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Induction of feline immunodeficiency virus specific antibodies in cats with an attenuated *Salmonella* strain expressing the Gag protein

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Salmonella typhimurium *aroA strains (SL3261), expressing high levels of the Gag protein of feline immunodeficiency virus (FIV) fused with maltose binding protein (SL3261-MFG), were constructed using an invertible promoter system that allows the stable expression of heterologous antigens at levels toxic for bacteria. A SL3261 strain expressing the B subunit of cholera toxin by a similar system (SL3261-CtxB) served as a control in FIV-immunization experiments. Cats immunized once orally or intraperitoneally with SL3261-MFG or SL3261-CtxB all developed serum antibodies to SL3261 lipopolysaccharide and against maltose binding protein or the B subunit of cholera toxin, respectively. Two intraperitoneal immunizations with SL3261-MFG also resulted in the development of Gag specific serum antibodies. Two oral immunizations with SL3261-MFG primed for a Gag specific response, which was demonstrated upon FIV challenge. All challenged cats became infected and no significant differences in viral loads were found between SL3261-MFG and SL3261-CtxB immunized cats. © 1997 Elsevier Science Ltd.*

Keywords: FIV; *Salmonella typhimurium*; invertible promoter

Feline immunodeficiency virus (FIV) infection of cats shows many similarities with human (HIV) and simian immunodeficiency virus (SIV) infections. It infects its natural host persistently, is T lymphotropic, causes a progressive loss of CD4$^+$ T cells, can infect macrophages and astrocytes, and eventually causes severe immunodeficiency or feline AIDS[1-6]. Because of these similarities, FIV infection of cats is considered a useful small-animal model for the evaluation of vaccine strategies relevant for controlling HIV-1 infection of humans.

In the search for effective lentivirus vaccines different strategies like inactivated complete virus[7], subunits[8-12], peptides[13], live attenuated virus[14-17], viral carriers[18-20], bacterial carriers[21,22] and nucleic acid immunizations[23,24] are being explored. In the FIV-cat system, vaccination of cats with paraformaldehyde fixed T cells (FL-4 cells) persistently infected with FIV and with paraformaldehyde inactivated FIV derived from the same cells, proved to be protective against homologous and to a lesser extent heterologous challenge[25,26]. In the SIV-macaque system, vaccination with whole inactivated virus induced partial protection against challenge with PBMC from a SIVmac infected macaque[27]. Earlier reported protection against challenge with cell-free virus after immunizing with inactivated whole SIVmac was almost certainly mediated by the immune response to cellular proteins incorporated into both the immunogen and the challenge viruses[27,28]. To date, the most effective protection of macaques against SIV infection has been obtained with live attenuated SIV[14,29]. However, safety issues are still a major subject of debate[17].

Live recombinant carriers like pox-viruses, BCG and attenuated *Salmonella* strains are being evaluated as alternative approaches for lentivirus vaccine development. Attenuated *Salmonella* strains have been used successfully to present heterologous bacterial[30,31], viral[32-34] and protozoal[35-37] antigens to the immune system of mammals. Systemic humoral and cellular responses, including class I restricted cytotoxic T lymphocytes (CTL), and mucosal humoral responses have been shown to result from vaccination with recombinant *Salmonella* strains[35,38-41].

The development of *Salmonella* bacteria as carriers for heterologous antigens has largely been hampered by problems with stability of expression or production levels caused by the toxicity of the antigens for the

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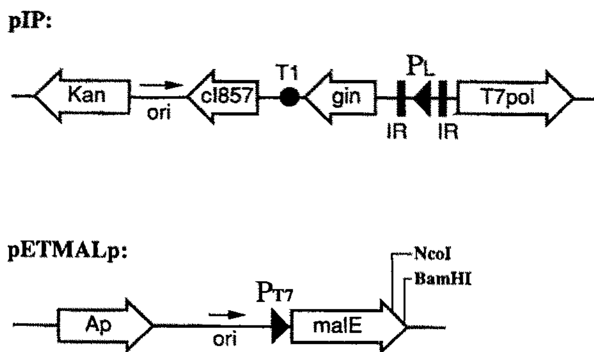

Figure 1 The two plasmid invertible promoter system. P_L, leftward promoter of bacteriophage lambda; IR, inverted repeat; T1, rrnB T1 transcription terminator; gin, invertase gene; cI587, phage lambda temperature sensitive repressor protein gene; Kan, kanamycin resistance gene; Ap, ampicillin resistance gene; malE, gene coding for maltose binding protein; T7pol, T7 RNA polymerase gene; P_{T7}, *gene-10* promoter of bacteriophage T7. The invertible promoter of pIP controls transcription of the T7 RNA polymerase gene, which in turn directs transcription of a gene located under control of the P_{T7} promoter on a second plasmid [e.g. pETMALp, pETMALgag (not shown) or pT7ctxB (not shown)]. In this study four different pIP-vectors (pIP2, -3, -4 and -5) were used. The vector pIP3 (=pYZ27GP) differs from pIP2 (=pYZ27bGP) by having an additional adenine in the untranslated leader sequence of the *gin* gene. The vectors pIP4 and pIP5 are derivatives from pIP2, respectively pIP3, in which the *p15A* origin of replication (*ori*) that results in an intermediate copy number, is replaced by the *ori* and partitioning (*par*) region of the low copy number plasmid pSC101. The *par* region ensures equal distribution of the plasmid over the daughter cells during cell division

producing *Salmonella* strains. Especially expression of non-bacterial antigens may cause problems due to different codon usage or the presence of toxic (e.g. hydrophobic) sequences. Therefore we[42–45] developed a system that allows the stable expression of antigens at levels toxic for individual bacteria. The system is based on an invertible promoter that controls the expression of T7 RNA polymerase. A single promoter inversion results in expression of T7 RNA polymerase, which in turn directs expression from genes located under the control of a T7 promoter present on another vector in the same cell (*Figure 1*). The key feature of the system is that the promoter controlling the T7 RNA polymerase expression inverts with a frequency that results in toxic levels of antigen expression in only a sub-population of the bacteria. Therefore the major part of the bacterial population will not be affected by expression of the heterologous antigen and will continuously segregate new antigen-producing bacteria.

Here we describe the use of this invertible promoter system to express high levels of FIV core protein as a fusion with the maltose binding protein (MBP) in the *S. typhimurium aroA* vaccine strain SL3261 and the use of this strain to induce specific immunity in cats.

MATERIAL AND METHODS

Bacterial strains, plasmids and media

Escherichia coli strain DH5α (BRL Life Technologies, Breda, The Netherlands) was used as a host for all plasmid constructions. Before introduction into the *S. typhimurium aroA* vaccination strain SL3261[46] plasmids were passaged through the restriction deficient, modification proficient *S. typhimurium* strain SL5283, a *galE503* derivative of LB5000[47,48].

Strain BL21DEpLysS[49] is a lysogen of the *E. coli* strain BL21 that contains a copy of the T7 RNA polymerase gene in the chromosome under control of the IPTG inducible *lac*UV5 promoter[50] and the pLysS plasmid which codes for T7 lysozyme; a natural inhibitor of T7 RNA polymerase. Strain BL21DEpLysS was used to express genes under the control of a T7 promoter. The T7 RNA lysozyme, encoded by pLysS, reduces the basal activity of T7 RNA polymerase in uninduced cells, thereby allowing the establishment of target genes whose basal expression would otherwise have been toxic to the cells. The vector pLysS confers resistance to chloramphenicol and is compatible with plasmids containing a ColE1 origin of replication[49]. All bacteria were grown aerobically at 37°C in Luria broth (LB), unless indicated otherwise. Ampicillin was used at 100 $\mu g\ ml^{-1}$ in LB agar plates and 200 $\mu g\ ml^{-1}$ in liquid media. Kanamycin and chloramphenicol were used at concentrations of 50 $\mu g\ ml^{-1}$ and 35 $\mu g\ ml^{-1}$, respectively, in agar plates as well as in liquid media.

Construction of the plasmids pYZ27bGP (=pIP2), pYZ27GP (=pIP3), pIP4 and pIP5 has been described previously[43,45]. The vectors pET3xa[49] and pMal-p were purchased from Invitrogen and New England Biolabs, respectively.

DNA manipulations

Isolation of plasmid DNA, preparation of DNA fragments and ligations were carried out using standard methods[51]. Plasmids were introduced into bacteria by electroporation as previously described[44].

Construction of plasmids used in this study

To increase the copy number of pET3xa[49] the *rop* gene, which negatively regulates plasmid copy numbers of ColE1 based plasmids, was removed by cleavage with *Bgl*II and *Pvu*II, followed by blunting of the ends with T4 DNA polymerase and religation of the plasmid. From the resulting plasmid, designated pET3xa1, a number of inconvenient restriction sites were removed by cleavage with *Eco*RI and *Eco*RV followed by blunting with T4 DNA polymerase and religation. The resulting plasmid was designated pET3xa2. The *malE* gene, which codes for precursor maltose binding protein (preMBP), was obtained by PCR using pMal-p (cat #800-61, New England Biolabs, Inc., Beverly, MA, USA) as template and the oligo nucleotides 5'-CGAGCATATGAAAATAAAAACAGGTGCACG-3' and 5'-GAATTCAGGCCTACCC TCGATGGATCC-3' as amplimers. The first amplimer was designed to introduce a *Nde*I site (underlined) at the position of the ATG start codon of *malE*. The second amplimer overlapped partially with the multiple cloning site (MCS) of pMAL-p, so that the PCR product would contain the 5'-part of the MCS, which includes a unique *Kpn*I site. The PCR product was cleaved with *Nde*I and *Kpn*I and cloned into the corresponding sites of pET3xa2 to generate pETMALp (*Figure 1*). In this plasmid transcription of the *malE* gene is under the control of the T7 promoter.

The vector pETMALgag was constructed by replacing the small NcoI-BamHI fragment of pETMALp (*Figure 1*) by the *Nco*I-*Bam*HI fragment of pMAL-gag, which contained the 3'-end of the *malE* gene in frame

Table 1 SL3261 strains used in this study

Strain	Plasmids	Heterologous antigen
SL3261	no	no
SL3261(pETMALgag)	pETMALgag	no
SL3261-MFG2	pETMALgag+pIP2	MFG[a]
SL3261-MFG3	pETMALgag+pIP3	MFG[a]
SL3261-MFG4	pETMALgag+pIP4	MFG[a]
SL3261-MFG5	pETMALgag+pIP5	MFG[a]
SL3261-CtxB	pT7ctxB+pIP2	CtxB[b]

[a]MFG, fusion protein of Maltose binding protein and FIV-Gag
[b]Ctxb, B subunit of cholera toxin

with the complete coding part of the *gag* gene of the molecular FIV clone 19k1. In pETMALgag, transcription of the *malE-gag* fusion is under the control of the T7 promoter.

Western blot analysis of bacteria

For Western blot analysis SDS–PAGE was immediately followed by transfer of the proteins to nitrocellulose. Blots were incubated for 30 min at room temperature with blocking buffer (BB) [0.5% Tween-20 and 0.5% low fat milk powder in PBS]. Subsequently, the blots were incubated for 1 h at room temperature with serum of an experimentally infected SPF cat (cat 89176), diluted 100 times in BB. After washing 3 times over a period of 30 min in PBS containing 0.5% Tween-20, the blot was incubated for 1 h at room temperature with a biotinylated anti-cat IgG monoclonal (Sigma Immuno Chemicals, clone CT-21, cat.nr. B-226) diluted in BB. After washing, the blot was incubated for 30 min at room temperature with alkaline phosphatase conjugated ExtrAvidin (Sigma Immuno Chemicals, cat.nr. E2636) diluted in BB. Finally the blot was washed three times, soaked for 10 min in substrate buffer (0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) and developed in substrate buffer containing Nitro Blue Tetrazolium (NBT) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP).

Detection of antigen expression by electron microscopy

To obtain an indication of the percentage of bacteria of strain SL3261-MFG4 (*Table 1*) that, due to a promoter inversion, expressed the MBP-Gag fusion protein, bacteria were grown in the presence of ampicillin and kanamycin until they reached the logarithmic phase. Subsequently, the bacteria were spun down (13,000 rpm) for 2 min, washed once with ice cold PBS and fixed with 4% paraformaldehyde in 0.1 M NaCacodylate (pH 7.4). Then the bacteria were transferred to 2% gelatine, impregnated in 2.3 M sucrose and frozen in liquid nitrogen. Finally ultra thin cryosections were made. The cryosections were subjected to immunogold labelling using the Gag specific moAB 2–11[52] diluted 100 × in PBS containing 0.5% BSA and 0.1% gelatine, and protein A conjugated to gold particles with a diameter of 10 nm (Aurion, Wageningen, The Netherlands, nr. 110.111) diluted 20 × in the same buffer. The sections were stained and embedded into methyl cellulose according to the method of Tokuyasu[53]. Subsequently, the sections were analysed by transmission electron microscopy (Phillips EM400). Bacteria were arbitrarily classified according to the amount of labelling. For this determination only complete longitudinal cross sections of bacteria were used.

Plasmid stability of bacterial strains during growth in thymocytes

The assay for determination of plasmid stability of SL3261 strains after invasion of eukaryotic cells was essentially performed as described previously[45]. In short, thymocytes derived from a specified pathogen free (SPF) cat were stimulated for 3 days with Concanavalin A, washed and subsequently maintained in culture medium (RPMI 1640 [Gibco] supplemented with 10% [v/v] FCS, 2 mM L-glutamine, penicillin [100 IU ml^{-1}], streptomycin [100 μg ml^{-1}], L-glutamine [2 mM] and IL2 [100 IU ml^{-1}, Cetus) at 37°C/5% CO_2. About 10^8 bacteria from logarithmic phase cultures were added to 10^6 thymocytes in a total volume of 1 ml RPMI 1640 without any additives. After 2 h incubation at 37°C the cells were washed and subsequently maintained at 37°C/5% CO_2 in 10 ml culture media containing gentamicin (50 μg ml^{-1}) to kill extracellular bacteria. After 24 h the plasmid stability was determined by lysing the thymocytes and comparing the number of CFUs that could be recovered on plates without and with the appropriate antibiotics.

Immunization and FIV challenge of cats

For immunization, bacteria from logarithmically growing cultures were harvested by centrifugation, washed, and resuspended in PBS. Subsequently, six months old specified pathogen free (SPF) cats, without detectable antibody levels against *Salmonella* LPS or CtxB, received 4 ml of 2.5×10^8 cells ml^{-1} by the intraperitoneal (i.p.) route or 10 ml of 5×10^{10} cells ml^{-1} directly into the stomach to simulate oral administration. This "oral" immunization was performed with anaesthetized cats using a syringe connected to a thin tube that reached into the stomach. Just prior to oral administration of the bacteria, gastric juices were neutralized by administration of 10 ml 10% $NaHCO_3$, using the same device.

Two cats were immunized with SL3261 expressing Gag, one cat by the oral route and the other by the i.p. route. The primary immunization (week 0) was performed with SL3261-MFG4, whereas the secondary immunization (week 9) was performed with equal amounts of strains SL3261-MFG2 and SL3261-MFG4 (*Table 1*). As a control, two cats were immunized with the CtxB expressing strain SL3261-CtxB (*Table 1*) at week 0 and 9. Again, one cat was immunized by the oral route and the other by the i.p. route. Eighteen weeks after the primary immunization all cats were challenged intramuscularly with 30 ID50 of the molecular FIV clone 19k1[54]. Serum samples were taken at 1, 2, 3, 4, 5, 9, 10, 13 and 15 weeks after the primary immunization, immediately prior to FIV challenge and 4, 7, 10, 14, 19, 24, 28, 33 and 67 days post challenge.

Detection of FIV infection

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood and cultured in limiting dilutions as previously described[54] to enumerate percentages of FIV infected cells. The presence of FIV

antigen in culture supernatants was detected with a FIV antigen capture ELISA as previously described[55].

ELISAs

LPS-ELISA. The detection of *S. typhimurium* LPS specific antibodies was essentially performed as previously described[44]. The incubation step with HRP conjugated anti-mouse antibody was replaced by an incubation for 90 min at 37°C with a biotinylated anti-cat IgG monoclonal antibody (Sigma Immuno Chemicals, clone CT-21, cat.nr. B-226) diluted 1:1000, followed by incubation for 1h at 37°C with a streptavidin–biotin–HRP complex (cat.nr. RPN1051, Amersham) also diluted 1:1000. The titer was defined as the highest dilution of test samples at which the O.D. 450 was higher than the mean O.D. 450+3 times S.D. of pre-immune sera.

CtxB-ELISA. The ELISA for the detection of CtxB specific antibodies differed from the LPS-ELISA in the coating step, which was performed in PBS containing 5.0 μg ml^{-1} CtxB (List Biological Labs, Campbell, CA, USA) and the dilutions of the biotinylated anti-cat IgG monoclonal antibody (1:2000) and the streptavidin HRP-conjugate (1:2500). In addition the ELISA-buffer in which the cat sera were diluted, was supplemented with 1% SL3261 sonificate prepared from a 50 times concentrated o/n bacterial culture to reduce background reactions. The titer was defined as the highest dilution of test samples at which the O.D. 450 was at least three times higher than that of the corresponding serum dilution of the control cat immunized in a similar way with SL3261 expressing MBP-Gag instead of CtxB.

MBP-ELISA. The ELISA for the detection of MBP specific antibodies was performed identical to that for CtxB except that for the coating step 3.0 μg ml^{-1} MBP was used. The MBP was isolated from strain DH5α(pMALc) by affinity chromatography with maltose–Sepharose using a commercially available kit (Protein Fusion and Purification System, cat #800, New England Biolabs, Inc., Beverly, MA, USA). The titer was defined as the highest dilution of test samples at which the O.D. 450 was at least two times higher than that of the corresponding serum dilution of the control cat immunized in a similar way with SL3261 expressing CtxB instead of MBP-Gag.

Gag-ELISA. Antibody titers against p17 and p24 core proteins were determined using a commercially available ELISA (FIV-p24/p17 antibody test kit, cat. nr. F1002-AB01, European Veterinary Laboratory BV, Woerden, The Netherlands).

RESULTS

Expression of FIV-Gag

For use in the two plasmid invertible promoter system (*Figure 1*), the *malE* gene was placed under the control of the T7 promoter. The resulting plasmid, designated pETMALp (*Figure 1*), was transferred to the *E. coli* strain BL21DEpLysS which contains on its chromosome the T7 RNA polymerase gene under control of the IPTG inducible *lac*UV5 promoter.

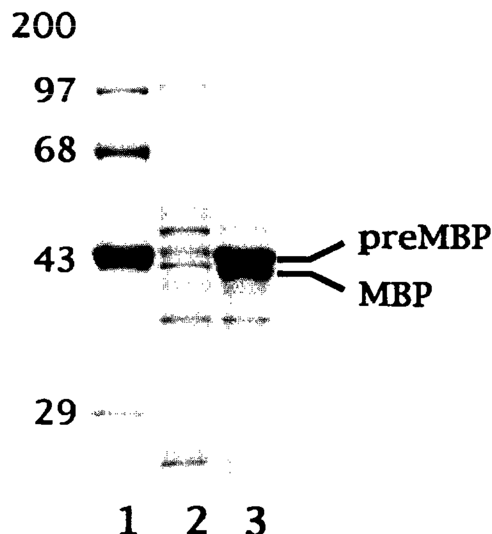

Figure 2 Expression of MBP by BL21DEpLysS(pETMALp). Five hours after the addition of 1 mM IPTG to logarithmically growing bacteria, cells were harvested and total bacterial lysates were subjected to SDS–PAGE followed by staining of the gel. Lanes: 1. molecular weight markers; 2. BL21DEpLysS; 3. BL21DEpLysS (pETMALp). Numbers on the left refer to the molecular sizes of the marker proteins in kDa

IPTG-induction of the T7 RNA polymerase gene in BL21DEpLysS(pETMALp) resulted in the expression of the *malE* gene (*Figure 2*). A prominent band with a Mw corresponding to preMBP, as well as a weaker band with a Mw corresponding to MBP from which the signal sequence had been cleaved-off, were visible on a Coomassie Blue stained SDS–PAGE gel.

To obtain high expression levels of the *gag* gene of the molecular clone FIV 19k1, it was cloned in frame with the *malE* gene of pETMALp and the resulting plasmid was designated pETMALgag. The plasmid pIP2 (=pYZ27bGP) (*Figure 1*) contains the T7 RNA polymerase gene under control of an invertible P_L-promoter. *S. typhimurium* strain SL3261, harbouring pETMALgag in combination with pIP2, designated SL3261-MFG2, expressed high amounts of the MBP-Gag fusion protein (*Figure 3*A). The presence of the Gag-moiety of this fusion protein was confirmed by Western blotting which shows the full-length MBP-Gag band and a number of break-down products (*Figure 3*B).

Inversion frequency and plasmid stability

Previously[45] it had been demonstrated that pIP3, which differs from pIP2 by having an extra nucleotide in the untranslated leader sequence of *gin*, reveals slightly lower expression levels of genes under the control of a T7-promoter than pIP2. The vectors pIP4 and pIP5 are lower copy number derivatives of pIP2 and pIP3, respectively, which reveal significantly lower expression levels than the parental plasmids. The pIP-vectors were analyzed by SDS–PAGE (*Figure 4*A) and Western blotting (*Figure 4*B). Three individual clones were tested for each of the plasmid combinations. The highest MBP-Gag production was obtained for SL3261-MFG2, followed by SL3261-MFG3 and SL3261-MFG4, which reveal comparable expression levels, and finally SL3261-MFG5.

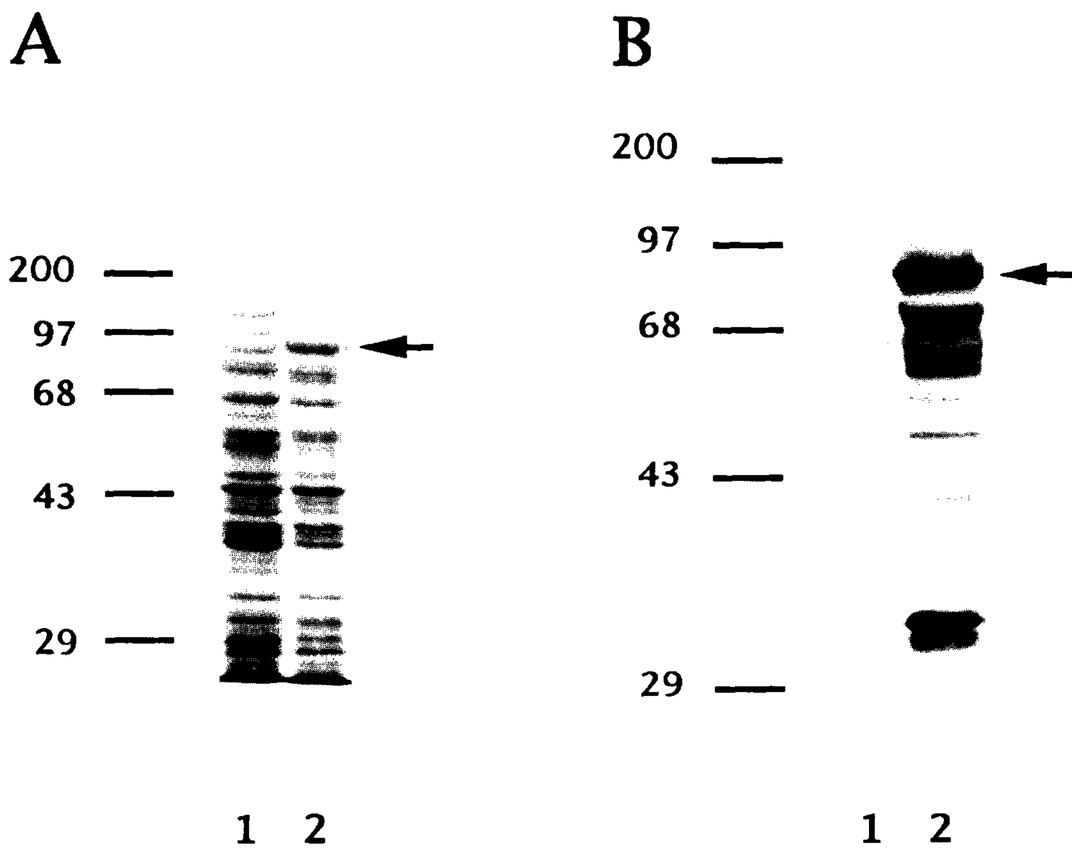

Figure 3 Expression of MBP-Gag by SL3261 containing the two-plasmid invertible promoter system. Cells were grown at 37°C, lysed and subjected to SDS–PAGE. Lanes: 1. SL3261; 2. SL3261-MFG2. (A) Coomassie-blue stained SDS–PAGE gel; (B) Western blot using serum of an experimentally FIV-infected SPF cat. The arrow indicates the position of the full-length MBP-Gag fusion protein

Figure 4 Expression of MBP-Gag by different SL3261-MFG strains. Total cell lysates of bacteria grown at 37°C are shown. For each strain three individual clones were analysed. Lanes: 1. SL3261; 2. SL3261(pETMALgag); 3-5. SL3261-MFG3; 6-8. SL3261-MFG2; 9-11. SL3261-MFG5; 12-14. SL3261MFG4; (A) Coomassie-blue stained SDS–PAGE gel; (B) Western blot using serum of an experimentally FIV-infected SPF-cat. The arrow indicates the position of the MBP-Gag fusion protein. Only the relevant part of the SDS–PAGE and WB is shown

The plasmid stability of SL3261 strains after growth in eukaryotic cell lines gives an indication of the *in vivo* plasmid stability[45]. Therefore the plasmid stability of the SL3261-MFG strains was determined in cat thymocytes cultured *in vitro* (*Table 3*). In this invasion assay pETMALgag alone was stably maintained in SL3261, but the combinations of pETMALgag with pYZ27bGP (=pIP2), and pYZ27GP (=pIP3) were not. The majority of the bacteria harbouring one of these combinations lost one or both plasmids within 24 h. Furthermore, the bacteria that still contained both plasmids, appeared to be mutants that did not express the fusion protein. In contrast, the combinations of pETMALgag with pIP4 or pIP5 were relatively stable and all the tested bacteria still expressed the MBP-Gag fusion protein.

Of the two stably expressing strains the highest expression levels were obtained with SL3261-MFG4. To obtain an indication of the percentage of the cells, that

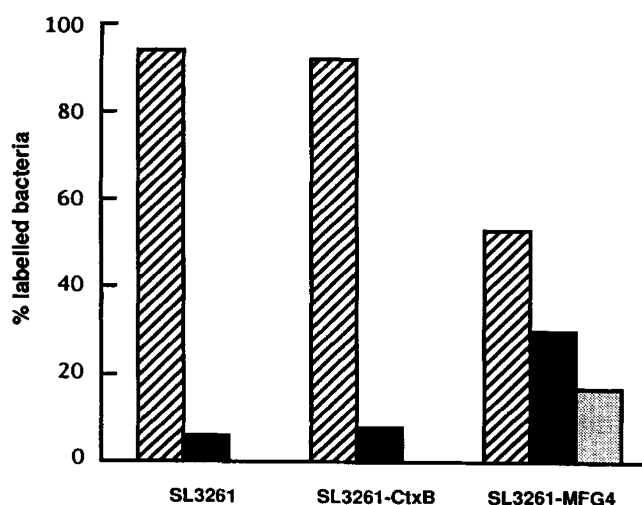

Figure 5 Percentage immunogold labelled bacteria using a Gag specific monoclonal antibody. Logarithmically growing bacteria were fixed, immunogold labelled and analysed by transmission electron microscopy. Bacteria were classified according to the amount of goldparticles/cell: 0 (striped); 1-3 (black); more than 3 (dotted)

Table 2 Immunization schedule of cats with SL3261 strains expressing heterologous antigens

Cat (no)	Strain	Route	Immunizations (week)	Challenge[a] (week)
1	SL3261-CtxB	oral	0 and 9	18
2	SL3261-CtxB	i.p.	0 and 9	18
3	SL3261-MFG	oral	0 and 9	18
4	SL3261-MFG	i.p.	0 and 9	18

[a]Challenge was performed i.m. with 30 cat ID50 of the molecular clone IV 19k1

expressed the MBP-Gag fusion protein in this strain, bacteria from logarithmic growth cultures were fixed, immunogold labelled on ultra-thin cryosections and analysed by means of transmission electron microscopy. Bacteria were arbitrary classified according to the amount of labelling (*Figure 5*). Of the cells of the negative control strains SL3261 and SL3261-CtxB, 94% and 92% were not labelled, respectively. The remaining 6% and 8% respectively, were labelled with only one to three gold particles. Of strain SL3261-MFG4 53% of the cells were not labelled, 30% contained one to three gold particles and 17% contained more than 3 particles, with an average of 10.5 particles/bacterium. Taking the 7% background labelling of the control strains into consideration, the percentage cells from strain SL3261-MFG4 expressing MBP-Gag is approximately 40% (=30 − 7+17) of the total population.

Immunogenicity of SL3261-MFG and SL3261-CtxB in the cat

Two cats were immunized with SL3261-MFG4 and two cats with strain SL3261-CtxB, which previously induced high titers of CtxB specific antibodies in mice[45]. Within each group one of the cats was immunized orally and the other one intraperitoneally (i.p.) (*Table 2*). All animals developed antibodies to *S. typhimurium* LPS within one week after the first immunization (*Figure 6*a). After administration of SL3261-CtxB, high anti-CtxB serum titers were induced (*Figure 6*b). After the primary immunization with SL3261-MFG4, considerable anti-MBP IgG titers were induced in the i.p. immunized cat (*Figure 6*c). The orally immunized cat developed a weak specific IgG response after the first immunization, but the second immunization at week 9 resulted in a considerably increased response.

After the primary immunization with SL3261-MFG4, no antibodies specific for the Gag-moiety of the MBP-Gag fusion protein could be detected. This strain was selected for the primary immunization, because the plasmids in this strain were relatively stable in the invasion assay (see *Table 3*). The second immunization was performed with equal amounts of SL3261-MFG4 and SL3261-MFG2. Although the plasmid combination of SL3261-MFG2 was not stably maintained in the invasion assay, it was incorporated in the second immunization because it expressed higher levels of MBP-Gag. The idea was that SL3261-MFG2 would present a higher amount of MBP-Gag early in infection, while SL3261-MFG4 would give a lower but more stable expression. One week after the second immunization Gag-specific antibodies were detected in the serum of the cat immunized by the i.p. route (*Figure 6*d). No Gag-specific antibodies were detected in the serum of the orally immunized cat.

To determine whether immunization with SL3261-MFG had induced protective cellular immune responses, all cats were challenged intramuscularly with 30 ID50 of the homologous molecular clone FIV 19k1, eighteen weeks after the first immunization. Between 14 to 19 days post challenge (p.c.) all the animals had developed a persistent viraemia (*Table 4*). No significant differences in viral load were observed between the cats immunized with SL3261-MFG or SL3261-CtxB (*Table 5*). Both cats immunized with the SL3261-MFG strains developed an anamnestic response to Gag as antibody titers to Gag started to rise two weeks earlier in these cats than in the cats immunized with SL3261-CtxB (*Figure 6*d).

DISCUSSION

In the present paper we have shown that the *S. typhimurium* strain SL3261 can be used efficiently as a vector to present heterologous antigens to the feline immune system. Cats immunized with SL3261-MFG developed a Gag specific immune response, but were not protected from intramuscular challenge with the homologous molecular clone FIV 19k1.

The development of candidate vaccines based on recombinant *Salmonella* as live carriers, has largely been hampered by the inability to stably express heterologous antigens at levels that are toxic for the producing bacteria. However, we previously[45] described a two plasmid invertible promoter system which may solve this problem. The system is based on the pIP-vectors which contain the T7 RNA polymerase gene under control of a promoter that inverts at random. When the promoter inverts to the ON position, T7 RNA polymerase is expressed which in turn directs expression of the gene of interest that has been positioned under the control of a T7 promoter on a second plasmid. The system can be stably maintained when a pIP vector is used that results in toxic antigen expression in only a minor part of the bacterial population.

In the present study we used the invertible promoter vectors pIP2 (=pYZ27bGP), pIP3 (=pYZ27GP), pIP4

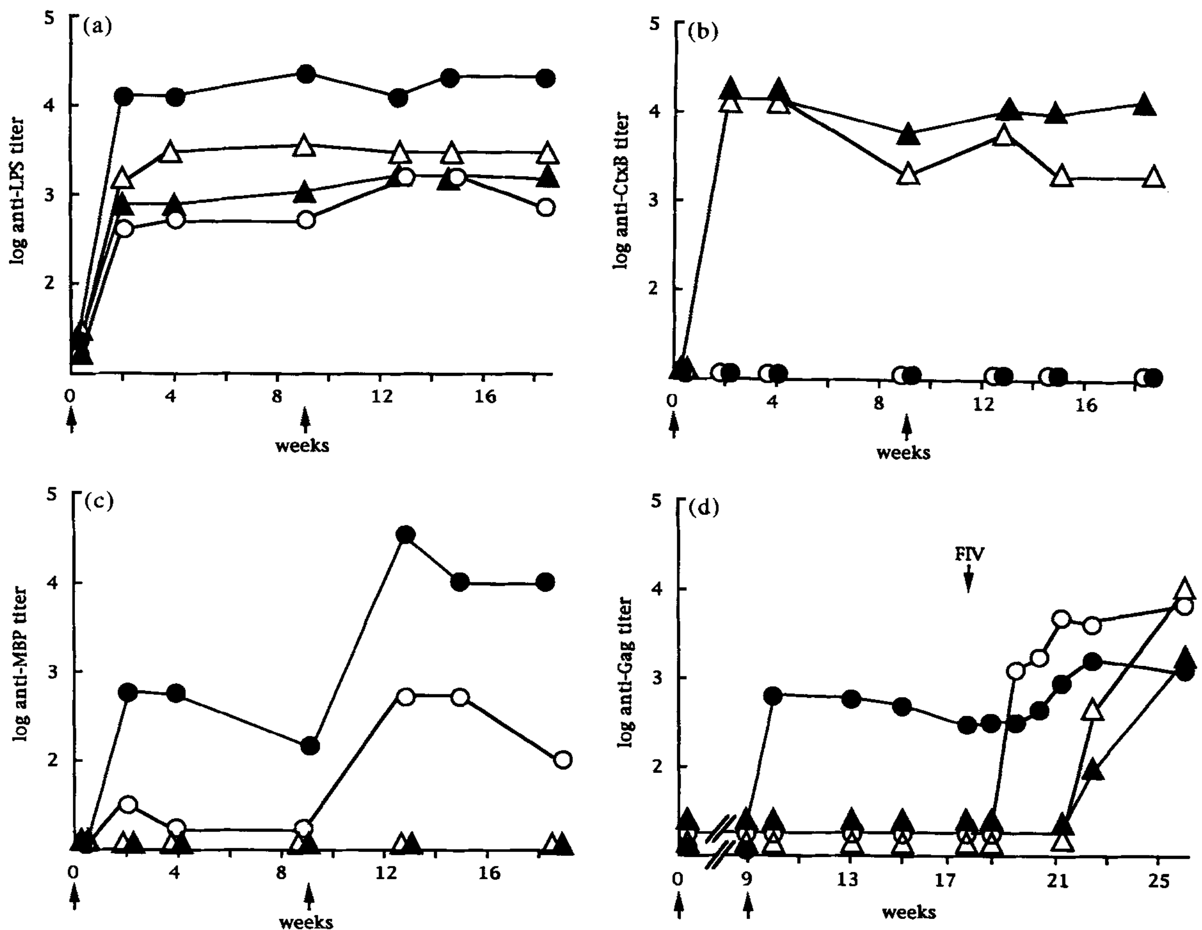

Figure 6 The IgG antibody titers in cat sera immunized with recombinant *S.typhimurium* vaccine strains and challenged with FIV. Cats were immunized orally (open symbols) or i.p. (closed symbols) at week 0 and 9 with SL3261-MFG (circles) or SL3261-CtxB (triangles). Eight weeks after the booster immunization the cats were challenged intramuscularly with a dose of 30 cat ID50 FIV. (a) LPS-specific antibody titer; (b) CtxB-specific antibody titer; (c) MBP-specific antibody titer; (d) core proteins p17/p24-specific antibody titer.

Table 3 Stability of recombinant SL3261 strains in cat thymocytes cultured *in vitro*

strain	Stability plasmids[a]	expression[b]
SL3261(pETMALgag)	99.5±0.5%	
SL3261-MFG2	17±2%	0/2
SL3261-MFG3	7±2%	0/2
SL3261-MFG4	71±21%	4/4
SL3261-MFG5	79±19%	3/3

[a]Cat thymocytes were infected with the different bacterial strains and cultured for 24 h in the presence of gentamicin to kill extracellular bacteria. The plasmid stability was determined by lysing the thymocytes and comparing the number of CFU on LB-agar plates with and without all the appropriate antibiotics.
[b]Number of MBP-Gag expressing colonies/total number of tested colonies harbouring both plasmids.

and pIP5 to express the Gag protein of FIV as a fusion product with MBP. *S. typhimurium aroA* strains SL3261-MFG (*Table 1*) expressed high levels of the MBP-Gag fusion protein. The highest expression levels were obtained with SL3261-MFG2 followed by SL3261-MFG3 and SL3261-MFG4, which revealed similar expression levels, and finally SL3261-MFG5. Of the different SL3261-MFG strains, only SL3261-MFG4 and SL3261-MFG5 stably maintained their plasmids after invasion of a cat thymocyte cell line (*Table 3*). Although

Table 4 Detection of FIV in cat PBMCs after challenge

Immunization: stain/route	Presence of FIV at day (p.c.): 4	7	10	14	19	24	60
SL3261-MFG/oral	-	-	-	+	+	+	+
SL3261-MFG/i.p.	-	-	-	+	+	+	+
SL3261-CtxB/oral	-	-	-	-	+	+	+
SL3261-CtxB/i.p.	-	-	-	-	+	+	+

PBMC were stimululated with 10 μg ml^{-1} ConA for three days, washed and maintained in culture medium containing 100 μ/ml^{-1} r-IL-2. The presence of FIV-antigen was detected using an antigen capture ELISA

Table 5 Viral load after FIV challenge

Immunization: antigen/route	Infected cells per 10^6 PBMC (days p.c.): 14	19	33	67
SL3261-MFG/oral	NT	12.5	58	75
SL3261-MFG/i.p.	<12.5	NT	133	375
SL3261-CtxB/oral	<12.5	NT	79	150
SL3261-CtxB/i.p.	<12.5	NT	79	112

SL3261-MFG3 and SL3261-MFG4 expressed similar amounts of the MBP-Gag fusion protein, indicating the presence of comparable percentages of antigen

expressing bacteria, a striking difference in plasmid stability between both strains was observed. The higher plasmid stability of SL3261-MFG4 compared to SL3261-MFG3, is most likely the result of the partitioning (*par*) region present on pIP4 which is absent on pIP3. This *par* region ensures equal distribution of the plasmid copies over the daughter cells during cell division, what greatly enhances plasmid stability[56].

Immunogold labelling of SL3261-MFG4 demonstrated that about 40% of the bacteria expressed MBP-Gag (*Figure 5*). This does not mean that all these expressing bacteria actually contain a pIP plasmid with the P_L promoter in the ON position. It may be speculated that a number of these cells have been derived from cells that divided after a promoter inversion occurred, but before antigen expression reached a level that prevented further cell divisions.

The *S. typhimurium* vaccination strain SL3261 appeared to be immunogenic in cats. After i.p. as well as after oral immunization, high specific antibody titers to *S. typhimurium* LPS were induced. Moreover, SL3261 can also be used as a vector to present heterologous antigens to the immune system of cats, as high serum antibody titers were induced to CtxB and MBP. The responses to FIV Gag were considerably weaker. A second immunization was necessary to induce detectable Gag specific serum antibodies upon i.p. immunization. After two oral immunizations no Gag-specific antibodies were detected, but the immune system had clearly been primed for Gag protein recognition, as after FIV challenge Gag-specific antibodies could be detected two weeks earlier than in the control cats. It is unlikely that this is the result of enhancement of FIV infection, as we observed with other FIV vaccine candidates[57], since the FIV loads of this cat at 33 and 67 days post challenge were even lower than those of the control cats. It should be stressed that the specific antibody responses were mainly determined as a marker to demonstrate that the heterologous antigens expressed by SL3261 are presented to the immune system of cats, since it cannot be expected that antibodies against the internal Gag proteins would neutralize FIV. In general *S. typhimurium* preferentially induces cellular immune responses, including cytotoxic T cell responses[35–38,40] which may contribute to protective immunity[58]. No FIV specific T cell responses were determined in the framework of these experiments. However, if indeed Gag specific T helper and CTL responses were induced by these immunizations, they were apparently not sufficient to protect the cats against the homologous challenge with 30 CID50 FIV.

We are currently investigating the immune responses and priming effects in cats, immunized with SL3261 strains expressing the Gag and envelope proteins of FIV in order to generate a more complete immune response to FIV proteins that will hopefully result in protection.

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