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Adverse effects of feline IL-12 during DNA vaccination against feline infectious peritonitis virus

Harrie L. Glansbeek,[1] Bart L. Haagmans,[1,2] Eddie G. te Lintelo,[1] Herman F. Egberink,[1] Véronique Duquesne,[3] André Aubert,[3] Marian C. Horzinek[1] and Peter J. M. Rottier[1]

[1] Virology Division, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

[2] Institute of Virology, Erasmus University Rotterdam, Rotterdam, The Netherlands

[3] Virbac Laboratories Inc., 06511 Carros Cedex, France

Cell-mediated immunity is thought to play a decisive role in protecting cats against feline infectious peritonitis (FIP), a progressive and lethal coronavirus disease. In view of the potential of DNA vaccines to induce cell-mediated responses, their efficacy to induce protective immunity in cats was evaluated. The membrane (M) and nucleocapsid (N) proteins were chosen as antigens, because antibodies to the spike (S) protein of FIP virus (FIPV) are known to precipitate pathogenesis. However, vaccination by repeated injections of plasmids encoding these proteins did not protect kittens against challenge infection with FIPV. Also, a prime–boost protocol failed to afford protection, with priming using plasmid DNA and boosting using recombinant vaccinia viruses expressing the same coronavirus proteins. Because of the role of IL-12 in initiating cell-mediated immunity, the effects of co-delivery of plasmids encoding the feline cytokine were studied. Again, IL-12 did not meet expectations – on the contrary, it enhanced susceptibility to FIPV challenge. This study shows that DNA vaccination failed to protect cats against FIP and that IL-12 may yield adverse effects when used as a cytokine adjuvant.

Introduction

Feline infectious peritonitis (FIP) is a progressive and lethal infection of domestic cats with feline coronaviruses (FCoVs). Members of the family *Coronaviridae* are enveloped, plus-stranded RNA viruses. The large genome (±30 kb) of a coronavirus is surrounded by a nucleocapsid (N) protein and the elongated nucleocapsid is enveloped by a lipoprotein membrane. Three membrane proteins have been identified: the spike (S) protein, the membrane (M) protein and the small envelope (E) protein. FCoVs are widespread in the feline population; antibodies are found in 80–90% of cats in catteries and in 10–50% in single cat households (Addie & Jarrett, 1992; Loeffler *et al.*, 1978; Pedersen, 1976b). However, the disease, FIP, occurs in only 5–10% of seropositive cats (Addie & Jarrett, 1992; Pedersen, 1976a, b) and is caused by virulent FCoV mutants that arise in individual animals (Vennema *et al.*, 1998). These mutants are conveniently designated FIP viruses (FIPV).

Attempts to vaccinate cats against FIP have been largely unsuccessful. Vaccination with an avirulent FCoV (Pedersen & Black, 1983) or a recombinant vaccinia virus expressing the S protein (Vennema *et al.*, 1990) failed to induce protection and even exacerbated the disease. Administration of closely related human, canine or porcine coronaviruses also failed to protect cats (Barlough *et al.*, 1984, 1985; Stoddart *et al.*, 1988; Woods & Pedersen, 1979). Currently, a temperature-sensitive strain of FIPV is marketed as a vaccine (Christianson *et al.*, 1989). Although its ability to protect cats against FIPV was demonstrated (Gerber *et al.*, 1990; Gerber, 1995), the efficacy of this vaccine is a matter of debate (Fehr *et al.*, 1997; McArdle *et al.*, 1995; Scott *et al.*, 1995).

A major obstacle for vaccine development is the fact that coronavirus antibodies are not protective – rather, they enhance disease progression, as demonstrated by passive immunization of kittens with anti-FIPV antibodies (Weiss & Scott, 1981). This effect is due to anti-S antibodies: vaccination of kittens with a recombinant vaccinia virus expressing the S

Author for correspondence: Harrie Glansbeek.
Fax +31 30 2536723. e-mail H.Glansbeek@vet.uu.nl

0001-7867

protein (Vennema *et al.*, 1990), but not with M or N protein recombinants (Vennema *et al.*, 1991), resulted in early death. Antibodies against the S protein were also shown to induce antibody-dependent enhancement of infection of macrophages *in vitro* (Corapi *et al.*, 1992; Hohdatsu *et al.*, 1998; Olsen *et al.*, 1992). On the other hand, cell-mediated immunity appears to play a protective role. Cats that have recovered from FIP exhibit strong blastogenic and delayed hypersensitivity responses (Pedersen & Floyd, 1985). In addition, the N protein was found to induce protective immunity in a vaccination protocol where kittens were primed with a recombinant raccoon poxvirus and boosted with an avirulent FCoV (Wasmoen *et al.*, 1995). In view of its internal position in the virion, it is unlikely that antibodies played a role in the protective immunity induced by this vaccination protocol.

A recent approach to induce cell-mediated immunity against infectious agents utilizes plasmids encoding the protection-relevant antigen(s) and their endogenous expression in the host organism. These DNA vaccines often efficiently prime antigen-specific $CD4^+$ T helper cells as well as $CD8^+$ cytotoxic T cells (CTLs). DNA vaccines have been shown to induce protective immunity against herpes simplex virus (Manickan *et al.*, 1995), pseudorabies virus (PRV) (Gerdts *et al.*, 1997; Haagmans *et al.*, 1999; van Rooij *et al.*, 2000), influenza A virus (Yokoyama *et al.*, 1997) and lymphocytic choriomeningitis virus (LCMV) infection (Yokoyama *et al.*, 1997).

Immunity induced by DNA vaccination is enhanced when the immune system is boosted with another vaccine formulation, e.g. recombinant vaccinia virus. This was elegantly shown in studies with the malaria parasite *Plasmodium berghei*. Repeated application of plasmid DNA encoding pre-erythrocyte antigens conferred only limited protection to mice. However, priming with DNA followed by a single boost with a recombinant vaccinia virus expressing the same antigen resulted in complete protection and high levels of $CD8^+$ T cells (Schneider *et al.*, 1998). A similar DNA/vaccinia virus protocol was found to elicit the highest CTL responses in a human immunodeficiency virus vaccination study (Hanke *et al.*, 1998).

Another improvement of DNA vaccine efficacy has been attained by the co-injection of cytokine-encoding plasmids. In this respect, plasmids encoding IL-12 are particularly promising in that they stimulate T helper 1 (Th1) responses (Chow *et al.*, 1998; Sin *et al.*, 1999a, b; Tsuji *et al.*, 1997) and enhance the induction of antigen-specific $CD8^+$ CTLs (Hamajima *et al.*, 1997; Kim *et al.*, 1997; Okada *et al.*, 1997; Tan *et al.*, 1999; Tsuji *et al.*, 1997). In several studies, co-delivery of IL-12-encoding plasmids with DNA vaccines resulted in enhanced protection against virus infections and tumours (Boretti *et al.*, 2000; Chow *et al.*, 1998; Sin *et al.*, 1999a, b; Tan *et al.*, 1999).

In view of these considerations, we have investigated the potential of DNA vaccination against FIP, adopting a prime–boost protocol as well as co-delivery of IL-12-encoding plasmids.

Methods

■ **Construction and purification of plasmid DNA expression vectors.** The gene encoding the M protein of FIPV strain 79-1146 was isolated from pSCF-M (Vennema *et al.*, 1991) by *Bam*HI/*Eco*RI digestion. The 3′ recessive ends were filled in using the large fragment of DNA polymerase I enzyme (Klenow) and the fragment was ligated into *Eco*RV-digested VR1012 (Hartikka *et al.*, 1996) (Vical) to yield VR1012-M. Thus, the gene was cloned behind the human cytomegalovirus (CMV) immediate-early promoter and intron A and in front of the bovine growth hormone polyadenylation-processing signal (BGH polyA). Similarly, the N gene of FIPV 79-1146 was excised from pSCF-N (Vennema *et al.*, 1991) by *Bam*HI/*Eco*RI digestion. The fragment was also blunt-ended using Klenow and ligated into *Eco*RV-digested VR1012 to yield VR1012-N.

To construct a vector encoding both FIPV-M and FIPV-N, the entire expression cassette of VR1012-M (containing the CMV promoter, FIPV-M cDNA and BGH polyA) was purified after *Apa*LI digestion and cloned into *Dra*I-digested VR1012-N to yield VRMVRN. To introduce the stimulatory CpG sequences present in the ampicillin resistance (Amp^r) gene (Roman *et al.*, 1997; Sato *et al.*, 1996), this gene was excised from pcDNA-3 (Invitrogen) by *Bsp*HI digestion, blunt-ended using Klenow and ligated into *Dra*III-digested VRMVRN to yield VRMVRN-CpG.

IL-12 is a heterodimeric protein which is composed of disulfide-bonded 35 kDa (p35) and 40 kDa (p40) subunits. The genes encoding the subunits of feline IL-12 have been cloned in our laboratory (Schijns *et al.*, 1997). Each cDNA was initially cloned separately into *Eco*RV-digested VR1012, yielding VR1012-p35 and -p40. To obtain a vector that encodes both the p35 and the p40 chain, the entire expression cassette from VR1012-p35 was excised by *Apa*LI digestion. The 3′ recessive ends were filled in using Klenow and the fragment was cloned into *Dra*I-digested VR1012-p40 to yield VR1012-fIL12.

Plasmids were grown in the PC2495 strain of *Escherichia coli* and purified on columns (Qiagen), according to the manufacturer's directions.

■ **Radioimmunoprecipitation of expressed FIPV-M and -N proteins.** COS-7 cells were seeded in 35 mm diameter dishes at 5×10^5 cells per dish. After a culture period of 16 h, cells were transfected with 1 μg of plasmid DNA using Lipofectamin Plus (Gibco BRL), according to the manufacturer's instructions. Cells were washed with PBS 24 h after transfection and kept for 30 min in cysteine- and methionine-free Dulbecco's minimal essential media (DMEM) containing 10 mM HEPES (pH 7·2) and 5% dialysed foetal calf serum (FCS). [^{35}S]Methionine (Amersham) was added to a final concentration of 11·1 MBq/ml and incubation was continued for 1 h at 37 °C. Subsequently, cells were lysed by a 10 min incubation on ice with lysis buffer (20 mM Tris–HCl pH 7·5, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and centrifuged for 15 min at 10000 ***g*** and 4 °C. For immunoprecipitation, 100 μl of the supernatant was diluted with 1 ml detergent solution (50 mM Tris–HCl pH 8·0, 62·5 mM EDTA, 0·4 % deoxycholate, 1% NP-40, 0·7% SDS, 0·1 mg/ml BSA), whereafter 3 μl ascites fluid obtained from an experimentally FIPV-infected kitten was added. After overnight incubation at 4 °C, 50 μl of a 10% (w/v) suspension of formalin-fixed *Staphylococcus aureus* cells (Pansorbin) (Calbiochem) was added and the incubation was continued for 30 min at 4 °C. The bacteria were spun down, washed three times with RIPA buffer (10 mM Tris–HCl pH 7·4, 150 mM NaCl, 0·1% SDS, 1% deoxycholate, 1% NP-40) and resuspended in 30 μl Laemmli's sample buffer containing 5% β-mercaptoethanol. Samples were heated for 1 min at 95 °C and analysed by SDS–PAGE in 15% gels, followed by fluorography.

■ **Radioimmunoprecipitation assay for the analysis of antibodies in cat sera.** *Felis catus* whole foetus (fcwf-D) cells were infected with FIPV strain 79-1146 at an m.o.i. of 10. After an incubation of 4·5 h, cells were washed with PBS and cultured for 30 min in cysteine- and methionine-free DMEM containing 10 mM HEPES (pH 7·2) and 5% dialysed FCS. [^{35}S]Methionine was added to a final concentration of 11·1 MBq/ml and the incubation was continued for 2 h at 37 °C. Subsequently, cells were lysed by a 10 min incubation on ice with lysis buffer and centrifuged for 15 min at 10000 ***g*** and 4 °C. For precipitation, 25 µl of the supernatant was diluted with 1 ml TESV (20 mM Tris–HCl pH 7·3, 1 mM EDTA, 100 mM NaCl) containing 1% Triton X-100, whereafter 25 µl cat serum was added. After overnight incubation at 4 °C, 50 µl of a 10% (w/v) suspension of formalin-fixed *S. aureus* cells was added and the incubation was continued for 30 min at 4 °C. The bacteria were spun down, washed three times with RIPA buffer and resuspended in 30 µl Laemmli's sample buffer containing 5% β-mercaptoethanol. Samples were heated for 1 min at 95 °C and analysed by SDS–PAGE in 10% gels, followed by fluorography.

■ ***In vitro* expression of recombinant feline IL-12.** COS-7 cells were seeded in 35 mm diameter dishes at 5×10^5 cells per well. After a culture period of 16 h, cells were transfected with 1 µg of plasmid DNA using Lipofectamin Plus, according to the manufacturer's instructions. Culture media were collected 72 h after transfection. Cytokine activity released into the culture medium was analysed using a bioassay, described previously by Gately *et al.* (1997). In short, human peripheral blood lymphocytes (PBLs), isolated using Lymphoprep (Nycomed), were cultured for 2 days in Iscove's medium containing 5 µg/ml concanavalin A. To stimulate the formation of blasts, recombinant human IL-2 was added (50 units/ml) and cells were cultured for an additional 3 days. Cells were washed, seeded in 96-well plates (2×10^4 cells per well) and cultured in the presence of the culture media for transfected cells. Recombinant human IL-12 (Genzyme) was used as a positive control. After 48 h, [^{3}H]thymidine (Amersham) was added and the incubation was continued for 4 h, whereafter the cells were harvested by an automated cell harvester. The incorporated radioactivity was quantified by liquid scintillation counting.

■ **Production of recombinant vaccinia virus stocks.** Construction of the recombinant vaccinia viruses vSC, vFN and vFM has been described previously (Vennema *et al.*, 1991). To produce new virus stocks, RK-13 cells were infected with recombinant virus at an m.o.i. of 0·1. After a culture period of 3–4 days, cells were harvested and disrupted in 10 mM Tris (pH > 9). The homogenate was centrifuged for 10 min at 1100 r.p.m. and the supernatant was collected. Virus stocks were titrated on RK-13 cells.

■ **Virus neutralization assay.** FIPV strain 79-1146 (50 µl of $1 \times 10^{6.5}$ $TCID_{50}$/ml) or PRV strain NIA-3 (50 µl of 2×10^5 p.f.u./ml) were incubated overnight at 37 °C with twofold dilutions of heat-inactivated plasma from kittens (50 µl), whereafter the viruses were added to fcwf-D cells (16000 cells per well in 96-well plates). After an incubation period of 18 h, cells were stained with crystal violet to visualize plaques.

■ **Design of vaccination/challenge trials.** To evaluate the efficacy of DNA vaccines, two vaccination/challenge experiments were performed using female, specific-pathogen-free HsdCpb:CADS(BR) kittens (Harlan). At the start of the experiments, the kittens were 14–16 weeks of age.

In the first experiment, three groups (A–C) of kittens ($n = 5$) were injected with different plasmids in 1 ml PBS. Kittens in group A received 200 µg of plasmid DNA encoding the PRV glycoprotein D (VR1012-gD) (Haagmans *et al.*, 1999). Group B kittens each received 200 µg VR1012-M and 200 µg VR1012-N. Group C kittens were injected with 200 µg VR1012-M, 200 µg VR1012-N, 200 µg VR1012-p35 and 200 µg VR1012-p40. Vaccinations were done four times at intervals of 3 weeks. Each vaccine dose was distributed equally over four sites by two intradermal injections and two intramuscular injections (upper hind limbs). Four weeks after the fourth vaccination, all kittens were challenged oronasally with 1000 $TCID_{50}$ FIPV 79-1146.

In the second experiment, three groups (A–C) of four kittens each were vaccinated with the following plasmids in 0·8 ml PBS. Group A kittens (control) were inoculated with 400 µg VR1012-gD, group B with 400 µg plasmid DNA encoding both FIPV-M and FIPV-N (VRNVRM-CpG) and group C with 400 µg VRMVRN-CpG and 400 µg of plasmid DNA encoding both subunits of feline IL-12 (VR1012-fIL12). Cats were vaccinated twice (3-week-interval) by intradermal injection. At 3 weeks after the second DNA vaccination, the kittens of group A received a subcutaneous injection of 1×10^8 p.f.u. of recombinant vaccinia virus vSC, while the kittens in groups B and C were boosted by a similar injection of a mixture containing 1×10^8 p.f.u. of recombinant vaccinia virus expressing FIPV-N (vFN) and 1×10^8 p.f.u. of recombinant vaccinia virus expressing FIPV-M (vFM). Kittens were challenged oronasally with 50 $TCID_{50}$ FIPV 79-1146 at week 3 after the last vaccination.

To avoid unnecessary suffering, kittens were euthanased once they had entered the irreversible terminal phase of FIP, as judged by the veterinary experts of the animal facility.

For both vaccination/challenge experiments, the approval of the Ethical Committee of Utrecht University was obtained.

■ **Statistical analysis.** The significance of the differences in the numbers of PBLs was analysed using Student's *t*-test. Evaluation of statistical differences in survival after FIPV challenge was performed using Cox's proportional hazard model.

Results

In vitro expression of FIPV-M and -N

The genes encoding FIPV-M and -N antigens were cloned into the expression vector VR1012 to yield VR1012-M and -N. COS-7 cells were transfected with these plasmids and metabolically labelled proteins were immunoprecipitated from cell lysates using ascites fluid from an FIPV-infected cat as the antibody source. As shown in Fig. 1 (lanes 2 and 3), a protein with a molecular mass of about 30 kDa was precipitated from cells transfected with VR1012-M, while a lysate of VR1012-N-transfected cells yielded a 45 kDa protein. The molecular masses of the precipitated proteins correspond to those of FIPV-M and -N. To reduce the amount of vaccine DNA, we also constructed an expression vector encoding both FIPV-N and -M. To this end, the entire expression cassette of VR1012-M (i.e. the fragment containing the CMV promoter, FIPV-M cDNA and BGH polyA) was isolated and ligated into VR1012-N to yield VRMVRN. In view of the expected adjuvant activity of CpG sequences present in the Amp^r gene (Roman *et al.*, 1997; Sato *et al.*, 1996), this gene was also inserted, yielding the plasmid VRMVRN-CpG. As shown in Fig. 1 (lane 4), both FIPV-M and -N were expressed in COS-7 cells transfected with VRMVRN-CpG.

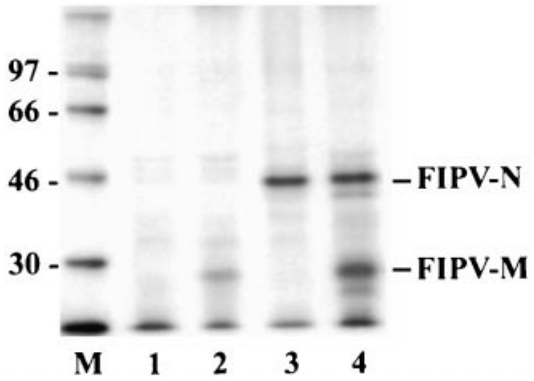

Fig. 1. Expression of FIPV-M and -N in transfected COS-7 cells. COS-7 cells were transfected with the plasmids VR1012 (lane 1), VR1012-M (lane 2), VR1012-N (lane 3) or VRMVRN-CpG (lane 4), whereafter the cells were incubated with ^{35}S-labelled amino acids and lysed. Lysates were subjected to immunoprecipitation analysis with ascites fluid from an FIPV-infected cat. Immunoprecipitates were analysed by SDS–PAGE in 15% gels.

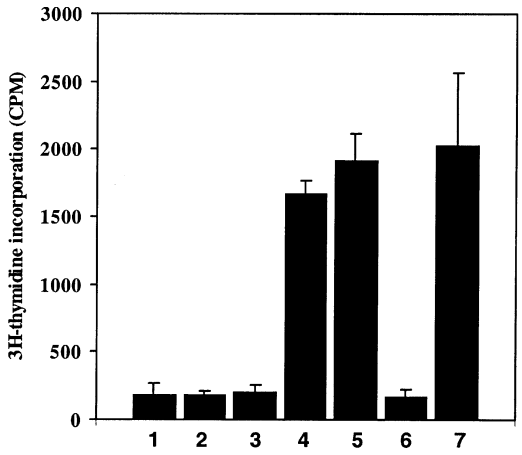

Fig. 2. Expression of biologically active feline IL-12 by transfected COS-7 cells. Culture media of cells transfected with VR1012 (lane 1), VR1012-p35 (lane 2), VR1012-p40 (lane 3), VR1012-p35+VR1012-p40 (lane 4) or VR1012-fIL12 (lane 5) were collected 72 h post-transfection. Media were diluted 20 times, whereafter the ability to stimulate proliferation of human PBLs was evaluated. Culture medium (lane 6) and medium containing 12 ng/ml recombinant human IL-12 (lane 7) were used as controls.

In vitro expression of biologically active feline IL-12

The sequences encoding the 35 kDa (p35) and 40 kDa (p40) subunits of IL-12 were initially cloned separately into the VR1012 expression plasmid, yielding VR1012-p35 and -p40, respectively. Although co-delivery of different vectors encoding each subunit during vaccination was found to be effective (Kim *et al.*, 1997; Tsuji *et al.*, 1997), we also constructed the vector VR1012-fIL-12, which encodes both the p35 and the p40 chains. Any cell receiving this plasmid should produce both subunits and, consequently, biologically active IL-12.

As shown in the results of a bioassay (Fig. 2), proliferation of human PBLs was stimulated by culture media both of COS-7 cells co-transfected with VR1012-p35 and -p40 (lane 4) and of cells transfected with VR1012-fIL12 (lane 5); no biological activity was found in culture media of cells transfected with VR1012-p35 (lane 2) or -p40 (lane 3) alone. These results demonstrate that biologically active feline IL-12 was produced as predicted.

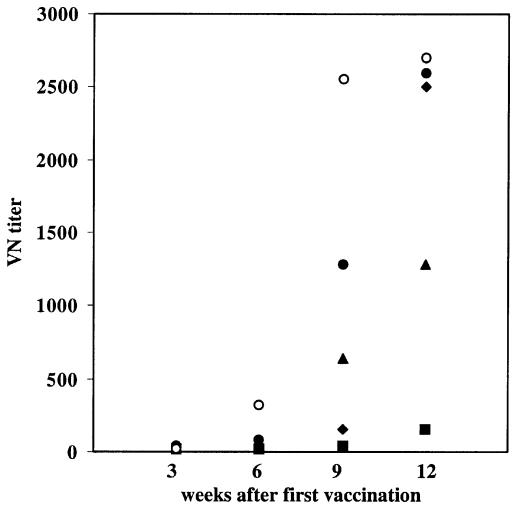

Fig. 3. Induction of PRV-neutralizing (VN) antibodies in sera of VR1012-gD-immunized kittens. Kittens were vaccinated four times at intervals of 3 weeks. At each vaccination, 200 µg of plasmid DNA was injected. Sera were analysed by an *in vitro* PRV neutralization assay.

DNA vaccination of kittens with plasmids encoding FIPV-M and -N

For the vaccination/challenge experiments, groups of five kittens each were injected four times at intervals of 3 weeks. The control group A received DNA encoding the PRV glycoprotein D (VR1012-gD). Animals in group B were vaccinated with a mixture of the plasmids encoding FIPV-M and -N.

As shown in Fig. 3, VR1012-gD induced neutralizing antibodies in all kittens from group A. No PRV-neutralizing antibodies were detected in kittens from the other groups (data not shown).

To evaluate the induction of coronavirus-specific antibodies, sera were analysed in radioimmunoprecipitation assays using lysates of metabolically labelled FIPV-infected fcwf-D cells as the antigen source (Fig. 4). As demonstrated in the group B panel, sera from three kittens clearly precipitated the N protein and two of them also precipitated some M protein. No specific precipitation was observed in the control group A. These data show that an FIPV-specific immune response was induced after vaccination.

At week 3 after the last vaccination, cats were challenged by inoculation with 1000 $TCID_{50}$ of the virulent FIPV strain 79-1146. In the control group A, three of five kittens died within 30 days after challenge; two kittens survived for more

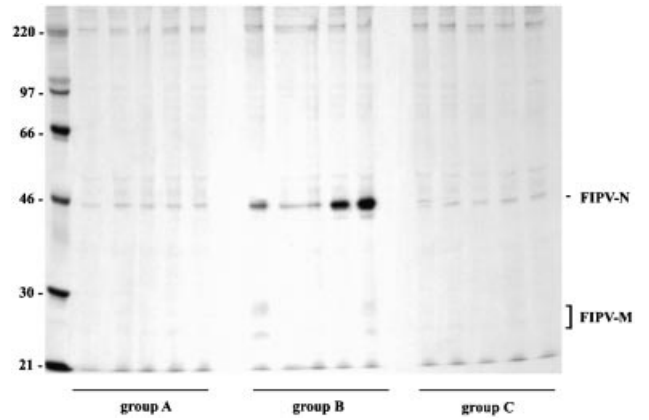

Fig. 4. Induction of FIPV-specific antibodies in kittens by DNA vaccination with plasmids encoding FIPV-M and -N. Kittens were vaccinated four times at intervals of 3 weeks with VR1012-gD (group A), VR1012-M + VR1012-N (group B) or VR1012-M + VR1012-N + VR1012-p35 + VR1012-p40 (group C). At each vaccination, 200 μg of each plasmid was injected. At 3 weeks after the last vaccination, sera were taken and subjected to immunoprecipitation analysis using cell lysates of metabolically labelled FIPV-infected fcwf-D cells. Samples were analysed by SDS–PAGE in 10% gels.

than 40 days (Fig. 5). Vaccination with plasmids encoding FIPV-M and -N resulted in similar survival rates. All kittens had high titres of FIPV-neutralizing antibodies at day 14 after challenge, indicating that also the survivors had been infected with the virus. No significant differences in neutralizing antibody titres were found between the vaccinated and control kittens (data not shown).

Co-delivery of IL-12-encoding plasmids during vaccination

The antibody responses observed for kittens vaccinated with VR1012-M and -N plus the plasmids expressing feline IL-12 are depicted in Fig. 4 (group C). In this experiment, VR1012-p35 and -p40 were co-injected. Whereas the vaccinations with VR1012-M and -N alone did induce antibodies in some kittens (group B), no antibodies were detected when VR1012-p35 and -p40 were co-administered (group C).

As shown in Fig. 5, the co-delivery of VR1012-p35 and -p40 did not improve protection – rather, it seemed to enhance the susceptibility to FIPV; all kittens died within 30 days after challenge, while in each of the two other groups, two of five kittens survived for more than 40 days. All kittens from group C had high titres of FIPV-neutralizing antibodies at day 14 after challenge. These titres were not significantly different from those of kittens from groups A or B (data not shown).

Vaccination of kittens using a prime–boost protocol

In a second trial, kittens were vaccinated twice with the plasmid encoding both FIPV-M and -N (VRMVRN-CpG) and boosted with recombinant vaccinia viruses expressing FIPV-N

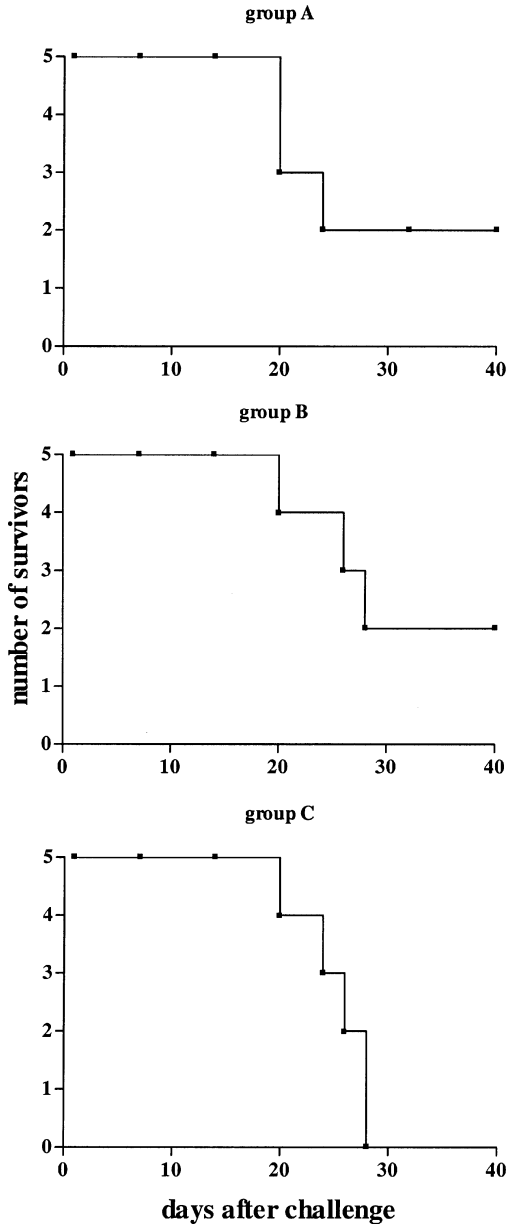

Fig. 5. Survival after challenge with FIPV. Kittens were vaccinated four times with VR1012-gD (group A), VR1012-M + VR1012-N (group B) or VR1012-M + VR1012-N + VR1012-p35 + VR1012-p40 (group C). At each vaccination, 200 μg of each plasmid was injected. At week 3 after the last vaccination, kittens were challenged oronasally with 1000 $TCID_{50}$ FIPV 79-1146.

(vFN) and -M (vFM). Control kittens were primed with the expression vector encoding the PRV glycoprotein D (VR1012-gD) and boosted using the control recombinant vaccinia virus vSC. All animals were challenged with FIPV 3 weeks after the poxviruses had been administered.

No antibodies against FIPV-M or -N could be demonstrated in the sera of kittens vaccinated with VRMVRN-CpG and boosted with vFN and vFM (data not shown). However, analysis of sera taken 1 week after the oronasal challenge with

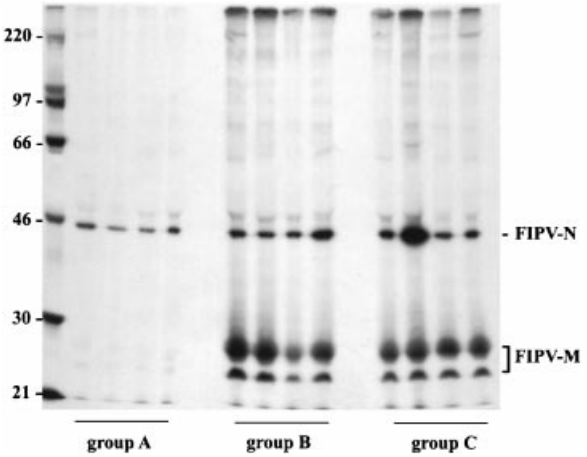

Fig. 6. Radioimmunoprecipitation analysis of cat sera taken 1 week after challenge with FIPV 79-1146. Kittens were vaccinated twice at intervals of 3 weeks with plasmids VR1012-gD (group A), VRMVRN-CpG (group B) or VRMVRN-CpG + VR1012-fIL12 (group C), as detailed in Methods. Cats were boosted 3 weeks after the second DNA vaccination by subcutaneous injection with control recombinant vaccinia virus (vSC; group A) or with recombinant vaccinia virus expressing FIPV-N (vFN) and -M (vFM; groups B and C). After 3 weeks, all kittens were challenged oronasally with 50 $TCID_{50}$ FIPV 79-1146. At day 7 after challenge, sera were taken and subjected to immunoprecipitation analysis using cell lysates of metabolically labelled FIPV-infected fcwf-D cells. Immunoprecipitates were analysed by SDS-PAGE in 10% gels.

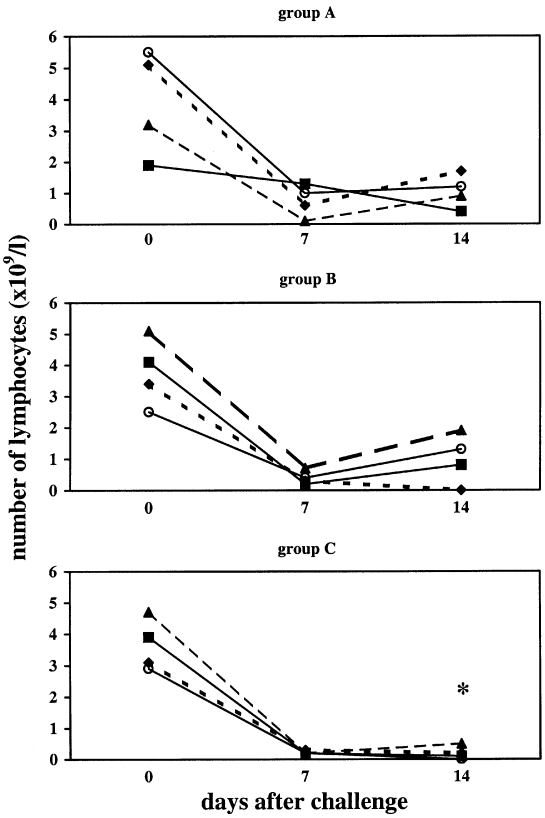

Fig. 7. Decrease in the numbers of PBLs after FIPV challenge. Kittens were vaccinated twice with VR1012-gD (group A), VRMVRN-CpG (group B) or VRMVRN-CpG + VR1012-fIL12 (group C) and boosted by injection with recombinant vaccinia viruses, as described in the legend to Fig. 6. After 3 weeks, all kittens were challenged oronasally with 50 $TCID_{50}$ FIPV 79-1146. Blood samples were analysed on the day of challenge (D0) and 7 and 14 days thereafter. The asterisk indicated the significantly low number of PBLs than that observed in group A ($P = 0{\cdot}04$, Student's *t*-test).

50 $TCID_{50}$ FIPV 79-1146 demonstrated clearly that the immune system had been primed. Unlike the control cats (group A), all kittens from groups B and C had significant antibody levels against the M protein. Due to the known non-specific precipitation of the N protein by cat sera (see group A), the interpretation of the responses to the N protein is less clear-cut. Yet, the observations of Fig. 6 show that at least one cat had seroconverted, while the same appeared likely for the other animals from groups B and C.

Because lymphopenia is a known feature of FIP, PBLs were counted at different time points. As shown in Fig. 7, PBL number in the control kittens (upper panel) dropped dramatically during the first days after challenge and the same was observed in the vaccinated group (Fig. 7, middle panel); no significant differences in the course and level of PBL numbers could be demonstrated.

Also, vaccination did not affect the survival rates (Fig. 8); three of four vaccinated kittens died within 30 days, while only one kitten survived FIPV challenge for more than 40 days (group B). The same result was found for the control group A. All kittens, including the survivors, developed high titres of FIPV-neutralizing antibodies, indicating that all kittens had been infected with FIPV (data not shown).

Co-delivery of IL-12-encoding plasmids during prime–boost vaccination

The VR1012-fIL12 plasmid (expressing both IL-12 subunits) was combined with the plasmid encoding the M and N proteins in a DNA vaccine. When sera taken 1 week after challenge were analysed for antibodies, no effect of VR1012-fIL-12 co-administration was observed (Fig. 6, group C). Neither did co-application improve protection against FIPV challenge – rather, it had an opposite effects. By day 7 after challenge, all kittens in group C had undergone a severe drop in the number of lymphocytes, comparable to that in the control kittens. While the levels of PBLs in groups A and B tended to increase during the second week after challenge, the levels in kittens from group C remained very low. At day 14 the number of PBLs in these kittens was significantly lower as compared to that in control kittens ($P = 0{\cdot}04$, Student's *t*-test). In addition, three of four kittens from group A showed a clear recovery of the numbers of PBLs between days 14 and 28 ($> 0{\cdot}5 \times 10^9$ PBL/l increase). None of the kittens vaccinated with VRMVRN-CpG and VR1012-fIL12 showed a similar increase (data not shown). The adverse effect of IL-12 co-expression on lymphocyte recovery was in line with the observed increased susceptibility to FIPV. All kittens from group C died within 30 days after challenge (Fig. 8). Survival analysis of the two independent vaccination trials demon-

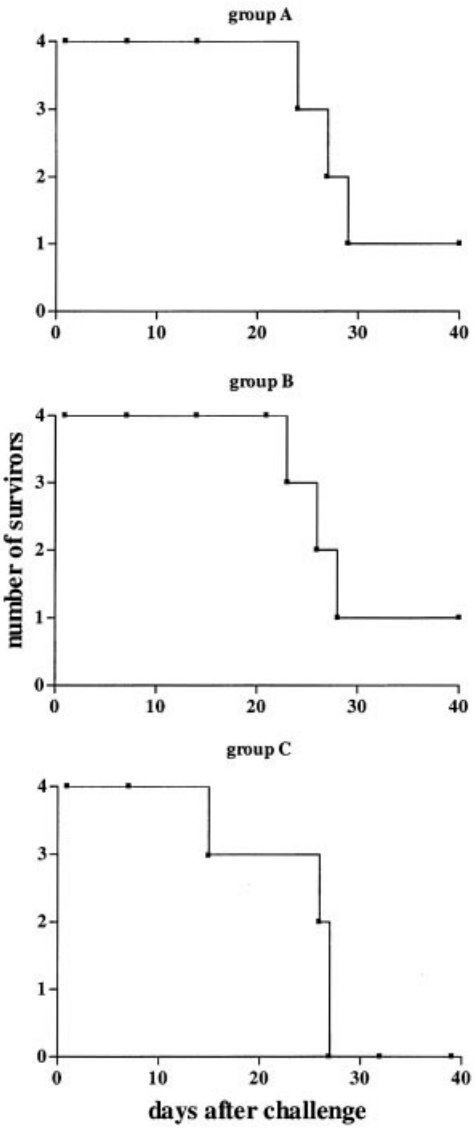

Fig. 8. Survival after challenge with FIPV. Kittens were vaccinated twice with VR1012-gD (group A), VRMVRN-CpG (group B) or VRMVRN-CpG + VR1012-flL12 (group C) and boosted by injection with recombinant vaccinia viruses, as described in the legend to Fig. 6. After 3 weeks, all kittens were challenged oronasally with 50 $TCID_{50}$ FIPV 79-1146.

strated that kittens vaccinated in the presence of IL-12 DNA had a significantly higher risk of death from FIP ($P = 0{\cdot}03$, Cox's proportional hazard model).

Discussion

We have explored different DNA vaccination approaches to protect kittens against FIP. Two trials were carried out, one in which the animals were injected with plasmids only and one in which a prime–boost protocol was used. In view of the known adverse effects of antibodies against the S protein (Corapi *et al.*, 1992; Hohdatsu *et al.*, 1998; Olsen *et al.*, 1992; Vennema *et al.*, 1990), the other major coronavirion proteins M and N were used as antigens. DNA vaccination induced antibodies against the M and N proteins in several kittens, indicating that both antigens were expressed *in vivo*. Although the proteins were immunogenic, neither vaccination protocol induced protection against FIPV challenge.

These observations leave us with the question as to why protection was not achieved. Obviously the quality and/or degree of the immune responses induced by our vaccinations were insufficient. Because assays to measure CTL responses are not established in our laboratory for the feline species yet, we had no opportunity to directly evaluate this parameter. However, the protective effects described following vaccinations with poxviruses expressing the FIPV M or N protein (Vennema *et al.*, 1991; Wasmoen *et al.*, 1995) suggest the existence of CTL epitopes on these antigens and we may assume that FIPV-specific cellular immune responses were induced but that their levels may have been just too low. In order to allow the quantitative analysis of these T cell responses in the future, we are presently establishing assays for feline CTLs.

While antibodies to the N protein obviously do not neutralize virus infectivity, those recognizing the exposed amino-terminal domain of the M protein potentially do. Indeed, a monoclonal antibody against the M protein can inhibit infection of feline macrophages *in vitro* (Kida *et al.*, 2000). Anti-M antibodies might induce complement-mediated neutralization of FIPV, since those directed against the homologous protein of transmissible gastroenteritis virus, a related coronavirus, do neutralize in the presence of complement (Laviada *et al.*, 1990; Woods *et al.*, 1988). However, our results indicate that anti-M antibodies are not important for protecting cats against FIP. At day 7 after challenge, all vaccinated kittens had high titres of these antibodies, while titres of (infection-enhancing) S-specific antibodies were still low.

Cytokines play a critical role in orchestrating immune responses and there is much interest in the use of plasmids encoding cytokines as genetic adjuvants. Co-delivery of plasmids encoding IL-12 along with DNA vaccine formulations has been shown to augment antigen-specific $CD4^+$ Th1 (Chow *et al.*, 1998; Sin *et al.*, 1999a, b; Tsuji *et al.*, 1997) and $CD8^+$ CTL responses (Chow *et al.*, 1998; Hamajima *et al.*, 1997; Kim *et al.*, 1997; Okada *et al.*, 1997; Tsuji *et al.*, 1997). In cats, IL-12 co-delivery improved protection against feline immunodeficiency virus (Boretti *et al.*, 2000; Leutenegger *et al.*, 2000). Contrary to our expectation, co-injection of IL-12-encoding plasmids did not contribute to protection – on the contrary, it clearly enhanced the susceptibility of the animal to FIPV challenge. The adverse effects of IL-12 were also demonstrated by the lower numbers of PBLs after challenge.

We can only speculate about how IL-12 may have caused the increase in susceptibility to FIPV. Factors known to diminish resistance of cats to FIPV are changes in the humoral

(enhanced production of antibodies against the S protein) or cellular (inhibited activity) immune responses (Hayashi *et al.*, 1983; Pedersen & Floyd, 1985; Vennema *et al.*, 1990). We have no evidence from our experiments that IL-12 caused an enhanced production of S antibodies, which are known to exacerbate FIPV infection of macrophages through the binding of FIPV/antibody complexes to Fc receptors (Corapi *et al.*, 1992; Olsen *et al.*, 1992). The titres of neutralizing antibodies measured after challenge did not differ significantly between the groups. Enhanced infection of macrophages could also have occurred through Fc receptor upregulation via induction of interferon (IFN)-γ (Horvath *et al.*, 1996; Mortola *et al.*, 1998; Puddu *et al.*, 1997). A similar mechanism has been described for IFN-γ-mediated enhancement of dengue virus infection (Kontny *et al.*, 1988).

More likely, our co-administration of IL-12 may have led to a suppression of cell-mediated immunity. The observation that the PBL counts during the recovery phase were lower in IL-12-treated cats than in the controls supports this idea. Moreover, in the first trial injections of IL-12 DNA diminished the induction of antibodies during vaccination, possibly through effects on specific helper responses. Although IL-12 is a potent adjuvant for the induction of cell-mediated immunity, several studies have shown dose-dependent effects, with high cytokine concentrations sometimes leading to a suppression of the immune response. For instance, induction of cell-mediated immunity after vaccination with a recombinant adenovirus expressing hepatitis C virus antigens was potentiated by co-administration of a recombinant adenovirus expressing IL-12; high-dose co-administration of this vector, however, inhibited the immune response (Lasarte *et al.*, 1999). Immunosuppression was accompanied by increased apoptosis in the spleen (Lasarte *et al.*, 1999).

Also, dose effects of IL-12 have been observed after DNA vaccination. Repeated injections with a low dose of IL-12 protein were found to enhance CTL induction, whereas a high dose suppressed generation of antigen-specific CTL responses (Lee *et al.*, 2000). Similarly, antigen-specific T cell responses were enhanced by co-delivery of a low dose of IL-12 DNA during priming, while high IL-12 expression during priming or during the boost with recombinant vaccinia viruses was strongly suppressive (Gherardi *et al.*, 2000). The immunosuppressive effects seem to result from nitric oxide (NO), since the effect could be overcome by specific inhibitors of inducible NO synthase (Lasarte *et al.*, 1999; Gherardi *et al.*, 2000). In addition to the suppressive effect on the induction of cell-mediated immunity after vaccination, Orange *et al.* (1994) also found that a high concentration of IL-12 can enhance susceptibility to viruses. They showed that treatment with a low dose of IL-12 enhanced immunity to LCMV infection, while the mice treated with high doses showed a dramatic decrease in CTL induction and a 2-log increase in LCMV titres in both spleen and kidneys. In view of the observations discussed above, we hypothesize that the adverse effects of IL-12 co-expression during our DNA vaccination against FIPV was caused by overexpression of the cytokine; lower levels might still enhance immunity against FIPV.

In summary, we show that DNA vaccination with vectors encoding the M and N proteins did not protect cats against FIP. Co-delivery of vectors encoding feline IL-12 also failed to induce protective immunity and even gave rise to adverse effects. Our study demonstrates that plasmids encoding IL-12 are no panacea for adjuvanting genetic vaccines.

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