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Primary Structure of the Membrane and Nucleocapsid Protein Genes of Feline Infectious Peritonitis Virus and Immunogenicity of Recombinant Vaccinia Viruses in Kittens

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Feline infectious peritonitis virus (FIPV) causes a mostly fatal, immunologically mediated disease in cats. Previously, we demonstrated that immunization with a recombinant vaccinia virus expressing the FIPV spike protein (S) induced early death after challenge with FIPV (Vennema *et al.*, 1990, *J. Virol.* 64, 1407–1409). In this paper we describe similar immunizations with the FIPV membrane (M) and nucleocapsid (N) proteins. The genes encoding these proteins were cloned and sequenced. Comparison of the amino acid sequences with the corresponding sequences of porcine transmissible gastroenteritis virus revealed 84.7 and 77% identity for M and N, respectively. Vaccinia virus recombinants expressing the cloned genes induced antibodies in immunized kittens. Immunization with neither recombinant induced early death after challenge with FIPV, strongly suggesting that antibody-dependent enhancement is mediated by antibodies against S only. Immunization with the N protein recombinant had no apparent effect on the outcome of challenge. However, three of eight kittens immunized with the M protein recombinant survived the challenge, as compared to one of eight kittens of the control group.

INTRODUCTION

The feline infectious peritonitis coronavirus (FIPV) causes a highly fatal disease in cats. In the pathogenesis of FIP the immune status of the animal is very important: cats with preexisting antibodies to FIPV may experience an accelerated infection upon challenge, resulting in survival times shorter than those of seronegative animals (Pedersen and Boyle, 1980; Weiss and Scott, 1981). This phenomenon is referred to as "early death." The underlying mechanism is thought to be an antibody-dependent enhancement of infection of macrophages (Porterfield, 1986). Although antibodies against the spike protein (S) neutralized viral infectivity *in vitro* (de Groot *et al.*, 1989; Vennema *et al.*, 1990a), immunization of kittens with a recombinant vaccinia virus expressing the S protein caused early death after challenge (Vennema *et al.*, 1990a).

The FIPV virion contains three protein species: The 200,000 mol wt S protein, the 25–30,000 mol wt membrane glycoprotein M, and the 45,000 mol wt nucleocapsid protein N (reviewed by Spaan *et al.*, 1988). During natural and experimental FIPV infections antibodies are found against all structural proteins (Boyle *et al.*, 1984; Horzinek *et al.*, 1986). For other coronaviruses it has been demonstrated that antibodies against M or N may inhibit virus replication, in addition to those directed against S. In the murine hepatitis virus (MHV) system monoclonal antibodies against both M and N which neutralize infectivity *in vitro* and were able to protect mice against lethal challenge have been found (Fleming *et al.*, 1989; Lecomte *et al.*, 1987). Protection was also demonstrated for a monoclonal antibody against N, which did not neutralize MHV *in vitro* (Nakanaga *et al.*, 1986). Monoclonal antibodies against the M protein of transmissible gastroenteritis virus (TGEV) of swine neutralized viral infectivity *in vitro* in the presence of complement, whereas those against N did not (Woods *et al.*, 1988). Fiscus and Teramoto (1987) described six anti-FIPV N protein and two anti-FIPV M protein monoclonal antibodies which failed to neutralize *in vitro*.

In this report we present the cDNA cloning and sequence analysis of the M and N protein genes of FIPV. The cloned genes were expressed using the recombinant vaccinia virus system. These recombinants were used to immunize kittens, which were subsequently challenged with FIPV.

MATERIALS AND METHODS

Cells and viruses

FIPV strain 79-1146 (McKeirnan *et al.*, 1981) was grown in *Felis catus* whole fetus cells (fcwf-D; obtained

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from Dr. N. C. Pedersen). For vaccinia virus (strain WR; obtained from Dr. G. Wertz) infections, HeLa, human 143 thymidine kinase-negative, and rabbit kidney (RK-13) cells were used. Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO Laboratories) containing 10% heat-inactivated fetal bovine serum.

Cloning and sequencing of the FIPV M and N genes

The preparation of a cDNA library of FIPV genomic RNA has been described (de Groot *et al.*, 1987b). Clones containing sequences derived from the 3′ end of the genome were identified as described before (de Groot *et al.*, 1988). Restriction fragments of clone B12 (de Groot *et al.*, 1988), were subcloned in M13 and sequenced, using the dideoxy chain termination procedure (Sanger *et al.*, 1977). Comparison with the published sequence of the 3′ one-third of the TGEV genome (Rasschaert *et al.*, 1987) allowed identification of the coding regions of both the M and N gene. Sequence data were analyzed using the computer programs of Staden (1982) and Devereux *et al.* (1984).

Construction of recombinant vaccinia viruses expressing the FIPV M and N protein

Recombinant DNA techniques were performed essentially as described by Maniatis *et al.* (1982). The FIPV M gene was isolated as an 870-bp Klenow-filled *Sty*I–*Mlu*I fragment and recloned in the *Sma*I site of vaccinia virus insertion vector pSC11 (Chakrabarti *et al.*, 1985; obtained from Dr. B. Moss), yielding plasmid pSCFM. The *Sty*I site (5′-C_CAAGG-3′) was located 48 nucleotides upstream of the translation initiation codon, and the *Mlu*I site (5′-A_CGCGT-3′) was located 30 nucleotides downstream of the termination codon. The N gene was isolated as a 1159-bp *Nde*I–*Sph*I fragment which was made blunt-ended with T4 DNA polymerase and recloned in pSC11, yielding plasmid pSCFN. The fill-in reaction of the *Nde*I site (5′-CA_TATG-3′), located 23 nucleotides upstream of the initiation codon of the N coding region, restored an AUG codon. The reading frame followed by this AUG is only two amino acids long and terminates just upstream of the N gene. The *Sph*I site (5′-GCATG_C-3′) was located 7 nucleotides downstream from the N gene termination codon. All restriction sites are underlined in Fig. 2.

The insertion plasmids, pSCFM and pSCFN were then used to construct the recombinant vaccinia viruses vFM and vFN respectively, using procedures described before (Chakrabarti *et al.*, 1985). For control experiments the recombinant vaccinia virus vSC was constructed using pSC11.

Immunization of kittens

Recombinant vaccinia viruses vFM, vFN, and vSC were used to immunize kittens. Specific-pathogen-free (SPF) kittens, 13 to 14 weeks of age, were injected subcutaneously with 10^8 PFU. A second immunization with 5×10^8 PFU of the appropriate virus was given 3 weeks later. Two weeks after the second immunization, all kittens were challenged orally with 3×10^5 PFU of FIPV strain 79-1146; they were examined daily for clinical signs. Euthanasia was carried out when the kittens became prostrate and a full postmortem examination was performed.

Radioimmunoprecipitation assays

Lysates of FIPV-infected fcwf-D cells or recombinant vaccinia virus-infected HeLa cells were prepared after metabolic labeling with L-[^{35}S]methionine. Radioimmunoprecipitation (RIP) and analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were carried out as described before (Vennema *et al.*, 1990b).

In vitro neutralization assays

Neutralizing antibody was determined in heat-inactivated sera using fcwf-D cells in a 96-well microplate assay. Titers were expressed as the maximum serum dilution that inhibited the cytopathic effect of 100 50% tissue culture infectious doses of FIPV.

RESULTS

Isolation and characterization of cDNA clones containing the M and N protein genes

The coding regions of the M and N proteins have been assigned to mRNAs 4 and 5, respectively (de Groot *et al.*, 1987a). Together with mRNA 6, these mRNAs correspond to the 3′ end of the FIPV genome. The preparation of a cDNA library of FIPV genomic RNA has been described (de Groot *et al.*, 1987b). Clones containing sequences derived from the 3′ end of the genome were identified by colony hybridization, using mRNA 6 as a probe (de Groot *et al.*, 1988). Clone B12 was selected for sequence analysis. The sequencing strategy is shown in Fig. 1. Additional cDNA clones were isolated from the library using fragments of pB12 as probes and analyzed to confirm the nucleotide sequence. By comparison with the published sequence of TGEV (Rasschaert *et al.*, 1987), the coding

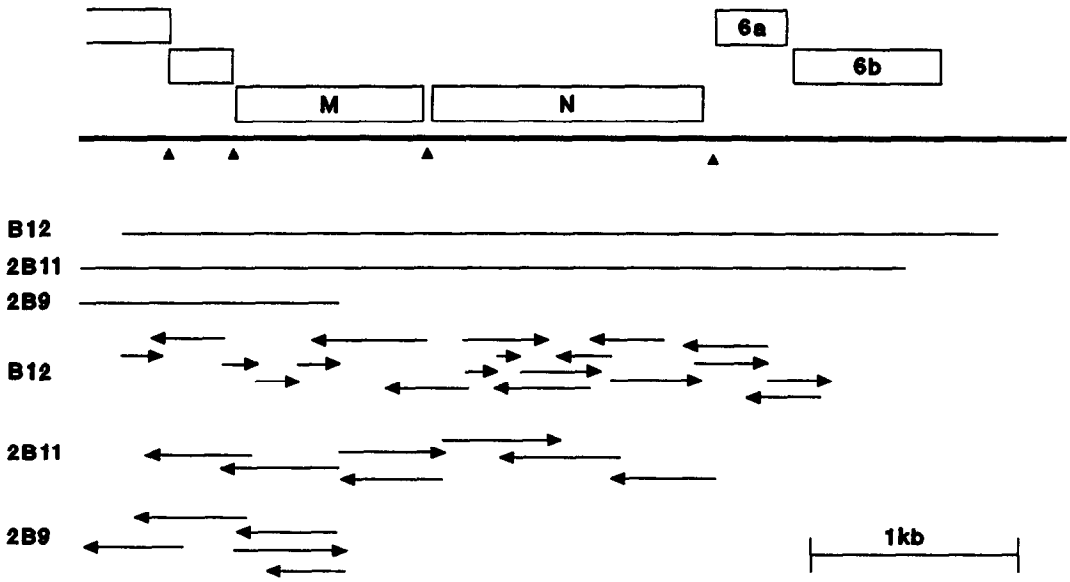

FIG. 1. Organization and sequencing of the 3′ end of the FIPV genome. Boxes indicate the open reading frames which were named according to the recently proposed guidelines (Cavanagh *et al.*, 1990). The solid bar represents the genome. The arrowheads indicate the positions of conserved intergenic sequences (CTAAAC). Horizontal lines represent the relevant parts of cDNA clones B12, 2B11, and 2B9. The direction and extent of the sequences obtained for each clone are represented by arrows in the lower part of the figure.

regions of both the M and N protein genes were identified. The nucleotide and derived amino acid sequences are shown in Fig. 2. The nucleotide sequence was 79% identical to the corresponding sequence of TGEV (Rasschaert *et al.*, 1987). Optimal alignments of the predicted amino acid sequences are represented graphically in Fig. 3. Among the M protein sequences most differences were found in the N-terminal region which is thought to protrude from the viral membrane. Between the N protein sequences domains with insertions/deletions and multiple differences were found around residues 200 and 350. Other differences were predominantly conservative changes. Overall identity was slightly lower for the N proteins (77%) than for the M (84.7%) and S proteins (82.2%; Jacobs *et al.*, 1987).

Expression of the M and N protein using recombinant vaccinia viruses

Recombinant vaccinia viruses containing the FIPV M and N protein genes were constructed as described above. Expression of the recombinant proteins was studied in an immunoprecipitation assay with lysates of L-[^{35}S]methionine-labeled cells (Fig. 4). The proteins produced in vFM- and vFN-infected cells comigrated with the authentic viral M and N proteins and were specifically precipitated by ascitic fluid from a cat that had died of FIP. The recombinant M protein was fully glycosylated as demonstrated by removal of the oligosaccharide side chains with endoglycosidase H (data not shown).

Immunization of kittens and survival after challenge

Kittens were immunized with recombinant vaccinia viruses and subsequently challenged with FIPV as described under Materials and Methods. Details of the immunized kittens are presented in Table 1, together with the survival times. After challenge the kittens developed fever during the first 3 days; they returned to normal body temperatures by Postchallenge Day (PCD) 9–10. Secondary pyrexia started on PCD 11–15 (exceptions: M81 on PCD 28, 721 on PCD 27; M89 had only a few days of pyrexia) and reached up to 41.2°. All kittens were anorectic from PCD 16 onwards. Fluid therapy was given where necessary. The mortality curves after challenge are represented in Fig. 5. Most kittens were euthanized between 3 and 4 weeks after challenge. In the groups immunized with vSC and vFN seven animals were euthanized during this period, in contrast to only three of those immunized with vFM. During the 6th week after challenge two animals immunized with vFM and one immunized with vFN were euthanized. Postmortem examination showed that all kittens, with the exception of 722, had peritoneal effusions and granulomatous lesions on the viscera. Cat 722 was apparently normal, apart from weight loss. It was euthanized on PCD 22 because of

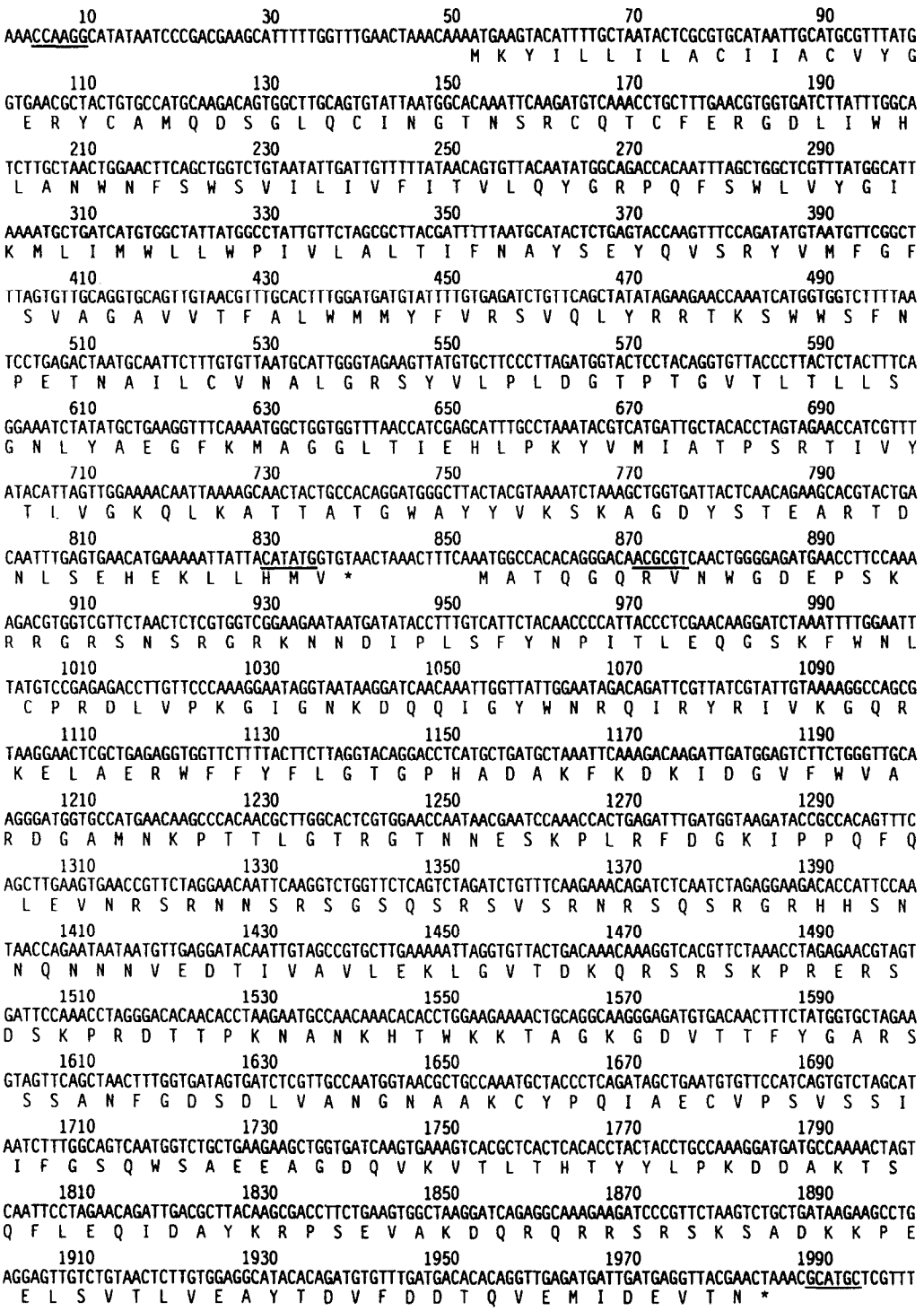

FIG. 2. Nucleotide sequence of a 2-kb segment of the FIPV genomic cDNA clone B12, encoding the membrane and nucleocapsid proteins. The derived amino acid sequences are presented below the nucleotide sequence. Restriction endonuclease sites used for recloning the M (*Sty*I, CCAAGG; *Mlu*I, ACGCGT) and N (*Nde*I, CATATG; *Sph*I, GCATGC) genes are underlined. The sequence data have been submitted to the EMBL Data Library and are available under Accession No. X56496.

neurologic signs. Cats 89S and 89P had pleural effusions and lesions on the lungs in addition to the peritoneal lesions. Histologically, all cats had lesions typical of FIP. Kitten 722 therefore was a case of noneffusive FIP, whereas all the others showed the exudative form of FIP.

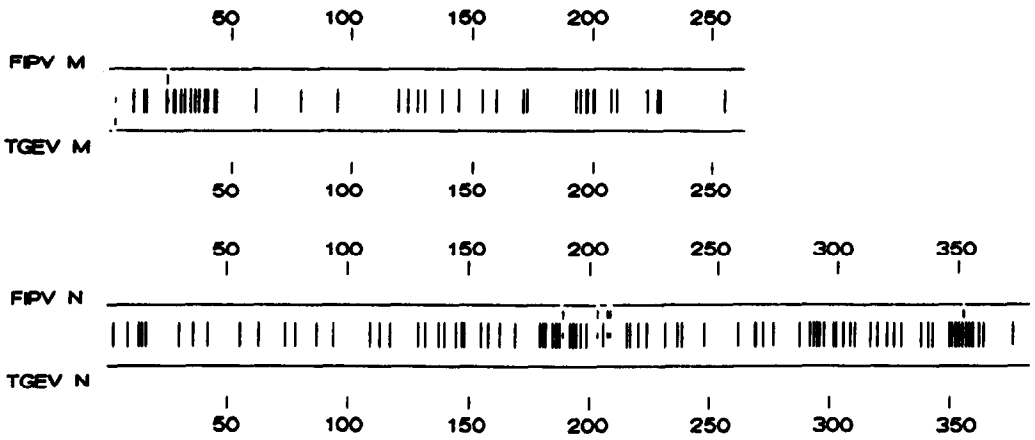

FIG. 3. Amino acid sequence alignments of the FIPV and TGEV membrane (M) and nucleocapsid (N) proteins, obtained with the UWGCG program Gapshow. Differences are indicated by vertical lines. Gaps are shown in the horizontal lines representing the sequences and are indicated by broken lines. Overall identity was 84.7% for the M proteins and 77% for the N proteins. Paired amino acid sequence alignments are available on request.

Humoral immune response to FIPV proteins

Serum samples were taken on the days of primary and secondary immunization, 3 days before challenge and at different PCDs, or on the day when euthanasia was performed. Aliquots of sera obtained from animals from one group were pooled for each time point and used in an RIP assay with a lysate of metabolically labeled, FIPV-infected cells. The amount of pooled antiserum used was such that each individual serum was diluted 100-fold in the final immunoprecipitation mixture. Pooled sera of the days of primary and secondary immunization gave a background level of precipitation (Fig. 6). Particularly, the nucleocapsid protein was precipitated nonspecifically, probably by binding to Pansorbin. Pooled sera from 3 days before challenge of the kittens immunized with vFM and vFN precipitated the M and N proteins, respectively. The same serum pool of the control group gave only background signal. After challenge, the amount of N and M protein precipitated increased, starting from PCD 5 and 7, respectively, as a result of secondary immune responses. The primary response against the S protein could be detected at low levels on PCD 9. The pooled sera of vFN-immunized kittens from before and shortly after challenge appeared to precipitate small amounts of M protein. Since the intensity of the signal correlated with the amount of N protein detected, precipitation of the M protein may be due to formation of complexes between M and N as was described previously for MHV (Sturman *et al.*, 1980). Coprecipitation of N protein with antibodies against M was not observed, probably because it was below the background level of N precipitation. Antibody against all structural proteins increased to high levels between PCD 9 and 17 in all groups.

In order to find out whether antibody levels and survival times correlated for the kittens immunized with vFM, individual sera were tested from the day of secondary immunization, 3 days before challenge and PCD 1, 9, and 17. Although differences in the levels of

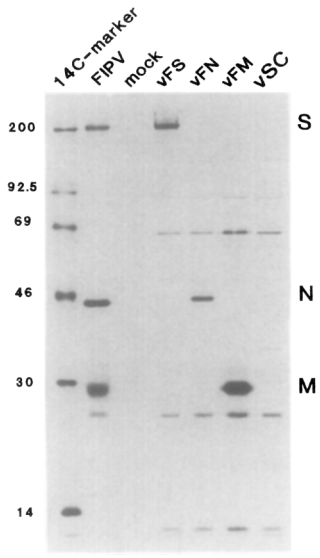

FIG. 4. SDS–PAGE analysis of L-[^{35}S]methionine-labeled proteins immunoprecipitated with ascitic fluid from a cat that died of FIP. Lanes FIPV and mock: lysates of FIPV- and mock-infected fcwf-D cells, respectively. Lanes vFM, vFN, and vSC: lysates of HeLa cells infected with the respective recombinants. Recombinant vFS, expressing the FIPV S protein (Vennema *et al.*, 1990b), was included (lane vFS). FIPV structural proteins and the recombinant vaccinia virus expression products have been indicated. ^{14}C-labeled marker protein molecular weights in kilodaltons have been indicated on the left side.

TABLE 1

GROUPS OF IMMUNIZED KITTENS AND INDIVIDUAL SURVIVAL TIMES AFTER CHALLENGE

rvv[a]	No.	Sex	Litter[b]	PCD[c]
vSC	M81	f[d]	1	n.a.[e]
	M89	m[f]	4	28
	M90	f	4	28
	89O	m	5	23
	89Q	f	5	21
	89V	f	7	25
	89W	f	7	22
	M85	f	8	28
vFM	M79	f	1	n.a.
	M87	f	2	n.a.
	M88	f	2	41
	721	f	3	n.a.
	M93	m	4	23
	M94	f	4	36
	87R	f	5	23
	89S	m	6	21
vFN	M80	f	1	41
	720	m	3	29
	722	f	3	22
	M91	m	4	22
	M92	m	4	18
	89P	f	5	21
	89T	f	6	22
	89U	m	7	28

[a] Recombinant vaccinia virus used to immunize groups of eight kittens.
[b] Corresponding numbers refer to the same litter.
[c] Postchallenge day the kittens were euthanized.
[d] Female.
[e] Not applicable, surviving kitten.
[f] Male.

antibodies were seen (Fig. 7), these did not correlate with the survival times. Similarly, small differences were found in the levels of antibody against the S and N proteins on PCD 17; again there was no correlation with survival times.

In vitro neutralizing activity of sera from immunized kittens

Sera were tested in a neutralization assay. Neutralizing activity was not detected in pooled sera until PCD 5. At this day sera delayed but did not completely inhibit CPE. From PCD 7 onwards neutralization titers gradually increased similarly in all groups, from 10^{-2} to 2×10^{-4} on PCD 23. No differences were observed between the vFM group and the other two groups which could be advocated to explain the differences in survival time. Also, among the kittens of the vFM group, only small differences were observed in the development of neutralization titers with respect to time (data not shown).

DISCUSSION

Sequence comparison

Comparison of the amino acid sequences of the M protein of FIPV and TGEV revealed an overall identity of 84.7%. The structural features predicted for the TGEV M protein (Laude *et al.*, 1987; Kapke *et al.*, 1988; Britton *et al.*, 1988), also apply to FIPV. Interestingly, a short stretch of highly divergent amino acid sequence was observed. A stretch of 23 amino acids (residues 23 to 45) between the signal sequence and the first hydrophobic domain had only 47.8% identity in an optimal alignment. The N-glycosylation site and three cysteine residues located in this region were conserved. In analogy to MHV, this part of the M protein is predicted to be exposed on the outside of virus particles (Rottier *et al.*, 1986). The observed divergence in this region is indicative of specific immunologic pressure. Presumably, an immune response against this portion of the M protein influences virus multiplication.

From the data of the M protein of FIPV an N-terminal signal sequence is predicted, similar to the one found in TGEV (Laude *et al.*, 1987; Kapke *et al.*, 1988; Britton *et al.*, 1988). The N-terminal signal sequence of the TGEV M protein is not absolutely required for translocation and glycosylation (Kapke *et al.*, 1988). However, after its removal only a small portion of the M protein produced by *in vitro* translation in the presence of microsomes became glycosylated (Kapke *et al.*, 1988). A construct of the FIPV M gene lacking the signal se-

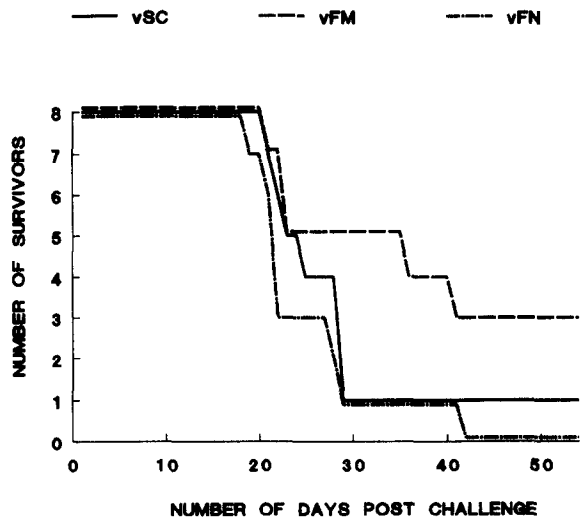

FIG. 5. Graphical representation of the mortality after FIPV challenge (Day 0); the groups of eight kittens had been immunized with vSC, vFM, and vFN, respectively.

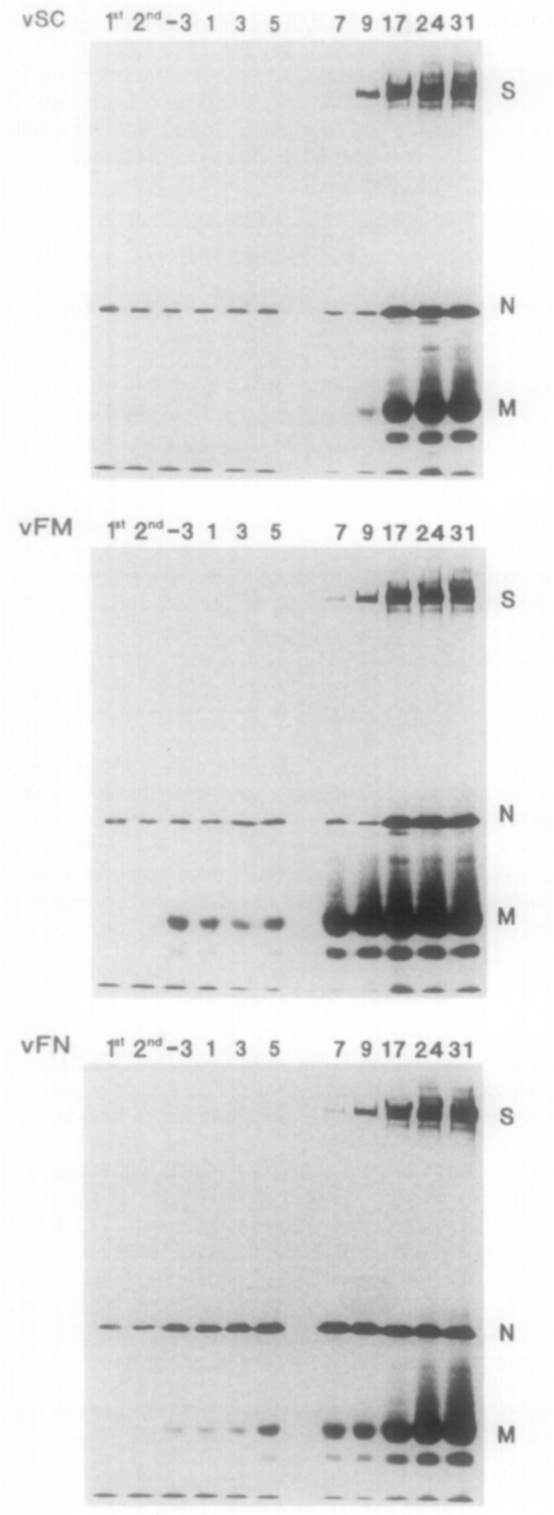

FIG. 6. SDS–PAGE analysis of RIP assays with pooled sera from experimentally infected kittens. FIPV-specific antibodies were detected using a lysate of metabolically labeled FIPV-infected fcwf-D cells as antigen source. Panels vSC, vFM, and vFN: pooled sera of eight kittens immunized with vSC, vFM, or vFN, respectively. The lanes marked 1st, 2nd, and -3 represent reactions at the day of the first and second immunization, and at 3 days before challenge. Subsequent numbers indicate the postchallenge day on which sera were taken. Sera taken postmortem from kittens euthanized between the indicated time points were included in the pool of the closest time point. Therefore, at 31 days after challenge $n = 4$ for pool vSC, $n = 5$ for pool vFM, and $n = 3$ for pool vFN and at PCD 24 $n = 7$ for pool vFN. For all other pools $n = 8$.

quence coding region was expressed in HeLa cells (data not shown), using the hybrid vaccinia virus T7 expression system (Fuerst *et al.*, 1986). The mutant M protein was fully glycosylated (data not shown), which illustrates that the N-terminal signal sequence of the FIPV M protein is not required for glycosylation.

After alignment of the N proteins of FIPV and TGEV two divergent regions, which may represent immunologically important domains in the N protein, were found. Since the N protein is not exposed on the outside of virus particles, these domains may represent T-cell epitopes rather than B-cell epitopes.

Immunization of kittens and challenge with FIPV

We have shown before that immunization of kittens with the FIPV S protein expressed by a live recombinant vaccinia virus made them more sensitive to challenge than control animals (Vennema *et al.*, 1990a). We now show that neither the N protein nor the M protein expressed by recombinant vaccinia viruses induced early death after challenge. Immunization with vFN induced N-protein specific antibodies in kittens but appeared to have no effect on the outcome of challenge. In contrast, immunization with vFM markedly affected the survival time after challenge in a number of kittens. However, all animals seroconverted after challenge (Fig. 7D) and showed clinical signs (pyrexia and anorexia) and only three of eight animals recovered from infection. Clearly, the significance of these results needs to be substantiated with additional immunization experiments. The serological assays performed with individual sera from the animals of the vFM-immunized group showed no correlation between antibody levels and survival times. It has been implied that immunity against FIPV is largely cell-mediated (Pedersen, 1989). Measurements of cell-mediated immunity in experimentally infected cats have been reported but were difficult to perform and showed considerable variation (Pedersen, 1987).

For further immunization experiments to evaluate the potential role of the M protein in protection we consider improvement of its immunogenicity with an approach similar to that shown recently for recombinant vaccinia virus-encoded HIV-1 gp160 (Earl *et al.*, 1990). It was demonstrated that removal of 2 cryptic vaccinia

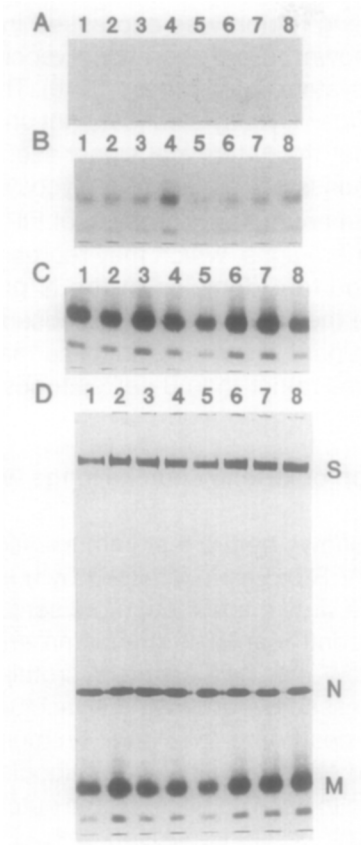

FIG. 7. SDS–PAGE analysis of RIP assays with individual sera of the vFM-immunized group. Kittens immunized with vFM were numbered from shortest to longest survival time after challenge; these were 21 days for 1 (89S), 23 days for 2 (89R) and 3 (M93), 36 days for 4 (M94), 41 days for 5 (M88), or more than 90 days for 6 (M87), 7 (721), and 8 (M79). Sera were from the day of the second immunization (A), from 3 days before challenge (B), and from 9 (C) and 17 (D) days after challenge; panels A through C represent only the lower portions of the gels containing the M protein bands. D illustrates the primary immune response against the S and N proteins. The structural proteins have been indicated.

virus early transcription termination signals (TTTTTNT; Rohrmann *et al.,* 1986) enhanced the immunogenicity considerably. The M protein gene contains 1 transcription termination signal. Expression of the M protein *in vitro* could be measured only after 6 hr p.i. (data not shown) although transcription is driven by the 7.5-kDa gene early-late promoter. Besides increasing immunogenicity, early expression is essential for the induction of cytotoxic T-lymphocytes (CTLs) by recombinant vaccinia viruses (Wachsman *et al.,* 1989). Specific CTLs are probably of major importance in recovery from FIPV infection but the viral antigens involved are not known. Enhancement of the induction of CTLs by removal of early transcription termination signals remains to be shown.

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