evidence that the accelerated virus replication in FIV-ΔEnv-iscom-vaccinated cats was antibody mediated, as was demonstrated with plasma from cats vaccinated with FIV-Env-iscoms (Siebelink *et al.*, 1995c). Since the virus replication kinetics in FIV-ΔEnv-iscom-vaccinated cats was similar to that in cats vaccinated with FIV-Env-iscoms, we speculate that in FIV-ΔEnv-iscom-vaccinated cats the accelerated virus replication was also mediated by antibodies. In contrast to the FIV-Env-iscom-vaccinated groups, enhancement of virus replication was observed in the absence of detectable VNA titres on the day of challenge in FIV-ΔEnv-iscom-vaccinated cats (Table 1). The absence of VNA in these animals is most likely explained by the absence of the regions HV3–5 from the vaccine preparation since we and others have shown that neutralization of FIV infection *in vitro* is mainly mediated by antibodies directed against (conformational) epitopes involving regions HV3 (Lombardi *et al.*, 1993, 1995) and/or HV4 and HV5 (Siebelink *et al.*, 1993, 1995a; Verschoor *et al.*, 1995). Although the HV3–5 deletion may have compromised the conformation of the envelope glycoprotein, the ΔEnv glycoprotein was still immunogenic and Env-specific antibodies were detected by ELISA. Since bacterially expressed proteins were used in the ELISA, antibodies against remaining conformational epitopes may have been missed.

Post-challenge serum VNA responses in the FIV-ΔEnv-iscom-vaccinated group developed more rapidly than in the control group. This may have been caused by the accelerated kinetics of virus replication, by a priming effect of the FIV-ΔEnv-iscom vaccine on the induction of VNA specific for epitopes outside HV3–5, or a combination of both mechanisms. HIV-1 vaccination studies in guinea pigs have demonstrated that masking of the envelope V3 region by the introduction of *N*-linked glycosylation sites and reduction of the net positive charge resulted in the induction of antibodies specific for the V1 region and with a broader neutralizing capacity *in vitro* than antibodies induced by the original protein (Garrity *et al.*, 1997). Furthermore, in an attempt to generate immunogenically superior HIV-1 envelope proteins, it has been shown that removal of one or more of the V1, V2 or V3 loops can result in the exposure of normally obscured, conserved neutralization epitopes (Sanders *et al.*, 2000). Thus, the antibody response induced by FIV-ΔEnv-iscoms may have been redirected towards regions on the envelope protein other than HV3–5 and have primed for the induction of post-challenge antibodies that can neutralize FIV *in vitro* but may predispose for accelerated infection *in vivo*. This is in agreement with the more efficient induction of antibodies specific for epitopes present in the TM region of the envelope glycoprotein observed after vaccination with FIV-ΔEnv-iscoms (see Fig. 3b, Env-TM response). As in HIV-1 neutralization, these epitopes may also be involved in *in vitro* virus neutralization of FIV.

It has been suggested that antibodies directed to the principal immunodominant domain (PID), located in the extracellular part of the TM region, are involved in enhancement of HIV-1 infection of cell cultures *in vitro* (Robinson *et al.*, 1990). The high similarity between HIV-1 and FIV PID structures (Pancino *et al.*, 1995) suggests that this region may be involved in the induction of antibodies mediating enhancement of FIV infection. However, the observation that immunization of cats with a peptide containing the FIV PID resulted in reduced virus loads upon challenge infection compared with control animals (Richardson *et al.*, 1998) does not support this option. Collectively, these results point towards major differences between virus neutralization *in vitro* and *in vivo*, and it should be stressed that data generated *in vitro* should be extrapolated cautiously to the *in vivo* situation. Indeed, the physiological microenvironment has been shown to have a major effect on the infectivity of HIV-1 isolates in PBMCs and monocyte-derived macrophages (Wu *et al.*, 1995). Considering the outcome of this study, epitope deletion as an approach to improve the efficacy of candidate lentiviral vaccines is not likely to result in a successful vaccine.

Although it has been shown for HIV-1 infection *in vitro* that antibody-mediated enhancement by a monoclonal antibody could be abrogated by mutating the targeted epitope (Mitchell *et al.*, 1998), this is less likely to occur in the context of a polyclonal immune response *in vivo*.

In vitro infection experiments have revealed possible mechanisms involved in enhancement of HIV-1 infection besides virus-specific antibodies. For example, it has been shown that more efficient virus entry was mediated by components of the complement system and their receptors (Boyer *et al.*, 1991; Tacnet-Delorme *et al.*, 1999). These mechanisms may also act *in vivo*. Since in most studies cats are challenged shortly (within 2–4 weeks) after the last booster vaccination, an additional role for other mechanisms, such as immune activation, in vaccine-induced acceleration of infection cannot be excluded (Richardson *et al.*, 1997, 2002; Karlas *et al.*, 1999). We have attempted to address the role of immune activation in another group of cats that was vaccinated with FIV-Env-iscoms by challenging these animals 12 weeks after the last booster vaccination instead of 2 weeks. Despite this adjourned challenge, both provirus and plasma virus load kinetics developed similar to groups 1 and 2 of the current study (data not shown). Both virus-neutralizing titres as well as envelope-specific antibody responses (ELISA) were similar to those described for the FIV-Env-iscom-vaccinated animals in the current study. Therefore, it was suspected that antibodies, not vaccine-induced immune activation, were at the basis of the observed accelerated virus replication. However, a control group vaccinated with simian immunodeficiency virus (SIV)-Env-iscoms and challenged 2 weeks after the last booster vaccination exhibited virus load kinetics in between those of the PBS controls and the groups with accelerated virus replication. Since an SIV-Env-iscom-vaccinated group challenged 12 weeks after the last booster vaccination was not included, we could not accurately assess the potential involvement of immune activation in this set-up and these groups were therefore not included in this study.

The difference in virus replication kinetics in vaccinated compared with control cats was more pronounced when virus loads were tested as the number of infected cells in the infectious centre test than when plasma loads were tested by real-time PCR (compare Fig. 4a and b). This suggests that in the vaccinated cats a more efficient entry of virus into susceptible cells took place. Recent studies suggest that the specific activation of lentivirus-specific $CD4^+$ T cells results in an increased susceptibility to infection of these cells (Richardson *et al.*, 1997, 2002; Douek *et al.*, 2002). Accordingly, the induction of FIV-specific $CD4^+$ T cells by vaccination could have supported a more efficient infection of these cells upon challenge infection, possibly through an increased expression of CXCR4 (Willett *et al.*, 1997b). However, earlier results showing that a factor responsible for vaccine-induced enhancement of virus replication could be transferred to naïve cats by plasma obtained from cats vaccinated with an FIV-Env-iscom vaccine (Siebelink *et al.*, 1995c) argue against a role for virus-specific $CD4^+$ T cells as a single determinant responsible for vaccine-induced enhancement of virus replication. Still, it cannot be ruled out that multiple vaccine-induced mechanisms are involved in the observed accelerated virus replication.

The present study has shown that vaccine-induced enhancement of FIV challenge infection is not exclusively mediated by antibody responses against epitopes within HV3–5 of the envelope glycoprotein. Since the envelope iscom vaccine from which these regions were deleted retained its property to induce an antibody response that predisposed for accelerated viraemia upon challenge infection, other regions of the envelope glycoprotein may be involved in antibody-mediated acceleration of infection as well. Until those regions are identified, the use of the envelope glycoprotein as a candidate subunit vaccine against FIV infection of cats should be considered with caution.

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