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Live, Attenuated Coronavirus Vaccines through the Directed Deletion of Group-Specific Genes Provide Protection against Feline Infectious Peritonitis

Bert Jan Haijema, Haukeline Volders, and Peter J. M. Rottier*

Institute of Virology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, 3584 CL Utrecht, The Netherlands

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Feline infectious peritonitis (FIP) is a fatal immunity-mediated disease caused by mutants of a ubiquitous coronavirus. Since previous attempts to protect cats under laboratory and field conditions have been largely unsuccessful, we used our recently developed system of reverse genetics (B. J. Haijema, H. Volders, and P. J. M. Rottier, J. Virol. 77:4528–4538, 2003) for the development of a modified live FIP vaccine. With this objective, we deleted the group-specific gene cluster open reading frame 3abc or 7ab and obtained deletion mutant viruses that not only multiplied well in cell culture but also showed an attenuated phenotype in the cat. At doses at which the wild-type virus would be fatal, the mutants with gene deletions did not cause any clinical symptoms. They still induced an immune response, however, as judged from the high levels of virus-neutralizing antibodies. The FIP virus (FIPV) mutant lacking the 3abc cluster and, to a lesser extent, the mutant missing the 7ab cluster, protected cats against a lethal homologous challenge; no protection was obtained with the mutant devoid of both gene clusters. Our studies show that the deletion of group-specific genes from the coronavirus genome results in live attenuated candidate vaccines against FIPV. More generally, our approach may allow the development of vaccines against infections with other pathogenic coronaviruses, including that causing severe acute respiratory syndrome in humans.

Coronaviruses generally cause mild respiratory or intestinal infections in mammals and birds. A notorious exception in veterinary virology is the feline infectious peritonitis virus (FIPV), a mutant of an innocuous virus that causes a highly lethal, immunopathological disease characterized by severe systemic inflammatory damage of serosal membranes and disseminated pyogranulomas. FIPV-infected macrophages play a prominent role in pathogenesis (for a review, see reference 7), presumably through the release of proinflammatory cytokines with subsequent cytokine dysregulation. Interestingly, the worsening of the respiratory symptoms in patients infected with the severe acute respiratory syndrome (SARS) coronavirus is also associated with severe immunopathological damage induced by stimulated (or possibly infected) macrophages (24, 34).

Members of the family *Coronaviridae* are enveloped viruses with a large positive-sense RNA genome. In the virion, the genomic RNA (about 30 kb) encased in the nucleocapsid (N) protein forms the nucleocapsid, which is surrounded by a lipid membrane containing the spike (S), membrane (M), and envelope (E) proteins (for a review, see reference 38). Trimers of the S protein (9) form the characteristic peplomers that protrude from the virion membrane. The S protein is responsible for viral attachment to specific host cell receptors, which are determinants of the host range, and for cell-to-cell fusion (for a review, see reference 4).

Feline coronaviruses are widespread, and antibodies are found in most cat populations worldwide (1, 20, 29). However, only 5 to 10% of the seropositive cats develop FIP (1, 29), which is caused by virulent mutants arising in individual animals (44). In young kittens, the primary infection only leads to mild enteritis and to an asymptomatic persistence of the coronavirus (15). When replication flares up, e.g., after immunosuppressive events, virulent mutants occur in the expanding quasispecies cloud and FIP may result. Interestingly, mutations unique to FIPV have been found among others in the group-specific genes *3c* and *7b* (44). The group-specific genes of feline coronaviruses occur in two clusters, the *3a*, *3b*, and *3c* genes (located between the S and E genes) and the *7a* and *7b* genes (at the 3′ end of the viral genome) (Fig. 1A). Group-specific genes are found in each of the four groups of coronaviruses, although their makeup and location differ among the groups (Fig. 1A). Except for the HE glycoprotein, a virion membrane component with hemagglutinin and esterase activity of group 2 coronaviruses, no functions have been established for any of the group-specific gene products. Studies of transmissible gastroenteritis virus (TGEV) (group 1) and mouse hepatitis virus (MHV) (group 2) have indicated that these genes are not essential for growth in cultured cells (6, 8, 28, 39).

The development of vaccines against FIPV has proven cumbersome. The administration of closely related heterologous live coronaviruses failed to induce protection (2, 3, 40, 46). Vaccination with feline enteric coronavirus, low-virulence FIPV, or sublethal amounts of virulent FIPV occasionally provided some protection (30–33), but results were too inconsistent to have clinical relevance. Subunit vaccines containing the M and/or N genes showed low and inconsistent protective capacities (13, 43). Immunization with a recombinant vaccinia virus expressing the S gene even showed an enhanced and

* Corresponding author. Mailing address: Institute of Virology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.165, 3508 TD Utrecht, The Netherlands. Phone: 31-30-2532462. Fax: 31-30-2536723. E-mail: P.Rottier@vet.uu.nl.

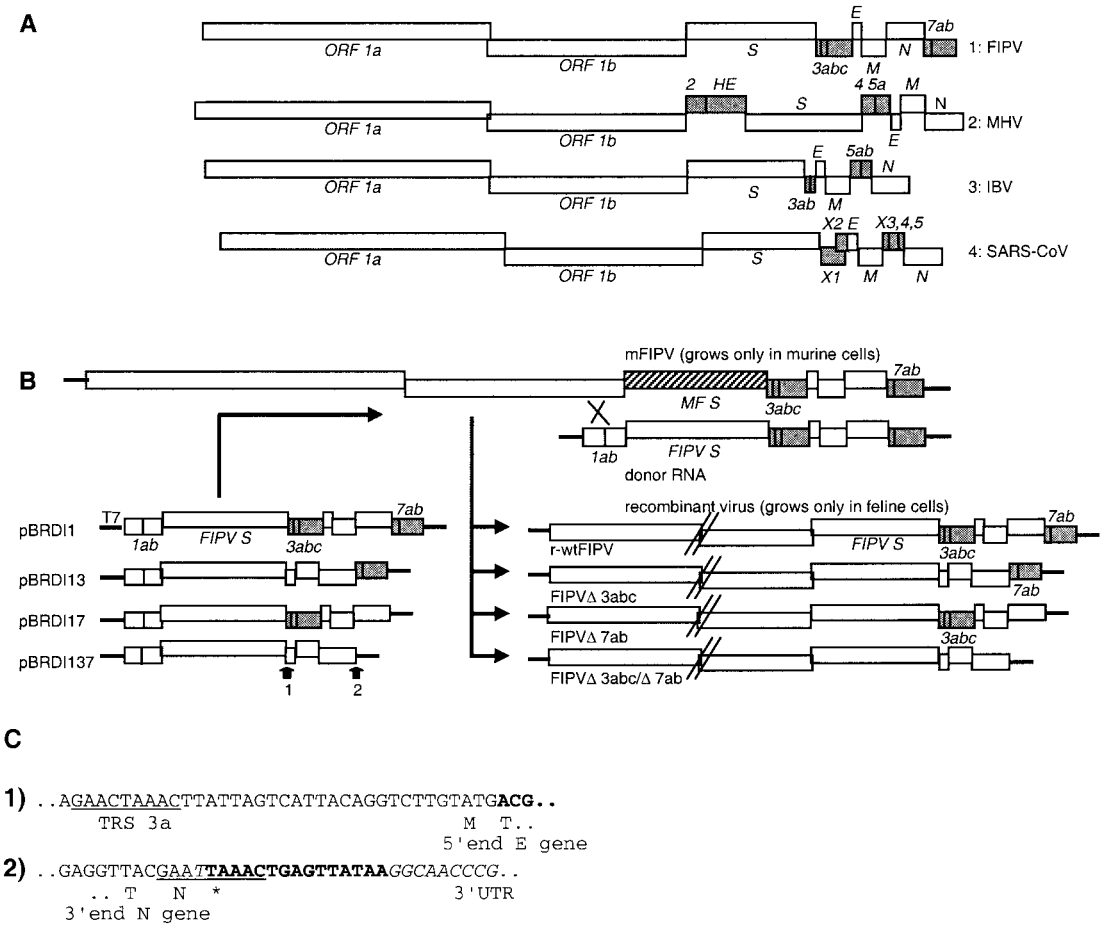

FIG. 1. Coronavirus genomic organization. (A) Genomic organization of the coronavirus group-specific genes. One member for each group is shown, namely the group 1 FIPV, the group 2 MHV, the group 3 infectious bronchitis virus (IBV), and the group 4 SARS coronavirus (SARS-CoV). The group-specific genes are shown in gray. (B) Plasmid constructs (left), targeted recombination (top), and recombinant viruses (right). The transcription vectors from which the synthetic RNAs were made in vitro by using T7 RNA polymerase are indicated at the left. Vector pBRDI1 has been described before (14), and the other vectors are derivatives thereof (see Materials and Methods). T7 indicates the position of the T7 promoter; the 1ab boxes represent the in-frame fusion between the 5′ segment of ORF1a and the 3′ domain of ORF1b. The group-specific genes are indicated in gray. Positions of the sequences shown in panel C are indicated by arrows. The scheme at the top of panel B shows the principle of targeted recombination using the interspecies chimeric virus mFIPV, which only grows in murine cells. The ectodomain-encoding region of the MHV S gene is shown as a hatched box in the mFIPV genome. A single crossover event (indicated by a cross) anywhere within the 3′ domain of ORF1b present in the donor RNA and viral genome generates a recombinant genome. Recombinant progeny can be selected on the basis of the ability to infect feline cells and the simultaneously lost ability to infect murine cells. Recombinant viruses are represented at the right. (C) Sequences in the transcription vectors indicated by the arrows in panel B are shown and are defined as follows: 1, the region between the S and E genes; 2, the region between the N gene and the 3′ UTR. The FIPV TRSs are underlined. 1, the initiating methionine encoded by the E gene is indicated; 2, the amino acids encoding the 3′ end of the N gene are indicated and the 5′ end of the 3′ UTR is in italics. The 3′ end of ORF7b is shown in bold and the C-to-T mutation within the TRS is in italics.

accelerated progression of the disease upon challenge, leading to an early death syndrome (42). Currently, a temperature-sensitive strain of FIPV is available as a vaccine in some countries. Although its ability to protect cats against FIPV was demonstrated (11, 12), the efficacy of this vaccine is a matter of debate (10, 21, 37).

For the present study, we took a different approach: we obtained live attenuated viruses through the directed deletion of the group-specific genes from an otherwise lethal FIPV strain. We hypothesized that these genes are involved in virulence and that their loss would therefore result in attenuated but protective viruses. To delete these genes, we used a novel targeted RNA recombination system for FIPV which was based on a host switching methodology (14).

MATERIALS AND METHODS

Viruses, cells, and antibodies. *Felis catus* whole fetus (FCWF) cells (American Type Culture Collection) were used to propagate, select, plaque titrate, and radiolabel FIPV, r-wtFIPV, FIPVΔ3abc, FIPVΔ7ab, and FIPVΔ3abc/Δ7ab. All of these viruses, as well as all FIPV cDNA constructs described below, were derived from FIPV strain 79-1146 (22). Mouse LR7 (19) cells were used to propagate murine FIPV (mFIPV) (14). Both LR7 and FCWF cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml (all from Life Technologies, Ltd., Paisley, United Kingdom).

Plasmid constructs. Transcription vectors for the production of synthetic donor RNAs for targeted recombination were derived from plasmid pBRDI1 (Fig. 1B), which carries a FIPV RNA transcript consisting of the 5′ end of the genome (681 nucleotides [nt]) fused to the 3′ 363-nt proximal end of ORF1b and running to the 3′ end of the genome (14). Transcription vector pBRDI13 lacks ORF3abc. For the deletion of ORF3abc, primers 1 (5′-GCCATTCTCATTGATAAC-3′) and 4 (5′-CATACAAGACCTGTAATGAC-3′) and primers 2 (5′-CTTCTGTTGAGTAATCACC-3′) and 3 (5′-GTCATTACAGGTCTTGTATGACGTTCCCTAGGGC-3′) were used to generate fragments of 375 bp (A) and 1,012 bp (B), respectively. Fragments A and B were fused by using the overlap between both fragments through primers 3 and 4 and were amplified with primers 1 and 2, resulting in a 1,366-bp fragment (C). Fragment C was digested with AflII and SnaBI and cloned into pBRDI1, resulting in pBRDI13. Transcription vector pBRDI17, which lacks ORF7ab, was constructed as follows: primers 5 (5′-GGTGATTACTCAACAGAAGC-3′) and 8 (5′-TTTAATTCGTAACCTC-3′) and primers 6 (5′-GCGGCCGCTTTTTTTTTTTT-3′) and 7 (5′-GAGGTTACGAATTAAACTGAGTTATAAGGCAAC-3′) were used to generate fragments of 1,215 bp (D) and 324 bp (E), respectively. Fragments D and E were fused by using the overlap between both fragments through primers 7 and 8 and were amplified with primers 5 and 6, resulting in a 1,524-bp fragment (F). Fragment F was digested with MluI and NotI and cloned into pBRDI1, resulting in pBRDI17. The correctness of the sequences of fragments C and F was confirmed by DNA sequencing. Plasmid pBRDI137 lacks both ORF3abc and 7ab. The targeted recombination procedure to construct the FIPV recombinants was performed as described previously (14).

Genomic analysis of candidate recombinants. After plaque purification, monolayers (25 cm^2) of FCWF cells were infected with the candidate recombinant viruses, the culture medium was harvested at 24 h postinfection, and viral RNAs were isolated by use of a QIAamp viral RNA mini kit (Qiagen) as specified by the manufacturer. Viral RNAs were reverse transcribed under standard conditions using Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) and primer 10 (5′-ATGGATCCGACGCGTTGTCCCTGTGTGGCCATTTGAAAGTTTAG-3′) or 13 (5′-GCGGCCGCTTTTTTTTTTTT-3′) to prepare cDNAs of the 3abc and 7ab regions, respectively. Primer pair 1 (5′-GCCATTCTCATTGATAAC-3′) and 9 (5′-CAGGAGCCAGAAGAAGACGCTAA-3′) and primer pair 11 (5′-GGTGATTACTCAACAGAAGC-3′) and 12 (5′-GACCAGTTTTAGACATCG-3′) were used to amplify the 3abc and 7ab regions, respectively. PCR amplifications were run for 30 cycles of 1 min at 94°C, 1 min at 48°C, and 2 min at 72°C with AmpliTaq DNA polymerase (Perkin-Elmer). Products were analyzed by agarose gel electrophoresis.

Animal experiments. Specific-pathogen-free HsdCpd:CAD(BR) kittens (Harlan Sprague Dawley, Inc.) were used in the infection and vaccination studies. At the start of the experiment, the kittens were approximately 17 to 20 weeks old. The animals were housed in separate groups in a closed facility. Kittens were inoculated oronasally with wild-type FIPV or recombinant derivatives. Kittens were examined for signs of disease on a daily basis. For avoidance of unnecessary suffering, kittens were euthanized once they had entered the irreversible terminal phase of FIP, as judged by the animal experts at the animal facility, and a full postmortem examination was performed. For all animal experiments, the approval of the Ethical Committee of Utrecht University was obtained.

Scoring system for clinical signs. Cats were scored for several clinical signs as follows. On a daily basis, they were scored for depression (inactivity for three consecutive days, 1 point), anorexia (not eating for three consecutive days, 1 point), and neurological disorders (swaggering, 1 point). On a weekly basis, they were scored for fever (>40.1°C, 1 point), jaundice (yellow plasma, 1 point), weight loss (loss of >2.5% of body weight per week, 1 point), and lymphopenia (lymphocyte count of $<0.5 \times 10^9$/liter).

Virus neutralization assays. For virus neutralization assays, equal aliquots of FIPV (50 μl of $10^{6.5}$ 50% tissue culture infective doses/ml) were incubated overnight at 37°C with twofold dilutions of heat-inactivated plasma from kittens (50 μl). The viruses were then added to FCWF cells (16,000 cells per well in a 96-well plate) and incubation was continued for 48 h, after which the development of cytopathic effects was determined (13).

RESULTS

Deletion of the group-specific genes of FIPV 79-1146. We first studied the requirement of the FIPV 79-1146 group-specific genes for replication in culture cells. We therefore set out to prepare recombinant viruses from which these genes were deleted by targeted RNA recombination (Fig. 1B). Three derivatives of the transcription vector pBRDI1 were generated, lacking either gene cluster 3abc (pBRDI13) or 7ab (pBRDI17) or both clusters (pBRDI137). As a consequence of the deletion of the 3abc cluster, the transcription regulatory sequence (TRS) preceding the E gene, which is located within the 3′ end of the *3c* gene, was also deleted. TRSs are found upstream of most coronavirus genes and are essential for the generation of subgenomic mRNAs. Therefore, the TRS of ORF3a (Fig. 1C, sequence 1) was designed to drive the generation of the mRNA expressing the E gene. To accomplish this, we fused the initiating methionine codon of ORF3a to the second codon (ACG) of the E gene (Fig. 1C, sequence 1).

We were unable to obtain a recombinant virus containing a deletion of ORF7ab when the stop codon of the N gene was fused to the second nucleotide (G) of the 3′ untranslated region (UTR). We therefore constructed vector pBRDI17, in which the 3′-terminal 15 nt of ORF7b were maintained and in which the TRS located at the 3′ end of the N gene was mutated by one nucleotide change (Fig. 1B, arrow 2) without affecting the coding sequence.

The deletions were introduced into the FIPV genome by RNA recombination between synthetic RNAs derived from the transcription vectors and the genome of the interspecies chimeric virus mFIPV (Fig. 1B) (32), which carries the MHV S glycoprotein ectodomain, allowing it to grow in murine cells. As a positive control, a recombination experiment between RNA derived from pBDRI1 and the genome of mFIPV was also performed. Due to the presence of the wild-type FIPV S gene in the donor RNAs, recombinant viruses, designated r-wtFIPV, FIPVΔ7ab, FIPVΔ3abc, and FIPVΔ3abc/Δ7ab, were easily selected by their regained ability to grow in feline cells (Fig. 1B).

Genomic analysis of the candidate recombinant deletion viruses. To examine whether the candidate recombinant viruses obtained had acquired the desired deletions, we performed reverse transcription (RT)-PCR on the genomic viral RNAs isolated from plaque-purified stocks, amplifying the 3abc or 7ab gene cluster. A schematic overview of the amplified regions and primers used is shown in Fig. 2A. Analyses of the RT-PCR products obtained for the 3abc region and for the 7ab region are shown in Fig. 2B and C, respectively. In the case of r-wtFIPV and FIPVΔ7ab, the RT-PCR products derived from the 3abc region migrated according to their predicted size of 1,491 bp, showing that these viruses still carried the 3abc region (Fig. 2B). In contrast, FIPVΔ3abc and FIPVΔ3abc/Δ7ab lacked this region, as judged by the sizes of the amplified fragments (397 bp) (Fig. 2B). For the 7ab region, RT-PCR products of the predicted size of 1,572 bp (Fig. 2C) were obtained with r-wtFIPV and FIPVΔ3abc, showing that they still carried the 7ab region. In contrast, FIPVΔ7ab and FIPVΔ3abc/Δ7ab lacked this region, as judged by the size of the obtained RT-PCR fragments (646 bp) (Fig. 2C). The 397- and 646-bp RT-PCR fragments derived from FIPVΔ3abc, FIPVΔ7ab, and FIPVΔ3abc/Δ7ab, indicative of a 3abc and a 7ab deletion, respectively, were cloned and sequenced to confirm the intended DNA sequence. These results demonstrate that the various FIPV recombinant viruses did indeed have the expected deletions and that both regions were dispensable for growth in culture cells.

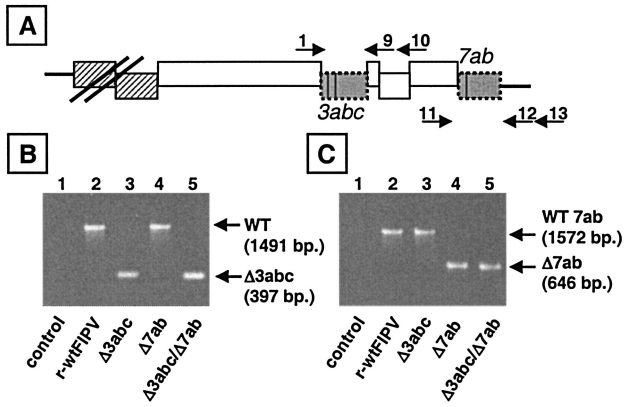

FIG. 2. RT-PCR analyses of the FIPV deletion recombinants. RT-PCR was used to amplify the ORF3abc region (B) and the ORF7ab region (C) using RNAs isolated from recombinant FIPV as a template. (A) Physical map of the genome of FIPV. Gray boxes indicate ORF3abc and ORF7ab; the locations of the primers used are indicated by arrows. For primer sequences, see Materials and Methods. (B) RT-PCR using primer 10 for the RT step and primers 1 and 9 for PCR. The products obtained in either the presence (WT; 1,491 bp) or absence (Δ3abc; 397 bp) of ORF3abc are indicated by arrows. (C) RT-PCR using primer 13 for the RT step and primers 11 and 12 for PCR. The products obtained in either the presence (WT; 1,572 bp) or absence (Δ7ab; 646 bp) of ORF7ab are indicated by arrows. Lane 1, no template; lane 2, r-wtFIPV; lane 3, FIPVΔ3abc (Δ3abc); lane 4, FIPVΔ7ab (Δ7ab); lane 5, FIPVΔ3abc/Δ7ab (Δ3abc/Δ7ab).

Tissue culture growth phenotype. All four recombinant viruses lost the ability to infect mouse LR7 cells but replicated efficiently in feline FCWF cells, showing similar cytopathic effects. Each virus induced extensive syncytia, to an extent similar to that with wild-type virus. Small but consistent differences were observed in one-step growth experiments: the virus titers reached with FIPVΔ3abc were generally two- to fivefold higher than those with r-wtFIPV. In contrast, FIPVΔ7ab, and even more so, FIPVΔ3abc/Δ7ab grew to lower titers (Fig. 3). These differences in growth were due to the genetic deletions and not to any fortuitous mutations acquired during passaging, as independently obtained recombinant viruses exhibited similar growth characteristics. These results demonstrate that the group-specific genes play no major role in growth on culture cells.

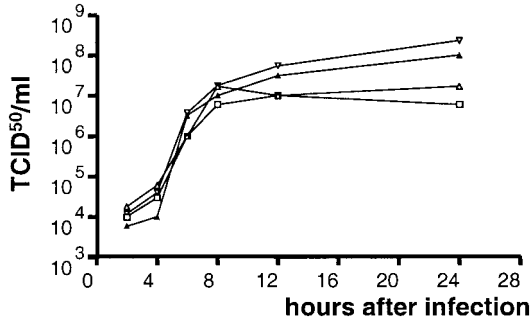

FIG. 3. Growth of FIPV deletion recombinants in feline cells. Single-step growth kinetics of r-wtFIPV (▲), FIPVΔ3abc (▽), FIPVΔ7ab (△), and FIPVΔ3abc/Δ7ab (□) are shown. Viral infectivity in the culture medium was determined at different times postinfection by a quantitative assay with FCWF cells, and 50% tissue culture infective doses ($TCID_{50}$) were calculated.

Virulence of recombinant viruses. The properties of the recombinant viruses were next characterized in their natural host, the cat. Animals were inoculated oronasally with a dose of 100 PFU of the parental virus FIPV 79-1146 ($n = 4$), r-wtFIPV ($n = 5$), FIPVΔ3abc ($n = 5$), FIPVΔ7ab ($n = 5$), or FIPVΔ3abc/Δ7ab ($n = 5$). FIPV 79-1146 served as a positive control; the lethal course of infection with this dose in young kittens has been demonstrated (13). Cats were monitored for 3 months for signs of clinical disease. Inoculation with FIPV 79-1146 or its recombinant equivalent r-wtFIPV resulted in a rapid onset (at 7 to 14 days postinoculation) of clinical disease signs. All cats within these two groups showed depression and anorexia, often accompanied by fever, jaundice, weight loss, and lymphopenia, as shown in Table 1. Three of four and five of five cats infected with FIPV 79-1146 and r-wtFIPV, respectively, had to be euthanized between weeks 2 and 6, resulting in similar survival curves for both viruses (Fig. 4). Postmortem analyses revealed that the livers, spleens, and/or kidneys within the peritoneal cavity were affected similarly, showing multiple dispersed pyogranulomas, sometimes accompanied by ascites formation, which is characteristic of classical FIP disease. These data show that r-wtFIPV is as virulent as the parental FIPV 79-1146 strain and therefore serves as a proper control.

In sharp contrast, cats inoculated with the various deletion viruses showed no clinical signs typical of FIP disease (Table 1), and all cats survived (Fig. 4). In each case, the deletion of the group-specific genes apparently led to a severe attenuation of the virus.

Induction of antibodies. To confirm that the deletion viruses had actually established an infection and to investigate whether a measurable antibody response had been induced, we determined the FIPV-neutralizing activity in cat plasma. Plasma samples were prepared from blood taken at days 0, 25, and 90 postinoculation and were heat inactivated, and the FIPV-neutralizing activity was determined by end-point dilution using FCWF cells. None of the cat plasma showed significant neutralizing activity at day 0 (Fig. 5). However, at day 25 postinoculation, all cats had seroconverted and showed high titers of neutralizing antibodies, except for cat 422 (FIPVΔ3abc/Δ7ab), which remained seronegative. Surprisingly, the titers observed in cats inoculated with virulent FIPV 79-1146 and r-wtFIPV were comparable to the titers induced by the avirulent viruses FIPVΔ3abc and FIPVΔ7ab (Fig. 5A to D). The titers remained high until day 90. In contrast, the titers measured in FIPVΔ3abc/Δ7ab-infected cats were clearly lower, especially at 90 days postinoculation (Fig. 5E). Altogether, the results demonstrate that all viruses induced significant antibody responses.

FIPV deletion viruses serve as attenuated, live vaccines. To study whether a previous infection with the attenuated deletion variants would protect the cats against a homologous FIPV challenge, we performed a vaccination and challenge experiment. Groups of five kittens each were vaccinated oronasally twice with FIPVΔ3abc, FIPVΔ7ab, or FIPVΔ3abc/Δ7ab (100 PFU at day 0 and 1,000 PFU at day 42). A control group was mock vaccinated with phosphate-buffered saline (PBS). Once again, the virus-inoculated kittens did not develop any signs of disease, while neutralizing antibodies were induced in all animals.

TABLE 1. Total clinical scores after inoculation with wild-type FIPV and FIPV deletion mutants

Virus or animal no.	Clinical score for symptom							Total clinical score	Day of death postinfection
	Fever	Depression	Anorexia	Jaundice	Neurological disorder	Weight loss	Lymphopenia		
FIPV									
249	2	3	3	4	0	3	2	17	
261	2	1	1	2	0	1	2	9	12
277	1	2	2	3	1	2	2	13	27
428	0	1	2	3	0	2	3	11	27
rFIPV									
251	1	1	3	1	0	2	0	8	35
263	0	2	3	1	1	3	0	10	35
376	0	1	3	1	0	1	1	7	25
380	2	1	2	0	1	0	0	7	19
424	0	1	3	2	0	0	1	8	43
Δ3abc									
241	0	0	0	0	0	0	0	0	
279	0	0	0	0	0	1	0	1	
283	0	0	0	0	0	0	0	0	
400	0	0	0	0	0	0	0	0	
440	0	0	0	0	0	0	0	0	
Δ7ab									
243	0	0	0	0	0	0	0	0	
281	0	0	0	0	0	0	1	1	
378	0	0	1	0	0	0	0	1	
426	0	0	0	0	0	0	0	0	
430	0	0	0	0	0	0	1	1	
Δ37									
245	0	0	0	0	0	0	0	0	
247	0	0	0	0	0	1	0	1	
420	0	0	0	0	0	0	0	0	
422	0	0	0	0	0	0	0	0	
432	0	0	0	0	0	0	1	1	

At day 70, all kittens were challenged by oronasal inoculation with 500 PFU of the virulent FIPV strain 79-1146 and were monitored for 3 months (Fig. 6). The control kittens were successfully infected: all cats showed severe clinical signs of FIP disease within 1 week (Table 2), including a severe loss of body weight (Fig. 7) and a dramatic drop in peripheral blood lymphocytes (Fig. 8). Four of the five kittens died within 30 days after challenge; the remaining animal (cat 77) survived for more than 3 months (Fig. 6). The postmortem findings for the four deceased cats were consistent with systemic, pathological FIP disease.

In contrast, the vaccination with either FIPVΔ3abc or FIPVΔ7ab provided strong protection against disease caused by the FIPV challenge. The cats remained healthy (Table 2) and gained body weight (Fig. 7), and no drop in peripheral

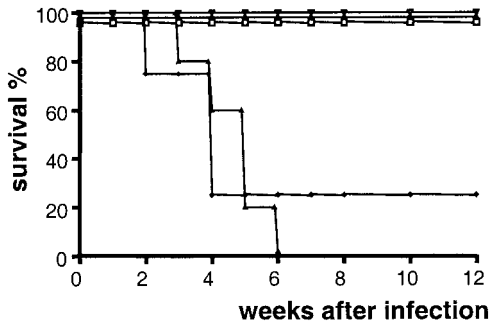

FIG. 4. In vivo survival after infection with FIPV deletion recombinants. Kittens (20 weeks old) were inoculated with 100 PFU of FIPV (n = 4) (♦), r-wtFIPV (n = 5) (▲), FIPVΔ3abc (n = 5) (▽), FIPVΔ7ab (n = 5) (△), and FIPVΔ3abc/Δ7ab (n = 5) (□), and percentages of survival are shown.

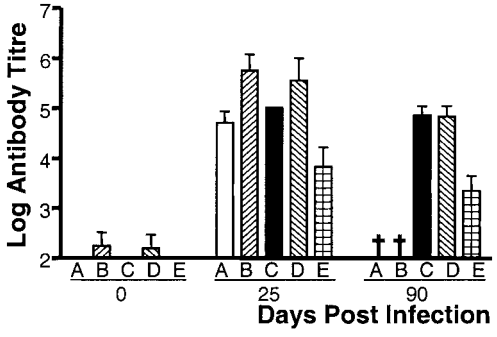

FIG. 5. Induction of FIPV-neutralizing antibodies in plasma of kittens inoculated with FIPV deletion variants at different days postinfection (0, 25, and 90 days). The means per group of kittens are given. (A) FIPV. (B) r-wtFIPV. (C) FIPVΔ3abc. (D) FIPVΔ7ab. (E) FIPVΔ3abc/Δ7ab. The error bars represent standard deviations.

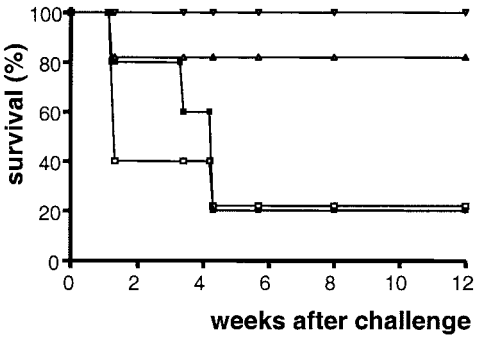

FIG. 6. Survival after challenge with FIPV. Kittens were vaccinated twice, at day 0 (100 PFU) and day 42 (1,000 PFU), with FIPVΔ3abc (n = 5) (▽), FIPVΔ7ab (n = 5) (△), or FIPVΔ3abc/Δ7ab (n = 5) (□) or were mock vaccinated with PBS (n = 5) (■). At day 70, kittens were challenged oronasally with 500 PFU of FIPV 79-1146.

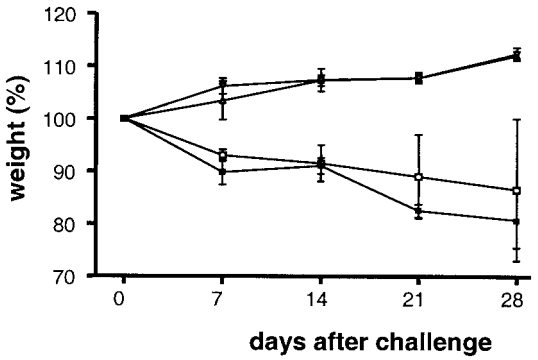

FIG. 7. Body weight of kittens after challenge. Kittens were vaccinated twice, at day 0 (100 PFU) and day 42 (1,000 PFU), with FIPVΔ3abc (n = 5) (▽), FIPVΔ7ab (n = 5) (△), or FIPVΔ3abc/Δ7ab (n = 5) (□) or were mock vaccinated with PBS (n = 5) (■). At day 28 after the last vaccination, the animals were challenged oronasally with 500 PFU of FIPV 79-1146 and their body weights were determined regularly. The figure shows the average body weights per group of kittens, taking the value at the day of challenge (0) as 100%. The error bars represent standard deviations.

blood lymphocytes was observed (Fig. 8). The animals survived for at least 3 months (Fig. 6), except for one animal (cat 133, vaccinated with FIPVΔ7ab). This cat developed severe FIP within a week and had to be euthanized at day 9 postinoculation. A postmortem examination revealed the typical signs of FIP. Cats 91 and 125 (vaccinated with FIPVΔ3abc) showed a slight body weight drop at one time point, indicating that the challenge was not entirely without burden (Table 2). However, both cats resumed growth and remained healthy. Upon postmortem macroscopic examination, none of the cats vaccinated with FIPVΔ3abc or FIPVΔ7ab (except cat 133) showed any of the characteristic FIP lesions.

Remarkably, the vaccination with FIPVΔ3abc/Δ7ab was not protective. All kittens developed signs of FIP disease within 1 week (Table 2; Fig. 7 and 8). Four of the five kittens died within 30 days after challenge, whereas the remaining one (cat 111) recovered and survived for more than 3 months (Fig. 6). The accelerated development of disease in some of the animals suggested that the vaccination with this attenuated virus was

TABLE 2. Total clinical scores after challenge with FIPV 79-1146

Virus or animal no.	Clinical score for symptom							Total clinical score	Day of death postchallenge
	Fever	Depression	Anorexia	Jaundice	Neurological disorder	Weight loss	Lymphopenia		
Control									
077	0	1	2	1	1	2	1	7	
081	0	1	1	2	0	1	1	6	8
113	2	1	2	1	0	4	1	11	30
119	1	1	1	2	0	3	3	11	30
123	0	1	1	2	0	2	1	7	30
Δ3abc									
079	1	0	0	0	0	0	0	1	
085	0	0	0	0	0	0	0	0	
091	0	0	0	0	0	1	0	1	
105	0	0	0	0	0	0	0	0	
125	0	0	0	0	0	1	0	1	
Δ7ab									
087	0	0	0	0	0	0	0	0	
095	0	0	0	0	0	1	0	1	
107	0	0	0	0	0	0	0	0	
117	0	0	0	0	0	0	0	0	
133	0	2	1	2	0	1	1	7	8
Δ37									
089	0	1	1	2	0	1	1	6	9
109	1	1	2	1	0	4	2	11	30
111	1	0	1	0	0	1	1	4	
121	1	1	1	0	0	1	0	4	9
143	0	1	0	2	0	1	1	5	9

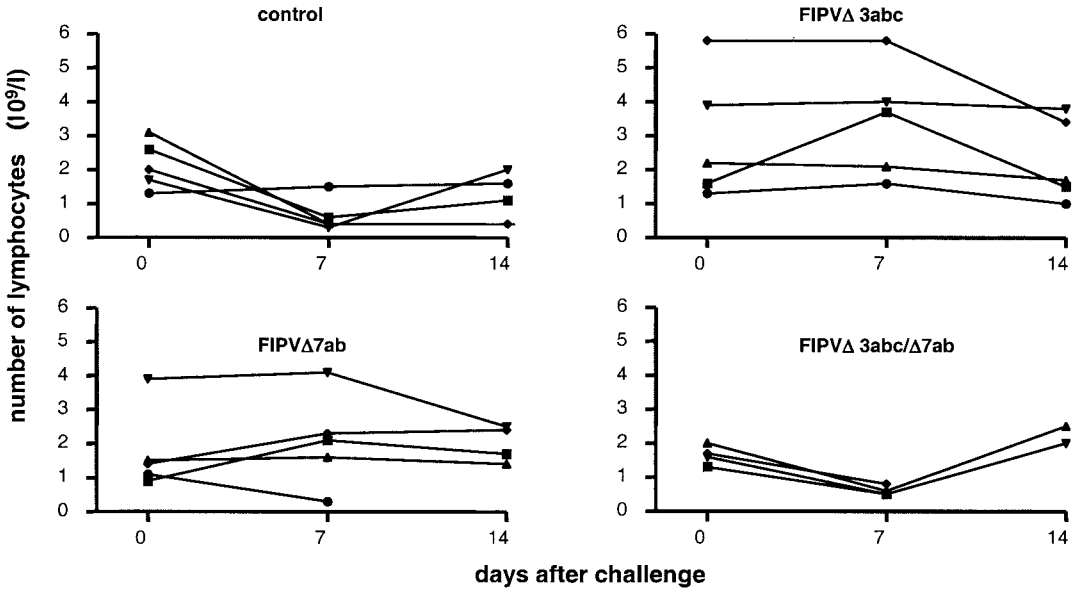

FIG. 8. Number of peripheral blood lymphocytes after FIPV 79-1146 challenge. Kittens were vaccinated twice with PBS (control), FIPVΔ3abc, FIPVΔ7ab, or FIPVΔ3abc/Δ7ab. At day 28 after the last vaccination, the animals were challenged oronasally with 500 PFU of FIPV 79-1146. Blood samples were analyzed for lymphocyte counts on the day of challenge (0) and 7 and 14 days thereafter.

detrimental rather than protective. The postmortem analysis of the four deceased cats confirmed the FIP diagnosis.

For another vaccination and challenge experiment with the recombinant deletion viruses, we used the animals from the virulence experiment described in Table 1. The cats of groups Δ3abc, Δ7ab, and Δ3abc/Δ7ab were challenged with FIPV 79-1146 at day 90 after the first inoculation. An age-matched control group of nonvaccinated cats was included. Essentially similar results were obtained. All of the cats in the control group and three of five cats in the FIPVΔ3abc/Δ7ab group became ill with FIP after the challenge, whereas none of the cats in the two other groups developed any detectable signs of disease.

DISCUSSION

Our studies demonstrate that the genomic deletion of the group-specific gene clusters 3abc and/or 7ab from FIPV strain 79-1146 hardly affects growth in culture cells but converts an otherwise lethal virus into an innocuous derivative. Moreover, prior infections with viruses lacking either one of these gene clusters protected animals against a homologous challenge, making these viruses promising live attenuated vaccine candidates. Live attenuated vaccines are generally the most powerful means of protection against viral diseases, as was demonstrated by the successful control of the smallpox, polio, and measles epidemics. Interestingly, the attenuation resulting from the simultaneous deletion of both FIPV gene clusters, as in FIPVΔ3abc/Δ7ab, compromised the immunogenicity to an extent insufficient to provide protection.

Our observations corroborate the concept that the coronavirus group-specific genes are not essential for growth in cell culture but function in virus-host interactions. A number of coronaviruses have been described that carry mutations in these genes, some in viral genomes isolated directly from animals (such as feline coronaviruses [44]), but often these alterations have been obtained during passaging in vitro (for feline coronaviruses, see reference 17). Recently, the advent of reverse genetics technologies allowed the manipulation of these genes in an isogenic background. Thus, the directed deletion of the group-specific genes from MHV (a group 2 coronavirus), which show no homology with those of FIPV, resulted in viruses that multiplied well in culture cells but exhibited strongly reduced virulence (8). Similar results were obtained with swine TGEV, which is, like FIPV, a group 1 coronavirus, when ORF3a and -3b (homologues of FIPV's 3a and 3c) were replaced by a green fluorescent protein gene (39). One should be aware that these attenuating effects are not necessarily due to the deletions per se. For instance, more subtle gene inactivations, such as that of ORF7 of TGEV (a homologue of FIPV ORF7a) (28) and of ORF4 of MHV (27), achieved through genetic manipulation of their TRSs, resulted only in a minor or no reduction of virulence. Obviously, in order to elucidate their real significance in virulence, these genes should be inactivated by site-specific mutagenesis.

FIPVΔ7ab and FIPVΔ3abc are the first highly efficacious live vaccines against FIP (90% survival compared to 20% in the control group). A major obstacle in previous vaccine attempts has been the fact that coronavirus antibodies are not protective, but rather they can enhance and accelerate the disease (45). The antibody-dependent enhancement (ADE) is caused by neutralizing spike protein-specific antibodies (42) that facilitate an enhanced (possibly Fc receptor-mediated) infection of macrophages (5, 18, 26). This phenomenon has been observed before with influenza virus, lentivirus, alphavirus, and flavivirus infections (25, 35, 36, 41). Remarkably, neither FIPVΔ7ab nor FIPVΔ3abc elicited ADE upon challenge, despite their induction of high titers of neutralizing antibodies, including antibodies against the S protein (radio-immunopre-

cipitations are not shown). In contrast, kittens vaccinated with FIPVΔ3abc/Δ7ab not only were unprotected, but the apparent accelerated progression of disease in some of these animals was suggestive of ADE. The significance of these observations is yet unclear. There are indications that cellular immunity is important for protection against FIP (33). Recently, strong cellular responses were detected in cats that survived a FIPV challenge (J. D. F. Mijnes and R. J. de Groot, unpublished results). The lack of protection provided by infection with FIPVΔ3abc/Δ7ab may thus be due to its insufficient induction of cellular immunity compared to FIPVΔ7ab and FIPVΔ3abc.

Feline coronaviruses occur in two serotypes with different serological and biological properties. The main differences are found in the S protein, for which type II (a field recombinant from a canine coronavirus [16]) shares only approximately 45% of the sequence with type I (23). It remains to be established whether our serotype II vaccines provide protection against a serotype I challenge. Such studies are under way and will obviously provide interesting insight into the importance of the spike protein for protection.

Mounting evidence suggests that the coronavirus group-specific genes are not essential for growth in cell culture and that their deletion results in reduced virulence; these mutants can be used as modified live vaccines. We propose the deletion approach as a basis for vaccine development against other pathogenic coronaviruses in animals and humans, including the SARS coronavirus.

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