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# The Sites of Early Viral Replication in Feline Infectious Peritonitis

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ABSTRACT

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The sites of early replication of feline infectious peritonitis virus were studied following oral inoculation of specific-pathogen-free (SPF) cats with virus grown in cell cultures. Viral antigen was first detected by immunofluorescence in the tonsils and small intestine within 24 h of inoculation, and was later found in caecum, colon, mesenteric lymph nodes and liver. However, histological changes in the gut did not appear until relatively late in the course of infection.

Virus was recovered from the oropharynx and the faeces from as early as the second or third day after inoculation, and shedding continued until euthanasia.

## INTRODUCTION

Feline infectious peritonitis (FIP) is a disease of cats caused by a coronavirus, feline infectious peritonitis virus (FIPV). However, the exact pathogenesis of the disease is not well understood, although there is some evidence to suggest it may be immune-mediated (Horzinek and Osterhaus, 1979; Pedersen and Boyle, 1980). The natural route of infection is unknown, but both the respiratory and oral routes have been suggested. Experimentally, most workers have used intraperitoneal inoculation to reproduce the disease, though a variety of other routes have also been used (Hardy and Hurvitz, 1971; Weiss and Scott, 1981a,b; Hayashi et al., 1982). Two studies have examined possible sites of early viral replication following infection with FIPV. Weiss and Scott (1981a,b) used an aerosol to administer the inoculum, and demonstrated initial replication in tracheobronchial lymph nodes, followed by generalised dissemination of the virus. Hayashi et al. (1982) showed that oral inoculation of the virus led to viral replication and lesions in the small intestinal mucosa.

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Both these studies used inocula prepared from tissue suspensions from infected cats.

After initial difficulties, FIPV was grown in cell culture (O'Reilly et al., 1979; Black, 1980; Pedersen et al., 1981a), and although such virus has now been used experimentally to reproduce the disease (O'Reilly et al., 1979; Black, 1980; Pedersen et al., 1981a), no detailed studies on its early pathogenesis have been reported. This study was therefore undertaken to determine the early sites of virus replication following oral inoculation of FIPV grown in cell culture. This route was chosen since it is likely to be the most important one in natural infections.

## MATERIALS AND METHODS

### *Cats*

Seven 9-month-old (Experiment 1) and three 18-month-old (Experiment 2) specific-pathogen-free (SPF) cats were used in these studies. The cats were housed in individual cages within barrier-maintained isolation units at the Universities of Bristol or Liverpool laboratory animal facilities.

### *Cell culture*

The feline embryo lung (FEL) cell line (O'Reilly and Whitaker, 1969) was obtained from Wellcome Research Laboratories, Beckenham, Kent, Gt. Britain. Cells were grown in the BHK modification of Eagles medium (Wellcome) supplemented with 10% tryptose phosphate broth, 10% foetal calf serum, 100 i.u. $ml^{-1}$ penicillin, 100 $\mu g$ $ml^{-1}$ 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 Research Laboratories. It was grown in FEL cells and used at the eleventh (FIPV-P11) passage.

### *Serum neturalisation test*

Serum neutralisation tests (SN) were performed in microtitre plates using a constant virus, varying serum technique (Povey and Johnson, 1969a,b) with duplicate 2-fold dilutions of serum and approximately $10^3$ $TCID_{50}$ of virus per well. 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% $CO_2$. Titres were expressed as the 50% end point

of the serum dilution which inhibited any specific cytopathic effect. In each test a known positive serum sample was used as a control, and a back titration of the virus stock was performed.

*Histological studies*

Samples for histology were fixed in 10% neutral buffered formalin, and embedded in polywax before sectioning. Sections were cut at 6 $\mu$m and stained with haematoxylin and eosin.

*Immunofluorescent staining*

Samples for examination by the immunofluorescent staining technique (IFT) were frozen by immersion in liquid nitrogen, and stored at $-70°$C until required. Sections were cut in a cryostat (SLEE) and fixed in absolute alcohol at room temperature for 10 min. The sections were then overlayed with a 1/25 dilution of anti-FIPV serum, normal non-immune cat serum, or porcine anti-transmissible gastroenteritis virus (TGEV) serum, and were incubated in moist conditions at 37°C for 1 h. Sections were washed twice in phosphate buffered saline (PBS), dipped in distilled water, air dried and stained for 45 min at 37°C in a moist chamber with fluorescein-conjugated anti-cat (or anti-pig) IgG (Miles Ltd, Stoke Poges, Gt. Britain) diluted 1/30 in PBS. They were then dipped in distilled water and mounted in glycerol before examination under a Leitz inverted microscope.

*Electron microscopy*

Samples for electron microscopy (EM) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight. The samples were rinsed twice in buffer, post-fixed in osmium tetroxide, dehydrated in an ethanol series and flat-embedded in Epon. Thin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate and examined under a Philips EM.

*Isolation of FIPV from inoculated cats*

Oropharyngeal swabs were placed into 2 ml of FEL growth medium. An approximately 20% suspension of faeces was made in 10 ml of FEL growth medium containing 5 times the usual antibiotic concentration. These were then centrifuged at $3000 \times g$ for 10 min to remove debris. Four 0.2-ml samples were immediately inoculated onto confluent monolayers of FEL cells grown in 24-well dishes (NUNC, Flow Laboratories, Irvine, Gt. Britain). After 1 h at 37°C, maintenance medium was added, and the monolayers were incubated further at 37°C. Cultures were examined for cytopathic effect (CPE) daily for 3 days, then the monolayers were fixed in absolute alcohol, and stored at $-20°$C, prior

to immunofluorescent staining. The presence of virus was also confirmed by specific neutralisation with FIPV antiserum.

Tissue taken from the cats at necropsy was ground in 1 ml of medium with a small amount of sterile sand using a Griffith's tube. This suspension was centrifuged at $750 \times g$ for 10 min to deposit debris. Each sample was inoculated onto a confluent monolayer of FEL cells grown in 24-well plates (NUNC). After adsorption for 1 h at 37°C maintenance medium was added, and the monolayers further incubated at 37°C. Cultures were examined daily for 3 days for cytopathic effect and then the monolayers were fixed prior to staining by IFT. Other pieces of tissue were co-cultivated with FEL cells. They were minced into small fragments and incubated on a confluent monolayer of FEL cells in growth medium for 3 days at 37°C. Supernatant fluid and tissue fragments were separately passaged 3 times in further FEL cultures. During passage the cultures were examined daily for CPE.

*Experimental design*

Five cats were inoculated orally with 3 ml of virus containing $10^{6.7}$ $TCID_{50}$ $ml^{-1}$ FIPV, and a further two cats were given 3 ml of uninfected cell culture fluid orally as a control (Experiment 1). All cats were examined daily for clinical signs and rectal temperature was recorded. Faecal samples and oropharyngeal swabs were taken 2 weeks and 1 week before inoculation, then daily thereafter for virus isolation. One FIPV-inoculated cat was killed at 1, 2, 3, 7 and 14 days after inoculation; the two control cats were killed at 7 and 14 days after inoculation.

The cats were deeply anaesthetised with incremental doses of Saffan (Glaxovet) and pieces of stomach, duodenum, jejunum, ileum, caecum and colon were removed for pathological examination. Blood was then taken from the heart, the samples were allowed to clot and the serum was removed. The cats were killed by injection of pentobarbitone into the heart. Macroscopic lesions were recorded, and representative pieces of kidney, mesenteric and popliteal lymph nodes, spleen, pancreas, liver, bladder, adrenal gland, gall bladder, heart, lung, trachea, tonsil, testes, brain and spinal cord were taken for histology and IFT; lung and small intestine samples were also examined by EM. Samples of small intestine, trachea and tonsil were taken for attempted isolation of virus by both tissue homogenisation and co-culture techniques.

In Experiment 2, two cats were inoculated with FIPV-P11 and one with control fluid as before, and were examined clinically for the development of clinical signs of FIP. Following euthanasia, one cat was submitted for post-mortem examination. A number of tissues and samples were taken from both infected cats and examined for virus by the homogenisation method.

RESULTS

*Clinical signs*

In Experiment 1, 2 days after inoculation with FIPV, all of the cats were pyrexic (40.2–41.7°C), and the cat killed on Day 14 showed a further episode of pyrexia on Days 12 and 13 after inoculation. The cats were killed before clinical signs of FIP occurred.

In Experiment 2, the 2 cats inoculated with FIPV developed clinical signs of FIP, 20 and 22 days after inoculation and were killed 2 days later. Clinical signs consisted of depression, anorexia, icterus, dyspnoea and vomiting.

No clinical signs were observed in the control cats.

*Isolation of virus*

Virus shedding patterns in oropharyngeal and faecal samples from cats in Experiment 1 are shown in Table 1. Virus was first detected in oropharyngeal swabs from two cats 2 and 4 days after inoculation and in the faeces of three cats 3, 4 and 5 days after inoculation, and shedding continued until euthanasia. Virus was detected by IFT in the first passage in FEL cells. Virus was also isolated in cell culture from both homogenised and co-cultured small intestinal and tonsil tissue samples from all cats. In the co-cultures, CPE appeared after 3 passages in FEL cells and the presence of virus was confirmed by IFT. Virus

TABLE 1

Virus shedding in cats inoculated orally with FIPV (Experiment 1)

| Day of sacrifice | Sample | Duration of virus shedding (days) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 1 | o/p | – | | | | | | | | | | | | | |
| | faeces | – | | | | | | | | | | | | | |
| 2 | o/p | – | – | | | | | | | | | | | | |
| | faeces | – | – | | | | | | | | | | | | |
| 3 | o/p | – | – | – | | | | | | | | | | | |
| | faeces | – | – | + | | | | | | | | | | | |
| 7 | o/p | – | + | + | + | + | + | + | | | | | | | |
| | faeces | – | – | – | + | + | + | + | | | | | | | |
| 14 | o/p | – | – | – | + | + | + | + | + | + | + | + | + | + | + |
| | faeces | – | – | – | – | + | + | + | + | + | + | + | + | + | + |

+virus detected; –virus not detected; o/p oropharyngeal swab.

TABLE 2

Virus isolation by homogenisation from tissues of cats inoculated orally with FIPV (Experiment 2)

| Tissue tested | Cat A (killed Day 22) | Cat B (killed Day 24) |
|---|---|---|
| Stomach | – | – |
| Jejunum | + | – |
| Duodenum | + | – |
| Ileum | + | – |
| Caecum | + | – |
| Colon | + | – |
| Kidney | – | – |
| Pancreas | – | – |
| Spleen | – | – |
| Liver | – | – |
| Mesenteric lymph node | + | N.D. |
| Peritoneum | + | – |
| Soft palate | – | – |
| Tonsil | – | – |
| Mandibular lymph node | – | N.D. |
| Maxillary lymph node | N.D. | – |
| Trachea | – | – |
| Brain | – | N.D. |
| Spinal cord | – | N.D. |
| Lung | – | – |
| Ovary | N.D. | – |
| Bone marrow | – | – |
| Oro-pharyngeal swab | – | N.D. |
| Urine | – | – |
| Faeces | – | – |
| Whole blood | – | – |
| Plasma | – | – |
| Peritoneal fluid (including cells) | +* | – |
| Peritoneal fluid (excluding cells)$^{+}$ | – | – |

N.D., not done; *positive on second passage; all other samples positive on first passage in cell culture; $^{+}$cells removed by centrifugation prior to inoculation of cultures.

was not isolated from the tracheal tissue samples nor from any of the samples from the control cats.

In Experiment 2, virus was isolated from a number of abdominal tissues taken at post-mortem examination from Cat A, killed 22 days after inoculation, but no tissues from Cat B yielded virus (Table 2). In addition, virus was isolated from the cellular fraction of peritoneal fluid from Cat A.

No virus was isolated from the control cat.

*Serology*

Serum samples taken at the time of sacrifice showed the following antibody titres: Day 1, <10; Day 2, <10; Day 3, <10; Day 7, 20; and Day 14, 360 (Experiment 1) and Day 22, 384; Day 24, 256 (Experiment 2). None of the control cats seroconverted.

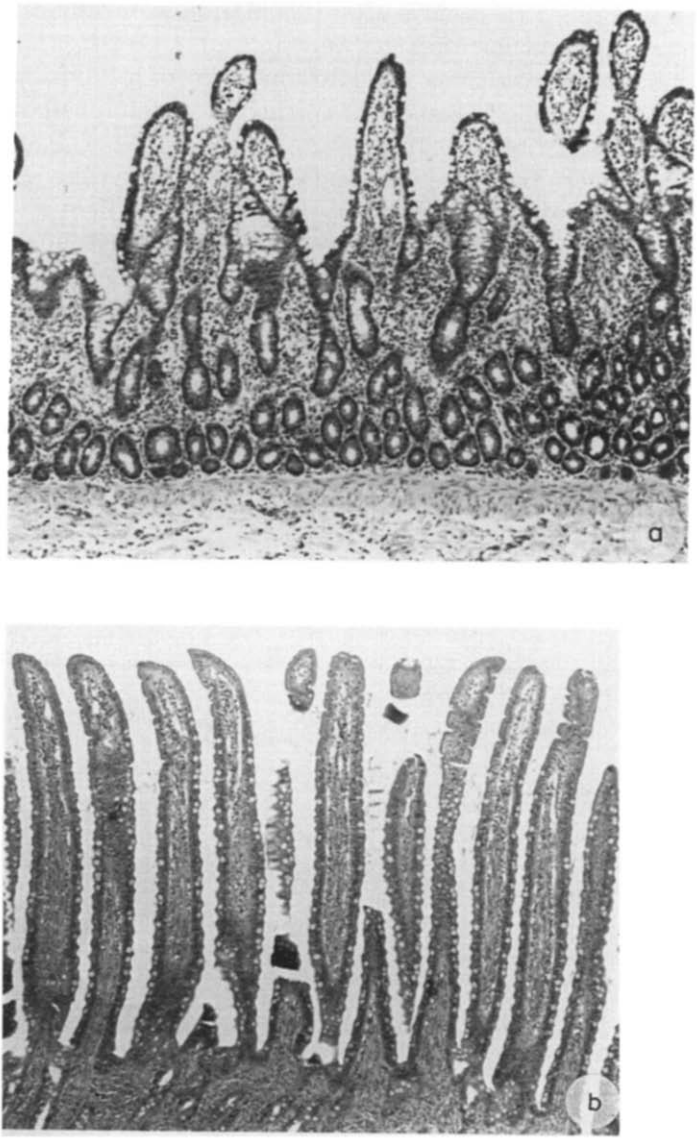


Fig. 1. (a) Blunting and fusion of villi in the ileum of cat killed 14 days after oral inoculation with FIPV. (b) Normal cat ileum.

*Pathological findings*

Gross abnormalities were not seen in any of the cats in Experiment 1.

On histological examination, there were changes in the tonsils of all cats inoculated with FIPV in this experiment. In the cat killed on Day 1 after inoculation there was a slight polymorphonuclear cell infiltration of the tonsil. The tonsils of the cats killed 2, 3, 7 or 14 days after inoculation were similar. Polymorphonuclear neutrophils and macrophages were present in the tonsillar lymphoid area and epithelium with a surface exudate consisting of inflammatory cells and fibrin. There was focal erosion of the epithelium of the tonsil of the cat killed 14 days after inoculation.

Lymph nodes were congested in the cats killed on Days 1 and 2 after inoculation, but lymph nodes of the remaining cats appeared normal. There appeared to be a reduction in thymic volume in the cat killed 7 days after inoculation, with loss of distinction between the cortex and medulla.

Changes were seen in the intestinal tract of the cat killed on Day 14 after inoculation. There was blunting and fusion of villi in the distal small intestine (Fig. 1). The remaining small intestine and colon were normal.

TABLE 3

Detection of FIPV antigen by immunofluorescence in tisses taken at necropsy after oral inoculation with FIPV (Experiment 1)

| Tissue tested | Killed on Day | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 7 | 14 |
| Peyers patches | – | – | – | + | + |
| Stomach | – | – | – | – | – |
| Duodenum | + | – | – | + | – |
| Jejunum | + | + | + | + | – |
| Ileum | + | + | + | + | – |
| Caecum | – | + | + | + | + |
| Colon | – | – | + | – | + |
| Kidney | – | – | – | – | – |
| Tonsil | + | + | + | + | + |
| Mes LN | – | – | + | – | – |
| Pop LN | – | – | – | – | – |
| Trachea | + | – | – | – | – |
| Lung | + | – | – | – | – |
| Spleen | – | – | – | – | – |
| Pancreas | – | – | – | – | – |
| Liver | – | – | – | – | + |

Mes LN, mesenteric lymph node; pop LN, popliteal lymph node; – no fluorescence seen; + virus antigen detected.

There was a trace of periportal polymorph infiltration in the liver of the cat killed on Day 14 after inoculation.

The kidneys of all the cats, including the two controls had prominent calcinosis in the interstitium of the medulla.

Focal congestion, haemorrhage and oedema were observed in the lungs of the cat killed 1 day after inoculation; no such changes were seen in any of the cats killed at later times.

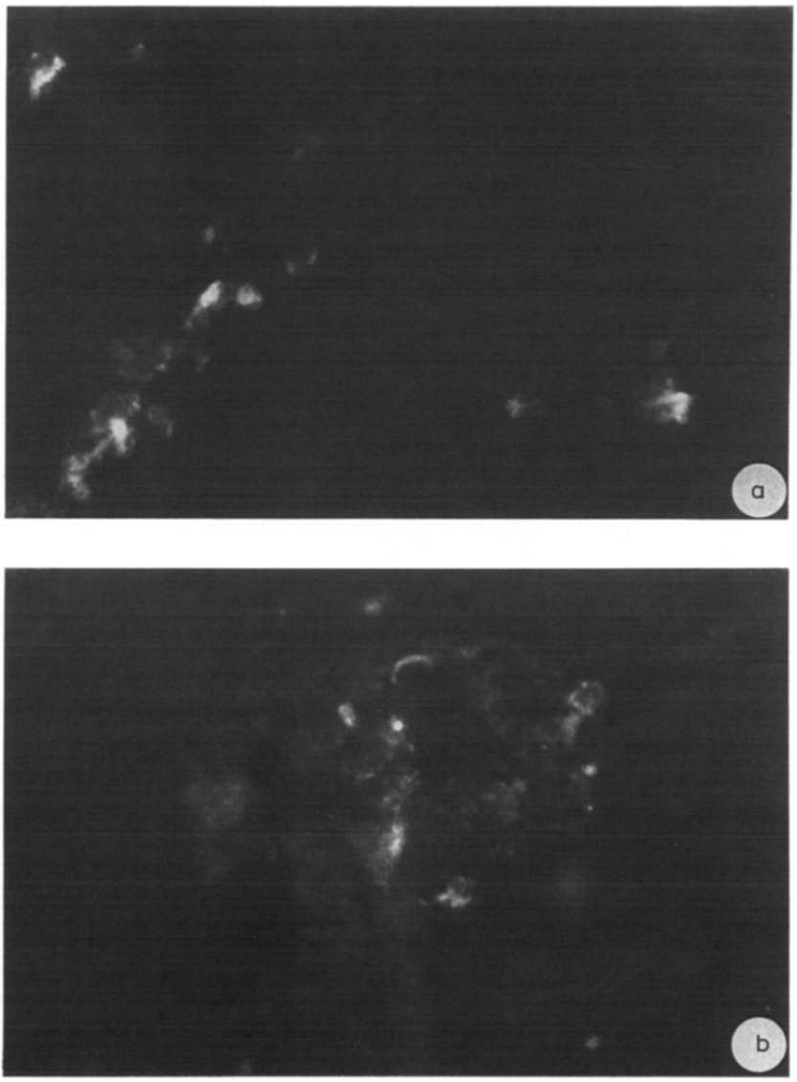


Fig. 2. (a) Immunofluorescence in tonsil of cat killed 1 day after oral inoculation with FIPV. (b) Immunofluorescence in the ileum of cat killed 3 days after oral inoculation with FIPV.

Remaining tissues from all cats were normal.

In Experiment 2, post-mortem examination of Cat A revealed lesions compatible with an early clinical case of FIP. On gross examination, the small intestine was slightly reddened, with enlargement of the mesenteric lymph nodes. Approximately 60 ml of fibrinous fluid was present in the abdominal cavity and 20 ml of similar fluid in the thoracic cavity. On histological examination, there was a thin band of fibrin and inflammatory cells on the mesenteric surface of the stomach, and in the jejunum a solitary mesenteric focus of inflammatory cells interspersed with cell debris was present. There was focal dilatation of duodenal crypts with small amounts of intraluminal debris. In mesenteric and mandibular lymph nodes there was diffuse and focal expansion on lymphoid tissue, congestion, oedema and infiltration of inflammatory cells, but histological changes were not present in the tonsils. There were no changes recognised in the liver to account for the icterus.

*Immunofluorescence*

The results of the immunofluorescence tests on tissues from the cats in Experiment 1 are summarised in Table 3. Viral antigen was detected in all the tonsils from the virus-inoculated cats; the fluorescence was particularly intense in the cat killed on Day 1 (Fig. 2a). Immunofluorescence was observed in lung tissue only once, in the cat killed on Day 1 after inoculation. Immunofluorescence was not detected in any of the stomachs, but it was observed in most of the jejunum and ileum samples (Fig. 2b), in the duodenum on Days 1 and 7 after inoculation, in all the caecal samples from Day 2 to Day 14 after inoculation and in the lumen of the colon on Days 3 and 14 after inoculation. The fluorescence was most intense in the intestinal samples obtained on Days 7 and 14 after inoculation. Viral antigen was detected in the mesenteric lymph nodes on Day 3 after inoculation and in Peyer's patches on Days 7 and 14, although it was not associated with any particular cell type. Fourteen days after inoculation, slight immunofluorescence was observed around the portal areas of the liver. Immunofluorescence was not seen associated with any other tissues examined, nor in any samples taken from the control cats.

*Electron microscopy*

Coronavirus-like particles were observed in the enterocytes of the jejunum in all virus-inoculated cats in Experiment 1. Similar particles were not observed in the lungs of the virus-inoculated cats nor in samples from the control cats.

## DISCUSSION

This study has demonstrated a number of findings in the early stages of infection following oral inoculation of FIPV, which may be helpful in elucidating aspects of the pathogenesis and epidemiology of the disease.

Firstly, it appears that following oral inoculation of the virus, initial viral localisation and subsequent replication probably occurs in the tonsil. Positive viral immunofluorescence and histological changes were seen in this site throughout the first 14 days after inoculation and virus was also isolated by means of co-culture and homogenisation techniques. In addition, FIPV shedding was recorded in oro-pharyngeal secretions. Evidence of viral replication or pathological changes were found only infrequently in the tonsils by Weiss and Scott (1981b), but they used an aerosol rather than the oral route for infection, which may have influenced initial site of virus localisation. There may also be differences between isolates or dosage or possibly the cats themselves may influence pathogenesis (Pedersen and Floyd, 1985).

It is not clear exactly which cell type supports replication of FIPV in the tonsil. Histologically there was a mixed cellular infiltrate, but not until Day 14 was any epithelial damage detected. There is some evidence that macrophages may be a target cell for FIPV (Pedersen, 1976; Horzinek and Osterhaus, 1979). By the time FIP was apparent clinically (Day 22), histological changes were no longer present in the tonsil, although changes were seen in mandibular lymph nodes at this time.

Evidence of viral replication was found by means of immunofluorescence and virus isolation in small intestine, caecum and colon, throughout the course of the disease. However, histological changes were not seen until Day 14 in the distal small intestine. These changes were similar to those described for FIP by Hayashi et al. (1982), and those observed in infection with other enteric coronaviruses: feline enteric coronavirus (FECV), transmissible gastroenteritis virus (TGEV), and canine coronavirus (CCV) (Keenan et al., 1976; Kerzner et al., 1977; Pedersen et al., 1981b). However in both TGEV and CCV infections, histological changes are usually apparent within a few days of infection whereas in this experiment the changes were not apparent until Day 14. It is possible that despite the presence of virus in enterocytes, demonstrated by EM and IFT, viral damage was insufficient to cause recognisable histological changes. It is also possible that the pathology of the viral infection in the intestine was immune-mediated, since it was not seen until Day 14 when high serum neutralising antibody levels had developed.

It was of interest that there were also slight histological changes and specific immunofluorescence on Day 14 in the liver. However, by Day 22, when FIP was clinically apparent, histological changes were mainly confined to duodenal mucosa and gut-associated lymphoid tissue, although changes characteristic

of FIP were seen in omentum and jejunal mesentery. Moreover, a fluid exudate typical of FIP was present in peritoneal and thoracic cavities.

Whether virus was present in the gut and Peyer's patches as a result of ingestion of virus, or as a result of haematological spread, is not known.

The changes in the lung of the cat killed on Day 1 may have been caused by inhalation of virus when the virus was administered, since no further evidence of viral replication or histological changes was seen in any respiratory tissues in the other cats. Hence the route of infection may determine the sites of the early changes seen in FIP, and may explain the differences between the observations of Hayashi et al. (1982) and Weiss and Scott (1981b).

The patterns of virus shedding in oro-pharyngeal secretions and faeces of virus-inoculated cats broadly confirm more detailed studies reported recently elsewhere (Stoddart et al., 1988). Such virus presumably is infectious to other cats and therefore is of epidemiological importance.

Thus, following oral inoculation, FIPV appears to replicate initially in the tonsil and in the small intestine, although associated histological changes in the gut do not appear until relatively late in the course of infection. Furthermore, enteric lesions are still present when classic histological signs of FIP are starting to develop although infectious virus is no longer being shed into the faeces at this stage of the disease (Stoddart et al., 1988). Evidence of viral replication in other tissues does not appear until later in the course of the infection.

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