Early interaction of feline calicivirus with cells in culture

by

Luiz Carlos Kreutz

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GENERAL INTRODUCTION

The family *Caliciviridae* comprises a group of small single-stranded RNA viruses that have a distinctive cup-shaped surface morphology and a capsid composed of a single protein . These features are unique among animal viruses (7, 58, 60) and are the major criteria used to define the family. Members of the *Caliciviridae* family include Vesicular exanthema of swine virus (VESV), San Miguel sea lion virus (SMSV), feline calicivirus (FCV), and rabbit hemorrhagic disease virus (RHDV) (47, 48, 57). Additional viruses with calicivirus-like morphology, or a single capsid protein, or both, may eventually be included in the family *Caliciviridae*, but none of these has as yet been sufficiently characterized for a definitive classification (5, 14, 15, 17, 19, 24, 25, 28, 30, 61, 67, 68, 70).

The study of the molecular biology of caliciviruses has been neglected in favor of other small RNA viruses mainly because of the relatively low impact caliciviruses have on the health of human and animal populations. However, in the past, caliciviruses which originated from marine mammals had a devastating effect on domestic swine populations with great losses to producers (66). According to some researchers, the reappearance of the disease is only a matter of time (63). Consequently, the study of the molecular aspects of these viruses has recently resumed and feline calicivirus has been the group model. In this regard, some progress has been made in the understanding of its genome organization and synthesis of specific viral induced proteins. Notably, the complete nucleotide sequence of one FCV strain is now available (12) and the gene encoding for the viral capsid protein has been identified for a number of isolates (12, 62, 74) and expressed in vitro (46).

As with other viruses, the study of the early events of caliciviruses replication cycle has been overshadowed by a greater interest in the molecular biology of the viruses and the proteins they synthesize. However, as information in both fields accumulates, the need to understand the

relationship of viruses with cellular receptors has become more evident.

This study was designed to further the understanding of FCV interaction with cells in culture. The principal objectives were: 1) Determine the optimal pH for the interaction of FCV and cellular receptor sites, 2) Determine the kinetics of FCV attachment to Crandell-Reese feline kidney (CRFK) cells in culture, 3) Determine the approximate number of virus attachment sites per cell, 4) Determine whether different FCV strains share the same attachment site on the surface of CRFK cells and if the interaction could be blocked by other animal calicivirus, 5) Investigate the biochemical nature of the receptor attachment site, and 6) Study the role specific receptor sites play on restricting FCV replication in nonpermissive cells.

Explanation of Thesis Format

This thesis includes a manuscript submitted to the Journal of Virology, in a style required by the American Society for Microbiology. A general introduction, objectives of study and a literature review precede the manuscript, and a general summary follows it. Additional references cited following the general summary refer to citations in the general introduction and literature review. The M. S. candidate, Luiz Carlos Kreutz, was the principal investigator of the study and is the senior author of the manuscript. All the experimental procedures reported in the manuscript were also completed by the M. S. candidate, with limited participation of the co-authors.

REVIEW OF THE LITERATURE

Introduction

Caliciviruses are a group of small, non-enveloped viruses that express a single major capsid protein of 60 to 70 Kilodaltons (58, 60). The name calicivirus is derived from its characteristic cup-shaped surface depressions observed by negative staining electron microscopy (from the Latin calyx = cup). Caliciviruses were formerly classified under the *Picornaviridae* family (27). However, physico-chemical properties and the strategy of RNA replication indicated a re-classification of caliciviruses as a separate family, the *Caliciviridae* (7, 9, 22, 23, 60).

The recognized and characterized members of this family include vesicular exanthema of swine virus, (VESV), San Miguel sea lion virus (SMSV), and feline calicivirus (FCV) (57). Recently, the rabbit hemorrhagic disease virus (RHDV), and human calici-like viruses were also included under the *Caliciviridae* family (14, 20, 47, 48). Isolation and partial characterization of calicivirus-like particles from a variety of animal species has been reported (5, 14, 15, 17, 19, 24, 25, 28, 30, 61, 67, 68, 70) but their importance as etiological agents of specific diseases is uncertain.

Retrospective studies indicated that caliciviruses were associated with the outbreaks of vesicular exanthema of swine (VES) in 1932. The clinical signs of VES resembled foot-and-mouth disease (FMD), and the outbreaks were related to the feeding of swine with raw garbage containing marine food waste (63, 66).

Caliciviruses were first isolated from cats with respiratory disease in 1957 (26), and subsequent isolations were reported throughout the world (29). Antigenically, FCV isolates are considered variants of a single serotype (6, 50, 52).

The sea lion calicivirus, San Miguel sea lion virus (SMSV) was isolated from aborting sea lions in 1972, and was in all aspects identical to VESV (64). When inoculated in susceptible pigs, SMSV caused a disease indistinguisable from vesicular exanthema of swine (64, 69).

Swine calicivirus

Vesicular exanthema of swine had its first outbreak in 1932 and it was thought to be foot-and-mouth disease (FMD) because of the clinical similarities (63). VES is an acute viral disease caused by VES virus (VESV) and is characterized by fever and the appearance of vesicles on the snout, lips, tongue and mucosa of the oral cavity and on the sole, interdigital space and coronary band of the foot of infected animals (66).

The outbreaks of VES were associated with the feeding of swine with uncooked garbage containing food waste of marine origin. Following legislation requiring the cooking of garbage fed to animals, VES gradually disappeared and was considered an exotic disease in 1959 (63). However, as marine mammals are considered the primary source of the virus, and serological studies demonstrated transmission of calicivirus between marine and terrestrial animals, the reappearance of the disease is likely to occur (54, 63, 65, 66).

Recently, calicivirus particles were isolated from feces of piglets with diarrhea (56). The virus was denominated porcine enteric calicivirus (PEC) and resembled the prototype FCV, displaying the characteristic cup-shaped morphology (56). Infection of gnotobiotic piglets with PEC induced mild to severe diarrhea that persisted for 3 to 7 days. No signs of vesicular disease could be observed and serological studies have shown that PEC is distinct from VESV (28).

Sea lion calicivirus

Caliciviruses were isolated from California sea lions in 1972, on San Miguel Island (64). The virus, called San Miguel sea lion virus (SMSV) was morphologically and biophysically identical to vesicular exanthema of swine virus (VESV).

Originally, SMSV was isolated from vesicular lesions of the flippers, and from the throat, nasal and rectal swabs of aborting sea lion females and their aborted fetuses and propagated in Vero cells. Human cells such as primary embryonic kidney and lines of Hela, and PK-15 also supported

viral replication (64). Experimentally, SMSV infects monkeys and possibly humans (68).

San Miguel sea lion virus was also isolated from ocean fishes (69) and serologic studies demonstrated that many marine and terrestrial animals may became infected with SMSV (54, 65). No information is available concerning studies on the experimental infection of sea lions with caliciviruses, and experimental infection of pigs with SMSV is highly restricted.

Human calicivirus

Human candidate caliciviruses have been found associated with diarrheal and vomiting disease (14, 20). There are two groups of human calicivirus: one group, which displays surface morphology characteristic of caliciviruses is described as human calicivirus (HCV). A second group with amorphous structure is referred to as small round structured viruses (SRSV) or "Norwalk-like agents". However, the single capsid protein in both groups has identical molecular weight (17). The clinical signs associated with infection by human candidate caliciviruses are similar for both groups. The incubation period, duration of symptoms and excretion period of detectable virus are also similar. However, HCV is more frequently associated with infections in infants whereas Norwalk agent is predominantly associated with disease in older children and adults (17). Furthermore, HCV has been replicated in cell culture, in the presence of small amounts of trypsin while Norwalk agent has not yet been propagated in cells in culture (18).

Rabbit calicivirus

Calicivirus has been implicated as the causative agent of a newly emerging hemorrhagic disease in rabbits (47, 48). Outbreaks of this apparently new viral disease was first reported in China in 1984 (80) and was described as rabbit hemorrhagic disease (RHD). The disease is characterized by high morbidity and mortality in adult animals. Animals under the age of two months usually survive an infection. Clinical signs are observed 24 to 48 hours after experimental infection and are

characterized by fever, abdominal distention, constipation or diarrhea and hematuria. Mortality may be as high as 100% of infected animals. Pathological changes are observed in almost all organs and are characterized by petechial hemorrhage, generalized congestion and poor blood coagulation (80). Viruses are readily isolated from tissue speciments, mainly liver and lungs, and have not yet been replicated in a cell system (47, 48).

Caliciviruses in other animal species

The significance of calicivirus infection on a variety of animal species is not clear. The criteria for classification as calicivirus or calici-like virus is based on the virus structure and biophysical properties. Caliciviruses have been isolated from calves with respiratory tract problems in which virus shedding was observed over a 7 week period, demonstrating the potential of the bovids to become persistently infected with caliciviruses (67). Calici-like viruses (Newbury agent) have been isolated from calves with enteric disease and its pathogenicity was demonstrated in gnotobiotic calves (5).

Caliciviruses are also implicated with enteric infection and vesicular genital disease in dogs, although their significance as etiological agents remains to be established (15, 24, 25,61). A chicken candidate calicivirus has been implicated with enteric problems in chickens and pheasants (19,30), and calicivirus has been isolated from a pygmy chimpanzee showing signs of upper respiratory disease (70).

Feline Calicivirus

Clinical signs and pathogenesis

Feline calicivirus (FCV) is widespread in the cat population and is primarily associated with upper respiratory tract disease. FCV was first isolated from a laboratory-induced panleucopenia infected, moribund cat (26). Because of its marked cytophathogenic effect in cultured feline kidney

cells, the agent was designated "kidney cell-degenerating virus" (KCD), and classified temporarily as feline picornavirus (27). Several feline caliciviruses were further isolated in many parts of the world (29), and considerable antigenic variation is observed by serum neutralization tests (6, 50, 52). Currently, FCV is considered a major health hazard to the feline respiratory system.

The severity of infection caused by FCV varies according to the virulence of specific strains, and is generally limited to the upper respiratory tract. FCV of low virulence causes little or no clinical disease, with a transient, short pyrexia (51). A typical FCV infection produces depression of appetite, conjunctivitis, nasal discharge, sneezing , coughing, and ulcerations on the tongue, hard palate and nostrils. A temperature response is inconsistent and pyrexia or subnormal temperature may be observed (32, 51). Infection with strains of higher virulence may be followed by tracheitis, pneumonia and death (37). Clinical signs may be observed from 2 to 10 days after experimental infection and may last from 2 to 14 days (29). Chronic stomatitis, characterized by ulcerative or proliferative lesions on the gingiva, and occasionally on the buccal mucosa and tongue, may also be associated with FCV infection (39, 40). However, FCV is not a primary cause of inflammatory oral lesions in cats. Rather, FCV causes stomatitis predominantly in cats immunocompromised by chronic infection with feline immunodeficiency virus (FIV) (73).

Infection by some strains of FCV may also cause a "limping" syndrome in kittens, characterized by fever, viremia and lameness. Infected kittens are reluctant to move and on clinical examination demonstrate hyperesthesia upon palpation, pain on manipulation of joints and mild redness on the skin upon the affected joint (1, 49).

Feline calicivirus can be consistently isolated from the oropharynx region of infected cats during the clinical phase of the infection. The isolation of viruses from conjunctival and nasal swabs corresponds to the presence of clinical signs (16, 78). Occasionally, FCV has also been isolated from

rectal swabs, urine and blood samples (36, 49, 78), although viremic spread is not a consistent feature of caliciviruses.

Following infection with FCV, cats may became persistently infected, shedding virus in oropharyngeal secretions for weeks, months or years after recovery from clinical infection (53, 77, 78). The long-term carrier state is more frequently developed in kittens exposed to non-aerosolised virus, when maternal antibodies decline (33, 35). Symptomless carrier cats have been implicated in the maintenance of FCV in the cat population. Virus transmission readily occurs to susceptible contacts (33, 53), and are thought to be the major cause of FCV disease outbreaks in kittens and breeding catteries.

The replication of feline calicivirus in persistently infected cats is basically restricted to the epithelium on the surface of the tonsils and surrounding region from which viruses can consistently be isolated (21). Chronically infected symptomless cats shed virus continuously despite the presence of protective immune response (21, 79). It was proposed that the sites of FCV replication may be partially protected from the immunological surveillance (53), and antigenically different viruses could arise because of the low level of immunological pressure at the tonsilar region (21, 77, 79).

Pathological findings during FCV infection are mainly restricted to the oral cavity and lungs. Irregularly shaped ulcers, 2 to 5 mm in diameter, with discrete defined margins may be found on the glossal and palatine area. Microscopically, a neutrophilic reaction is observed at the periphery and underneath the lesion (31, 51). Mandibular and parotid lymph nodes are usually enlarged and upon microscopic examination reveal an increase in the cellular component of the diffuse area around the lymphoid follicles. The lung may show small foci of consolidation, particular at the apical lobe. Microscopically, consolidation areas may be plugged with cell debris including alveolar macrophages and neutrophils (31, 51, 79).

Vaccination

Little information is available concerning vaccination procedures against FCV infection. Currently, inactivated and live attenuated feline calicivirus vaccines, usually in combination with feline viral rhinotracheitis (FVR), and killed feline panleucopenia (FPL) virus are used to confer protection against infection (2, 37, 41, 74).

Early vaccination experiments demonstrated that proper immunization required the application of two doses of the vaccine (2). Accordingly, the low virulence FCV strain F9 protected vaccinated cats against challenge with the high virulence strain F255. The vaccine has been marketed and used for vaccination programmes

Tham and Studdert (74) showed that a betapropiolactone-inactivated FCV vaccine was able to protect vaccinated cats against virus challenge six months after vaccination. The vaccine was administered twice, with 14 day intervals and had its effect potentiated by adjuvant. Vaccinated animals showed mild clinical signs and had a shorter period of virus shedding than unvaccinated control animals, that showed a severe clinical infection after virus challenge (74). However, a poor antibody response was obtained against a heterotypic FCV.

A triple vaccine containing live attenuated FCV, feline viral rhinotracheitis (FVR), and killed feline panleucopenia (FPL) virus is also currently used. The protocol recommends two doses at 21 day intervals. Measurements of the antibody production in specific pathogen free (SPF) cats after vaccination and challenging procedures demonstrated that cats immunized against the triple commercial vaccine had protective levels of antibody which protected against virus challenge at 21 days after the second vaccine dose (41).

However, because of great antigenic variation among FCV, vaccines containing only one serotype may hasten the emergence of new serotypes and induce protection in small percentage of the population. Recently, it has been shown that immunization of cats with a commercial vaccine containing FCV strain F9 neutralized only 54% of the field isolates, and the most cross reactive

field isolate neutralized only 29% of the other field isolates. Consequently, the use of polyvalent FCV vaccines was recommended rather than the current monovalent types (39).

Virus isolation

Isolation of FCV is promptly achieved in cell culture of feline origin, in which cytopathic effect (CPE) is observed as early as 6 hours post infection (PI). The growth curve of FCV indicates a maximal concentration of intracellular virus at 6 h PI. Following cell lysis, extracellular virus concentration reaches a maximun at 8 h PI. Cytopathic effect is characterized by areas of shrunken, rounded, refractile cells that detach freely from the monolayers. Inclusion bodies have not been observed in caliciviruses infected cells. Virus particles are found only in the cytoplasm, arranged irregularly or in linear arrays (72).

Virus characteristics

Feline calicivirus is a small, non-enveloped virus which belongs to the *Caliciviridae* family. Caliciviruses range in size from 35 to 40 nm in diameter and have a buoyant density of 1.36 to 1.39 g/ml in cesium chloride gradients (7, 71). The sedimentation coefficient of caliciviruses range from 160 to 207 S in sucrose gradients (72). Members of this family possess a positive sense, non-segmented, polyadenylated RNA genome of 7.69 Kb (4, 12). Naked viral RNA has a sedimentation coefficient of 36 to 38 S and molecular weight of 2.5 to 2.8 $\times 10^6$. Total viral RNA comprises approximately 18% of the virus weight (58). A protein (VpG) of 10,000 to 15,000 Da is covalently attached to the 5', uncapped viral RNA genome, and is necessary to confer infectivity (8, 59). Virus particles are roughly spherical and have a characteristic surface morphology with cup-shaped depressions, from which the virus group derives its name (from the Latin calyx = cup). Virion particles are formed by a single major capsid polypeptide which varies in size among family members, but is in the range of 60,000 to 71,000 in molecular weight (7, 9, 60).

Virus genome organization

Currently, little information is available regarding the calicivirus genome organization. The complete sequence of the FCV strain F9 genome has recently been reported (12) and consists of 7690 nucleotides in length. The virus genome contains 3 open reading frames (ORF). The larger, ORF I encodes for non-structural proteins and extends from position 2 to 5308. The initiation codon in the viral RNA of this ORF is located at position 20, and encodes for a polypeptide of 1763 amino acids (12). The region encoding for the non-structural proteins was previously partially sequenced and characterized (43). The amino acid sequence of this region revealed a high degree of similarity with the 3C cysteine protease, 2C polypeptide and 3D RNA-dependent RNA polymerase of picornaviruses. These proteins are located at the C-terminus of the FCV nonstructural polypeptide. A 40 KDa region located between the FCV 2C and 3C protease-like polypeptides, that have no amino acid sequence similarities to any picornavirus protein was also reported (43).

At the 3' end of the FCV genome, ORF II extends from residues 5314 to 7362 and encodes for a 671 amino acid polypeptide (12). Sequence analysis for the 3' end of the genome were also completed for FCV strain F4 (76) , CFI-68 (46), KCD and NADC (62). In these strains, ORF II contains 2004 nucleotides and encodes a protein of 668 amino acids, which differs primarily in the E region among different strains. This ORF encodes for the capsid protein (46). The capsid protein gene is currently the only gene well characterized in caliciviruses. A small ORF III is located at the extreme 3' end of the viral genome and extends from position 7362 to 7644 (12). Protein synthesis from this ORF has not yet been demonstrated. However, the predicted amino acid sequence of this product argues for a functional role as a small nucleic acid binding protein comparable to plant RNA viruses (46). This protein may be synthesized in fusion with the capsid protein by a -1 frameshift. Such a mechanism would allow regulated translation of specific peptides required at much lower copy number than the capsid protein (46). However, this hypothesis needs to be confirmed. Following

the stop codon of the third ORF, there are 40 to 50 nontranslated nucleotides before the poly (A) tail (12, 46, 76).

The nucleotide sequence of a calicivirus candidate, the RHDV, has also been reported. The complete nucleotide sequence encompasses 7437 residues (42) and reveals a considerable degree of similarity and homology with the overall sequence with FCV. A major difference between the FCV and RHDV genome sequence concerns the number of ORFs. The RHDV genome has two ORFs. The first ORF starts with ATG at position 10-12 ending at position 7042-7044. The coding capacity of this ORF is for 2344 amino acid (257 KDa protein). A second, small ORF its located at the 3' end, overlapping the first ORF in 17 nucleotides and consists of 351 residues. The capsid protein of RHDV is encoded by the 3' region , and is synthesized as a fusion protein with the non-structural proteins (42). Furthermore, the non-structural proteins encoded for by the 5' region also show a high degree of similarity with FCV proteins. Accordingly, the 5' end of RHDV genome encodes for a picornavirus 2C-like domain, a not yet characterized protein, a cysteine protease and finally the RNA polymerase.

Virus specific RNAs

Currently, the number and forms of virus-specific RNAs in feline calicivirus infected cells are not well characterized. However, it is well known that FCV mRNAs are synthesized as a 3' co-terminal nested set of subgenomic molecules (3, 11, 45). The number of proteins synthesized in FCV infected cells far exceeds the coding capacity of the mRNAs observed in the infectious cycle.

Currently, it is accepted that a full length complementary RNA is the first molecule synthesized from the incoming viral genome (11). Subgenomic RNAs are transcribed from this negative sense genomic RNA and subsequently synthesized in their negative form. These transcripts are then replicated independently via negative stranded forms (11, 45). Viral RNAs synthesized in infected cells are, in order of size, the 7.6 Kb genomic RNA, and species of 5.3 Kb, 4.3 Kb, 3.6 Kb, the capsid

subgenomic RNA of 2.4 to 2.7 Kb, 1.9 Kb, 1.5 Kb and a small transcript of 550 nucleotides (11, 45). The 2.4 to 2.7 Kb transcript is the most abundant subgenomic RNA synthesized and encodes the FCV capsid protein (45, 46). No proteins are yet identified with the remaining subgenomic RNAs. The 1.9 Kb, 1.5 Kb and 550 nucleotides transcripts have no negative sense counterpart (12). The mechanisms and strategy of transcription are not yet fully characterized. However, the synthesis of subgenomic RNAs provides a fast and powerful mechanism of amplification, which is responsible for the rapid accumulation of positive sense RNA species observed approximately at 2 hr post infection onward, which coincides with the time when viral encoded proteins begin to be synthesized.

Virus specific proteins

The synthesis of proteins in FCV infected cells is also not well characterized. Proteins synthesized during the replication cycle are under a temporal regulation which may be divided into two phases (10). The early phase is characterized by the synthesis of 2 proteins, of 75,000 and 73,000 in molecular weight, respectively. The function of these proteins is still unknown. The second, late phase begins at approximately 3 hr post infection and coincides with a rapid increase in protein synthesis and intracellular accumulation of viral proteins (10). The capsid protein of 62 Kd in molecular weight (cP62) is the most abundant product of this phase and is synthesized as a polypeptide of 76 Kd which rapidly undergoes proteolytic cleavage. Several other viral induced proteins with molecular weights of 96 Kd, 84 Kd, 39 Kd, 36 Kd and 27.5 Kd are also detected in the late phase. However, functions for these proteins remain unknown. At the onset of cytopathic effect, two minor species of 70 Kd and 49 Kd in molecular weight are also detected (10).

The capsid protein (cP62) is synthesized as a precursor of 76 Kd (cP76) and is not detected unless inhibitors of proteolytic cleavage are added to infected cells. Under such conditions, and also at increased temperatures (42° C), larger proteins, of 125 Kd, 123 Kd and 98 Kd are observed (10),

which may be the product of the four transcripts observed at this time (45). However, a clear maturation pathway of FCV induced polypeptides is still unknown. Nevertheless, polypeptides of molecular weights of 96, 84, 75, 73, 70, 62, 49, 39, 36 and 27 KD appear to be final forms of viral-induced polypeptides (10).

The capsid protein

The single capsid protein of FCV is a unique feature among animal viruses. The capsid protein gene is located at the 3' end of the viral genome (10, 46) and is transcribed from a full length negative sense genomic RNA (11) as a 2.4 to 2.7 Kb transcript, which may be detected as early as 2 h PI (11, 45). This early transcript is abundantly replicated and transcribed, which accounts for the rapid accumulation of the capsid protein in viral infected cells (45). The capsid genes for FCV isolates CFI/68 (46), F9 (12), F4 (76), KCD and NADC (62) have been cloned and sequenced, and the amino acid sequence for the capsid protein has been determined.

The calicivirus capsid protein is hypothetically divided in six distinct regions designated A to F (44). The criteria used to define the borders was based on sequence homologies and the amount of amino acid sequence variability between each region. For FCV, the N-termini of the capsid precursor, which is cleaved from the final product, comprises region A. Region B extends from amino acids 121 to 396 and starts with the conserved FRLE-D cleavage sequence. A stretch of 5 amino acids, 397 to 401 forms a sequence divergent region C, followed by a highly conserved region D, which extends from residues 402 to 425. Region E is highly variable and correspond to residues 426 to 520, which most likely contains the serotype determinants. The carboxy-termini comprises the highly conserved region F (62).

The overall amino acid sequence of the FCV capsid protein is highly homologous among different strains. Using the capsid protein of strain NADC as a standard, similarities of 88 to 91% of the amino acid sequences are found among the 5 isolates (62). However, specific regions may have

higher or lower degrees of homology. Specifically, the E region contains two sequence areas, from amino acids 426 to 458, and from amino acid 491 to 520 which diverge by 55 to 68% among isolates. At the center of these two regions, from amino acids 459 to 490, the sequence of amino acids is highly conserved among all isolates. Furthermore, amino acid residues 466 to 473 and 484 to 490 are highly hydrophobic and have a low surface probability, whereas the core of this region, from amino acids 474 to 483 is hydrophilic and displays a high surface probability, as well as the remaining surrounding region, forming two pockets on region E (62). Such characteristics suggest antigenicity and interaction with cellular receptors. The surrounding hypervariable region would allow enough antigenic drift to evade the immune response but the interaction with cellular receptors would be guaranteed by the conserved amino acids underlying the pockets, as observed and described for other viruses (55). This hypothesis fits perfectly with the observation concerning the persistence of FCV in sites of low immunologic pressure in carrier cats and the variation in the antigenic spectrum among feline calicivirus (34, 38).

Several neutralization epitopes have been identified in the FCV capsid protein distributed in four antigenic sites (13, 75). Interestingly, one epitope was conserved in all FCV strains tested and did not generate resistant variants as the other epitopes. Accordingly, such epitopes had poor immunogenicity and may be critical for virus replication. It would be of interest to identify the amino acids composing this epitope and its location on the capsid protein. Based on amino acid sequence analysis (62), it is likely that neutralizing epitopes are located in the variable region E, at the vicinity of the putative receptor binding sequence. Such studies would provide valuable information regarding critical sequences necessary for stimulating the immune system, interaction with cellular receptors, uncoating mechanisms and viral assembly.

PAPER. EARLY INTERACTION OF FELINE CALICIVIRUS WITH CELLS IN CULTURE

EARLY INTERACTION OF FELINE CALICIVIRUS WITH CELLS IN CULTURE

Running Title: Calicivirus cell interaction

LUIZ C. KREUTZ, BRUCE S. SEAL,* AND WILLIAM L. MENGELING

Virology Swine Research Unit

National Animal Disease Center

USDA, Agricultural Research Service

P.O. Box 70, 2300 Dayton Avenue

Ames, Iowa 50010

*Corresponding Author

Phone: 515-239-8465

FAX: 515-239-845

ABSTRACT

The kinetics and biochemical properties of feline calicivirus (FCV) attachment to Crandell-Reese feline kidney cells were determined. Maximal binding was observed at pH 6.5 and was independent of the temperature. However, cells in suspension at 4°C bound virus more efficiently than did cells in monolayers at 4°C or 37°C. High initial binding rate was observed either in monolayers or cells in suspension and proceeded to a maximum at 90 min, although half maximal binding was observed as early as 15 min. Binding was specific and competitively blocked by serotypically homologous or heterologous FCV as well as by San Miguel sea lion virus. Treatment of cells with proteases increased FCV binding, whereas phospholipase had no effect on virus attachment. Conversely, cells treated with neuraminidase followed by O-glycanase treatment showed a decreased binding ability. Cells of feline origin bound FCV very efficiently, whereas non-permissive cells showed a poor binding ability. Following viral genomic RNA transfection, infectious virus could be recovered from all non-permissive cells, with the exception of Madin-Darby canine kidney cells. These results suggest that FCV binds selectively to cells expressing a specific receptor in which carbohydrates may be an important component of the attachment site and that FCV replication in non-permissive cells is primarily restricted by the absence of appropriate attachment sites on the cell surface.

INTRODUCTION

Caliciviruses are a family of small non-enveloped viruses ranging in size from 35 to 40 nm in diameter that express a single major capsid protein of 60 to 70 Kilodaltons (Kd) (53, 54). Caliciviruses posses a positive-sense, non-segmented, polyadenylated RNA genome of approximately 7.69 Kb (12) to which a protein (VpG) is covalently attached to the 5' end (11). Members of the *Caliciviridae* family include feline calicivirus (FCV), San Miguel sea lion virus (SMSV), vesicular exanthema of swine virus and rabbit hemorrhagic disease virus (48, 50, 52). Caliciviruses have also been identified in other animal species but are not well characterized (15, 18, 19, 20, 24, 57, 58).

No published information is available concerning the early interaction of caliciviruses with cells in culture. The first step of the viral infection cycle is interaction of the viral attachment protein with specific structures on the cell surface. These viral receptors are thought to be the major determinants of tissue tropism (14, 21, 22, 23, 29, 31, 38, 43, 47). Recently, however, it has been suggested that host range may also be virus related. Amino acid substitutions can alter the glycosylation pattern of the influenza virus HA1 subunit hemagglutinin, changing its receptor binding properties. Accordingly, such changes result in an increased virus yield from partially resistant cells and would allow virus host expansion in nature (5). The ability of certain poliovirus strains to infect mice may be determined by specific amino acids located on the B-C loop of the VP1 protein N-terminus (46). Mutants of porcine parvovirus have their tissue tropism altered by a few amino acid substitutions on the non-structural and capsid protein (61). Intracellular factors may also govern virus replication. Poliovirus infective cycle is restricted at different steps after internalization by some Hela cells (34). Porcine parvovirus and gibbon ape leukemia virus replication and host range may be controlled by intracellular factors as well, rather than at the surface level (49, 51, 62). Receptor binding characteristics of some viruses have been reported (1, 3, 9, 28, 37, 39, 63), and some virus receptors have been partially or extensively characterized

(2, 4, 8, 10, 13, 27, 30, 35, 44, 55, 59). Pursuant to these accomplishments, the interaction of FCV with a possible cellular receptor was investigated. In this study, we investigated the binding characteristics of FCV with feline cells and its interaction with various non-permissive cells in culture.

MATERIALS AND METHODS

Cells and virus. Crandell-Reese feline kidney cells (CRFK) (16) were grown in 490 cm^2 roller bottles on Hank's minimal essential media (MEM) and supplemented with 5% fetal bovine serum. Feline calicivirus (FCV) strain CFI/68 (17), and KCD (25) were obtained from American Type Culture Collection. The FCV strain NADC-1 was isolated from a cat at the Ames, Iowa, animal shelter (W.L.M.). To determine cell type tropism, various cell lines were obtained from the American Type Culture Collection or the Cytology Group of the National Veterinary Services Laboratory (NVSL, Ames, IA). Cells examined were African green monkey Vero cells, canine A-72 cells, porcine PK-15, murine EMF-P, canine MDCK, feline AK-D, feline Fc3Tg, feline FCWP, feline WWC and feline KKp. The KKp is a primary kitten kidney cell line propagated at the NVSL. The FCWP and WWC cells are primary whole embryo cell lines provided by Dr. Roger Woods of the National Animal Disease Center (NADC, Ames, IA).

Virus purification. Feline calicivirus was propagated by infection of CRFK cells, replicated in 490 cm^2 roller bottles, at a multiplicity of infection of 10 PFU per cell. The virus was allowed to adsorb for 1 hr at 37°C. The inoculum was removed and 10 ml of serum-free media were added, and incubation was continued at 37°C until cytopathic effect (CPE) was complete. To generate radioactively-labeled virus, CRFK cells were incubated in methionine-cysteine free Hanks MEM without serum. Trans 35-S label (ICN Biomedicals, Inc.) containing radioactive methionine and cysteine was added to a concentration of 30 μCi/ml 4 hrs after infection with FCV strain CFI/68. Infected cells were harvested when cells detached freely from the flask surface. The supernatant fluid was frozen and thawed twice to release virus particles, and the cell debris was removed by centrifugation at 12,000 x g for 30 min at 10°C in a Beckman JA-20 rotor. The supernatant was layered onto a step gradient containing 1 ml of 55% sucrose and 6 ml of 27.5% sucrose, both of which

were prepared in R-buffer (10 mM Tris-hydrochloride [pH 7.2], 150 mM NaCl, 50 mM $MgCl_2$) (1). The virus was banded at the 27.5:55 interface by centrifugation at 100,000 x g for 2 hrs in a SW 40 swinging bucket rotor. The virus band fraction was collected, dialyzed overnight against R-buffer and layered over a linear density gradient of 1.28 to 1.44 g/ml of CsCl in R-buffer. Isopycnic banding of the virus was achieved by centrifugation at 150,000 x g for 18 hrs in a SW 40 rotor. The viral band was removed from the gradient and dialyzed against R-buffer for 18 hrs with three changes of the dialysate. For radioactively labeled virus, the gradient was fractionated from the bottom into 0.5 ml fractions, and 5 µl of each fraction was used for the determination of radioactivity by liquid scintillation counting. A second aliquot of 5 µl was removed from each fraction for slot-blot hybridization of viral RNA as described (56). The density of each fraction was determined by refractometry (Fisher Scientific Co.) and infectivity profiles were performed across these gradients by plaque assay on 35 mm petri dishes. Purified virus was dialyzed against R-buffer and stored in aliquots at -70°C. Following purification, viruses were examined by electron microscopy.

Virus quantification. The number of PFU/ml across the gradient was determined. Briefly, 100 µl of a tenfold dilution of each fraction was adsorbed on CRFK cells grown in 35 mm petri dishes. After 1 hr at 37°C, the inocula was removed, the cells washed, and 5 ml of 0.5% agarose in serum-free MEM layered on top. Incubation proceeded at 37°C for 36 to 48 hrs, after which plaques were visualized by staining the cells with crystal violet (0.02%). Because of the similarities between caliciviruses and picornaviruses, the number of virus particles stored in solution were determined by absorption at 259 nm in a 1 cm path using E 1%/259 = 76 (6).

Virus binding assay. Measurements of virus attachment were performed on monolayer or cells in suspension by a modification of the procedure described by Baxt et al. (9).

Method 1. To determine binding of virus to monolayers, Crandell-Reese feline kidney cells were grown in 24 well plates to a concentration of approximately 8 x 10^5 cells/well. After removing the

media and washing the cells with serum-free MEM, 10 µl of MEM containing radioactively labeled virus were added. The multiplicity of infection (particles/cell) used are indicated in the figures and legends. Plates were incubated at 4°C or other temperatures as indicated, and gently rocked every 10 min. At various times post infection (P.I.), the inoculum was removed and the plates washed with 0.5 ml of cold phosphate buffered saline (PBS). The intact cell monolayer was solubilized with 0.2 ml of PBS containing 1% Triton-X-100 and transferred to scintillation vials in which 1.5 ml of scintillation fluid were added. The extent of bound virus was determined in a Packard TRI-CARB 2200 CA liquid scintillation analyzer. All experiments were repeated in triplicate on different days, and equivalent results were consistently obtained.

Method 2. To determine binding of virus to cells in suspension, CRFK cells grown in 490 cm^2 roller bottle were washed once with PBS and removed with 5mM EDTA in PBS without Ca^{2+} or Mg^{2+}. After detachment, cells were washed once in MEM 5% calf serum, followed by two washes in cold serum-free MEM in which they were resuspended to a concentration of 5×10^7 cells/ml. Aliquots containing 5×10^5 cells were placed individually in 1.5 ml conical plastic tubes and centrifuged for 5 s at 12,000 x g in a microcentrifuge. The supernatant was carefully removed, and the cells were resuspended in 20 µl of MEM containing radioactively labeled virus at concentrations indicated in the figure legends. Binding was allowed to occur at 4°C, or as otherwise stated. The reaction was quenched at specific times by the addition of 0.2 ml of cold PBS and centrifugation at 12,000 x g for 5 s. The supernatant fluid was removed and a second wash step was performed. The pellet was collected as described for method 1. Binding of radioactive FCV CFI/68 to other mammalian cells, as specified in results, was also assessed by the same method.

Receptor saturability. To determine the number of attachment sites to FCV on the cell surface, CRFK cells were prepared as described in method 2 and incubated with increasing amounts of radioactive virus. Bound radioactivity was determined after incubation at 4°C for 90 min.

Competition experiments. Cells were prepared as described in method 2 and incubated with 20-fold excess of unlabeled homologous (CFI/68) or serologically heterologous (KCD and NADC) FCV. San Miguel sea lion virus (53), a calicivirus from pinnipeds, and the non-enveloped parvovirus feline panleucopenia virus (FPV) (33) were also used in competition experiments. After incubation at 4°C for 90 min with non-radioactive virus, cells were pelleted, washed, and incubated with radioactively labeled virus for an additional hour at 4°C. The amount of radioactively labeled virus bound was determined as described above.

Transfection of cells. Viral genomic RNA was obtained as described previously (40). Aliquots containing 5 μg/ml were pelleted and resuspended in 100 μl of OPTI MEM 1 (Gibco-BRL). For transfection, 20 μg of Lipofectin (Gibco-BRL) was diluted to a final volume of 100 μl of OPTI MEM 1 and mixed with the solution containing the viral RNA (26, 42). The mixture was gently vortexed and incubated on ice for 5-10 min when OPTI MEM 1 was added to a final volume of 1 ml. Confluent monolayers of cells on 24 well plates were washed twice with serum-free MEM and incubated with OPTI MEM 1 for 15 min before transfection. The media was removed, and the cells transfected with 200 μl/well of the RNA-Lipofectin preparation. After 3 hrs at 37°C, the inoculum was replaced by 0.5 ml of serum-free MEM and incubation was allowed to proceed for 24-48 hrs (complete CPE on permissive cells). The supernatant was collected and viral progeny measured by plaque assay. Lipoinfection using viral particles was also completed using a multiplicity of infection of 20 (7). After 2 hrs, the inocula was replaced by MEM containing anti FCV neutralizing polyclonal antisera at a dilution of 1:500 and incubated for 30 min. Control viral infections were conducted simultaneously without Lipofectin in each cell line, as described in Fig. 7. Viral progeny was measured by plaque assay as described.

Enzyme treatment of cells. To determine the nature of the FCV attachment site components, aliquots of cells in suspension were treated with the enzymes listed in Table 1 at the specified conditions. Cells were then washed twice in MEM containing 2% fetal calf serum and 2 μM of

phenylmethylsulphonyl fluoride (PMSF) to inhibit the enzymes and the remaining binding ability was determined by incubating the cells with radioactively labeled virus for 60 min at 4°C. The cell-associated radioactivity in each treatment was measured and compared with untreated cells.

Enzymes, chemicals, and isotopes. Translabeled 35-S methionine/cysteine was purchased from ICN Biomedicals, Inc. The following enzymes were purchased from Boehringer Mannheim Biochemicals: chymotrypsin, pronase, proteinase K, neuraminidase, and papain. Trypsin, α-glycosidase, β-glycosidase, α-amylase, chondroitinase ABC lyase, acylase, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. Phospholipase A was obtained from Calbiochem-Behring Corp. O-glycanase was purchased from Genzyme Corp.

RESULTS

Virus purification. Virus preparations used to examine the early interaction of FCV with CRFK cells were obtained from lysates of infected cells by two centrifugation steps through sucrose and CsCl, respectively. A predominant peak of radioactivity was observed in the CsCl fraction with a density of 1.37 g/ml (Fig. 1A), which corresponded to the previously reported density of FCV virion (53). Higher virus titers were also observed in fractions having a peak of radioactivity, and slot-blot results clearly demonstrated a greater amount of viral RNA in these fractions (Fig. 1A). Gel electrophoresis of radioactively labeled virus showed a single band of radioactivity (data not shown) corresponding to the single capsid protein of FCV. Viruses obtained by this method consisted of uniform and characteristic particles as demonstrated by electron microscopy (Fig. 1B). A typical morphology consisting of small circular cup-shaped uniform particles were observed.

Effect of pH on virus attachment. The effect of pH on the binding of FCV to CRFK cells was determined for a series of values between pH 5.0 and 9.0 (Fig. 2). Maximal binding of FCV was observed at pH 6.5, although there was no large difference in the binding ability of the virus in the pH range between 6.0 and 8.0. Binding occurred to a lesser extent at pH 5.0 to 6.0 and decreased sharply at pH 9.0. The pH was maintained between 6.5 and 7.0 by using Hepes-buffered media for subsequent virus binding experiments. These results correlated well with those observed with plaque assays in which a maximal number of plaques was consistently observed at pH 6.0 (Fig. 2).

Kinetics of FCV attachment to CRFK cells. Figure 3 illustrates the binding of ^{35}S methionine labeled FCV strain CFI/68 to CRFK cells. Viruses readily associated with cellular receptors and half maximum of total binding was complete by 15 min. However, binding of FCV to cells in monolayers (method 1) occurred to a lesser extent than binding to cells in suspension (method 2). Adsorption of viruses occurred at a greater initial rate and to a greater extent at 4°C than at 37°C. The percentage of virus binding to the cells increased progressively with time, independently of the

temperature. Cells in suspension at 4°C bound viruses more efficiently and a plateau was reached as soon as 30 min after virus addition to CRFK cells.

Saturability of FCV binding. To determine whether FCV binding to CRFK cells was saturable, increasing amounts of radioactively-labeled CFI/68 were incubated with a constant number of CRFK cells in suspension by method 2. The number of viral particles which became cell associated increased linearly with viral input (Fig. 4). When expressed as a percentage of input, the amount of radioactivity bound was high for low multiplicities of infection and the relative amount bound decreased at higher multiplicities of infection. A plateau was reached at multiplicity of infection of approximately 1.5 to 3 x 10^3 virus/cell, indicating a finite number of receptors on the cell surface (Fig. 4).

Specificity of FCV binding. To determine whether FCV attachment was specific and if different isolates of FCV share the same receptor, the ability of unlabeled FCV isolate CFI/68 to compete with 35-S labeled CFI/68 for attachment to CRFK cells was assessed (Fig. 5). Unlabeled FCV strains KCD and NADC, SMSV, and FPV were also assessed for their ability to block attachment of radioactively-labeled FCV CFI/68. The attachment of radiolabeled CFI/68 was inhibited by excess of homologous unlabeled virus (Fig. 5). Feline calicivirus isolates KCD and NADC were also able to reduce attachment of CFI/68 to a similar extent as the homologous virus. Interestingly, San Miguel sea lion virus 1 (SMSV1), a calicivirus from pinnipeds, was also able to prevent binding of CFI/68 to CRFK cells, even though it does not replicate in feline cells. Binding of FCV CFI/68 to CRFK cells was in no way affected by the presence of FPV.

FCV cell tropism and replication in non-permissive cells. Cell tropism of FCV was assessed by incubation of radioactively-labeled virus with cells in suspension for 60 min at 4°C. The amount of bound virus is represented as percentage of virus input. FCV bound readily and efficiently to all feline cell lines tested (Fig. 6). Highest binding was observed with WWC and CRFK cells. Somewhat lower binding was observed with the feline tongue cells, Fc3Tg, and with the primary

cells, KKp and FCWP. Binding to the other mammalian cells could not be demonstrated at levels higher than background except MDCK cells, which bound FCV at approximately 30% that of CRFK cells (Fig.7 A).

Viruses possessing a positive-sense RNA genome may replicate in cells lacking specific receptors once they bypass the cell membrane barrier. To investigate the role of receptors on FCV replication and correlate it with FCV cell tropism, the cationic phospholipid Lipofectin was used to transfect a variety of non-permissive cells either with viral RNA or viral particles. CRFK cells were used as a control for comparisons of viral progeny, which was measured by plaque assay. Virus yield in the various transfected cell lines were as specified in Fig. 7B. Viral replication, detected by cytopathic effect (CPE), was readily observed in CRFK cells lipoinfected either with viral RNA or viral particles, as well as in CRFK cells transfected with whole virus particles. Non-permissive cells transfected with viral RNA showed dispersed cells which rounded up and detached from the monolayer, except MDCK cells. Control non-permissive cells infected with virus only showed no CPE and virus could not be recovered from the supernatant at 24 hrs P.I. (data not shown).

Effect of enzymes on FCV binding. To determine the biochemical nature and possible components involved with the virus attachment site, CRFK cells in suspension were submitted to a variety of enzymatic treatments. The remaining binding ability was assessed and compared with that of untreated cells. Cells, virus, and enzyme concentrations, as well as reaction conditions, were as specified in Table 1. Binding of FCV to CRFK cells was enhanced on cells treated with proteases (trypsin, chymotrypsin, proteinase K, pronase, and papain). Phospholipase A had no effect on virus binding. Glycosidases, except O-glycanase, had little or no effect on FCV binding,. When CRFK cells were treated with neuraminidase followed by O-glycanase treatment, virus binding ability of the cells was reduced by approximately 30%.

DISCUSSION

Specific macromolecules on the cell surface have been used by viruses to gain entrance into the cellular environment where they replicate. These macromolecular receptors frequently are the sole barrier that restrict viral replication and tissue tropism. The virus-receptor interaction is influenced to some extent by a variety of factors such as temperature, ion concentration, divalent cations, and electrical charges. No information regarding these early events of viral replication is available for caliciviruses. Furthermore, no information on the interaction of caliciviruses with non-permissive cells had been reported.

The binding characteristics of FCV to CRFK cells and its interactions with other mammalian cells were examined during this study. The binding of FCV CFI/68 to CRFK cells occurred independently of the temperature, either in monolayers or cells in suspension. However, overall FCV binding was greater at 4°C. Similar results have been reported for foot-and-mouth disease (FMDV) (9), encephalomyocarditis virus (45), and mengo virus (41). Viruses attached to the cells progressively with time to a maximal binding rate of 40 to 60% depending on the method used. The initial binding rate occurred rapidly, and half maximum of total binding was observed as early as 15 min. Furthermore, cells in suspension bound virus at a greater initial rate than cells in monolayers, probably because more receptors on the cell surface are available for interaction with the viral attachment protein. Lower binding observed with cells in monolayers at 37°C may be accounted for by spontaneous virus elution at the physiological temperature.

The rate of FCV attachment to CRFK cells was apparently highly dependent on diffusion. When the volume of the binding media was increased, but not the virus or cell concentration, the rate of attachment decreased significantly, lowering the overall slope of the attachment curve. The interaction of FCV and cellular receptors was also dependent on divalent cations. Increasing

concentrations of EDTA greatly reduced the percentage of virus that became cell-associated (data not shown).

Binding of FCV to CRFK cells did not vary significantly from pH 6.0 to 8.0, but was slightly lower at pH values of 5.0 to 6.0. Maximaland minimal binding was observed at pH 6.5 and 9.0, respectively. Similar pH dependence on the interaction of virus and cell receptors were also observed for FMDV (9), rabies virus (63), and simian virus 40 (13). Furthermore, the ability of FCV to form plaques on a plaque assay was consistently higher on cells held at pH 6.0 during the adsorption period.

It was demonstrated in this study that FCV exhibits specific saturable binding to CRFK cells. The number of attachment sites was estimated from the relationship between input multiplicity and attachment of specific amounts of radioactive virus. Approximately 1.5 to 3 x 10^3 virus particles bound to CRFK cells. The adsorption curve indicated that the efficiency of attachment reached a plateau after 1.6 x 10^3 virus particles had been adsorbed to cells. The number of binding sites observed in this study correlates well with data reported for other virus-cell systems (9, 36, 63). Scatchard analyses of the binding data were not performed in that it assumes a monovalent interaction of ligand and receptor, which is most likely not the case with FCV. It has been assumed and extensively reported that viruses from the same group share or compete for the same receptor site on permissive cells (9, 28, 60, 63). Similarly, our experiments have demonstrated that different FCV isolates bound to the same receptor on the CRFK cell surface. Small differences were observed in the ability of different FCV virus isolates to compete for the same attachment sites. It is assumed, therefore, that FCV binding has a specific requirement for receptor-mediated interaction with cells. Non-specific binding observed in the presence of an excess of unlabeled homologous virus averaged approximately 10% and is thought not to lead to the normal infective pathway. Surprisingly, SMSV 1 prevented FCV from binding to cells in culture even though CRFK cells are non-permissive to SMSV 1. Conversely, FCV did not bind to Vero cells, in which SMSV 1

replicates, suggesting that SMSV 1 may recognize a related receptor on CRFK cells. No efforts were made to determine SMSV 1 internalization on CRFK cells. Reciprocal experiments were not carried out and the binding characteristics of SMSV will be investigated in future studies. Binding of FCV to CRFK cells was in no way abolished by the presence of FPV, an unrelated non-enveloped virus.

Binding of FCV was observed with all feline cells tested. Differences observed on the percentage of attachment to an individual cell type may reflect the number and distribution of the attachment sites on the cell surface. FCV binding to non-permissive mammalian cells occurred to a lesser extent and any binding may be non-specific, leading to a nonproductive infection. However, MDCK cells showed a higher binding ability, but no attempts were made to assess binding specificity or virus internalization.

Viral replication in non-permissive cells may be restricted by the absence of specific receptors on the cell surface and lack of virus internalization or viral uncoating. Such inhibition of replication may be overcome by transfecting non-permissive cells with viral particles or with viral genome. The preformed liposome, Lipofectin, has been used to bypass the membrane lipid bilayer, delivering virus particles (7, 32) or nucleic acids into cells (26, 42), greatly improving the understanding of the early events on the virus infectious cycle. To address the question of receptor specificity and its relationship with tissue tropism, FCV particles and FCV RNA were transfected into permissive and non-permissive cells. Our results showed that the positive sense RNA of FCV could be replicated once it reached the cell translation system of the non-permissive cells. Cells transfected with viral particles provided similar results (data not shown). However, viral progeny could not be recovered from MDCK-transfected cells, which was the sole non-permissive cell line that bound virus to a larger extent. Such cells probably restricted viral replication at the translation or post-translational level, rather than at the receptor level as observed for the remaining non-permissive cells. Such information emphasizes the importance of receptors and intracellular factors on viral replication, tissue tropism, and pathogenesis.

Information on the biochemical nature of viral attachment sites is commonly sought by determining the effect of enzymatic treatment of cells on virus binding. Binding assays were completed on CRFK cells treated with a variety of different enzymes. The results of these assays suggest that a carbohydrate moiety may be an important component of the attachment site. The enzyme O-glycanase, with specifically removes the Gal-ß (1, 3) GalNac core disaccharides from serine and threonine residues of glycoproteins, reduced FCV binding to CRFK cells by approximately 30%. Neuraminidase treatment of the cells prior to the addition of O-glycanase is recommended to remove potential inhibitors of O-glycanase such as N-acetyl neuraminic acid, a common substituent of O-linked carbohydrates. Treatment of cells with O-glycanase alone had no effect on FCV binding. Further evidence for the involvement of carbohydrates on the attachment site for FCV came from studies wherein sodium periodate treated cells had a reduced binding ability (data not shown). Interestingly, a variety of other small non-enveloped viruses have been shown to require O-linked sugar residues for adequate ligand binding (8, 13, 28, 29).

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Table 1. Effect of enzymatic treatment of CRFK cells on FCV binding to CRFK cells in culture

Enzyme	Conc	% binding compared with control
None		100
Phospholipase A[a]	200 μg/ml	105
Trypsin[b]	50 μg/ml	122
Chymotrypsin[b]	100 μg/ml	118
Proteinase K[b]	50 μg/ml	132
Pronase[b]	25 U /ml	115
Papain[c]	200 μg/ml	136
Neuraminidase[d]	400 mU/ml	101
α-glucosidase[e]	100 μg/ml	95
β-glucosidase[e]	1 mg/ml	92
α-amylase[e]	100 μg/ml	102
O-glycanase[f]	2 mU/ml	69
Chondroitinase ABC lyase[e]	1 U /ml	97
Acylase[e]	100 μg/ml	103

[a]Reaction conditions; pH 7.5, 25°C, 30 min
[b]Reaction conditions; pH 7.5, 25°C, 15 min
[c]Reaction conditions; pH 5.5, 25°C, 15 min
[d]Reaction conditions; pH 5.0, 25°C, 60 min
[e]Reaction conditions; pH 7.0, 37°C, 60 min
[f]Cells were treated with 400 mU of neuraminidase per ml at pH 5.0, 25°C 60 min prior to the treatment with O-glycanase at pH 6.8, 37°C, 2 h.
Buffer: [a,b,c,d]serum-free MEM; [e]PBS; [f]20 mM sodium phosphate.

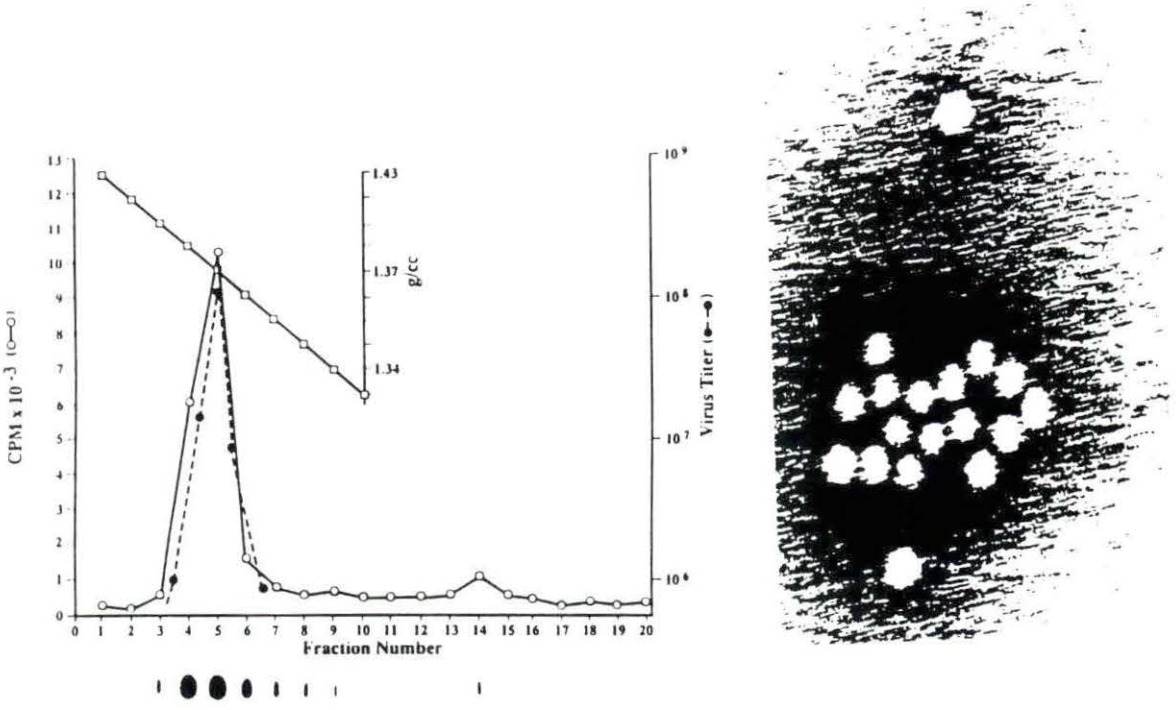

Figure 1. Feline calicivirus purification for virus binding assays. (A) [35S]-methionine labeled feline calicivirus serotype CFI/68 was propagated as described in the text and purified by through CsCl linear gradient (1.28 to 1.44 g/cc). Fractions of 0.5 ml were collected from the bottom to the top. A portion of each fraction was analyzed by liquid scintillation spectrometry (—o—) or infectivity (—•—). Slot blot analysis of each fraction is depicted below the fraction number. Density determination on the gradient fractions containing the virus particles were accomplished as described in the text. Infectivity titrations were performed as described in the text. (B) Electron micrograph of FCV particles purified as described in panel A from fractions 4, 5, and 6.

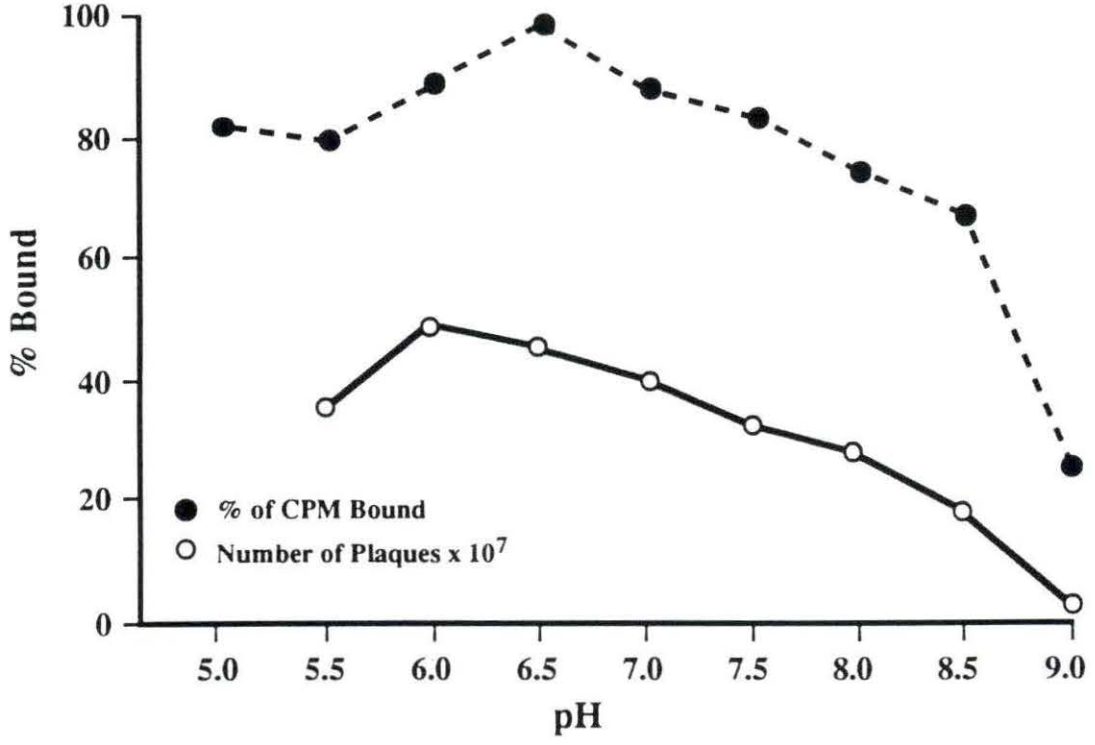

Figure 2. Effect of pH on FCV binding and plaque formation. Cells in suspension were incubated at the indicated pH at 4°C with saturable amounts of radioactively-labeled virus. After 1 hr, cells were rinsed and associated radioactivity was determined as described in the text. For plaque formation, monolayers of CRFK cells were preincubated for 15 min with MEM and further incubated with virus for 1 hr at the indicated pH at 37°C. Petri dishes were overlaid with MEM 0.5% agarose and incubated at 37°C. Values indicated are average of two or more experiments.
(—•—) % of maximum cpm bound.
(—o—) Number of plaques x 10^7.

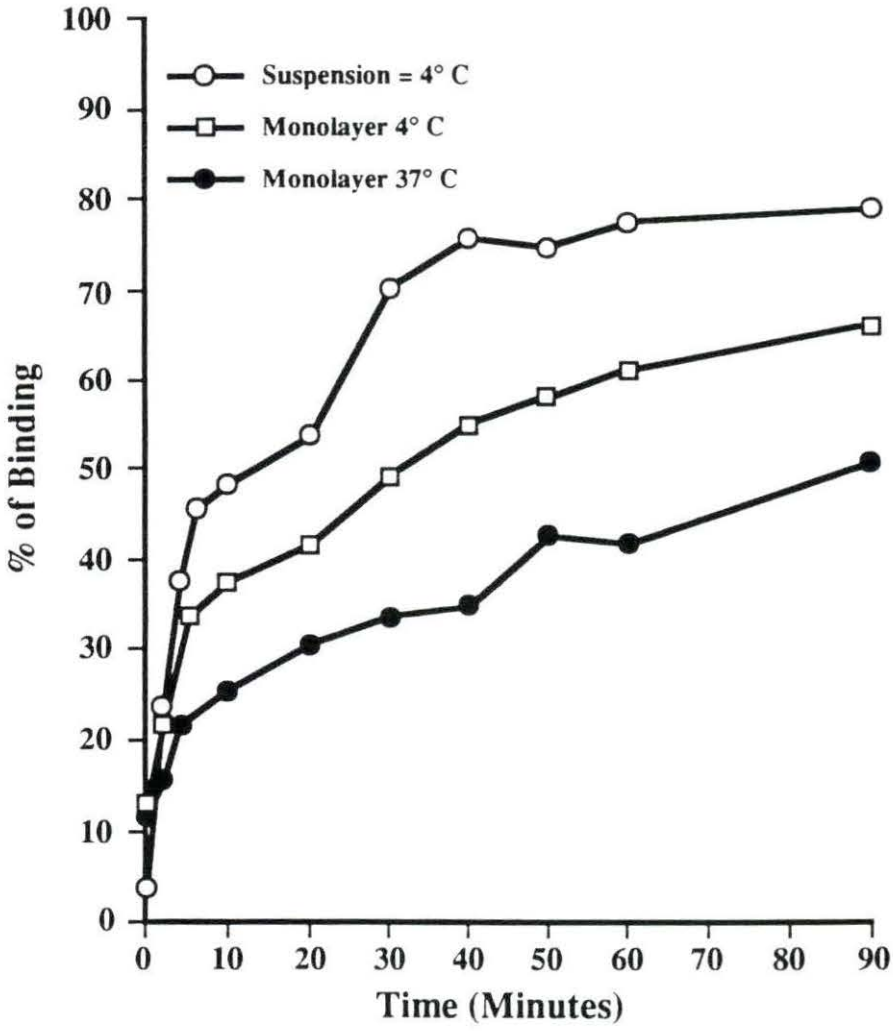

Figure 3. Kinetics of FCV attachment to CRFK cells. Ra dioactively labeled FCV serotype CFI/68 was bound to CRFK cells in suspension at 4°C (—o—), in monolayers at 4°C (—□—) or in monolayers at 37°C (—•—). The reaction was quenched at each time point by washing the cells with cold MEM. The cell-associated radioactivity was measured by liquid scintillation spectrometry and is expressed as a percentage of total input. Results are average of two or more experiments.

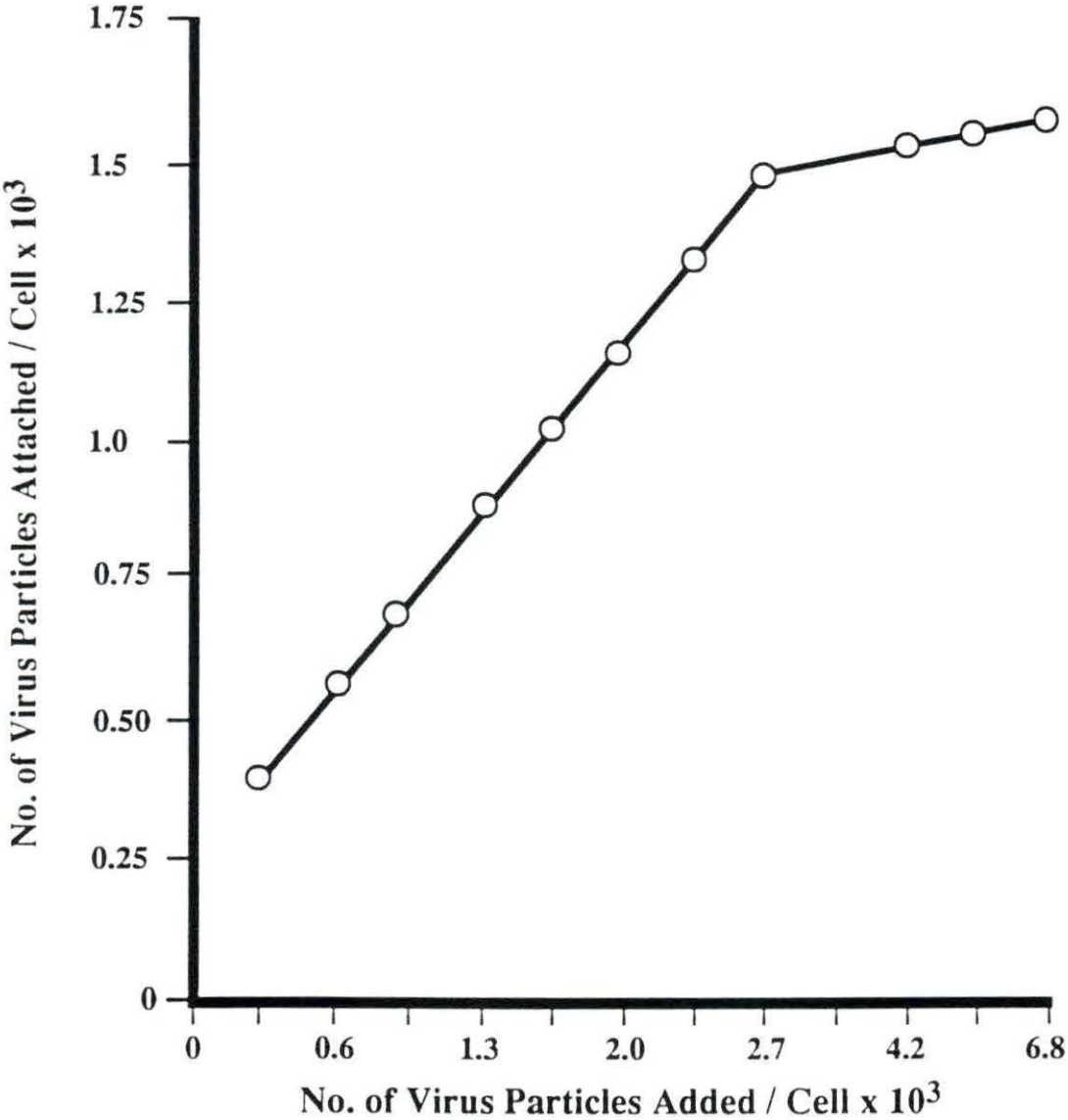

Figure 4. Number of putative attachment sites for FCV on CRFK cells. An increasing number of radioactively-labeled FCV particles were added to a constant number of cells (5×10^5 cells) in suspension. After 90 min at 4°C, cells were washed twice and cell-associated radioactivity was determined. The number of bound virus was estimated as a percentage of total input.

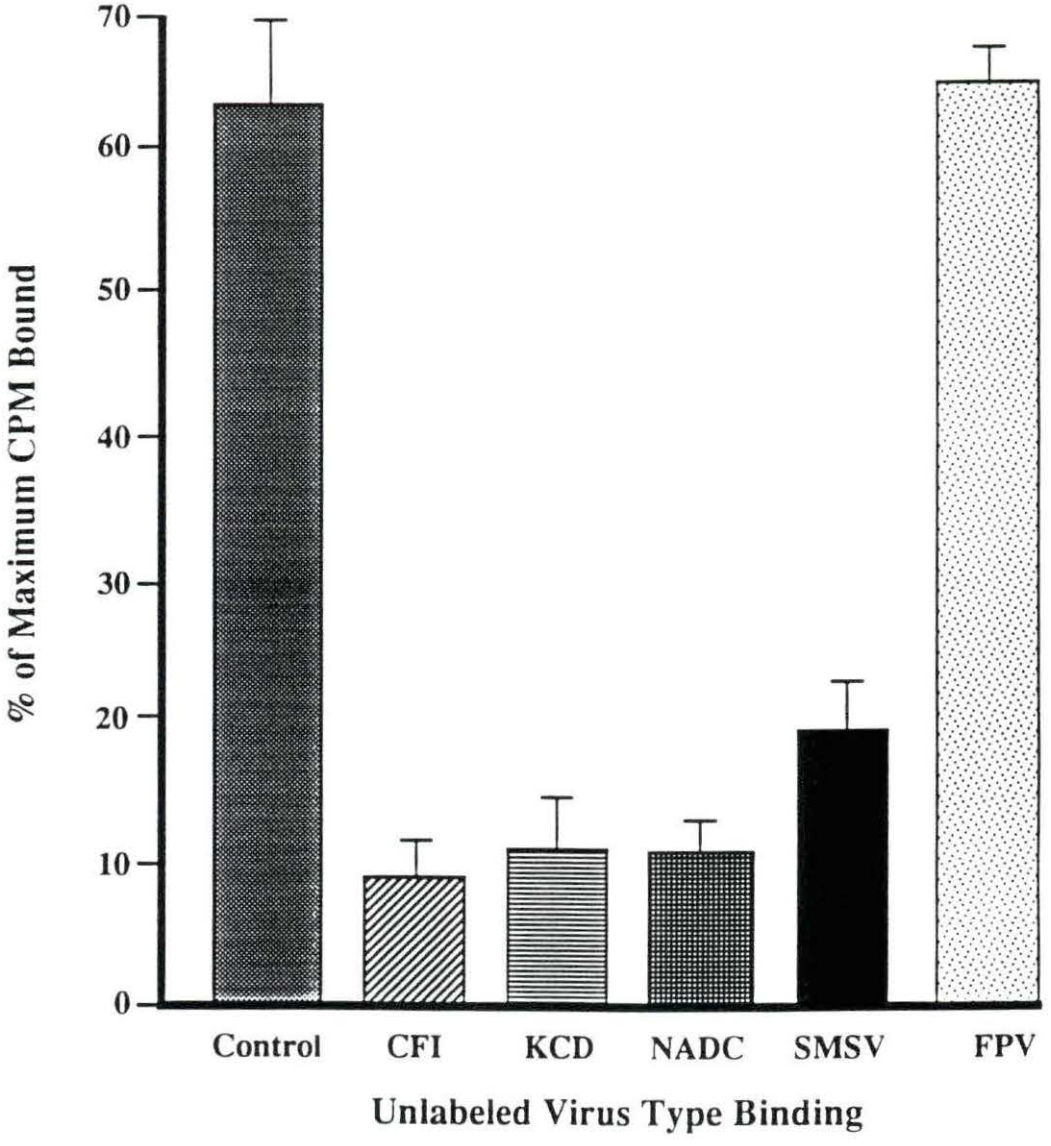

Figure 5. Competition binding experiments among caliciviruses. CRFK cells in suspension (5×10^5 cells) were allowed to react with an excess (1×10^9 particles) of homologous (CFI/68) or heterologous (KCD, NADC) unlabeled virus for 90 min at 4°C. A related calicivirus from pinnipeds, San Miguel sea lion virus (SMSV), and the non-enveloped feline parvovirus (FPV) were also used in competition experiments. Radioactively-labeled virus was added after washing cells twice with cold MEM, and allowed to react for additional hr at 4°C. Cell-associated radioactivity was estimated as a percentage of total input and represents the average ± S EM of two or more experiments.

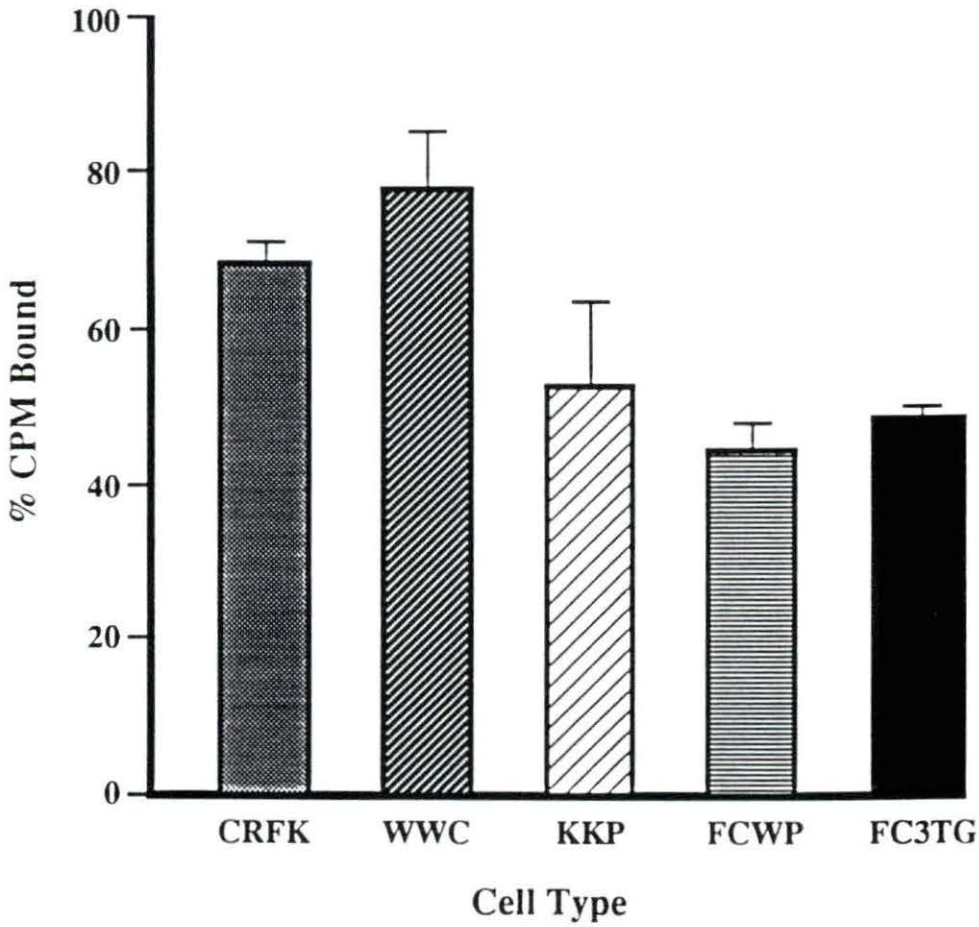

Figure 6. Binding of FCV to different cells of feline origin. Cells in suspension (5 x 10^5 cells) were allowed to react with 1 x 10^8 virus particles at 4°C for 60 min. Cells were rinsed, and associated radioactivity was determined by liquid scintillation spectrometry. Values indicated are in percentage of total input and represent the average ± SEM of two or more experiments.

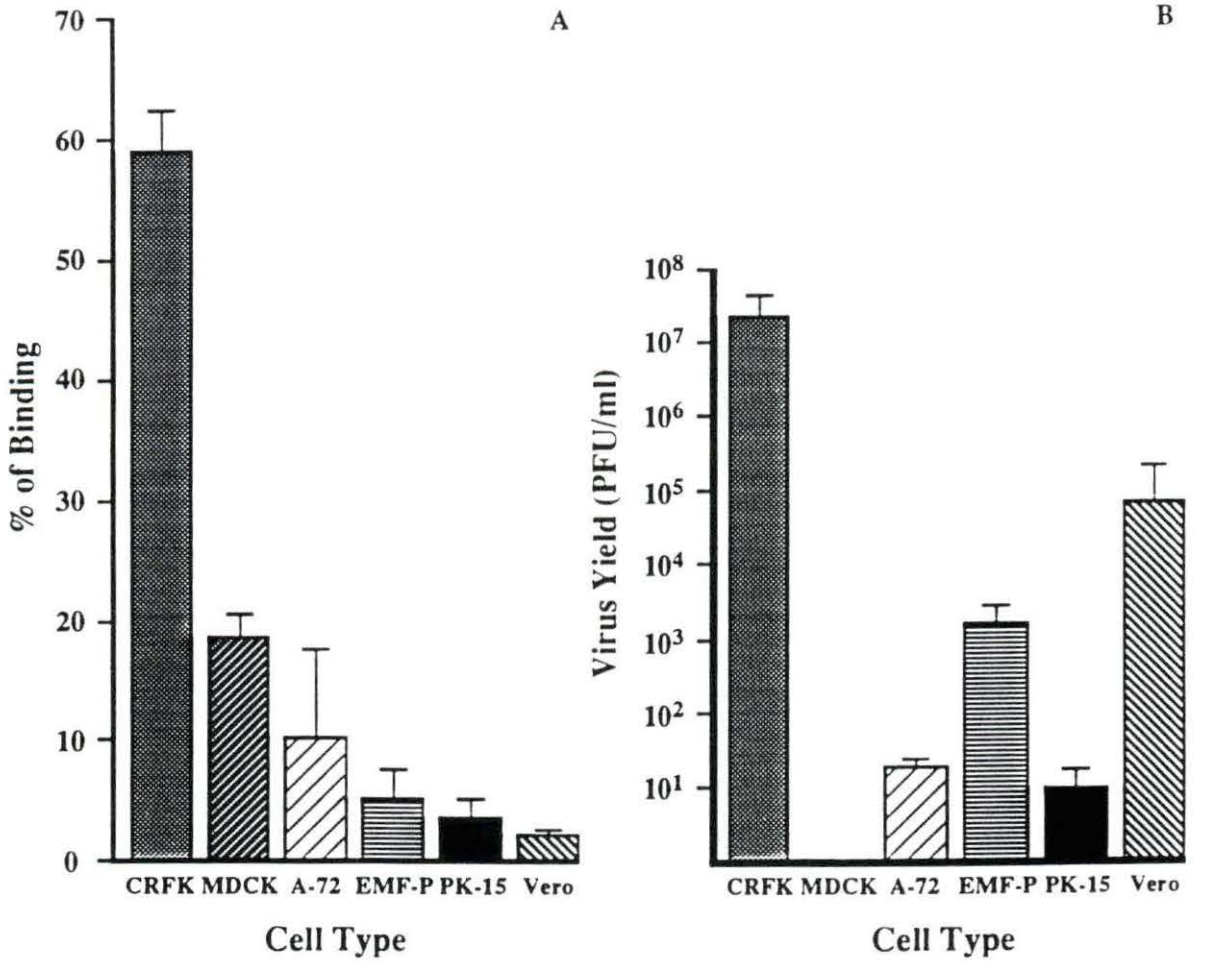

Figure 7. Feline calicivirus interaction with non-permissive cells. (A) Cells in suspension (5×10^5 cells) were allowed to react with radioactively-labeled FCV CFI/68 at 4°C for 1 hr. Cell-associated radioactivity was determined after washing the cells twice with cold MEM. Results are represented as percentage of total input and are the average ± SEM of two or more experiments. (B) Monolayers of cells in 24-well plates were transfected with viral RNA as described in the text. Virus yield was measured by standard plaque assay procedures on monolayers of CRFK cells grown in 35 mm petri dishes.

GENERAL SUMMARY

Feline calicivirus is widespread in the cat population in which it causes an upper respiratory disease. The study of the molecular biology of feline calicivirus has been updated mainly because of the relatively little information which was available concerning caliciviruses. Feline calicivirus has been used as a model for the *Caliciviridae* family which also includes San Miguel sea lion virus (SMSV), vesicular exanthema of swine virus (VESV), and rabbit hemorrhagic disease virus (RHDV).

The study of the molecular biology of a virus group usually overlooks the early events of the virus replicative cycle. The first step leading to a productive viral infection is the interaction of the viral attachment protein with specific molecules on the cell surface, acting as virus receptors. The concept of "receptor" implies the interaction between two entities and has been misused in experimental virology. Rather, the term virus receptor site should be applied, in that it defines the interaction of a virus with a specific region on the cell surface composed of many molecules. This view is mainly supported by the nature of the virus surface structure, composed of repeated molecules with the potential of multivalent interactions. This is specifically true for FCV, and the term virus receptor site has been used in this dissertation.

The early events of FCV interaction with CRFK cells was investigated with the main objective of further the understanding of calicivirus tissue tropism, replication and pathogenesis. Feline calicivirus interaction with receptor sites was optimized at mild acidic conditions and occurred independently of the temperature. Following a high initial binding rate, the association of virus with cells proceeded slowly up to 90 minutes, which reflects a lower receptor site availability after a short period of time. Binding of FCV to CRFK cells was specific and competitively blocked by homologous virus, a primary requirement for ligand-receptor interaction. Biochemically, the receptor attachment site for FCV may possess carbohydrates moieties which play an important role in the interaction with the viral attachment protein. Receptor sites for FCV were observed in

various cells of feline origin which bound virus very efficiently and supported viral replication. Binding of FCV was also observed with a non-permissive cell line, MDCK, although to a lesser extent than to feline cells. Binding to other non-permissive cells occurred at levels not higher than 10%, which is considered non-specific, and may lead to a non-productive infection. The role a specific receptor attachment site plays in tissue tropism and viral replication was demonstrated by transfecting non-permissive cells with viral genomic RNA, which is infectious per se. Interestingly, MDCK cells did not support viral replication even though it bound virus quite efficiently. However, infectious virus could be recovered from the other non-permissive cells, demonstrating that FCV replication in non-permissive cells is mainly restricted by the absence of attachment sites on the cell surface rather than by intracellular factors.

Following specific binding, viruses are internalized, uncoated and the virus genome replicated. The intracellular pathway of FCV infectious cycle is currently being investigated to further the understanding of the various stages associated with virus replication. Evidence suggests that caliciviruses require passage through acidic endocytic vesicles for proper uncoating and replication. The role of an acidic environment or enzymes activated by acidic pH in FCV uncoating will be further investigated. Future work with FCV will also be directed towards determining the nucleotide sequence of the capsid gene and the amino acid sequence of the capsid protein of a series of variant progeny virus isolates that originated from a single parental strain, after persistent infection in the host. Such studies will be helpful in the understanding of virus mutation during replication under low immunologic pressure, and in the determination of amino acid sequences composing epitopic regions of the capsid protein. Data obtained from these studies may be used for the formulation of peptides or multivalent vaccines in substitution of the current monovalent types, which may no longer provide good immunization.

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