Salmonella typhimurium aroA recombinants and immune-stimulating complexes as vaccine candidates for feline immunodeficiency virus


1 National Institute of Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands
2 Erasmus University and University Hospital Rotterdam, Institute of Virology, PO Box 1738, 3000 DR Rotterdam, The Netherlands
3 Academic Medical Centre, University of Amsterdam, Department of Human Retrovirology, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands
4 European Veterinary Laboratory, PO Box 198, 3440 AD Woerden, The Netherlands

Two experimental feline immunodeficiency virus (FIV) vaccines were tested, either alone or in combination, in four groups of cats (A–D). One vaccine (SL3261-FIV) was composed of live attenuated Salmonella typhimurium aroA (SL3261) strains expressing the capsid (Gag) and part of the envelope (Env) proteins of FIV. The other was composed of FIV Gag and Env proteins incorporated into immunostimulating complexes (iscom-FIV). Cats of group A were immunized four times with SL3261-FIV. Cats of group B were immunized twice with SL3261-FIV and then twice with iscom-FIV. Cats of group C were immunized twice with SL3261 expressing the B subunit of cholera toxin (SL3261-CtxB) and then twice with iscom-FIV. Cats of group D, which served as negative controls, were immunized twice with SL3261-CtxB and then twice with iscom into which the Gag and Env proteins of simian immunodeficiency virus (SIV) had been incorporated (iscom-SIV). Two weeks after the last immunization, all cats were challenged with FIV. At this time, cats immunized with iscom-FIV (groups B and C) showed strong plasma antibody responses to Gag and Env, whilst these responses were weak or undetectable in the cats immunized four times with SL3261-FIV (group A). Seven weeks after FIV challenge, Env-specific antibody responses had increased considerably in cats of all groups except group A. The mean virus loads in the cats of this group proved to be lower than those of the other groups at all time points, indicating partial protection.

Introduction

Feline immunodeficiency virus (FIV) infection causes a disease in cats similar to AIDS in humans. Like human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), it is a T-lymphotropic lentivirus that persistently infects its natural host and causes loss of CD4+ T cells, finally resulting in severe immunodeficiency (Brunner & Pedersen, 1989; Ackley et al., 1990; Dow et al., 1990; Torten et al., 1991; Hoffmann-Fezer et al., 1992). These similarities make FIV infection of cats a useful animal model for the evaluation of HIV vaccination strategies.

Many of the vaccine strategies that have been explored to induce protective immunity against lentiviruses have been without or with limited success. Inactivated whole-virus vaccines have been effective in the FIV system. Cats vaccinated with paraformaldehyde-fixed T cells (FL-4 cells) persistently infected with FIV and cats vaccinated with paraformaldehyde-inactivated FIV derived from the same cells proved to be protected against homologous and, to a lesser extent, against heterologous FIV challenge infection (Yamamoto et al., 1991, 1993; Hosie et al., 1995). Similar approaches with vaccine preparations derived from other cells were unsuccessful (Hosie et al., 1992; Verschoor et al., 1995). The most impressive
protection against SIV infection in macaques has been obtained by vaccination with live attenuated virus (Daniel et al., 1992; Clements et al., 1995), but safety concerns (Dittmer et al., 1995) may limit the use of this approach.

Live carriers such as canary pox, BCG and attenuated Salmonella strains are being extensively evaluated as alternative lentivirus vaccine candidates. Attenuated Salmonella strains have been used successfully as live carriers to elicit mucosal and systemic humoral responses, as well as cellular responses, including class I restricted cytotoxic T cells (CTL), against a number of bacterial (Wick et al., 1993), viral (Tite et al., 1990; Charbit et al., 1993; Schön et al., 1994; Valentine et al., 1996) and protozoal (Aggarwal et al., 1990; Flynn et al., 1990; Yang et al., 1990; Gonzalez et al., 1994) antigens. Induction of mucosal immunity may be of particular relevance to HIV-1 vaccine development since the most common route of infection is via mucosal surfaces of the genital tract.

Although attenuated Salmonella strains may present antigens to the immune system, the development of vaccines using Salmonella strains as live carriers has been hampered by low and/or unstable expression of heterologous antigens. To solve this problem, an expression system was developed that allows abundant and stable expression of heterologous antigens by a bacterial population, even if antigen expression is toxic for individual bacteria (Yan et al., 1990; Yan, 1992; Tijhaar et al., 1994; E. J. Tijhaar, J. A. Karlas, Z. X. Yan, T. F. Meyer, A. D. M. E. Osterhaus & F. R. Mooi, unpublished results). The system is based on an invertible promoter that randomly inverts. After switching to the ‘on’ position, it directs the expression of T7 RNA polymerase. The polymerase in turn directs expression of genes located under the control of a T7 promoter present on another vector in the same cell. The bacterial population consists therefore of two subpopulations, one of which expresses the gene of interest due to the promoter inversion, whilst the other major subpopulation does not produce the antigen. Unhindered by the expression of the heterologous antigen, the non-producing subpopulation may disseminate within the host and segregate new antigen producers, allowing a widespread expression of the antigen in the body.

Previously, we have used this invertible promoter system to express the gag gene of FIV in Salmonella typhimurium (Tijhaar et al., 1997). Cats immunized intraperitoneally (i.p.) with these S. typhimurium strains developed a clear antibody response to the Gag protein. Oral administration did not induce Gag-specific antibodies, but did prime the immune system, as became evident by the considerably faster and stronger response to Gag after challenge with FIV as compared to the control cats. However, none of the cats immunized in this way proved to be protected. In the present study, we extended the immunization regimen by co-administering S. typhimurium strains that express part of the env gene and subsequent boosting with recombinant Gag and Env proteins incorporated into immune-stimulating complexes (iscom).

Here we demonstrate that immunization with iscom-FIV, either alone or in combination with S. typhimurium strains expressing FIV Env and Gag, resulted in the induction of higher specific plasma antibody titres. However, this did not correlate with reduced virus loads upon challenge. In contrast, repeated immunization with the FIV Env- and Gag-expressing S. typhimurium strains alone did result in lower mean cell-associated FIV loads upon challenge.

Methods

■ Bacterial strains and media. The bacterial strains (Table 1) and their growth conditions have been described previously (Tijhaar et al., 1997).


The oligonucleotide 5’ AAAACAGGTGCACGCGAAGAAGGTAAA-GACCGATGCCCGCAGCAATGGC 3’ was used for introducing the deletion, whilst oligonucleotide 5’ ACCACGATGGCGCAATGGC 3’ was used for selection. This oligonucleotide introduced a silent mutation into the malE gene of pETMALp, which code for the stretch of hydrophobic amino acids in the signal sequence of pre-maltose binding protein (MBP) that is essential for transport to the periplasm. This mutation was introduced because the toxicity of cytoplasmically localized MBP fusion proteins, as encoded by pETMALc, is usually less and the expression higher than of MBP fusion proteins that are directed to the periplasm. The mutation was performed using the Transformer Site-Directed Mutagenesis kit (catalogue number K 1600-1; Clontech Laboratories).

■ Western blot analysis of bacteria. Western blots were performed as described previously (Tijhaar et al., 1997).

■ Plasmid stability in bacterial strains during growth in thymocytes. Thymocytes derived from a specified pathogen-free (SPF) cat were stimulated for 3 days with 5 μg/ml concanavalin A (ConA) and subsequently maintained in culture medium [RPMI 1640 (Gibco-BRL) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), l-glutamine (2 mM), and β-mercaptoethanol (2 × 10−3 M)] containing 100 IU/ml IL-2 (Cetus). Prior to infection with the SL3261 strains, thymocytes were washed twice in RPMI 1640 without any additives. Bacteria from exponentially growing cultures were harvested by centrifugation and washed twice with RPMI 1640. Approximately 108 bacteria were added to 109 thymocytes in a total volume of 1 ml RPMI 1640. Subsequently, cells were incubated for 2 h at 37 °C, during which period they were resuspended every 20 min. Finally, the cells were washed twice in RPMI and suspended in 10 ml culture medium supplemented with gentamicin (50 μg/ml) to kill extracellular bacteria and IL-2 (100 IU/ml). After 24 h at 37 °C, cells from 1 ml of culture were harvested, washed twice in PBS and lysed in PBS containing 1% Triton X-100 to release intracellular bacteria. The bacteria were plated on media without antibiotics and colonies were allowed to form at 28 °C. Subsequently, at least 100 colonies of each sample were tested for resistance on plates containing ampicillin and kanamycin. Plasmid
Table 1. Main features of the plasmids and bacterial strains used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Main features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pETMALp</td>
<td>malE (MBP), T7 promoter, AmpR</td>
<td>Tijhaar et al. (1997)</td>
</tr>
<tr>
<td>pETMALc</td>
<td>Δ signal sequence of malE, T7 promoter, AmpR</td>
<td>This study</td>
</tr>
<tr>
<td>pETMALgag</td>
<td>malE–gag (MBP–Gag), T7 promoter, AmpR</td>
<td>Tijhaar et al. (1997)</td>
</tr>
<tr>
<td>pETMALenv</td>
<td>malE–env (MBP–Env), T7 promoter, AmpR</td>
<td>Tijhaar et al. (1997)</td>
</tr>
<tr>
<td>pT7ctxB</td>
<td>ctxB (CtxB), T7 promoter, AmpR</td>
<td>Tijhaar et al. (1997)</td>
</tr>
<tr>
<td>plP2/plP4⁺</td>
<td>T7 RNA polymerase gene, invertible promoter, KanR</td>
<td></td>
</tr>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL3261</td>
<td>Salmo nella typhimurium aroA mutant</td>
<td>Hoiseth &amp; Stocker (1981)</td>
</tr>
<tr>
<td>SL3261-CtxB</td>
<td>SL3261 (pT7-CtxB + plP2)</td>
<td>Tijhaar et al. (1997)</td>
</tr>
<tr>
<td>SL3261-FIV</td>
<td>Equal amounts of:</td>
<td></td>
</tr>
<tr>
<td>SL3261 (pETMALgag + plP2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL3261 (pETMALgag + plP4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL3261 (pETMALenv + plP2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL3261 (pETMALenv + plP4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Plasmid plP4 is a lower copy number derivative of plP2 (previously also designated pYZ27bGP), which directs lower expression levels of genes positioned under control of a T7 promoter than plP2 (Tijhaar et al., 1997).

Stability was expressed as the percentage of bacteria that were still resistant to both antibiotics.

- **Preparation of iscom.** FIV Env iscom were prepared as described previously (Rimmelzwaan et al., 1994) from lectin-purified protein derived from BHK cells infected with the recombinant vaccinia virus vGR657x15. This recombinant vaccinia virus expressed a form of the envelope protein from which the cleavage site between the surface (SU) and transmembrane (TM) part of the protein had been deleted to facilitate incorporation into iscom.

  For the preparation of FIV Gag iscom, the complete coding sequence of the gag gene of molecular FIV clone 19k1 (Siebelink et al., 1992) was cloned in-frame with the bacterial expression vector pMALc (New England Biolabs) using PCR. Escherichia coli containing the resulting plasmid expressed an MBP–Gag fusion protein after IPTG induction, which was affinity-purified on an amylose column according to the procedure recommended by the manufacturer. The MBP–Gag fusion protein was coupled to palmitic acid (Reid, 1992) and then incorporated into the iscom via this hydrophobic anchor by a procedure similar to that described for the preparation of the FIV Env iscom.

  Iscom containing vaccinia-derived SIV Env and iscom containing SIV Gag derived from an MBP–Gag fusion protein, which were both a kind gift from E. Hulskotte, have been described elsewhere (Hulskotte et al., 1995).

- **Immunization of cats and FIV challenge.** Immunizations were performed as shown in Table 2. Two Env-expressing strains [i.e. SL3261(pETMALenv + plP2) and SL3261(pETMALenv + plP4)] and two Gag-expressing strains [i.e. SL3261(pETMALgag + plP2) and SL3261(pETMALgag + plP4)] were used. Although only the plasmid combination of pETMALgag with plP4 was stably maintained in the invasion assay (Tijhaar et al., 1997), SL3261(pETMALgag + plP2) was included in the vaccine formulation because of the higher expression levels obtained by this strain. The idea was that the combination with plP2 would present a higher amount of antigen early in infection, while the combination with plP4 would result in a lower but more stable expression. Equal amounts of the four different strains were used. For convenience, this combination of Gag- and Env-expressing strains is in short referred to as SL3261-FIV. As a control, the CtxB-expressing strain SL3261(pT7ctxB + plP2) (Tijhaar et al., 1997), in short referred to as SL3261-CtxB, was used. Per immunization, each cat received a total of 5 × 10¹¹ S. typhimurium SL3261 strains expressing the gag gene and strains expressing part of the env gene of FIV 19k1. † S. typhimurium SL3261 strain expressing CtxB. # Iscom containing FIV Env and Gag. § Iscom containing SIV Env and Gag.

Table 2. Immunization schedule

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Immunization at weeks 0 and 4</th>
<th>Immunization at weeks 10 and 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SL3261-FIV⁺</td>
<td>SL3261-FIV⁺</td>
</tr>
<tr>
<td>B</td>
<td>SL3261-FIV⁺</td>
<td>Iscom-FIV⁺</td>
</tr>
<tr>
<td>C</td>
<td>SL3261-CtxB⁺</td>
<td>Iscom-FIV⁺</td>
</tr>
<tr>
<td>D</td>
<td>SL3261-CtxB⁺</td>
<td>Iscom-SIV§</td>
</tr>
</tbody>
</table>

* Equal amounts of S. typhimurium SL3261 strains expressing the gag gene and strains expressing part of the env gene of FIV 19k1.
† S. typhimurium SL3261 strain expressing CtxB.
# Iscom containing FIV Env and Gag.
§ Iscom containing SIV Env and Gag.
Semi-quantitative PCR of proviral DNA. PBMC taken 16 weeks post-challenge (p.c.). Ten point titration was performed on chromosomal DNA prepared from E. J. Tijhaar and others

° at 60

70 'down' reactions, starting with two cycles at an annealing temperature of GAGACTTAGC (nucleotides 1440–1461). Both PCRs were 'touch-

GGACTAGGAGG (nucleotides 1106–1126) with GCACAGCTCGAG-

tides 1670–1692), and for the nested PCR reaction GGCAAGAGAA-

leotides 1014–1033) with GCATTTTATATCCTGGTGAGCC (nucleo-

reaction were GGCCCTCCACAGGCATATCC (corresponding to nuc-

uninfected cells was used. The primer combinations used in the first PCR as a negative control, chromosomal DNA isolated in parallel from

the first PCR reaction was used as template in the second (nested) PCR.

Detection of FIV infection. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood and cultured as previously described (Siebelink et al., 1990). Each iscom-immunized cat received 10 µg of each protein by the subcutaneous route. All cats were challenged intramuscularly with 30 50% cat-infectious doses (CID₅₀) of the homologous molecular FIV clone 19k1 (Siebelink et al., 1992) 2 weeks after the last immunization. Plasma samples were taken immediately before the primary immunization and after 2, 4, 6, 9-5, 10-5, 11, 13, 15, 17, 18-7, 18-5, 20, 22, 26 and 32 weeks.

■ Detection of FIV infection. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood and cultured as previously described (Siebelink et al., 1992). The presence of FIV antigen in the culture supernatant was detected using an FIV antigen capture ELISA (Siebelink et al., 1990). The cell-associated virus load was based on those dilutions that resulted in wells positive and negative for FIV antigen, assuming that one infected PBMC gave rise to antigen production after 3 weeks of cocultivation.

■ ELISA. The ELISAs to determine specific antibody responses against lipopolysaccharide (LPS) from S. typhimurium and against MBP were performed as described previously (Tijhaar et al., 1997).

Antibodies against the Gag proteins p24 and p17 were detected with a commercially available test kit, using recombinant p24 and p17 proteins (catalogue number F1002-AB01; European Veterinary Laboratory).

An ELISA based on the detection of antibodies to the bacterial fusion proteins SU1 and SU3 (see Fig. 1) was performed as described elsewhere (De Ronde et al., 1994). Plasma samples were used at a 1 : 100 dilution in this assay.

Results

Construction and characterization of pETMALc and pETMALenv

The vector pETMALp (Table 1) contains the malE gene under control of a T7 promoter. When present in bacteria that

5 × 10¹¹ bacteria by the oral route and 10⁸ bacteria by the i.p. route. Preparation and administration of the bacteria were performed as described previously (Tijhaar et al., 1997). Each iscom-immunized cat received 10 µg of each protein by the subcutaneous route.

Fig. 1. Representation of different malE-env fusions and their relative expression levels in S. typhimurium SL3261(pIP4). The top bar represents the env gene of FIV encoding the envelope protein, including the leader (L), surface (SU) and transmembrane (TM) proteins. The hypervariable regions are represented as black boxes. The bacterial fusion products SU1 and SU3 used in the Env-ELISA are indicated. The relative expression levels of the different malE-env fusions as determined by SDS–PAGE and Western blotting is shown on the right. The photograph shows a stained gel of bacterial lysates subjected to SDS–PAGE. Lanes: 1, molecular mass markers (in kDa); 2, SL3261; 3, SL3261(pETMALenv+pIP4) (pETMALenv = construct D). The arrowhead indicates the position of the MBP–Env fusion protein.

The ELISAs to determine specific antibody responses against lipopolysaccharide (LPS) from S. typhimurium and against MBP were performed as described previously (Tijhaar et al., 1997).

Antibodies against the Gag proteins p24 and p17 were detected with a commercially available test kit, using recombinant p24 and p17 proteins (catalogue number F1002-AB01; European Veterinary Laboratory).

An ELISA based on the detection of antibodies to the bacterial fusion proteins SU1 and SU3 (see Fig. 1) was performed as described elsewhere (De Ronde et al., 1994). Plasma samples were used at a 1 : 100 dilution in this assay.

Results

Construction and characterization of pETMALc and pETMALenv

The vector pETMALp (Table 1) contains the malE gene under control of a T7 promoter. When present in bacteria that
immunization experiments. Before the immunization experiments were started, plasmid stability in SL3261 strains after invasion of cat thymocytes was determined. Plasmid pETMALenv proved to be stable in combination with plp2 as well as with plp4 (Table 3).

**Plasma antibody responses after immunization**

The day following the first *Salmonella* immunization (Table 2), two out of ten cats appeared to be feeling unwell as they were much less active than usual. Two to three days later these cats seemed to have recovered completely. No obvious side-effects were observed following the *Salmonella* booster immunizations. All the cats developed plasma antibodies against *S. typhimurium* LPS within 2 weeks after the first immunization (Fig. 3). Considerable differences were observed between individual cats. Two weeks after the second immunization, titres ranged from 800 to 50000. The cats immunized with the MBP–Env- and MBP–Gag-expressing SL3261 strains (groups A and B) developed antibodies specific for the MBP moiety of the fusion protein within 2 weeks after the first immunization. Two weeks after the second immunization, the plasma titres were around 10000 for most of the cats of groups A and B. In spite of the strong antibody responses to the MBP moiety, no clear responses were detected against the Env or Gag moieties of the MBP fusion proteins. After the third immunization with SL3261-FIV, Gag-specific responses were detected in two of the three cats of group A (Fig. 3). After the first immunization with iscom-FIV (groups B and C), anti-Gag titres rose faster in the group that had previously been immunized with SL3261-FIV (group B) as compared to the group that had received SL3261-CtxB (group C) (Fig. 3), indicating a priming effect of the Gag-expressing SL3261 strains. The second iscom-FIV immunization of groups B and C at week 13 resulted in a considerable increase in the anti-Gag response (Fig. 3). Titres in the plasma samples of cats that had not been primed with SL3261-FIV (group C) were similar to those that had (group B). Even after a fourth immunization with SL3261-FIV, the Gag-specific antibody titre remained weak in the cats of group A. On the day of challenge, only plasma samples of the cats vaccinated twice with iscom-FIV

---

**Table 3. Plasmid stability after invasion of cat thymocytes**

Cat thymocytes were infected with the different bacterial strains and cultured in the presence of gentamycin to kill extracellular bacteria. After 2 days the percentage intracellular bacteria still resistant to both ampicillin and kanamycin was determined. Values are means ± SD of three independent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL3261(pETMALenv + plp2)</td>
<td>93 ± 7%</td>
</tr>
<tr>
<td>SL3261(pETMALenv + plp4)</td>
<td>92 ± 3%</td>
</tr>
</tbody>
</table>

---

**Fig. 2.** Expression of MBP from malE with and without a functional signal sequence, using the two-plasmid invertible promoter system. Total lysates of bacteria grown until late exponential phase at 37 °C were subjected to SDS–PAGE, followed by staining of the gel. Lanes: 1, SL3261(pETMALc + pIP4); 2, SL3261(pETMALp + pIP4); 3, SL3261(pETMALp); 4, SL3261; 5, molecular mass markers (in kDa).

---

produce T7 RNA polymerase, large amounts of the MBP are expressed from this gene (Tijhaar et al., 1997). The pIP vectors (Table 1), which contain the T7 RNA polymerase gene under control of an invertible promoter, allowed the continuous production of an MBP–Gag protein by a bacterial population (Tijhaar et al., 1997). The MBP–Gag protein was encoded by an in-frame fusion between the malE gene of pETMALp and the gag gene of FIV. Attempts to express parts of the FIV env gene using pETMALp were unsuccessful. In an attempt to reduce the toxicity of MBP fusion proteins, the region of malE coding for the hydrophobic part of the signal sequence was deleted so that the protein would remain localized within the cytoplasm. Expression of MBP by bacteria containing pIP4 (Table 1) in combination with the mutated plasmid, designated pETMALc, was at least as great as that from the unmutated plasmid pETMALp (Fig. 2). However, expression of the KpnI–KpnI env fragment of the molecular clone FIV 19k1 (Siebelink et al., 1992) in-frame with the malE gene of pETMALc (Fig. 1A) remained problematic. To determine which parts of the env gene were predominantly responsible for this lack of expression, different gene truncations were made (Fig. 1B, C and D). The malE fusion with the KpnI–BamHI env fragment was well expressed (Fig. 1B). Introduction of a frameshift by filling in the HindIII site (Fig. 1C) resulted in a translational stop codon within seven codons of this site. No expression was observed from this truncated malE–env fusion. The Bcl–KpnI fragment (Fig. 1D), which encodes the variable regions V-3, V-4 and V-5 in which several neutralizing B cell epitopes are localized, was well expressed (Fig. 1). Therefore, this plasmid, designated pETMALenv, was selected for immunization experiments.

Before the immunization experiments were started, plasmid stability in SL3261 strains after invasion of cat thymocytes was
Fig. 3. Development of antibody responses in cats after vaccination. In each graph, filled triangles, open squares and filled circles represent cats nos 1, 2 and 3, respectively. The small arrows at the bottom of the figure indicate days of immunization and the large arrow indicates the day of challenge.
Fig. 4. Development of antibody responses in cats after challenge. The OD_{450} values shown were obtained with plasma samples diluted 1:100. In each graph, filled triangles, open squares and filled circles represent cats nos 1, 2 and 3, respectively.
(groups B and C) showed clear reactivity with the bacteria-derived FIV envelope fusion protein SU3 (Fig. 4; 0 weeks p.c.). No significant antibody response to SU3 was detected after a single iscom-FIV immunization (results not shown). Cats of groups C and D showed an antibody response to MBP upon immunization with the respective iscom preparations, which contained MBP either as part of the Gag fusion protein (group C) or as a contaminant (group D) (Fig. 3).

Plasma antibody responses after FIV challenge

Two weeks after the last immunization, all cats were challenged with homologous molecularly cloned FIV (FIV 19k1). Gag-specific antibody titres in the plasma samples from cats vaccinated with iscom-FIV (groups B and C) rose within 3 weeks p.c. Cats of the control group (group D) and the group vaccinated four times with SL3261-FIV (group A) started to develop Gag-specific antibodies 5 weeks p.c.

Within 2-5 weeks p.c., the antibody responses to bacteria-derived FIV Env fusion proteins started to increase in the cats immunized with iscom-FIV (groups B and C) (Fig. 4). At this time, both cats immunized with SL3261-CtxB/iscom-FIV (group C) and one out of three cats immunized with SL3261-FIV/iscom-FIV (group B) had also developed responses to SU1. At 5 weeks p.c., the plasma samples of all the cats of groups B and C displayed plasma antibodies specific for SU1 and SU3. Both cats of the negative control (group D) developed antibodies specific for SU3 between 2 and 7 weeks p.c. and one cat also developed antibodies to SU1. In contrast, the cats only immunized with SL3261-FIV (group A) did not develop detectable antibody responses to SU1 during the whole 16 week period of the experiment. Only one cat (no. 1) of this group developed antibodies to SU3, but later than the control cats (group D).

Development of viraemia after FIV challenge

At 19 days p.c., FIV-infected PBMC were detected in both cats of the control group (group D), in two out of three cats of group B and in one of the two cats of group C (Fig. 5a). At this time, no infected PBMC were detected in the cats immunized four times with SL3261-FIV (group A). At 25 days p.c., FIV-infected PBMC were detected in all cats, except for one cat of group A. At 110 days p.c., FIV-infected PBMC were demonstrated in all cats of groups B, C and D, but in only one cat of group A. The average cell-associated virus loads at this time were higher in group C than in the other groups. The data for virus load at 110 days post challenge are shown in Fig. 5b.

Fig. 5. Virus load in cats after challenge. Loads are expressed (a) as the numbers of FIV-infected cells per 10^6 PBMC of individual cats at different time points and (b) as the minimum amount of chromosomal DNA isolated from PBMC 110 days after challenge required as template to obtain a positive FIV-specific PCR. In each graph, filled bars, hatched bars and white bars represent cats nos 1, 2 and 3, respectively.
time point were 8, 217, 38 and 31 infected cells per $10^6$ PBMC for groups A, B, C and D, respectively. PCR on chromosomal DNA isolated from PBMC taken at this time point demonstrated the presence of proviral FIV DNA in all cats (Fig. 5b). The overall lowest levels of proviral DNA were observed in the cats immunized four times with SL3261-FIV (group A).

**Discussion**

In the present study we have shown that cats immunized four times with FIV Gag- and Env-expressing *S. typhimurium* strains developed lower mean cell-associated virus loads upon homologous FIV challenge than the other groups of cats studied. Immunization with iscom-FIV, either alone or in combination with recombinant *S. typhimurium*, did not result in a decrease in cell-associated FIV load upon challenge. Due to the relatively small group sizes, the differences observed between the four groups can only provide an indication of a protective effect of vaccination in the first group of cats.

The Gag and Env proteins were expressed as fusions with MBP in the attenuated *S. typhimurium araA* strain SL3261. The expression levels of the different *env* gene truncations indicated that a region located between the BamHI and Bell site of *env* (Fig. 1) had a prohibitive effect on expression. It is likely that the region encoding the highly hydrophobic putative leader of the SU protein is mainly responsible for this effect. Antibodies to MBP, Gag and Env were induced more efficiently when these proteins were incorporated into iscom than when presented by SL3261 (Figs 3, 4). In fact, three immunizations with SL3261-FIV were required to induce detectable titres of Gag-specific antibodies and even four immunizations failed to induce Env-specific antibodies. However, the MBP fusion proteins expressed by the SL3261 strains were presented to the immune system of these animals since MBP-specific antibodies were detected within 2 weeks after the first immunization in five out of six cats immunized with SL3261-FIV (groups A and B; Fig. 3). Although after the second immunization Gag-specific antibodies were still not detected, the immune system had clearly been primed for this protein by SL3261-FIV as specific antibodies developed faster after the first iscom-FIV immunization in group B (SL3261-FIV/iscom-FIV) than in group C (SL3261-CtxB/iscom-FIV). Priming with SL3261-FIV apparently had no beneficial effect on the level of the MBP-, Gag- or Env (SU1/SU3)-specific plasma antibody responses (compare groups B and C; Figs 3, 4).

None of the evaluated immunization strategies protected the cats from FIV infection upon homologous challenge, but the reduced mean cell-associated virus loads (Fig. 5a), the reduced mean proviral DNA loads (Fig. 5b) and the much weaker induction of antibodies against SU1/SU3 after challenge in the cats immunized four times with SL3261-FIV as compared to cats of the other groups, suggested that this immunization regimen did have a protective effect. It is unlikely that the observed reduced FIV loads were the result of induced antibody responses as no significant Env-specific antibodies and only weak Gag-specific antibody titres were present at the time of challenge. Furthermore, no reduced virus load was observed in the iscom-FIV-immunized cats, which had much higher Gag- and Env-specific plasma antibody titres. This is not surprising in view of our recent observations that enhancement of FIV infection may occur after immunization with Env iscom (Siebelink et al., 1995). It seems therefore more likely that, if indeed the cats of group A were partially protected, a cellular rather than a humoral immune response was the basis of this protection. In our previous study, cats immunized with a *Salmonella* strain expressing only the FIV gag gene had a comparable virus load after challenge as control cats (Tijhaar et al., 1997). The reduced virus load in cats immunized with Salmonella strains expressing the gag and env genes observed in this study indicates that the *env*-expressing *Salmonella* strains are mainly responsible for the observed reduced virus load. Interestingly, Flynn et al. (1996) found that vaccine-induced protection against FIV infection correlated with Env- but not Gag-specific CTL responses. Alternatively, the reduced virus load observed in this study may not (only) have been due to the *env*-expressing *Salmonella* strains, but to the increased number of immunizations, the different route of administration (oral or i.p. in the previous study vs. oral and i.p. in this study) or a combination of these factors.

In conclusion, this study provides an indication of a protective effect of immunization with the *S. typhimurium* recombinants expressing the FIV Gag and Env proteins. However, further studies are required to evaluate the full potential of this approach for lentivirus vaccine development.

We thank Geert van Amerongen and Nico Schmidt for biotechnical assistance, Ellen Hulsotke for the SIV Gag and Env iscom and Conny Kruyssen for assistance in preparing the manuscript. This investigation was supported by a grant from the ‘Raad voor Gezondheidsonderzoek’, The Hague, The Netherlands (grant number 88-108/89027).

**References**


Clements, J. E., Montelaro, R. C., Zink, M. C., Amedee, A. M., Miller, S.,


Received 8 April 1997; Accepted 7 July 1997