STUDIES ON A TEMPERATURE-SENSITIVE MUTANT OF FELINE CALICIVIRUS

by

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DECLARATION

All of the work described in this thesis was carried out by the author apart from the derivation of FCV-K virus, described in Chapter 1, and the results in Table 6.3 in Chapter 6 which were obtained from Prof. O. Jarrett.

SUMMARY

A temperature sensitive (ts) mutant of feline calicivirus (FCV) was isolated from a vaccine strain of FCV. Virus-infected feline embryo cells were treated with the mutagen 5-fluorouracil (5-FU). The resulting virus was cloned and 192 clones were tested for growth at 31°C (permissive temperature) and 37°C (non-permissive temperature). Twelve clones which appeared to grow at 31°C but not at 37°C were selected and one clone was isolated, the growth of which was 760-fold lower at 37°C than at 31°C. This virus was designated FCV-ts.

The characteristics of growth of the mutant were examined. The growth of the virus at 31°C was prolonged compared to wild type FCV at 37°C with an eclipse period of 10 hours and maximum yield of virus was at 20 hours, compared to 2 hours and 3 hours respectively for wild type FCV.

The nature of the ts lesion was investigated. The virus particles were not inactivated faster at 37°C than at 31°C over an incubation period of 6 hours. Virus adsorption to cells during 2 hours was as efficient at 37°C as at 31°C. Temperature sensitivity was found, however, when the temperature of incubation was shifted from 31°C to 37°C after only 2 hours of incubation following adsorption. These results suggested that the virus capsid protein might not be thermolabile but that an early event in viral growth was affected.

RNA synthesis in FCV-ts infected cells was examined in the presence of actinomycin-D to inhibit cellular RNA production. A 4-fold decrease in RNA synthesis was found 24 hours after infection when the temperature was shifted from 31°C to 37°C for 2 hours. This result suggested that the ts lesion affected RNA synthesis or survival and might be in a viral RNA polymerase activity.

The usefulness of FCV-ts as a vaccine in cats was investigated. Four kittens were given 1 x 10^6 plaque forming units

intranasally and examined at intervals for the presence of FCV in the oropharynx. Virus was isolated over a period of 26 days which compared to 40 days for the original FCV vaccine virus found in an earlier experiment. The reason for the prolonged growth might be that a revertant virus had arisen which grew well at 37°C. A clone of a revertant was subsequently isolated from one of the kittens which grew as well at 37°C as at 31°C. Similar viruses were isolated when FCV-ts was passaged 3 times in cell culture. These results indicated that this particular ts mutant would offer no substantial advantage over the parental virus as a vaccine.

ABBREVIATIONS

CPE	Cytopathic effect
cpm	Counts per minute
EDTA	Ethylenediaminotetracetate
DFC-1	Dulbecco's modification of Eagle's medium containing 1% FBS.
DFC-10	Dulbecco's modification of Eagle's medium containing 10% FBS.
FBS	Foetal bovine serum
FCV	Feline calicivirus
FEA	Feline embryo cells of the FEA strain
FHV	Feline herpesvirus
FITC	Fluorescein isothiocyanate
5-FU	5-fluorouracil
PBS	Dulbecco's phosphate buffered saline
pfu	Plaque-forming unit
TCA	Trichloroacetic acid

GENERAL INTRODUCTION

1. Feline respiratory disease

Feline respiratory disease is a clinical syndrome that may be caused by one or several infectious agents so that it is similar in this respect to the common cold of humans or kennel cough of dogs (Arnett and Green, 1984). The infectious agents involved in feline respiratory disease include feline herpesvirus (FHV) and feline calicivirus (FCV) which have been reported to be responsible for up to 90% of infectious respiratory disease in cats (Ott, 1975). Infection with these viruses is discussed below in detail.

The remaining cases are caused by a variety of other agents. Feline reovirus type 3 has been isolated in the USA but the prevalence of the infection is unknown although it has been suggested that the virus is widespread in that country (Gillespie and Scott, 1973). This virus has been shown to cause a conjunctivitis and photophobia in experimentally infected cats.

Feline pneumonitis caused by Chlamydia psittaci was described in the USA by Baker (1944) but has not been shown to be of importance in the UK as a cause of respiratory disease (Povey and Johnson, 1971). However, the strain found in cats can cause conjunctivitis (Hoover et al., 1978).

Bacteria have been isolated from cases of respiratory disease in cats. For example, mycoplasma have been claimed to be involved particularly in conjunctivitis (Campbell et al., 1973; Tan and Miles, 1974). However, members of the mycoplasma group, including M.felis, M.gateae, M.feliminutum, M.arginini, M.acholeplasma and M.laidlawii have been isolated from the saliva of clinically healthy cats (Wilkinson, 1980). M.felis has also been isolated from the saliva of cats showing signs of conjunctivitis, which suggests a possible pathogenic role for the organism (Cole et al., 1967).

Other bacteria acting alone or in various combinations may be secondary pathogens such as Streptococcus pyogenes, Staphylococcus

aureus, Pasteurella multocida, Bordetella bronchiseptica and Escherichia coli (Povey and Wilson, 1971; Powey, 1976).

2. Feline viral rhinotracheitis

Feline viral rhinotracheitis (FVR) was first described by Crandell and Maurer (1958). Subsequent experimental studies were carried out by Johnson and Thomas (1966) and Povey and Johnson (1967) which characterised feline herpesvirus type 1 as a species-specific member of the herpesviruses group sharing many biological and physicochemical properties with other members of that family (Crandell, 1973; Mekercher, 1973) Thus, there is only one serotype of the virus. Also FHV was found to be of reasonably uniform pathogenicity in susceptible cats (Crandell et al., 1961, 1973; Gaskell and Wardley, 1978). The virus is stable at pH between 6 and 7, at which it remains infectious for 5 months. It is heat-labile and sensitive to both ether and chloroform and is readily destroyed by many commonly used commercial disinfectants (Povey and Johnson, 1970; Scott, 1980). The virus grows readily in feline cell cultures in which it produces a characteristic slow, local cytopathic effect with intranuclear inclusion bodies in infected cells (Paul and Pratt, 1983). The virus forms plaques in cell culture. FHV is labile, surviving for only a few hours outside the cat (Povey and Johnson, 1970) and depends on direct or close contact of animals for transmission (Gaskell and Povey, 1982).

Clinically, FVR can present a varied picture depending on levels of immunity, age and general health of the cat. Young kittens are most severely affected. There is often a febrile response and ocular involvement with conjunctivitis. In the second stage of the disease ocular and nasal discharges are copious and often become purulent signifying increased bacterial activity and an inflammatory response. Coughing may be a dramatic feature. Also FHV has been associated with skin ulcers although it was not determined whether the virus acted as the primary pathogen or as a secondary invader (Flecknell et al., 1979; Bistner et al., 1971).

The incubation period may be as short as 2 days or as long as 10 days (Crandell, 1973), the difference being related mainly to infecting dose of virus. The acute stage of the disease lasts for 2 to 3 weeks when recovery occurs.

3. Feline calicivirus disease

Feline calicivirus disease (FCD) is of even more frequent occurrence although generally less severe than FVR. FCD is common in all cat populations and the severity of the condition varies with pathogenicity of the infecting strain of virus. In kittens between 3 and 12 weeks of age the disease is more severe and frequently fatal due to extensive pneumonia (Love and Baker, 1972). This is thought to correlate with declining levels of maternal immunity (Kahn and Gillespie, 1971). In contrast, in mature cats FCV infection may be subclinical or seen as a mild disease of the upper respiratory tract and oral cavity (Povey, 1976).

Pathology and clinical signs

Clinical signs may be quite variable depending upon the strain of calicivirus involved, route of exposure, severity of the exposure and resistance of the exposed cat. FCV isolates generally can be grouped into 3 types, based on their virulence although it is often difficult to draw clear lines of distinction. An avirulent strain was found to produce no significant changes (Ott, 1964). Calicivirus of moderate virulence produce ulcers of the tongue, hard palate and nostrils in exposed cats. FCV of high virulence produce pneumonic signs, with or without oral and nasal ulcers (Ott, 1983). Ulcers of the tongue, hard palate, and nose are more commonly seen with FCV, although they may also be caused by FHV as well (Arnett and Green, 1984). Some strains of FCV can cause gingivitis without other signs of respiratory disease (Gruffydd-Jones, 1985).

Different plaque types of FCV were shown to vary in their virulence and hence, the disease conditions they cause. Ormerod

(1979) found that an extra-large plaque FCV strain was more virulent than a minute plaque strain and that the lesions it caused were more extensive. All of these observations suggested that FCV isolates of varying degrees of virulence cause a range of disease states in cats ranging from mild lesions of the oral mucosa and upper respiratory passages to quite marked cell destruction in the epithelial of the tongue and buccal cavity and in the interstitial tissue of the lungs (Hoover and Kahn, 1975; Povey, 1976; Holzing and Kahn, 1970; Povey and Hale, 1974).

The major clinical features of the disease caused by highly virulent strains of FCV are fever, anorexia, depression, dyspnoea and death (Kahn and Gillespie, 1971). The disease is first manifested as fever (104-105°F) and anorexia. The fever may fluctuate after the initial elevation but the temperature tends to remain elevated through the course of the disease. Depression is often marked. Death may occur within 5 days of the onset of signs. Recently Pedersen et al. (1983) described a transient syndrome of fever, generalised stiffness and hyperaesthesia and pain on manipulation of joints caused by certain strains of FCV.

FCV has a greater predisposition for the lower respiratory tract and lung than other agents associated with feline respiratory disease, and oculonasal discharge in less frequent with FCV than with FHV infections (Povey and Hale, 1974) although in some cases sneezing and nasal ocular discharges may make it impossible to differentiate calicivirus infection clinically from FVR. (Cats with respiratory signs may be concurrently infected with FHV and FCV).

The diagnosis of FCV infection is based on the typical clinical signs of oral and occasionally nasal ulceration, with or without pneumonic signs. Virus isolation and identification of a calicivirus from lung tissue of a kitten that has died of a severe pneumonic disease is important in establishing the diagnosis of calicivirus infection in a cattery. However, isolation of calicivirus from the mouth of a cat without clinical signs of

infection is of less diagnostic significance, since it is impossible to be sure that the virus isolated is pathogenic.

Carrier states

Animals surviving the initial FCV or FHV attack usually turn into carriers. Such cats are known to play an important epidemiological role in outbreaks of severe FCV disease in kittens and breeding catteries (Love and Baker, 1972). Surveys have shown as high as 40% of animals to be viral carriers in colonies where FCV is endemic (Wardley et al., 1974) and even more (up to 80%) of cats which recover from FHV infection remain as viral carriers (Gaskell and Povey, 1973, 1977).

The carrier state of FCV is characterised by more or less continuous shedding of virus from the oropharynx for a period of weeks, months or even years (Povey et al., 1973; Wardley, 1977; Wardley and Povey, 1977). Viral shedding has been reported for long periods from the tonsil (Almeida, 1968), faeces and urine (Rich and Fabricant, 1969; Povey and Hale, 1974; Stein, 1980), as well as nasal or conjunctival secretions (Povey, 1976).

Although FCV shedding from high level excretor carriers was shown by many workers to be continuous, it is episodic with FHV, usually following natural stress due to re-housing, pregnancy, lactation and cat shows or artificially after corticosteroid treatment (Gaskell and Povey, 1973; 1977; Ellis, 1981). The site of FHV latency in carrier animals is not known, though there is some evidence that nervous tissue may be involved (Gaskell and Povey, 1979).

Transmission

Carrier cats are considered to be a major source for transmitting the infection. Natural transmission by these cats takes place through licking.

Wardley and Povey (1977) suggested many factors which affect the transmission of FCV similar to those shown by Gaskell and Povey (1979; 1982) concerning FHV. These authors found a relationship between the level of infecting dose and the length of the incubation period as well as the severity of the resulting syndrome. They also showed that the duration of contact plays a role in transmitting the disease. Povey and Ingersoll (1975) showed that FCV was readily transmitted to cats in close contact as well as indirectly on the hands of attendants. The distance between infected and susceptible cats was found to be of critical importance in transmitting the disease. As it is not likely to have virus-containing microdroplets sneezed by infected cats spreading beyond 4 feet, it was necessary for a successful airborne viral transmission to have the infected and susceptible cats within this range (Wardley and Povey, 1977).

The age of disease establishment in newborn kittens was shown to vary depending on the level of maternal immunity and to the amount of virus to which these kittens are exposed. Kittens exposed to high level excretors would pick up infection and succumb earlier (Johnson and Povey, 1984).

Little is known of the interaction between FCV and maternally immune kittens during the period of declining passive immunity (Johnson and Povey, 1984). Maternal antibody which persists for approximately 13 weeks (Povey, 1970; Johnson and Povey, 1983) appears to provide incomplete protection against FCV infection (Gillespie and Scott, 1973). Kahn and Gillespie (1971) observed that weanling kittens with low titres of virus neutralising antibody to FCV were susceptible to experimental aerosol challenge with a virulent strain but the mortality rate was lower than in seronegative kittens. However, signs of FCD have been shown in 6-day old kittens born to a seropositive, apparently uninfected dam (Kahn and Walton, 1971). Gaskell and Wardley (1978) have suggested that many kittens under conditions of natural exposure experience subclinical and immunising infections. Johnson and Povey (1984) studied the clinical, virological and immunological events

occurring in kittens born to FCV carrier queens and found that all the kittens became infected between 3 and 4 weeks after birth and shed FCV consistently for periods of 3 to 11 weeks.

The persistence of maternally derived antibody is dependent upon the amount of antibody acquired from the mother and the rate of catabolism of immunoglobulins in offspring (Brambell, 1970). The estimated half-life of 15 days for maternal FCV antibody agrees with reports on maternal antibody to feline leukaemia virus (Jarrett et al., 1977) and FHV (Gaskell, 1975) but is greater than the 9.5 day half life determined for maternal antibody to feline panleukopenia virus (Scott et al., 1970). It appears, however, that this balanced host-virus interaction during the transition from passive to active immunity is sensitive to factors such as virus dose and the limited nature of protection afforded by maternal immunity. Ormerod et al. (1979) suggested that the extent of experimental infection not only depends on virus dose but also on the route of administration. For example aerosol dosage favoured deposition of virus in lungs, an important event in the development of pneumonic FCV infection, while nasal installation tended to produce oral and nasal lesions only.

4. Isolation of FCV

Fastier (1957) in New Zealand was the first to isolate FCV. In the same year Bolin (1957) also isolated the virus in the USA. Other workers have recovered the virus from cats in different parts of the world: USA (Sinha, 1957; Bittle et al., 1960; Crandell and Madin, 1960; Kahn and Gillespie, 1970), Switzerland (Bürki, 1963), Italy (Torlone, 1960), England (Prydie, 1966; Povey, 1969), Australia (Studdert et al., 1970) and Scotland (Ormerod and Jarrett, 1978). The virus has been isolated from cases of disease, convalescent carriers (Gillespie and Scott, 1973) or from the oropharynx of clinically healthy cats (Piercy and Prydie, 1963; Bürki, 1971; Walton and Gillespie, 1970; Povey and Johnson, 1971; Wardley et al., 1974; Ellis, 1981). Wardley et al. (1974) found the prevalence of FCV to be 8% of normal household pet cats, 24% of

cats at shows and 41.5% of cats in multicat households.

The virus has been isolated in monolayer cultures of feline kidney cells (Crandell, 1960; Fastier, 1957), diploid feline tongue and feline thymus cell lines (Lee, et al., 1969) cell lines from embryonic feline lung (Crandell, et al., 1960). FCV has a rapid cytopathic effect that tends to distinguish it from FHV which induces a slower focal cytopathic effect and also produces inclusion bodies in cell culture. Cell cultures of heterologous species usually do not support replication of FCV and the virus does not propagate in embryonated hens' eggs or in common laboratory animals.

5. Characteristics of calicivirus

FCV belongs to the family Caliciviridae (Studdert, 1978; Schaffer, 1979; Schaffer et al., 1980). Caliciviruses were first recognised in 1932 when they were shown to cause vesicular exanthema of swine in Southern California (Traum, 1936). Other caliciviruses, of which the prototype is San Miguel sealion virus, were isolated from pinnipeds in California with ulcerative lesions of the mouth (Smith et al., 1973). More recently caliciviruses have been isolated from fish in the Pacific Ocean (Smith et al., 1981). It has been suggested that both vesicular exanthema and San Miguel sealion virus are caliciviruses of fish which have spread through the food chain to sealions, seals and pigs.

Other caliciviruses have been isolated from calves, a rock rattlesnake, walrus and dolphins, (Smith et al., 1983b) and from pygmy chimpanzee (Pan paniscus) (Smith et al., 1983a). There are probably at least three human serotypes which will not grow in foetal kitten cells and recently a human candidate calicivirus was described which replicates in human embryo kidney cells (Cubitt and Barrett, 1984). Particles resembling calicivirus have also been detected in faeces of children showing no clinical symptoms (Madley and Cosgrove, 1976; Flewett and Davis, 1976). Similar viruses were implicated in outbreaks of vomiting and diarrhoea in England and Japan in which many school children as well as adults

were involved in small epidemic outbreaks (Sellers, 1984).

FCV was originally assigned to the family picornaviridae like all the other members of the genus because of their resemblance to picornaviruses: for example, small size, ether and chloroform resistance, virions composed of a single-stranded, non-segmented RNA and replication in the cellular cytoplasm. However, there are some clear differences. Thus, their virion is larger with a diameter of 35 to 40 nm, and their capsid is composed of a single major polypeptide (Bachrach and Hess, 1973) with 32 cup-shaped depressions arranged in icosahedral symmetry (Studdert, 1978; Schaffer, 1979; Hagan and Bruners, 1981).

6. Structure and properties of FCV

The virion of FCV is non-enveloped (Fastier, 1957; Bartholemew, 1968), icosahedral in shape, contains linear (non-segmented) single-stranded RNA (Adldinger et al., 1969; Khan, 1969) and protein (Wawrzkiewicz et al., 1968; Newman et al., 1973; Bachrach and Hess, 1973). The RNA forms 18% of the virion mass with a molecular weight of 2.8×10^6 and the protein comprises 80% with a molecular weight of 12.4×10^6 so that the total virion mass is 15.2×10^6 daltons (Burroughs et al., 1978). The virus particle size has been determined by filtration (Bürki, 1965; Crandell, 1967) and electron-microscopic examination (Zwillenberg and Bürki, 1966; Almeida, 1968; Strandberg, 1968). The virus has a diameter of 35-40 nm (Zwillenberg and Bürki, 1966; Almeida et al., 1968) and the surface resembles a Star of David made from two interlocking equilateral triangles with a central hole (Madley, 1979). The pattern of the holes or depressions suggests a 32 subunit structure. In negatively stained preparations, the capsid shows a number of large, dark stain penetrated cup-shaped structures (Zwillenberg and Bürki, 1966; Almeida et al, 1968). Strandberg (1968) described the capsomers as thin and rod-like 5 nm long and 2-5 nm wide.

Physiochemical properties

FCV has a buoyant density of 1.36 - 1.39 g/cm^2 in caesium chloride (Studdert et al., 1970; Newman et al., 1973) and the sedimentation coefficient is 160-170S. The virus is resistant to lipid solvents such as ether or chloroform (Bürki, 1966; Bartholemew and Gillespie, 1968) and also to hydroxybenzylbenzimidazole, guanidine hydrochloride and 0.2 per cent sodium deoxycholate (Bürki, 1965). FCV is labile at pH 3 but not at pH 7 (Oglesby et al., 1971) and is readily inactivated at 50°C in the presence of Ca^{++} or Mg^{++} (Khan and Gillespie, 1970). The virus is known to retain its infectivity at low temperature (-65°C) for at least 4 years (Gillespie and Scott, 1973; Holmes and Gillespie, 1973). Among different temperatures for storing purified FCV Komolafe (1979) found that 4°C was better than -70°C for retention of infectivity.

No haemagglutinin has been associated with FCV (Crandell et al., 1960; Gillespie et al., 1971).

FCV produces plaques in cell cultures overlayed with agar, agarose or methyl cellulose (Bürki, 1965; Crandell, 1967; Bartholemew, 1968; Ormerod and Jarrett, 1978). There is a correlation between plaque size and virulence, large plaque strains being more pathogenic than minute plaque strains. (Ormerod and Jarrett, 1978).

The virus replicates in the cytoplasm of infected cells, and is observed as single particles, extensive single particles, non-regular accumulations as paracrystaline arrays or a single or multiple linear arrays associated with microfibrils (Strandberg, 1968; Studdert and Oshea, 1975; Ormerod and Jarrett, 1978).

7. Proteins of FCV

Black and Brown (1977) identified two polypeptides in infected cells with molecular weights of 100 x 10^3 (p100) and 80 x 10^3 (p80)

which had no relationship to the major capsid protein of molecular weight 68,000 (p68) isolated from the virus. Komolafe (1980) confirmed the presence of p80 and p68 but not p100 in FCV infected cells, and reported the presence of two other apparently virus-specific polypeptides of molecular weights 80,000 and 40,000.

In an infected cell the virus-specific proteins and nucleic acid are synthesized separately and consequently must be brought together and assembled into mature viral particles. The mechanism of assembly of these particles from progeny RNA and protein is not known with any certainty and little work has yet been done on FCV in this direction. Komolafe et al. (1978) described a protein subunit of the virus with a sedimentation coefficient of 15S which was generated when purified virus was disrupted at low pH. A component with identical physical characteristics to this subunit was produced in cells infected with FCV and was apparently a precursor in the assembly of the viral capsid.

8. Antigenic properties of feline caliciviruses

Investigation of the antigenicity of FCV has been carried out by many workers (Bittle et al., 1960; Bürki, 1965; Crandell, 1967; Prydie, 1966; Povey, 1971; Parker, 1972; Studdert et al., 1970; Povey, 1974 and Povey and Ingersoll, 1975). Results based on the use of the neutralisation test which is most widely used for serotype identification, showed considerable serological differences between isolates but with much cross- reaction. These results were further confirmed by the use of complement fixation tests (Tan, 1970; Gillepsie and Scott, 1973) and immunodiffusion (Tan and Miles, 1971; Gillespie and Scott, 1973). Gillespie et al. (1971) in a study on FCV-infected feline cells in culture by immunofluorescence showed that there was cross-reactivity between strains. Finally, Povey (1974) studied the serological relationships among a total of 46 strains of FCV isolates by serum neutralisation and found that all isolates tested could be regarded as serological variants of a single serotype. Using an arbitrary 20 antibody-units concept as is used in human rhinovirus serology

(Kapikian et al., 1967), he concluded that no isolate could be distinguished as a separate serotype.

Komolafe (1978) described 2 surface antigenic determinants of FCV. These were suggested to be group-specific and strain-specific determinants, one of which was responsible for the production of neutralising antibodies found in their homologous immune sera.

9. Vaccination against feline viral respiratory disease

As described above, FHV and FCV constitute the major cases of respiratory disease in cats. Several vaccines are available for protection against both of these diseases which contain either individual viruses or are a combination of both viruses, sometimes with feline panleukopenia virus. These forms include inactivated virus which is injectable and modified live virus products which are available for injection or for intransal instillation.

In the UK the first of several vaccines against FVR and FCD became commercially available in 1976. Table 1.1 is a summary of the vaccines currently available in the UK.

Isolation of the FVR virus and cross-immunizing with FCV strains (Kalunda, 1975; Povey, 1979) eventually led to the development of vaccines for prevention of both diseases. The original vaccines contained modified live viruses and were given intramuscularly or subcutaneously. Later studies indicated, however, that intranasal administration of FHV and FCV antigens might be more effective. Kahn et al. (1975) obtained distinctly better protection using a single intranasal dose of the F-9 strain of FCV which was already known to be broadly antigenic and of low virulence (Kalunda et al., 1975). This virus was also used in several other vaccine preparations either singly (Bittle and Rubic, 1976) or in combination with attentuated FHV and attenuated or inactivated panleukopaenia virus vaccine (Bittle and Rubic, 1975; Davis and Beckenhauer, 1976).

TABLE 1 Vaccines currently available in the UK for the protection of cats against viral respiratory disease

Trade name	Manufacturer	Disease covered	Vaccine type	Vaccine route	Dosage regime	Booster vaccination
Feliflu FVR-C	C-Vet	FVR FCD	modified live	sc or im	2 doses 3-4 weeks apart	1 dose annually
Felocell CVR	Smith Kline	FVR FCD	modified live	sc or im	2 doses 3-4 weeks apart	1 dose annually
Katavac CH	Duphar Veterinary	FVR FCD	modified live	in	1 dose	1 dose annually
Nobi-vac Tricat	Intervet	FVR FCD FP	modified live	sc or im	2 doses 3-4 weeks apart	1 dose annually
Purtect RC	Glaxovet	FVR FCD	modified live	sc	2 doses not less than 3 weeks apart	1 dose annually
Purtect RCkP	Glaxovet	FVR FCD FP	modified live	sc	2 doses not less than 3 weeks apart	1 dose annually
Vaxicat Plus	Coopers Animal Health	FCD FP	inactivated oil adjuvanted	sc	2 doses 3-4 weeks apart	1 dos annually

FVR = Feline viral rhinotracheitis
FCD = Feline calicivirus disease
FP = Feline panleucopenia
sc = subcutaneously
im = intramuscularly
in = intranasal

In North America, Povey and Wilson (1978) developed a combined FHV and FCV inactivated vaccine and concluded that protection afforded against challenge was comparable to that achieved by modified live virus vaccines. In the UK there is one killed feline respiratory virus vaccine available.

Some evidence was found in an earlier study that previously unexposed cats vaccinated intramuscularly with a combined live attenuated vaccine against FCV infection and FHV could subsequently become carriers of FHV following challenge and subclinical infection with a field isolate of the virus (Orr et al., 1978). Virus isolated from one such carrier animal was found to be virulent and such animals would clearly be of epidemiological importance. These findings contrasted with the intranasally administered combined live modified vaccine with which there appeared to be evidence for the development of an FHV carrier state following challenge.

Gaskell et al. (1982) in a study on cats vaccinated intramuscularly with a combined inactivated FCV, FHV and feline panleucopenia vaccine (Vaxicat Plus; Tasman Ltd) found that no clinical signs appeared following vaccination and there was no evidence of local reaction or of viral shedding following vaccination compared with that shown for other FCV vaccines.

The intramuscular attentuated virus vaccines have the advantage of not producing clinical signs or viral shedding at the time of vaccination (Bittle and Rubic, 1974; 1975) compared with intranasal attenuated virus vaccines which not uncommonly produce mild clinical signs in the first week following vaccination. Thus, mild sneezing may be observed 5-9 days later in up to 60 per cent of cats and some ocular and nasal discharge, and oral and na al ulceration have been reported (Davis and Beckenhauer, 1977; Pov y, 1977; Povey and Wilson, 1978; Wilson, 1978; Orr et al., 1980). Although intranasal vaccine virus replicates locally and therefore may spread to in-contact animals, no evidence of disease in such animals has been reported in the UK (Gaskell, 1981). In North

America mild signs have been reported with in-contact cats following the use of intranasal vaccine (Kahn, 1977).

Bittle and Rubic (1975; 1976), Kahn and Hoover (1976) and Orr et al. (1978) found that no shedding of virus or spread to in-contact cats occurred in intramuscularly vaccinated cats and therefore vaccination by this route usually appears to be free from any adverse effects. However, Cox (1978), North (1978) and Robinson (1978) suggested that the disease may appear in endemic colonies following vaccination.

The modified FHV in the intranasal vaccine may be shed by the vaccinated cat for up to 16 days after vaccination and may spread to susceptible cats closely associated with the vaccinated cats (Orr et al., 1980). However, calicivirus in a combined intranasal vaccine may be shed for 3-83 days post vaccination (Orr et al., 1980). Povey and Wilson (1978) reported in their study that cats vaccinated with inactivated vaccine and challenged appeared to become FCV carriers and then infect susceptible in-contact animals. The reason why vaccination by the intramuscularly route appears to protect against infection is not clear, though it may be related to differences in local immunity and the ability of the challenge virus to survive and replicate (Gaskell, 1981).

Following intranasal vaccination both viruses replicate locally and are shed in the oropharynx (Orr et al., 1980). Locally replicating virus probably stimulates a better immunity at the actual site of infection than does systemically administered virus. Intranasal vaccines stimulate local resistance to both viruses within 24 hours (York and Slater, 1976) or 48 hours after a single vaccination (Davis and Beckenhauer, 1977; Folkers and Hoogenboon, 1978) compared with intramuscular vaccines which need a longer time to establish protective immunity. These vaccines produce a humoral antibody response within 14 days compared with 7 days for the intranasal vaccine. Scott (1977) suggested that intranasal vaccine stimulates a rapid localized as well as a systemic humoral immune response. These suggestions have led to

the recommendation of the intranasal vaccination to stimulate rapid immunity in outbreaks of disease in catteries and in chronically or recurrently diseased cats. McDermott and Bienenstock (1979) and Povey (1977) concluded that the intranasal route gives more complete protection but at the expense of post-vaccination complications which can be severe.

It is probably true that intranasal vaccination gives better protection than intramuscular vaccination, though few challenge studies with the modified live intranasal vaccine (Katavac CH, Duphar Veterinary Ltd) currently available in the UK have been published. Orr et al. (1980) suggested that vaccination via the intranasal route, however, may prevent the subsequent development of an FHV carrier state following challenge with virulent field virus, at least in the short term. FHV was recovered for up to only 13 days following vaccination and attempts to stimulate shedding of any latent virus with a corticosteroid course 58 days after vaccination were unsuccessful. The FHV vaccine strain used in this work was a temperature sensitive mutant (Slater and York, 1976) and it may be that by confining replication of the virus to the lower temperature of the nasal chamber, no carrier state can develop. The site of FHV latency in carrier cats is not known, though there is some evidence that the nervous tissue may be involved (Gaskell and Povey, 1979). Levels of serum neutralising antibody are currently used for determining efficacy though the level of protection is almost certainly related to other components of the immune response as well.

Immunity to FHV and FCV following vaccination may last up to a year (Bittle and Rubic, 1974). Orr et al. (1980) found that 11 weeks after vaccination, protection was complete in all 8 vaccinated and challenged cats, whereas all 4 unvaccinated and challenged controls showd severe signs characteristic of FVR.

Vaccine efficacy and recommendation for the age of initial vaccination in young kittens depends on the rate of maternal antibody decay. However, vaccination programmes for the

preventation of FVR of cats usually commence at 9 to 12 weeks of age since kittens that have obtained colostrum from immune mothers can have FHV antibody for up to 10 weeks although only approximately 50% of them have minimal or non-detectable amounts of serum neutralising antibody by 6 weeks of age (Povey and Johnson, 1970; Edwards et al., 1977).

Recent studies have successfully introduced an intramuscularly, killed virus adjuvanted vaccine against FHV which operates in the presence of maternal antibody. Johnson and Povey (1985) indicated that early vaccination is potentially useful in the control of FVR in kittens in catteries in which respiratory tract disease develop before the recommended vaccination age of 9-12 weeks. With the live intranasal vaccine results were consistent with earlier studies (Slater and York 1976; Ott, 1978; Pickering, 1981) which provided evidence that protection could be achieved in kittens even during the presence of maternal antibody. However, in the recent study, multiple vaccine doses were found unnecessary. Thus it may be particularly useful for the protection of young kittens in catteries where the disease is endemic.

All these factors make it important that a combination of management and vaccination is employed in the control of FCV and FHV infections (Gaskell and Wardley, 1978). Cats should be vaccinated annually and should be separated as far as possible from contact with the virus (Gaskell and Wardley, 1978). Animals at particular risk should be boosted 6-monthly. In breeding colonies where the disease is endemic breeding queens should be regularly vaccinated and their immunity boosted before mating with an inactivated vaccine. Kittens should be vaccinated as soon as maternal antibodies are at a non-interfering level and certainly before exposure to adult stock (Gaskell, 1981).

10. Aims of the present study

The major aim of this study was to isolate a ts mutant of FCV which might be useful as a vaccine against FCV infection in cats.

The efficacy of a ts mutant of FHV has been impressive in controlling FVR. This virus grows for only a short period in the upper respiratory tract and so the possibility of spread of vaccine virus to other cats is limited. However current attenuated FCV vaccine virus given intranasally grows for periods of up to 40 days and can be transmitted to in-contact cats (O. Jarrett, personal communication). A ts mutant of FCV might solve this problem.

Another reason for wishing to isolate ts mutants was to examine the genetic composition of FCV. Only one gene product, the capsid protein, has been unequivocally identified. Mutants might help to identify other gene products such as an RNA polymerase and open the way for a genetic analysis of caliciviruses.

In this thesis Chapter 1 gives the general materials and methods which were used. Chapter 2 describes the isolation of a ts mutant of FCV. Chapter 3 contains details of the growth cycle of this mutant. In Chapter 4, experiments to characterise the mutant are described and in Chapter 5 evidence is presented that the ts lesion might be in the viral RNA polymerase. Chapter 6 is an account of the intranasal administration of the ts mutant to cats and the isolation from one of these animals, and from mutant virus grown in cell culture, of a revertant virus.

CHAPTER 1

GENERAL MATERIALS AND METHODS

Derivation of FCV-K

FCV-K was derived by O. Jarrett from a vial of Katavac-CH vaccine (Duphar Ltd.) which contained FCV and FHV components. The freeze-dried vaccine was reconstituted with sterile diluent and removed from the vial into a 5 ml glass bottle. In order to inactivate the herpesvirus a volume of 0.5 ml vaccine was mixed with an equal volume of chloroform and was shaken for 5 minutes. The mixture was poured into a glass conical centrifuge tube and spun at 2000 rpm for 10 minutes. The aqueous phase was removed, dispensed in 3 0.1 ml lots and frozen at -70°C. One aliquot was thawed and tenfold dilutions were made in medium. One ml of each dilution was inoculated into a confluent FEA monolayer in 5 cm plates. After 3 days there was extensive cytopathic effect (CPE) in all dilutions down to 10^{-3}. The medium from the plate inoculated with the 10^{-3} dilution was harvested, centrifuged at 2000 rpm for 10 minutes and the supernatant fluid was removed and stored in 0.5 ml lots at -70°C.

Cells

Feline embryo cells of the FEA strain (Jarrett et al., 1973) were used between passage numbers 10 and 80.

Media

Dulbecco's modification of Eagle's Minimal Essential Medium (Gibco, Ltd) was used to grow the FEA cells. Growth medium was enriched with 10% foetal calf serum (FCS) (Imperial Laboratories) (DFC-10). Maintenance medium contained 1% FCS (DFC-1).

Agarose overlay medium

A stock solution of 4% agarose was prepared by dissolving 20 gm of agarose powder (LMT Seaplaque, Miles Laboratories) in 500 ml of deionised water in an autoclave at 15 lbs/in^2 pressure for 15 minutes. The solution was dispensed in 25 ml volumes and

re-autoclaved at 15 lbs/in^2 pressure for 15 minutes. Agarose was added to the overlay medium to a final concentration of 1%. To prepare this, 25 ml of stock 4% agarose was melted in a microwave oven then cooled to 42°C or 37°C in a water bath. This solution was then mixed with 75 ml of Dulbecco's MEM in which the water content had been reduced by one-third.

Cell cultures

FEA cells were grown in 2.5 litre rotating bottles containing 100 ml of DFC-10 and were maintained at 37°C. Cells were subcultured 1:3 at weekly intervals. Monolayers were detached by removing the growth medium and rinsing the cells first with 20 ml of 0.02% EDTA in PBS followed by 25 ml 0.01% trypsin in 0.02% EDTA. The cells were resuspended in DFC-10, then transferred into fresh bottles which were flushed with 5% CO_2 in air and returned to 37°C.

Plaque assays

Plaque assays were carried out in either 5 cm polystyrene plates (Nunclon Delta) with 4 ml DFC-10 or in 12-well cluster plates (Costar) with 2 ml DFC-10 in each well. The 5 cm plates were seeded with 2 x 10^6 cells and 12-well plates with 1 x 10^6 cells in each well. After 24 hours' incubation at 37°C, these plates were infected with a volume of 1 ml virus per 5 cm plate or 0.5 ml per well of a 12-well plate. After an adsorption period of 1 hour, the inoculum were removed and the plates were washed twice with 2 ml DFC-1 medium. An agarose overlay was added to the plates which were then incubated for 2-3 days. The plates were fixed and stained by adding 2 ml (to 5 cm plates) or 0.5 ml (to cluster plates) of a solution of 0.5% crystal violet, 10% formaldehyde and 5% methanol. The plates were incubated overnight at room temperature. The overlay was then washed off, the plates were washed in water, dried, and the plaques were counted.

CHAPTER 2

ISOLATION OF A TEMPERATURE SENSITIVE MUTANT OF FCV

CONTENTS

Introduction

Mutation is defined as the discontinuous event which results in a change in information content of a genome. This could arise in two ways: a point mutation which includes the substitution of one base by another, and a frame-shift mutation which occurs by insertion or deletion of single base pairs. This latter event is more extensive because it alters the entire gene from the site of the change (Pringle, 1984). Mutation can happen spontaneously or artificially by using chemical or physical agents (mutagens). Spontaneous deletion mutation is common to all viral groups. These mutant viruses may interfere specifically in the process of replication of normal genomes and may play a natural role in stimulating the immune system (Pringle, 1984).

In viruses with RNA genomes high rates of spontaneous mutation have been measured, in the order of 10^{-3} to 10^{-4} per incorporated nucleotide. For example, cloned populations of the paramyxovirus, Newcastle disease virus, contained small plaque mutants at a frequency of 3 to 7% (Granoff, 1961) and in the rhabdovirus, vesicular stomatitis virus, frequencies of 1 to 5% have been reported for spontaneous mutation to the ts phenotype (Pringle, 1975). In contrast, the rates of spontaneous mutation are quite low in DNA genomes being as low as 10^{-8} to 10^{-11} per incorporated nucleotide. For example, less than 0.1% spontaneous ts mutants were reported for rabbitpox virus (Sambrook et al., 1966).

Artificial mutation can be induced by using mutagens. Mutagens are used to increase the frequency of mutants in the population that is subsequently screened by the use of appropriate selection pressures. A large number of different mutagens has been used in animal virus systems, but these fall into a small number of classes defined by the mechanism of mutagenesis (Drake, 1969, 1976; Freese, 1963). The base analogues 5-fluorouracil (5-FU) and 5-azacytidine for RNA viruses, and 5-bromodeoxyuridine for DNA viruses, are incorporated into the viral genome and mutations are produced by miscoding during replication (Pringle, 1984). The

major problem associated with the use of mutagens is selecting an appropriate dose so that the frequency of mutation with the desired phenotype is increased. Mutants are picked from the lowest mutagen dose giving as reasonable frequency of mutations with the desired phenotype (Ramig, 1985). Other mutagens induce mutation by direct chemical change. For example, heat inactivation mutation occurs when the mutants are unable to multiply in susceptible cells at high temperature but are unrestricted at low temperature.

The generation of mutants has been attempted by many workers for vaccine manufacture. Attempts have been made to develop mutants from feline respiratory viruses. An attentuated FHV has been reported by Bittle and Rubic (1975) and Slater and York (1976). Also Bearle (1971), Kim et al. (1971) and Brown et al. (1975) found that it is possible to attenuate a virus without altering its antigenicity by propagation at low temperatures such as 26°C or 32°C. Bittle and Rubic (1976) attentuated the F9 strain of FCV after passage in cell culture at low temperature (30 or 32°C) over 148 passages.

A number of workers have used 5-FU to increase mutation. Such mutants of wild type strains of vesicular stomatitis virus have been isolated following mutagenesis (Pringle, 1970; Pringle et al., 1971; Pringle and Wunner, 1973). Richmond (1975) and Richmond and Polatnick (1976) isolated a ts mutant of foot-and-mouth disease virus mutagenized with 5-FU which produced plaques at the permissive temperature of 33°C but not at 38.5°C. Chanock and Murphy (1980) used 5-FU to increase mutation rates and produced a series of ts mutants for experimental influenza and respiratory syncytial virus vaccines. These have not gained acceptance, mainly because they have shown a tendency to revert to virulence. Georgine et al. (1976). Gadkari and Pringle (1980) also isolated ts mutants of respiratory syncytial virus. ts mutants have also been isolated from Bunyumwera virus (Iroegbu and Pringle, 1981) and Chandipura virus, a human rhabdovirus (Gadkari and Pringle, 1980) by treatment with 5-FU. Eastman and Blair (1985) isolated 10 ts mutants of the togavirus, Japanese encephalitis virus, after

mutagenesis by growth of wild-type virus in the presence of 5-FU or 5-azacytidine. Mutants were selected which grew at least 100-fold better at 33°C than at 41°C. The 5-FU was found to be more effective at inducing ts mutation than was 5-azacytidine.

A property associated with most mutation is the ability to revert to the wild type. Reversion can be induced by mutagens and often knowledge of the mechanism of the mutagen inducing the forward mutation along with that of the mutagen inducing reversion can allow deduction of the base change involved in the original mutation (Ray and Fields, 1979).

The aim of the work described in this chapter was to isolate ts mutants from a vaccine strain of (FCV-K) by using a low temperature (31°C) as the permissive temperature. A chemical agent (5-FU) was used in an attempt to increase the mutation rate.

Materials and Methods

5-fluorouracil (5-FU)

5-FU (Sigma) was used as a mutagen. A stock solution was prepared by dissolving 2 mg 5-FU in 10 ml distilled water to make a concentration of 200 µg/ml which was stored at 4°C.

Toxicity of 5-FU

Confluent FEA cell monolayers in 5 cm plates seeded with 2 x 10^6 cells and incubated for 24 hours at 37°C were used to determine the toxicity of the 5-FU. Serial dilutions covering a range from 500 to 30 µg/ml of 5-FU were prepared in medium, then the inoculum was removed from the plates and a volume of 4 ml from each dilution was added. These plates were incubated at 37°C for 24 hours and examined for survival of the cells.

Growth of FCV-K in presence of 5-FU

Confluent monolayers in 5 cm plates were obtained by the incubation of 2 x 10^6 FEA cells in 4 ml of DFC-10 for 24 hours at 37°C. The incubation medium was removed and 1 ml of FCV-K stock (1.4 x 10^7 pfu/ml) was added. The infected cells were incubated at 37°C for 2 hours, then the inoculum was removed and the plates were washed twice with 2 ml of DFC-1. Serial dilutions covering a range from 500 to 30 µg/ml of 5-FU were prepared in medium and 4 ml from each dilution was added to a plate which was incubated at 31°C until a CPE was observed in virus-infected control plates not containing the mutagen. The culture fluids were then harvested and centrifuged at 2,000 rpm for 10 minutes to remove cellular debris. The supernatant fluid was collected and 1.0 ml volumes were transferred to small screw-capped vials which were stored at -70°C for further use.

Assay of virus treated with 5-FU

Confluent FEA cell monolayers in 12-well cluster plates were obtained by seeding with 1 x 10^6 cells and incubating for 24 hours at 37°C. The incubation medium was removed and ten-fold dilutions from each sample of 5-FU treated virus were added at 0.5 ml/well. The infected plates were incubated at 31°C for 2 hours to allow the virus to adsorb. The inocula were then removed and agarose overlay medium was added. Infected cultures were incubated for 3 days at 31°C. The monolayers were then stained and the plaques were counted.

Isolation of FCV ts mutant virus

Forty confluent FEA cell monolayers in 5 cm plates were made by incubation of 2 x 10^6 FEA cells per plate for 24 hours at 37°C. The medium was removed and a suitable dilution of FCV-K which had been grown in the presence of 200 µg/ml 5-FU, was made. One ml was inoculated into each plate and the infected plates were incubated at 31°C for 2 hours. The inoculum was removed and the plates were washed twice with medium. A volume of 4 ml agarose overlay medium was added and the infected cultures were incubated for 3 days at 31°C.

Plates containing only 4-10 plaques were selected and suitable plaques were marked on the underside of the plate. A total of 192 plaques were picked through the overlay using an automatic pipette (Gilson P200) set to take up 100 µl and each agarose plug was added to 100 µl DFC-10 medium in a 96-well flat-bottomed cell culture plate (NUNC). A volume of 50 µl was then removed into a duplicate well on another plate. A volume of 50 µl DFC-1 containing 5 x 10^4 FEA cells was added to each well. The plates were then incubated at 31°C or 37°C for 3 days. Clones demonstrating CPE at 31°C but not at 37°C were identified as potential ts mutants and were collected and stored at -70°C. These samples were recultured in 5 cm plates containing 2 x 10^6 FEA cells with 4 ml DFC-10 then after 3 days' incubation in 31°C the plates were harvested, transferred

to conical centrifuge tubes and spun at 2,000 rpm for 10 minutes. The supernatant fluids were transferred into screw-cap vials and stored at -70°C then tested for temperature sensitivity by plaque assay.

Plaque purification of mutant virus

Mutant virus clones were produced by plaque purification. FEA monolayers in 5 cm plates were incubated with terminal dilutions of virus. Adsorption for 2 hours was followed by removal of the inoculum and washing twice in DFC. An agarose overlay medium was applied. Plaques developed and could be visualised after 3 days' incubation at 31°C. Plates containing only 5-10 plaques were selected and suitable plaques were marked on the underside of the plate. The plaques were transferred by using an automatic pipette into 5 cm plates containing FEA monolayers with 4 ml DFC-10. After 3 days' incubation at 31°C a CPE developed. The medium was removed, centrifuged at 2,000 rpm for 10 minutes and the supernatnant fluid was filtered through a 0.22 μm Millipore filter to remove viral clumps. The suspension was then transferred into a screw-capped vial and stored at -70°C.

Dilutions were prepared from the stock of cloned virus suspension and cell monolayers were infected as above. A single plaque was picked to produce a second cloned virus suspension and by repeating the process three times the cloned virus preparation was derived. The virus was retested at 31°C and 37°C before and after virus stocks were made by plaque assay. This virus was designated FCV-ts.

Stock FCV-ts virus

Ten 9 cm plates each containing approximately 5×10^6 FEA cells were infected with 1 ml containing 3.8×10^6 pfu/ml of FCV-ts. Adsorption for 2 hours at 31°C was followed by adding 3 ml DFC-10 to each plate. These plates were incubated at 31°C for 3 days. The infected cell suspension was centrifuged at 2,000 rpm for

10 minutes to remove cell debris and the fluid was filtered through a 0.22 μm filter. The supernatant fluid was dispensed in 0.5 ml volumes and stored at -70°C.

Results

Toxicity of 5-FU

The cell monolayers which were incubated at 37°C for 24 hours with different concentrations of 5-FU showed complete cellular destruction with the higher concentration of 500 µg/ml 5-FU and less with 200 µg/ml. The cell monolayers grew normally with the other concentrations of 5-FU (Table 2.1).

Growth of FCV-K in presence of 5-FU

A CPE was observed in the plates containing different dilutions of 5-FU after 3 days' incubation at 31°C. The extent of the CPE was obviously higher with lower concentrations of 5-FU. This was confirmed when plaque assays were carried out on the harvested fluids as shown in Table 2.2.

Isolation of FCV-ts mutant virus

Twelve clones showed CPE at 31°C but none or apparently less at 37°C. These clones were selected and assayed at the two temperatures. Only one showed a difference in titre (3.0×10^6 pfu/ml at 31°C and 1.7×10^3 pfu/ml at 37°C) as shown in Table 2.3. The other eleven clones showed the same titre at 31°C or 37°C (approximately 1×10^6).

Plaque purification of mutant virus

The titre of the FCV-ts mutant stock prepared as described in the Materials and Methods was 3.8×10^6 at 31°C and 5.0×10^3 at 37°C after three rounds of plaque purification, as shown in Table 2.4.

TABLE 2.1

Toxicity of 5-FU for FEA cells

Concentration of 5-FU (μg/ml)	Cell lysis
500	Complete
250	+
200	+
125	±
100	-
62	-
50	-
30	-

+ = some cell lysis.

- = no cell lysis.

TABLE 2.2

Plaque assay of FCV-K treated with 5-FU

Concentration of 5-FU (μg/ml)	Virus titre (pfu/ml)
500	2.5×10^4
250	1.7×10^4
200	1.6×10^4
125	1.5×10^5
100	1.4×10^5
62	2.0×10^5
50	2.2×10^5
30	3.0×10^6

TABLE 2.3

Plaque assay of cloned FCV-ts

Virus dilution (log_{10})	31°C Plaques/Well	37°C Plaques/Well
- 2	Confluent	8, 9
- 3	Confluent	3, 4
- 4	Confluent	0, 0
- 5	14, 16	0, 0
- 6	8, 10	0, 0
- 7	6, 15	0, 0
Titre (pfu/ml)	3.0×10^6	1.7×10^3

TABLE 2.4

Plaque assay of plaque purified FCV-ts

Virus dilution (log_{10})	31°C plaques/plate	37°C Plaques/plate
- 3	Confluent	2, 3
- 4	Confluent	0, 0
- 5	19, 19	0, 0
- 6	0, 0	0, 0
- 7	0, 0	0, 0
Titre (pfu/ml)	3.8×10^6	5.0×10^3

Discussion

The mutagen 5-FU was titrated on FEA cells and the highest concentration which was found to be relatively non-toxic was 200 µg/ml. Higher concentrations showed toxic effects and cell lysis. This mutagen was also found to inhibit the growth of the virus, FCV-K, in FEA cell monolayers and the degree of inhibition was found to increase with increasing doses of mutagen.

Virus grown from cultures exposed to 200 µg/ml of 5-FU was cloned and 12 clones which appeared to grow at 31°C and not at 37°C were selected. These clones which were isolated from mutagenized stock were propagated in cells until screening for temperature sensitivity had been completed. Only one of these clones was found to be temperature sensitive when the viruses were titrated at each temperature. This might be attributed either to the existence of revertant viruses or to the quantitative differences in the viral doses which were plated in the original screening process. The difference in the titre at 31°C or 37°C was approximately 760-fold. Since only one ts mutant was isolated it is not known whether this was a pre-existing mutant in the FCV-K virus stock or whether the 5-FU increased the rate of generation of mutants.

The small number of stable ts mutants of FCV which kept their ts characteristic was also shown for other viruses. Eastman and Blair (1985) in a study of a togavirus, Japanese encephalitis virus, using 5-FU in concentrations between 100 and 200 µg/ml, managed to recover only 18% of the potentially temperature sensitive clones after purification. They attributed this small proportion to the high rate of reversion to wild type with this virus. However the mutants which were selected grew at least 100-fold better at 33°C than at 41°C.

The concentration of 5-FU has been suggested in some studies to be connected to the number of mutants, while in other studies it was found to be unrelated. Gadkari and Pringle (1980) in a study on Chandipura virus found no association between the concentration

of 5-FU and the number of ts mutants when using BHK-21 cells but on using BSC-1 cells there was a significant increase in the frequency of isolation of ts mutants with increasing concentration of 5-FU up to 200 µg/ml. In another study on vesicular stomatitis virus Pringle et al. (1971) found a comparable increase in mutant number with increase in 5-FU concentration.

The stock of FCV-ts which was made was used subsequently in the studies to characterise the mutant described in Chapters 3, 4 and 5 and in the experiment in which kittens were exposed to the virus which is described in Chapter 6.

CHAPTER 3

GROWTH CURVE OF THE FCV ts MUTANT

CONTENTS

Introduction

In order to carry out experiments to characterise the FCV *ts* mutant it was first necessary to establish the growth curve of the virus, as far as possible under one-step growth conditions. In this chapter a growth curve experiment is described in which feline embryo cells were infected with the virus at a multiplicity of infection of 1. The amounts of extracellular and cell-free virus were determined at intervals after infection by plaque assay and the proportion of infected cells was found by immunofluorescence.

Materials and Methods

FCV-ts growth curves

FEA cell monolayers in 12-well cluster plates were obtained by incubation of 1 x 10^6 FEA cells in each well for 24 hours at 37°C. The cells were infected with 1 x 10^6 pfu of FCV-ts in 0.5 ml. After an adsorption time of 60 minutes at 31°C the inocula were removed and the cultures were washed twice with DFC-1. Three ml of DFC-1 were added to each well before re-incubation at 31°C. Immediately, and at 2, 4, 6, 10, 12, 14, 18, 20, 22 and 24 hours afterwards the medium was removed from 2 wells, transferred to a small screw-cap vial and stored at -70°C. These samples represented the extracellular virus. Two ml of fresh DFC-1 was then added to each well and by scraping the monolayers off into medium a cell suspension was produced. The suspension was frozen and thawed in a dry ice/ethanol bath, then centrifuged for 10 minutes at 2,000 rpm. The supernatant fluid was stored at -70°C. These samples represented the cell-associated virus.

The extracellular and cell-associated virus were then titrated by plaque assay.

Immunofluorescence

An indirect immunofluorescence method was used at the same time as the growth curve experiment to determine the proportion of infected cells. Monolayers were produced on 8-well microscope slides (Flow Laboratories). Each 7 mm well was seeded with 2.5 x 10^4 FEA cells in 25 ml DFC-10 and virus suspension diluted to contain 2.5 x 10^4 pfu in 25 ml DFC-1 was added (i.e. a virus-cell ratio of 1). After 10 hours' incubation at 31°C, the slides were washed twice for 2 minutes with PBS and the cells were fixed in methanol for 3 minutes at room temperature and dried in air. A volume of 25 µl of anti-FCV cat serum diluted 1:10 in PBS was added to each well. This serum was from a cat which had been infected 14 days previously with the G-1 strain of FCV by Ormerod et al.

(1978). The slides were incubated at 37°C for 1 hour in a humidified box. The serum was then removed from each well and the slides were washed twice in PBS for 3 minutes, dipped in water and dried at 37°C.

A volume of 25 µl of FITC-conjugated anti-cat globulin (Miles Laboratories) diluted 1:30 in PBS was then added to each well. The slides were re-incubated at 37°C for 1 hour in a humidified box. The antibody was removed and the slides were washed twice in PBS and once in water and then dried. Each well was examined for the presence of immunofluorescence in a Leitz Orthoplan microscope using an ultraviolet light source. A 25X water immersion objective was used.

The proportion of cells containing FCV antigen was determined by counting at least 400 cells.

Results

Growth curve of FCV-ts on FEA cells

This experiment was designed to examine the rate of synthesis of the ts mutant virus, the efficiency of virus release from infected cells and the total virus yields.

The growth of FCV ts is shown in Tables 3.1 and 3.2 and in Fig. 3.1. The extracellular virus growth curve showed that the end of the eclipse phase occurred at around 10 hours after infection (Table 3.1). The extracellular virus titre increased rapidly between 8 and 12 hours but continued to rise up to 20 hours.

The cell-associated virus growth curve is shown in Table 3.2. The initial rise in the cell-associated virus occurred 8 hours after infection and continued until 24 hours after infection. At this time the CPE was extensive, involving almost all the cells.

Immunofluorescence

FEA cells were infected with FCV ts mutant and stained 10 hours after infection with anti-FCV cat serum followed by FITC-conjugated anti-cat globulin. Fluorescence was limited to cell cytoplasm and specific fluorescence was observed in approximately 37% of the cells as shown in Table 3.3.

TABLE 3.1

Growth curve of FCV ts: extracellular virus

Time in hours	Dilution of virus (log_{10})	Number of plaques/plate	Titre (pfu/ml)
0	-1	0, 0	10^1
2	-1	0, 0	10^1
4	-1	0, 0	10^1
6	-1	0, 0	10^1
8	-1	0, 0	10^1
10	-4	4, 6	1.0×10^4
12	-4	6, 11	1.6×10^5
14	-5	3, 4	7.0×10^5
18	-5	2, 7	9.0×10^5
20	-5	12, 13	9.0×10^6
22	-6	5, 2	7.0×10^6
24	-6	4, 3	7.0×10^6

TABLE 3.2

Growth curve of FCV ts: cell-associated virus

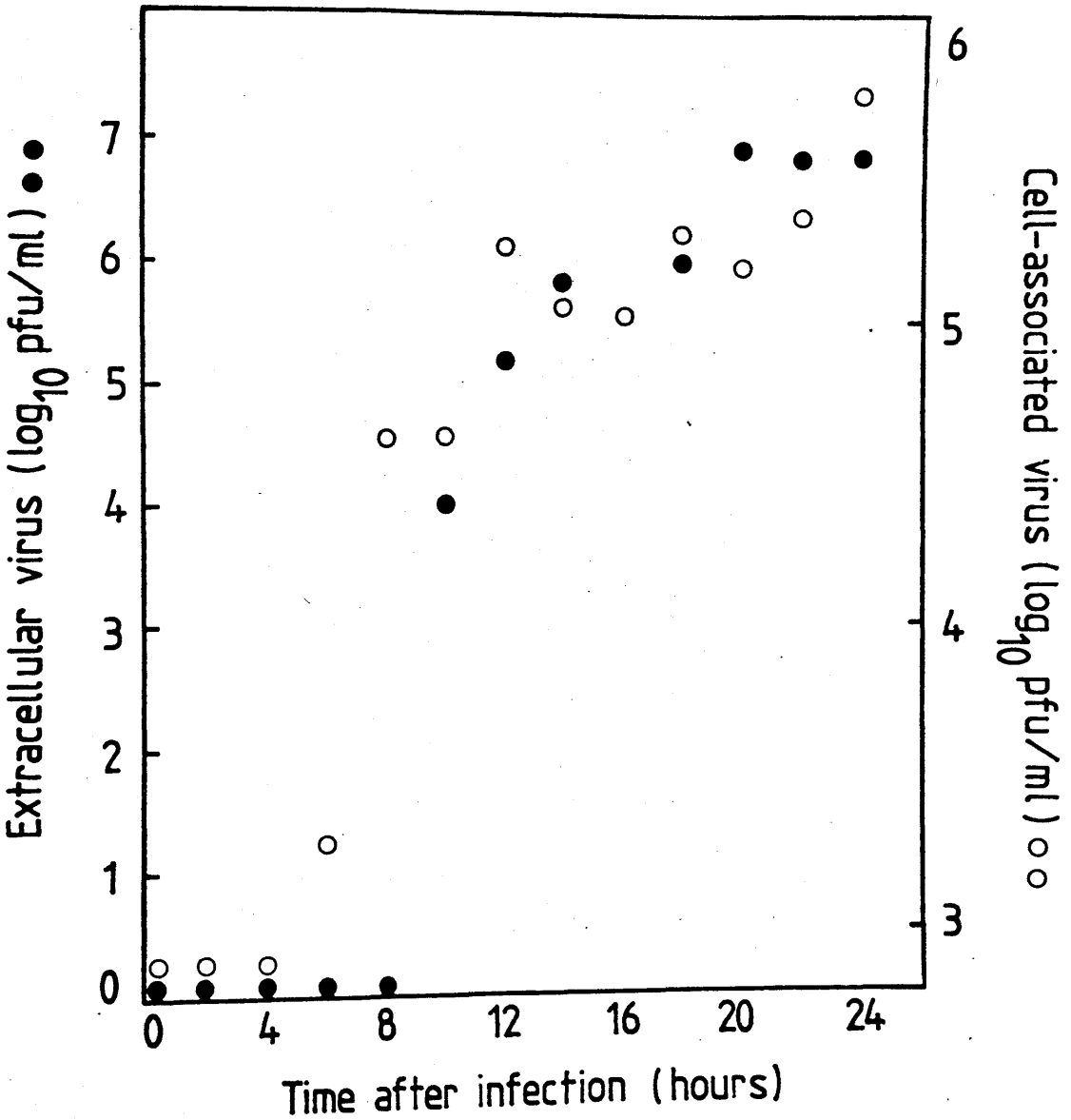
Time in hours	Dilution of virus (log_{10})	Number of plaques/plate	Titre (pfu/ml)
0	-2	3, 5	8.0×10^2
2	-2	4, 4	8.0×10^2
4	-2	2, 6	8.0×10^2
6	-2	10, 10	2.0×10^3
8	-3	19, 22	4.1×10^4
10	-3	16, 29	4.0×10^4
12	-4	9, 9	1.8×10^5
14	-4	6, 5	1.1×10^5
16	-4	5, 5	1.0×10^5
18	-4	7, 13	2.0×10^5
20	-4	8, 7	1.5×10^5
22	-4	13, 10	2.3×10^5
24	-4	25, 35	6.0×10^5

TABLE 3.3

Proportion of FCV ts infected cells

Microscope field	Number of cells with FCV antigen	Total number of cells
1	31	86
2	51	116
3	24	86
4	49	126
Total	155	424
% with antigen	37%	

Fig 3.1 Growth curve of FCV-ts



Discussion

The majority of conditional lethal mutants of animal viruses which have been described are ts mutants. These mutants are unable to multiply in susceptible cells at high temperatures but are unrestricted at lower temperatures. The plaque morphology and growth characters can often be used to distinguish mutant and wild type virus (Pringle, 1984). In this study the growth curve of the FCV-ts mutant was studied at the permissive temperature of 31°C. Cell associated and extracellular virus were measured.

As indicated by the extracellular virus growth the end of the eclipse occurred at around 8 hours after infection. In a study of the growth of the wild type strains G_1, G_2 and G_{10} at 37°C Ormerod (1978) found that the latent and eclipse periods ended at approximately 2 hours after infection with a rapid rise in intracellular virus between 2 and 3 hours. This experiment served to demonstrate the characteristic, rapid single step growth curve of FCV which was very similar to that of picornaviruses (Howes and Melnick, 1957).

The growth curve of FCV-ts at 31°C was prolonged compared to that of wild type strains at 37°C, taking 18-24 hours to reach maximum yields. However the curve had the general appearance of a one-step growth curve.

One problem about carrying out this experiment was that the titre of the stock FCV-ts was quite low (3.8×10^6 pfu/ml) compared to that of wild type FCV grown at 37°C which is often in excess of 10^8 pfu/ml. Therefore the multiplicity of infection in the experiment was only 1 compared to up to 100 in the experiments of Ormerod (1978). However even at this relatively low multiplicity of infection the proportion of cells which was estimated to be infected on the basis of containing FCV antigen by immunofluorescence was 37%. This figure was comparable to the 28% achieved with wild type virus by Ormerod (1978).

These experiments described the general characteristics of the growth of FCV-ts in FEA cells and the results permitted the design of experiments to find the stage of viral growth which was temperature sensitive, as described in Chapters 4 and 5.

CHAPTER 4

INITIAL CHARACTERISATION OF THE FCV ts MUTANT

CONTENTS

Introduction

From the result of the experiments described in Chapter 2 it was clear that the growth of FCV-ts was severely inhibited at the non-permissive temperature of 37°C compared to the permissive temperature of 31°C. In this chapter, experiments are described which aimed to find the stage of the growth cycle of the virus at which the temperature-sensitive event occurred.

Three experiments were done. In the first, the virus was incubated at each temperature for 6 hours to find whether or not the virus particles themselves were thermolabile. In the second, the virus was inoculated on to feline embryo cells to examine the effect of temperature on the earliest stage of the growth cycle, virus adsorption. In the third experiment, cells were infected with the mutant, incubated at 31°C and then at intervals after infection were shifted to 37°C and incubated until 24 hours after infection. It was expected that this experiment would show whether an early or late event in the growth cycle was temperature sensitive.

Materials and Methods

1. Effect of temperature on the FCV-ts particle.

A stock of FCV-ts was diluted to 1 x 10^6 pfu/ml, using DFC-1 as a diluent and was dispensed in 2 ml volumes in 5 ml plastic bottles (Sterilin). The samples were placed in a waterbath at 37°C or 31°C. Immediately afterwards and then after 6 hours, 1 ml volumes of virus were removed and stored at -70°C. The infectivity of the samples was subsequently determined by plaque assay.

2. Effect of temperature on adsorption of FCV-ts

Two series of FEA cells in 12-well plates were infected with 0.5 ml per well of a ten-fold dilution of FCV-ts (3.8 x 10^5 pfu/ml). These cells were incubated for 2 hours at 31°C or 37°C, the inoculum was removed and the cells were washed twice in DFC-1. Two ml of overlay medium were applied and the infected cells were re-incubated at 31°C for 2 days. The cells were then fixed and stained and the plaques were counted.

3. Temperature shift experiment

Five-cm plates were seeded with 2 x 10^6 FEA cells in 4 ml DFC-10 and were incubated for 24 hours at 37°C. The cells were then infected with 3.8 x 10^6 pfu in 1.0 ml. After an adsorption time of 2 hours at 31°C the inocula were removed and the cultures were washed twice in DFC-1. Four ml of DFC-1 were added to each plate before re-incubation at 31°C. Immediately and at 2, 4 or 6 hours afterwards plates were transferred to 37°C. At 24 hours after infection the cultures were harvested. The infected cells were scraped off into the medium and were centrifuged at 2,000 rpm for 10 minutes. The supernatant fluids were dispensed in 0.5 ml volumes and stored at -70°C then later assayed to obtain the titre of extracellular virus.

Results

1. Effect of temperature on the FCV-ts particle

Table 4.1 shows the effect of incubating the FCV-ts particle at 31°C or 37°C for 6 hours. During this incubation period there was a fall in the titre of virus by a factor of about 10 but there was little difference in titre between incubation at 31°C (2.2×10^4 pfu/ml) or 37°C (4.3×10^4 pfu/ml).

2. Effect of temperature on the adsorption of FCV-ts

As shown in Table 4.2, after adsorption for 2 hours at 31°C or 37°C followed by incubation for 2 days under an overlay agarose at 31°C, there was very little difference in titre between virus adsorbed at 31°C (1.8×10^6 pfu/ml) or 37°C (2.9×10^6 pfu/ml).

3. Temperature shift experiment

In this experiment the infected cells were incubated at 31°C for 2, 4, 6 or 24 hours after inoculation. At each time a plate was removed and the culture was harvested to determine the titre of extracellular virus as described in Chapter 3. At the same time another plate was shifted to 37°C and was incubated at that temperature until 24 hours after infection when it was harvested.

As shown in Table 4.3 the titre of virus produced after 24 hours of continuous incubation at 31°C was 1.4×10^6 pfu/ml. However, when the virus infected cultures were shifted to 37°C the amount of virus produced after 24 hours was only moderately greater than at the time when the cultures were shifted, at 2, 4 or 6 hours following infection.

TABLE 4.1

Effect of temperature on the FCV-ts virus particle

Time of incubation (hours)	Dilution of virus (log_{10})	No. of plaques per well		Titre of virus (pfu/ml)	
		31°C	37°C	31°C	37°C
0	-4	10, 19	16, 18	2.9×10^5	3.4×10^5
6	-3	9, 13	21, 22	2.2×10^4	4.3×10^4

TABLE 4.2

Effect of temperature on adsorption of FCV-ts

Dilution of virus (log_{10})	No. of plaques per well		Titre of virus (pfu/ml)	
	31°C	37°C	31°C	37°C
-5	7, 11	14, 15	1.8×10^6	2.9×10^6

TABLE 4.3

Temperature shift experiment

Time after infection (hours)	31°C			31°C - 37°C		
	Dilution of virus (log_{10})	No. of plaques	Titre (pfu/ml)	Dilution of virus (log_{10})	No. of plaques	Titre (pfu/ml)
2	-2	32	3.2×10^3	-3	12	1.2×10^4
4	-3	18	1.8×10^4	-3	45	4.5×10^4
6	-3	10	1.0×10^4	-3	59	5.9×10^4
24	-5	14	1.4×10^6			

Discussion

Three experiments were done in this chapter to begin to characterise the FCV-ts mutant. The first experiment showed that the FCV-ts virus particle was not itself temperature sensitive because it was quite stable when incubated at the non-permissive temperature over a period of 6 hours.

Ormerod (1978) used the same method to measure the stability of his wild type FCV strains at 37°C and found that the three isolates (G_1, G_2, G_{10}) were apparently of equal stability at this temperature with a half life of approximately 5 hours. A similar finding was reported for vesicular exanthema virus variants (McChain and Hackett, 1958).

In principle, conditional lethal mutation can be obtained in any gene whose function is essential for normal replication. This is because a change in an amino acid may produce a conformational change which affects the stability of the whole molecule at high temperature (Pringle, 1984). Baltimore (1968) in a study of poliovirus mutants described the characterisation of the phenotypic properties of the virus and found that the virions from three of these mutants were much more heat sensitive at the non-permissive temperature of 45°C than wild type virions. On the other hand, virions from another mutant were as heat resistant as wild type virions. It was also found that one of these mutants was probably a capsid mutant.

The second experiment indicated that the first stages of infection of cells by the FCV-ts virus were also not temperature sensitive. The adsorption of virus to FEA cells over a period of 2 hours was equally efficient at 31°C and 37°C. From these results it appeared that the temperature sensitive event occurred early in the growth cycle of the virus but after virus adsorption to the cells.

Generally ts mutation is found to block a specific viral function. The time in the virus life cycle at which that function is required can often be determined by temperature shift experiments (Ramig, 1985). The third experiment in this study suggested that the temperature sensitive stage in viral growth began soon after virus adsorption. Shifting the temperature from 31°C to 37°C even 2 hours after infection resulted in only a 3-fold increase in virus yield after 24 hours compared to a 500-fold increase in yield after 24 hours' growth at 31°C. Similar results were obtained when the temperature was shifted 4 and 6 hours after infection.

The ts lesion might be in a viral polymerase molecule or in the capsid protein. However the results obtained in this chapter could not distinguish between these alternatives. The experiments described in the next chapter were designed to determine whether the ts lesion affected RNA synthesis and therefore might be in a polymerase gene.

CHAPTER 5

ATTEMPTS TO DEFINE FURTHER THE TEMPERATURE SENSITIVE LESION IN FCV-ts

CONTENTS

CONTENTS

Introduction

The site of the temperature sensitive lesion in FCV-ts might be in the gene coding for the capsid protein or a non-structural protein such as an RNA polymerase. In this chapter attempts were made to distinguish between these possibilities. The capsid protein of FCV is well-defined although there is controversy about whether or not a larger precursor molecule is synthesised in infected cells (Black and Brown, 1975, 1977; Komolafe, 1978). If the ts lesion were in the capsid protein it might be expected that the virion itself would be thermolabile by comparison with studies on other small RNA-containing viruses (Polatnick and Richmond, 1980). However, the results in the previous chapter indicated that the infectivity of the virion was not affected by incubation at the non-permissive temperature.

In this chapter virus-specific RNA synthesis was studied in FCV ts-infected cultures. Very little is known about the calicivirus-induced RNA polymerase. Such an enzyme must be synthesised in calicivirus-infected cells in order that viral RNA is replicated. If the ts lesion were in this molecule it might be expected that RNA synthesis in infected cells would be reduced at the non-permissive temperature. Accordingly RNA synthesis in FCV-ts infected cells was studied by investigating the incorporation of ^{3}H-uridine into acid-precipitable radioactivity in infected cells at the permissive and non-permissive temperature. This incorporation was compared with that observed in FCV strains which grew at 37°C. These were the vaccine FCV-K from which the ts mutant was derived and FCV-ts R which was a revertant of FCV-ts, the isolation of which is described in Chapter 6.

Materials and Methods

Cell culture

Plastic 3 cm plates were seeded with 6 x 10^5 FEA cells in 2 ml DFC-10 medium and incubated at 37°C for 24 hours.

Viruses

FCV-ts at 1 x 10^6 pfu/ml was used for the following experiments.

FCV-K was derived from Katavac-CH vaccine as described in Chapter 1.

FCV-tsR was isolated from a cat inoculated with FCV-ts as described in Chapter 6.

Experiment 1: Effect of actinomycin-D on RNA synthesis in FEA cells

Two sets of samples were prepared according to the actinomycin-D concentration. In the first, concentrations were 0, 2, 10 and 50 µg/ml and in the second, 0, 0.6, 2.5 and 10 µg/ml.

After removing the media from the plates of confluent FEA cells, 2 ml of different actinomycin-D concentrations was added to the cells in each plate, which were then incubated at 31°C. After one hour this medium was replaced by 2 ml of fresh actinomycin-D at the same concentration together with 5 µCi/ml ^{3}H-uridine of specific activity 40 Ci/mmol (Amersham International). These plates were incubated at the same temperature for another hour. After incubation the cells were scraped off, the remaining cells were washed off with 2 ml PBS and the whole culture was transferred into a 10 ml plastic conical tube. The suspensions were centrifuged at 2000 rpm for 5 minutes. The supernatant fluids were discarded carefully and the sediments were resuspended in 10 ml of

5% cold trichloracetic acid (TCA). After vigorous mixing, the suspensions were incubated at 4°C for 10 minutes, after which time they were collected on 2.5 cm Whatman GF/C filters using a Millipore filter manifold. The filters were washed twice with 5 ml cold 5% TCA and then with 5 ml of ethanol. After drying at 60°C for 30 minutes the filters were transferred to plastic vials to which was added 10 ml of Aqualuma (LKB) scintillation fluid. The radioactivity in the samples was counted in a Packard scintillation spectrometer. Each sample was counted so that the standard error was less than 5%.

Experiment 2: Effect of FCV-ts infection on RNA synthesis in FEA cells

Uninfected and cells infected with FCV-ts were used and RNA synthesis was measured. After 16 hours of incubation at 31°C at which time there was developing CPE but while the cells were still attached to the plates, 200 µl of a mixture of actinomycin-D (2.5 µg/ml) and ^{3}H-uridine (5µci/ml) was added. After another period of incubation for 2 hours the cells were scraped off and processed as in the previous experiment.

Experiment 3: Effect of temperature shift on virus-specific RNA synthesis in FCV-ts infected FEA cells

Uninfected and infected FEA cells were incubated for 24 hours at 31°C. The 200 µl actinomycin-D (2.5 µg/ml) and ^{3}H-uridine was added. Half of the plates of cells were re-incubated at 31°C and half were shifted to 37°C for 3 hours. The TCA-precipitable cpm was then established as before.

Experiment 4: Effect of temperature shift on virus-specific RNA synthesis in FCV-K or FCV-tsR infected FEA cells

The effect of temperature shift on two FCV strains which grew at 37°C was studied. The two viruses were FCV-K, the vaccine virus from which the FCV-ts mutant was derived, and FCV-tsR the revertant

virus isolated from FCV-ts, described in Chapter 6, which grew at the non-permissive temperature. The experiment was carried out as for Experiment 3. The cells were infected at a multiplicity of infection of 2 pfu/cell and the temperature was shifted after incubation at 31°C for 24 hours.

Results

Experiment 1: Effect of actinomycin-D on cellular RNA synthesis

Control samples which had not been incubated with actinomycin-D showed the highest incorporation of ^{3}H into acid-precipitable material which reflects the amount of RNA synthesis in the cells during the period of exposure to ^{3}H-uridine. Increasing the actinomycin-D concentration decreased the incorporation of radiolabel.

From these results it was decided to use 2.5 µg/ml in the experiment to investigate the incorporation of ^{3}H-uridine into FCV-infected cells. (Table 5.1).

Experiment 2: Effect of FCV-ts infection on RNA synthesis in FEA cells

The aim of this experiment was to find if virus-specific RNA synthesis could be detected in FEA cells treated with actinomycin-D, following infection with FCV-ts.

The result of this experiment, shown in Table 5.2, was that there was no increase in RNA synthesis in the infected cells. Therefore, the next experiment was to investigate RNA synthesis later after infection, at 24 hours when the CPE was more advanced. Also in this experiment the effect on RNA synthesis of shifting the temperature from 31°C to 37°C was studied.

Experiment 3: Effect of temperature shift on virus-specific RNA synthesis in FCV-ts infected FEA cells

The purpose of this experiment was to find if virus-specific RNA synthesis in FCV-ts infected cells was affected by shifting the temperature of incubation from 31°C to 37°C. The results are in Table 5.3.

Table 5.1

Effect of actinomycin-D on cellular RNA synthesis

Experiment	Actinomycin-D concentration (µg/ml)	^{3}H incorporation (cpm per sample)	Mean cpm
A	0	17694, 17791, 12334	15939
	2	1685, 1899, 2812	2132
	10	797, 3591, 639	598
	50	466, 4681, 441	458
B	0	6819, 7135	8977
	0.6	1232, 1352	1292
	2.5	425, 524	474
	10	660, 440	550

Table 5.2

Effect of FCV-ts infection on RNA synthesis in FEA cells

Treatment of cells	^{3}H uridine incorporation (cpm)			
	uninfected		infected	
	cpm/plate	mean	cpm/plate	mean
Actinomycin-D (2.5 µg/ml)	725, 526	550	516, 572	600
No actinomycin	4281, 3555	3500	3095, 3909	3600

Table 5.3

Effect of temperature shift on RNA synthesis in FCV-ts infected cells.

Temperature shift	^{3}H-uridine incorporation (cpm) uninfected cpm/plate	mean	infected cpm/plate	mean	Ratio*
31°C - 31°C	2600, 2432	2516	9655, 9707	9681	3.8
31°C - 37°C	1459, 1352	1405	2032, 2026	2029	1.4

* Ratio of infected cell cpm : uninfected cell cpm.

The conclusions of this experiment were first, that at 24 hours after infection, virus-specific RNA synthesis can be detected because there is 4-fold increase in incorporation of ^{3}H-uridine into TCA-precipitable cpm in the infected cells compared to uninfected cells. Secondly, by shifting the temperature from the permissive temperature (31°C) to the non-permissive temperature (37°C), the incorporation of ^{3}H-uridine into TCA-precipitable cpm was decreased by a factor of 5. This indicated that RNA synthesis was affected by the increase in temperature and suggested that the temperature-sensitive lesion involved decreased RNA synthesis perhaps as a result of a thermolabile RNA polymerase.

Experiment 4: Effect of temperature shift on virus-specific RNA synthesis in FCV-K or FCV-tsR infected cells

This experiment was designed to examine viral RNA synthesis in FEA cells infected with viruses which grew as well at 37°C as at 31°C under conditions of temperature shift. The results are shown in Table 5.4.

For FCV-K there was a slight reduction in the amount of ^{3}H which was incorporated into acid-precipitable material when the temperature was shifted suggesting that this virus may be slightly ts. For the revertant virus, FCV-tsR, there was a very slight increase in the ^{3}H which was incorporated suggesting that it could direct the synthesis of viral RNA at 37°C as well as at 31°C.

Table 5.4

Effect of temperature shift on virus-specific RNA synthesis in FCV-K or FCV-tsR infected cells

Virus	Temperature shift		^{3}-H uridine incorporation cpm/plate	mean
FCV-K	31°C	31°C	1599, 1871	1735
	31°C	37°C	946, 963	958
FCV-tsR	31°C	31°C	1319, 1500	1409
	31°C	37°C	1735, 1788	1761

Discussion

The experiments described in this chapter were designed to find if virus-specific RNA synthesis was affected when the ts FCV mutant was grown at the non-permissive temperature. If so, it would suggest that the ts lesion was in the gene which codes for an RNA polymerase, as yet unidentified.

The first experiment confirmed that, as expected, actinomycin-D inhibited RNA synthesis in FEA cells and the concentration required to reduce synthesis to less than 5% of the level in uninfected cells was established. RNA synthesis was measured by the incorporation of ^{3}H-uridine into acid-precipitable radioactivity. This concentration (2.5 μg/ml) of actinomycin-D was then used in subsequent experiments.

In the second and third experiments it was found that infection of FEA cells with FCV-ts dramatically increased RNA synthesis after 24 hours' incubation at 31°C by a factor of 4 in the presence of actinomycin-D. It is assumed that this increase was due to virus-specific RNA synthesis although the RNA was not characterised further.

Also in the third experiment the effect on virus-specific RNA synthesis of shifting the temperature of incubation from 31°C to 37°C was investigated. It was found that this temperature shift reduced RNA synthesis in FCV-ts infected cells by a factor of 5. These results indicated that the ts lesion was in a site which affected RNA synthesis.

The most likely reason for this result is that the ts lesion is in an RNA polymerase gene and that the RNA polymerase does not function properly at the non-permissive temperature. Many examples of such mutants are known in other RNA-containing viruses: for example, togaviruses (Eastman and Blair, 1985), foot-and-mouth disease virus (Richmond and Polantnick, 1976) and the rhabdovirus, vesicular stomatitis virus (Wunner and Pringle, 1972).

On the other hand McCahon (1981) reported that in foot-and-mouth disease virus approximately 83% of the mutations were in the capsid protein region and a similar distribution was found with poliovirus (Cooper, 1968, 1971).

Another possibility is that viral RNA is synthesised normally but is not shielded from degradation after shift-up to the non-permissive temperature as suggested for mutants of foot-and-mouth disease virus by Polantnick and Richmond (1980), perhaps because association with capsid protein is affected. In this case the ts lesion might be in the capsid protein gene although phenotypically it is the integrity of the viral RNA which is affected by the higher temperature.

CHAPTER 6

INFECTION OF CATS WITH THE FCV-ts MUTANT

CHAPTER 6. Page No.

Introduction

At the beginning of this study it was believed that a ts mutant of FCV might be useful as a vaccine. Temperature sensitive mutants of FHV are used in cats as intranasal vaccines and have been very successful (Slater and York, 1976). These viruses grow locally in the upper respiratory tract and induce a good immune response. Also their growth is limited to a few days (Orr et al., 1980) which reduces the chance of virus spreading to in-contact cats. In contrast, the non-temperature sensitive FCV component in the same vaccine grows for a period of several weeks in the oropharynx (O. Jarrett, personal communication). It was hoped that the FCV-ts virus might behave in a similar way to the vaccine FHV.

In this chapter an experiment is described in which FCV-ts was administered to kittens by the intranasal route and the kittens were examined subsequently for virus excretion. Unexpectedly virus was found to be present in the oropharynx for up to 26 days following infection. Virus isolated from a kitten at the end of this period was checked for temperature sensitivity and was found to have reverted, growing as well or better at 37°C than 31°C. A similar revertant was then found when FCV-ts was passaged in cell culture.

Materials and Methods

Animals

Four kittens, 13 weeks old, were obtained from the breeding colony of the University of Glasgow, Department of Veterinary Pathology. These kittens were separated from the queens when they were 12 weeks old and transferred to experimental accommodation. They were housed together in a room with a floor area of 12 m^2.

Infection of cats

FCV-ts was diluted in DFC-1 medium to a concentration of 1.0×10^6 pfu/ml and a volume of 1 ml was given intranasally to each kitten using a syringe without a needle. A volume of 0.5 ml of virus inoculum was instilled into each nostril.

Sampling of cats

Oropharyngeal swabs were taken on the day before infection, on the day of infection (day 0), and then on each day for the first 2 weeks of the experiment, after which samples were taken twice weekly until the end of the experiment.

Rectal temperatures were taken before infection as above and for the first 12 days following infection.

Processing of samples

The oropharyngeal swabs were taken with the aid of sterile cottonwool swabs on wooden sticks (Exogen). The swab was broken off into a plastic bottle containing 2.5 ml of transport medium (Leibovitz L-15 medium with 20% FBS, 500 µg/ml lincomycin sulphate and 2.5 µg/ml amphotericin B) which was then sealed. In the laboratory the fluid was transferred from the bottles to conical centrifuge tubes and centrifuged at 2,000 rpm for 10 minutes. The upper 2 ml of the supernatant fluid was removed and a volume of

1 ml was transferred to each of 2 vials which were stored at -70°C prior to virus isolation.

Isolation of virus

Samples from each cat were diluted to 2 ml and were inoculated into each of two confluent FEA cell cultures in 5 cm plates and adsorption was allowed for 2 hours at 31°C followed by replacement of the medium with 4 ml DFC-1. The plates were incubated at 31°C and were examined daily until a CPE was observed, or 4 days had elapsed. At the end of 4 days plates not showing CPE were regarded as negative.

Revertant virus isolated from cats

An isolate made from cat 2 at 26 days after infection was passaged once by inoculating 1.0 ml of the culture fluid into a confluent FEA cell culture in a 5 cm plate and incubating at 31°C for 2 days. The infected cells were harvested, centrifuged at 2000 rpm for 10 minutes and the supernatant fluid was filtered (0.22 μm) and stored at -70°C until use. This sample was then cloned once as described in Chapter 3 and subsequently titrated at 31°C or 37°C by plaque assay. For comparison the virus used to inoculate the cat was titrated simultaneously at each temperature.

Revertant virus isolated from cell culture

Confluent FEA cells in a 5 cm plate were infected by 2.0 ml of FCV-ts containing 3.8×10^6 pfu/ml and left 2 hours for adsorption after which the plate was incubated in 31°C for 2 days. The cells were harvested and above procedure was repeated twice. Then the infected cell suspension was centrifuged at 2000 rpm for 10 minutes to remove cell debris and the fluid was filtered through a 0.22 μm filter. The supernatant fluid was dispensed in 0.5 ml volumes and stored at -70°C. The virus was assayed at 31°C or 37°C.

Results

Clinical examination

No clinical signs or elevation of rectal temperature was noted following infection as shown in Table 6.1.

Virus recovery from the oropharynx

Oropharyngeal swabs taken from the 4 cats one day before inoculation, on the day of inoculation and after one day following inoculation were negative for FCV. Table 6.2 shows the results of the isolation of the virus from the oropharynx of infected cats for a period of 43 days. After 24 hours all the cats began to excrete the virus except Cat 1 which excreted virus after 48 hours. All the cats continued to excrete virus for 26 days. Subsequent oropharyngeal swabs taken from the cats proved negative.

Revertant virus isolated from cats

The results of this experiment showed that virus could be isolated from the oropharynx of cats inoculated with FCV-ts over a priod of 26 days. It had been expected that the period of virus excretion would be much shorter and more like the excretion of the temperature sensitive FHV in the Katavac-CH vaccine as shown in Table 6.3. In fact, the duration of FCV excretion was quite similar to that of the wild type FCV Katavac-CH vaccine virus (up to 40 days) as shown in Table 6.3. Data for Table 6.3 were obtained form O. Jarrett and are based on a previous experiment carried out as above.

One reason for this prolonged excretion might be that a revertant had arisen during growth of the virus in the cats which could grow at 37°C and might be more invasive in the oropharynx. Accordingly, samples taken from Cat 2 were inoculated on to FEA cells which were incubated at 31°C or 37°C. The virus used as the inoculum for the cats was also titrated at the two temperatures.

TABLE 6.1

Rectal temperatures of inoculated cats

Cat No.	Rectal temperature (°C) Days after infection										
	-1	0	1	2	3	4	5	6	7	9	12
1	38.0	38.6	39.3	38.2	38.4	38.7	38.8	38.4	39.3	38.6	38.0
2	38.8	38.5	39.1	38.2	38.6	39.1	38.4	38.4	39.0	39.0	38.2
3	38.8	38.6	38.2	38.3	39.1	39.3	39.5	40.2	39.7	38.8	38.3
4	38.6	38.6	38.3	38.4	38.6	38.8	39.4	38.6	39.0	38.6	38.2

TABLE 6.2

FCV isolation from oropharyngeal swabs

Days after infection	FCV isolated from cat number: 1	2	3	4
-1	-	-	-	-
0	-	-	-	-
1	-	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	-	-
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+
12	+	+	+	+
14	+	+	-	-
16	+	+	+	+
19	+	+	+	+
22	+	+	+	+
26	+	+	+	+
34	-	-	-	-
45	-	-	-	-
51	-	-	-	-

TABLE 6.3

Isolation of FCV or FHV from kittens given Katavac-CH vaccine

Days after infection	Isolation of virus from oropharynx of cats			
	5	6	7	8
0				
1				
2				
3				
4				H
5		H	CH	H
6	H	H	CH	H
7	H	H	C	H
8	CH	H	C	H
9	CH		H	
10	C	C	C	C
11	C			
12	C		C	
13	C	C	C	C
14	C	C	C	C
15	C	C	C	C
16	C	C	C	C
17	C	C	C	C
18	C	C	C	C
19	C	C	C	C
20	C	C	C	C
21	C	C	C	C
22	C	C	C	C
23	C	C	C	C
24	C			C

TABLE 6.3 (Continued)

Isolation of FCV or FHV from kittens given Katavac-CH vaccine

Days after infection	Isolation of virus from oropharynx of cats			
	5	6	7	8
25			C	C
26			C	C
27		C		
28		C		
29			C	
30			C	
31		C	C	
32			C	
33			C	
34			C	
35	C	C	C	
36	C		C	
37	C		C	
38	C			
39	C			
40	C			
41				
42				
43				
44				
45				

C = FCV isolated

H = FHV isolated

Where no result is given, no virus was isolated.

The results in Table 6.4 show that the titre of the excreted virus at 37°C was 1.4×10^7 pfu/ml which was approximately the same as at 31°C (9.0×10^6 pfu/ml). In contrast, the virus used to inoculate the cats had a titre of 5×10^3 pfu/ml at 37°C which was 760-fold less than the titre at 31°C (3.8×10^6 pfu/ml).

The revertant virus clone which was studied was designated FCV-tsR.

Revertant virus isolated from cell culture

The virus stock which had been used to inoculate the cats was passaged 3 times in FEA cell cultures and was then titrated at 31°C and 37°C without further cloning. The results are shown in Table 6.4. It was found that the difference in titre of the resulting virus between 31°C and 37°C was only 2.8 (1.3×10^6 : 4.5×10^5) compared to 760 for the original virus stock. This result indicated that viruses had been selected during the three passages of growth in vitro which were able to grow well at 37°C.

TABLE 6.4

Testing for revertant of FCV-ts to wild type

Virus	Dilution of virus (log_{10})	No. of plaques per well		Titre of virus (pfu/ml)	
		31°C	37°C	31°C	37°C
FCV-ts	-5	19, 19	0, 0	3.8×10^6	
	-2		3, 2		5.0×10^2
Excreted Virus	-6	5, 4	4, 10	9.0×10^6	1.4×10^7

TABLE 6.5

Revertant virus from cell culture

Temperature	Dilution of virus	No. of plaques	Titre of virus (pfu/ml)
31°C	10^{-5}	10, 3	1.3×10^{6}
37°C	10^{-4}	15, 30	4.5×10^{5}

Discussion

In this Chapter the FCV-ts mutant which was given intranasally to the cats was found to grow for a period of 26 days in all 4 cats. This compared with periods of 26-40 days for Katavac-CH FCV which was derived from an attenuated FCV (O. Jarrett, personal communication). Also Orr et al. (1980) found that FCV was recovered for up to 83 days following vaccination with Katavac CH.

The relatively long period during which FCV was recovered from the cats in the present experiment compared to temperature sensitive FHV in these previous experiments might be attributed to one of two main reasons. First the biology of FCV and FHV might be different. For example the difference in the length of time of growth in the oropharynx might be an indication that the immune response of the cat to FHV is more efficient than that against FCV. A second alternative reason might be that a revertant FCV emerged which was able to grow at 37°C and was therefore similar in its growth properties in vivo to the 'wild type' FCV vaccine component in Katavac-CH.

When virus isolated 26 days after infection from one of the cats (cat 2) was tested, it was found to grow as well at 37°C as at 31°C. Also virus passaged in vitro gave rise to similar viruses. The reason for the appearance of this virus might be that it was a real revertant. Revertants occur with high frequency in RNA virus mutants (Ramig, 1985). Reversion can occur in a number of ways. For example by a back mutation at the nucleotide of the original mutation, restoring the wild type nucleotide sequence (true reversion); or a second mutational event can occur at another site in the same gene which restores a frame shift mutation to the original correct reading frame (suppression). Mackenzie (1975) suggested that most reversions in foot-and-mouth disease virus ts mutants were the result of a second independent mutation in the same gene rather than true reversion since ts revertants did not always show restoration of virulence.

GENERAL DISCUSSION

An alternative reason for the appearance of a FCV which grew at the non-permissive temperature might be that the original mutant virus stock was contaminated with wild type virus carried through the cloning procedure. While it is not possible to completely exclude this possibility, it seems unlikely since the virus was plaque purified 3 times and was filtered between each cycle of cloning through a 0.22 μm filter to try to eliminate viral clumps. The difference in titre at 31°C and 37°C of the resulting virus was 760. A much smaller difference might have been expected if a wild type virus had been carried through each cycle of cloning in a viral aggregate.

In an attempt to overcome the difficulties encountered with some previous live virus vaccines, the use of the ts mutant as a vaccine strain was investigated. This FCV-ts mutant was developed from attenuated virus vaccine to minimize the probable risk of pathogenic infection. However, FCV-ts was found in this study to revert to the wild type characteristic which was to grow as well at 37°C as at 31°C and the virus survived for a long period in cats. However, the virus did not appear to revert to virulence since no pathogenic effects were observed following infection. Studies which were done previously on the FCV virus in the Katavac vaccine showed that reversion to virulence of the vaccine viruses does not occur (Katavac-CH International Technical Manual). The present work indicates that ts mutants of FCV may not be more suitable as vaccines than wild type attenuated vaccines.

However, this property may not apply to all FCV ts mutants since in other RNA viruses ts mutants have been used as vaccines. For example Mills et al. (1971) found that a ts mutant of influenza virus was useful as a live, attentuated vaccine and recently Caplan et al. (1985) derived an attenuated strain of Rift Valley Fever virus after serial mutagenesis with 5-FU for use as a live virus vaccine. Their work suggests that it may be necessary to induce multiple mutations in the same gene of RNA viruses to minimise the risk of reversion to the wild type phenotype.

The aim of this study was to isolate a ts mutant of FCV and to investigate its use as a vaccine similar to the ts FHV in existing vaccines and for genetic analysis of the virus.

A ts mutant was isolated from FCV-K which was an avirulent vaccine virus by treating infected cells with 5-FU in an attempt to increase the number of mutants. One mutant was isolated from 192 clones after selection at 31°C which is near the nasal passage temperature. It was not clear if this clone was a pre-existing mutant or whether 5-FU increased the proportion of mutants in the population as in vesicular stomatitis virus (Pringle, 1970). To confirm this would require experiments in which the proportion of mutants would be determined at different concentrations of the mutagen to find if increasing concentrations increased the proportion of mutants.

The FCV ts mutant was cloned by 3 cycles of plaque purification and this virus was passed through a 0.22 μm filter at each stage in an attempt to reduce viral clumps which might have contained residual wild type virus. This process seemed to be successful in getting rid of the wild type virus since there was a difference of 760 between the virus titre at 31°C and 37°C.

As expected the growth of the mutant was found to be prolonged at 31°C compared to the wild type of FCV at 37°C. The eclipse phase lasted 10 hours and the maximum yield was at 24 hours after infection. However these yields were in general found to be low compared to those reported for wild type virus (Ormerod, 1979).

With lower titred virus stocks it was originally considered that it might be difficult to carry out one-step growth experiments. However, using immunofluorescence it was found that 37% of the cells were infected by the ts mutant when the multiplicity of infection was about 1 compared to 28% of the wild type FCV under conditions where a multiplicity of infection of up to 100 was used (Ormerod, 1979). It is not clear why the remaining cells resist the infection even at the high multiplicities of infection used by Ormerod (1979).

In temperature shift experiments the mutant virus was found not to be thermolabile over a period of 6 hours. Also adsorption to FEA cells was equally efficient at 31°C and 37°C. However an early event appeared to be ts since temperature shift to 37°C even 2 hours after adsorption reduced the yield of virus dramatically.

The only FCV-specific protein identified for certain is the capsid protein with a molecular weight of 68,000 (Komolafe, 1978). These results might indicate that this protein was not thermolabile since the virus particle was stable at 37°C. This has been shown to correlate with the ts lesion in some mutants in other viruses although the correlation is not absolute (Polatnick and Richmond, 1980). Future work on the ts lesion should be to examine in detail protein synthesis in infected cells by pulse labelling with radiolabelled amino acids and analysing the labelled products by polyacrylamide gel electrophoresis (Komolafe, 1978). Protein synthesis in FCV-infected cells should be mainly virus-specific (Komolafe, 1978). Virus specificity could be further defined using radioimmune precipitation with anti-capsid protein antibodies.

However, other results suggested that the lesion might be in another gene, the RNA polymerase. No such enzymatic activity has been investigated in FCV-infected cells and a viral polymerase has not been identified. The results showed that RNA synthesis was reduced at the non-permissive temperature by a factor of 4. The simplest explanation of this finding is that viral RNA polymerase is ts. This could be investigated further using an assay for polymerase activity similar to that used for foot-and-mouth disease virus by Polatnick and Richmond (1980) to find if in vitro, as well as in vivo, RNA synthesis is ts.

An alternative explanation of these results is that FCV RNA is synthesised normally at the non-permissive temperature but is then degraded by ribonuclease because it is not protected by, for example, a ts capsid protein. This has been suggested for mutants of foot-and-mouth disease virus by Polatnick and Richmond (1980).

Future experiment to investigate protein synthesis in FCV ts infected cells as described above might also identify a polymerase. Although there is no antibody available which is specific for polymerase, the sera of convalescent cats should contain antibody to all virus-specific proteins, not only capsid protein as shown by Komolafe et al. (1980). Demonstration of a protein produced at 31°C but not at 37°C might identify a potential polymerase molecule although it is quite likely that the enzyme might be synthesised at the non-permissive temperature but remain non-functional.

A major aim of this work was to find if a ts FCV could be used as a vaccine against FCV infection of cats in the same way that a ts FHV has been used. The idea of the vaccine was that the viral mutant would grow in the nasopharynx but would not be invasive and would only survive a short time before being eliminated by the immune response. When kittens were inoculated with FCV-ts intranasally, the virus was subsequently isolated from the oropharynx over a period of 26 days. This result was unexpected since this period is similar to the 40 days during which the FCV in the original vaccine was found, but shorter than the 83 days found by Orr et al. (1980). Therefore the ts lesion did not appear to influence the period during which the virus could grow in the upper respiratory tract.

One possible reason for this long period of growth was that the virus might have reverted and be able to grow again at 37°C. When tested, this was found to be the case since virus isolated from one of the kittens 26 days after infection was shown to grow at 37°C and when a clone was purified it was shown to grow as well at 37°C as at 31°C. This result suggested that a revertant had arisen during growth in the cat and was responsible for the prolonged growth of the virus in the oropharynx. A similar situation was found in vitro when FCV-ts was passaged 3 times in FEA cells and the progeny was found to grow well at 37°C compared to the original ts isolate. As discussed in Chapter 6, it was considered unlikely that this result was due to a carry-over of

virus in clumps during the original cloning procedure and was more likely to be caused by a true revertant virus.

Therefore it was considered that this particular ts mutant of FCV would not be useful as a vaccine since because of reversion it showed no advantage over the parent vaccine virus. However, in future it might be useful to try to isolate other mutants and to test them in vitro for reversion before testing them in cats. In addition it would be preferable to try to obtain mutants with multiple ts lesions in the same gene so that the chance of reversion was reduced.

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