Antigenic Change in Feline Calicivirus During Persistent Infection

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ABSTRACT

To determine if antigenic variation occurred during persistent infection of cats with feline caliciviruses (FCV), nine persistent (progeny) isolates from nine different carrier cats were compared antigenically to the original infecting parent strain, FCV 255, by two-way cross-neutralization tests with rabbit antisera. Five of the nine progeny viruses isolated 35 to 169 days after initial infection were antigenically different from the parent strain. These five isolates represented four distinct antigenic phenotypes. The emergence of four distinctly different antigenic variants from a single parent strain indicates that FCV, like many other RNA viruses, exhibits considerable antigenic heterogeneity during replication in its natural host, and supports the hypothesis that antigenic variation contributes to chronic FCV infection.

RÉSUMÉ

Le but de cette étude était de vérifier si la souche infectante de calicivirus (FCV 255) avait subi des variations antigéniques chez neuf chats affectés chroniquement. Un test de neutralisation croisé à l'aide d'un antisérum de lapin a été mené. Cinq des neuf isolats viraux étaient antigéniquement différents entre 35 et 169 jours suivant l'inoculation. Ces cinq isolats représentaient quatre différents phénotypes antigéniques. Cette étude démontre que ce virus, comme plusieurs virus ARN, peut présenter plusieurs formes antigéniquement différentes après réplication virale dans l'hôte et ces variations pourraient contribuer aux infections chroniques. (*Traduit par Dr Pascal Dubreuil*)

INTRODUCTION

Feline caliciviruses (FCV) are a common cause of ororespiratory infections of cats (1–4). They vary in virulence, with clinical manifestations ranging from mild or inapparent upper respiratory tract infections to severe and sometimes fatal pneumonitis (5,6). Early attempts to classify FCV serologically indicated the existence of numerous serotypes (1,7,8), however subsequent investigations provided convincing evidence for a single serotype of FCV, with varying degrees of antigenic relatedness between isolates (9,10). These studies led to production of vaccines incorporating broadly cross-reactive FCV strains. Despite widespread vaccination of cats over the past 15 years, a recent study revealed the overall prevalence of FCV has remained relatively constant at about 20% of cats sampled (4).

One attribute of FCV important to successful persistence in the cat population is their ability to establish chronic infection in cats recovering from the acute phase of infection (11). In chronically infected cats, virus replication continues at low levels in superficial epithelial cells of the tonsils and adjacent mucosa despite the presence of an otherwise protective immune response (9,12). Carrier cats shed virus continuously in oropharyngeal secretions and readily transmit FCV to susceptible contacts (11,13). This mode of transmission is particularly effective between carrier queens and their kittens (14), providing generation to generation transfer of the virus.

The mechanisms by which FCV escape immune elimination in carrier cats have not been determined, although suggestions include replication at sites partially protected from immune effectors, and emergence of antigenic variants under immunological pressure (11,15,16). Antigenic change in two persistent strains has been reported (16), but data were not presented, and no other investigations of this phenomenon have been described. Consequently this study was conducted to determine if antigenic change occurs during chronic FCV infection. Nine cats persistently infected after inoculation with a single FCV strain (the "parent" strain) were held in strict individual isolation and monitored for viral persistence by virus isolation from oropharyngeal swabs. One isolate from each carrier cat (termed "progeny" strains) and the original parent strain were used to prepare rabbit antisera for cross-neutralization tests to determine the antigenic relationships between the parent and progeny strains, and among the nine progeny strains.

MATERIALS AND METHODS

CELLS

Virus isolation, propagation and neutralization tests were conducted with Crandell's feline kidney (CRFK) cells grown in Eagle's minimum essential medium supplemented with 2 mM L-glutamine, 0.5% lactalbumin hydrolysate, 100 units mL^{-1} penicillin, 50 μg mL^{-1} streptomycin and 6% heat-inactivated fetal bovine serum.

VIRUS ISOLATION

Samples for virus isolation were obtained by vigorous swabbing of the oropharynx with sterile cotton swabs. The contents of the swab were eluted into 2 mL of transport medium (cul-

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ture medium without fetal bovine serum and with 10× antibiotics) and frozen at −70°C. Duplicate 0.5 mL volumes of the thawed samples were inoculated onto confluent monolayers of CRFK cells in tubes and processed as described previously (3). Development of typical calicivirus cytopathic effects within five days was indicative of presence of the virus in the test specimen.

VIRUSES

The parent virus was FCV strain 255, obtained from Dr. R.C. Povey, University of Guelph, and isolated originally from a kitten dying of respiratory disease (17). Stock virus was prepared as clarified supernatant from infected CRFK cell cultures frozen and thawed 18 h after inoculation with plaque purified 14th cell culture passage of the virus.

The nine progeny strains were isolates from nine different kittens in which persistent infection developed after inoculation with FCV 255. All procedures were conducted according to guidelines established by the Canadian Council on Animal Care. Unvaccinated kittens six to 16 weeks old were obtained from a breeding colony at the University of Guelph where cats are free of known pathogenic feline respiratory viruses, and breeding stock are vaccinated against feline parvovirus, feline herpesvirus 1 and FCV with an inactivated vaccine containing antigens of FCV 255. Two to five oropharyngeal swabs taken prior to inoculation did not contain FCV or other cytopathic viruses. Serum titers of FCV 255 neutralizing antibody, determined as described below, were 1:8 or less and were consistent with residual levels of maternal antibodies (18). Each kitten was inoculated intranasally and intraocularly on day 1 with a total of 10^5 50% cell culture infectious doses ($CCID_{50}$) of FCV 255 in 0.5 mL of PBS and five days later was transferred to a Horsfall unit and maintained in strict isolation. Infection was monitored by virus isolation from oropharyngeal swabs collected at least weekly, and persistence was identified by consistent isolation of FCV for more than 30 days (16). Sera collected at intervals after inoculation and tested as below for FCV neutralizing antibodies had moderate to high titers typical of responses of persistently infected cats (14). The progeny viruses (P1-9) were the last isolate from each cat before termination of the study and were obtained on day 35 (P1, P2), day 36 (P3, P4), day 58 (P5), day 91 (P6), day 105 (P7), day 161 (P8) and day 169 (P9). Stock of each isolate was prepared as for the parent virus from one passage in CRFK cells without plaque purification.

PRODUCTION OF ANTISERA

Stock parent and progeny viruses were purified as described previously (19). Antiserum specific for each virus was produced by intramuscular inoculation of two rabbits with 0.2 mL volumes of purified virus emulsified in an equal volume of Freund's complete adjuvant, followed at monthly intervals by two further inoculations with the same antigen dose in Freund's incomplete adjuvant. Sera collected from the two rabbits two weeks after the third inoculation were pooled, heated to 56°C for 30 minutes and stored in small aliquots.

NEUTRALIZATION TESTS

Virus neutralization tests of cat sera and two-way cross-neutralization tests of rabbit sera were carried out using a microneutralization technique (20) in which duplicate serial twofold dilutions of sera were tested against 50–100 $CCID_{50}$ of virus, confirmed by back titration of the virus inoculum. Fifty percent neutralization endpoint titers were determined by the method of Reed and Meunch (21). Results for cross-neutralization tests are reciprocal geometric mean titers of three replicate tests. The antigenic relatedness of the progeny viruses to the parent strain and to each other was determined by analysis of these results with the titer ratio method described by Archetti and Horsfall (22) and applied previously to FCV (20). In the equation $r = \sqrt{r_1 \times r_2}$, r is the geometric mean of the titer ratios r_1 and r_2, where r_1 is found by dividing the titer of antiserum to virus 1 tested against virus 2 by its titer against virus 1, and r_2 is found by dividing the titer of antiserum to virus 2 tested against virus 1 by its titer against virus 2. The value of r estimates the degree of antigenic difference between virus 1 and virus 2. For identical viruses, $r = 1$, while a value for r of ≤ 0.5 or ≥ 2 indicates significant antigenic difference (22).

The relationship of the progeny strains to the parent virus was also tested by the 20-antibody unit procedure used initially to compare human echoviruses (23) and rhinoviruses (24) and later applied to numerous FCV strains (1,20). In two replicate experiments, antiserum to FCV 255 diluted to 1/20 of its end point titer against homologous virus was tested by the above procedure in eight replicate wells for its ability to neutralize 50–100 $CCID_{50}$ of each of the progeny strains. The number of wells showing virus growth (typical FCV cytopathic effect) after four days was recorded and the results of the two replicate tests were averaged. Results were scored as total neutralization where 0/8 wells showed evidence of virus growth, partial neutralization if virus growth occurred in one to four wells, and non-neutralization if virus growth occurred in five to eight wells.

RESULTS

Mean titers of the 10 rabbit antisera tested against their homologous viruses ranged from 1:801 to 1:9066, which for 7/10 were greater than titers against other strains (Table I). Both one-way and two-way cross-neutralization tests indicated that five of the nine progeny viruses differed substantially from the parent strain. Antiserum to the parent strain, FCV 255, readily neutralized isolates P2, P3, P5 and P6, whereas its titers against P1, P4, P7, P8 and P9 were more than three- or fourfold lower than against homologous virus. Reciprocal test results correlated well with these findings, in that titers of antisera to P2, P3, P5 and P6 tested against FCV 255 approached or exceeded their homologous titers, while sera raised to viruses P1, P4, P7, P8 and P9 showed relatively weak neutralizing activity against FCV 255. These observations were substantiated by Archetti and Horsfall analysis (Table II). Progeny viruses P1, P4, P7, P8 and P9 were each found to be antigenically distinct from FCV 255 ($r = 3.7 - 19.7$), while P2, P3, P5 and P6 were essentially identical to the parent strain ($r = 0.9 - 1.5$). When tested by the less discretionary

20-antibody unit procedure against antiserum to FCV 255 (diluted 1:453), viruses P2, P3, P5 and P6 were totally neutralized, P7 was partially neutralized (1/8 wells with virus growth), and P1, P4, P8 and P9 were not neutralized (8/8 wells with virus growth).

Among the progeny viruses, cross-neutralization tests (Table I) and Archetti and Horsfall analysis (Table II) indicated isolates P2, P3, P5 and P6, which were similar to the parent strain, were closely related to one another ($r = 0.7 - 1.7$) and different from the other progeny viruses ($r \geq 3.5$). Of the five progeny strains antigenically different from the parent virus, isolates P1 and P4 were essentially identical ($r = 1.6$), yet different from strains P7, P8 and P9 ($r \geq 6.8$), while the latter viruses were each antigenically distinct ($r \geq 5.7$).

TABLE I. Two-way cross-neutralization tests of rabbit antisera to FCV 255 and nine progeny strains (P1–P9) from cats persistently infected with FCV 255

FCV strain	Day of infection	Virus neutralizing titers[a] of antisera to FCV strains									
		255	P1	P2	P3	P4	P5	P6	P7	P8	P9
255	0	**9066**	304	3663	2512	314	1348	1603	222	283	1255
P1	35	694	**3547**	373	192	1348	512	119	200	146	1254
P2	35	9742	119	**3812**	2695	186	953	1261	111	200	1133
P3	36	6889	93	4533	**4533**	200	1448	1348	198	200	431
P4	36	373	2267	264	222	**2109**	245	119	152	142	292
P5	58	6411	87	2109	2436	119	**1491**	1603	91	169	337
P6	91	5793	132	2983	1218	119	1348	**801**	167	206	443
P7	105	2896	71	694	373	283	746	373	**953**	132	800
P8	161	132	71	111	111	111	146	132	54	**1603**	401
P9	169	272	119	222	256	100	283	186	142	674	**5391**

[a]Data are reciprocal geometric means of 50% neutralization titers from three replicate tests. Homologous titers are in underlined, bold type

TABLE II. Antigenic comparison of FCV 255 and nine persistent progeny strains by Archetti and Horsfall analysis of results of two-way cross-neutralization tests

FCV strain	Day of infection	Geometric mean of titer ratios[a] of antisera to strains									
		255	P1	P2	P3	P4	P5	P6	P7	P8	P9
255	0	1									
P1	35	12.4	1								
P2	35	1.0	17.5	1							
P3	36	1.5	30.0	1.2	1						
P4	36	12.8	1.6	12.8	14.7	1					
P5	58	1.3	10.9	1.7	1.4	10.4	1				
P6	91	0.9	13.5	0.9	1.5	10.9	0.7	1			
P7	105	3.7	15.4	6.9	7.7	6.8	4.6	3.5	1		
P8	161	19.7	23.4	16.6	18.1	14.7	9.8	6.9	14.6	1	
P9	169	12.0	11.3	9.0	14.9	19.7	9.2	7.2	6.7	5.7	1

[a]Geometric mean (r) = $\sqrt{r_1 \times r_2}$, where
r_1 = titer of antiserum to virus 1 tested against virus 2 divided by titer of antiserum to virus 1 tested against virus 1, and
r_2 = titer of antiserum to virus 2 tested against virus 1 divided by titer of antiserum to virus 2 tested against virus 2
Values for r of ≤ 0.5 or ≥ 2 indicate significant antigenic difference between the two compared strains (22)

DISCUSSION

The results of this study confirm the occurrence of antigenic variation in FCV during persistent infection. Five of nine progeny viruses isolated from nine carrier cats held individually in strict isolation were found to be antigenically different from the inoculated parent strain, FCV 255. In addition, identification of four antigenically distinct phenotypes among the progeny strains different from the parent strain provides an indication of the potential for antigenic heterogeneity in FCV derived from a single strain.

Antigenic similarities and differences between FCV 255 and its progeny strains were suggested by titers obtained in two-way cross-neutralization tests and were confirmed by two methods with different levels of stringency. The first, Archetti and Horsfall analysis, can detect slight antigenic differences between strains (20,22), and by this method, progeny strains P1, P4, P7, P8 and P9 were significantly different from the parent strain. In examining antigenic relatedness of numerous FCV strains, Povey (20) regarded Archetti and Horsfall analysis as too stringent and preferred the less discretionary 20-antibody unit method developed for rhinoviruses. Even by this technique, the same finding held for isolate P1, P4, P8 and P9 with high Archetti and Horsfall r values, while isolate P7, with an r value closer to the range indicating identity, was neutralized partially by 20 antibody units of antiserum to FCV 255. This may indicate the extent of antigenic difference between P7 and FCV 255 was indeed less. Alternatively, since the progeny strains were not plaque purified, isolate P7 may have been a mixed population of viruses, some being the same as the parent strain and others antigenically different. Had this been the case however, antiserum to P7 would be expected to have had a higher titer against FCV 255 than 1:222.

Without comparing sequential isolates from each cat to the parent strain, it was not possible to determine the frequency or consistency of antigenic change in persistent FCV. However this study does show that antigenic variants can arise relatively early in the course of infection. While three of the antigenically different progeny viruses were from cats infected for the longest periods (105–169 days), two were isolated 35 days (P1) or 36 days (P4) after initial infection. As the immune response to FCV peaks four to six weeks after infection (25,26), this period is one of increasing immunological pressure when the first selection of antigenic variants would be most likely to occur. Subsequent immune responses to the new antigenic phenotype would create another wave of immunological pressure, perhaps resulting in emergence of a second population of antigenic variants.

The finding that four of the progeny strains were identical to the parent strain is difficult to interpret. On one hand, it implies that antigenic variation does not occur in all persistent FCV infections, and that other mechanisms are responsible for or contribute to FCV persistence. Alternatively, it is

possible that antigenic variation might have been detected had sequential isolates from the same cats been investigated, or that infection may soon have been eliminated. These possibilities should be considered for strains P2 and P3, isolated on days 35 and 36, in view of the increasing immunological pressure they would face in their hosts at that time. It is also possible, though speculative, that isolates P5 (day 58) and P6 (day 91) had been preceded by one or more antigenically different populations and reversion to the original phenotype afforded the best chance of survival.

Comparison of the progeny strains by Archetti and Horsfall analysis of their cross-neutralization titers revealed four distinct variants among the five strains antigenically different from FCV 255. Strains P1 and P4, isolated on days 35 and 36 respectively, were identical while strains P7, P8, and P9, isolated after more than 100 days of infection were each unique. Archetti and Horsfall *r* values of 5.7 or greater for these comparisons suggest that differences would also have been evident by the 20-antibody unit method, although this was not attempted.

Recent monoclonal antibody analysis of FCV strain F4 has revealed at least seven epitopes involved in neutralization, four being located on the 67 kDa capsid protein (27,28). One noncapsid epitope was conserved in 20 FCV strains from different continents, including North America, although FCV 255 was not tested. Tohya *et al* (27,28) also reported the generation of antigenic variants in FCV cultured in the presence of neutralizing monoclonal antibodies, and concluded that the seven epitopes were grouped into four antigenic sites. Hence in the present study, emergence of antigenic variants distinguishable with polyclonal rabbit antibodies during persistent infection indicates that the conserved epitope is not immunodominant in cats or rabbits, and suggests that these naturally arising variants differ in at least one and probably several neutralization epitopes. Since all were derived from a single plaque purified clone, FCV 255 appears capable of a considerable antigenic heterogeneity in its carrier host. The finding that the earliest detected variants, P1 and P4, were of the same antigenic phenotype may indicate that within this heterogeneity, certain mutations affecting the involved epitopes occur more frequently than others.

Early investigators likened persistent FCV infection to chronic foot-and-mouth disease virus infections in cattle, and postulated that antigenic change was responsible for FCV persistence (11,16). Results of the present study provide strong evidence in support of this hypothesis, and indicate that FCV should be included among the numerous RNA viruses now known to be capable of rapid genetic and antigenic change (29,30). Further research, particularly with sequential isolates from carrier cats, will help to define more clearly the role of antigenic variation in FCV persistence. These findings also have important implications for the effective control of FCV. The strain used in this study, FCV 255, was shown previously to be moderately cross-reactive with other FCV strains (20), yet gave rise to four antigenically distinct progeny viruses. Hence parenteral vaccines containing one or two broadly cross-reactive strains are unlikely to provide protection against all variants of FCV circulating in cat populations. Antigenic and genetic analysis should however identify the antigenic repertoire of key epitopes involved in FCV immunity, enabling construction of more effective vaccines.

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