Further Development of a Recombinant Feline Herpesvirus Type 1 Vector Expressing Feline Calicivirus Immunogenic Antigen

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Vaccines are the most effective and cheap prophylactic tools in the field of veterinary medicine. Recently, genetic engineering techniques have been applied to design new vaccines in order to reduce some problems which the present vaccines have [27]. A recombinant viral vector vaccine for delivery of foreign antigens is also a type of the genetically engineered vaccine. The vector vaccines contain an attenuated live vector in which foreign genetic information derived from one or more other microbial pathogens has been incorporated. The vector vaccines are applied as recombinant polyvalent vaccines to induce protective immunity against the pathogen(s) derived from the heterologous genetic information, in combination with immunity against field-type variants of the vector [27].

Feline herpesvirus type 1 (FHV-1) is a member of the alphaherpesvirus subfamily of \textit{Herpesviridae} and causes primarily an upper respiratory tract disease, known as feline viral rhinotracheitis (FVR) [12]. The thymidine kinase (TK) gene [29], ORF2 gene downstream of the gC homologue [22], and gI and gE homologous genes [7, 16, 23] of FHV-1 were reported as target regions for the deletion and some of these non-essential genes have been used as insertion sites for expression of foreign antigens. Up to now, the genes derived from feline calicivirus (FCV) [28], feline leukemia virus [1, 21, 24] and feline immunodeficiency virus [14, 20] have been stably introduced into the FHV-1 genome for development of recombinant polyvalent vaccines for feline medicine.

We previously reported the attenuation of TK deficient mutant, C7301\textsuperscript{dl}TK, of FHV-1 [29] in cats and the construction of a recombinant FHV-1, C7301\textsuperscript{dl}TK-Cap, in which the entire ORF of cDNA encoding the capsid protein of FCV was inserted into the TK deletion locus of C7301\textsuperscript{dl}TK in cats and the construction of a recombinant FHV-1, C7301\textsuperscript{dl}TK-Cap [28], of FHV-1 [29] in cats and the construction of a recombinant FHV-1, C7301\textsuperscript{dl}TK-Cap, in which the entire ORF of cDNA encoding the capsid protein of FCV was inserted into the TK deletion locus of C7301\textsuperscript{dl}TK [28]. FCV immunogenic antigens were expressed mainly as a fusion capsid protein with the N-terminal part of TK under the control of the FHV-1 early promoter by the C7301\textsuperscript{dl}TK-Cap. In vivo experiments showed that cats vaccinated twice intraocularly, intranasally and orally with the C7301\textsuperscript{dl}TK-Cap were protected to a significant degree against subsequent virulent challenges with both parent FCV F4 and FHV-1 C7301 strains [25]. However, cats inoculated with C7301\textsuperscript{dl}TK-Cap excreted the recombinant viruses with lower levels than cats with C7301\textsuperscript{dl}TK and only two of the five cats developed detectable virus neutralizing (VN) antibody against FCV in cats inoculated with C7301\textsuperscript{dl}TK-Cap before FCV challenge [25]. We consider that the induction of protective immunity by recombinant polyvalent vaccines is attributable to their full replication and expression of the foreign immunogenic antigen \textit{in vivo}. Therefore, further improvement of recombinant FHV-1 was required to increase their efficacy.

In this study, we constructed a further improved recombinant FHV-1 carrying a putative FHV-1 gC promoter sequence upstream of the FCV precursor capsid gene of C7301\textsuperscript{dl}TK-Cap and evaluated the vaccine efficacy of the recombinant virus \textit{in vivo} experiments.

**MATERIALS AND METHODS**

**Viruses and cells:** The three recombinant FHV-1, C7301\textsuperscript{dl}TK [26], C7301\textsuperscript{dl}TK-Cap [28] and a recombinant virus constructed in the present study, and the parent FHV-
1 C7301 [11] and FCV F4 [17] strains were grown in Crandell feline kidney (CRFK) cells [2], as described previously [25, 26, 28, 29].

Construction of transfer vector: We previously reported the nucleotide sequence of the FHV-1 gC gene and the flanking regions [10]. In this study, we decided to use a 781 bp SalI fragment, containing the 5′-coding signal sequence of gC and the upstream region, as a putative gC promoter sequence for expressing foreign antigen. The fragment was subcloned into pBluescript SK- as described previously [10]. A 2032 bp EcoRI fragment, containing an entire ORF of cDNA encoding the precursor capsid protein of FCV F4 strain [15], was inserted into the EcoRI site located immediately downstream of the SalI fragment in the subcloned plasmid and the resulting plasmid was designated as pf(gCp)-Cap. After the pf(gCp)-Cap was digested with Asp713 and XbaI, a 2885 bp Asp713-XbaI fragment, containing both of the putative gC promoter sequence and the FCV precursor capsid gene, was isolated, blunt-ended by T4 polymerase and inserted into a unique SmaI site at the end of the TK deletion region of pfTK-SBAS E (Fig. 1E), which was previously constructed as a transfer vector to produce a TK deficient mutant, C7301dlTK [26] (Fig. 1C). The plasmid was designated as pfTK(gCp)-Cap and used as a transfer vector.

Generation and identification of recombinant FHV-1: The viral DNA of FHV-1 C7301 strain was cotransfected with the transfer vector, pfTK(gCp)-Cap, into CRFK cells and the recombinant viruses were selected from the propagated viruses as described previously [26, 28]. One of these selected viruses was plaque-purified three times.

To examine whether the recombinant virus has the intended insertion in the TK gene, we compared the TK gene region of the recombinant virus with those of parent FHV-1 C7301 strain and the recombinant C7301dlTK by Southern blot and polymerase chain reaction (PCR) amplification analyses as described previously [26, 28].

Time course of viral growth: One step growth curve of the recombinant FHV-1 constructed in the present study was determined as described previously [28].

Analyses of expressed antigen: Seven anti-FCV monoclonal antibodies (MAbs) (1D7, 16B11, 8C7, 8C10, 4B1, 15H3 and 13B14) used in this study recognize seven different neutralizing epitopes on the capsid protein of FCV F4 strain [15, 18, 19], and three anti-FHV-1 MAbs (22F4, 17C11 and 41G4) recognize three different major glycoproteins of FHV-1 C7301 strain, gB, gC and gD [6, 8–10]. All MAbs were used for indirect immunofluorescence assay (IFA) and some of them were also used for enzyme-linked immunosorbent assay (ELISA).

To examine the antigenic property of the foreign product expressed by the recombinant virus in IFA, infected- or uninfected-cells were smeared on glass slides, air-dried and then fixed in acetone. The fixed cells were incubated for 30 min at 37°C with the MAbs described above. After incubation, the slides were washed three times with phosphate-buffered saline (PBS), and then anti-mouse IgG goat antibody conjugated with fluorescein isothiocyanate was applied. After incubation for 30 min at 37°C, the slides were washed again, mounted in buffered glycerol and

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**Fig. 1.** Construction of transfer vectors. A, SalI restriction endonuclease map of FHV-1 genome [13]. B, BamHI-SalI fragment of pfTK-SB containing the TK gene [26]. C, BamHI-SalI fragment of pfTK-SBAS E. pfTK-SBAS E was used to produce C7301dlTK [26]. D, BamHI-SalI fragment of pfTK-Cap, pfTK-Cap was used to produce C7301dlTK-Cap [28]. E, BamHI-SalI fragment of pfTK(gCp)-Cap. As described in this text, an Asp713-XbaI fragment, containing a putative gC promoter sequence and an FCV precursor capsid gene, was blunt-ended and inserted into a unique SmaI site of pfTK-SBAS E. pfTK(gCp)-Cap was used to produce dlTK(gCp)-Cap in this study. On the construction of pfTK(gCp)-Cap, the sequence from initiation codon of the signal sequence of gC to termination codon of capsid gene is in frame. B, BamHI; Ev, EcoRI; Sm, SmaI; El, EcoRI; S, SalI.
examined by a fluorescence microscopy.

To prepare the antigens for ELISA, monolayers of CRFK cells were prepared on 60 mm culture dishes and inoculated with viral samples a multiplicity of infection of 3 plaque-forming units (PFU) per cell. At various intervals, the infected cells were collected, washed twice with PBS and then lysed in lysis buffer (1% Triton X-100, 1% sodium deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide and 0.2 units/ml aprotinin in 0.01 M Tris-Cl (pH 8.0), 0.14 M NaCl and 0.025% NaN3 solution). After the lysate was centrifuged at 15,000 rpm for 1 hr, the supernatant was used as the antigen for ELISA at a dilution of 1:400.

For ELISA, each well of a 96-well flat-bottom ELISA plate (Nunc, Denmark) was coated with 50 µl of the antigen solution at 4°C overnight. After the wells were blocked with 100 µl of PBS containing 0.1% bovine serum albumin for 2 hr at room temperature (RT), they were washed with PBS containing 0.05% Tween-20 (PBST). Fifty microliter of an MAAb 4B1 against FCV capsid protein, 22F4 against FHV-1 gB or 17C11 against FHV-1 gC at a dilution of 1:100 were added to each well. After incubation for 1 hr at RT, the wells were washed with PBST. Fifty microliter of an anti-mouse IgG goat antibody conjugated with horseradish peroxidase at a dilution of 1:200 were then added to each well and reacted for another 1 hr at RT. The wells were washed again with PBST and reacted with 100 µl of substrate containing 3, 3', 5, 5'-tetramethylbenzidine (BIO-RAD, U.S.A.) for 30 min at RT. After reaction, optical densities were measured at 655 nm with ELISA reader (BIO-RAD, Model 3550, U.S.A.). As a negative control, the antigens derived from uninfected CRFK cells were used and independent experiments were performed in triplicate.

Animal experiment: A total of 12 specific pathogen-free cats of approximate 3 months of age were obtained from Harlan Sprague Dawley, Inc., Madison, WI, U.S.A. and divided into three groups. Each group of cats (n=4) was kept in each isolation room.

Each group was inoculated once intraocularly, intranasally and orally with a total of 1.0 ml of supernatant fluid from each of dITK(gCp)-Cap-, C7301/dITK-Cap, or C7301/dITK-infected CRFK cells containing 1.0 × 10^6 PFU per cat, as vaccines, and designated as dITK(gCp)-Cap, C7301/dITK-Cap or C7301/dITK group, respectively. Four weeks (Days 28) after the vaccination, all groups were challenged by the same routes with a total of 1.0 ml of supernatant fluid from FCV F4 strain-infected CRFK cells containing 1.0 × 10^9 PFU per cat. During 8 weeks after the vaccination, all cats were observed daily for clinical signs and scored using the point system as described previously [25]. Virus isolation and VN assays were also performed as described previously [25, 28, 29].

RESULTS

**Generation and identification of a recombinant FHV-1:**

As described in Materials and Methods, we constructed a transfer vector, pITK(gCp)-Cap, carrying a blunt-ended fragment, containing a putative gC promoter sequence and an FCV precursor capsid gene, in the Smal site of pITK-SBASE (Fig. 1E). On the construction of pITK(gCp)-Cap, the sequence from initiation codon of the signal sequence of gC to termination codon of the capsid gene was in frame. By using the transfer vector, we obtained a recombinant FHV-1, dITK(gCp)-Cap. By Southern blot and PCR amplification analyses, the recombinant dITK(gCp)-Cap was confirmed to have the intended insertion at the TK deletion locus (data not shown).

Next, we examined whether the insertion of a putative gC promoter sequence influences the virus growth. As a result, the growth kinetics of dITK(gCp)-Cap was similar to those of other recombinant viruses, C7301/dITK-Cap and C7301/dITK, in CRFK cells (data not shown), indicating that the insertion had no influence on the virus growth.

Expression of FCV immunogenic antigen by the recombinant dITK(gCp)-Cap: To analyze whether the recombinant dITK(gCp)-Cap expresses the FCV immunogenic antigen, dITK(gCp)-Cap-, C7301/dITK-Cap-, or C7301/dITK-infected CRFK cells were examined by IFA, using seven anti-FCV and three anti-FHV-1 MAbs. With all anti-FCV MAbs, a positive fluorescence was observed in cytoplasm of CRFK cells infected with the recombinant dITK(gCp)-Cap and C7301/dITK-Cap, but not in C7301/dITK-infected CRFK cells. In particular, the fluorescence was stronger in dITK(gCp)-Cap-infected cells than C7301/dITK-Cap-infected cells (Fig. 2). On the other hand, with the three anti-FHV-1 MAbs, a positive fluorescence was detected on the surface of CRFK cells infected with the three recombinant FHV-1 (data not shown).

Further, the expression of FCV antigen by each of the recombinant viruses in CRFK cells was chronologically determined by ELISA. With a MAAb 4B1 against FCV capsid protein, the optical densities of the antigens prepared from CRFK cells inoculated with C7301/dITK-Cap increased rapidly and reached the maximum at 6 hr, while the density with the dITK(gCp)-Cap reached the maximum at 24 hr and was higher than that with C7301/dITK-Cap (Fig. 3). On the other hand, time course of the syntheses of gB and gC by each of the recombinant viruses was also determined as positive controls and showed that these glycoproteins were synthesized in the late phase by these recombinant viruses in parallel (data not shown).

**Vaccine efficacy of the recombinant dITK(gCp)-Cap against FCV challenge:** Following vaccination, none of the vaccinated cats developed clinical signs (data not shown). Virus shedding in ocular, nasal and oral mucuses was monitored at appropriate intervals in three groups until FCV challenge (Fig. 4). As a result, cats in C7301/dITK-Cap group excreted the recombinant viruses with lower titers than cats in C7301/dITK group. The data of C7301/dITK-Cap corresponded to our previous report [25]. On the other hand, although cats in dITK(gCp)-Cap group also excreted the viruses with lower titers than cats in C7301/dITK group,
these titers were not as low as those of C7301/dTK-Cap group.

The vaccine efficacy of the recombinant dTK(gCp)-Cap against FCV infection was demonstrated by subsequent virulent FCV challenge at 4 weeks after the vaccination. In C7301/dTK group, three of the four cats developed typical severe clinical signs of FCV infection, including ulceration or erosion of the hard palate, tongue, lips or nose, and another cat (Cat No. TK-3) showed mild eruptions on the surface of tongue and lip. Compared with these cats, cats in dTK(gCp)-Cap and C7301/dTK-Cap groups were protected to a significant degree in clinical responses against FCV challenge (Fig. 5). In addition, vaccination with dTK(gCp)-Cap showed a better protection in their clinical scores than that with C7301/dTK-Cap. However, two cats (Cats No. GC-1 and 2) in dTK(gCp)-Cap group showed mild clinical signs after FCV challenge, including erosion or eruption on the surface of tongue and lips.

The developments of VN antibody against FCV and FHV-1 during observation period of 56 days were shown in Tables 1 and 2, respectively. Before FCV challenge, no VN antibody against FCV was detected in cats of C7301/dTK and C7301/dTK-Cap groups, but the VN antibody was detected in three cats of dTK(gCp)-Cap group (Table 1).
Table 1. Virus neutralizing antibody titer against FCV which inhibited CPE formation completely. Titers were expressed as the reciprocal of the highest serum dilution.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cat No.</th>
<th>Days after vaccination</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>dTK(gCp)-Cap</td>
<td>GC-1</td>
<td>≥2</td>
</tr>
<tr>
<td></td>
<td>GC-2</td>
<td>≥2</td>
</tr>
<tr>
<td></td>
<td>GC-3</td>
<td>≥2</td>
</tr>
<tr>
<td></td>
<td>GC-4</td>
<td>≥2</td>
</tr>
<tr>
<td></td>
<td>(Mean)</td>
<td>≤2</td>
</tr>
<tr>
<td>C7301/dTK-Cap</td>
<td>TC-1</td>
<td>≥2</td>
</tr>
<tr>
<td></td>
<td>TC-2</td>
<td>≥2</td>
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<tr>
<td></td>
<td>TC-3</td>
<td>≥2</td>
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<td></td>
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<tr>
<td></td>
<td>(Mean)</td>
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<tr>
<td>TK-2</td>
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<td>TK-3</td>
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<tr>
<td>TK-4</td>
<td>≥2</td>
<td>≥2</td>
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<tr>
<td>(Mean)</td>
<td>≤2</td>
<td>≥2</td>
</tr>
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</table>

a) Before vaccination. b) Before FCV challenge. c) VN antibody titer was expressed as the reciprocal of the highest serum dilution which inhibited CPE formation completely.

Additionally, after the challenge, VN antibody response against FCV developed earlier in dTK(gCp)-Cap and C7301/dTK-Cap groups than C7301/dTK group and the mean titer was higher in dTK(gCp)-Cap group than that in C7301/dTK-Cap group. On the other hand, VN antibody against FHV-1 was detected in all cats at 56 days after vaccination, except one cat in C7301/dTK-Cap group. The mean titer of VN antibody against FHV-1 in each group corresponded to the results of virus shedding examination.

DISCUSSION

Both FHV-1 and FCV induce upper respiratory signs such as sneezing, ocular and nasal discharges, and conjunctivitis in cats. FHV-1 infection is generally severer than FCV infection which is often associated with mouth ulceration [4]. For development of recombinant polyvalent vaccines using an FHV-1 vector, FCV which invades respiratory routes similar to FHV-1 might be a suitable donor pathogen in terms of the FHV-1 replicative tropism. Additionally, since conventional FHV-1 vaccine is presently used as a combination with FCV vaccine [3], it might also encourage the development of recombinant polyvalent vaccines which can induce protective immunities against these pathogens at the same time.

In this study, we succeeded in construction of a further improved recombinant FHV-1, dTK(gCp)-Cap, carrying a putative FHV-1 gC promoter sequence upstream of the FCV precursor capsid gene, by using a transfer vector pTK(gCp)-Cap. Growth kinetics of dTK(gCp)-Cap in CRFK cells was similar to those of C7301/dTK-Cap and C7301/dTK. Duplication of the putative FHV-1 gC promoter sequence in the FHV-1 genome does not seem to influence the virus growth in cell cultures. In IFA, an MAb 4B1 against FCV capsid protein reacted more strongly with CRFK cells infected with dTK(gCp)-Cap than those with C7301/dTK-Cap (Fig. 2). In addition, CRFK cells infected with dTK(gCp)-Cap reacted with all seven anti-FCV MAbs recognizing seven different neutralizing epitopes on the capsid protein of FCV (data not shown). These results indicate that the promoter activity of the putative gC promoter sequence is higher than that of the TK promoter and that the product expressed by the dTK(gCp)-Cap conserves sufficiently the immunogenic property of the FCV capsid protein, respectively.

By ELISA, the expression of FCV immunogenic antigen by dTK(gCp)-Cap became the maximum at 24 hr, while the expression by C7301/dTK-Cap reached the maximum at 6 hr. Our result suggests that the recombinant dTK(gCp)-Cap expresses the FCV immunogenic antigen in the late phase by using the gC promoter sequence as an FHV-1 late promoter. In addition, the dTK(gCp)-Cap showed a better expression in the late phase than C7301/dTK-Cap. Gene expressions of alphaherpesviruses are divided into three classes, immediate early, early and late phases, and regulated in cascade fashion [5]. The early genes follow the immediate early genes in the cascade and encode several proteins involved in viral DNA synthesis, including a TK. On the other hand, the late genes encode the majority of structural components of the virion, including a gC, and the transcriptions proceed only after the viral DNA synthesis has occurred [5]. We speculate that the synthesis of foreign structural protein by herpesvirus vector should also be regulated in the late phase in terms of their cascade fashion and the late promoter is stronger than the early promoter.

From the construction of the transfer vector, pTK(gCp)-
Cap, the capsid protein expressed by d/TK(gCp)-Cap is expected to be fused with the signal peptide of gC and released as a secreted protein from the infected cells. However, we could not prove it in this study. For the proof, we need to generate other recombinant viruses furthermore. For example, it might be a recombinant FHV-1 carrying the putative FHV-1 gC promoter sequence lacking the signal sequence of gC upstream of the FCV precursor capsid gene. Further analyses will be required.

As shown in Fig. 4, cats in d/TK(gCp)-Cap group, as well as C7301/d/TK-Cap group, excreted the recombinant viruses with lower titers than cats in C7301/d/TK group. However, the mean titers of the re-excreted virus and the induced VN antibodies against FCV and FHV-1 were higher in d/TK(gCp)-Cap group than those in C7301/d/TK-Cap group. These results suggest that the expression of foreign antigen in the late phase does not influence replication of the vector in cats significantly and induce immune responses against target pathogens more effectively when compared with the expression in the early phase. However, further study will be required.

To evaluate whether the recombinant d/TK(gCp)-Cap is more effective as an FCV vaccine than C7301/d/TK-Cap, all cats were exposed with virulent FCV F4 strain. The result showed that one vaccination with the d/TK(gCp)-Cap by natural infection routes protected cats more effectively than those vaccinated with C7301/d/TK-Cap. The vaccine efficacy corresponded to their replication data in cats. However, two cats in d/TK(gCp)-Cap group showed mild but certain FCV clinical signs and the vaccination could not provide 100% protection against FCV challenge. Further improvement of recombinant FHV-1 will be required. Such improvement might be accomplished by finding more effective insertion sites in the FHV-1 genome and more powerful late promoters for expression of other foreign antigens.

The present study demonstrates the construction of a better recombinant polyvalent vaccine against FCV and FHV-1 infections for cats than that reported previously. These results will be applicable for the further development of recombinant polyvalent vaccines using herpesvirus vectors.

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