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Virus Shedding and Immune Responses in Cats Inoculated with Cell Culture-Adapted Feline Infectious Peritonitis Virus

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ABSTRACT

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Eight specific pathogen-free cats were inoculated orally or parenterally with a cell culture-adapted strain of feline infectious peritonitis virus (FIPV). Faeces and oropharyngeal swabs were monitored daily for infectious virus by inoculation of feline embryo lung cells. Virus was recovered from both sites for ~ 2 weeks after inoculation, before clinical signs of disease developed.

Peripheral blood lymphocytes collected from these cats were tested in an in-vitro blastogenic assay using concanavalin A (con A) and FIPV antigen. All cats showed a profound suppression of the response to con A which only recovered to pre-inoculation levels in 2 cats, one of which survived. These 2 cats also responded to FIPV antigen on the 21st day after infection, the greater response being in the survivor. The other cats, surviving 16–18 days, developed no response to FIPV antigen.

Antibody titres, measured by immunofluorescence and by virus neutralization, rose rapidly to very high levels in all cats, regardless of the route of inoculation.

INTRODUCTION

Feline infectious peritonitis (FIP) is a fatal disease of cats which occurs in two forms, wet and dry. The wet form is characterised by the development of fluid in one or more of the body cavities, and the dry form by the development of granulomatous lesions in various organ systems. The disease is caused by a coronavirus, FIPV. The virus has proved difficult to isolate from infected cats in cell cultures, and this has hampered studies on the virus-host interactions; until relatively recently, most experiments were performed using tissue suspensions from infected cats. However, in recent years several successful iso-
lations in cell cultures have been made (O'Reilly et al., 1979; Black, 1980; McKeirnan et al., 1981; Hitchcock et al., 1981; Pedersen et al., 1981). Although these isolates have been used to reproduce the disease experimentally, the virus has proved difficult to recover from experimental animals, so there is little information on the shedding patterns exhibited by infected animals, which would be helpful in determining the epidemiology of the disease.

The pathogenesis of FIP is complex and not fully understood. It has been suggested that the disease is immune mediated since Jacobse-Geels et al. (1982) showed the presence of circulating immune complexes in cats with FIP and depletion of the C3 component of complement. In addition, it appears that humoral immunity to the virus may be non-protective. Thus, in experimental studies, the majority of inoculated cats develop clinical signs and these animals, like naturally occurring cases, generally have high titres of antiviral antibody (Pedersen et al., 1981). Moreover, it has been shown that vaccination with sub-lethal doses of FIPV, or administration of antibody to cats prior to infection with FIPV, exacerbates the disease (Pedersen and Boyle, 1980). This has led to the suggestion that cell-mediated immunity is probably more important than humoral immunity in protection from this disease (Pedersen and Black, 1983).

However, only limited investigations have been carried out to test this hypothesis. These include the examination of the possible role of T cells in cats by thymectomy (Hayashi et al., 1983) and also by using a mouse model (Takenenouchi et al., 1985). Recently, there have been preliminary reports on delayed hypersensitivity and blastogenic responses in FIP-recovered cats (Pedersen and Floyd, 1985).

We now report the results of a study of the virus-shedding patterns following oral and parenteral inoculation with a cell culture-adapted strain of FIPV. The blastogenic response to FIPV and concanavalin A (con A) mitogen and the serological responses of both groups of cats were also monitored.

MATERIALS AND METHODS

Cats

Twelve 1-year-old specific pathogen-free (SPF) cats were used in these studies. The cats were housed in individual cages within a barrier-maintained isolation unit in the University of Bristol laboratory animal facilities.

Cell culture

The feline embryo lung (FEL) cell line (O'Reilly et al., 1979; Hitchcock et al., 1981) was obtained from Wellcome laboratories. Cells were grown in the BHK modification of Eagle's medium (Wellcome) supplemented with 10%
tryptose phosphate broth, 10% foetal calf serum, 100 i.u. ml$^{-1}$ penicillin, 100 μg ml$^{-1}$ streptomycin and 50 i.u. ml$^{-1}$ nystatin (Squibb). For maintenance, the serum concentration was reduced to 2%.

**Virus**

The "Wellcome strain" of FIPV (O'Reilly et al., 1979; Hitchcock et al., 1981) was obtained from Wellcome Laboratories. It was grown in FEL cells and used at the 11th (FIPV-P11) and 40th (FIPV-P40) passages.

**Serum neutralisation test (SN)**

Serum neutralisation tests were performed in a microtitre system using a constant virus, varying serum technique with duplicate 2-fold dilutions of serum and ~10$^3$ TCID$_{50}$ of virus per well; 2 control wells to which medium was added instead of virus were also included. After incubation at 37°C for 1 h, a suspension of FEL cells (10$^4$ cells ml$^{-1}$) was added to the wells and the plate incubated for 2–3 days at 37°C in an atmosphere containing 5% CO$_2$ (Povey and Johnson, 1969a, b). Titres were expressed as the 50% end point of the serum dilution which inhibited any specific cytopathic effect (CPE). In each test, a known positive serum sample was used as a control and a back titration of the virus dilution was performed.

**Indirect immunofluorescent staining technique (IFT)**

The test was performed directly in the wells of tissue culture plates in which the infected cells were growing. After removal of medium, the cells were washed with phosphate-buffered saline (PBS) and fixed in absolute alcohol, in which they were also stored at −20°C if necessary. Fixed cells were washed twice in PBS for 15 min, rinsed in distilled water, and air dried. For detection of viral antigens, an antiserum to FIPV prepared in an SPF cat was diluted 1:50 and added to the cells which were then incubated in a moist atmosphere for 1 h at 37°C, washed twice in PBS for 15 min, rinsed in distilled water and air dried. FITC-conjugated anti-cat IgG (Miles) diluted 1:30 in PBS was added for 45 min at 37°C, after which the cells were washed twice in PBS, dipped in distilled water, air dried and examined under an inverted UV microscope (Leitz).

When antiviral antibody was being measured, FEL cells infected with FIPV were used as the antigen. Doubling dilutions of the test serum were made, and the endpoint was taken to be the highest dilution of serum that showed fluorescence. Known positive and negative sera derived from SPF cats were included as controls with each test.
Isolation of FIPV from inoculated cats

Oropharyngeal swabs were placed into 2 ml of FEL growth medium. Faecal samples were collected into plastic pots, and an ~20% suspension made in 10 ml of FEL growth medium containing 5 times the usual antibiotic concentration. These were then centrifuged at 3000 x g for 10 min to remove debris; 0.2 ml of each sample was then immediately inoculated in quadruplicate onto confluent monolayers of FEL cells grown in 24-well dishes (Nunc). After 1 h at 37°C, maintenance medium was added, and the monolayers were incubated at 37°C. Cultures were examined for CPE daily for 3 days, then the monolayers were fixed in absolute alcohol, and stored at −20°C, prior to immunofluorescent staining. In addition, both the supernatant and the cells from the first passage of samples obtained 3, 5, 7, 11 and 13 days after infection were passed twice more, both with cell lysates produced by freezing and thawing whole cultures and also by trypsinisation and passage of cells to enhance the development of CPE. The presence of virus was also confirmed by specific neutralisation with FIPV antiserum.

The blastogenic assay

Stock con A (Sigma) solution was made up at a concentration of 1 mg ml⁻¹ in RPMI 1640 medium containing 20 mM HEPES, 1.5 g l⁻¹ sodium bicarbonate, 10% foetal calf serum and antibiotics (RPMI medium), and was stored at −20°C until used. To determine the optimal time of culture, FIPV was grown in FEL cells for various times, irradiated with UV light to inactivate the virus, scraped off into a small amount of RPMI medium, and used at a concentration of 4 x 10⁴ cells ml⁻¹.

A single whole-blood technique was used for all the blastogenic tests (Goddard, 1984). Blood was collected in preservative-free heparin (Pularin: Duncan, Flockhart and Co., Ltd.), then diluted 1:20 in RPMI medium and dispensed into the wells of a U-bottomed microtitre plate (Nunc). Mitogens were added to the blood to the desired concentration, and a similar volume of RPMI medium was added to each control. Six replicates of each test were set up, and 6 control wells. After incubation in a CO₂-enriched atmosphere for up to 7 days, 0.5 μCi of tritiated thymidine (Amersham) were added to each well. The plate was then incubated for a further 6 h, after which time the cells were harvested onto paper discs using a cell harvester (Skatron Titertek). Each disc was suspended in 10 ml of scintillation fluid (Scintran cocktail T, BDH Ltd.) and counted in a scintillation counter. Results were expressed as stimulation ratio (test dpm/control dpm).

Initial experiments showed that the optimum time of culture of FIPV in FEL cells for the production of FIPV mitogen was 16 h. The optimum time of in-
Incubation of the assay was 7 days. The optimal concentration of con A was 10 μg ml⁻¹ and the optimal time of incubation was 5 days.

Experimental design

The cats were divided into 3 groups of 4 animals. Each cat was bled 6 times at weekly intervals before administration of the virus, to study the variability in the blastogenic responses in each cat. Oropharyngeal swabs and faecal samples were taken at the same times to test for virus excretion.

The cats in the first group (Group A) were inoculated with 10⁶.7 TCID₅₀ of FIPV-P11 intravenously (i.v.) and 10⁷ TCID₅₀ of FIPV-P40 intraperitoneally (i.p.). Those in the second group (Group B) received 10⁶.7 TCID₅₀ of FIPV-P11 orally on 3 consecutive days. Two cats in the third group (Group C) received uninfected cell culture fluid orally; the other 2 were inoculated i.v. and i.p. Faecal samples and oropharyngeal swabs were taken daily and the cats bled at intervals over 30 days for the blastogenic assay and estimation of the antibody titre.

In this study, clinical signs of FIP were used as an indication of disease, following inoculation with virus. The cats were examined daily for general health, and body temperature was measured. The abdomen was palpated every other day for signs of fluid. Once a week the eyes of the cats were examined with an ophthalmoscope for ocular signs of FIP. Euthanasia was carried out on the cats when clinical signs developed and a full post-mortem examination was performed.

RESULTS

Clinical signs

All 4 cats in Group A and 3 of the cats in Group B developed the effusive form of FIP (Table I). The remaining cat in Group B did not develop clinical signs and survived. Three of the cats in Group A developed both pleural and peritoneal fluid, the other affected cats developed peritoneal fluid only. All the cats in Groups A and B showed pyrexia 48 h following inoculation, but no further episodes of pyrexia occurred. All the cats in both groups that succumbed to FIP developed a wide spectrum of clinical signs with jaundice being the most consistent marker of disease (Table I). None of the cats in Group C developed any clinical abnormality (not shown).

Virus isolation

Virus was detected in oropharyngeal secretions and faeces of all the FIPV-inoculated cats (Groups A and B), irrespective of the route of administration and the patterns of virus shedding in both groups were similar (Table II). In all cases, virus shedding was first detected in the oropharynx on the 2nd day.
TABLE I

Clinical signs in experimental cats following parenteral or oral inoculation with FIPV

| Number | Onset of signs | Day of death | Cachexia | Jaundice | Vomiting | Dyspnoea | Anorexia | Lethargy | Fluid       |
|--------|----------------|--------------|----------|----------|----------|----------|----------|----------|-------------|
|        |                |              |          |          |          |          |          |          |             |
| Group A| Parenteral     |              |          |          |          |          |          |          |             |
| 1      | 14             | 16           | +        | +        | +        | –        | +        | +        | Peritoneal  |
| 2      | 16             | 16           | +        | +        | +        | +        | +        | +        | Pleural/peritoneal |
| 3      | 16             | 18           | +        | +        | +        | +        | +        | +        | Pleural/peritoneal |
| 4      | 14             | 18           | +        | +        | +        | +        | +        | +        | Pleural/peritoneal |
|        | Oral           |              |          |          |          |          |          |          |             |
| Group B|                |              |          |          |          |          |          |          |             |
| 5      | 14             | 16           | +        | +        | –        | –        | +        | +        | Peritoneal  |
| 6      | 16             | 27           | +        | +        | +        | +        | +        | +        | Peritoneal  |
| 7      | 14             | 18           | +        | –        | –        | –        | +        | –        | Peritoneal  |
| 8      |                |              |          |          |          |          |          |          | None        |

*Number of cats with clinical sign/total number of cats inoculated.
TABLE II

Virus shedding in the oropharynx and faeces in experiment cats following parenteral or oral inoculation with FIPV

| Cat number | Day of death | Sample | Duration of virus shedding (days) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 onwards |
|------------|--------------|--------|----------------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group A parenteral inoculation | 1 | 16 | o/p | + | + | + | + | - | - | - | - | - | + | - | - | - | - | - | - |
| | | | Faeces | - | - | - | - | + | + | + | + | - | - | - | - | - | - | + | - | - |
| | 2 | 16 | o/p | + | + | + | + | + | + | + | + | - | - | - | - | - | - | + | - | - |
| | | | Faeces | - | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - |
| | 3 | 18 | o/p | no faeces | - | + | + | + | + | + | + | + | - | - | - | - | - | + | - | - | - |
| | 4 | 18 | o/p | no faeces | - | + | + | + | + | + | + | + | - | - | - | - | - | + | - | - | - |
| Group B oral inoculation | 5 | 16 | o/p | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| | | | Faeces | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 6 | 27 | o/p | no faeces | - | + | + | + | + | + | + | + | + | - | - | - | - | + | - | - | - |
| | | | Faeces | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 7 | 18 | o/p | no faeces | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 8 | o/p | no faeces | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

+, positive virus isolation confirmed by immunofluorescence (see text).
-, negative (all cats tested until death or Day 40 post-infection).

After infection and in most cases continued up to Day 9 or 10 (Table II). A second short episode of shedding from the oropharynx occurred at about Day 14 in most animals. This was about the time of the onset of clinical signs. The one cat (Cat 8) that did not show this shedding episode on Day 14 was the one cat that did not develop clinical signs of FIP.

The onset of virus shedding in the faeces was more variable, ranging from 2 to 7 days after infection and continuing until Day 10-14 (Table II). The mean duration of shedding, where known, was ~9 days. No secondary episode of shedding was detected in the faeces. No virus shedding was detected in the control cats.

**Serology**

The cats infected with FIPV showed a rapid increase in antibody titre (Fig. 1a and b) in both the IFT and SN tests, irrespective of the route of inoculation. By Day 9, the antibody titre in both Groups A and B was >2000 by the SN test. However, these levels were not achieved using the IFT in either group by the time the majority of the cats were dead (Day 18), although the titres were still rising. The cat that survived (Cat 8) had similar titres to the other cats with both the SN and IFT tests. The control cats did not seroconvert.
**Oral Group** (2/4 dead on day 18)

**Parenteral Group** (4/4 dead on day 18)

Fig. 1a. Development of the SN antibody titre following parenteral or oral inoculation with FIPV.

Fig. 1b. Development of the IF antibody titre following parenteral or oral inoculation with FIPV.
Fig. 2a. Response to con A mitogen in Group A prior to and following parenteral inoculation with FIPV.

Fig. 2b. Response to con A mitogen in Group B prior to and following oral inoculation with FIPV.

The blastogenic response

All the cats showed week-to-week variation in the response to con A in the period before inoculation with the virus (Fig. 2a and b) or cell lysate (Fig. 2c). No response to FIPV was seen during this time.

Following inoculation with FIPV, the response to con A markedly decreased
in all the cats in Groups A and B within the first 2 weeks (Fig. 2a and b). The response increased above pre-inoculation levels in only 2 cats (Cat 6 and 8, the survivor) by the third week after infection. However, by this time all the other cats had developed the disease and had been killed. The blastogenic response in Cat 8 continued to increase until the end of the observation period. The
blastogenic response to FIPV-infected cells had developed only in Cats 6 and 8 (Fig. 3), although by this time all the other cats were dead. The stimulation ratio in Cat 8 (the survivor) was greater than that of Cat 6, which subsequently died.

The control cats did not show any decline in their response to Con A (Fig. 2c) or develop any response to FIPV.

DISCUSSION

This study on experimentally-induced FIP broadly confirms the findings of Pedersen and Boyle (1980) in terms of incubation period and development of the characteristic clinical signs. The pattern of the disease was similar in both the parenterally and orally inoculated cats, except that one orally-inoculated cat experienced only sub-clinical infection. The reason why some cats develop disease and others do not is not yet known; apart from possible host factors, virus strain, dose or route of infection may play a role (Pedersen and Floyd, 1985).

Recovery of FIPV from experimentally-infected cats by isolation in cell cultures has not been well documented. In this study, virus was shed from the oropharynx and the faeces irrespective of the route of administration. Virus shedding was generally detected in the oropharyngeal swabs before being detected in faeces. The start of virus shedding from the oropharynx coincided with pyrexia. The exact significance of this is unknown, but it suggests that virus generalization had occurred with subsequent localisation in tissues of the oropharynx and then the gut. Indeed, in the parenterally-inoculated group, this would presumably be the only mechanism by which virus could arrive at these sites, whereas in the orally-inoculated group direct contact with target tissues cannot be ruled out.

It is interesting that nearly all the cats that developed FIP showed a secondary episode of virus shedding from the oropharynx prior to the development of clinical signs, and the cat that did not fail to develop clinical signs of the disease. The reasons for this are unclear, and it would be interesting to unravel the immune mechanisms operating at this time. No virus was detected in oropharyngeal swabs or faecal samples taken later than the 15th day after infection. However, in all but 2 cats, monitoring ceased shortly after this time when the cats developed clinical signs and were destroyed. Since viral shedding occurred before most cats showed any clinical signs of FIP, this may partly explain why the virus is difficult to isolate from clinical cases, since the majority of them are probably examined after viral shedding has ceased. It is also possible that the virus may be entirely cell-associated when the clinical signs arise (Weiss and Scott, 1981a, b). Re-isolation of virus in the present study may have been facilitated by the use of a strain of FIPV which grows readily in cell culture to a high titre, and by prompt inoculation of culture with fresh material.
It is also interesting to note that Pedersen et al. (1984) have described a similar shedding pattern from faeces for an enteric coronavirus isolate.

The shedding of virus from both the oropharynx and in faeces for several days suggests that the natural route of infection may be either faecal–oral or oral–oral or possibly oral–nasal. However, likely sources of infection for other cats cannot be easily identified before clinical signs appear, unless methods to facilitate the isolation of field strains of virus are developed.

The existence of carriers in FIP has been postulated from epidemiological evidence (Pedersen, 1981; Gaskell, 1984). Recent corroborative evidence has come from the stimulation of clinical FIP in previously exposed but asymptomatic cats superinfected with feline leukaemia virus and treated with methyl prednisolone (Pedersen and Floyd, 1985). The nature of such a carrier state and whether or not such animals are infectious to other cats is not known. However, in the present study, no virus shedding was detected in the one recovered cat monitored for 4 weeks after the initial episode of virus shedding had ceased. In addition, in other studies (Stoddart, 1986) an SPF cat placed in contact for 2 years with another recovered experimental cat failed to seroconvert. In contrast, when several other SPF cats were placed in contact with a colony with endemic FIP, but with no overt disease, seroconversion occurred over a period of 6 months, but no disease (Stoddart, 1986). Further work is needed to determine whether or not a carrier state can readily be established and under what conditions virus may be shed from these animals.

In the present study, antibody titres rose rapidly to very high levels. This finding is similar to that described by Pedersen and Boyle (1980). No difference was found between the groups of cats inoculated orally or parenterally. Interestingly, both the SN and IF antibody titre gradually fell over the next 6 months in the cat that survived (Stoddart, unpublished observation).

The whole blood blastogenic assay used in these experiments has the great advantage, with respect to cat studies, in that it does not require large volumes of blood. These assays may also have other advantages; they have been reported to give greater cell viability (Pauly and Han, 1976) and an equivalent or greater stimulation ratio compared to conventional assays (Pauly and Han, 1976; Shifrine et al., 1978; Tham et al., 1982). However, they have the disadvantage that leucocyte numbers are not standardised, and Kristensen et al. (1981) have suggested that the cell concentration should be standardised whenever possible.

The stimulation ratios obtained with con A in the whole blood blastogenic assays were similar to those described by other workers for isolated populations of feline lymphocytes (Cockerell et al., 1976a, b; Taylor and Siddiqui, 1977; Rojko et al., 1982). Following infection with FIPV, the con A response which gives an indication of non-specific cellular activity was profoundly reduced, and only recovered to and maintained pre-inoculation levels in the cat
that survived the infection. The reason for the disappearance of con A responsiveness is not known at present.

The 2 cats that survived longest developed a response to FIPV mitogen, and of these the cat that survived developed the greater response. The development of a blastogenic response to FIPV may, therefore, correlate with an ability to combat infection, but this type of assay merely measures the ability of cells to respond to antigen. However, even allowing for the limitations of the test, the results presented here provide some support for the hypothesis that cell-mediated immunity is important in protection against FIPV (Pedersen and Black, 1983). Further work using more sophisticated tests for cell-mediated immunity needs to be done.

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