Intranasal immunization with inactivated feline calicivirus particles confers robust protection against homologous virus and suppression against heterologous virus in cats

Hiroaki Sato,1,* Go Sehata,1 Nobutaka Okada,1 Kayo Iwamoto,1 Katsuo Masubuchi,1 Risa Kainuma,1 Tatsuki Noda,1 Tatsuhiko Igarashi,1 Takuo Sawada,2 Taichi Noro1 and Eiji Oishi1

Abstract
The protective efficacy of intranasal (IN) administration of inactivated feline calicivirus (FCV) vaccine against homologous or heterologous FCV infection was investigated. Groups of cats immunized with the experimental inactivated, non-adjuvanted FCV vaccine via either the IN or subcutaneous (SC) route were exposed to homologous or highly heterologous FCV. Both the IN and SC immunization protocols established robust protection against homologous FCV infection. Although neither immunization regimen conferred protection against the heterologous strain, clinical scores and virus titres of oral swabs were lower in cats in the IN group compared to those in the SC group, accompanying a faster neutralizing antibody response against the heterologous virus in cats in the IN group. The IN group secreted more IgA specific to FCV proteins in oral washes (lavage fluids from the oral cavity) than the SC group. IN immunization with an inactivated whole FCV particle, which protects cats from homologous virus exposure and shortens the period of heterologous virus shedding, may serve as a better platform for anti-FCV vaccine.

INTRODUCTION
Feline calicivirus (FCV) is a major pathogen in cats and typically causes a variety of clinical manifestations, such as oral and upper respiratory tract disease, stomatitis and lameness [1–5]. Severe systemic disease caused by highly virulent strains has also been reported in North America and Europe over the past decade [6–12].

The structural capsid protein of FCV is divided into six regions, A–F, based largely on sequence variability among isolates [13–16]. Region A is cleaved to produce the mature capsid protein [17]. While regions B, D and F are relatively conserved among isolates, regions C and E are highly variable [13, 15]. Region E is considered immunodominant and contains neutralizing epitopes [15, 16, 18, 19]. This region is further divided into two hypervariable regions (HVRs) – the amino-terminus HVR (N-HVR) and the carboxy-terminus HVR (C-HVR) – and a relatively conserved region (ConE) [15, 20].

Although several vaccines have been commercially available for the past 30 years, FCV infections remain highly prevalent in cat populations [21–23]. One reason is likely to be the inability of vaccines to confer protective immunity against antigenically diverse field viruses [24, 25]. None of the vaccines currently commercially available are thought to protect cats against all existing strains of the virus. In an effort to broaden the spectrum of the vaccine, multiple strains capable of inducing a wider range of cross-reactive antibodies have been selected and combined as immunogens [26, 27], since it has been shown that levels of serum-neutralizing antibodies (NABs) against the viral agent correlate with the degree of viral suppression [28–30].

There are also safety concerns regarding currently available anti-FCV vaccines. In addition to occasional inflammatory reactions at the injection site [31] and vaccine-associated sarcoma [32, 33], isolated cases of vaccine-induced FCV disease have been reported in animals immunized using live vaccines [34–36]. Clinical signs associated with replication of the vaccine strain at the inoculation site have occurred in cases of intranasal (IN) administration of live vaccine [37].
Live attenuated IN FCV vaccines [37], some feline herpesvirus recombinant vectored vaccines [38], and DNA vaccines [39], which induce mucosal immunity, have been reported. However, there are no reports on the efficacy of an IN-inactivated FCV particle vaccine. Furthermore, little has been reported on the correlation between the induction of mucosal antibodies and protection against FCV infection.

In this study, we evaluated the protective effect of IN immunization with inactivated FCV vaccine against homologous or heterologous virus exposure and found a correlation between induction of secretory IgA (sIgA) and protective responses against heterologous virus infection. This is also the first study to report on the efficacy of IN immunization using inactivated FCV vaccine.

RESULTS

Antibody response following immunization

IN immunization of cats with FC-7 vaccine induced measurable titres of NAb against the vaccine strain after the second immunization (Figs 1a and 2a). The titres were significantly lower in the IN cohort than in the subcutaneous (SC) cohort. By contrast, both cohorts produced barely detectable levels of NAb against the highly heterologous strain FC-64 (Figs 1b and 2b).

IN immunization induced more anti-FCV IgA antibodies than SC immunization in the oral cavity following the second immunization (Figs 1c and 2c). Similar results were obtained by the ELISA assay when strain FC-64 was used as the antigen (data not shown). FC-7, but not FC-64, was neutralized at minimally detectable titres in the lavage fluid from the oral cavity (oral wash) of the IN cohort at two weeks after the second immunization, as illustrated in Figs 1(c) and 2(c); the titres were 1.0 log4 (Fig. 1c) and 1.3 log4 (Fig. 2c).

None of the animals in the control cohort produced antibodies during the immunization period (Figs 1 and 2).

Homologous protection test

As expected, following exposure, the animals in the control cohort exhibited typical clinical signs of FCV infection such as hyperthermia, oronasal ulceration and discharge. By contrast, the animals in both immunization cohorts manifested barely recognizable clinical signs of FCV infection upon exposure to homologous virus (Fig. 3a). No exposure virus was recovered from any of the animals in the IN cohort and low titres of the virus (mean titres ranged from 1.5 to 1.6 log TCID₅₀ ml⁻¹) were detected in the SC cohort on days 1 and 3 post-exposure (p.e.). Consistent with the clinical manifestations of FCV infection, all animals in the control cohort continuously shed measurable amounts of infectious virus (mean titres ranged from 1.6 to 4.0 log TCID₅₀ ml⁻¹) in saliva during the observation period (Fig. 3b).

Heterologous protection test

After exposure to the heterologous virus, typical clinical signs of FCV infection were observed in all of the cohorts, which exhibited virtually indistinguishable clinical scores up to 9 days p.e. The mean clinical scores of the IN cohort decreased to a level that was significantly lower than those of the control cohort on days 11 and 13 p.e. (Fig. 4a).

All of the cohorts shed comparable amounts of virus in the oral cavity until day 5 p.e. The amount of virus shed in the IN cohort gradually decreased subsequently, but the amount of virus shed in the other two cohorts remained approximately the same until day 21 p.e. (Fig. 4b). There were statistically significant differences between the mean virus titres of the IN and SC cohorts on days 15, 17 and 19 p.e. and between the IN and control cohorts on days 7, 9, 11, 13, 15, 17, 19 and 21 p.e. (Fig. 4b).

Antibody response following virus exposure

In the homologous protection test, the control cohort produced NAb directed against homologous FC-7 and/or highly divergent FC-64 in the circulation as early as 1 week p.e. The titres increased with time during the observation period (Fig. 1a, b). By contrast, the antibody titres of both the IN and SC cohorts directed against FC-7 did not increase further upon virus exposure, suggesting that the exposure virus either did not replicate or did not replicate enough to stimulate further antibody production (Fig. 3b). In the immunization cohorts, neutralizing activity against FC-64 gradually decreased following virus exposure from minimally detectable to undetectable levels (Fig. 1b). Only the IN cohort produced additional anti-FCV IgA in the oral cavity upon strain FC-7 exposure (Fig. 1c). The samples also contained IgA reactive to strain FC-64, as described in Antibody response following immunization. Neutralizing activity against strain FC-7 was detected in the oral wash of the IN cohort at 1 week p.e., as shown in Fig. 1(c) (titre of 0.8 log4).

In the heterologous protection test, in response to FC-64 exposure, both the IN and SC cohorts produced gradually increasing amounts of NAb directed against vaccine strain FC-7 (Fig. 2a). After a slight delay, the control cohort produced low titres of the antibody that were detectable from 2 weeks p.e. onward (Fig. 2a). All three cohorts produced NAb directed against FC-64 as early as 1 week p.e. (Fig. 2b). The kinetics of the NAb production of the IN cohort stood out, as the neutralizing titre of this cohort at 1 week p.e. was significantly higher than those of the SC and control cohorts (Fig. 2b).

The immunized cohorts generated the anti-FCV IgA antibody in the oral cavity at 2 weeks p.e. whereas the control cohort did not (Fig. 2c). Samples from the immunized groups also contained IgA reactive to FC-64 as described above. Neutralizing activity against strain FC-7 was detected in the oral wash of the IN cohort at 1 week p.e., as shown in Fig. 2(c); the titre was 0.8 log4. By contrast, no neutralizing activity against FC-64, the exposure strain, was detected during the observation period.
FC-7 vaccine-induced IgA recognized the capsid protein of heterologous strain FC-64

Serum IgA induced by IN or SC immunization with FC-7 vaccine reacted not only with capsid proteins of FC-7 but also with those of FC-64. By contrast, IgA in the oral cavity of IN cohort bound both protein, while no detectable binding against either proteins was observed in samples of oral fluid from the SC-vaccinated cats (Fig. 5).

DISCUSSION

In the field, FCV consists of a wide variety of isolates with discrete antigenicity [24, 25]. A host protective immunity conferred by vaccination or natural infection serves as a selective pressure to spawn escape variants, which establish persistent infection in cats [22, 40, 41]. Currently available anti-FCV vaccines are generally effective and control FCV disease to some extent [26, 28, 42]. However, their effectiveness and safety can be improved. An ideal anti-FCV vaccine should confer cross-protective immunity against a wide range of field isolates with distinct antigenicity, minimize the emergence of antigenic variants by decreasing the duration of virus replication in the event of unsuccessful protection, and induce active immunity in the oral and respiratory mucosae, since FCV replicates primarily in these
anatomical compartments [43]. In terms of safety, the ideal FCV vaccine should not lead to adverse reactions [31–37].

In this study, we found that IN immunization of the whole inactivated FCV achieved equal or better protection against homologous virus exposure than SC immunization of the same preparation. Furthermore, IN administration limited replication of a highly divergent strain of FCV to a shorter duration than SC administration.

IN immunization induced better protective immunity in the vaccinees than SC, based on the elimination of virus shedding and prevention of clinical manifestations following inoculation with a homologous strain. Among the immunological parameters examined, virus-neutralizing sIgA in saliva was detected only in the IN cohorts. The blood NAb titre, the major contributor to control FCV replication, was unexpectedly higher in SC than in IN animals. Therefore, we believe that sIgA contributed to the observed protection in IN animals. In the case of other viruses that replicate in the oral and respiratory mucosae, such as influenza virus, sIgA neutralizes the incoming viral agent at the portal of entry and replication [44–46]. Transfer of sIgA isolated from respiratory tract washings of mice intranasally immunized with influenza A virus haemagglutinin conferred protection against exposure to both homologous and heterologous viruses [46]. Therefore, IN immunization conferred superior protection against virus

![Fig. 2. Kinetics of NAbs in the blood (a, b) and sIgA in the oral cavity (c) of cats after immunization with the whole inactivated FCV particle vaccine of strain FC-7 and exposure to the highly heterologous virus FC-64. Cats (n=5) were immunized (I) intranasally (Intranasal) or subcutaneously (Subcutaneous) with FC-7 vaccine and exposed (I) to strain FC-64. Unimmunized cats (n=5) served as controls (Control). Sera and oral washes were collected at each immunization, at exposure, and at 1, 2 and 3 weeks p.e. NAb titres were determined and anti-FCV IgA levels in the oral cavity were assessed by ELISA, as described in Measurement of antibody titres. (a) Serum NAb titres against vaccine strain FC-7. (b) Serum NAb titres against highly divergent strain FC-64. (c) Anti-FCV IgA antibody response in the oral cavity and neutralizing activity of oral washes against strain FC-7. § denotes the time point at which neutralizing activity against FC-7 was detected. The plots represent mean values of samples in each group with error bars of standard deviation. Statistical significance was determined using Tukey’s test (P<0.05; *: Intranasal vs. Subcutaneous; †: Intranasal vs. Control; and ‡: Subcutaneous vs. Control).]
strains that can be cross-neutralized by a vaccine-induced antibody, likely through induction of functional sIgA in the mucosa, compared to SC immunization, which induces a NAb primarily in the circulation. Thus, effective induction of vaccine-induced mucosal immunity would enhance the control of FCV infection.

Following exposure to an antigenically divergent strain (FC-64), IN immunization, like SC immunization, failed to induce protection. IN immunization, however, restricted virus replication such that the virus was shed for a shorter period than SC immunization. By comparing immunological parameters induced by these two immunization protocols, we found that kittens in the IN cohort produced NAbs directed against the exposure strain earlier than those in the SC cohort. Indeed, IN animals exhibited measurable amounts of NAbs as early as 1 week p.e. Based on this prompt antibody response against the heterologous exposure virus, it is conjectured that IN immunization induces an immunological condition that can readily respond to an incoming related but new agent. The IN group secreted a greater amount of sIgA than the SC group; this may
explain why virus replication was lower in this group. sIgA promotes reverse transcytosis, the uptake of antigens on the mucosal surface. This is mediated by M cells that reside in mucosal-associated lymphoid tissue (MALT) through the formation of antigen–antibody complexes [47–49]. The antigen–sIgA complexes are then introduced to antigen-presenting cells, such as dendritic cells, that reside beneath the M cells. It is likely that mucosal sIgA directed against epitope(s) on capsid proteins shared by FCV isolates introduce replicating exposure virus in the process of reverse transcytosis, leading to the observed prompt NAb response, resulting in the observed reduction of virus shedding.

It is important to experimentally corroborate whether the above mechanism was involved in the immune response to heterologous strains in IN animals. For this, it is necessary to demonstrate that passively administered sIgA directed against capsid protein epitope(s) shared by FCV strains would accelerate antigen presentation. It is, however, technically infeasible to collect a volume of oral lavage fluid sufficient for antibody preparation to apply to the oronasal mucosa of recipient cats. Another difficulty is the narrow host range of FCV; this precludes use of an alternative animal model, such as a rodent. For these reasons, we were unable to investigate this matter.

Fig. 4. Clinical scores (a) and virus titres in the oral cavity (b) of cats after exposure to the highly heterologous FCV strain FC-64. Cats (n=5) were immunized intranasally (Intranasal) or subcutaneously (Subcutaneous) with FC-7 vaccine and exposed to strain FC-64. Unimmunized cats (n=5) served as controls (Control). Clinical scores and virus titres of oral swabs of cats were examined at exposure and every other day thereafter for 21 days, as described in Homologous and heterologous virus protection tests and Measurement of virus titres, respectively. The bars represent mean values of samples in each group with error bars of standard deviation. Statistical significance was determined using Tukey’s test (P<0.05; *: Intranasal vs. Subcutaneous; †: Intranasal vs. Control; and ‡: Subcutaneous vs. Control).

It is assumed that antigen presentation in MALT is more effective and relevant for the control of FCV infection than presentation in the lymph nodes and spleen because the virus primarily replicates in cells of the oral and respiratory mucosae [43]. Previous research concerning host defence against, and development of vaccines for, FCV infection has associated with HRP-mediated colour development of a substrate solution. The membrane was first incubated with diluted sera (S) (lanes 1 and 5, IN cohort; lanes 3 and 7, SC cohort) or oral lavage fluid (O) (lanes 2 and 6, IN cohort; lanes 4 and 8, SC cohort) collected at 2 weeks after the third immunization with strain FC-7, immediately before exposure, followed by incubation with a horseradish peroxidase (HRP)-conjugated anti-cat IgA antibody. The bound antibody was visualized with HRP-mediated colour development of a substrate solution. Lane M: protein molecular mass marker.

In addition to the aforementioned advantages of IN immunization, the use of inactivated particles as the immunogen in IN immunization, as in the current study, negates the possibility of the vaccine serving as a seed for escape variants. Furthermore, IN immunization is less likely to cause vaccination-related adverse effects, which occasionally arise with currently available vaccines, because IN immunization is a non-invasive procedure.

We demonstrated that IN immunization with whole inactivated FCV particles has greater efficacy and safety than SC immunization, but we are aware of issues with this regimen. One major concern is the durability of the slgA resulting from the vaccination. We observed that mucosal slgA did not persist as long as NAb in the circulation (Figs 1 and 2). Investigations on the persistence of slgA and the protective effect bestowed by this vaccination will offer further insights into a safe and effective immunization regimen against FCV disease. Another issue is the lower NAb response in the IN cohort compared with the SC cohort following immunization. It is also desirable to explore the use of adjuvants intended for mucosal application [52] because of their ability to induce persistent induction of mucosal immunity, including slgA, and superior NAb responses.

This study demonstrated that IN immunization with an inactivated whole FCV particle, which protects cats from homologous virus exposure and shortens the period of heterologous virus shedding, may serve as a better platform for anti-FCV vaccine.

**METHODS**

**Viruses**

Two antigenically distinct FCV strains, FC-7 and FC-64, were isolated in Japan from two cats exhibiting upper respiratory distress [26]. Cross-neutralization tests showed that the antigenicities of the two strains were very different [26]. The homologies of the deduced amino acid sequences of N-HVR, C-HVR and ConE in region E of the capsid protein are 52.9, 65.6 and 89.3 %, respectively [26], indicating a distant antigenic relationship. These viruses were propagated in Grancell Rees feline kidney (CRFK) cells. The infectious titres were determined by an end-point dilution assay using CRFK cells and calculated using the Spearman–Karber formula [53]. The titres were expressed as TCID50 ml\(^{-1}\).

**Vaccine preparation**

An experimental vaccine: formalin-inactivated whole FCV FC-7, non-adjuvanted vaccine, designated FC-7 vaccine, was prepared as follows. The virus was propagated in CRFK cells at 37 °C for 24 h. The culture supernatant was clarified by centrifugation at 3000 g for 30 min and inactivated with 0.2 % (v/v) formalin at 37 °C for 24 h. The supernatant was concentrated 25-fold using a tangential flow filter (Mini-Kros Module, Toyobo, Japan). The virus titre of the culture supernatant before inactivation was 2.0×10⁶ TCID50 ml\(^{-1}\). One millilitre of the vaccine contained FCV particles equivalent to 5.0×10⁹ TCID50.

**Animals**

In the current study, 30 kittens that were free from specific pathogens including FCV and reared in the experimental animal farm of Kyoto Biken Laboratories, Japan, were used. The age of all animals was between 3 and 5 months.

**Homologous and heterologous virus protection tests**

The 15 kittens in each group were subdivided into three cohorts of five kittens: IN, SC and unimmunized cohorts. The IN and SC cohorts received 1 ml of FC-7 vaccine three times via the respective routes at 2-week intervals. The unimmunized cohorts did not receive any control materials. Two weeks after the last immunization, all of the animals were inoculated intranasally with 0.1 ml of 1×10⁷ TCID50 of strain FC-7 as the homologous exposure virus, or FC-64 as the heterologous exposure virus.
Clinical signs were objectively monitored by two persons before exposure and every other day of a 21-day observation period using a clinical scoring method derived from the European Pharmacopeia [54] to compare clinical manifestations among the cohorts.

**Measurement of virus titres**

Oral swabs were collected before exposure and every other day during the observation period. The swabs were resuspended in 2 ml of Eagle’s minimum essential medium supplemented with 400 µg ml⁻¹ streptomycin and 400 U ml⁻¹ penicillin. The swab samples were stored at −70 °C until measurement. Virus titres were determined as described above.

**Measurement of antibody titres**

Blood samples were collected at each immunization, at exposure, and at 1, 2 and 3 weeks p.e. Serum samples were inactivated at 56 °C for 30 min prior to analysis. Oral wash samples were collected by wiping oral fluid with Eye Sponge (Becton, Dickinson and Company, USA) pre-wetted with 0.1 ml of phosphate-buffered saline (PBS), and processed following the manufacturer’s instructions.

Serum and oral wash NAb titres were measured as described previously [26], except that the samples were serially diluted fourfold.

Anti-FCV IgA antibodies in the oral wash were assessed by ELISA. ELISA microplates (Thermo Fisher Scientific, USA) were coated with formalin-inactivated whole virus particles of strain FC-7 at 4 °C for 16 h, followed by washing to remove unbound antigen. The oral wash fluids were diluted to 1 : 100 with PBS with 0.05 % (w/v) Tween20 (Sigma-Aldrich, USA) and applied to the microplates. The plates were incubated at 37 °C for 1 h and then washed. A horse-radish peroxidase (HRP)-conjugated goat anti-cat IgA antibody (Bethyl Laboratories, USA) was added to each well. Following incubation at 37 °C for 1 h, the plates were washed and incubated with an HRP substrate at 37 °C for 30 min. The reaction was terminated by the addition of 1 N sulphuric acid. An ELISA plate reader (Thermo Fisher Scientific, USA) was used to determine the optical density (OD) at 492 nm with a standard at 630 nm.

**Immunoblot analysis**

The reactivity of IgA induced by FC-7 vaccine to proteins of strains, FC-7 and FC-64, was assessed by immunoblotting [55]. Proteins of strains FC-7 and FC-64 were separated in 10 % (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels under non-reducing conditions. The resolved viral proteins were electrophoretically blotted onto nitrocellulose membranes. The membranes were first incubated with dilutions of serum or the oral wash of cats from the IN and SC groups collected immediately before the challenge, followed by peroxidase-conjugated anti-cat IgA antibodies. Bound antibodies were visualized by adding a dianinobenzidine-hydrogen peroxide substrate (Sigma-Aldrich, USA).

**Statistics**

Comparisons among groups were made using Tukey’s test. P<0.05 was deemed statistically significant.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

All animal experiments were in compliance with the Animal Experiment Guidelines of Kyoto Biken Laboratories, Japan, approved by the Animal Care and Use Committee of the institution, and conducted following the guidelines provided by Ministry of Agriculture, Forestry and Fisheries, Japan.

**References**
