Functional Differences in the Peplomer Glycoproteins of Feline Coronavirus Isolates

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The feline coronaviruses can be divided into two distinct antigenic groups on the basis of antigenic differences found on the peplomer (E2) glycoprotein of the virus. Because the E2 glycoprotein is responsible for many of the biological functions of coronaviruses, experiments were done to determine whether there were any E2 functional differences between these two antigenic groups. The avirulent feline infectious peritonitis virus (FIPV) isolate FIPV-UCD-2, which has one antigenic type of E2, was less rapidly internalized and could not spread from cell to cell in the presence of neutralizing antibody. Two virulent isolates, FIPV-DF2 and FIPV-79-1146, as well as the non-FIP-causing feline enteric coronavirus (FECV) isolate FECV-79-1683, all of which have the second antigenic type of E2, were very rapidly internalized and were able to spread from cell to cell despite the presence of neutralizing antibody. The avirulent FIPV-UCD-2 and FECV-79-1683 isolates were more labile at 37°C at pHs of 6.5 and above than were the virulent FIPV-DF2 and FIPV-79-1146 isolates.

Feline infectious peritonitis virus (FIPV) is a coronavirus of cats which has been isolated at least eight times in the past 8 years (1, 4, 7–10). Another coronavirus, feline enteric coronavirus (FECV) has also been isolated from cats (7, 9). It has been recently noted that some of these isolates differ dramatically in their abilities to cause feline infectious peritonitis (FIP) (10). From previous studies, a hierarchy of virulence has been proposed, with FIPV-UCD-2 being completely avirulent, followed in ascending order of virulence by FIPV-UCD-4 and FIPV-UCD-1 and by FIPV-TN406, FIPV-DF2, and FIPV-79-1146; the latter three isolates are extremely virulent and almost always cause fatal FIP. The FECV isolates FECV-UCD and FECV-79-1683 occasionally cause a mild enteritis in cats but have never been shown to cause FIP.

We have recently demonstrated that the avirulent isolate of FIPV (FIPV-UCD-2) has a peplomer (E2) glycoprotein that is antigenically different from those of other FIPV isolates (5). Virulent FIPV isolates included FIPV-DF2, FIPV-79-1146, FIPV-TN406, FIPV-UCD-1, and FIPV-UCD-4 and were recognized by seven anti-E2 monoclonal antibodies (MAbs), whereas FIPV-UCD-2 was recognized by a different set of seven anti-E2 MAbs. None of the 14 anti-E2 MAbs reacted with all isolates. The FECV isolate FECV-79-1683 peplomer was very similar antigenically to the peplomers of virulent FIPV isolates, reacting with six of the seven anti-FIPV-DF2 E2 MAbs.

During coronavirus replication, virions are assembled intracellularly and are released via secretory vesicles (12). The virions do not bud from the plasma membrane, although some of the E2 glycoprotein does migrate to the cell surface, where it is responsible for causing cell fusion with adjacent cells. The E2 glycoprotein is responsible for many of the biological properties of the virus, including binding to the cellular receptor, inducing neutralizing antibodies, eliciting cell-mediated cytotoxicity, causing pH-dependent thermo-

lability, inducing cell fusion, and fusing the viral envelope with the cellular membrane for infection (12).

We compared the avirulent FIPV-UCD-2 and non-FIP-causing FECV-79-1683 isolates with the virulent FIPV-DF2 and FIPV-79-1146 isolates in several in vitro assays. Several functional differences were found among the peplomers of these antigenically and pathogenically diverse feline coronaviruses. These differences included rate of internalization into permissive cells, ability to spread from cell to cell in the presence of neutralizing antibody, and pH-dependent thermolability.

Feline whole fetus cells (fcwf), a gift of N. C. Petersen, University of California, Davis, were grown in RPMI supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 2 mM L-glutamine. Established monolayers of fcwf cells in 96-well flat-bottom tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.) were inoculated with approximately 1,000 PFU of virus per well. At the indicated times, the inoculum was removed and polyclonal neutralizing antibody was added to neutralize any noninternalized virus. At 9 h postinfection (p.i.), the monolayers were fixed in 70% acetone and stained in an indirect immunoperoxidase assay by using the antinucleocapsid MAb (13G1) and diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) at 0.06 mg/ml with 0.01% H_2O_2 filtered through a 0.45-μm-pore-size filter (Gelman Sciences, Inc., Ann Arbor, Mich.). Foci of infection were counted for each time point and calculated as a percentage of those seen at 9 h p.i. which had had no neutralizing antibody added to the well. These assays were done in triplicate, and the results are shown in Fig. 1. When neutralizing antibody was added at 60 min p.i., 70 to 85% of the FIPV-DF2, FIPV-79-1146, and FECV-79-1683 viruses had been internalized, compared with only 25% of the avirulent FIPV-UCD-2 isolate. By 2 h, 100% of the virulent FIPV isolates as well as the FECV isolate had been internalized, compared with 35% of the FIPV-UCD-2 isolate. It was not until 5 h p.i. that 100% of the FIPV-UCD-2 virus was internalized.

The peplomer glycoprotein of coronaviruses has been shown to be responsible for fusing cells and producing the characteristic syncytia observed with many coronaviruses. To determine whether FIPV-UCD-2 differed from the other

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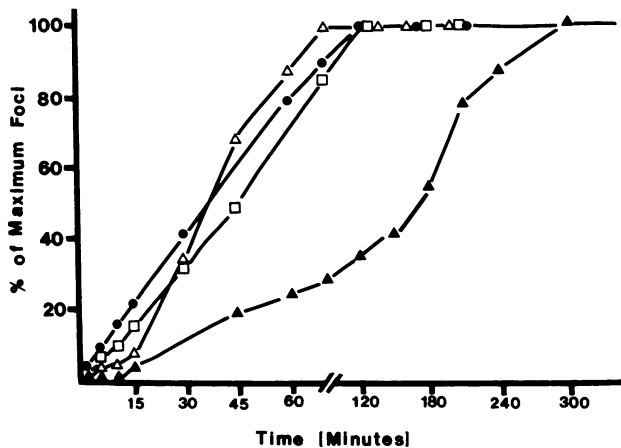‡ Present address: Triton Biosciences, Inc., 1501 Harbor Bay Parkway, Alameda, CA 94501.

FIG. 1. Kinetics of internalization. Monolayers of fcwf cells were incubated with 1,000 PFU of virus per well (△, FIPV-DF2; □, FIPV-79-1146; ●, FECV-79-1683; ▲, FIPV-UCD-2). At various times, the virus was removed and neutralizing antibody was added to neutralize noninternalized virus. At 9 h p.i., the cells were examined for the presence of nucleocapsid antigen by indirect immunoperoxidase staining.

isolates, the ability to spread from cell to cell was investigated. Monolayers of fcwf cells were inoculated with sufficient virus to form approximately 60 foci of infection per well and incubated for 1 h at 37°C. After virus adsorption, the inoculum was removed and neutralizing antibody was added to the wells. The plates were incubated for 24 to 96 h, and at daily intervals some cells were fixed in acetone and stained in the indirect immunoperoxidase assay. The percentage of infected cells was determined at earlier time points by counting the number of stained cells in the entire monolayer. At later time points, the percentage of the monolayer which was either stained or destroyed was estimated by observing the entire monolayer. These assays were done in triplicate. At 24 h p.i., from 1 to 25% of the monolayer was infected as determined by indirect immunoperoxidase staining (Fig. 2). For the FIPV-DF2, FIPV-79-1146, and FECV-79-1683 isolates, the percentage of infected cells steadily increased during the course of the experiment, although the number of foci of infection remained constant. Altering the amount of inoculating virus (5 to 1,000 foci per well) changed the slope of the lines but not the inevitable outcome of eventual complete destruction of the monolayer with these three isolates. A marked difference, however, was seen with the avirulent FIPV-UCD-2 isolate. At 24 h p.i., approximately 60 isolated infected cells per well were observed. As the experiment progressed, the number of infected cells decreased until at 96 h p.i. no cell demonstrated staining with the anti-nucleocapsid MAb. This experiment was repeated by using an inoculum designed to give approximately 1,000 foci per well at 24 h. By 48 h the number of infected cells had decreased to approximately 100, and at 72 h the number had decreased to 10. The virus seemed unable to spread and form syncytia in the presence of neutralizing antibody, unlike the other isolates tested.

Another biological function of the E2 glycoprotein is its role in the pH-dependent thermolability of infectivity (11). Four of the virus isolates were diluted (1:10) in media of differing pHs (pHs 4.0 to 8.0) and incubated at either 37 or 4°C for 24 h or titrated immediately in a plaque assay (5). Plaques were visualized after 2 to 3 days by staining with 0.25% crystal violet in 20% methanol. At the end of the 24-h

incubation period, the remaining samples were titrated and the number of plaque-forming units per milliliter for each treatment was expressed as a percentage of the time zero control at that particular pH (Fig. 3). All of the viruses were relatively stable at 4°C, and all were relatively unstable at 37°C. The most dramatic differences were observed with FIPV-UCD-2 and FECV-79-1683 at pHs of 6.0 and above. After being subjected to pH 6.0, only 0.01% of the original infectious FIPV-UCD-2 remained, compared with 1 to 10% for the other isolates. At pH 6.5, no infectious virus was detected for FIPV-UCD-2, whereas the other isolates demonstrated 0.5 to 1% of their control values. At pH 7.0, no infectious virus could be detected for either FIPV-UCD-2 or FECV-79-1683, whereas FIPV-DF2 and FIPV-79-1146 showed 0.08 to 0.8% of their respective control values. At pH 8.0, both FIPV-79-1146 and FIPV-DF2 demonstrated about 0.08% of the infectivity of the time zero sample, whereas neither FIPV-UCD-2 nor FECV-79-1683 had detectable infectious virus.

The suggestion of a hierarchy of virulence of FIPV isolates (10), our demonstration of antigenically different peplomers on feline coronavirus isolates (5), and the known importance of the E2 glycoprotein in biological functions of coronaviruses (12) prompted this study to determine whether differences in the pathogenicity of feline coronaviruses can be correlated with functional differences in the peplomer. Antigenic differences in the E2 glycoprotein of mouse hepatitis virus, another coronavirus, have been associated with differences in pathogenicity. Some neutralizing MAbs specific for the E2 of mouse hepatitis virus were able to convert a fatal case of encephalitis to a nonfatal demyelination in vivo (2), and anti-E2 MAb-resistant mutants of MHV have been shown to be less virulent (3, 6).
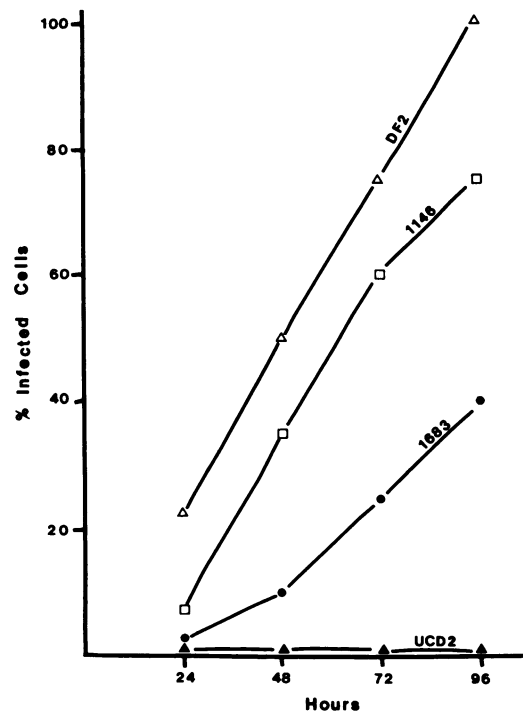

FIG. 2. Cell-to-cell spread of feline coronavirus isolates. Monolayers of fcwf cells were incubated with 60 PFU of virus per well. After the addition of neutralizing antibody at 1 h p.i., infected cells were examined at 24, 48, 72, and 96 h p.i. for the presence of viral antigen by an immunoperoxidase assay.
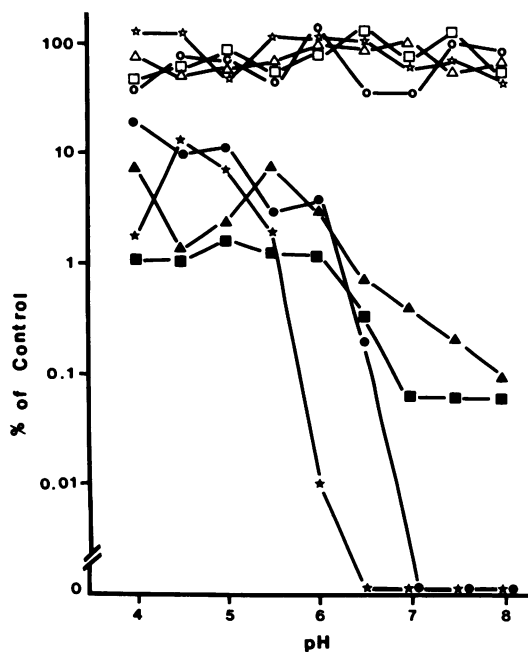
FIG. 3. pH-dependent thermolability of four feline coronavirus isolates. Purified virus diluted in media of different pHs was incubated at 4 or 37°C for 24 h and then titrated in a plaque assay. Results after 24 h of incubation at each pH are expressed as a percent of the time zero titration results at that pH. Open symbols, incubated at 4°C for 24 h. Closed symbols, incubated at 37°C for 24 h. Symbols: □ and ■, FIPV-DF2; △ and ▲, FIPV-79-1146; ○ and ●, FECV-79-1683; ☆ and ★, FIPV-UCD-2.

The nonpathogenic FIPV-UCD-2 isolate was shown to have a biologically different E2 glycoprotein from the other isolates tested in kinetics of internalization, cell-to-cell spread, and pH-depenent thermolability. These biological differences may explain its avirulence in cats. One of the functions of the peplomer is to bind to cellular receptors during the adsorption process. Differences in the speed with which virus isolates are internalized by the cell might suggest differences in structure, number, or avidity of the cellular receptors for different virus isolates. The FIPV-DF2, FIPV-79-1146, and FECV-79-1683 isolates were internalized more rapidly into fcwf cells than was the FIPV-UCD-2 isolate (Fig. 1). The FIPV-UCD-2 isolate thus may have fewer receptors or less affinity for its receptors than the virulent isolates and FECV-79-1683.

There seemed to be no cell-to-cell spread or syncytium formation of the avirulent FIPV-UCD-2 isolate in the presence of neutralizing antibody compared with the other three isolates (Fig. 2). The FIPV-79-1146, FIPV-DF2, and FECV-79-1683 isolates gradually spread from cell to cell until eventually the entire monolayer was involved in virus production and ultimately destroyed despite the presence of neutralizing antibody. This may also explain the avirulence of FIPV-UCD-2 in cats, especially because many naturally exposed cats (68%) produce neutralizing antibodies to FIPV-UCD-2 but few field cats appear to produce neutra-

lizing antibodies to the virulent FIPV isolates (data not shown).

The pH of the extracellular fluid in which the virus is located before encountering a permissive cell may play a role in pathogenicity. The avirulent FIPV-UCD-2 isolate and the non-FIP-causing FECV-79-1683 isolate were completely inactivated after 24 h at 37°C at pHs of 7.0 and above (Fig. 3). The virulent isolates retained some infectivity even at pH 8.0. Therefore, the greater thermolability of the avirulent FIPV isolate may play a role in its nonpathogenicity in cats, especially since it is less rapidly internalized than the other isolates (Fig. 1).

In the case of feline coronaviruses, differences in pathogenicity may in part be determined by the peplomer glycoprotein as demonstrated here. Yet it is difficult to explain why FECV-79-1683 does not cause FIP if the peplomer is the critical polypeptide for pathogenicity. It may be that there are additional antigenic differences in the FECV-79-1683 E2 that correlate with cell tropisms or that the pH-dependent thermolability is the critical biological difference relating to pathogenicity. Alternatively, the E1 glycoprotein, N polypeptide, some nonstructural protein, or any combination may form the basis of pathogenicity in feline coronaviruses.

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