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Characterization of a temperature sensitive feline infectious peritonitis coronavirus

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Summary. The characteristics of a temperature sensitive feline infectious peritonitis virus (TS-FIPV) were examined. TS-FIPV, unlike its parent strain, DF2 wild type FIPV (WT-FIPV), propagated at 31 °C (permissive temperature) but not at 39 °C (nonpermissive temperature). This temperature preference of TS-FIPV was also demonstrated in cats by the ability of the virus to replicate only at the lower temperature in the upper respiratory tract and not at systemic sites where higher temperatures (38–39 °C) prevail. Viral structural proteins and RNA were synthesized at 39 °C but some undefined maturational defect prevented the formation of infectious TS-FIPV at its nonpermissive temperature. TS-FIPV was more thermolabile than WT-FIPV which indicated alterations in the structural proteins of TS-FIPV, and a difference in the envelope protein of the two viruses was revealed by Western blot analysis. Plaque assay characterization showed that TS-FIPV produced small plaques in comparison to the large plaques of WT-FIPV. These unique characteristics possessed by TS-FIPV may account for its nonvirulent nature and ability to stimulate protective immune responses in cats.

Introduction

Feline infectious peritonitis (FIP) is a complex and fatal disease of cats caused by infection with feline infectious peritonitis virus (FIPV). Previous attempts to consistently protect cats by immunization with other antigenically related coronaviruses such as porcine transmissible gastroenteritis virus (TGEV) [26], canine coronavirus (CCV) [2], and human coronavirus [1] have been unsuccessful. Inconsistent protection was found when cats were given a sublethal dose of virulent FIPV and cats vaccinated with an avirulent FIPV were more easily infected than were nonvaccinated cats [14]. Recently, it has been demonstrated that an intranasally (IN) administered temperature sensitive FIPV (TS-FIPV) vaccine is efficacious and safe upon FIPV challenge [5].

The pathogenesis of FIP is complicated and not fully understood. Evidence indicates that FIP is an immune-mediated disease [10]. The virus has been shown to replicate initially in the upper respiratory tract and small intestine [20]. Macrophages, the primary FIPV target cell, may then cross the mucosal barrier and spread virus throughout the cat [8, 24, 25]. Furthermore, a correlation between FIPV virulence in vivo and ability to infect macrophages in vitro has been observed [18]. It has been suggested that a strong cell-mediated immune response to FIPV may be more important than humoral immunity in protecting cats from this disease [15, 19]. However, the role of local immune responses in the upper respiratory tract and intestinal tract has not been carefully evaluated and may represent an important immune defense mechanism against FIP.

TS-FIPV was developed by serial passages at a reduced temperature, followed by ultraviolet irradiation. The selected virus will propagate at its permissive temperature (31 °C) but not at its nonpermissive temperature (39 °C). Temperature sensitivity was accompanied by the appearance of various characteristics that distinguish TS-FIPV from its virulent parent strain. The purpose of this report is to present these distinguishing characteristics which include plaque size, temperature stability, ability to synthesize RNA, expression of structural proteins and temperature dependent replication in vitro and in vivo.

Materials and methods

Cell culture

Norden Laboratories feline kidney (NLFK) cells were used from passage 80 to 92. Cells were propagated in Basal Medium Eagle (BME) supplemented with 5% fetal bovine serum (FBS) and 10 mM Hepes buffer.

Virus isolation

The DF2 wild type FIP virus (WT-FIPV) was originally isolated from a cat liver explant. After several passages of tissue homogenates in specific pathogen free (SPF) cats, the virus was adapted to NLFK cells by cocultivation with infected primary spleen cells. The DF2 WT-FIPV strain was grown on NLFK cells at 39 °C for passages 1 through 60 and then passed at 31 °C up to passage 99. The virus collected at passage 99 was ultraviolet irradiated (5 cm distance with a Westinghouse 782-30 lamp, 118 V, 60 cycles, 0.5 amps) for 5 min. The virus was plaque purified and designated TS-FIPV. Passage 13 of the plaque purified TS-FIPV was used in the characterization studies.

Plaque assay, growth curve, and virus titration

The plaque assay was done with confluent cell monolayers inoculated with 0.2 ml of a tenfold virus dilution. After adsorption for 1 h at 31 °C or 39 °C, the cells were overlaid with 4 ml of 0.75% carboxymethylcellulose in 199 medium. Monolayers were fixed and stained with a solution of 10% formalin, 1% crystal violet and 20% ethanol 72 h post-infection.

Viral growth curves were conducted to compare the growth of TS-FIPV and WT-FIPV at both 31 °C and 39 °C. TS-FIPV and WT-FIPV were inoculated onto confluent cell monolayers at a multiplicity of infection (m.o.i.) of approximately 0.1. At predetermined

times over 65 h, viral fluids were aseptically removed, stored at −80 °C, and titrated for infectivity at the completion of the growth curve. The $TCID_{50}$ assay for virus infectivity was done in 96-well microtiter plates by infection of 5 wells each with 100 µl of a tenfold virus dilution. TS-FIPV was titered at 31 °C and WT-FIPV was titered at 39 °C. Wells were examined for cytopathic effects (CPE) on day 5 and the method of Reed and Muench [16] was used to calculate the $TCID_{50}$.

Thermolability

Thermolability studies were performed at 54 °C with WT-FIPV and TS-FIPV. Each virus was diluted 1 : 5 in serum-free BME. At intervals between 5 and 75 min, samples were removed, immediately cooled in an ice bath and titrated for residual infectivity.

Viral RNA synthesis

NLFK cells were used to determine TS-FIPV virus-specific RNA synthesis. Cell monolayers were infected at an m.o.i. of approximately 10 for 1 h at 31 °C. After removal of the inoculum, 2 ml of BME containing 2% dialyzed FBS, 1 µg/ml actinomycin D and 25 µCi [5-^{3}H]-uridine was added to duplicate cultures. Following incubation for 12 h, the radiolabeled cellular RNA was extracted according to Miller et al. [12]. The RNA pellets were solubilized in Readi-Protein (Beckman, Fullerton, CA) and counted in a liquid scintillation counter (Model LS3801, Beckman Instruments, Fullerton, CA).

A parallel set of cultures without radiolabel was used to monitor infectivity. The unlabeled cells and medium collected at 12 h were titrated after one freeze-thaw cycle.

Monoclonal antibodies

Spleen cells from BALB/c mice immunized with either CCV or WT-FIPV were fused with NS1/Ag4 mouse plasmacytoma cells (American Type Cell Culture, Rockville, MD) using standard procedures [13]. Hybridoma culture fluids were screened for specific antibody production by ELISA and Western blot. Selected hybridomas were cloned twice by limiting dilution and ascites was produced by intraperitoneal injection of 5×10^6 hybridoma cells into pristane-treated BALB/c mice.

The monoclonal antibodies chosen for use in this study were broadly cross-reactive against CCV, FIPV, and feline enteric coronavirus (FECV). Three antibodies designated Mab-P, Mab-N, and Mab-E were selected based on their specificities for the peplomer protein (Mab-P), nucleocapsid protein (Mab-N) and envelope protein (Mab-E). Mab-P and Mab-N were obtained from a fusion performed with a CCV-immunized mouse and Mab-E originated from a mouse immunized with WT-FIPV. All three antibodies were used as the primary antibody in the Western blot and indirect immunofluorescence assays (IFA) (see below).

Comparison of WT-FIPV and TS-FIPV structural proteins

The structural proteins of WT-FIPV grown at 39 °C and TS-FIPV grown at 31 °C were examined by SDS-PAGE and Western blot. SDS-PAGE was performed by a modified Laemmli system [9]. After electrophoresis, the proteins were blotted to Immobilon PVDF Transfer Membrane (Millipore, Bedford, MA) by the procedure of Towbin et al. [22]. Following transfer, the blot was blocked with 2% nonfat dried milk in PBS, incubated with Mab-P, Mab-N, or Mab-E and then reacted with alkaline phosphatase-conjugated goat anti-mouse IgG (H + L) (Kirkegaard and Perry, Gaithersburg, MD).

TS-FIPV proteins at permissive and nonpermissive temperature

TS-FIPV proteins in supernatants from cells infected at 31 °C and 39 °C were examined by Western blot. NLFK monolayers were infected at an m.o.i. of approximately 1 or 10 for

1 h at 31 °C or 39 °C. The inocula were removed, the cultures were washed twice with BME and finally 2 ml of BME was added. At intervals between 0 and 96 h post-adsorption, the culture medium was removed and centrifuged (13,000 × g) for 10 min. The supernatants were frozen at −80 °C until Western blot analysis.

Immunofluorescence microscopy

WT-FIPV and TS-FIPV intracellular and surface antigens expressed at 31 °C and 39 °C were examined by an indirect IFA [7]. Cell monolayers were infected with WT-FIPV and TS-FIPV at an m.o.i. of approximately 0.1. At the appropriate time point the cells were rinsed with PBS and then fixed in acetone for 20 min at −20 °C. The slides were then exposed to Mab-P, Mab-N, or Mab-E for 30 min at 37 °C. Following incubation, the slides were rinsed in PBS and then exposed to fluorescein-conjugated goat anti-mouse IgG (H + L) (Kirkegaard and Perry, Gaithersburg, MD) for 30 min at 37 °C. Detection of viral surface antigens by indirect IFA was done as described for detection of intracellular antigens except the monolayers were not fixed. The cells were observed and photographed with a Zeiss Axioskop photomicroscope equipped with a Zeiss × 40 objective.

In vivo fate of the virus

SPF cats (Liberty Labs, Liberty Corner, NJ), 10 to 20 months old, were used in this study. Three cats were vaccinated IN with 1 ml of TS-FIPV ($10^{6.75}$ $TCID_{50}$/ml). At 1, 2, and 4 days after inoculation one TS-FIPV vaccinated cat was sacrificed. One cat was infected orally with WT-FIPV ($10^{6.23}$ $TCID_{50}$/ml) and sacrificed on day 4.

After sacrifice, tissues were aseptically removed from the cats and frozen at −80 °C. Tissues were then thawed and pulverized with a manually operated tissue grinder to make a 5% to 20% (w/v) suspension using BME with antibiotics as a diluent. The suspensions were clarified by centrifugation (10,000 × g) for 3 min and 0.2 ml was then added to the cell monolayers for 1 h at 31 °C for TS-FIPV and 39 °C for WT-FIPV. After incubation the tissue suspensions were removed and the monolayers were washed once with BME followed by the addition of 1 ml BME. The plates were incubated at the optimal temperature for each virus and were observed for viral CPE daily for 7 days following inoculation. A standard virus neutralization test [7] was done with Mab-P on the virus positive tissue homogenates to confirm the presence of coronavirus. Cat tissues were examined by direct IFA for viral antigen.

Results

In vitro temperature sensitivity

The effect of incubation temperature on the in vitro growth of TS-FIPV is shown in Table 1. TS-FIPV had optimal infectivity when propagated and titrated

Table 1. Temperature sensitivity of TS-FIPV grown for 48 h at 31 °C or 39 °C and then assayed for infectivity at both temperatures

Growth temperature	Infectivity ($TCID_{50}$/ml)	
	31 °C	39 °C
31 °C	2.1×10^6	4.7×10^1
39 °C	2.1×10^4	4.7×10^1

at 31 °C. The CPE produced at 31 °C was characterized by formation of syncytia. When grown at 31 °C, TS-FIPV showed infectivity initially when titrated at 39 °C. However, after 4 days at 39 °C CPE was observed on less than ten percent of the monolayer and all plaques that formed were healed within 5 days. When grown at 39 °C, TS-FIPV had no detectable infectivity when assayed at 39 °C, but showed minimal infectivity when assayed at 31 °C.

Growth curves

The growth curves (Fig. 1) show that TS-FIPV grown at 31 °C appeared in the culture supernatant at approximately 8 h and then increased rapidly, reaching a maximum yield of virus at about 48 h. This contrasts considerably with TS-FIPV grown at 39 °C which reached a titer of four logs by 18 h and then remained essentially stationary. It appears that TS-FIPV infected initially at 39 °C but a defect expressed at 39 °C prevented the virus from propagating as it would at 31 °C. WT-FIPV propagated at 39 °C has optimum virus production by 42 h followed by a rapid decline in virus yield. In contrast, WT-FIPV did not grow at 31 °C.

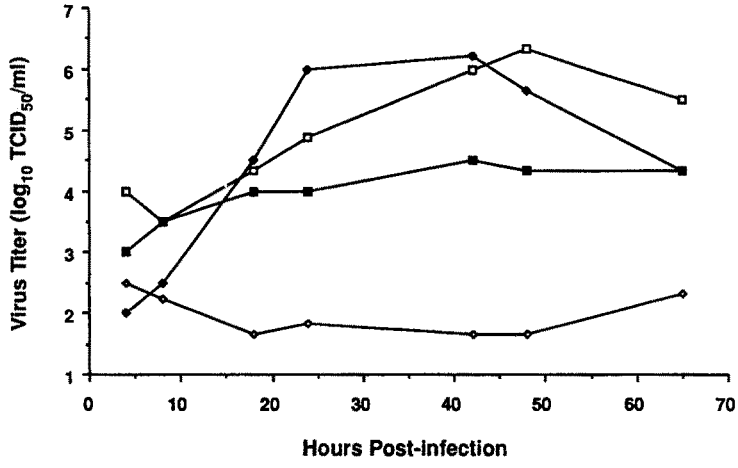

Fig. 1. Growth of FIP viruses on NLFK cells: TS-FIPV at □ 31 °C and ■ 39 °C and WT-FIPV at ◇ 31 °C and ◆ 39 °C

Plaque size

Plaque assay results showed two distinct plaque types for WT-FIPV and TS-FIPV at their optimal temperatures. WT-FIPV had large distinct plaques at 39 °C, 1.0 to 2.2 mm, but none at 31 °C. Plaques for TS-FIPV at 31 °C ranged in size from 0.5 to 1.0 mm and at 39 °C no plaques were detected.

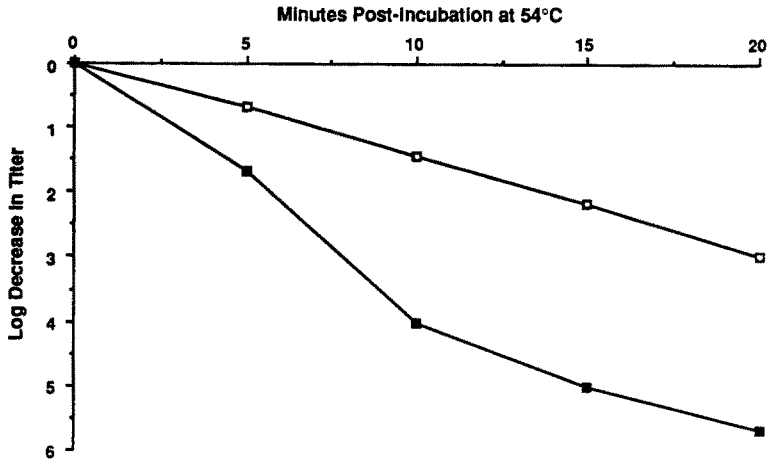

Fig. 2. Thermal inactivation of □ WT-FIPV and ■ TS-FIPV at 54 °C

Thermolability

As can be seen in Fig. 2, TS-FIPV was rapidly inactivated at 54 °C. TS-FIPV infectivity decreased by five logs in 15 min. WT-FIPV was more stable at 54 °C than TS-FIPV since only two logs of infectious virus were inactivated within 15 min.

RNA synthesis

The synthesis of TS-FIP virus-specific RNA, as measured by ^{3}H-uridine incorporation, was compared at permissive and nonpermissive temperatures. Viral RNA synthesis at 31 °C and 39 °C was similar 12 h following infection, indicating that normal viral entry and initial synthesis occurred at the nonpermissive temperature. The ^{3}H-uridine incorporation at 31 °C was 98,920 cpm and at 39 °C was 95,913 cpm. However, a difference in the number of mature, infectious virions was apparent by 12 h post-infection. Virus grown for 12 h at the permissive temperature had a titer of 1.0×10^5 $TCID_{50}$/ml, while virus grown at the nonpermissive temperature had a titer of only 2.1×10^3 $TCID_{50}$/ml. Thus, at the nonpermissive temperature, it appears that early viral RNA synthesis occurred without a concomitant virus maturation process.

Analysis of viral antigens

The structural proteins of WT-FIPV and TS-FIPV grown at 39 °C and 31 °C, respectively, were compared by Western blot using coronavirus-specific monoclonal antibodies. The Western blots showed that WT-FIPV and TS-FIPV had structural protein profiles characteristic of that reported for other coronaviruses [17]. The peplomer protein band had a molecular weight of 200 kDa and the apparent molecular weight of nucleocapsid was 58 kDa for both viruses (Fig. 3). Conspicuous differences appeared in the pattern of blotted envelope proteins for the wild type and temperature sensitive viruses. The WT-FIPV envelope

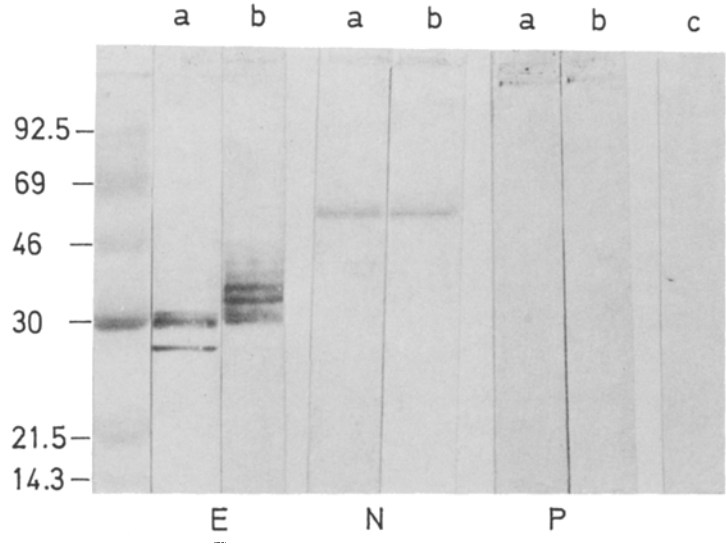

Fig. 3. Western blot comparison of *E* envelope, *N* nucleocapsid, and *P* peplomer structural proteins of *a* WT-FIPV and *b* TS-FIPV. *c* The monoclonal antibodies do not react with NLFK cell extract. Molecular weight markers (× 1,000) are at the left

protein consisted of 28 kDa and 30 kDa components (Fig. 3, E, a), whereas the envelope protein of TS-FIPV produced several protein bands from 30 kDa to 46 kDa (Fig. 3, E, b). The 28 kDa component of TS-FIPV was present but not at the same intensity as it was for WT-FIPV. The differences in molecular weight of the envelope protein of the two viruses were confirmed using immune cat sera. Immune sera from both TS-FIPV vaccinated cats and WT-FIPV challenged cats showed the same differences in the envelope protein of the two viruses as did the monoclonal antibody (data not shown). Thus, the differences in the molecular weights of the envelope polypeptides were not due to differences in reactivity to the monoclonal antibody. There was not a difference in the molecular weights of the envelope polypeptides from TS-FIPV grown at 31 °C or 39 °C (data not shown). The specificity of the monoclonal antibodies used for antigen detection is demonstrated by the lack of reactivity with NLFK cell extracts (Fig. 3, c).

The culture supernatant from TS-FIPV infected cells was monitored for the appearance of structural proteins at both the permissive and nonpermissive temperatures. TS-FIPV grown at 31 °C for 24, 48, 72, or 96 h had all three structural proteins detected in the culture supernatant (Fig. 4A). When TS-FIPV was grown at 39 °C for similar lengths of time, only nucleocapsid was found (Fig. 4B). Nucleocapsid may be the only protein released at 39 °C or the less abundant peplomer and envelope proteins were not detected. Figure 4 also shows that the nucleocapsid protein released at 39 °C may be associated with infectious RNA or a few viral particles were released at 39 °C because these culture supernatants were infective at 31 °C. However, at 24 h the infectivity of

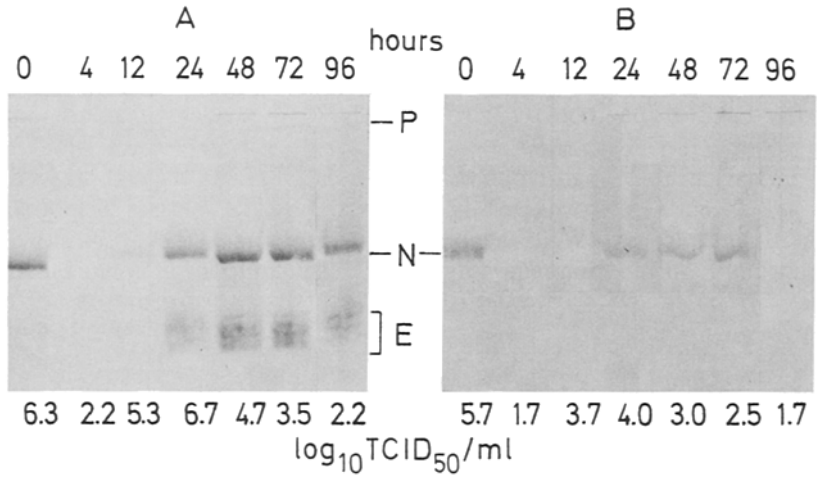

Fig. 4. Western blot detection of TS-FIPV structural proteins in culture supernatants of NLFK cells infected with TS-FIPV at a MOI of 10 for 4 to 96 h at **A** 31 °C **B** 39 °C. The virus titer (log_{10} $TCID_{50}$/ml) for each sample is shown at the bottom of each lane

the supernatant fluids from 39 °C was almost three logs less than the supernatant fluids from 31 °C. When assayed at 39 °C, no detectable virus titer was found.

Intracellular synthesis of TS-FIPV and WT-FIPV proteins was compared at 31 °C and 39 °C. Synthesis was monitored by IFA using coronavirus-specific monoclonal antibodies on acetone-fixed, infected cells. All three structural proteins of TS-FIPV were detected in the cells by IFA at both the permissive and nonpermissive temperatures (Table 2). The TS-FIPV proteins appeared at both temperatures within 6 h post-infection. Similar results were observed with WT-FIPV infected cells at 39 °C. When incubated at 31 °C, WT-FIPV proteins appeared somewhat later. Nucleocapsid was visible by 8 h while peplomer and envelope proteins were apparent by 9 h.

Examination of the surface of infected cells by immunofluorescence using a peplomer monoclonal antibody indicated that TS-FIPV was present on the surface of living cells by 12 h post-infection at 31 °C but not 39 °C (Table 2). Small areas of envelope surface fluorescence were observed for TS-FIPV by

Table 2. Indirect immunofluorescence detection of TS-FIPV and WT-FIPV proteins in acetone-fixed cells and on the surface of unfixed cells infected at 31 °C and 39 °C

	TS-FIPV		WT-FIPV	
	Acetone-fixed 31 °C/39 °C	Surface 31 °C/39 °C	Acetone-fixed 31 °C/39 °C	Surface 31 °C/39 °C
Peplomer	+/+	+/−	+/+	+/+
Nucleocapsid	+/+	−/−	+/+	−/−
Envelope	+/+	+/−	+/+	−/+

18 h at its permissive temperature only. After 12 h at 39 °C peplomer and envelope antigens were detected on the surface of WT-FIPV infected cells, but at 31 °C only slight peplomer fluorescence was found after 15 h. Nucleocapsid was not detected on the surface of cells infected with either virus.

In vivo fate of the virus

In order to determine if the temperature sensitive characteristics of TS-FIPV observed in vitro were reflected by growth of the virus in vivo, cats were inoculated with either TS-FIPV or WT-FIPV. At predetermined times post-inoculation, cats were sacrificed and tissues were examined for the presence of virus. Evidence of TS-FIPV replication in the upper respiratory tract was found by virus isolation and immunofluorescence (Table 3). Virus was isolated from the cervical lymph node, tonsil, trachea and turbinate at 1, 2, or 4 days post-vaccination. TS-FIPV antigen was identified by direct IFA in the mandibular lymph node and the tonsil. In contrast, by 4 days after oral infection with WT-FIPV, the virus had disseminated throughout the cat. WT-FIPV was isolated from four different lymph nodes (cervical, mandibular, mediastinal and mesenteric), the oral/nasal/pharyngeal area, as well as from the thymus and spleen. All of these tissues except the thymus were positive for viral antigen by direct IFA.

Table 3. Virus isolation and direct immunofluorescence assay on tissues of cats vaccinated intranasally with TS-FIPV or inoculated orally with WT-FIPV

Tissue	TS-FIPV		WT-FIPV	
	VI[a]	IFA[b]	VI	IFA
Kidney	−	−	−	−
Liver	−	−	−	−
Lung	−	−	−	−
Cervical lymph node	+	−	+	+
Mandibular lymph node	−	+	+	+
Mediastinal lymph node	−	−	+	ND[c]
Mesenteric lymph node	−	−	+	+
Pharynx	−	−	+	ND
Salivary gland	−	−	−	+
Spleen	−	−	+	+
Thymus	−	−	+	−
Tonsil	+	+	+	+
Trachea	+	−	+	+
Turbinate	+	−	+	+

[a] Virus isolation
[b] Direct immunofluorescence assay
[c] Not done

Discussion

The differences in the characteristics of TS-FIPV and WT-FIPV may account for the protective immunity afforded by TS-FIPV [5] and the onset of disease by WT-FIPV. TS-FIPV and WT-FIPV differ in temperature specificity and stability, plaque size, and structural protein expression. In addition, TS-FIPV and WT-FIPV disseminate differently in the cat. Although the lesions responsible for the temperature sensitive defect have not been located, the characteristics of TS-FIPV in relation to its parent strain suggest a maturation defect.

TS-FIPV growth is impaired at the optimal temperature (39 °C) for WT-FIPV but TS-FIPV replicates normally at its permissive temperature (31 °C). This temperature preference allows TS-FIPV to grow in the upper respiratory tract of cats but retards systemic growth in cats where the temperature is 39 °C. Replication of TS-FIPV in the upper respiratory tract may stimulate mucosal responses that may be required to protect cats against FIPV challenge. Mucosal immunity provided by TS-FIPV may be important in stopping the primary infection of FIPV since Stoddart et al. [20] has shown that FIPV administered orally replicated initially in the tonsil and small intestine. Also, the temperature preference of TS-FIPV may prevent a vaccine-induced sensitization of the cat. This contrasts with a previous study by Pedersen and Black [14] in which vaccination with a modified-live FIPV not only failed to protect cats against disease, but appeared to sensitize cats so they were more susceptible to FIPV challenge than were nonvaccinated cats.

WT-FIPV produced larger plaques in NLFK cells than did TS-FIPV. Tupper et al. demonstrated that two virulent FIPV strains (79-1146 and NOR 15, a plaque purified DF2 WT-FIPV) produced larger plaques than the nonvirulent FECV strain 79-1683 [23]. McKeirnan and coworkers also documented this difference in plaquing profiles between these same feline coronavirus strains [11]. It appears that avirulent feline coronaviruses produce small plaques, whereas virulent FIPV produces large plaques.

TS-FIPV RNA synthesis occurred normally at 39 °C for approximately 12 h, even though viral growth did not proceed normally. The absence of intact virion production in the presence of RNA synthesis at 39 °C suggests a defect in the maturation and assembly of the virion which has been shown in other temperature sensitive viruses [3, 4, 21].

The detection of TS-FIPV structural proteins by indirect IFA in NLFK cells at 39 °C without concomitant virus production also indicates a maturation defect at 39 °C. Surface expression of TS-FIPV and WT-FIPV peplomer and envelope proteins but not nucleocapsid at the optimal temperature for each virus resembles the situation in FIPV-infected macrophage-like cells [8]. The expression of viral antigen on the cell surface may be important in the pathogenesis of FIPV. The absence of TS-FIPV surface antigen at 39 °C may account for the lack of sensitization in TS-FIPV vaccinated cats.

A difference was evident in the molecular weights of the envelope polypep-

tides of TS-FIPV and WT-FIPV as shown by Western blotting. The low molecular weight (28 kDa) component was detected to a lesser degree for TS-FIPV than WT-FIPV. This same kind of difference was observed with two virulent strains of FIPV; UCD 1 did not have the low molecular weight component that was present in the Dahlberg strain of FIPV [6]. These observed differences in the envelope protein may be due to differences in glycosylation. Further investigation by two-dimensional gels is needed to clearly differentiate the envelope proteins of TS-FIPV and WT-FIPV.

Different characteristics are apparent between TS-FIPV and its parent strain WT-FIPV. The importance of these TS-FIPV characteristics in relation to the protective response of the virus in cats is under investigation.

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