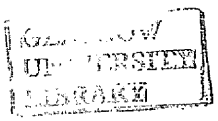
A STUDY OF FELINE CALICIVIRUS PLAQUE TYPES

by

Edward Ormerod

Thesis presented to The University of Glasgow, Department of Veterinary Pathology, for the degree of Doctor of Philosophy, May 1979.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346



CONTENTS

List of Tables

List of Figures

Acknowledgements

I am very grateful to Professor W.F.H. Jarrett, in whose department this work was carried out, for the use of the department's facilities.

I should like to express my sincere thanks to Dr. O. Jarrett for his invaluable advice, encouragement and assistance.

I am very grateful to other colleagues in the department for their help and advice and in particular to Dr. I.A.P. McAndlish who collaborated in the live animal experiments, Dr. H. Laird for her instruction and guidance in electron microscopy and Dr. D. Taylor for bacteriological support.

Special words of thanks are due to Dr. M. Stewart and Miss J. Cole for their splendid and considerable assistance in establishing and maintaining a specific pathogen free cat unit.

Mrs C. McClay was extremely helpful in preparing thin section material for electron microscopic examination.

I am very much obliged to Mr A. Finnie, Mr A. May and Mr C. Wilson for providing the photographs.

The manuscript was typed by Mrs M. McAuley, Mrs J. Downie and Miss J. Cole to whom I am indebted.

Finally, I should like to thank the Wellcome Trust for providing me with a scholarship which enabled this work to be carried out.

Summary

Genetic variation among Feline caliciviruses (FCV) was studied using the plaque character of an isolate as a marker. The basis for differences between isolates in plaque size and the relationship between plaque type and virulence was examined.

Initial work, reported in Chapter 3, was required to formulate a plaque assay method which was efficient and statistically acceptable as a particle counting system. To facilitate the differentiation of different plaque size populations it was found necessary to treat cultures with anti-virus serum or wash them with medium after virus adsorption.

It was observed that there was wide variation in plaque size between FCV strains (Chapter 4). Strains were arranged into four groups based on plaque size which were designated minute plaque (mp), small plaque (sp), large plaque (lp) and extra-large plaque (ep). The development of plaques of a number of strains, and significantly all mp forming strains, was found to be inhibited by the sulphated polysaccharide-containing fraction of agar. Evidence was presented which indicates that the plaque morphology of FCV is a highly mutable characteristic and that selection of smaller plaque variants (mp and sp) frequently occurs when isolates are passaged in cell culture.

A correlation between plaque size and virulence was noted (Chapter 5): isolates belonging to the mp group were all of low virulence whereas two highly virulent isolates belonged to the ep group. This correlation was investigated in detail by comparing the disease produced by two cloned isolates, G1 (ep forming) and G10 (mp forming) in specific pathogen free cats and by comparing their *in vitro* biological characteristics (Chapter 6). *In vivo*, G1 produced a more widespread infection than G10 with higher titres of virus present in turbinate and lung tissues sampled at necropsy. *In vitro*, the main reason for the large difference in plaque size between these isolates was demonstrated to be polyanion inhibition of the mp forming isolate although differences were also detected in growth curves which probably contributed to the plaque size difference. It was postulated that these different biological characteristics might also explain the observed difference in virulence between mp forming and ep forming isolates.

An electron microscopic study of isolates G1 and G10 and a number of FCV strains revealed inter-strain variation in the cytopathology induced by virus in cell culture, particularly in the way virions accumulated in infected cell cytoplasm prior to release. For example, G1 virus accumulated in large paracrystalline arrays whereas G10 virus remained in loose aggregates and occasionally in linear arrays. Inter-strain differences in antigen distribution were detected by immuno-fluorescent microscopy and it was suggested that these might be, at least in part, produced by different types of virion accumulation. There was no apparent correlation between the cytopathology induced by a virus strain and its plaque type or virulence. These observations did, however, suggest an ultrastructural basis for differences observed in single step growth curves.

Abbreviations

cpe	cytopathic effect
DEAE-dextran	diethylaminoethyl dextran
EDTA	ethylenediamino tetracetic acid
EFC	Eagle's medium plus foetal bovine serum
EMC	encephalomyocarditis virus
ep	extra-large plaque
FBS	foetal bovine serum
FCD	feline calicivirus disease
FCV	feline calicivirus
FEA	feline embryo A
FHV	feline herpesvirus
FITC	fluorescein isothiocyanate
FMDV	foot-and-mouth disease virus
FVR	feline viral rhinotracheitis
ic	infective centre
lp	large plaque
MEM	(Eagle's) Minimal Essential Medium
mpd	mean plaque diameter
PBS	phosphate buffered saline
PFU	plaque forming unit
PI	post infection
SMSV	San Miguel sea-lion virus
SN	serum neutralisation
sp	small plaque
SPF	Specific pathogen free
TS	Tris saline buffer
VESV	vesicular exanthema of swine virus.

CHAPTER 1

GENERAL INTRODUCTION

Feline respiratory disease

Feline caliciviruses: Isolation and classification
Prevalence

Epidemiological factors:
Antigenic variation
Variation in virulence
The carrier state

The present study

Feline Respiratory Disease

Respiratory infections are common in cats being most prevalent in cat breeding and boarding establishments and in cats which have been present at shows. Feline respiratory disease is highly contagious and present evidence suggests that the majority of these infections are caused by either of two viruses: feline herpesvirus (FHV) or feline calicivirus (FCV). Both of these produce, primarily, an upper respiratory tract disease which has become colloquially known as "cat flu". The clinical syndromes produced by each virus are superficially similar but it is possible to distinguish them, particularly under controlled experimental conditions. This is often much more difficult in the field where secondary bacterial and mycoplasma infections are common.

FHV is a typical herpesvirus, sharing many biological and physico-chemical properties with other members of the herpesvirus family. Only a single serotype is known to exist (Crandell, 1973). The disease it produces is called feline viral rhinotracheitis (FVR). This commonly recognised upper respiratory tract infection is characterised by its sudden onset, serous ocular and nasal discharges with sneezing and coughing accompanied by a rise in body temperature. Ocular and nasal discharges may become purulent indicating secondary bacterial infection. Often there is total cessation of food and fluid intake which together with profuse salivation at an earlier stage leads to dehydration. In most cases recovery begins after seven to ten days.

Experimental FHV infection tends to be severe (Crandell *et al.*, 1961; Povey and Johnson, 1967; Walton and Gillespie, 1970b) and many regard FHV as clinically the most important infectious agent involved in feline respiratory tract disease. Mortality may be high in kittens and in old or debilitated animals.

Feline calicivirus disease (FCD) in most cases is less severe than FVR but FCV is very common in all cat populations and some strains are highly virulent. FCD is characterised again by a sudden onset, dullness and anorexia accompanied by a rise in body temperature. Often the most prominent feature of the disease is ulceration of the dorsum and anterior border of the tongue (referred to as ulcerative glossitis) and of the hard and soft palate and external nares (Povey and Hale, 1974; Hoover and Kahn, 1975). Pneumonia is a common sequel under certain experimental conditions (Hoover and Kahn, 1975). By contrast to FVR, ocular and nasal

discharges, coughing and sneezing are not features of FCD. Again, recovery can be expected to begin after seven to ten days. FCD is generally difficult to characterise since there is marked strain variation in virulence and bacterial and mycoplasma superinfection is common.

It appears that other infectious agents which have been associated with feline respiratory disease may play only a minor role to FCV and FHV, some perhaps as secondary invaders. Among these are Chlamydia psittaci, feline reovirus, Mycoplasma felis and various bacteria.

Chlamydia psittaci, the feline pneumonitis agent, is only very rarely isolated from respiratory cases and has not been shown to be of importance in the UK (Povey and Johnson, 1971). Feline pneumonitis was originally described by Baker (1944) and is characterised by ocular and nasal discharges and sneezing, with pneumonia usually detected at necropsy. Elementary bodies can be found in monocytes in alveolar exudates but this could be the result of secondary infection after an initial viral pneumonia. A recent investigation in which feline chlamydial infection was experimentally induced, demonstrated that pulmonary lesions and lower respiratory tract disease did not occur although conjunctivitis and mild rhinitis was observed (Hoover, Kahn and Langloss, 1978).

Feline reovirus has been suggested as another cause of respiratory disease but has not yet been isolated in the UK. A type 3 reovirus was isolated in 1968 in America (Scott, Kahn and Gillespie, 1970) and the results of a limited serological survey carried out at the time indicated that feline reovirus infection (both type 3 and type 1) was common in cats. The experimental infection is associated with conjunctivitis, lacrimation, photobia and gingivitis (Scott, Kahn and Gillespie, 1970). Further isolations of feline reovirus have been infrequent (reviewed by Gillespie and Scott, 1973) and at present its importance is dubious.

Mycoplasma organisms can also be isolated from a large proportion of cases of feline respiratory disease (Tan and Miles, 1973) and there is serological evidence which supports an important role for Mycoplasma felis. M.felis has also been associated with conjunctivitis alone (Campbell et al, 1973). Experimental infections have been carried out by Blackmore and Hill (1973) and Tan (1974) and have produced very mild clinical signs. Therefore it seems likely that mycoplasmas play only a secondary role in feline respiratory disease.

Similarly, there are no indications of a primary bacterial cause of feline respiratory infection. The isolation rate of certain bacteria is seen to increase with respiratory disease, for example, Pasteurella multocida and Staphylococcus aureus (Povey and Johnson 1971), but again this is due to secondary invasion.

Isolation and classification of feline caliciviruses

In 1957 Fastier working in New Zealand isolated a new feline virus in cell culture. Later in the same year Bolin (1957) reported the successful cultivation of what appeared to be panleukopaenia virus in tissue culture. These along with further isolates were later shown to belong to a group of viruses composed of several antigenic types (Crandell and Madin, 1960; Bittle et al., 1960; Piercy and Prydie, 1963) and distinct from the feline rhinotracheitis virus isolated by Crandell and Maurer (1958).

Later it was shown that the viruses of this group exhibited the major properties of picornaviruses (Melnick et al., 1963; Bürki, 1965; Prydie, 1966; Crandell, 1967). However, these feline picornaviruses were found to differ from the other picornaviruses (enteroviruses, rhinoviruses, cardioviruses, foot-and-mouth disease virus and equine rhinoviruses) in several respects. Different physico-chemical properties were reported by Bürki (1965), Crandell (1967) and Prydie (1973). For example, the stability of the feline picornaviruses at pH 3.0 was intermediate between that of the enteroviruses and rhinoviruses; the buoyant density in caesium chloride was likewise intermediate; and the feline viruses were unstable in the presence of molar concentrations of magnesium ions at 50°C and insensitive to 2-(hydroxybenzyl) benzimidazole, in contrast to the other picornaviruses.

The most striking differences were found in the morphology of the virus: the virus particle was larger (35-40nm compared to 24-30nm) and had a characteristic appearance when negatively stained and examined by electron microscopy (Zwillenberg and Bürki, 1966; Almeida et al., 1968). Unlike other picornaviruses the subunit structure of the particles could be visualised with relative ease. The feline virus was seen to be composed of 32 structural subunits, each apparently concave in shape thus trapping the negative stain and allowing the bridging protein sides of the subunit to be outlined clearly. The other picornaviruses had convex shaped subunits and the subunit structure did not stand out in relief. The same appearance was shared by a virus isolated from pigs: vesicular exanthema of swine virus (VESV).

In 1971 the International Committee on Viral Nomenclature designated a new genus Calicivirus within the family Picornaviridae. The genus included both the feline virus and VESV, the name originating from the cup-like structure of the capsid subunits. (The prefix "calici" is derived from the Latin "calix" meaning cup).

Recent evidence has prompted some to suggest that caliciviruses are sufficiently distinct from the other picornaviruses to be removed from that family. (Black and Brown, 1975/1976). Caliciviruses have only one major structural polypeptide compared to four in the other picornaviruses; also the RNA synthesised in caliciviruses-infected cells is atypical of picornaviruses (Ehresmann and Schaffer, 1977; Black and Brown, 1977). Monocistronic translation with the formation of a large polyprotein molecule and post-translational cleavage is characteristic of picornavirus protein synthesis whereas in calicivirus replication subgenomic mRNA is first synthesised and then translated to provide the capsid protein.

Prevalence of feline caliciviruses

It is now clear that feline caliciviruses are ubiquitous in cat populations throughout the world and reports on their isolation are numerous (Bittle et al., 1960; Crandell and Madin, 1960; Bürki, 1963; Prydie, 1966; Povey 1969; Kahn and Gillespie, 1970).

Several surveys have been carried out to investigate the prevalence of FCV in sick cats and in healthy cats of different population categories. Walton and Gillespie (1970 a) found, in field studies on ill cats, that approximately equal proportions of them were shedding either FCV or FHV and a similar finding was made by Povey and Johnson (1971) when examining field cases in the UK. Wardley, Gaskell and Povey (1974) carried out a large survey involving a total of 1,500 healthy cats which produced some striking results regarding the numbers excreting FCV: 41.5% of cats in colonies; 24% of cats attending cat shows and 8% of household pet cats. A significant age distribution was noted: animals 1 year old and younger were infected in highest numbers. There was no distinction based on sex but neutered animals had a lower incidence than entire animals.

Serological surveys have been limited by the apparent antigenic heterogeneity of the FCV group but a small survey reported by Bürki (1971) indicated that cats sequentially become infected with one strain of FCV after another: kittens less than 6 months old were generally seronegative, cats up to 2 years old reacted to a single strain and older cats to several strains.

Epidemiological factors in feline calicivirus infection

There are three major factors which dominate the epidemiology of FCV. These are: variation in antigenicity resulting in a very large number of strains; variation in virulence between strains; and a carrier state characterised by persistent excretion of virus for long periods after primary infection.

Antigenic variation The most widely used test in the serological comparison of FCV isolates has been the serum neutralisation (SN) test. Using cross-neutralisation tests it was recognised that cross-reactions, of varying degrees and often only one-way, were common. (Bittle et al., 1960; Bürki, 1965; Kahn and Gillespie 1970).

Thus it was seen that antigenic variation existed between many isolates but there was some similarity between all strains. By applying the commonly used formula of Archetti and Horsfall (1950) many different serotypes could be differentiated. However, this type of analysis discredits anything less than almost complete antigenic homogeneity.

Povey (1974) carried out a large scale comparison of FCV isolates and using an arbitrary 20 antibody-units concept as is used in human rhinovirus serology (Kapikian et al., 1967), concluded that no isolate could be distinguished as a separate serotype. This has been supported by results of others (Kalunda et al.,1975; Bürki, Starustka and Ruttner, 1976). Most, if not all, FCV isolates can be regarded as serological variants of a single serotype.

The basis for this antigenic variation remains to be established. There is some recent evidence that the virus may contain two antigenic determinants, possibly a group-specific and a strain-specific determinant (Komolafe, 1978). The complement fixation test (Tan 1970) and the immunofluorescent test (Gillespie, Judkins and Kahn, 1971) give broad cross-reactions and possibly involve the group-specific determinant.

Serological studies have, in addition to clarifying the complex antigenic organisation of these viruses, revealed certain strains which exhibit a broad spectrum of antigenicity. Some of these strains have been tested in vaccination experiments with some success (Kahn, Hoover and Bittle, 1975). Therefore although antigenic variation occurs, the earlier concept of multiple FCV serotypes, and consequently a poor prospect of immunoprophylaxis, has been revised and, indeed, there are now two commercial FCV vaccines available in the UK.

Variation in virulence There are several reports comparing the virulence of FCV strains (Hoover and Kahn, 1973; 1975; Povey and Hale, 1974; Wardley and Povey, 1977a) and these have demonstrated that there is considerable variation among strains in this character.

For example, Hoover and Kahn (1973) compared the virulence of two strains, FPV 255 and KCD. Groups of specific pathogen free cats were exposed to aerosols of each strain. Strain FPV 255 infection resulted in anorexia, depression, lingual and palatine ulceration and a proliferative interstitial pneumonia. Infection by strain KCD resulted only in transient pyrexia with tiny vesicles and ulceration of the lingual and tonsilar mucosa. These two strains represent FCV isolates of high and low virulence. In further comparisons of isolates (Hoover and Kahn, 1975) strains of intermediate virulence were found and also a few strains which could be considered to be avirulent: for example, strain F 10.

The carrier state An indication of a persistent infection occurring in the natural disease was provided by Walton and Gillespie (1970a) who recovered FCV in oral swabs from apparently healthy adult cats in a cattery at the same time as kittens in the same cattery had a fatal respiratory infection. A FCV carrier state has been suspected by other workers (for example, Bürki, 1965; Prydie, 1966). Kahn and Gillespie (1971) reported the recovery of FCV from the tonsillar tissues of cats that had recovered from all clinical signs of the infection 34 days after experimental administration of virus.

The epidemiological importance of the carrier state was demonstrated by Povey, Wardley and Jessen (1973). This report and that of Wardley (1976) provided evidence that the period of asymptomatic excretion could be very long: two carrier cats continuously excreted virus for at least 2 years. In the latter study it was observed that the amount of virus excreted fluctuated to a significant degree and that the excreted virus underwent considerable antigenic change with time (virus collected at various points in time were compared in SN kinetic studies). From these results it would seem likely that antigenic drift occurs during the carrier state. It is possible that antivirus antibody is responsible for this drift by applying selection pressure.

To summarise the epidemiological situation: a large number of healthy cats apparently excrete FCV. Presumably most of these animals are carriers but some may be acutely infected by an avirulent strain. Susceptible animals (that is, young adults and kittens with little or

no specific immunity) are therefore always at risk but particularly when grouped together. Recurrent infection occurs since although there is antigenic similarity between strains there is sufficient difference between many of them to allow infection.

The present study

One factor of particular note about the feline caliciviruses is marked inter-strain variation in certain characters and it was the main aim of this study to examine the basis of this variation, particularly variation in virulence. Diversity in antigenicity and pathogenicity has been described above and these strain-to-strain differences may be considered as the observed effect of genetic variation. If a marker for genetic variation could be found and studied, it might help define the basis for this diversity.

The plaque character of an isolate provides such a marker and previous studies by other (Prydie, 1973; Kalunda *et al.*, 1975) have indicated that FCV isolates produce various and distinct plaque types. In the present study, plaque-type variation among FCV isolates was examined further and the relationship between plaque morphology and virulence was investigated.

The principle objectives of the study were as follows:

1) To examine a large number of FCV isolates, from different sources and to discern the various plaque characteristics; and to develop a classification of FCV isolates based on these characteristics.

2) To investigate possible correlations between plaque morphology and virulence.

3) To investigate differences between the plaque variants which might explain the observed difference in plaque morphology. Also, if plaque morphology and virulence were found to be related, these differences might provide an insight into why some strains are virulent and others not.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Cells

Media

Cell culture

Viruses

Virus isolation, cloning and stock production

Virus purification

Immune sera

Neutralisation (plaque reduction) tests

Experimental cats

Cells

A feline embryo cell line, FEA (Jarrett, Laird and Hay, 1973), was used exclusively. These cells were used between passage number 10 and 40.

Media

Cell monolayers were grown in Eagles Minimal Essential Medium (MEM), Glasgow modification (Macpherson and Stoker, 1962), which was prepared at the Institute of Virology, University of Glasgow. Growth medium was supplemented with 10% foetal bovine serum, (FBS, Gibco Biocult Ltd) (EFC/10) and maintenance medium with 5% FBS (EFC/5).

Leibovitz L-15 medium (Gibco Biocult Ltd) was used in place of MEM in certain situations where the atmospheric CO_2 concentration could not be maintained at 5%; for example, in growth curve experiments involving repeated sampling at short time intervals. The medium used in plaque assays was supplemented with agar (bacto-agar, DIFCO). Stock agar was prepared by adding 72g agar powder to 2 litres of deionised water (3.6% w/v). This suspension was autoclaved at 15lbs/in^2 pressure for 15 min. at which point the powder had dissolved. While the solution was still molten it was dispensed in 25 ml volumes and re-autoclaved at 15lbs/in^2 for 15 min. Agar was incorporated into the overlay at a final concentration of 0.9%. To achieve this, 25 ml of stock agar was heated in a water bath to boiling point, then cooled to 44°C. FBS was then added to a concentration of 5%.

Cell culture

Stock FEA cells were grown in 2.5l rotating bottles containing 100 ml of EFC/10 and maintained at 37°C. Cells were subcultured at 1:3 or 4, generally at weekly intervals. Monolayers were dispersed by removing the growth medium and rinsing, first with 20 ml of 0.02% EDTA, followed by 25 ml 0.01% trypsin in 0.02% EDTA. The cells were resuspended in EFC/10 and transferred into fresh bottles which were gassed with 5% CO_2 in air and returned to the 37°C hot room. The bottles were seeded with approximately 2-3 x 10^7 cells and yielded 6-8 x 10^7 cells when confluent.

For use in plaque assays and other procedures, cells were cultured in 5 cm polystyrene plates (Nunclon-Delta) with 4 ml EFC/10. The plates were seeded with 2 x 10^6 cells and confluency was obtained in 24 hours. The plates were maintained in a humidified incubator, in an atmosphere of 5% CO_2 in air and at 37°C.

Viruses

FCV isolates FC, CFI, 17FRV, KCD, FJ, FS, F5, F10, F11, F17 and F19 were obtained from E. Davies, Norden Laboratories, Lincoln, Nebraska: isolates FPL, F9, M8 and 68-2024 from R.C. Povey, Ontario Veterinary College, Guelph, Canada: isolates 135/62, 277/62, 337/61, 344/61 and 377/61 from F. Bürki, Institute of Virology, Veterinary University, Vienna, Austria: and isolate FPV255 from E.A. Hoover, Ohio State University, Columbus. The original references and passage history (where known) of these isolates are given in Table 4.1.

Local isolates (designated FCV-G and numbered) were obtained by swabbing cats attending a Glasgow veterinary clinic and from cats in our experimental colony (see Table 4.2).

Virus isolation and cloning

Local isolates were obtained from both ill and apparently healthy cats of various ages (Table 3) by oral swabbing. Cottonwool-tipped, sterile swabs were inserted to the tonsillar region and gently rotated until thoroughly wetted, mainly with saliva. The swabs were deposited into glass bottles containing 3 ml MEM in which they were transported to the laboratory. For each sample, the swab was removed and the medium was centrifuged at 2,500 rpm for 15 mins to remove any gross debris. The supernatant was collected and used to inoculate 5 cm plates containing confluent monolayers of FEA cells. After an adsorption time of 60 mins at 37°C in an atmosphere of 5% CO_2 in air, the inoculum was removed and the monolayers were washed three times in MEM. Four ml of EFC/10 was then added and the plates were incubated at 37°C and were observed 24 and 48 hours later. The presence of FCV was recognised by the appearance of a characteristic rapid, cytolytic cytopathic effect (cpe). This was initially focal: cells became rounded, more refractile and soon lost contact with the surface of the plate. Later, small satellite, secondary foci developed and the cpe spread until at 48 hours all cells were in suspension, usually as single cells or occasionally in small clusters. Negative plates were passaged once before discarding.

Virus clones were produced by plaque purification. FEA monolayers were inoculated with the terminal dilution of an isolate and after a 60 min adsorption period, a semi-solid agar overlay was applied. Plaques developed and could be visualised with ease after 48 hours incubation at

37°C. Plates containing only one plaque were selected and the position of a suitable plaque was marked in ink on the underside of the plate. The plug of agar over the plaque and the cells forming the plaque itself were transferred with a sterile Pasteur pipette into a vial containing 1 ml of MEM. Dilutions were made of this first clone virus suspension and monolayers were infected as above. A single plaque was picked to produce a second clone virus suspension and by repeating the process for a third time the resulting virus suspension was considered to be genetically purified: i.e., derived from a single infectious particle.

Virus stocks were made from the third clone virus suspension by passage in FEA cells. For the three clones which have been used frequently in this study (FCV G1, G2 and G10), nucleus stocks were produced by two passages in FEA cells, each time infecting monolayers and harvesting the culture after 24 hours. The suspension obtained was ultrasonicated for 30 seconds (24.78 K^c/s), centrifuged at 2,000 rpm for 15 min to remove cell debris and the supernatant fluid was dispensed in 0.5 ml volumes and stored at -70°C. From nucleus stocks, working virus stock was made by a further passage in FEA cells. Working stocks were stored at -20°C.

As described in Chapter 4, a single FCV isolate may contain more than one plaque variant. Clones G1 and G2 were plaque variants which originated from a single mouth swab. When medium from this mouth swab was assayed under agar, two types of plaque could be distinguished which differed in size. A larger plaque was cloned and designated FCV G1 and a smaller plaque cloned and designated FCV G2 . Clone G10 also originated from a mixed plaque type population contained in a single isolate.

Virus purification

Each of fourteen 2.5 l rotating bottles (containing a total of approximately 10^9 cells) was infected with 1 ml of stock virus at a virus-cell ratio of 2:1. After an adsorption period of 60 min at 37°C, 19ml of EFC/10 was added to each bottle. The cultures were harvested after 18 hours and the infected cell suspension was centrifuged at 7,000 rpm for 15 min to remove cell debris. Subsequent procedures were carried out at 4°C. Virus was precipitated from the supernatant fluid by the addition of an equal volume of cold saturated ammonium sulphate neutralised with sodium hydroxide. The precipitate was collected by centrifugation at 5,000rpm for 10 min and resuspended in a small volume of TS buffer (0.1M NaCl, 0.01M tris, 0.001M EDTA, pH 7.4).

Radiolabelled virus was prepared as follows: the monolayer contained in a 2.5 l bottle was initially starved of leucine for a 20 min period by incubating in leucine-free MEM (Gibco Biocult Ltd) and was then infected with 20ml of a virus suspension in the same medium containing ^{3}H-leucine of specific activity 50μCi per m mole. (The Radiochemical Centre, Amersham). Virus was collected by ammonium sulphate precipitation as above and was added to the non-labelled suspension in TS.

The concentrated virus was then purified by isopycnic centrifugation. Virus was centrifuged through a linear sucrose gradient (20-54%) at 40,000 rpm for 120 min in the SW 50.1 rotor in a Beckman L265B ultracentrifuge. The visible virus band was collected and centrifuged through a CsCl gradient (density 1.15-1.50g.cm^{-3}) at 40,000 rpm for 16 hours in the SW 50.1 rotor. The gradient was then fractionated and the ^{3}H activity in the gradient was monitored. Samples of 25μl of each fraction were mixed with 1.0 ml of water in a polythene vial and 5.0 ml of scintillation fluid (NE260; Nuclear Enterprises, Edinburgh) was added. The vials were capped and shaken and the radioactivity in each was determined in an Intertechnique SL40 scintillation counter. This revealed the characteristic two peaks of activity as observed by Komolafe (1978); a virus peak and, at a lower density, a viral subunit peak. The virus peak was collected and recycled through a CsCl gradient. A single peak was obtained at a density of 1.375 g.cm^{-3}.

Immune sera

Rabbit sera Antisera, primarily for use in immunofluorescence, were prepared in young adult Dutch rabbits. Purified isolate FCV G1 was suitably diluted in distilled water, emulsified with an equal volume of Freund's complete adjuvant and inoculated into the hind leg muscles of two rabbits. Each rabbit received approximately 10^{8} plaque forming units (PFU) of virus. Four weeks later a second injection of purified virus in distilled water was administered by the subcutaneous route. After a further 10 days the rabbits were bled. The immune sera were inactivated (50°C for 30 min), dispensed in 1ml volumes and stored at -20°C.

Cat sera Serum was collected from specific pathogen free (SPF) cats by cardiac puncture under general anesthesia, immediately before necropsy (see Chapter 5). These animals had been infected by isolates FCV G1 or G10, administered by aerosol or intranasal instillation 10-14 days previously.

Neutralisation tests

In early experiments in which sera from conventionally reared cats were screened for FCV-G1 antibody (to determine their suitability as experimental animals), a simple plaque reduction neutralisation test was used. Later a less laborious, accurate and reproducible, semi-micro, plaque reduction neutralisation test was developed. Serum dilutions were made in a 96-well complement fixation plate (Nunclon-Delta). Volumes of 50µl of the sera under test were placed in the first row of wells and 50µl of diluent (L15 medium) into the remaining wells. Using a microdiluter (Cooke Engineering Co.), twofold dilutions of sera were made. Stock virus was diluted in L15 medium to a concentration of 10^5PFU/ml and 50µl (containing $5x10^3$ PFU) was added to each well. After a reaction period of 1 hour at 37°C the serum-virus mixtures were diluted 1:100 (20µl from each well into 2 ml MEM) to prevent further neutralisation of the remaining virus. The residual infectivity was measured by plaque assay. A virus control well was included in each test: 50µl of virus dilution was added to 50µl of L15 medium and was incubated, diluted and assayed as above. Neutralisation titres were expressed as the reciprocal of the serum dilution which produced a 50% reduction in plaque numbers.

Experimental cats

Except for a pilot study where conventionally reared, seronegative kittens were exposed to a FCV G1 infection, specific pathogen free cats were used.

A group of 20 adult SPF cats of both sexes were obtained from the MRC Laboratory Animals Centre, Carshalton. Until required for experiment, the cats were housed as a free-running group in an isolated building. Sterile precautions were taken for the entry of personnel, food and litter. Facilities for positive pressure ventilation were not available. However, random swab sampling for bacteriological and virological examination, along with pre-inoculation swabs taken from all cats used in experiments, revealed that no cytopathogenic virus was present and there was no significant change in bacterial flora.

Animals were removed in groups of 3 at intervals over a 16 week period from this building and transported in individual, plastic, sterile containers to a separate experimental building.

CHAPTER 3

THE PLAQUE ASSAY

Introduction

Materials and Methods

1. Optimal conditions of the assay.
2. Suitability for use as a particle counting system.
3. Plaque size variability.

Results

1. Optimal conditions of the assay.
2. Suitability for use as a particle counting system.
3. Plaque size variability.

Discussion

INTRODUCTION

The 50% endpoint quantal technique has been the most commonly employed procedure for titrating feline calicivirus infectivity (for example: Bürki, 1965; Bartholomew and Gillespie, 1968).

Reports of a plaque assay method have been less frequent. Crandell (1967) described plaque production by several FCV isolates in cell monolayers in prescription bottles using an agar overlay. Bartholomew and Gillespie (1968) produced localised viral lesions in monolayers using a methylcellulose overlay. Prydie (1973) and Kalunda et al., (1975) used monolayers in Petri plates with an agar overlay and Love (1973) used an agarose overlay in an "open" plaque assay system utilising a zwitterionic buffering medium.

In this work a plaque assay was used extensively, both for quantitative studies and for the detection of different plaque types. In this section some technical aspects of the plaque assay are examined with the aim of defining the optimal conditions where maximal plaque counts could be obtained from any given virus suspension.

A statistically acceptable plaque assay should satisfy certain criteria and requirements as described by Dulbecco and Vogt (1954) and Cooper (1961, 1967). The assay formulated in the present study is examined by these criteria and by two important requirements in particular: firstly, that the plaque count must be proportional to the concentration of the inoculated virus; and secondly, that plaques should be spread among cultures of one batch according to the Poisson distribution.

Thus a maximised assay was obtained which was shown to be statistically acceptable as an infectivity-unit counting method.

As described in the following chapter, a large number of FCV isolates were examined by this plaque assay method to investigate variation in plaque characteristics. It was observed that isolates could be classified according to plaque size. However it was noted in early work that when an isolate was cloned and examined by plaque assay the range of plaque sizes observed was large. This led to initial difficulties in discriminating plaque variants. The factors causing such a wide distribution of size, in plaques containing genetically identical virus, were investigated with the aim of decreasing the scatter and so facilitating plaque variant recognition.

MATERIALS AND METHODS

1. Optimal conditions

Effect of volume on plating efficiency A constant quantity of virus (FCV G2) in volumes of diluent (MEM) varying from 0.1 to 1 ml was applied to FEA monolayers in 5 cm plates. Three plates were used for each volume. Virus was adsorbed at 37°C for 1 hour after which the inocula were removed and the plates were washed with MEM. The monolayers were overlaid with EFC/5 containing 0.9% bacto agar and reincubated at 37°C. After 48 hours they were fixed and stained with 0.5% crystal violet in 10% formalin and the plaques were counted.

Effect of adsorption time on plaque count Cultures were inoculated with a suitable dilution of virus (FCV G1) and incubated at 37°C. At intervals of 10 min, the inoculum was removed from sets of 3 plates, the cultures were washed with MEM and overlaid with agar containing medium. Plates were fixed and stained as above and the plaques were counted.

Effect of different overlays on plaque count FEA cultures in 5 cm plates, infected with a suitable dilution of virus (FCV G1), were overlaid with medium containing agar, agarose (Miles Laboratories) or methylcellulose. Agar and agarose were used at a final concentration of 0.9% in EFC/5. The preparation of agarose and its incorporation into the overlay was the same as described for agar.

Methylcellulose powder was soaked overnight in distilled water at 4°C and then autoclaved at 5lb/in^2 for 5 min. After cooling (to 4°C), the solution was mixed with appropriate concentrations of MEM supplemented with 5% FBS. Three final concentrations were tested: 0.75%, 0.9% and 1.8%.

Effect of temperature on plaque count FEA cultures in 5 cm plates, infected with a suitable dilution of virus (FCV G1), were overlaid with medium containing agar as above and placed in separate, humidified, 5% CO_2-air incubators at temperatures of 30°, 34°, 37°, or 39°C. Plates were fixed and stained as above.

2. Suitability for use as a particle counting system

The relationship between plaque count and virus concentration Virus stock (FCV G1) was diluted in MEM to approximately 1,000 PFU/ml. From this suspension, serial twofold dilutions were made in MEM. Sets of 3 FEA cultures in 5 cm plates were inoculated with 0.1 ml from each

dilution. After an adsorption time of 60 min at 37°C., the inocula were removed and the plates were washed with MEM. An agar overlay (EFC/5+0.9% bacto agar) was applied and the cultures were reincubated at 37°C. The plates were fixed and stained as before but after 30 hours to prevent plaques becoming confluent at lower dilutions.

The distribution of plaques among cultures of one batch Virus stock (FCV G10) was diluted in MEM to approximately 30 PFU/ml and 0.1 ml was inoculated into FEA cultures in 5 cm plates. After virus adsorption, removal of inocula and the rinsing of plates with MEM, an agarose overlay (EFC/5 + 0.9% agarose) was applied and the cultures were incubated at 37°C The plates were stained after 48 hours.

3. Plaque size variability

Plaque size distributions a) FEA cultures in 5 cm plates were infected with a suitable dilution of FCV G1 and G2 stock viruses and after adsorption the inocula were removed and an agar overlay was applied. The plates were fixed and stained at 48 hours as above. Plaques were magnified x 2 by projection through a photographic enlarger and their diameters measured.

b) FEA cultures infected with FCV G1 and G2 were treated with specific immune serum after virus adsorption. (The serum was obtained from a FCV infected field cat and its virus neutralising ability had previously been demonstrated against FCV G1 and G2). One ml of serum (1:100 dilution in MEM) was added to each plate after removal of the inoculum and the plates were incubated at 37°C for 1 hour. The serum was then removed and an agar overlay was applied. In half of the FCV G1 infected cultures, the monolayers were rinsed twice with MEM before application of the overlay. The plates were stained and plaques were measured as above.

To demonstrate plaque initiation in the presence of agar FEA cultures in 5 cm plates were overlaid with 1 ml, 1.5 ml and 2 ml of normal agar overlay. This was allowed to solidify and acted as an agar barrier to virus (FCV G1) which was later applied in a second overlay layer (200 PFU/ml agar overlay). In each plate, sufficient virus-containing agar was added to make the total overlay volume 4 ml. The plates were incubated at 37°C for 48 hours and fixed and stained as above.

1. Optimal conditions

Effect of volume on plating efficiency As shown in Fig. 3.1 plaque counts decreased markedly with increase in volume of inoculum dropping from an average of 54 to 12 on increasing the volume from 0.1 to 1.0 ml.

Effect of adsorption time on plaque count Plaque counts increased as the time allowed for virus adsorption increased from zero to 60 min (Fig. 3.2). Further adsorption, up to 120 min did not significantly increase the number of plaques per plate.

Effect of different overlays on plaque count An overlay incorporating agarose in place of agar produced an approximately 30% reduction in the plaque count when used in the assay of FCV G1. The rate of plaque development was also reduced resulting in a smaller plaque size at 48 hours. As will be seen later (Chapter 4) this reduction in plaque count and size with agarose is not true of all FCV isolates. For example, the plaque development of isolate G10 under an agarose overlay was found to be more rapid than when agar was used, with an increase in plaque count of approximately 50%.

Methylcellulose in the overlay at concentrations of 0.75 and 0.9% was ineffective at localising the cytopathic effect (although the actual final concentration may have been lower since it was difficult to obtain a homogeneous gel of methylcellulose in water). At a concentration of 1.8% in the overlay there was apparently no development of plaques.

Effect of temperature on plaque count There was no significant difference in the plaque counts obtained at the temperatures tested. However the largest plaques were seen in plates which were incubated at 37°C and, apparently, the rate of plaque development was slower at other temperatures: at 30°C it was necessary to incubate cultures for 4-5 days for sufficient plaque development to take place, in contrast to 2 days at 37°C.

A further experiment, designed to investigate the efficiency of virus replication at these temperatures (30°, 37° and 39°C) under liquid medium, was carried out. Four ml EFC/10 was added to FEA cultures in 5 cm plates, infected with 100 PFU of FCV G1 virus. The plates were incubated at different temperatures and at 24 hours the total amount of virus present in the cultures was determined by plaque assay. At 37°C the virus titre was 4.8×10^6 PFU/ml; at 30°C, 2.7×10^4 PFU/ml; and at 39°C, 6.2×10^5 PFU/ml.

Fig. 3.1. Effect of volume of inoculum on plating efficiency.

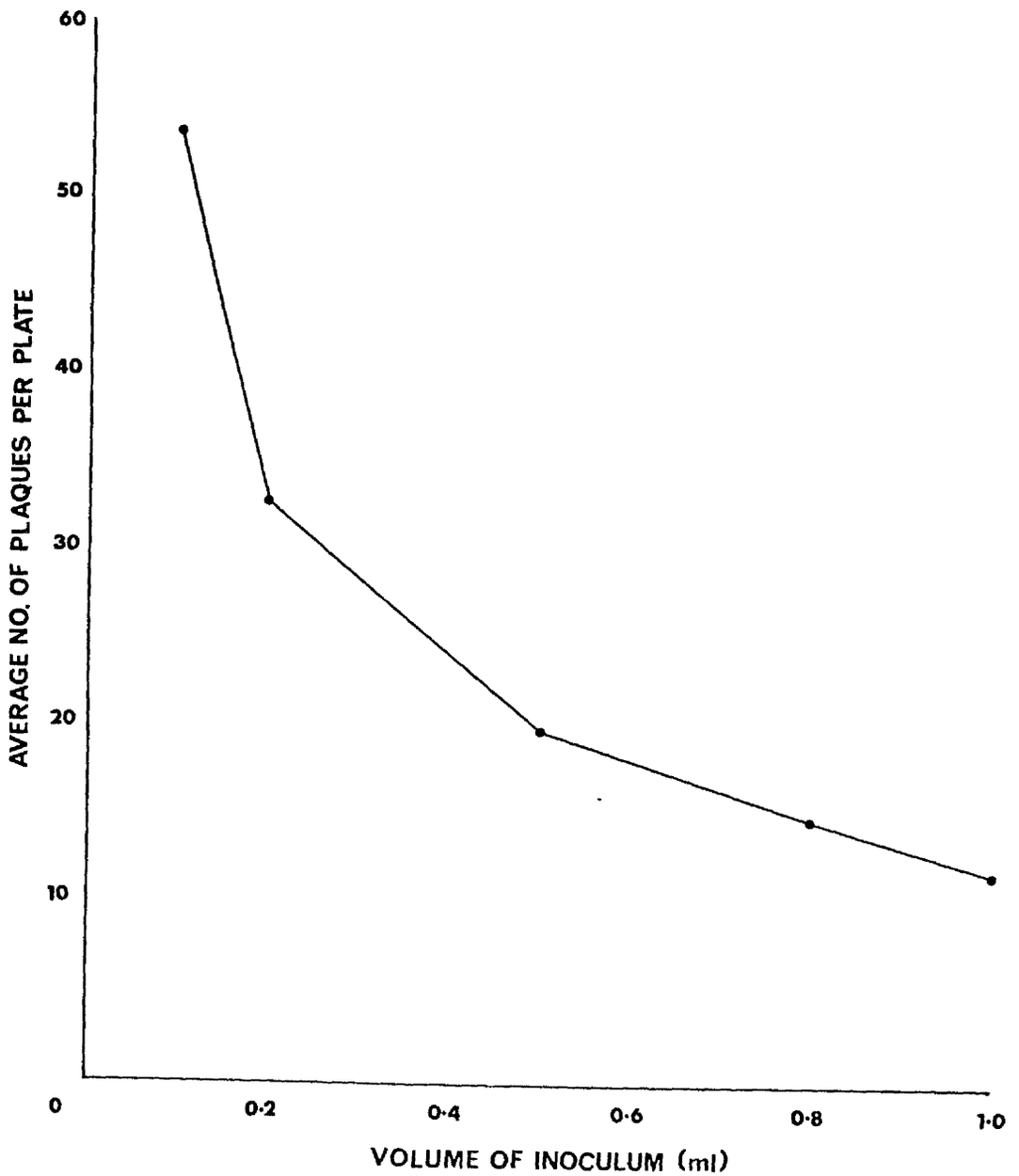
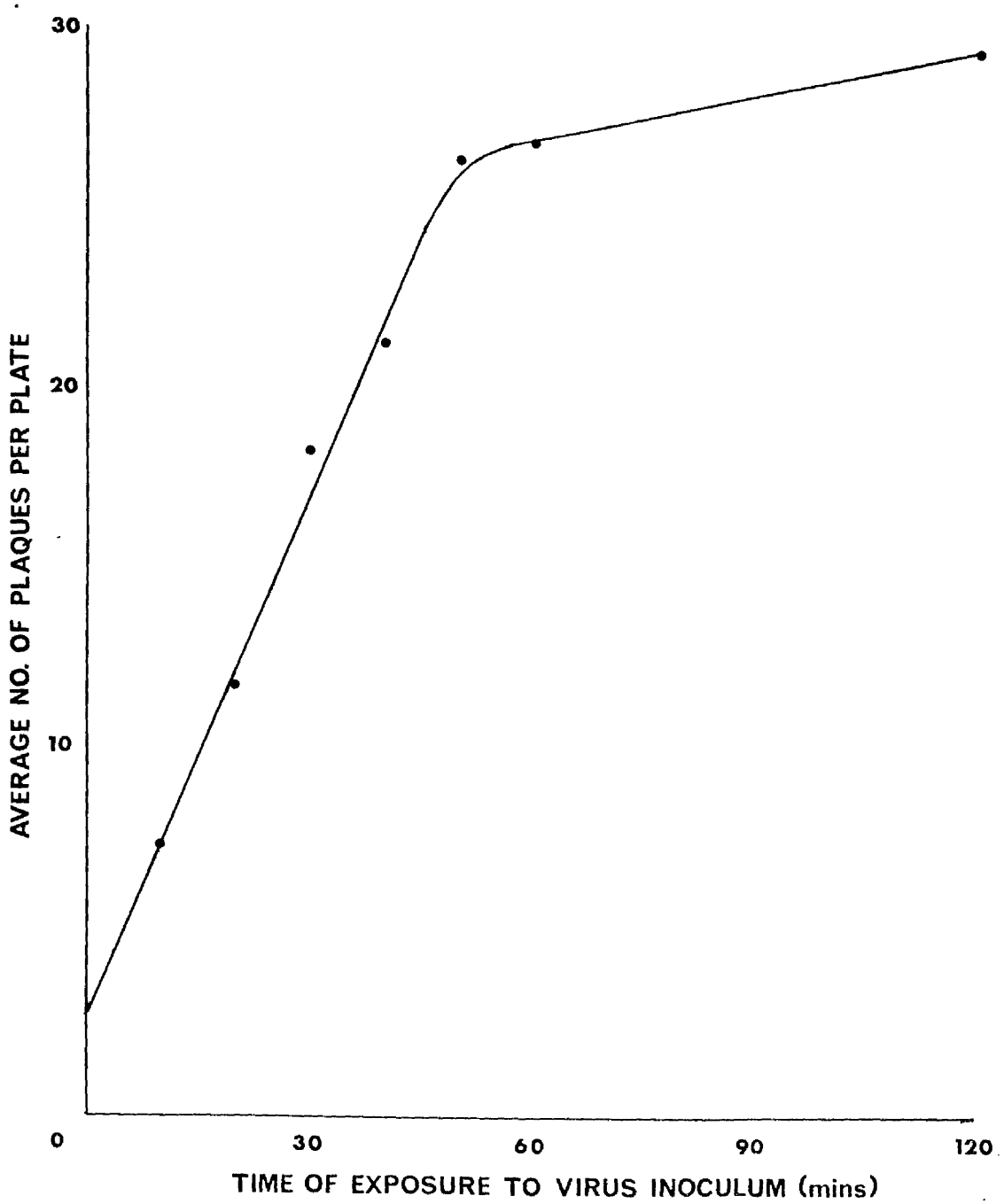


Fig. 3.2 Effect of adsorption period on plaque count.



2. Suitability for use as a particle counting system

The relationship between plaque count and virus concentration A linear relationship between relative virus concentration and the number of plaques per plate was obtained (Fig. 3.3).

The distribution of plaques among cultures of one batch A frequency distribution was made of the number of plaques per plate and is shown in Table 3.1. The mean number of plaques per plate was 3.85. The expected Poisson distribution was calculated and a goodness of fit test yielded a probability of 0.50 - 0.25 for the chance occurrence of the calculated X^2 value. Therefore the fit to a Poisson distribution was satisfactory.

3. Plaque size variability

Plaque size distribution Frequency histograms for the diameter of FCV G1 plaques are shown in Fig. 3.4. In the absence of post-adsorption antiserum treatment, a positively skewed frequency distribution was obtained (Fig. 3.4a). Several of the smaller plaques contained in the tail of the distribution were cloned and plated at suitable dilutions for measurement of plaque diameters. It was found that these smaller plaques did not breed true and in fact a distribution very similar to that produced by the parent clone was obtained. This indicated that the tail was caused by the later initiation or slower development of primary plaques (plaques developing from the initial inoculum) or by secondary plaques (plaques developing from newly formed progeny virus diffusing away from primary plaques).

The skewness was reduced by antiserum treatment after virus adsorption (Fig. 3.4b) and removed by a further two rinses with MEM (Fig. 3.4c). In both cases it was noted that there was a slight overall reduction in plaque size and also an approximately 50% reduction in plaque count.

Frequency histograms for the diameter of FCV G2 plaques were then compared to those already obtained for FCV G1. As shown later, these clones produce two distinct populations of plaque sizes. However there was an initial difficulty in distinguishing these plaque variants and this is demonstrated in Fig. 3.5a: without post-adsorption antiserum treatment, the two plaque populations merge; with antiserum treatment after virus adsorption, followed by two rinses with MEM, the overlap is much reduced (Fig. 3.5b).

Fig. 3.3 The relationship between plaque count and virus concentration.

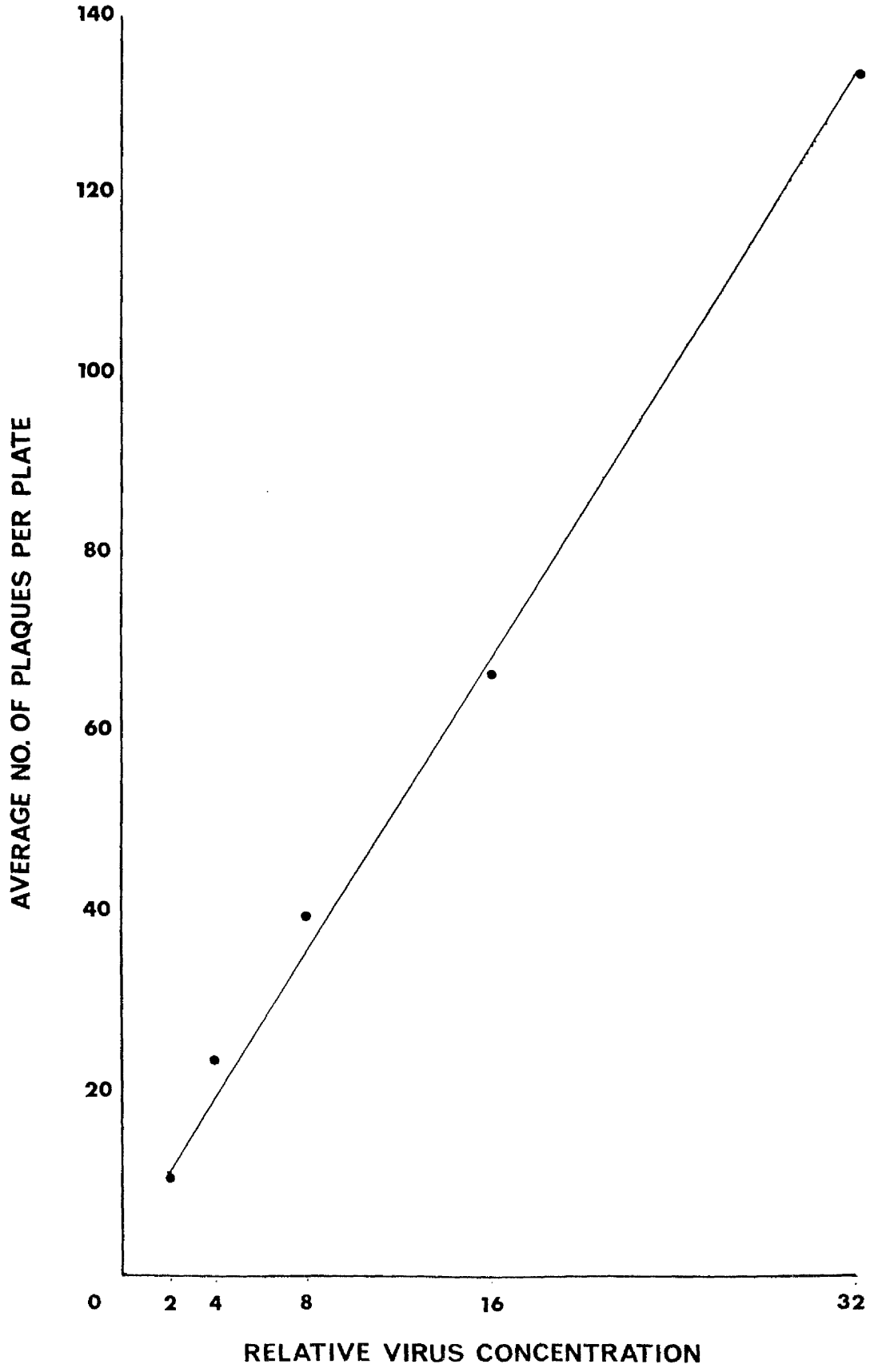


TABLE 3.1: Frequency distribution of plaques among cultures of one batch and the fit to a Poisson distribution.

NO. OF PLAQUES IN PLATES	OBSERVED FREQUENCY (f)	EXPECTED FREQUENCY (F)	(f-F)	CONTRIBUTIONS TO $x^2(\frac{(f-F)^2}{F})$
0	3	1.130	+1.87	3.09
1	5	4.349	+0.651	0.10
2	7	8.370	-1.370	0.22
3	12	10.739	+1.261	0.15
4	8	10.334	-2.334	0.53
5	5	7.955	-2.955	1.10
6	6	5.103	+0.897	0.16
7	3	2.806	+0.194	0.01
8	2	1.350		
9	1 → 4	0.577 → 2.149	+1.851	1.59
10	1	0.222		
TOTAL:	53	52.94	+0.065	6.95

d.f. = 7; p = 0.5- 0.25

Fig. 3.4 FCV G1 plaque size frequency distribution. A. Without post adsorption antiserum treatment. B. With post-adsorption antiserum treatment. C. With post-adsorption antiserum treatment plus two washes with MEM.

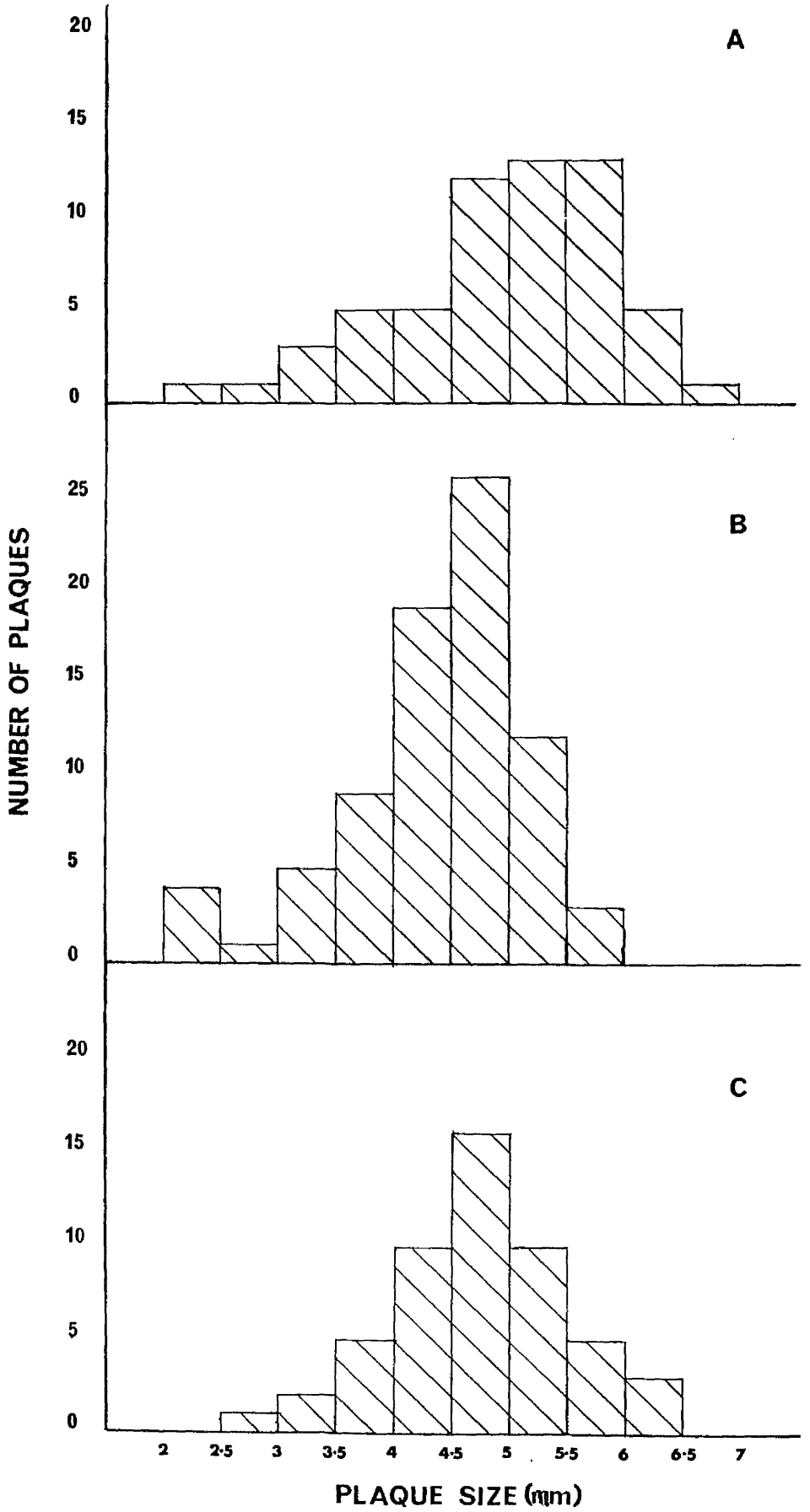
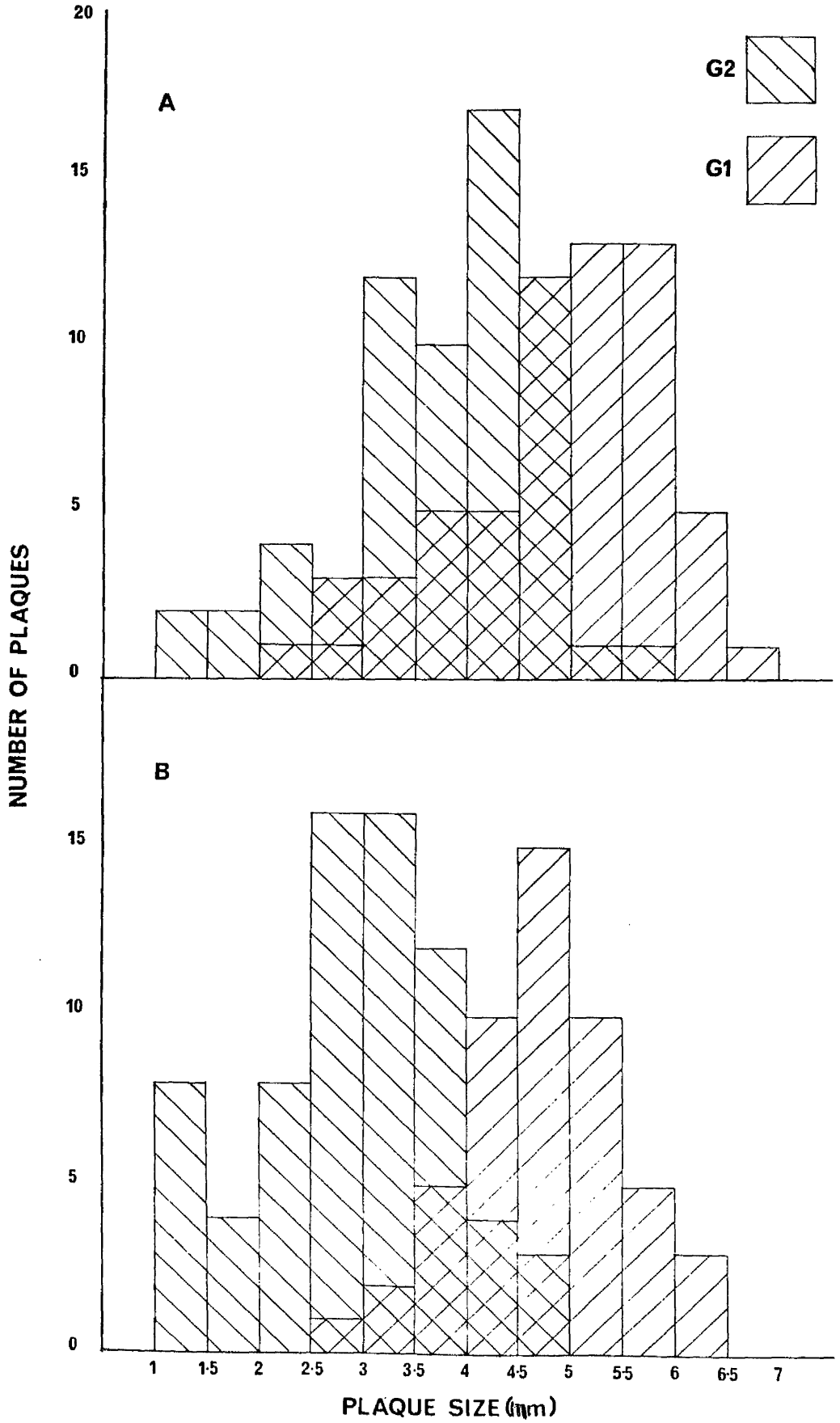


Fig. 3.5 FCV G1 and G2 plaque size frequency distributions.
A. Without post-adsorption antiserum treatment.
B. With post-adsorption antiserum treatment.



To demonstrate plaque initiation in the presence of agar FCV G1 virus was able to diffuse through the agar barriers and initiate plaques in the monolayers below. Confluent plaques were obtained in plates containing 1 ml and 1.5 ml barriers and an average of 60 plaques per plate was obtained with a 2 ml barrier. The same result was also obtained for FCV G2 virus which produced confluent plaques with a 1 ml agar barrier.

The ability of FCV to diffuse through agar and initiate cell infection meant that an agar cell suspension assay method was feasible and one was developed which was suitable for infectious centre assay. Base layers of 3 ml EFC/5 + 0.9% bacto agar were poured previously in 5 cm plates. The agar cell suspension was formed by mixing 3-4 x 10^6 FEA cells in 0.5 ml EFC/10 with 1 ml agar overlay at 44°C. Then 0.1 ml of the infected cell suspension was added and the mixed cell suspension was poured onto the base layer. Plaques developed after 48 hours and were contrasted by vital staining with neutral red (50µg/ml). This method could also be used for virus titration and gave comparable results to the standard monolayer assay.

DISCUSSION

The results of the work reported in this section indicated that the most efficient plaque assay system for the titration of FCV was as follows: using FEA cultures in 5 cm plates, virus should be diluted in MEM and inoculated onto plates in 0.1 ml volumes. Virus should be adsorbed for 50-60 min before removal of the inoculum and an overlay of EFC/5 + 0.9% bacto agar applied. Plates should be fixed and stained after 48 hours with crystal violet in formalin. This method was adopted and is referred to hereafter as the "standard plaque assay".

An inoculum of 0.1 ml produced higher titres than any larger volume (Fig. 3.1) presumably because virus was at its highest concentration and the number of virus-cell collisions was maximum. If an even lower volume of inoculum was used, there would be an increased danger of the monolayer drying out and decreased accuracy. It should be noted here that for virus isolation an inoculum of 0.5 ml was used. It was found that if the volume of the inoculum was increased with the concentration of virus constant, the plaque count increased from 0.1 - 0.5 ml but then began to decrease although with each increase in volume the total virus on the plate was increased proportionally. This would suggest that if presented with a virus suspension containing only a few infectious units (for example mouth swab medium) using an inoculum of 0.5 ml should increase the chance of contact between infectious unit and cell sheet, resulting in CPE production and the recognition of the presence of virus. The increase in plaque count using volumes from 0.1 - 0.5 ml was as expected; although the efficiency of plating was decreasing, this was overweighed by the increase in total virus. One might expect that a plateau would be reached where further increases in volume would have no effect on plaque count since the efficiency of plating of the additional virus would be very low. However, a decrease in plaque count was observed with inoculum volumes in excess of 0.5 ml. The cause of this phenomenon was not investigated but could possibly involve the physical movement of virus in large volumes (e.g., convection currents) or a reduced oxygen tension at the cell-liquid interphase. Another possibility is that at low volumes evaporation causing hypertonicity may be an important factor.

Lindenmann and Gifford (1963) while studying vaccinia virus in chick embryo cell monolayers observed that the diameters of plaques

grew linearly with time and all at the same rate; and also that plaque diameters were normally distributed since under experimental conditions plaques at a given time were not all of the same age. A similar finding was made here: for example, FCV G1 plaques, on average approximately 1 mm in diameter after 24 hours, grew linearly with time from that point, adding a growth ring approximately 2 mm wide every 24 hours. However, under simple assay conditions, at 48 hours, the plaque diameters were only near normal in distribution, being positively skewed (Fig. 3.4a). The incubation of cultures with specific antiserum after virus adsorption reduced the tail of smaller plaques. If these plaques were secondary, residual antiserum remaining in the overlay would prevent the diffusion of virus from primary plaques and the initiation of infection at another site. However, there were other results which indicated that these smaller plaques were not secondary: washing after the antiserum treatment, thereby removing much of the residual antiserum, produced a further decrease in the tail of the histogram; in other experiments, it was shown that rinsing of cultures with MEM after virus adsorption reduced the tail without the use of antiserum; also there was no increase in plaque count after 48 hours and the smaller plaques were not satellite to larger ones.

A likely explanation, accounting for the above findings, is that after the adsorption period some virus remains loosely associated with cell membranes and so can be removed by rinsing or neutralised with specific immune serum. Application of the agar overlay delays close association and penetration; possibly virus is suspended in the overlay but can still initiate plaques at some later time as was demonstrated in the agar barrier experiment. These findings are of some importance in the differentiation of plaque variants and this is demonstrated in Fig. 3.5 where, once the tail of smaller plaques is removed, the two plaque size populations become distinct.

A useful plaque assay system should satisfy 6 points as described by Cooper (1967). That virus concentration and plaque count are linearly related and that plaques are spread among cultures according to the Poisson distribution have been demonstrated here. The remaining 4 requirements have also been satisfied in other sections of this study. These are : 1) there was no plaque development in the absence of virus; 2) virus was regularly reisolated from a plaque and not from areas away from a plaque (observed during plaque cloning experiments); 3) plaque

initiation must be inhibited by low concentrations of heated specific antiserum that will not inhibit growth of another virus in the same cell system and not be inhibited by normal serum of the same source species (see cross-neutralisation tests with experimental cat sera in Chapter 5 and 4) repeated assays of virus stock in cultures of different batches should give the same titre.

CHAPTER 4

THE PLAQUE VARIANTS OF FELINE CALICIVIRUS

Introduction

Materials and Methods

1. A classification of isolates based on plaque morphology.
2. Observations on apparent *in vitro* plaque mutation.

Results

1. A classification of isolates based on plaque morphology.
2. Observations on apparent *in vitro* plaque mutation.

Discussion

INTRODUCTION

The increasing use of plaque techniques for quantitative studies of viruses led to the observation that plaque type mutants (variants) are common among animal viruses. A review describing the various types of plaque mutants of animal viruses has been given by Takemoto (1966).

Plaque mutants have been described most commonly by the size of plaques formed but other descriptive terms have been used such as round, irregular or diffuse (referring to plaque shape and margin) and clear or turbid (referring to the degree of cell killing).

Differences in plaque size among caliciviruses have been reported previously. McClain, Hackett and Madin (1958) observed two plaque variants occurring with vesicular exanthema of swine virus (VESV). Marked variation in plaque morphology was observed not only between virus types but also within virus types. The two variants were termed large plaque (lp) and minute plaque (mp). Two intermediate sized variants were later discovered by Walen (1963) using a single-burst technique followed by plaque assay. It was apparent that VESV stocks of the several virus types were heterogeneous with regard to plaque type.

Plaque size differences have been found between isolates of FCV but there have been no reports on plaque-type heterogeneity within an isolate. Crandell (1967) during an investigation of 8 FCV isolates observed that the plaque development of one (strain KCD) was slower than the others. Prydie (1973) found that FCV isolates could be assigned to one or other of 3 groups according to plaque size using an agar overlay; plaques at 48 hours measured 1mm, 3-5mm or 5-7mm. Kalunda *et al.* (1975) also reported 3 classes of isolates based on plaque size; using their assay method, the plaques at 72 hours measured 1mm (termed small plaque), 1-3mm (medium plaque) or 3mm (large plaque).

In the present study, the plaque morphology of FCV strains was examined by the technique described in Chapter 3. The strains were obtained from laboratories in Europe, Canada and the U.S.A. and had been characterised antigenically and in other biological aspects in previous reports. Variation in plaque size between strains as reported by Prydie (1973) and Kalunda *et al.* (1975) was found but in the present work four groups could be distinguished based on this character.

The viruses used in previous studies of FCV plaque variation have been passaged several or many times in cell culture. There has been no

investigation, to date, of plaque variants as they occur in vivo. Such an investigation was undertaken here and the incidence and types of variants isolated directly from cats was compared to the situation described previously for tissue culture passaged virus. Of particular interest was the observation of earlier work that a single isolate may contain more than one plaque type (O. Jarrett; unpublished results).

This investigation led to the conclusion that plaque mutation and/or plaque variant selection may occur during cell culture passage of FCV. This was investigated by reconstruction experiments and by analysing the distribution of mutants in clones. Also, some specific problems encountered in plaque cloning were examined.

MATERIALS AND METHODS

1. A classification of isolates based on plaque morphology

Viruses examined The original reference and cell culture history of each FCV strain examined is given in Table 4.1.

A number of local isolates were also examined and their origin and designation is given in Table 4.2. These isolates were obtained from the random swabbing of cats attending a veterinary clinic. Mouth swab medium was divided into two: the first aliquot was used for initial screening in which FCV isolation was recognised by the production of the characteristic CPE in FEA monolayers. If positive for FCV, the second aliquot, which had been stored at -20°C until required, was examined by the standard plaque assay.

Plaque measurement and classification Initially, several FCV strains along with three local clones, FCV G1, G2 and G10, were titrated by the standard plaque assay method. The cultures were rinsed twice with MEM after removal of the inocula to remove unadsorbed virus. Plaques were measured as before and for each isolate the mean plaque diameter was calculated from the measurement of between 50 and 100 plaques.

Subsequently, other strains and isolates were assayed concurrently with 4 isolates, G1, G2, KCD and G10, which represented the 4 plaque size groups of FCV ("extra-large" plaques (ep), large plaques (lp), small plaques (sp) and minute plaques (mp) respectively). The classification of additional strains and isolates was therefore by visual comparison with "control" plaques.

However, for Glasgow isolates in which the virus titre was sufficiently high to allow direct examination (i.e., without cell culture passage), plaques were measured and frequency distributions of their diameters were produced in an attempt to locate mixed plaque populations. Isolates with very low titres were passaged once in FEA cell culture and then plaques were examined as above.

2. Observations on apparent in vitro plaque mutation

Passage of isolates in cell culture Twenty local isolates were passaged 6 times in FEA cell cultures. At each passage, 0.1 ml of virus-containing fluid was inoculated into FEA monolayers in 5 cm plates and, after a 60 min adsorption period, 3 ml EFC/10 was added. Plates were

incubated at 37°C for 24 hours at which time the CPE was complete. Then 0.1 ml of infected cell virus-suspension was transferred to a fresh culture and the procedure was repeated. The 6th passage virus suspension was partially clarified by centrifugation (2,500 rpm for 10 min) and the virus suspension along with 1st passage virus and "control" isolates G1, G2, G10 and KCD were assayed by the standard method.

Apparent minute plaque (mp) to large plaque (lp) mutation (a) FCV G10 (mp forming virus) stock was suitably diluted in MEM so that an inoculum added to FEA cultures in 5 cm plates contained 1-2 PFU. After virus adsorption and removal of the inoculum an agarose overlay was applied and the cultures were incubated for 48 hours. Sub-clones were obtained from 40 single plaques as described before and the agarose plug was deposited into 1 ml MEM. The mp content of each sub-clone was determined by standard plaque assay and for those clones containing more than 1,000 PFU the lp content was estimated: 2 plates were each inoculated with 0.1 ml of each sub-clone and after adsorption and removal of the inoculum an agar overlay was applied. The remaining 0.7 ml of each clone was stored at -20°C for several weeks and then assayed. However, a large drop in the titre was noted presumably as a result of storage and these results were discounted.

(b) A mp variant, found in FCV G1 stocks was plaque cloned twice. From this, a further 30 clones were obtained as described immediately above. The sub-clones were deposited into 0.5 ml MEM and 10% of each (50µl) was used to determine the mp content of the clone. The remaining 90% was divided between 4 plates containing FEA monolayers and the lp content of the clone was determined after incubation under agar.

TABLE 4.1 Origin and history of FCV strains

Strain	Original reference	History (where known)
F5	Bittle *et al.*, 1960	
F10		
F11		
F17		
F19		
FJ		11th passage — Initial passage in feline kidney cells followed by passage in a feline tongue diploid cell line.
17FRV		25th passage
FC	Sinha 1958	11th passage
FS	" "	21st passage
CFI	Crandell and Madin 1960	18th passage
KCD	Fastier 1957	9th passage
68-2024	Povey 1970	14th passage in feline kidney cells.
M8	Povey and Hale 1974	9th passage in feline kidney cells.
337/61	Bürki 1965	
344/61		
377/61		
135/62		
277/62		
F9	Bittle *et al.*, 1960	
FPL	Bolin 1957	15th passage in feline kidney and feline tongue cells.
FPV 255	Kahn and Gillespie 1970	

TABLE 4.2 Origin of local FCV isolates

Isolate	Origin *
+G1 & 2	Kitten; glossal ulceration
G3	18 months; clinically healthy
G4	Adult; sneezing with nasal discharge
G5	6 months; coughing
G6	8 months; diarrhoea
G7	Kitten (6 weeks); oculonasal discharge
G8	Kitten (4 weeks); purulent oculonasal discharge
G9	Adult; coughing with oculonasal discharge
+G10 & 11	11 months; clinically healthy
G12	Adult; dehydrated and tachypnoeic
G13	Kitten (5 weeks); diarrhoea
G14	Kitten (4 weeks); pyrexia, sneezing, nasal discharge
G15	5 months; clinically healthy
G16	5 months; anorexia, coughing and sneezing
G17	9 months; surgical case
G18	Adult; clinically healthy
G19	Adult; surgical case
G20	Kitten (4 weeks); sneezing with muco-purulent ocular discharge
G21	Adult; anorexia, glossal ulceration
G22	4 months; coughing, sneezing, oculonasal discharge
G23	Kitten (11 weeks); muco-purulent oculonasal discharge
G24	Adult; anorexia and gingivitis
G25	Adult; clinically healthy
G26	9 months; surgical case

* Origin = Age of animal, followed by main clinical finding(s). (All isolations made from oro-pharyngeal swabs).

+ Isolates plaque cloned from a single mouth swab.

1. A classification of isolates based on plaque morphology

There was wide variation in plaque size under agar between FCV strains. Fig. 4.1 (a and b) shows representative plates from the assay of four isolates. Plaque sizes of G1, G2 and KCD were found to occur in a normal or near normal distribution, whereas the frequency distribution for plaques of G10 was positively skewed, most likely due to an "infraplaque effect" (Lindenmann and Gifford, 1963): that is, a proportion of plaques were hidden below the threshold of visibility. These strains produced four distinct populations of plaque sizes (P=0.01) and represent the four plaque size groups of FCV (see Tables 4.3 and 4.4). The minute plaque (mp), small plaque (sp) and large plaque (lp) groups are more or less equally represented among available strains (Table 4.4a), but strains belonging to the fourth group, producing 'extra-large' plaques (ep), are uncommon.

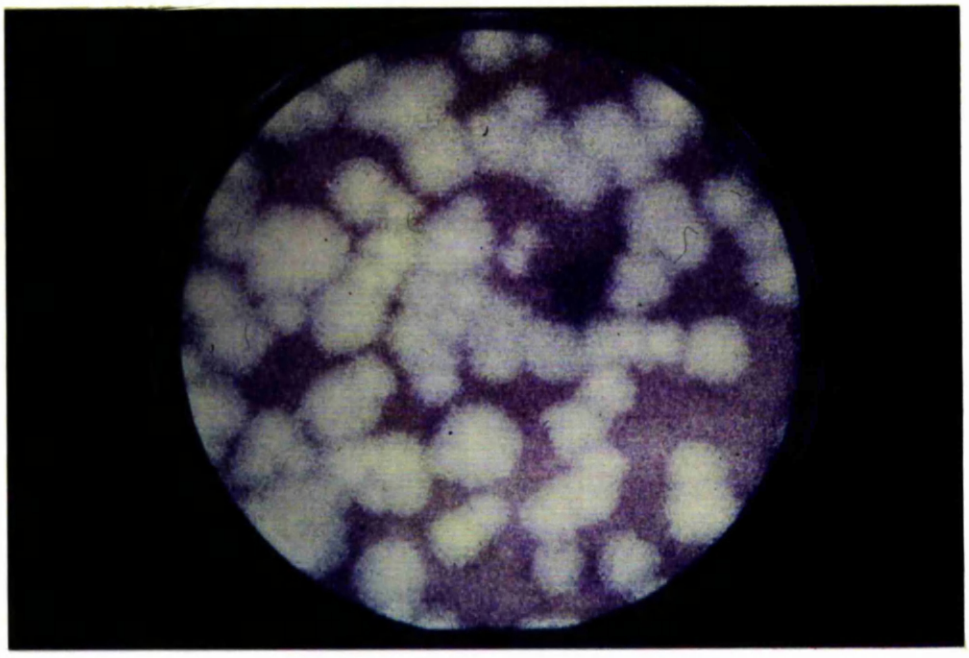
Strains classified as ep, lp and sp produced round regular plaques with relatively sharp borders. Minute plaques were more irregular in shape and more turbid. The turbidity was caused by incomplete cell killing; when stained and examined microscopically, plaques were seen to be crossed with a sparse matrix of thin, spindly cells.

Agarose overlays were used to determine whether plaque size might be influenced by inhibitory substances present in agar: agarose is the agar fraction free of sulphated polysaccharides which are known to inhibit virus multiplication in several virus-cell systems (reviewed by Takemoto, 1966). Agarose forms firmer gels than agar at the same concentration, therefore, unless inhibition occurs with agar, the plaque size tends to decrease under agarose overlays. However, with certain strains the plaque size was dramatically increased, for example G10 (see Fig. 4.2). If the mean plaque diameter increased by twofold or more, the sensitivity to agar was scored "++" (see Table 4.3). The sign "+" indicates an increase in size, but less than twofold. When more strains were examined under agarose overlays, it was found that all of the mp group, along with some of the sp group, were sensitive to agar inhibition. Only one strain of the lp group, strain FPL, has been found to be sensitive.

Isolates obtained directly from local cats belonged predominantly to the lp group (Table 4.4b) and, in contrast to the available FCV strains, isolates of the sp and mp groups were uncommon. Again, isolates of the ep group were seldom encountered. Additional isolates, originating from

Fig. 4.1a: The plaques produced by isolates G1 and G2

FCV G1



FCV G2

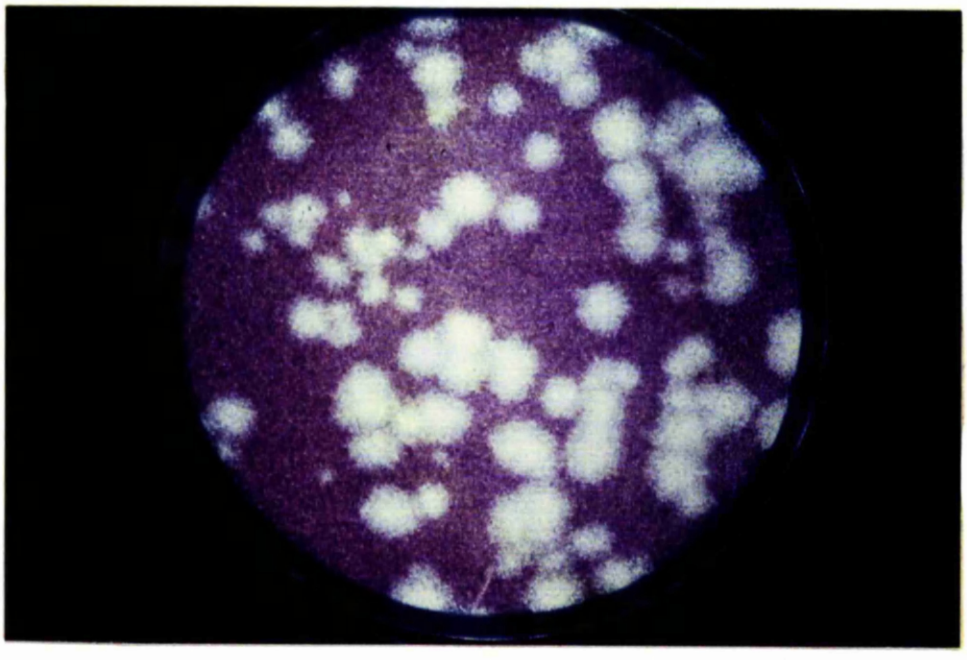
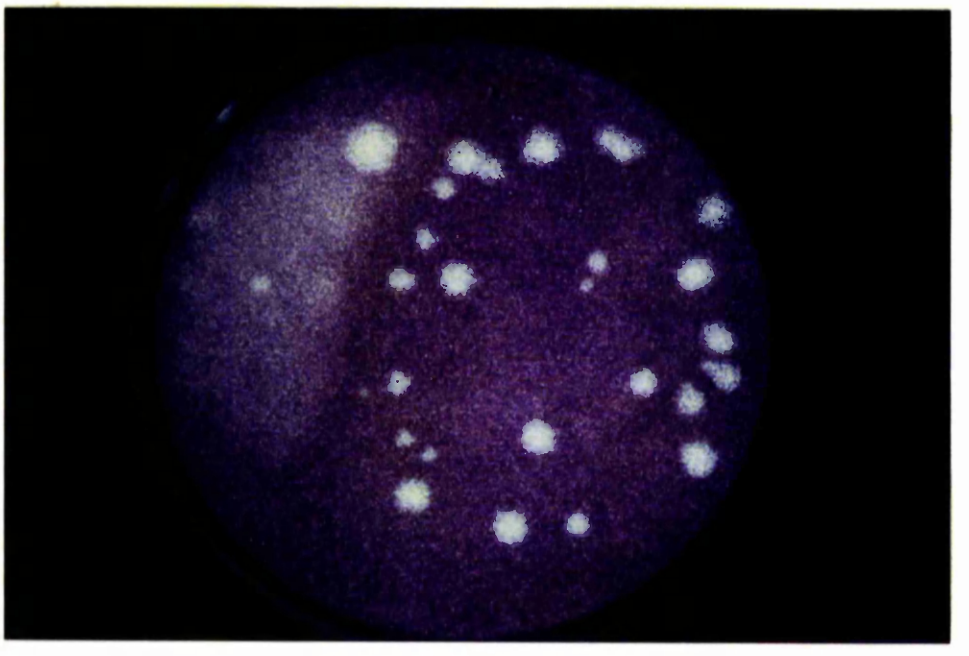


Fig. 4.1b: The plaques produced by isolates KCD and G10

KCD



FCV G10

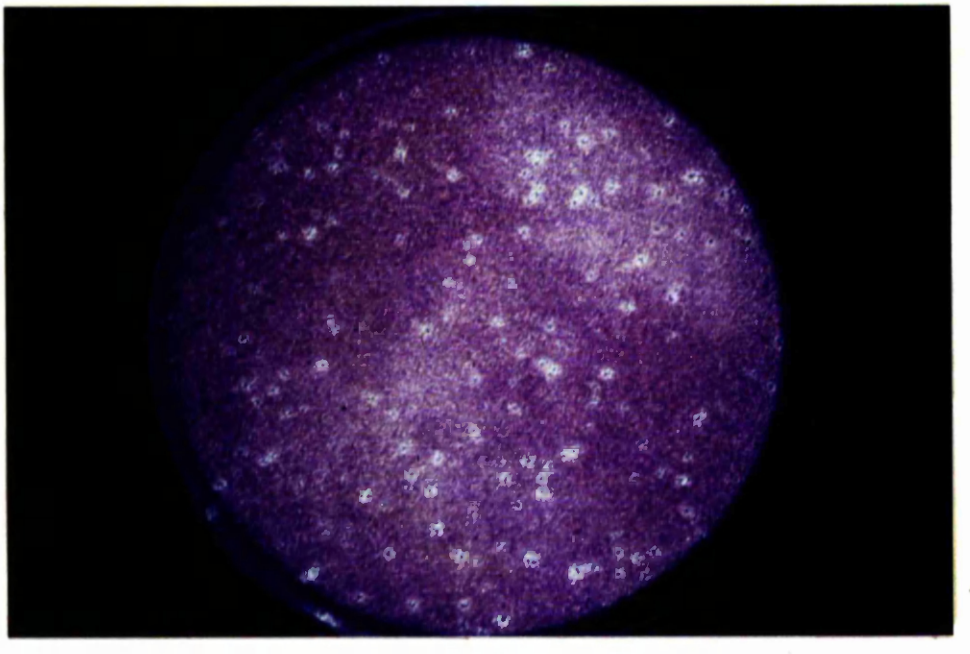
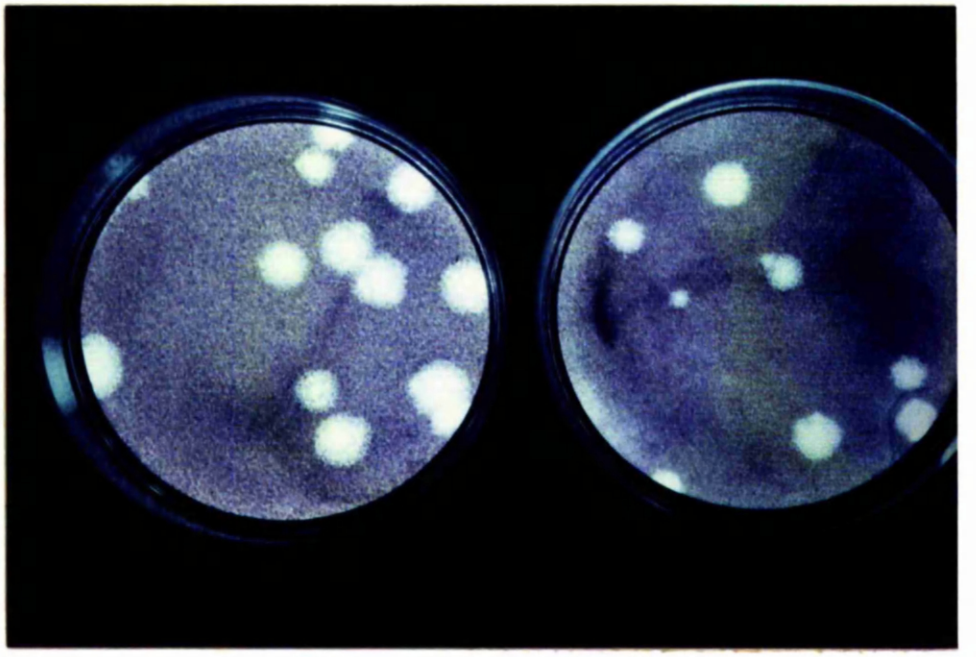


Fig. 4.2: The plaques produced by isolates G1 and G10 using agar and agarose overlays

FCV G1 Agar Agarose



FCV G10 Agar Agarose

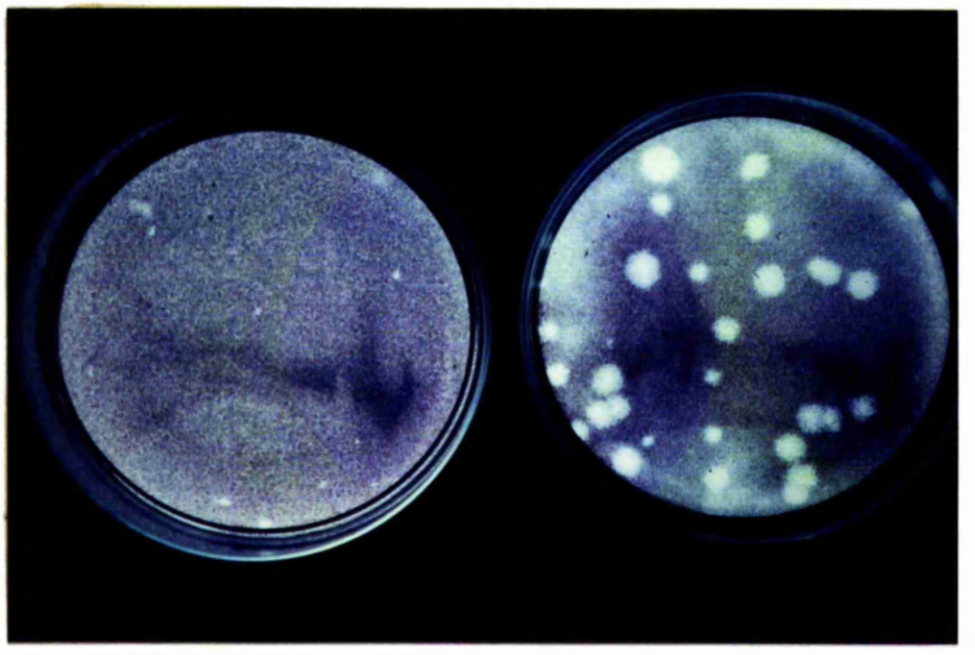


TABLE 4.3 Plaque sizes of isolates.

Plaque Group	Isolate	Mean Plaque Diameter (mm)	Sensitivity To Agar Inhibitor
ep	G1	3.6	-
lp	F9	3.1	-
	CFI	2.9	-
	G2	2.5	-
sp	KCD	1.7	+
	F11	1.7	-
	F5	1.3	+
mp	F17	1.0	++
	FJ	<1	+
	G10	<1	++

TABLE 4.4: The plaque types of FCV strains compared to the plaque types of virus directly isolated from cats.

(a) Plaque size of FCV strains:

PLAQUE GROUP	STRAINS
ep	FPV 255
lp	F9, CFI, FS, FPL, 68-2024, 337/61
sp	KCD, F11, F5, FC, 135/62
mp	F17, FJ, 17FRV, F10, F19, M8, 227/62, 344/61, 377/61

(b) Plaque size of viruses directly isolated from cats+:

PLAQUE GROUP	ISOLATES
ep	G1*, G16
lp	G4, 5, 6, 7, 8, 11*, 13, 14, 15, 18, 19, 22, 24, 25, 26
sp	G20*, G21
mp	G12, G17

+ Where titres were sufficiently high, the mouth swab medium was assayed. For lower titre isolates, one passage in FEA cells was permitted before assay.

* These isolates were shown by cloning to be composed of 2 plaque variants. Only the predominant variant is included.

various parts of the U.K., have also been examined and all have been classed as lp.

A number of isolates contained viruses which produced plaques of two different types. Clones G1 (ep) and G2 (lp) originated from a single mouth swab as did G10 (mp) and G11 (lp). Isolate G20 yielded two plaque types (sp and mp) which were cloned and bred true. Also isolates G15 and 24, after one passage in cell culture, appeared to be heterogeneous (lp and mp) on the basis of plaque size although clones were not collected. Isolate G14 contained FHV and FCV; FHV plaques were distinguished by their small size (1-2 mm), sharpness and clarity. Microscopically, syncytia and giant cells could be seen at the plaque margin.

A FCV isolation rate of 20% was obtained from cats attending this particular veterinary clinic. A total of 200 cats were sampled and it was observed that approximately 35% of them had been presented with respiratory illness or conjunctivitis or ulcerative glossitis or a combination of these signs; FCV was isolated from 60% of these cases of clinical disease.

2. Observations on apparent in vitro plaque mutation

Passage of isolates in cell culture In a number of cases, this was associated with an emergence of sp and mp variants. Of the isolates tested (G4-8, 12-22 and 24-26), a change of this type was observed in nine of them (see Table 4.5).

Fig. 4.3 shows representative plates infected with G19 before and after passage in FEA cell culture and demonstrates the most consistent finding, a plaque type of lp originally and a lp-mp mixture of roughly equal proportions after cell culture passage.

The plaques from 3 other isolates, G6 (lp/mp), G12 (sp/mp), and G22 (lp/sp), were similarly cloned and bred true. In all 4 cases the newly arisen plaque type was more sensitive to agar inhibition than the original plaque type.

Minute plaque variants were also observed after cloned viruses G1 and G2 were passaged seven times in FEA monolayers. These plaques were cloned and bred true.

Apparent mp/lp mutation This was observed in two situations: (a) Large plaques were occasionally seen in the plaque assay of mp virus stock (G10). For every 500-1000 minute plaques, one large plaque developed. These plaques could have arisen by spontaneous mutation (either during plaque development or more likely during virus stock production) or by directed mutation (that is, virus adaptation under the conditions of

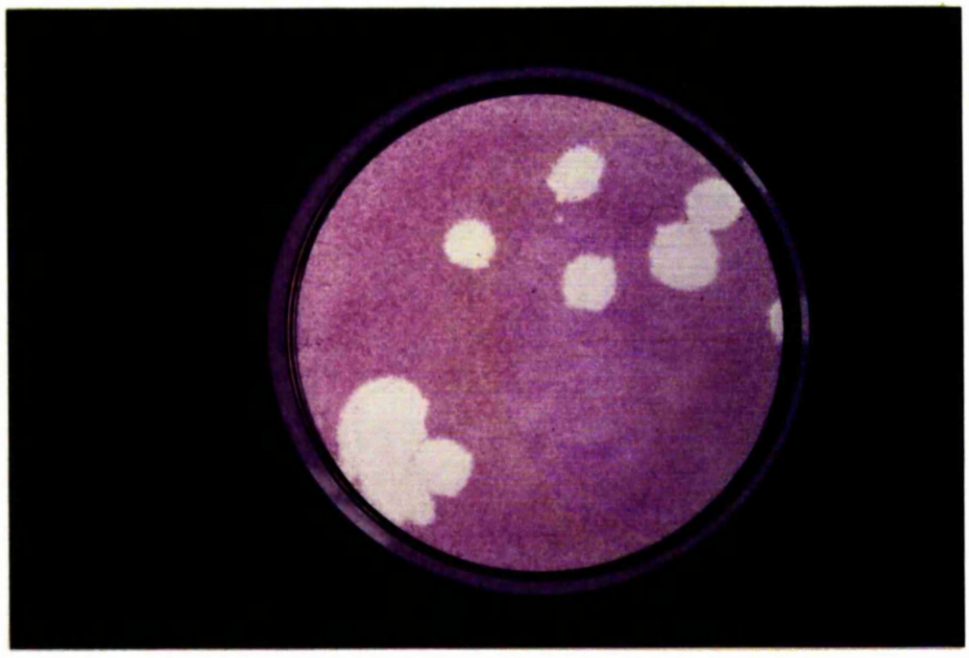
TABLE 4.5: Isolates in which passage in cell culture produced an altered plaque population.

ISOLATE	ORIGINAL PLAQUE TYPE (ZERO OR 1 PASSAGE IN CELL CULTURE)	PLAQUE TYPE AFTER 6 PASSAGES IN CELL CULTURE
G6	lp	lp/mp
7	lp	lp/mp
12	mp	sp/mp
15	*lp	mp
18	lp	lp/sp
19	lp	lp/mp
20	sp/mp	mp
22	lp	lp/sp
24	*lp	lp/mp

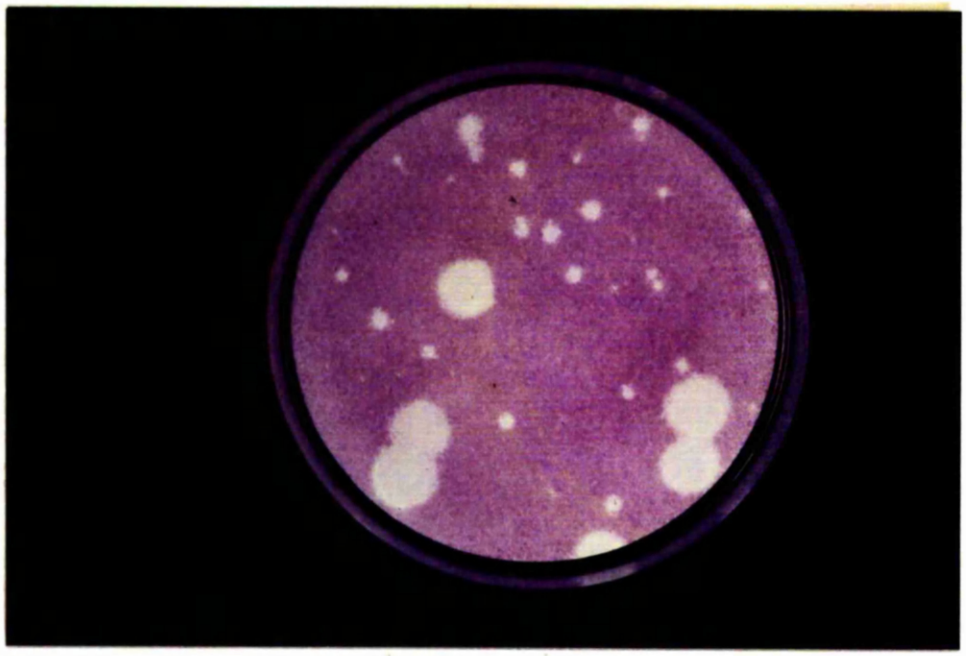
* lp/mp mixture suspected after 1 passage.

Fig. 4.3: The plaques produced by isolate G19 before and after passage in cell culture

Original plaque type (lp)



Plaque type after passage in FEA cell culture (lp-mp mixture)



the plaque assay) or possibly by inefficient plaque cloning, resulting in contamination from the original lp/mp mixture. In an attempt to resolve this question, sub-clones were obtained from the G10 stock and their content was determined by plaque assay (see Table 4.6). Some sub-clones contained lp variants and the titres of the sub-clones varied greatly. There was a large fluctuation in the ratio of numbers of variants to total number of PFU in a clone (i.e., the variants were clonally distributed). Several variant plaques were cloned at 48 hours and bred true. A number of cultures were reincubated and additional lp were seen to develop; for example, in sub-clone 6 an additional lp was cloned at 96 hours. This clone did not breed true but plated as mp. Similarly, an additional lp was cloned at 96 hours in sub-clone 24 and plated out as a lp/mp mixture. Since variant plaques bred true at 48 hours, it was assumed that plaque scoring at this time led to the recognition of lp variants already present in the sub-clone and precluded plaques originating from mp virus but converted to the lp phenotype by in-plaque mutation.

It was also assumed that, during plaque development under agarose (i.e., before cloning), the selection of one variant over the other was minimal; results presented in Chapter 6 indicate that the main cause of the mp phenotype (for isolate G10 at least) is polyanion inhibition and this does not occur with agarose: G10 mp and lp variants produced plaques of the same size under agarose.

It was not possible to carry out a complete fluctuation analysis (Luria and Delbruck, 1943) since only a few clones of a similar size could be examined. However, the results indicated spontaneous mutation rather than contamination or directed mutation.

(b) During the original plaque purification procedure from which two mp virus clones, G10 and G1-mp variant were eventually obtained, difficulty was found in obtaining three-times cloned virus which was phenotypically homogeneous. In both cases, a minute plaque was initially cloned from a plate containing a number of large plaques ("extra-large" plaques in the case of G1-mp variant) and, as might be expected, first clone virus was predominantly mp forming but contained some contaminating lp forming virus, but second clone virus was apparently all mp forming. However, when clones containing three-times plaque purified virus were examined, a number of them contained a large quantity of lp forming virus.

Table 4.7 shows the results of an investigation of 30 sub-clones obtained from second clone, G1-mp variant virus. The lp content of the

sub-clones varied greatly (from zero in several sub-clones to 2.3×10^4 in clone 12). Such a result could not have arisen by spontaneous mutation during sub-clone development.

Again, as in (a), it must be assumed that selection factors are negligible and that the sub-clone assay, when scored at 48 hours, gives a true representation of the number of variants already in the sub-clone.

Using an agar overlay, the plaques produced from second clone virus were phenotypically mp and yet many of them must have contained a high proportion of lp forming virus. It was later observed that, when second clone virus was assayed, using agar in the overlay, and incubated for longer periods, a number of large plaques appeared (at 72 and 96 hours). These plaques were presumably equivalent to sub-clones containing a high proportion of lp forming virus.

The physical nature (monodispersed or aggregated) of the virus in second clone G1-mp variant was investigated by filtration and rate zonal centrifugation. Second clone virus was diluted in MEM and passed through filters of pore size 220 and 450 µm (Millipore Co). Filtered and non-filtered virus was assayed. The 450 µm pore filter reduced the infectivity titre by 60% and the 220 µm pore filter by over 80%.

A volume of 0.2 ml containing 10^4 PFU of second clone virus was layered onto a 15-30%sucrose gradient and centrifuged at 45,000 rpm for 30 min in an SW 50.1 rotor. The gradient was fractionated and the fractions were assayed for infectious virus by the standard plaque assay. Infectivity was spread over a number of fractions in contrast to the single peak obtained when purified, monodispersed virus was centrifuged in the same way (Komolafe, 1978). The peak sample which occurred at the expected single-particle position in the gradient was re-assayed using an agarose overlay and 20 sub-clones were picked from suitable plaques as before. The sub-clones were screened at a dilution of 1:10 and each was apparently composed purely of mp forming virus. It could be speculated that these sub-clones originated from plaques of single particle origin and hence a high lp variant/mp ratio was not present.

TABLE 4.6: Investigation of 26 sub-clones of FCV G10 for total PFU content and number of lp variants present in sub-clone.

CLONE	P.F.U.	lp VARIANTS IN 20% OF CLONE
1	1,500	0
2	51,000	3
3	2,600	0
4	13,000	2
5	12,000	0
6	40,000	0
7	13,000	0
8	1,900	1
11	11,000	0
12	45,000	0
13	35,000	9
14	8,000	0
15	50,000	2
16	1,000	0
17	1,900	0
18	1,600	0
22	5,500	12
24	4,800	0
25	1,600	0
26	1,000	0
27	1,100	2
28	1,600	0
30	14,500	6
32	7,600	3
36	1,500	0
37	1,400	0

TABLE 4.7: Investigation of 30 sub-clones of second clone, G1-mp variant for total mp and lp content.

CLONE	TOTAL mp	TOTAL lp
1	3.7×10^4	0
2	4.4×10^4	1
3	1.1×10^5	1.8×10^3
4	1.3×10^4	4
5	5.7×10^4	1.8×10^3
6	1.3×10^5	3.5×10^3
7	0	0
8	5.2×10^4	2×10^3
9	5.0×10^4	0
10	1.3×10^4	5.8×10^2
11	2.4×10^4	5.0×10^2
12	1.5×10^4	2.3×10^4
13	7.3×10^3	10
14	2.7×10^4	20
15	3.4×10^4	1×10^2
16	1.3×10^4	1
17	2.9×10^4	4.5×10^3
18	3.6×10^2	0
19	2.5×10^3	3
20	1.9×10^4	0
21	0	0
22	1.1×10^4	1.5×10^2
23	3.4×10^3	0
24	5.0×10^3	0
25	2.5×10^4	2×10^2
26	5.5×10^3	4
27	2.0×10^4	3.3×10^2
28	1.0×10^4	2.3×10^2
29	4.0×10^2	0
30	2.5×10^2	3.5×10^2

DISCUSSION

FCV isolates can be divided into four groups according to plaque size under an agar overlay. Isolates belonging to the plaque groups lp, sp and mp probably represent the three groups reported by Kalunda et al (1975) and Prydie (1975). Isolates of the fourth group, ep, have not been distinguished in previous reports and are uncommon; of over 30 isolations made in this laboratory, together with 21 strains from elsewhere, only 3 were classed as ep. It is of interest that these plaque sizes correspond to the four different plaque types of VESV as described by Walen (1963).

There have been no previous accounts of inhibition of FCV plaque formation by agar. The inhibition of minute and small plaque mutants of picornaviruses was discovered by Takemori and Nomura (1960) for poliovirus and Takemoto and Liebhaber (1961) for encephalomyocarditis virus (EMC). As reported in these investigations, the inhibition of virus replication by the sulphated polysaccharide fraction of agar was important in producing the observed variation in plaque size. However for FCV the degree of inhibition is variable within any one plaque group and under an agarose overlay, plaques were still observed to vary in size. In this context, if an agarose overlay had been used initially, a different classification based on plaque size would have been obtained. The same may also be true if a different type of agar had been used in the overlay; it has been reported that different preparations of commercial agar may vary in their inhibitory properties (Wallis, Melnick and Bianchi, 1962). Therefore a standardised assay method, such as the one detailed here, is required. Sub-classification, based on sensitivity to agar inhibitors, helps to characterise further any new isolate.

In addition, new isolates should be plaque cloned. It would appear from this study that isolates of a mixed plaque type are not uncommon and obviously an erroneous result would be obtained if an attempt was made to describe and then classify such a mixed population in terms of plaque size. It should be noted that, using the standard plaque assay, only mixed populations with plaque type ratios (lp/mp or sp) of between 25:1 and unity can be identified with reasonable certainty on visual examination. If, for example, a new isolate containing a plaque mixture of lp and mp at a ratio of 50:1 was titrated in 5 cm plates, one minute plaque would be contained in plates along with an average of 50 large

plaques and these would approach confluency at 48 hours. Therefore the identification of the minute plaque would be very difficult.

Another factor which could lead to the underestimation of the frequency of occurrence of mixed plaque type isolation is that some mutants may have a low plating efficiency. Inapparent heterogeneity of this type has been reported for VESV (Walen, 1963).

In the cat, plaque morphology heterogeneity has arisen presumably either from a mixed FCV strain infection (which is perhaps not uncommon for such a ubiquitous virus) or as a result of mutation during virus replication in respiratory or tonsillar mucosae.

Passage of new isolates at high multiplicity of infection in cell culture in many cases was associated with the emergence of mp and sp variants. The isolates were not cloned beforehand and each could have been originally heterogeneous in plaque type. Therefore the emergence of new variants did not necessarily demonstrate plaque mutation but it did indicate selection, favouring sp and mp variants. When cloned virus was similarly passaged, the same result was found. This indicated plaque mutation and selection (contamination could be excluded since mutation was observed in earlier work before sp and mp variants from other sources were encountered). By comparing a mp variant (G10) with ep (G1) and lp (G2) variants in single step growth curves, it was found that, although the total yield per cell was lower, mp forming virus was released from cells earlier than ep or lp forming virus (see Chapter 6). It was assumed that this replication profile was representative of other mp, lp and ep types. When an isolate is passaged at high multiplicity there is a limited number of non-infected cells available for second, third and subsequent cycles of replication and conceivably the early release of virus leading to infection of these cells is a selection factor. Also, it might be expected that passage at low multiplicity would cancel this advantage since there would be a large number of cells available for subsequent replication cycles and the total cell yield of virus could become an important selection factor. A small experiment comparing the results of passage of an "artificial" mp/lp mixture (G10/G1) in cell culture, at high and low multiplicity of infection, produced a conflicting result: in both cases, G1 was apparently selected for. However, this result is not conclusive since only 2 passages in cell culture were performed and the initial G10 titre was overestimated.

Selection of sp and mp variants might also explain the observed difference in plaque types between established strains, which have been

passaged several or many times in cell culture, and new isolates (Table 4.4a and b). Established strains belonging to the sp and mp groups are common (67% of those available) but new isolates are most frequently grouped as lp with sp and mp types being uncommon (13% of isolates examined to date). Such a difference, arising simply from local variation is considered unlikely.

Mutation from ep or lp to sp or mp is difficult to study since, as discussed above, it is not possible to recognise a small amount of mp forming virus (or sp forming virus) within a large concentration of lp or ep forming virus. The reverse mutation can be studied more easily as the mutants can be visualised with relatively little difficulty. A lp variant was observed in G10 (mp forming virus) stock and this apparent mutation was studied. The lp variant was found to be clonally distributed indicating spontaneous mutation. Although the data were insufficient to calculate the mutation rate the number of variants observed and the clone sizes were of the same order as those reported by Breeze and Subak-Sharpe (1967) investigating small plaque (r+) to large plaque (r) mutation in EMC virus. They calculated a mutation rate of between 1-2 x 10^4 per particle per duplication. A similar, very high rate of mutation could therefore be expected for FCV G10 mp to lp mutation.

A more complex situation involving mp-lp "mutation" was encountered when mp forming virus was cloned from an original ep/mp and lp/mp mixture. The plaques formed by twice cloned virus were phenotypically minute when stained at 48 hours, under an agar overlay, but when these plaques were progeny tested a number were found to contain a high ratio of larger plaque variants. Spontaneous mutation at the same locus and in the absence of differential selection during plaque development could not account for such a result: at a very high mutation rate of 1-2 x 10^4 per particle per duplication the chance of obtaining 8 clones from 30 with a ratio of mutant to wild type of greater than 20% is almost infinitely high. As discussed earlier, the selection of lp forming virus in favour of mp forming virus using an agarose overlay is probably negligible. Mutation producing a large plaque phenotype may not be restricted to a single genetic locus. There are several examples where it is known that virus mutation at several loci produce a single phenotypic change. For example, the ulcerated (u+) pock to white (u) pock mutation of rabbitpox virus (Gemmell and Fenner, 1960) where white pocks are produced at a frequency of approximately 1% from u+ stocks. Also spontaneous ts mutants of vesicular stomatitis virus which are

produced at a frequency of 2.3% (Flamand, 1970). In the present study, it was observed that the phenotype of the "larger plaque" mutants differed, not only between sub-clones but sometimes within a sub-clone: for example, sub-clone 25 obtained from second clone G1-mp variant, apparently contained lp and sp size variants (all larger plaque variants were scored "lp" for convenience). It is significant that a similar high frequency of plaque variants was not observed when clones of mp-forming virus stock were examined.

A better explanation might be that the plaques formed from twice-cloned virus were of multiple origin. During plaque cloning a plug of overlay containing not only virus in suspension but also effete cells and cell debris was transferred to 1 ml MEM. Under these conditions it is likely that virus aggregates, either alone or in association with cellular elements could be present. The aggregation of other picornaviruses in certain media, particularly the smaller plaque mutants of these viruses, has recently been demonstrated (Totsuka, Ohtaki and Tagaya, 1978). Aggregation of G10 (mp forming) virus has been observed by electron microscopy in thin sections of infected cell culture pellets (see Chapter 7). The results of the experiments involving filtration and rate zonal centrifugation of twice-cloned mp forming virus also suggest particle aggregation. Such aggregates could conceivably contain lp forming virus as such, or be phenotypically mixed (Ledinko and Hirst, 1961). Pre-existing lp forming genome in the twice-cloned virus suspension could have arisen by mutatiom during the development of the parent plaque or possibly the parent plaque itself could have been of multiple origin, i.e. from an aggregate containing lp forming genome arisen by in-plaque mutation during the growth of the grandparent plaque or possibly even as a contaminant from the original plaque type mixture.

During the plaque purification process, preceding G10 and G1-mp variant stock formation, a relatively pure, three-times plaque purified, mp virus stock was eventually obtained, presumably by the chance cloning of a plaque containing no, or very few, lp variants (as in sub-clones 1, 2, 4, and 9 (Table 4.7)for example). It should be emphasised that this purity was only relative and, as described above, lp variants were detected in mp virus stock, in the standard plaque assay, at a ratio of 0.1-0.2% .

It is considered likely that G10 stock virus was monodispersed since, as described in Chapter 2, virus stocks were made by harvesting cultures, ultrasonicating them and then removing cellular debris by

centrifugation. G10 stock virus of known titre was ultrasonicated (24.78 K^c/s) for additional periods of 30 sec 1, 2 and 3 min. An increase in titre, which may be expected if virus was originally aggregated, was not observed. Also G10 stock virus, negatively stained and examined by electron microscopy was monodispersed. Therefore it is likely that the plaques cloned in the original experiment, using G10 stock virus, were of single particle origin and, this being so, the observed lp variants are assumed to have arisen by spontaneous mutation during plaque development.

CHAPTER 5

THE RELATIONSHIP BETWEEN PLAQUE MORPHOLOGY AND VIRULENCE

Introduction

Materials and Methods

Results

Discussion

INTRODUCTION

There have been a number of attempts to correlate the plaque size of mutants of various animal viruses with virulence (reviewed by Takemoto, 1966). While the number of strains studied for each virus has been limited, the general finding is that the small plaque type appears to be more frequently associated with attenuation: i.e., lower virulence. This has been found to be true for a number of picornaviruses. For example, certain small plaque mutants of poliovirus were associated with attenuation (Sabin, 1957); small plaque forming EMC virus was found to be relatively avirulent for mice compared to large plaque forming virus (Craighead, 1965); a small (irregular) plaque variant of foot-and-mouth disease virus (FMDV) was less virulent for pigs compared to a large plaque variant (Sellers et al., 1959). Also in a study of the plaque variants of VESV, the other major virus of the calicivirus genus, a correlation was found between plaque morphology and virulence: a cloned LPF (large plaque former) was pathogenic whereas a cloned MPF (minute plaque former) was not (McClain, Hackett and Madin, 1958).

There has been no attempt to correlate plaque size and virulence for FCV variants although Prydie (1973) did note that 3 strains of similar virulence varied in plaque size. It was the object of this study to investigate such a possible correlation.

Experimental infection of cats with FCV has been widely used to characterise the type of clinical disease produced and to investigate the pathogenesis of the infection (Bartholomew and Gillespie, 1968; Kahn and Gillespie, 1971; Love, 1975). Other studies have compared the virulence of several FCV strains (Hoover and Kahn, 1973; Povey and Hale, 1974; Wardley and Povey. 1977a) and have shown that there are considerable differences between strains: some are avirulent while others produce severe respiratory disease. Also, as described in the previous chapter, there is inter-strain variation in plaque size. If a number of strains of known virulence could be examined for plaque morphology, a correlation, if it existed, should be revealed. A study of this type was carried out and indicated a possible relationship: several strains of the mp group were of low virulence and a strain of the ep group was of high virulence.

The correlation was investigated further by comparing the disease produced by two local, cloned isolates G1 (ep) and G10 (mp). As an adjunct to this study, a comparison was also made of the aerosol and intranasal routes of infection: in all of the studies above, virus was administered

either by aerosol (e.g., Hoover and Kahn, 1975) or by intranasal instillation of virus-containing fluid (e.g., Povey and Hale, 1974). When this study began it appeared that investigators using an aerosol of virus often observed a more severe disease than those using intranasal instillation. It was not known if a strain which appeared to be, for example, relatively virulent on aerosol infection was of the same virulence when administered by intranasal instillation. For the purpose of categorising FCV strains according to their virulence characteristics, it was necessary to determine if the disease produced after infection by these routes of administration differed. Also it would be helpful to know which method was the more suitable for use in future studies in which it was required to simulate natural infection.

FCV strains of known virulence

The following strains are of known virulence in that their pathogenicity has been studied in comparison to other FCV strains under the same experimental conditions: FC, CFI, F17, 17FRV, KCD, F9, F10, FJ, FPL, M8, 68-2024 and FPV255. The reference to the study of the virulence of each is given in Table 5.2. Their classification according to plaque morphology was described in Table 4.4.

Experimental infection with isolates G1 and G10

Fifteen cats were used in 5 groups as shown in Table 5.1. After transfer to the experimental building each group was observed for a period of at least 3 days prior to infection to ensure that each cat was clinically healthy. Immediately before infection, serum samples were taken along with sets of swabs (mouth, nares, conjunctival and rectal) for virological examination. Tonsillar swabs were taken for bacteriological examination.

The cats were anaesthetised by intramuscular inoculation of 0.3-0.5 ml of ketamine hydrochloride (Vetalar: Parke, Davis & Co.). Groups 2 and 4 were infected by the intranasal instillation of 0.3 ml of stock virus suitably diluted using MEM with 2% FBS. The drops were delivered by micropipette and the dose per cat was $2\text{-}5\text{x}10^5$ PFU.

Groups 1 and 3 were infected by the aerosolisation of virus-containing fluid. Stock virus was diluted as above. Aerosols were produced by forcing air through a CF1 nebuliser producing particles less then 8μm in diameter. Each cat was enclosed in a polythene bag and the aerosol stream was directed towards the head of the animal for a period of two minutes. The concentration of virus in the fluid was $2 - 8\text{x}10^6$ PFU/ml and 0.7 ml was aerosolised in 2 min so that the approximate virus dose was $1 - 6 \times 10^6$ PFU.

Group 5 (control group) was subjected to an aerosol of MEM with 2% FBS but free of virus.

Each day, clinical observations were made and swabs from mouth, nares and conjunctiva were collected. Each cottonwool swab was deposited into a bottle containing 2 ml MEM.

Necropsies were performed at three periods after infection: 3-4 days, 7 days and 10-14 days. Samples from the following tissues were collected for virology: turbinates, tongue, tonsil, retropharyngeal lymph node, third eyelid, trachea, lung parenchyma, tracheobronchial

TABLE 5.1: Experimental plan for the infection of specific pathogen free cats.

GROUP	CAT NO.	ROUTE OF INFECTION	VIRUS	NECROPSY TIMES (DAYS)
1	1,2,3	Aerosol	G1	4, 7, 12
2	4,5,6	Intranasal	G1	3, 7, 10
3	7,8,9	Aerosol	G10	4, 7, 12
4	10,11,12	Intranasal	G10	3, 7, 14
5	13,14,15	Aerosol	-	4, 7, 12

lymph node, kidney and spleen. For bacteriology, swabs were taken from turbinates and tonsil and samples were collected from trachea, lung parenchyma, liver, kidney and spleen. Photographs were taken to record the macroscopic appearance of the lungs. Representative portions of the tissues listed above were fixed in 10% formol saline and processed for histological examination. In each case the left lung lobes were also fixed by instillation of fixative via the bronchial tree.

Virus isolation

Virus isolation from swabs has been described in Chapter 2.

For virus isolation from tissues, a 10% or 20% w/v suspension of tissue in MEM was prepared in a Stomacher (Seward Ltd.). The suspension was centrifuged at 2,500 rpm for 15 min and the supernatant was collected and inoculated into cell cultures as described above for swab medium.

Bacterial isolation

Tonsillar and turbinate swabs and samples obtained asceptically from tracheal mucosa, lung parenchyma, liver, kidney and spleen were inoculated on to paired blood agar and MacConkey agar plates. The plates were incubated aerobically at $37^{\circ}C$ for 24 to 48 hours and examined for bacterial growth. Bacterial colonies were identified according to the methods of Cowan and Steel (1974).

FCV strains of known virulence

Table 5.2 shows several FCV strains along with local isolates G1 and G10 arranged in approximate order of decreasing virulence.

It must be emphasised that this order can only be approximate: virulence is, of course, difficult to quantify and involves a certain amount of subjective judgement. In addition, Table 5.2 is a compilation from sources using different experimental methods. For example, Hoover and Kahn (1975) produced infection by the aerosolisation of virus fluid whereas Povey and Hale (1974) and Povey and Ingersol (1975) used the direct intranasal instillation method. As is shown in this study the route of infection may affect the extent of the disease. Nevertheless, certain trends can be seen. Strains belonging to the mp group predominate in the lower portion of the Table, whereas the most virulent strains belong to the ep group. Also, there may be a loose correlation between agar sensitivity and low virulence.

Experimental infection with isolates G1 and G10

Clinical observations The clinical signs and symptoms and their duration are shown in Table 5.3.

Infection with G1 produced relatively severe clinical signs. Administration by intranasal drop produced dullness, pyrexia, reduced appetite and ulceration of the tongue (cat 6 - see Fig. 5.1) and nares (cat 5 - see Fig. 5.2). Administration by aerosol produced more pronounced signs of dullness and anorexia along with hyperpnoea in 2 of the 3 cats (cat 1 and 2).

By contrast, G10 produced a very mild clinical disease following infection by either route. Administration by intranasal drop produced pyrexia in one animal (cat 10). An aerosol of G10 produced pyrexia in one cat (cat 9) and later this cat developed very small vesicles and ulcers on the tongue. Another cat (cat 8) had a transient reduced appetite.

The control animals remained clinically normal.

The daily, mean rectal temperatures of the 5 groups of cats are shown in Fig. 5.3 which demonstrates the febrile response observed in cats infected with G1. The rectal temperatures of G10 infected cats remained low similar to the controls.

Pathological findings In general, aerosol infection produced a more severe pathological picture than the intranasal drop method and, by either route, infection with G1 resulted in more severe and extensive

TABLE 5.2: The plaque morphology and agar sensitivity of FCV strains of known virulence.

ISOLATES ARRANGED IN DECREASING ORDER OF VIRULENCE	REFERENCE	PLAQUE SIZE	SENSITIVITY TO AGAR
FPV 255	Hoover and Kahn (1975)	ep	-
G1	-	ep	-
FPL	Hoover and Kahn (1975)	lp	+
FC	" "	sp	-
F9	" "	lp	-
17FRV	" "	mp	+
CFI	" "	lp	-
F17	Povey and Ingersol (1975)	mp	++
KCD	Hoover and Kahn (1975)	sp	+
G10	-	mp	++
M8	Povey and Hale (1974)	mp	+
FJ	Hoover and Kahn (1975)	mp	+
68-2024	Povey and Hale (1974)	lp	-
F10	Hoover and Kahn (1975)	mp	+

TABLE 5.3: Clinical response of SPF cats to inoculation with FCV isolates G1 and G10.

ROUTE OF INFECTION	VIRUS	CAT NO.	PM DAY	DAYS ON WHICH THESE CLINICAL SIGNS WERE OBSERVED					
				PYREXIA (39°C)	REDUCED APPETITE	DULLNESS	ULCERATION (ORAL NARES)	ABNORMAL RESPIRATION	COUGH/ SNEEZE
Aerosol	G1	1	4	1-4	4	1,4	-	4(a)	-
	G1	2	7	1, 4-6	4-7	4-7	-	4-7(a)	7
	G1	3	12	1	1-3,6-12	2, 8-12	6-12	-	-
	G10	7	4	-	-	-	-	-	-
	G10	8	7	-	2-4	-	-	-	-
	G10	9	12	1,4	-	-	11,12	-	-
Intranasal	G1	4	3	1-3	2,3	-	-	-	-
	G1	5	7	1-6	3-7	4-7	3-7	5,6(b)	-
	G1	6	10	1-5	2,4-7	-	5-10	-	-
	G10	10	3	1,2	-	-	-	-	-
	G10	11	7	-	-	-	-	-	-
	G10	12	14	-	-	-	-	-	-

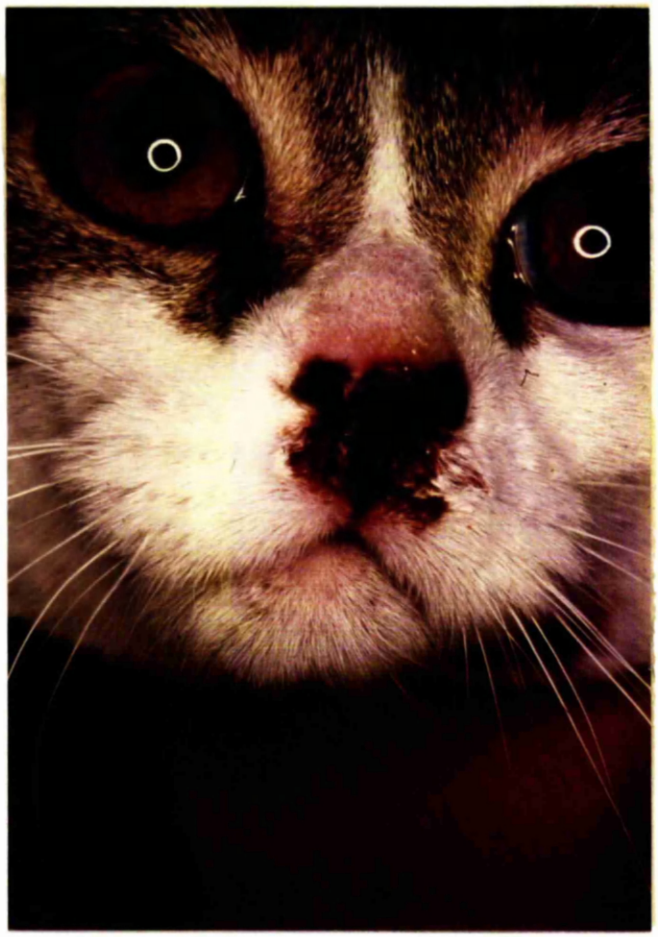
(a) hyperpnoea.

(b) mouth breathing.

Fig. 5.1: Tongue ulceration associated with FCV G1 infection.

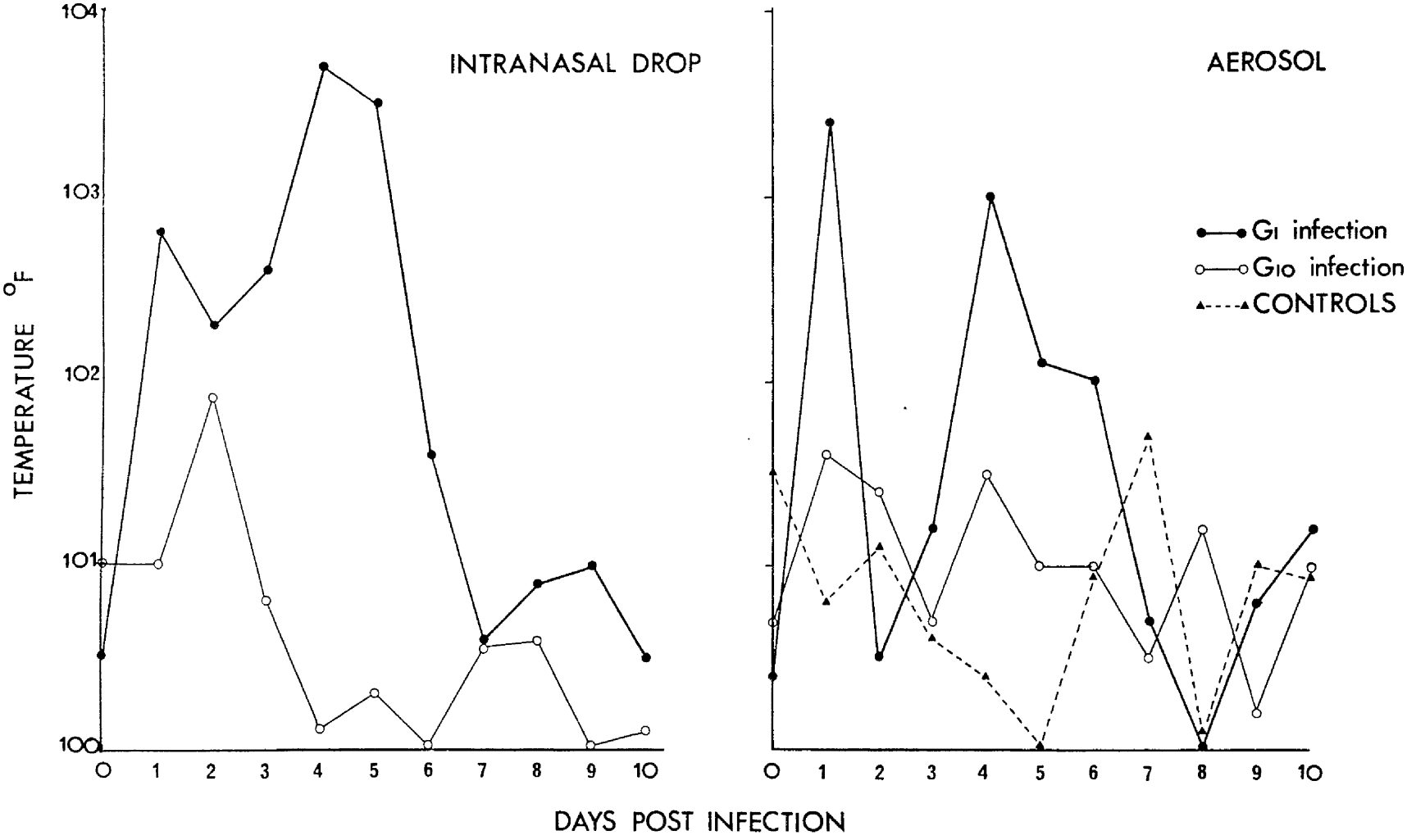
An ulcerated area is seen in the middle of the dorsum of the tongue (cat 6, 10 days P.I.).

Fig. 5.2: Ulceration of external nares associated with FCV G1 infection.



Ulceration and scab-formation produced blockage of the nostrils (cat 5, 7 days P.I.).

Fig. 5.3 Mean rectal temperatures of the five groups of cats.



lesions than occurred after exposure to G10. At necropsy significant findings were limited to the oropharynx, respiratory tract and the associated lymphoid structures.

Cats infected by aerosol of virus Aerosol infection with isolate G1 produced the most widespread and extensive lesions. The main finding in the oropharynx was the presence, in cat 3, of small vesicles and ulcers on the dorsum of the tongue. In all 3 cats, the palatine tonsils were congested with superficial areas of epithelial degeneration and neutrophil infiltration. In the upper respiratory tract there was oedema and focal neutrophil infiltration of the maxillary turbinate mucosa in cats 2 and 3. In the lower respiratory tract pneumonia was present in all cats. The areas of lung affected were the dependent portions of all lobes and the dorsal area of the caudal lobes. Affected lung tissue was consolidated and mottled grey-pink in colour. In cat 2, there was, in addition, a severe, left-sided, fibrinous pleurisy (Fig. 5.4) and in all 3 cats the tracheobronchial lymph nodes were enlarged and congested.

Aerosol infection with isolate G10 again produced pneumonia although findings in the upper respiratory tract and oropharynx were minimal: small lingual ulcers were present in cat 9.

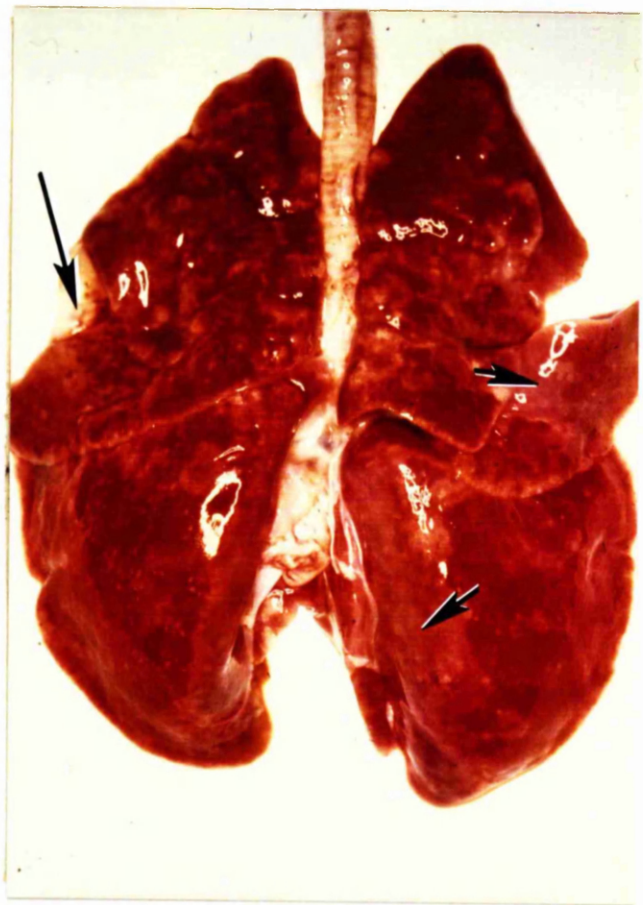
Cats infected by intranasal instillation of virus After intranasal instillation of virus, macroscopic lesions were confined to the upper respiratory tract and oropharynx except for cat 6 in which gross pneumonia was present. Intranasal infection with isolate G1 resulted in congestion and oedema of the turbinate mucosa, ulceration of the rhinarium and hard palate (cat 5) and lingual ulceration and microvesicles in the rhinarium (cat 6). Although macroscopic lung lesions were found in only one animal, microscopic evidence of pneumonia was present in all three cats.

The disease resulting from intranasal instillation of isolate G10 was apparently very mild and no lesions could be detected throughout the respiratory tract or oropharynx.

Pneumonic lesions Gross pneumonia developed in all cats exposed to an aerosol of either virus isolate, but only in one of the six cats exposed to intranasal instillation of virus. Fig. 5.5 shows the lesion distribution in the lungs. It should be noted that, after aerosol infection by either isolate, the same areas of lung were affected but the lesions were more extensive in animals exposed to G1.

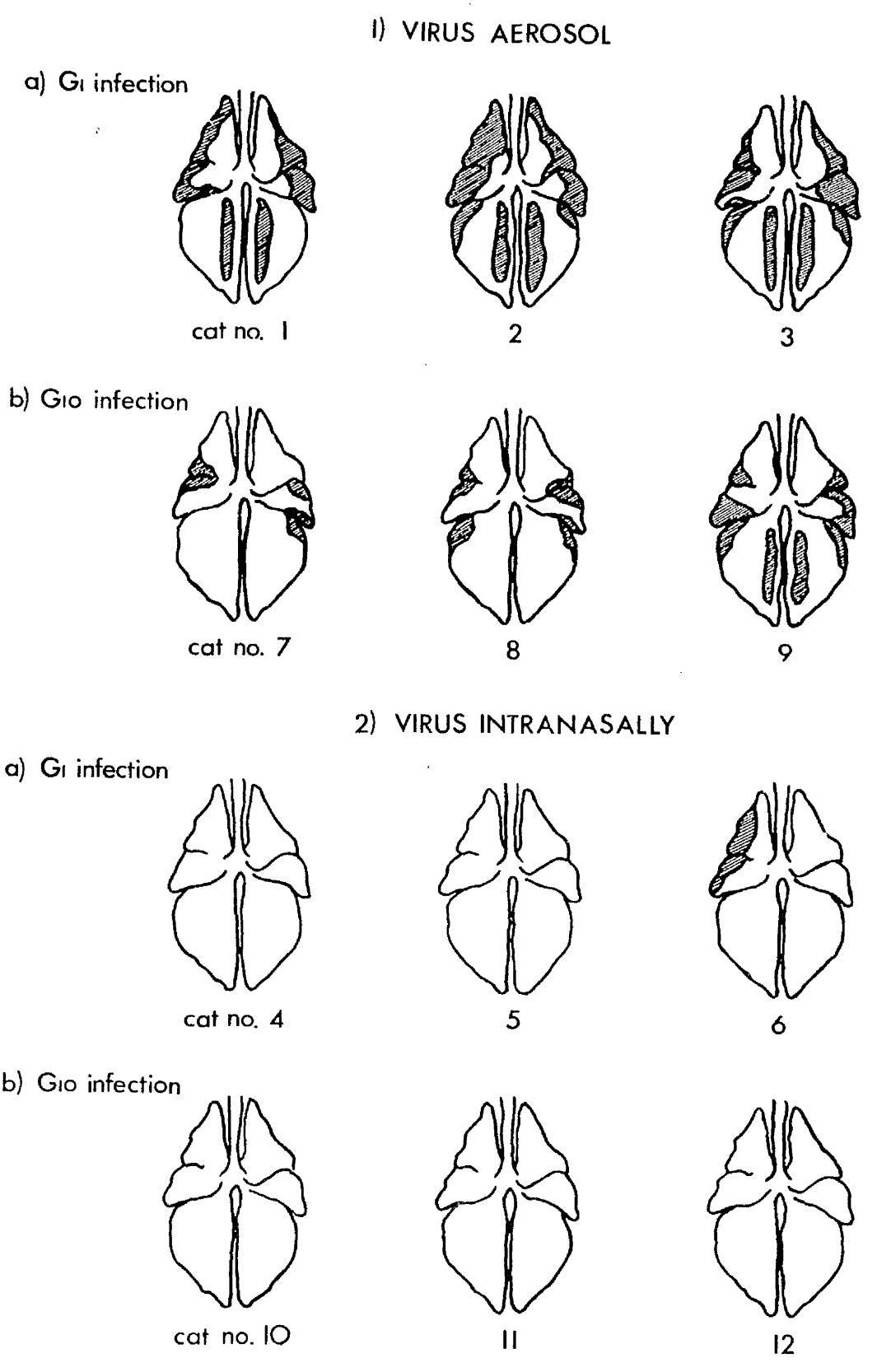
In the three groups of cats in which pneumonia was present microscopically, the pathogenesis of the pneumonic lesion appeared to be identical and results are in general agreement with those originally

Fig. 5.4: The gross appearance of the lungs of cat 2 (infected by FCV G1 aerosol).



Pneumon tissue is mottled grey-pink in colour (short arrows). There is fibrinous pleurisy on the left side (long arrow).

Fig. 5.5 Lesion distribution in the lungs after exposure to virus by aerosol or intranasal instillation.



described by Holzinger and Kahn (1970). The initial lesion was a focal alveolitis in which there was degeneration and sloughing of alveolar epithelial cells with congestion and neutrophil infiltration of alveolar walls. This was followed by the development of areas of acute exudative pneumonia with exudation of serofibrinous fluid and large numbers of neutrophils and macrophages into alveolar air spaces (Fig. 5.6a): in severely affected areas there was necrosis and localised haemorrhage. The pleura overlying pneumonic lung tissue was often oedematous and infiltrated by neutrophils. These exudative changes were found on day 3/4 and day 7 but on day 7 there was also a marked interstitial pneumonia which was the main change present on day 10/12.

This interstitial pneumonia appeared first at the periphery of exudative lesions and was characterised by hypertrophy and hyperplasia of Type II pneumocytes. The alveolar walls in many areas were completely lined by these large, cuboidal cells in which occasional mitotic figures could be seen (Fig. 5.6b). The epithelial cells of neighbouring alveolar ducts were also hyperplastic. At day 10/12 a cellular infiltrate was present alveolar air spaces but was composed mainly of macrophages and desquamated epithelial cells, with only a small number of neutrophils. The alveolar septae were also thickened by infiltration of lymphocytes and mononuclear cells. In some areas of lung in cats killed on day 10/12 there appeared to be residual lesions consisting of focal accumulations of lymphocytes mononuclear cells and occasional neutrophils in interstitial tissue and an increased number of macrophages in alveolar spaces.

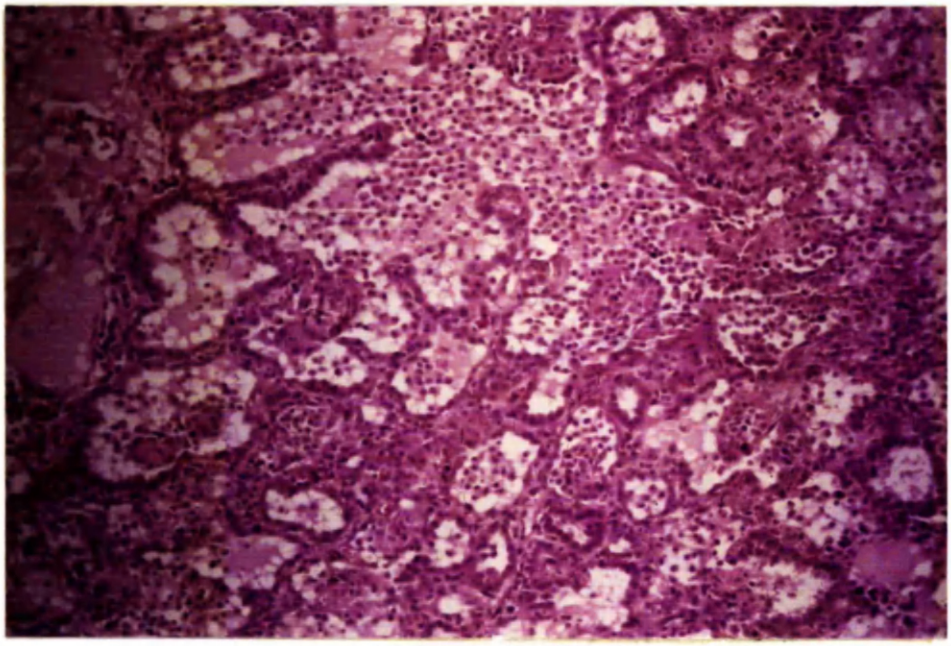
There were no significant findings in the control animals.

Virus isolation The results of attempted isolation of virus from swab sets taken from the cats are shown in Table 5.4. Virus was recovered most frequently from mouth swabs. Isolations from cats infected with G10 were confined to the mouth and there was little difference between aerosol and intranasally infected animals. Cats infected intranasally with isolate G1 provided the largest number of isolations from the nares and conjunctiva.

The results of virus isolations made from tissues are shown in Table 5.5. Each route of administration gave a different distribution of virus among the tissues sampled. Virus was isolated from the lung tissue of all cats infected by aerosol but from only one of the 6 cats infected by intranasal drop. In some cases aerosol administration resulted in systemic infection: virus was isolated from kidney (3/6) and

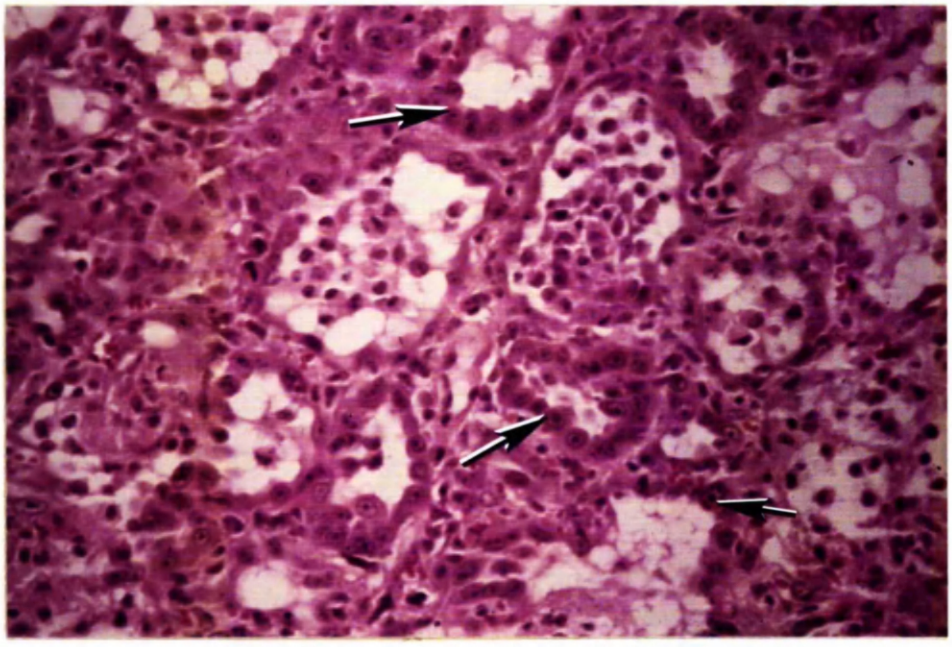
Fig. 5.6: Histopathology of FCV G1 induced pneumonia

a) Pneumonic area from the lung of cat 2 (PM day 7)



Alveolar air spaces are filled with serofibrinous fluid and large numbers of neutrophils and macrophages (Haemotoxylin and eosin, X 150)

b) Higher power of the same field.



Large cuboidal cells (arrows) can be seen lining alveoli and alveolar ducts. (X 375).

spleen (2/6). In the intranasally infected animals, virus was more commonly recovered from the turbinates (5/6) and retropharyngeal lymph nodes (4/6). The tongue and particularly the tonsil were common sites of virus replication with infection by either route. Virus was not isolated from swabs and tissues collected from control cats nor from pre-inoculation swab sets.

From each experimental group, several mouth swab samples, known to contain virus, were assayed by the standard method for plaque-typing. Virus recovered from G1 infected cats was ep forming and that recovered from G10 infected cats, mp forming, although, as in G10 stock, an occasional lp variant was observed in plates at mp confluency.

Turbinate, tonsil and lung samples were also titrated by plaque assay and the results are given in Table 5.6. G1 titres, particularly in turbinate and lung samples, were higher than G10 titres.

A different plaque type to that inoculated, was recovered from the lung of cat 9 (G10 aerosol) at necropsy, 12 days post-infection (P.I.). This isolate was passaged in FEA cells to obtain a sufficiently high titre and assayed using both agar and agarose overlays: plaques were of the lp type and not sensitive to agar inhibition. Lp forming virus was also recovered from the tongue of this animal whereas the virus recovered from tonsil and kidney (after 2 passages in culture) was mp forming.

Bacterial isolation The following bacteria were isolated from the pre-inoculation tonsillar swabs and from oral swabs taken randomly from the SPF group as a whole: Bordetella bronchiseptica, Pasteurella multocida, Pseudomonas aeruginosa and various coliform organisms.

There was no essential change after infection (at necropsy). Tonsil and turbinate swabs contained combinations of the above species along with staphylococci, streptococci and Proteus species which appeared of little significance. Similarly there were no significant isolations made from samples of trachea, bronchus, pneumonic and non-pneumonic lung parenchyma, liver, spleen or kidney.

Serological response Serum neutralisation titres (50% plaque reduction) for cats exposed to a virus aerosol are shown in Table 5.7. Neutralising antibody was detected at 7 days (cat 2) in the experiment using G1 virus. With both viruses a high neutralising titre against homologous virus was found at day 12 (cat 3 and 9).

Also there was one-way cross reactivity; anti-G10 serum (cat 9) neutralised G1 virus but anti G1 serum had no effect on G10 virus.

Seroconversion was similarly demonstrated after intranasal infection although with G10 the titre found at day 14 was relatively low (80). This serum neutralised G1 virus to a titre of 5.

TABLE 5.4: Virus isolated from swabs.

ROUTE OF INFECTION	VIRUS	CAT NO.	PM DAY	DAYS AFTER INFECTION 1	2	3	4	5	6	7	8	9	10	11	12	13	14
Aerosol	G1	1	4	-	M	M	M										
	G1	2	7	-	-	-	-	M	-	-							
	G1	3	12	-	M	-	M	M	M	M	MN	MN	MNC	MN	MN		
	G10	7	4	-	-	-	-										
	G10	8	7	M	M	M	M	M	-	M							
	G10	9	12	-	-	-	M	-	-	M	M	M	M	M	-		
Intranasal	G1	4	3	M	M	M											
	G1	5	7	-	M	M	MN	MN	MN	M							
	G1	6	10	M	MNC	MNC	MNC	MNC	MNC	MNC	MNC	MNC	MN				
	G10	10	3	-	M	M											
	G10	11	7	-	-	-	M	M	M	M							
	G10	12	14	-	-	-	M	-	M	M	M	M	M	-	-	-	-

M = mouth N = nasal C = conjunctival

TABLE 5.5 Virus isolated from tissues

ROUTE OF INFECTION	VIRUS	CAT NO.	PM DAY	VIRUS ISOLATED FROM THESE TISSUES									
				TURBINATES	TONGUE	TONSIL	RLN[a]	E[b]	TRACHEA	LUNG	BLN[c]	KIDNEY	SPLEEN
Aerosol	G1	1	4	+	+	+	-	-	+	+	+	+	+
	G1	2	7	-	-	+	-	-	+	+	+	-	-
	G1	3	12	+	+	-	+	-	-	+	-	+	+
	G10	7	4	-	-	+	-	-	-	+	+	-	-
	G10	8	7	-	-	-	-	-	-	+	-	-	-
	G10	9	12	-	+	+	+	-	-	+	-	+	-
	TOTAL			2	3	4	2	0	2	6	3	3	2
Intranasal	G1	4	3	+	+	+	+	+	+	+	+	-	-
	G1	5	7	+	+	+	+	-	+	-	-	-	-
	G1	6	10	+	-	+	+	-	-	-	-	-	-
	G10	10	3	+	+	+	+	-	-	-	-	-	-
	G10	11	7	+	+	+	-	-	-	-	-	-	-
	G10	12	14	-	-	-	-	-	-	-	-	-	-
	TOTAL			5	4	5	4	1	2	1	1	0	0

a = Retropharyngeal lymph node. b = third eyelid. c = bronchial lymph node.

TABLE 5.6: Titre and plaque type of virus recovered from turbinate, tonsil and lung tissue samples.

ROUTE OF INFECTION	VIRUS	CAT NO.	VIRUS TITRE (PFU/g) AND PLAQUE TYPE RECOVERED		
			TURBINATES	TONSIL	LUNG
	G1	1	7×10^2 (ep)	2×10^2 (ep)	5×10^7 (ep)
Aerosol	G1	2	3×10^4 (ep)	2.3×10^3 (ep)	4.5×10^6 (ep)
	G1	3	-	-	7.5×10^3 (ep)
	G1	4	3×10^3 (ep)	1×10^3 (ep)	5×10^2 (ep)
Intranasal	G1	5	<50 (ep)	1×10^2 (ep)	-
	G1	6	<50 (ep)	3×10^3 (ep)	-
	G10	7	-	1×10^2 (mp)	2.5×10^4 (mp)
Aerosol	G10	8	-	-	5×10^5 (mp)
	G10	9	-	1×10^2 (mp)	1.2×10^2 (lp)
	G10	10	<50 (mp)	1.5×10^4 (mp)	-
Intranasal	G10	11	<50 (mp)	4×10^2 (mp)	-
	G10	12	-	-	-

TABLE 5.7: Serum neutralisation (plaque reduction) titres of cats exposed to an aerosol of G1 or G10.

VIRUS	SERA *					
	G1 INFECTED			G10 INFECTED		
	CAT 1	CAT 2	CAT 3	CAT 7	CAT 8	CAT 9
G1	0	20	640	0	0	20
G10	0	0	0	0	0	>640

* Collected at necropsy.

DISCUSSION

The major clinical features of virulent FCV infection as reported by Hoover and Kahn (1975) are fever, anorexia, depression and dyspnoea or hyperpnoea. The FCV of low virulence cause little or no clinical illness and pyrexia is absent or transient. Clinical signs common to isolates of high and low virulence are ulcers of the tongue, hard palate or external nares.

This study indicates that there is a relationship between plaque morphology and virulence. Using the above criteria to determine if an isolate is of high or low virulence, all FCV classed as mp were of low virulence. FCV of the lp and sp groups were of high virulence (e.g., FPL and FC) or of low virulence (68-2024 and KCD). The only two isolates classed as ep were of high virulence. There was a loose correlation between sensitivity to agar inhibition and low virulence but this might be expected since all of the available mp isolates are inhibited by agar.

Two main conclusions were drawn from the experimental infection of cats with isolates G1 and G10. First, that these isolates differed in pathogenicity: G1 (classed as ep) was of high virulence and G10 (classed as mp) was of low virulence. Secondly, that with these isolates and by the methods of aerolisation and intranasal application used here, the route of administration markedly affected the extent of the acute disease. These results are in agreement with the general finding that aerosol infection with FCV produces a more widespread and serious disease than infection by intranasal instillation.

Isolate G10 administered by aerosol produced pneumonia in all infected cats and lingual ulcers in one cat. However, when the same isolate was administered by direct intranasal instillation there was a marked absence of lesions and the cats remained healthy throughout the period of the experiment although virus could be re-isolated from mouth swabs. Similarly isolate G1 was apparently less virulent when administered intranasally rather than by aerosol.

In both these experiments the infecting dose of virus may have varied along with the route of administration. The amount of virus aerosolised was ten-fold greater than the intranasal dose. However, an unknown but presumably large proportion of the aerosolised virus would have come into contact with the sides of the polythene enclosure and the

coat of the animal. Therefore the actual infecting dose was probably smaller by aerosol than by intranasal instillation.

At necropsy the distribution of virus in the tissues sampled was related to the lesion distribution. Significantly, lesions were present and virus was recovered from all six lungs after aerosol administration but after intranasal infection gross lesions were present in only one lung and virus was recovered from one other. Virus was re-isolated from the daily swab sets taken from all cats infected by either route but no attempt was made to compare the development of the carrier state.

Isolates G1 and G10 differed in pathogenicity. By either route of administration G1 produced a more widespread infection with higher titres of virus present in the tissues which were sampled at necropsy. Even after intranasal administration G1 virus reached the lungs: virus was re-isolated at day 3 from pulmonary tissue and histological evidence of a FCV induced pneumonia was present at days 3, 7 and 10 along with gross lung lesions at day 10. This pattern suggests that G1 replication extended to the lungs at an early stage in the infection; possibly the virus underwent several replication cycles in the upper respiratory tract and then spread to the lungs via the air passages. Following intranasal administration of isolate G10, infection was restricted to the upper respiratory tract and oropharynx. If isolate G10 is delivered directly to alveolar tissue, as in the aerosol administration experiment, virus can replicate and produce pneumonia. An explanation for the localisation of G10 virus after intranasal instillation may be that insufficiently large amounts of virus were produced in the upper respiratory tract mucosae to initiate the extension of infection to tissues in the lower respiratory tract.

The results of virus isolation from nasal swabs and virus titres in turbinate samples at necropsy, indicate a relative inefficiency of G10 replication in the nasal mucosa.

The slightly more severe disease observed in cat 9 compared to the other 2 cats also exposed to an aerosol of G10 might be explained by the isolation of lp forming virus from the lung and tongue at necropsy. Presumably this change in plaque type arose from disproportionate multiplication of the 0.1 percent lp forming virus in the inoculum.

In order to determine which method of virus administration simulates natural infection more closely, it is necessary to consider recent findings on the mode of transmission of FCV. Previously it was

believed that, apart from direct and fomite transmission, aerosol formation might be important in the spread of virus (Povey and Johnson, 1971; Kahn and Gillespie, 1971). However, Wardley and Povey (1977b) have recently shown that aerosol transmission is of little significance in natural transmission: no aerosolised virus was detected from acutely infected cats using a May sampler under optimum sampling conditions; and susceptible cats could be housed in the same airspace as infected animals and remain free of FCV. Thus the main modes of transmission (direct cat to cat contact, sneezing and fomite) are likely to involve the direct impaction of virus on the nasopharynx.

Undoubtedly pneumonia may occur as part of the natural infection (Love and Baker, 1972). However, the importance of FCV-induced pneumonia will be exaggerated if experiments involving aerosols are used: for example, Hoover and Kahn (1975) produced pneumonia with 9 out of 10 FCV strains on infection of SPF cats by aerosol although several of these strains were considered to be of low virulence. This would suggest that a small volume of virus-containing fluid administered by direct intranasal instillation is the method of choice although virus aerosolisation may be useful in circumstances where maximum pathological change is sought.

In a pilot study in which conventionally reared kittens were infected with FCV G1 by aerosol, a very different syndrome was observed. The clinical disease was characterised by moderate to profuse, muco-purulent oculo-nasal discharges which later resulted in the development of crusts around the eyes and nose. Sneezing and coughing were features with fluctuating and often high ($>40^{\circ}C$) rectal temperatures, depression, anorexia and dyspnoea.

The experiment was terminated at 21 days post infection and at this time there was no evidence of recovery.

At necropsy (performed at day 5, 13 and 21 post infection), there was a severe purulent rhinitis, tracheitis and bronchitis superimposed on a FCV associated proliferative pneumonia. Virus (ep type) was recovered from daily mouth swabs and from areas of the respiratory tract and associated tissues. Massive and pure cultures of Bordetella bronchi-septica were obtained from trachea and bronchus and histologically bacteria were observed at these sites between the cilia. A control animal (exposed to an aerosol of tissue culture medium) showed no clinical illness although B. bronchiseptica was recovered from trachea

and bronchus samples. This study indicated the need for careful bacterial monitoring during future experiments. It was interesting to find the presence of B. bronchiseptica in SPF cats in pre-inoculation and randomly taken tonsilar swabs. These cats were clinically healthy and after virus exposure there was no evidence of bacterial involvement in the resulting disease.

CHAPTER 6

THE BIOLOGICAL CHARACTERISTICS OF FCV ISOLATES AND THE BASIS FOR PLAQUE SIZE DIFFERENCES

Introduction

Materials and Methods

Results

Discussion

INTRODUCTION

The basis for plaque size differences is known for a number of plaque mutants of animal viruses (Takemoto, 1966). For example, differences in the rate at which virus is synthesised and released from infected cells have been reported for plaque size mutants of several viruses: the lp variant of VESV was released more efficiently from infected cells than the mp variant (McClain and Hackett, 1959); similarly, small plaque polyoma virus was possibly related to a greater adherence to cellular membranes compared with large plaque virus (Diamond and Crawford, 1964). The sulphated polysaccharide fraction of agar is known to inhibit the plaque formation of a number of small plaque mutants, particularly among viruses of the picornavirus, myxovirus, herpesvirus and poxvirus groups. With some mutants an acid overlay causes plaque inhibition. Thus, plaque formation of attenuated poliovirus strains was delayed under acid agar (Vogt, Dulbecco and Wenner, 1957) and in an acid environment free of sulphated polysaccharides and other polyanions (Wallis and Melnick, 1968). Other factors such as the temperature of incubation and the sensitivity of virus to interferon or serum inhibitors have been shown to play a role in determining the plaque size and development of mutants of the same virus (Takemoto, 1966).

This section of the present study describes attempts to explain the observed difference in plaque size among isolates of FCV. The study concentrates on two isolates in particular: FCV G1 representing the ep group and FCV G10 representing the mp group. A correlation has been found between strains of the mp group and low virulence and therefore it is possible that the same or similar factors which are involved in restricting G10 plaque formation under standard assay conditions also restrict G10 replication, dissemination and tissue damage in the cat. Differences in the biological characteristics of G1 and G10 might explain differences, not only in plaque size but also in relative virulence. The factors investigated included: polyanion inhibition, the optimum temperature and pH for plaque development, virus stability at 37°C, the rates of virus adsorption to FEA monolayers and the intracellular maturation and release of virus in single step growth.

The inhibition of plaque development by polyanions and the effect of DEAE-dextran

The inhibitory effect of heparin (a polyanion) was examined. FEA cultures were infected with suitable dilutions of FCV G1 and G10 virus stock and overlays were applied, either containing agarose (0.9% agarose in MEM with 1% FBS) or agarose and heparin (Sigma Chemical Company) at 100 µg/ml. The cultures were incubated at 37°C, fixed and stained after 48 hours and plaques were measured as described previously.

A polycation, diethylaminoethyl dextran (DEAE-dextran: Sigma Chemical Company) was added to the standard agar overlay and the effect on plaque size was examined. Overlays with DEAE-dextran concentrations of 0-1000 µg/ml were used in plaque assays of FCV G1, G10 and KCD and the effect on plaque formation and development was determined.

The optimum temperature and pH for plaque development

The optimum temperature for plaque development and for virus replication in liquid medium was determined for G2 and G10 by the same method as described in Chapter 3, for G1.

The effect of pH was determined by performing assays under agar overlays made up with medium of pH 7.0, 7.4 and 7.8. The pH of MEM is 7.4. The pH was decreased by the addition of Na H_2 PO4 (14 mg/ml) or increased by the addition of Na HCO_3 (35 mg/ml). The titres and plaque sizes of FCV G1 and G10 were compared using the 3 types of overlay.

Inactivation at 37°C

FCV G1, G2 and G10 virus stocks were diluted to 1-5 x 10^5 PFU/ml, using L15 + 1% FBS as diluent and were dispensed in 0.5ml volumes which were placed in a waterbath at 37°C. At hourly intervals for 6 hours and then at 12 and 24 hours, aliquots of virus were removed and stored at -70°C. The infectivity of each was later determined by plaque assay.

Adsorption to FEA monolayers

The adsorption rates of FCV G2 and G10 were found using the method described in Chapter 3, for G1.

Single step growth curves

1) FEA monolayers: growth curves FEA cultures in 5 cm plates were infected with G1, G2 or G10 virus suspensions (in MEM) at a virus-cell ratio of 10. After an adsorption time of 50 mins at 37°C, inocula were removed and the cultures were rinsed twice with MEM. Two ml of EFC/10 was

added to each plate before re-incubation at 37°C. Immediately, and at 2, 3, 4, 5 and 6 hours thereafter, 3 plates, each infected with one of the above isolates, were removed. The medium from each plate was transferred to a small screw-cap vial, stored at -20°C and later assayed to obtain the extra-cellular virus titre. Two ml of fresh EFC/10 was added to each plate and, by scraping monolayers off into the medium using a sterile silicon bung followed by agitation by rapid pipetting, a cell suspension was produced. The suspension was frozen and thawed twice, ultrasonicated for 30 seconds (24.78 k^c/s) and assayed to obtain the cell-associated virus titre.

2) FEA monolayers: length of eclipse and latent periods FEA cultures in 5 cm plates were infected with G1, G2 or G10 virus in L15 medium at a virus-cell ratio of 1:20. After an adsorption period of 3 hours at 4°C, the inocula were removed and cultures were washed twice with MEM. Two ml of EFC/10 was added to each plate, which were incubated at 37°C. At intervals of 15 mins between 2 and 5 hours after the end of the adsorption period, plates were removed, the medium was taken off and centrifuged at 2000 rpm for 5 mins. The supernatant was collected, stored at -20°C and later assayed to obtain the extra-cellular virus titre. Two ml of fresh EFC/10 was added to each plate and the monolayers were scraped off and suspended in this medium as above. After freezing and thawing and ultrasonication, as before, the suspensions were assayed to obtain the cell-associated virus titres.

3) FEA-cell suspension Using the following methods, the replication cycle of isolate G1 was compared to that of G10 and in a separate experiment strain FPL was compared to strain F11.

Approximately 10^8 PFU of each isolate (G1 or G10) was inoculated into a 5 cm plate containing a FEA monolayer (virus-cell ratio of 50). Strain FPL and F11 virus stocks were of lower titre and used at a virus-cell ratio of 5. Virus was adsorbed for 45 mins at 37°C and afterwards the inoculum was removed and the monolayer was rinsed three times with MEM. Four ml of EFC/10 was added and the culture was re-incubated at 37°C for a further 45 mins. At this point the medium was removed, the monolayer was dispersed with trypsin-versene solution and the cells were collected in 4 ml of L15. The cells were counted in a haemocytometer and diluted tenfold in L15 supplemented with 1% FBS. The cell suspension was placed in a waterbath at 37°C and gently agitated by magnetic stirrer.

Samples were removed at 0, 120, 180, 210, 240, 270, 300, 330, 360, 420 and 480 mins afterwards (zero time was taken as the end of the adsorption period). At each sample point, 1 ml of the cell suspension was removed and centrifuged at 3,000 rpm for 10 mins. The supernatant was collected, stored at -20°C and later assayed to obtain the extra-cellular virus titre. The cell pellet was resuspended in 1 ml L15, frozen and thawed twice, ultrasonicated for 30 secs. (24.78 K^c/s) and assayed to obtain the cell-associated virus titre.

4) Infective centre assay The method, employing a cell suspension in agar, was described in Chapter 3. Comparable results were obtained by the following method: an infected cell suspension was serially diluted in tenfold steps, using MEM as diluent. FEA cultures in 5 cm plates were inoculated with 0.1 ml from each dilution and after a 60 min adsorption period a standard agar overlay was added. Controls were included. Cells were removed by centrifugation at 3,000 rpm for 10 mins and the supernatant was titrated for non-cellular virus. The entire procedure was carried out during the virus eclipse period.

RESULTS

The inhibition of plaque development by polyanions and the effect of DEAE-dextran

As described earlier, all FCV isolates classified as mp along with certain other isolates were sensitive to inhibitors present in agar. To demonstrate that the inhibition of these strains was associated with the sulphated polysaccharide (polyanion) fraction of agar, heparin, another naturally occurring sulphated polysaccharide, was added to agarose and the effect on plaque size was observed. The addition of heparin made no difference to the size of plaques produced by isolate G1. However, the plaques produced by isolate G10 were of decreased size in the presence of heparin; under an ordinary agarose overlay, the mpd was 3.5 mm and the addition of heparin at 100 µg/ml resulted in a mpd of <1 mm.

The addition of DEAE-dextran to agar is known to enhance plaque formation and plaque development for a number of virus mutants (Leibhaber and Takemoto, 1961). DEAE-dextran is a polycation which, it has been suggested, neutralises the agar polyanions which cause inhibition, forming insoluble polyanion-polycation complexes.

DEAE-dextran was found to have an enhancing effect on G10 plaque development: under standard agar and at DEAE-dextran concentrations of 0-100 µg/ml the mpd was <1 mm, at 250 µg/ml the mpd was 2.0 mm and at 1000 µg/ml the mpd was 3.8 mm. In contrast the plaques produced by isolate G1 were of similar size under standard agar (mpd = 4.4 mm) and agar with DEAE-dextran at 1000 µg/ml (mpd = 4.6 mm). There was a slight enhancing effect on strain KCD plaque development: under standard agar the mpd was 1.4 mm and with DEAE-dextran at 1000 µg/ml the mpd was 1.8 mm.

The optimum temperature and pH for plaque development

For isolates G2 and G10, as for G1, the optimum temperature for plaque development and virus replication was found to be 37°C. Using media of pH 7.0 or 7.4 in the overlay had no significant effect on plaque size or on the number of plaques formed. However, with medium of pH 7.8 the plaque formation of isolate G10 was enhanced. The apparent titre of the inoculum using a standard agar overlay was 1.5×10^6 PFU/ml but, using medium of pH 7.8 the titre was 4.7×10^6 PFU/ml. There was no significant change in G10 plaque size at this pH.

The plaque development of isolate G1 was retarded at pH 7.8; the mpd under standard agar was 5.4 mm but, with medium of pH 7.8 in the overlay, the mpd was 4.0 mm.

Inactivation at 37°C

Fig. 6.1 shows the surviving fractions of virus infectivity at 37°C plotted against time. The three isolates are apparently of equal stability at this temperature with a half-life of approximately 5 hours. In an earlier experiment, in which virus was diluted in MEM instead of L15, a more rapid rate of inactivation was observed (a half-life of approximately 1 hour). The aliquots of virus in MEM were not gassed with 5% CO_2 in air and subsequently became alkaline so that presumably the increased pH resulted in decreased stability. A similar finding was reported for VESV variants (McClain and Hackett, 1959).

Adsorption to FEA monolayers

The adsorption rates for isolates G1, G2 and G10 were the same (see Fig. 6.2). In all three, there was no significant increase in plaque count when the adsorption time was extended from 50 or 60 min to 120 min.

Single step growth curves

1) Growth curves in FEA monolayers

The single step growth curves for isolates G1, G2 and G10, cell-associated and extra-cellular virus, are shown in Fig. 6.3. The curves for all three isolates are similar: the latent and eclipse periods end at approximately 2 hours with a rapid rise in intracellular virus between 2 and 3 hours. After 4 hours (G10) and 5 hours (G1 and G2), there was an apparent fall in the intracellular virus titre but this can be explained in terms of cells rounding up and losing contact with the surface of the plate. These suspended cells would contribute (as infective centres) towards the extra-cellular virus titre rather than the cell-associated titre. This fall in intracellular virus titre was not observed in other experiments where the cells were collected by centrifugation. The earlier appearance of the G10-associated cpe, compared to the G1 cpe, was also noted in other experiments.

2) The length of eclipse and latent periods in FEA monolayers

This experiment was undertaken to find whether there were small differences between isolates which would not necessarily be detected by sampling at hourly intervals or by the technique used above. Virus adsorption was carried out at 4°C for 3 hours in an attempt to increase

Fig. 6.1 Inactivation curves of FCV G1, G2 and G10 at 37°C.

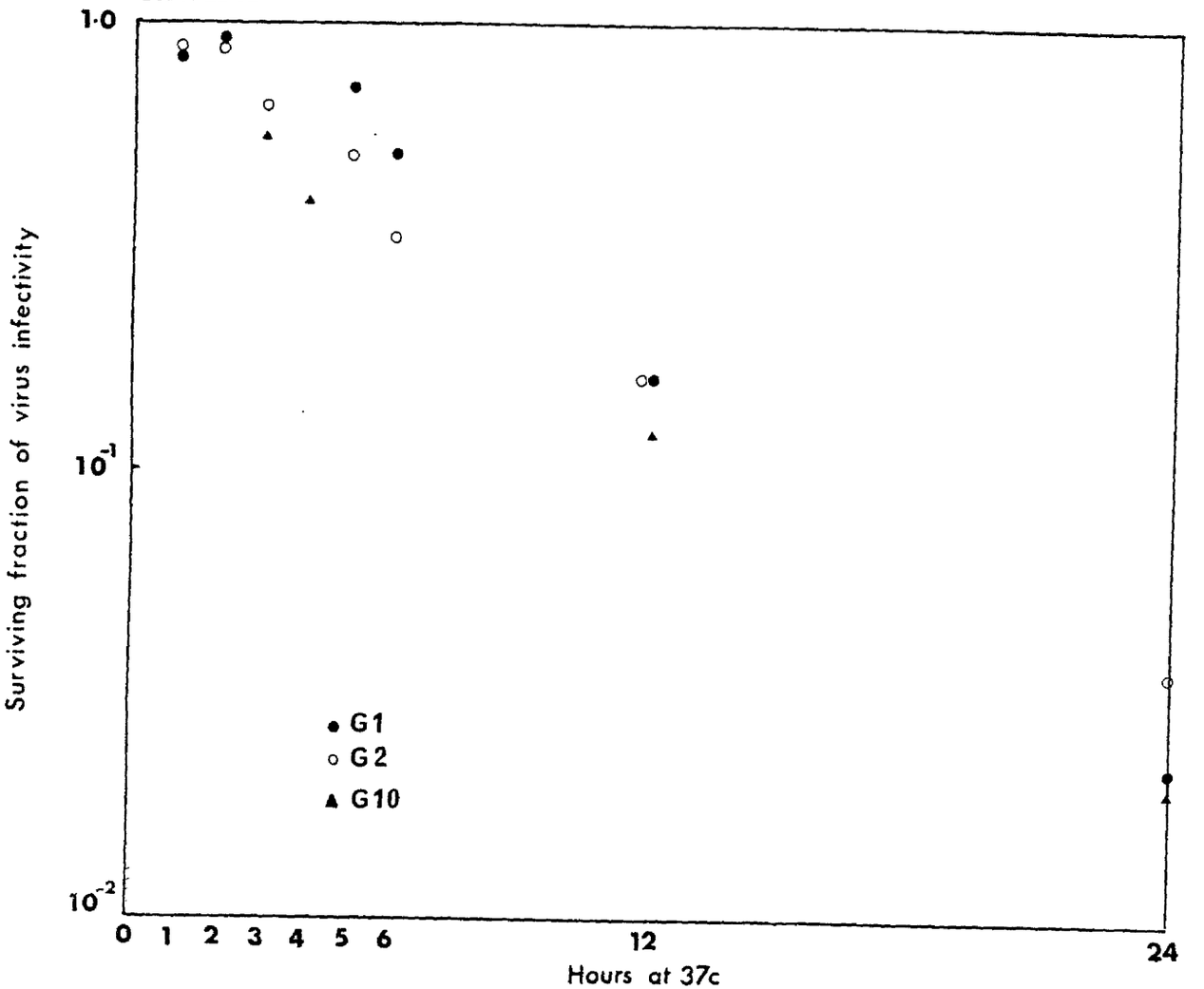


Fig. 6.2 Adsorption rates of FCV G1, G2 and G10 to FEA monolayers.

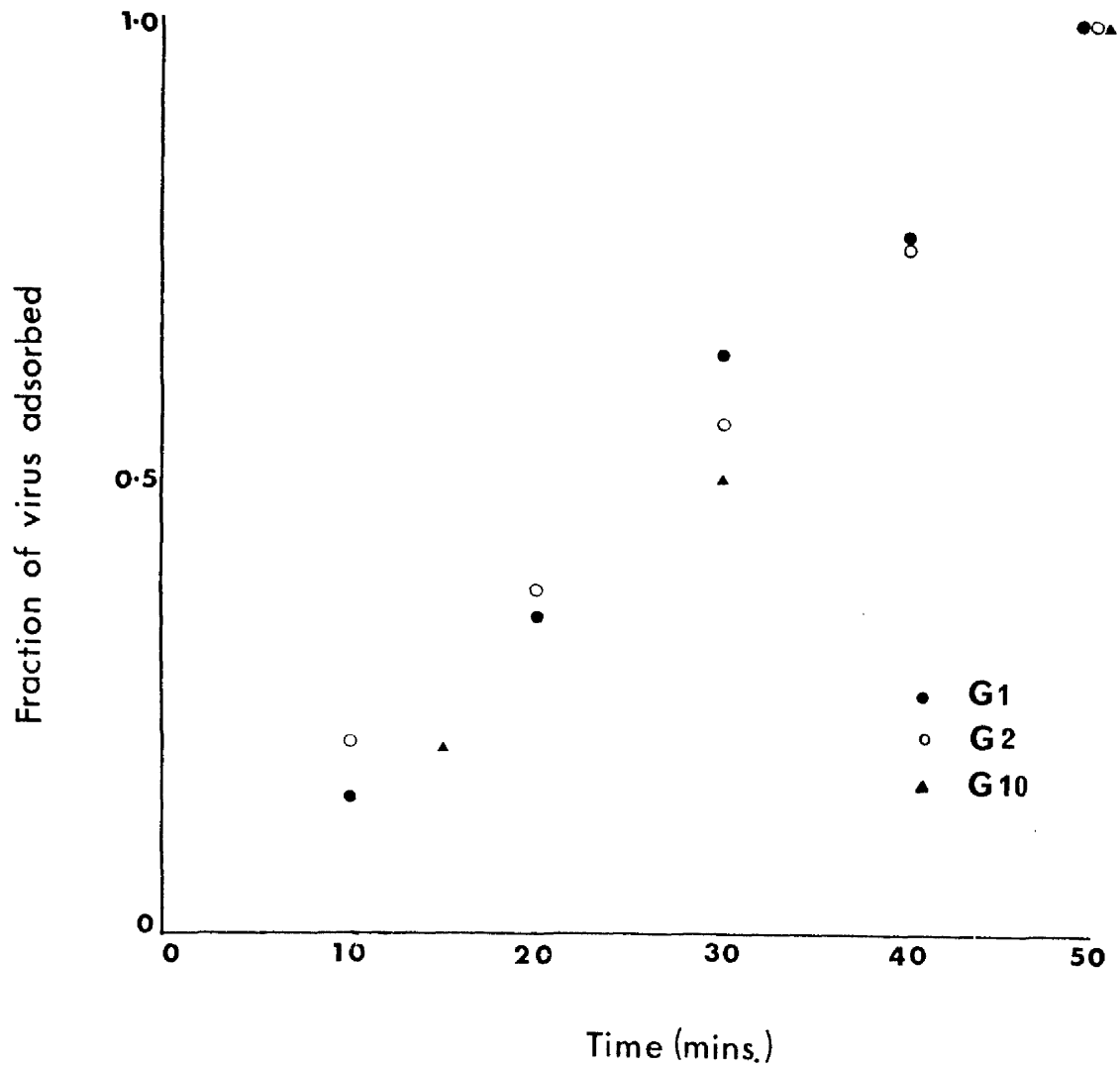
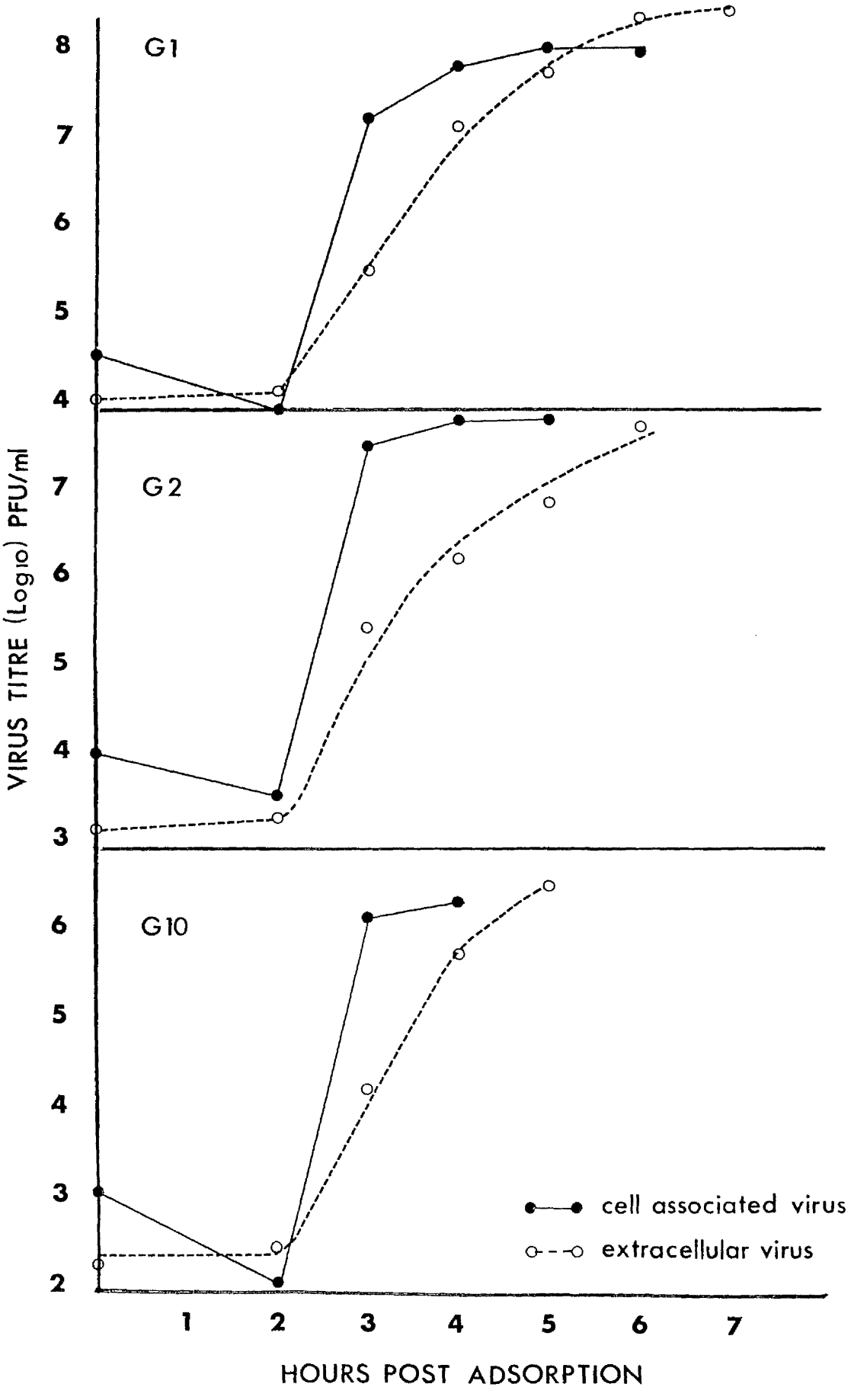


Fig. 6.3 Single step growth curves of FCV G1, G2 and G10 in FEA monolayers.



the synchrony of growth and samples were collected at 15 min intervals.

Fig. 6.4 shows that there is no difference in the length of the eclipse period for the three isolates. The first significant rise in virus titre occurred between 150 and 180 mins after adsorption.

A small difference was detected in the latent period of G10 compared to G1 and G2 (Fig. 6.5). Virus was released from G10-infected cells after 210 mins, 15 mins earlier than from cells infected with G1 or G2.

3) Growth curves in FEA cell suspension

These experiments, along with infective centre (i.c.) assays, were designed to examine the rate of synthesis of virus, the efficiency of virus release from infected cells and the total virus yields.

The single step growth of isolates G1 and G10 were compared and the results are shown in Figs. 6.6 and 6.7. The cell-associated virus growth curves are very similar (Fig. 6.6) in that the end of eclipse occurred between 2 and 3 hours after infection. However, the initial rise of 2 logs in the G10 titre compared to less than 1 log in the G1 titre perhaps indicates a slightly earlier appearance of newly synthesised G10 virus. With both isolates, maximum cell-associated virus titres were found after 6 hours. Infective centre assay indicated that 3.0×10^4 cells/ml were infected with G1 (69% of the total number of cells exposed) and 2.9×10^4 cells/ml were infected with G10 (64% of the total). The amount of infectious virus present in each of these cells was therefore approximately 10^3 PFU for G1 and G10.

However, from the slope of the curve during the rise phase, the rate of synthesis of G10 was apparently marginally slower than the rate of synthesis of G1.

A greater difference was observed in the extra-cellular virus growth curve (Fig. 6.7). G10 virus was apparently released earlier than G1 virus (as in (2) above) and the rate of release, as indicated by the slope of the curve, was slower. The burst from G1-infected cells was larger and occurred at least 1 hour before that from G10-infected cells.

The efficiency of release was calculated to be greater for G1 than G10: 20% of synthesised G1 virus was released into the medium (at 6 hours) compared with 7% of synthesised G10 virus.

The single step growth of isolates FPL and F11 are compared as shown in Figs. 6.8 and 6.9. The cell-associated virus growth curves are almost identical (Fig. 6.8). In this experiment the lower virus-cell

ratio during the initial infection of cells resulted in a smaller number of infective centres; 4.1 x 10^3 cells/ml were infected with F11 virus (10% of the total number of cells exposed) and 1 x 10^3 cells/ml were infected with FPL virus (only 3% of the total). This means that, although the curves are similar, the amount of FPL virus synthesised per cell was greater than the amount of F11 synthesised (2.5 x 10^3 PFU/cell compared to 10^3 PFU/cell).

Fig. 6.9 shows the extracellular virus growth curve. FPL virus was apparently released earlier than F11 virus and more was released (3.6 x 10^2 PFU per cell compared to 2.2 x 10^2 PFU per cell). However, the ratio of released to cell-associated virus at the burst phase was lower for FPL than for F11 (14% compared to 22%).

4) Infective centre assay

Two alternative methods for estimating infective centres were investigated mainly to ascertain if the results obtained by the plaque method (used in the above calculations of virus yield) were authentic: i.e., that plaques developed solely from virus-producing cells.

In the first method, infected cells were recognised by immunofluorescence. FEA monolayers, grown on microscope slides, were infected at a virus-cell ratio of 10. Five hours later the slides were washed twice in PBS and fixed in acetone. An indirect immunofluorescence technique was used, as described in Chapter 7, and specific fluorescence was observed in approximately 20% of the cells. A similar percentage of infectivity was found, using an agar cell-suspension infective centre assay, in another experiment with a virus-cell ratio of 10.

In the second method, single cells were cloned and the yield of virus from each was assayed. Virus (G1) was added to an FEA monolayer contained in a 5 cm plate (virus-cell ratio of 50). After an adsorption time of 50 mins at 37°C., the inoculum was removed, the culture was rinsed several times with MEM, the monolayer dispersed with trypsin-versene solution and the cells were resuspended and diluted to 5 x 10^3 per ml in L15. The cell suspension was divided into two aliquots and from one single cells were picked out by micropipette from drops of the cell suspension on a microscope slide. Fifty cells were cloned and deposited into vials containing 0.5 ml L15. The vials were incubated at 37°C for 6 hours to allow completion of the virus replication cycle, then stored at -20°C and ultrasonicated for 30 secs before each was assayed. Thirty-six vials contained no virus and the remaining 14 contained 750 to 1000PFU i.e., the proportion of infected cells was 28%. The other aliquot was

assayed by the plaque method which gave a result of 30%. These results are in good agreement and indicate that infective centre estimation by plaque assay gives an accurate figure for the number of virus-producing cells. Also the G1 virus yield per cell as calculated in (3) above was confirmed by this method.

Fig. 6.4 The eclipse periods of FCV G1, G2 and G10.

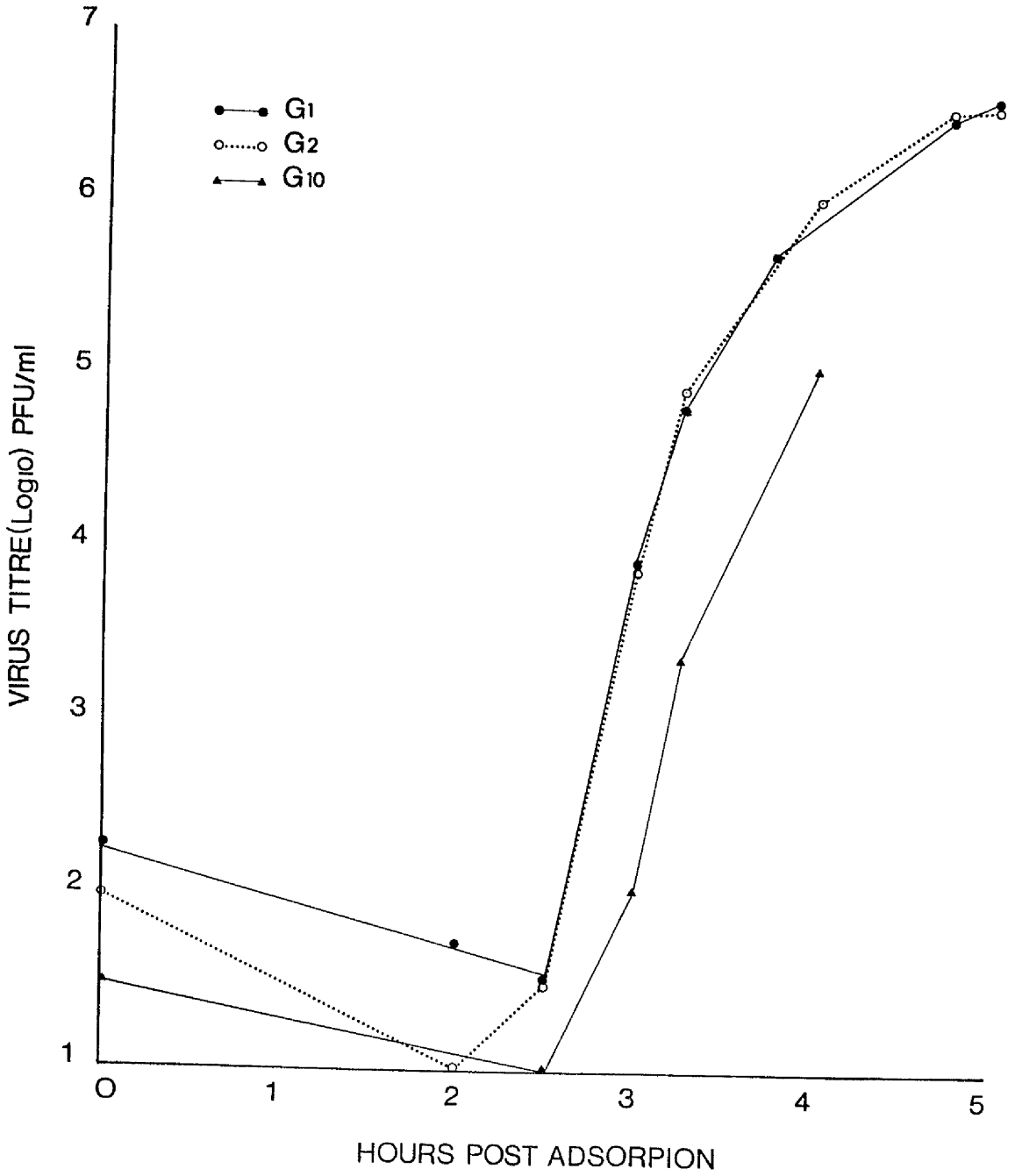


Fig. 6.5 The latent periods of FCV G1, G2 and G10.

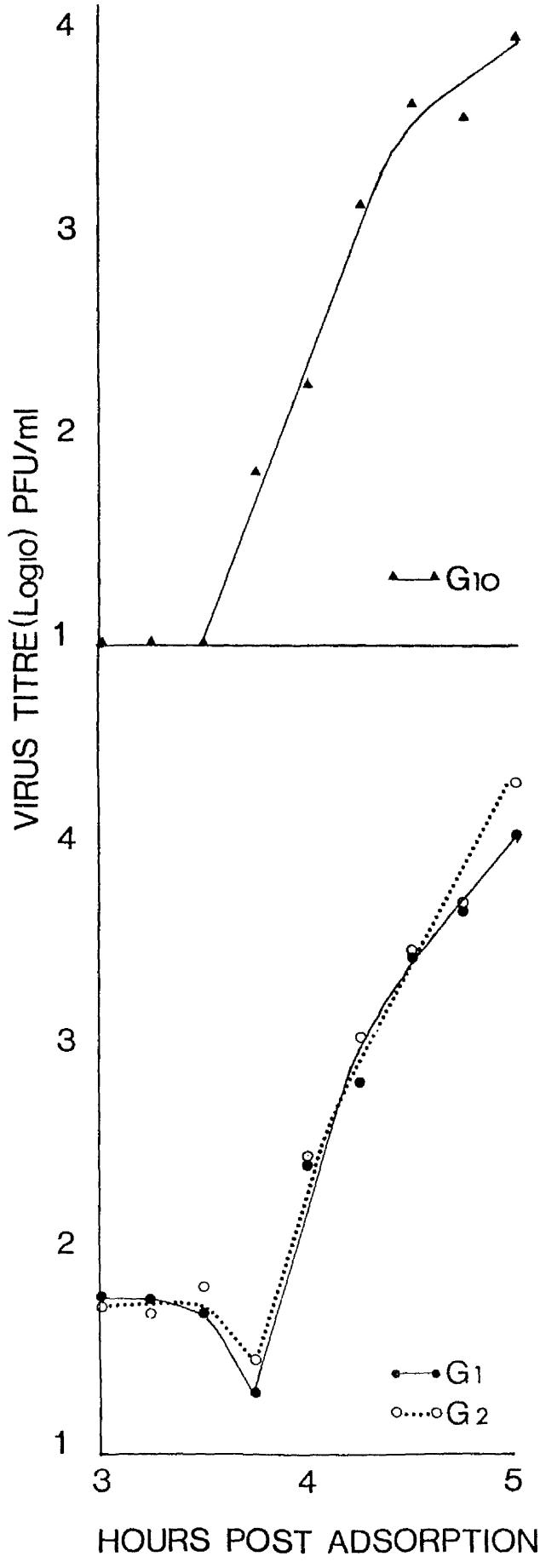


Fig. 6.6 Single step growth curves of FCV G1 and G10 in FEA cell suspension; cell associated virus.

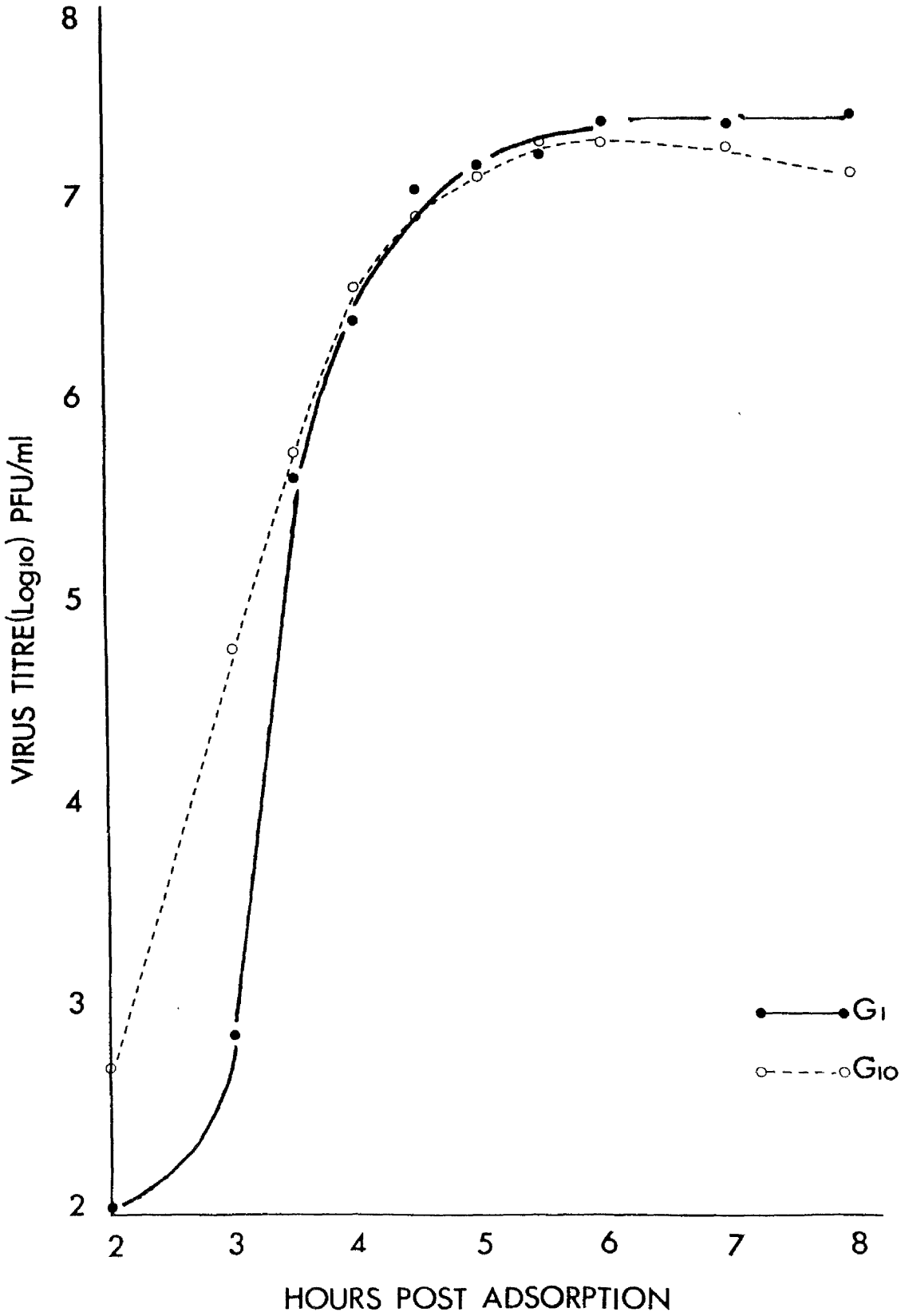


Fig. 6.7 Single step growth curves of FCV G1 and G10 in FEA cell suspension; ..... released virus.

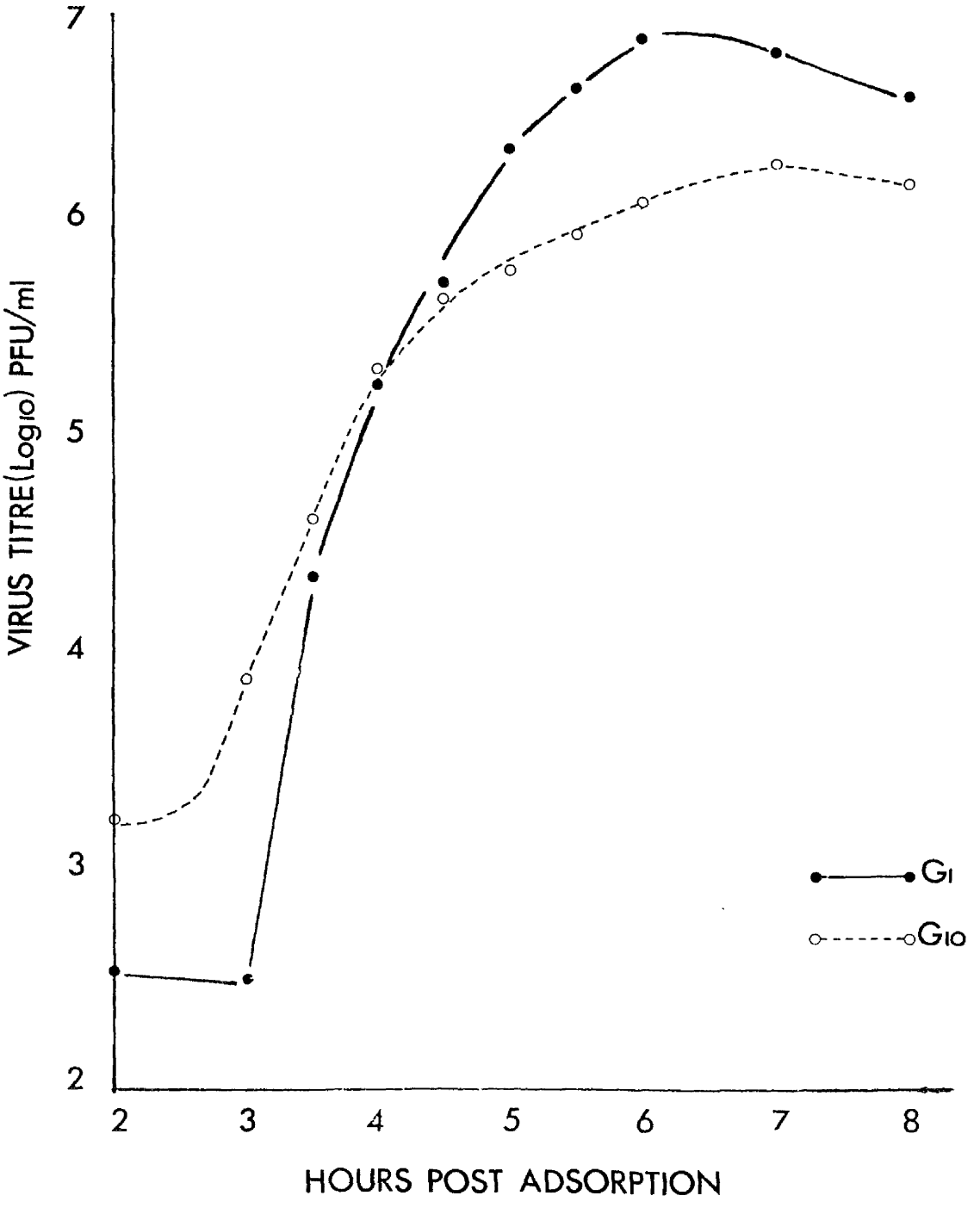


Fig. 6.8 Single step growth curves of F11 and FPL; cell associated virus.

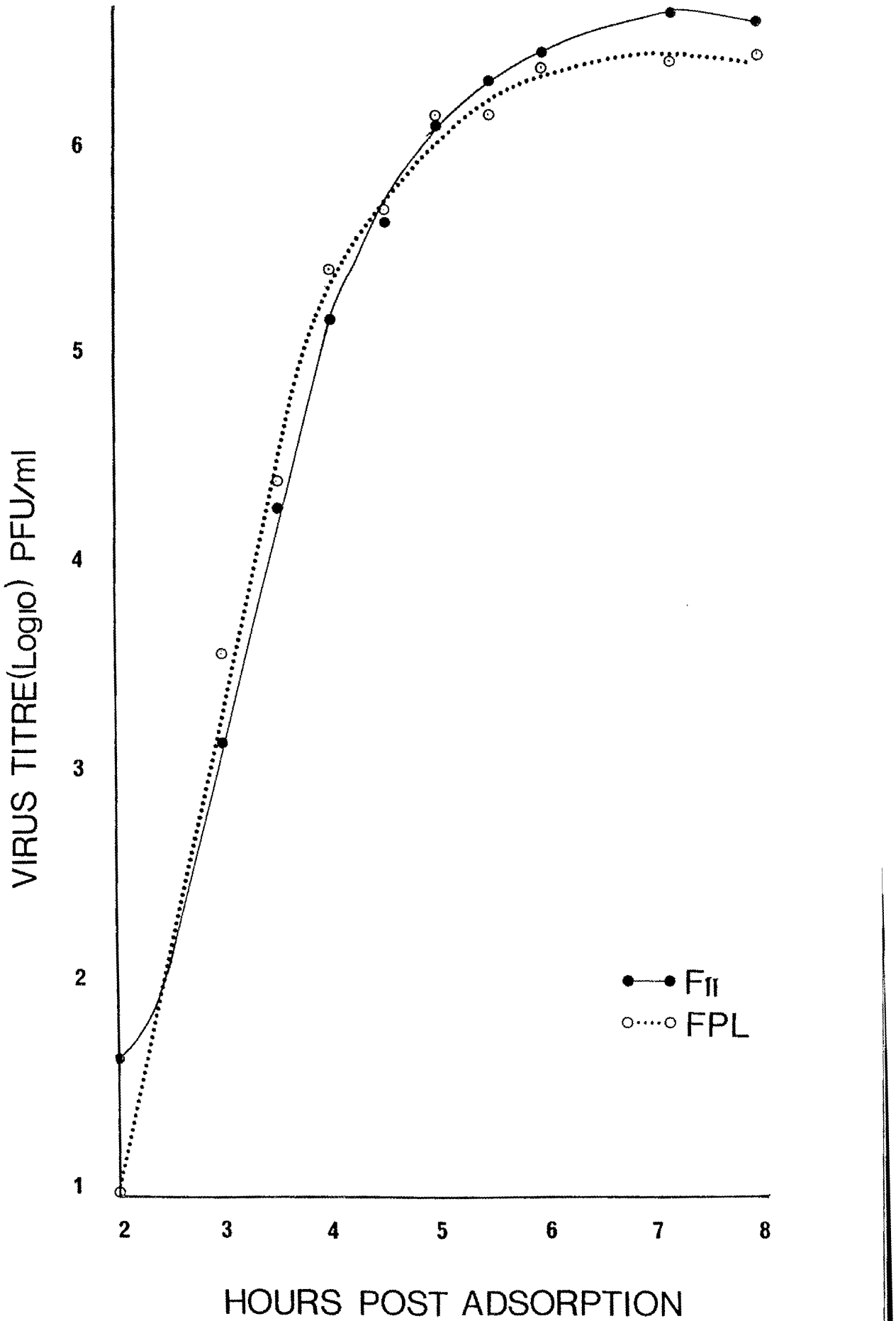
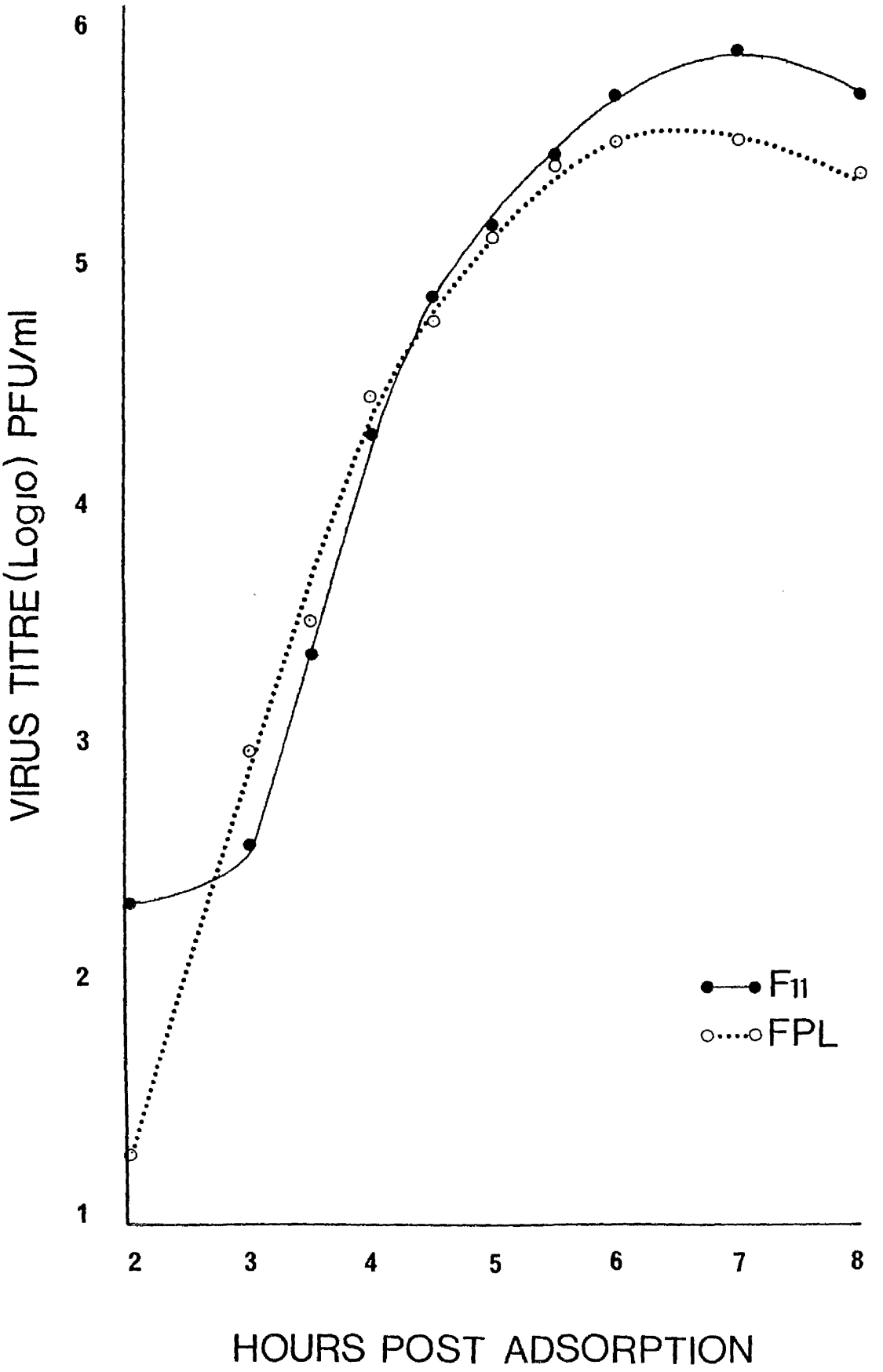


Fig. 6.9 Single step growth curves of F11 and FPL; released virus.



DISCUSSION

FCV isolates G1 and G10 produce plaques of very different size under standard plaque assay conditions (see Chapter 4). In the present section the biological characteristics of these two isolates, along with certain others, were examined to determine the basis for their plaque size difference and it was speculated that results might also relate to differences in virulence (see Chapter 5).

Using an agar overlay, G10 plaque development was inhibited, presumably by the sulphated polysaccharide (polyanion) fraction of agar. The results presented here demonstrate further that inhibition was associated with polyanions: G10 plaque development in agarose was inhibited by the addition of heparin (a polyanion) and the inhibitory activity in agar was removed by the addition of DEAE-dextran (a polycation). Inhibition of minute and small plaque mutants of picornaviruses by agar is not uncommon and has been demonstrated for poliovirus (Takemori and Nomura, 1960; Agol and Chumakova, 1962; 1963), for EMC virus (Takemoto and Liebhaber, 1961), for Coxsackieviruses (Choppin and Eggers, 1962) and for Mengovirus (Colter, Davies and Campbell, 1964). The inhibitory action was thought to result from the binding of virus to the polyanions, thereby preventing virus adsorption to cells (Liebhaber and Takemoto, 1963). Other polyanions have been shown to produce plaque inhibition (Takemoto and Kirshstein, 1964) and it has previously been demonstrated that certain viruses can be enhanced by the incorporation of cationic polymers (DEAE-dextran) in overlays supposedly by neutralising the polyanions in the overlay (Takemoto and Liebhaber, 1961). It was later reported (Wallis and Melnick, 1968) that cationic polymers enhanced the diffusion of certain viruses through agar and work by Balayan et al (1970) and recently by Totsuka, Ohtaki and Tagaya (1978) suggested an alternative mechanism for plaque inhibition by polyanions and the enhancing effect of polycations. It now seems likely that virus aggregate formation and consequently reduced diffusion takes part in delaying the appearance or reducing the rate of development of plaques and the tendency to aggregate is influenced by the constituents of the overlay, including sulphated polysaccharides. The results of Wallis and Melnick (1968) could be explained in terms of polycations preventing aggregate formation. For FCV, isolates such as G10 may form aggregates under an agar overlay whereas isolates such as G1 may tend to remain non-aggregated. In the

presence of polyanions G10 virus diffusion would be restricted and so, consequently, would G10 plaque size. In this context, G10 virus aggregation was observed by electron microscopy; when examining infected cell cultures, virus aggregates were found amidst cellular debris (see Chapter 7). These cultures had been maintained, during virus infection, in an ordinary, polyanion-free, liquid medium and this observation may indicate at least a tendency for this virus to aggregate.

The difference in the tendency to aggregate between G1 and G10 could be explained by assuming that these isolates had different ionic atmospheres to their capsid surface. A simple model might be that G10 virions have a greater net positive capsid surface charge than G1 virions and when released from cells tend to react or form complexes with polyanions and diffusion is restricted. G10 virions therefore accumulate locally. If polyanions are absent or if polycations are added to the overlay in sufficient amounts, local accumulation would be counteracted and virus diffusion increased. Differences in virion surface properties have been found between plaque variants of other viruses: for example, small plaque forming (r+) and large plaque forming (r) variants of EMC virus differ in their surface properties as measured by electrophoresis (Breeze, 1964). Differences were also detected by calcium phosphate chromatography (Burness, 1967) in which the large plaque variant eluted at a lower ionic strength than the small plaque variant, again thought to be due to the adsorption of virus to the column by its surface charge. It would be of interest to compare FCV G1 and G10 isolates by these techniques or by other techniques now available such as isoelectric focusing which separate particles on the basis of charge.

Since G10 plaque size dramatically increases in the absence of polyanions it is considered likely that polyanion inhibition is the main cause for the plaque size difference between G1 and G10. However, it was noted that under agarose or agar containing DEAE-dextran, G1 plaques still developed faster than G10 plaques. This remaining disparity may result from differences in virus replication kinetics which were detected between the two isolates. G1 virus was released at a faster rate and more completely than G10 virus and, although G10 virus was detected at an earlier time in the extracellular fraction, this would presumably contribute little to the extension of a local virus lesion compared to later events in the replication cycle.

It should be noted that differences were not detected initially by a simple monolayer growth curve experiment in which samples were taken

at hourly intervals. This experiment did serve to demonstrate the characteristic, rapid single step growth curve of FCV which is very similar to that found for picornaviruses (Howes and Melnick, 1957). It was evident that only major differences between the viruses examined would be detected by this method and for a more detailed analysis the G1 and G10 growth cycles were examined in two further experiments. Firstly, their eclipse and latent periods were determined and secondly their rates of synthesis and release, and total virus synthesis and release were obtained. It was necessary to increase the number of sample points and to obtain good synchrony of infection. In the former experiment virus was adsorbed at $4^{\circ}C$ for 3 hours in an attempt to attain synchronous virus replication. The rationale of this is that virus adsorption is a result of electrostatic attraction and independent of temperature (except for the effect of temperature on Brownian movement) but after adsorption further steps in the replication cycle are temperature dependent (penetration, uncoating etc.). After 3 hours at $4^{\circ}C$ most of the virus in the inoculum should be in the adsorbed state and when the temperature is raised to $37^{\circ}C$ penetration should begin synchronously. In the latter experiment synchronisation of infection was obtained by using a high virus to cell ratio of infection (Cooper, 1964).

The difference between the size of plaques produced by strains FPL and F11 under agarose may also be explained in part by the greater quantity of FPL virus released from infected cells.

In an alkaline agar overlay G10 plaque formation was enhanced (although there was no increase in the rate of plaque development) and G1 plaque size was reduced. It may be relevant that agarose is more alkaline than agar and a similar enhancement of G10 plaque formation and G1 plaque size reduction occurs under agarose. The basis for these observations was not investigated.

It should be noted that only isolates G1 and G10 have been examined in detail in this study and it has been assumed that their biological characteristics are representative of other isolates classed as ep and mp. This may not be strictly true since, for example, all mp forming isolates are not inhibited to the same degree by polyanions (see Chapter 4). However, a correlation does apparently exist between mp forming isolates and low virulence and possibly ep forming isolates and high virulence and it may be salutary to examine the biological characteristics of G1 and G10 and consider how these characteristics

could be related to virulence. FCV G10 and all other mp forming isolates examined were found to be inhibited in plaque development by polyanions. It is therefore conceivable that the same factor plays a role in mp virus inhibition *in vivo*. Cat respiratory tract (tracheal) mucous has been shown by electrophoresis to contain two negatively charged glycoproteins (Gallagher *et al.*, 1975). To investigate the possible mp virus plaque inhibitory property of cat respiratory tract mucous, the following experiment was carried out. Two seronegative, SPF, adult cats were anaesthetised and intubated. Respiratory tract secretions were stimulated by the intravenous administration of pilocarpine (5 mg/lb bodyweight) and collected by lung lavage using PBS as the washing agent. When a sufficiently large sample was obtained, hypersecretion was stopped by giving atropine intramuscularly (1 mg/lb bodyweight). The cats were allowed to recover and showed no ill effects. Secretions in PBS were homogenised in a Sylverson blender and the resultant suspension was added to molten agarose overlay at concentrations of 0-25%. This was used to overlay FEA cultures infected with approximately 25 PFU of G1 and G10 virus. Plaque diameters were measured in the usual way after 48 hours of incubation at 37°C. Respiratory tract secretions at a concentration of 10% in the overlay caused a reduction in size of both G1 and G10 plaques and there was no significant difference in the degree of reduction of each. At higher concentrations cell toxicity was observed. Therefore no polyanion-like inhibition was demonstrated by this rather unsophisticated method. It would be worthwhile to repeat this type of experiment using dialysed respiratory tract secretions (and possibly non-pilocarpine stimulated secretions) possibly with a more sensitive test for mp virus inhibition: for example, examining the effect on single or multi-cyclic growth. The secretions obtained were predominantly tracheal in origin and it may be that mucous from other regions of the cats respiratory tract, for example nasal mucous (see below), differs in its polyanion content.

Results presented in Chapter 5 indicate a relative inefficiency of G10 replication in nasal mucous compared to G1 replication. It was postulated that the amount of virus synthesised at this site may be important in determining the degree of extension (e.g., to the lungs) of infection. Single step growth curve experiments have provided results which demonstrate the relative inefficiency of G10 replication in cultured cells and it is conceivable that this factor alone restricts G10 virulence.

CHAPTER 7

THE CYTOPATHOLOGY INDUCED BY DIFFERENT FELINE CALICIVIRUSES

Introduction

Materials and Methods

1) Electron Microscopy
2) Immunofluorescence

Results

1) Electron Microscopy
2) Immunofluorescence

Discussion

INTRODUCTION

Electron microscopic studies of FCV have concentrated on the definition of the structure of the virus particle (Zwillenberg and Burki, 1966; Almeida et al., 1968) and there have been few reports on the fine structure of cells infected with these viruses. Peterson and Studdert (1970) and Studdert and O'Shea (1975) described ultrastructural changes associated with FCV infection in low passage feline embryo kidney cell cultures and in a feline embryo cell line (FEmb) respectively. In the latter study, in which the result of infection with a single FCV strain (10/66) was described, virus was found in the cytoplasm as single particles, in extensive non-regular accumulations, in crystalline arrays and in single or mutliple linear arrays associated with microfibrils. In an earlier report (Strandberg, 1968) the ultrastructural changes in primary cultures of feline kidney cells infected with six different strains of FCV were described. Strain differences were reported: some strains (e.g., 17 FRV and F2G) gave rise to large crystalline arrays, whereas with other strains (e.g., FPL and KCD) crystalline arrays were rarely found and virus-like particles were observed free in the cytoplasm (FPL) or in rows or chains bounded by fine filamentous structures (KCD). These filamentous structures were of a similar appearance to the "microfibrils" described by Studdert and O'Shea (1975).

In the present study, electron microscopic observations are reported which confirm that FCV strains differ in their cytopathogenesis. The possibility that cytopathology might be related to some other strain characteristic such as plaque type or rate of synthesis or mechanism of virus release, was investigated. The virus-cell relationships of a number of FCV strains were examined but again, as in Chapters 5 and 6, emphasis was placed on a comparison of isolates G1 and G10 since they were known to differ markedly in other characteristics.

A previous immunofluorescence study on FCV infected feline cells in culture revealed that there was cross-reactivity between strains and that specific fluorescence was limited to the cell cytoplasm (Gillespie, Judkins and Kahn, 1971). In the present study, immunofluorescent staining was used to examine the distribution of virus antigen in FCV infected cells, primarily to determine if this was related to strain differences already observed by electron microscopy. The distribution of virus antigen was found to differ in cells infected with different isolates and the ultrastructural basis for this was considered.

MATERIALS AND METHODS

1) Electron microscopy

Viruses and infection of monolayers The following viruses were used: FCV G1, G2 and G10, 17FRV, FPL, KCD, M8, FPV255, F11, F17 and G19. FEA monolayers in 5 cm plates were inoculated with 0.1 ml of virus suspension at a virus-cell ratio of 1-10:1. After an adsorption period of 60 mins, the inocula were removed and 4 ml of EFC/10 was added to each plate. By 24-30 hours, the majority of cells had rounded up and had become suspended in the medium; cells still attached to the plate were suspended by agitation. The cell suspension was centrifuged at 1,500 rpm for 5 mins, the supernatant was poured off and the cell pellet was fixed for electron microscopy.

Cell cultures were infected with isolates G1 and G10 and at hourly intervals from 0-6 hours after virus adsorption one cell culture of each was fixed. In these experiments, cultures were infected as above but at a virus-cell ratio of 50.

Isolates G1 and G10 were prepared for electron microscopic examination by negative staining: FEA cultures were infected as above and at 24 hours the cell suspension was harvested and ultrasonicated for 45 secs (24.78 K^c/s). Gross cellular debris was removed by centrifugation at 3,000 rpm for 10 mins and the supernatant was further clarified by centrifugation at 10,000 rpm for 15 mins. The supernatant was collected and virus was pelleted by centrifugation at 40,000 rpm for 120 mins in a Beckman SW50.1 rotor. The pellet was resuspended in 50 µl of PBS.

Electron microscopical techniques

a) Infected cell pellets

1) Fixation: Cells were fixed in paraformaldehyde/glutaraldehyde at 4°C for 4 to 6 hours (Karnovsky, 1965). The fixative mixture consisted of 1.3% paraformaldehyde (BDH, Poole, Dorset) and 1.6% glutaraldehyde prepared in cacodylate buffer at pH 7.2-7.4. The cell pellets were then rinsed in 0.1 M cacodylate buffer with 0.1 M sucrose in which they were left overnight. They were then post-fixed in 1% osmium tetroxide in Millonig's phosphate buffer for 90 mins.

2) Embedding: Dehydration was through an ascending series of 70%, 90% and absolute alcohol. The cell pellets were then rinsed in propylene oxide before being embedded in Araldite.

3) Staining: Ultrathin sections were cut on a LKB Mark III ultratome using glass knives. Sections were mounted on copper mesh grids and double stained with 20% uranyl acetate in methanol (Watson, 1958), followed by lead citrate (Reynolds, 1963). Stained sections were examined with an AEI EM 6B electron microscope.

b) Negative staining

A drop of virus-containing fluid was deposited onto a parlodion coated grid and allowed to stand for several seconds before excess fluid was removed by blotting from the side of the grid with filter paper. A drop of 2% phosphotungstic acid (pH 7.2) was deposited onto the grid and, after 30 secs, the grid was blotted and dried in air. The negatively stained preparations were examined in the EM 6B electron microscope.

The microscope was calibrated using a diffraction grating ruled at 2,160 lines per cm. Measurements of the diameters of virus particles and of other structures were made at a magnification calculated to be of 63,700 x and magnified a further 4 x photographically.

2) Immunofluorescence

Using an indirect immunofluorescence method, the pattern of fluorescence in virus infected FEA monolayer cells was examined for isolates G1, G10, 17FRV, FPL, KCD, M8, FPV255, F11, F17 and F19. Monolayers were produced on 12-well microscope slides (C.A. Hendley & Co). Each well was seeded with 5×10^4 cells in 50 µl EFC/10 and a confluent monolayer was present in each after incubation for 24 hours at 37°C. Medium was removed from each well and a suitably diluted virus suspension in 50 µl MEM was added at a virus-cell ratio of approximately 10. After 6 hours, the virus inocula were removed, the slides were washed twice with PBS and the cells were fixed in absolute alcohol for 3 mins at room temperature. The slides were then rinsed in PBS, dried in air and antiserum was added. Four different sera were used: 1) Rabbit anti-G1 virus prepared as described in Chapter 2; 2) Rabbit anti-G1 15S subunit kindly supplied by O. Komolafe, the preparation of which was described by Komolafe (1978); 3) Cat anti-G1 virus obtained from SPF cat no. 3 at necropsy; and 4) Cat anti-G10 virus obtained from SPF cat no. 9 at necropsy. All sera were inactivated by heating at 50°C for 30 mins and diluted in L15 medium where required. A volume of 25 µl of antiserum was added to each well and the slides were incubated at 37°C for 1 hour in a humidified box. The antiserum was then removed from each well and the slides were washed; slides were rinsed in de-ionised water, placed

in two consecutive baths of PBS (5 mins each), rinsed in de-ionised water and partially dried in air.

FITC-conjugated anti-globulin antibody was then added to each well. When rabbit serum had been used initially, a conjugated goat globulin preparation against rabbit globulin (Miles Laboratories) was used at a dilution of 1:40 in PBS. Where cat serum had been used initially, a conjugated rabbit serum against cat globulin was used at a dilution of 1:5 in PBS (kindly supplied by P. Rogerson). A volume of 25 µl of conjugated antiserum was added to each well and the slides were re-incubated at 37°C for 1 hour in a humidified box. The antiserum was then removed and the slides were washed by the same procedure as above.

Each well was examined for the presence of immunofluorescence by ultraviolet light microscopy using water immersion objectives at magnification of x 25 and x 50.

Control wells were included in each experiment and consisted of uninfected cell controls, pre-inoculation serum controls and FITC-conjugated serum or globulin only controls.

RESULTS

1) Electron microscopy

Electron microscopy of FEA cells infected with various FCV isolates

General findings By 24-30 hours after viral infection, most of the cells were small and round to oval in shape with a very pronounced decrease in the number of cellular projections compared with uninfected cells which had prominent cytoplasmic processes (Fig. 7.1 and Fig. 7.17).

It was assumed that all stages in the virus replication cycle would be represented in these sections. Some cells, infected during the initial adsorption period would have been infected for 24-30 hours while others would have become infected from virus produced in the initial replication cycles. It was observed that cells varied in appearance and it was considered likely that this was due to the asynchrony of viral infection. Thus, scattered sparsely among the small rounded cells were uninfected-type cells, similar to those in Fig. 7.1. Some cells were observed to be intermediate, rounded but still retaining short cytoplasmic projections while others were found to have ruptured cell membranes, presumably produced as a result of viral infection or processing for electron microscopy or both. There was a considerable amount of debris, of cellular origin, present in most sections composed mainly of vesicular structures and fragments of endoplasmic reticulum.

There was an increased electron density in the cytoplasm of the rounded cells. At low magnification the cytoplasm was of a coarse appearance although in some cells, infected with certain isolates (e.g. FCV G1), there were less electron dense, homogeneous and amorphous areas which, at higher magnifications, often were found to contain crystalline arrays of virus (Figs. 7.2 and 7.4).

A finding, previously reported by others (Strandberg, 1968; Studdert and O'Shea, 1975), was the presence of large numbers of membrane bound cytoplasmic vesicles (Fig. 7.17). These were usually located in the central area of the cell, their diameters varied from 100-150 nm and the majority contained an irregular, dark staining mass connected to the vesicle membrane by a thin, branching, filamentous structure. These vesicles were found in cells infected with all of the isolates examined but with certain isolates (e.g., G10 and FPL) they were more abundant. Accumulations of virus were often surrounded by vesicles of this type.

Cells in which virus particles and cytoplasmic vesicles were present, invariably contained very large numbers of ribosomes measuring approximately 20 nm in diameter but polyribosomes, typically abundant in non-infected cells, were not present. Ribosomes were scattered throughout the cytoplasm of infected cells and frequently extensive accumulations were observed (Fig. 7.17). Smaller groups of ribosomes were often observed nearby cytoplasmic vesicles and virus accumulations (Fig. 7.3) and ribosome accumulations were often found close to and following the course of the endoplasmic reticulum (Fig. 7.2). Also, large ribosome-like bodies were frequently located along the membranes of the endoplasmic reticulum.

Virus was found in the cytoplasm of cells and amidst cellular debris in intercellular spaces. In the cell cytoplasm, virus particles could only be recognised with relative ease when arranged in regular arrays; single particles could not be positively identified. The complete virus particle, as seen in negatively stained preparations, was appreciably larger than a ribosome (approximately 35 nm compared to 20 nm). However, since the viral core has a much higher affinity than capsid protein for the stains used, the apparent diameter of the positively stained particle is very close to that of a ribosome. Fig. 7.3 provides a comparison between virus particle and ribosome size. Single viruses could be more easily distinguished from ribosomes when viewed against the low electron dense background of extracellular material (Fig. 7.8). Here, the central dark staining area (or core) was seen to be surrounded by a less well stained capsid.

Other cellular morphological changes, previously described by others (Strandberg, 1968; Studdert and O'Shea, 1975; Love and Sabine, 1975) and observed in these experiments included rounding of the nucleus with central condensation of chromatin and separation of the inner and outer nuclear membranes, dilation of the cisternae of the endoplasmic reticulum, swelling of mitochondria, cell vacuolation, the presence of lysosome-like bodies, myelin figures and elongated fibrillar structures and the production of large quantities of smooth surfaced membranes.

Cells infected with isolate G1 The predominant feature of G1 infected cells was the presence of crystalline virus arrays (Figs 7.2 and 7.3) most frequently observed near the cell periphery and often large and multi-faceted (Fig. 7.4). Crystals were apparently cubic with lattice axes either 60° or 90° to one another (Fig. 7.5). The centre-to-centre

particle distances varied from 30 to 39 nm. The distances varied presumably according to the orientation of the plane of the section in relation to the planes of the crystal lattice. Small crystals were observed at 4 hours after virus adsorption and by 6 hours they were large and multi-faceted.

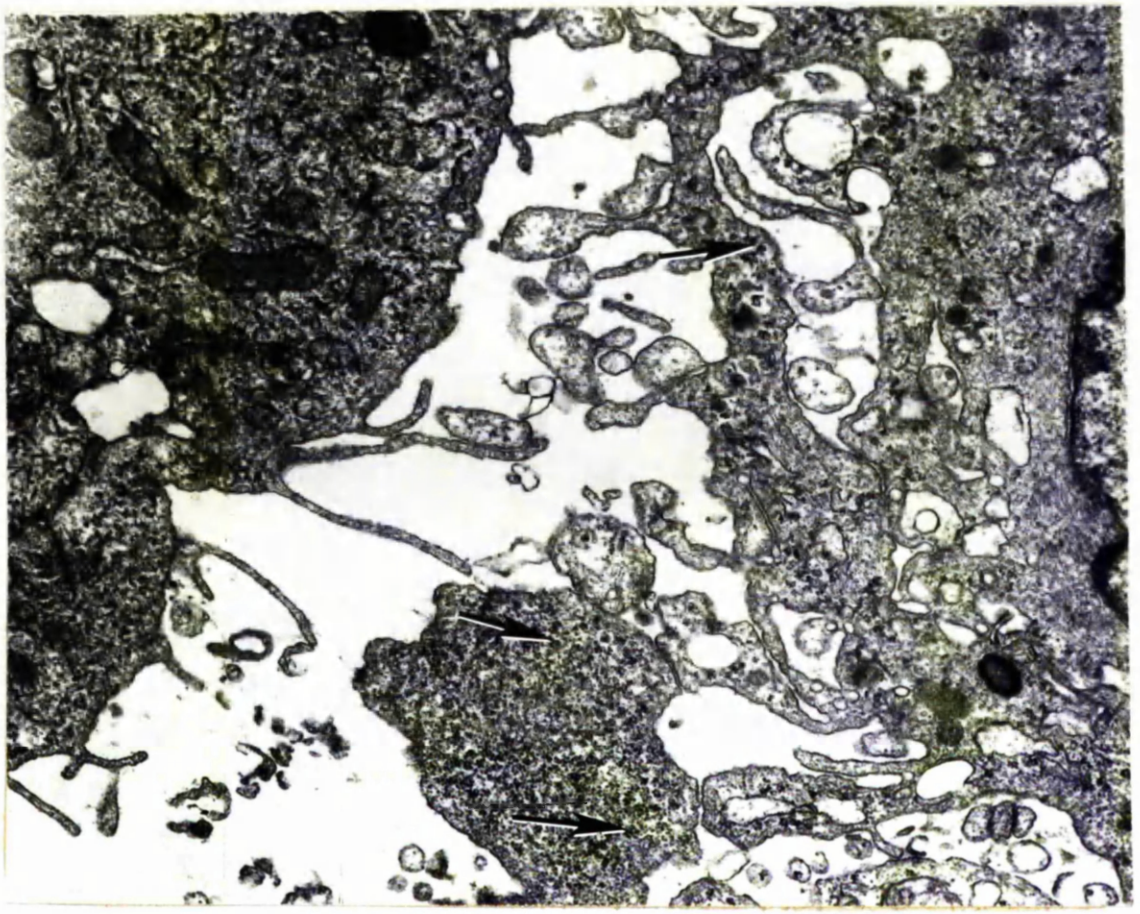
Close to many crystals were amorphous areas, free of cytoplasmic vesicles, ribosomes or other organelles which at high magnification were found to consist of fine granular material (Figs. 7.2 and 7.3). These areas were given the name "grey areas" and have apparently not been described before. The fine granular texture of these areas was of a similar nature and was, in many cases, continuous with the inter-core substance of the virus crystal (Fig. 7.6). Occasionally, circular structures, composed of fine granular material and of approximately virus diameter, were found (Fig. 7.7). These structures were found in groups and frequently a number contained dark staining centres; such particles were identical to virus particles in crystalline arrays. Also, structures of the same appearance were seen occupying "holes" in the crystal lattice.

No single particles, linear arrays or non-regular accumulations of particles were seen in G1-infected cells; virus was found only in the form of crystalline arrays.

Crystalline arrays were not observed in the cytoplasmic material found in inter-cellular spaces or in cells where the cell membrane was incomplete and it appeared that crystals broke up after cell membrane rupture (Fig. 7.8).

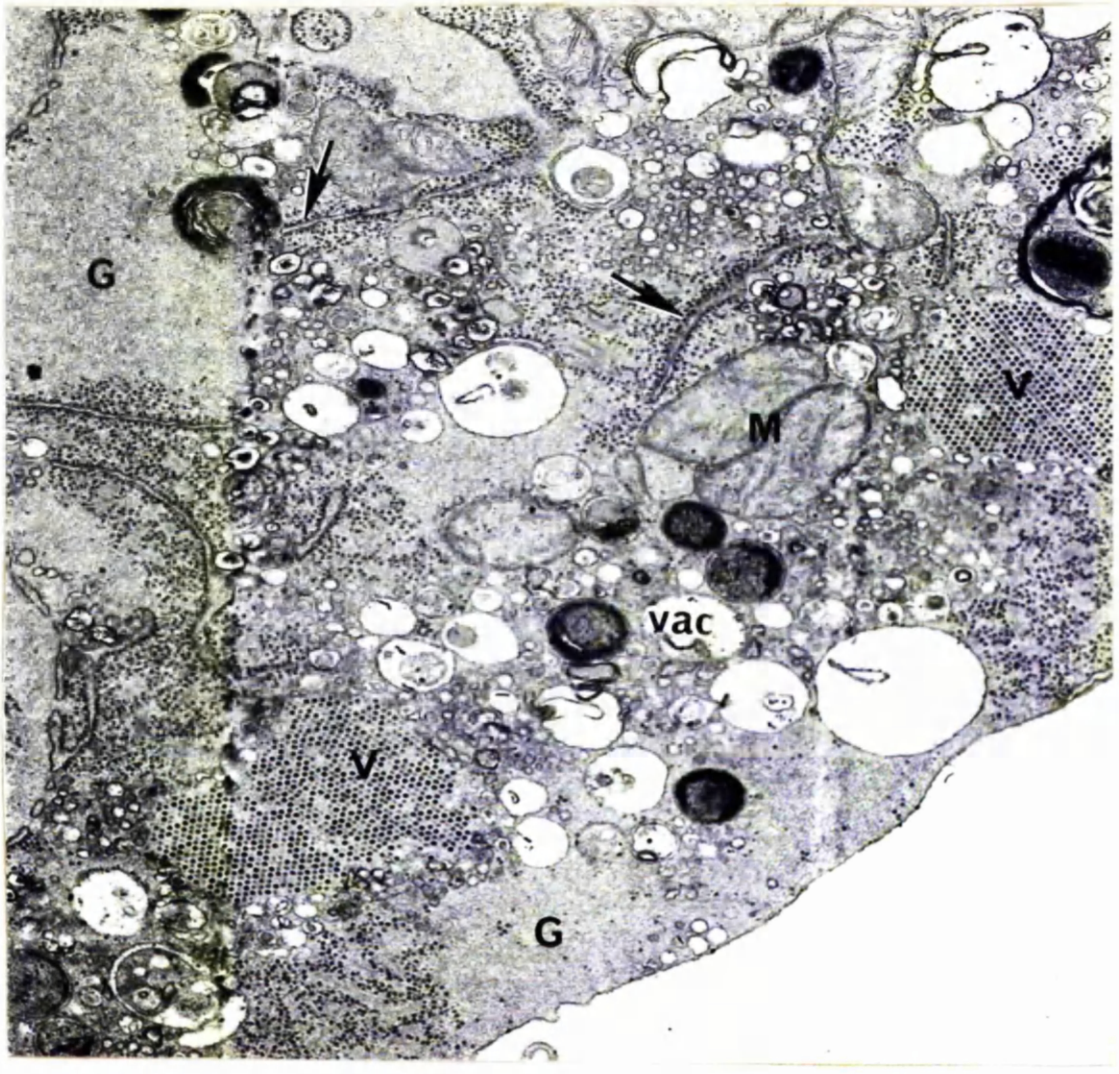
Cells infected with isolate G10 These cells had an overall greater electron density than G1-infected cells. In many of them cytoplasmic vesicles were particularly abundant. Virus was located in small pockets between vesicles (Fig. 7.9) or near the cell periphery (Fig. 7.10). Virus was not detected until 5 hours post adsorption. Large crystalline particle arrays and "grey areas" were not found in G10 infected cells. Occasionally small linear arrays were observed (Fig. 7.11) but these were not associated with any fibrillar structures. Most virus was present in non-regular or semi-crystalline aggregates. It was observed that, unlike G1 virus, virus relationships were maintained after rupture of the cell membrane. Fig. 7.12 shows small semi-crystalline virus aggregates, commonly found in the intercellular spaces, 30 hours after infection; Fig. 7.13 shows some linear virus arrays, less commonly found.

Fig. 7.1: Uninfected FEA cells.



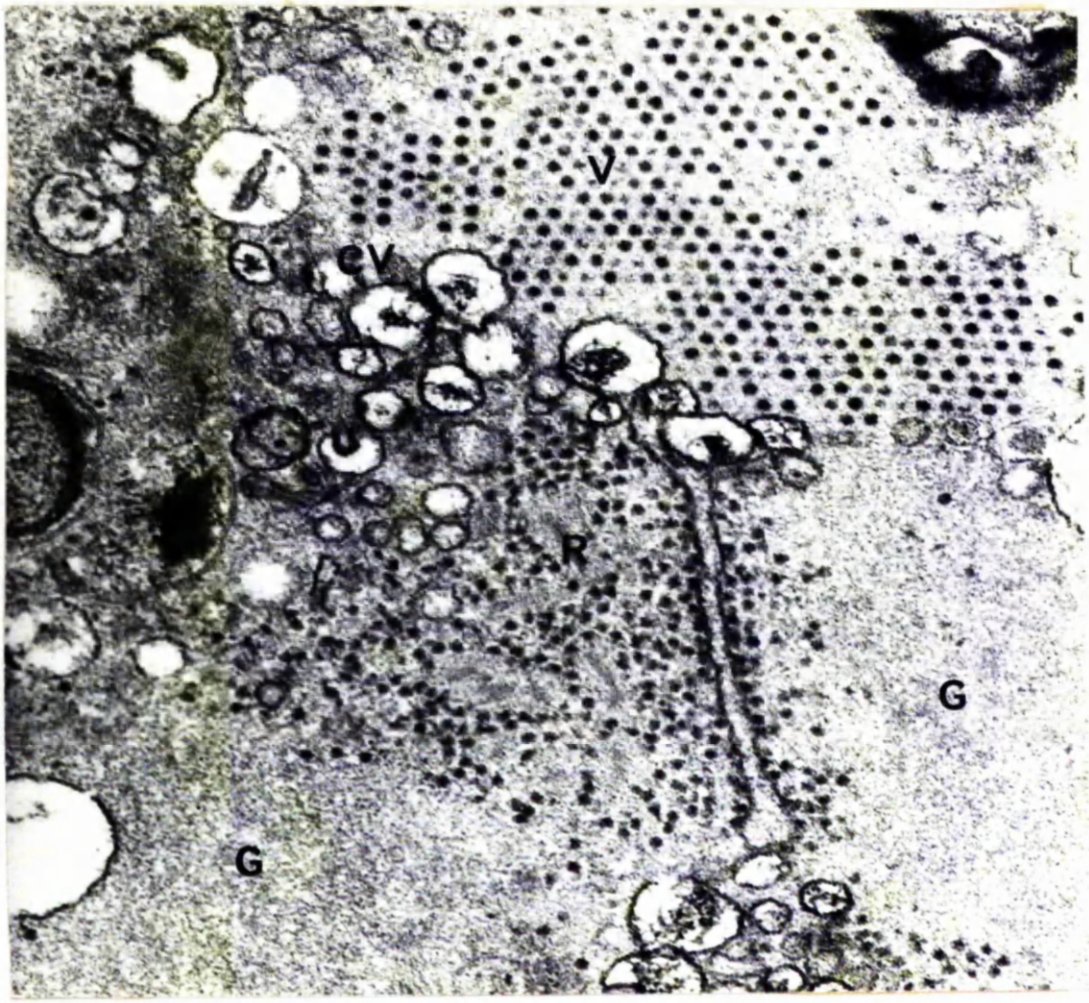
Cytoplasmic projections and invaginations, as seen here, are a common feature of normal FEA cells. Polyribosomes (arrows) are abundant in the cell cytoplasm (X 20,000).

Fig. 7.2: FCV G1 infected cell.



Two virus crystalline arrays (V) can be seen separated by a "grey area" (G) of low electron density containing vacuoles (vac) and swollen mitochondria (M). Large numbers of ribosomes are present adjacent to virus crystals. Some ribosomes are found along the course of the endoplasmic reticulum or associated with the membranes of the endoplasmic reticulum (arrows).
(X 30,000).

Fig. 7.3: FCV G1 infected cell.

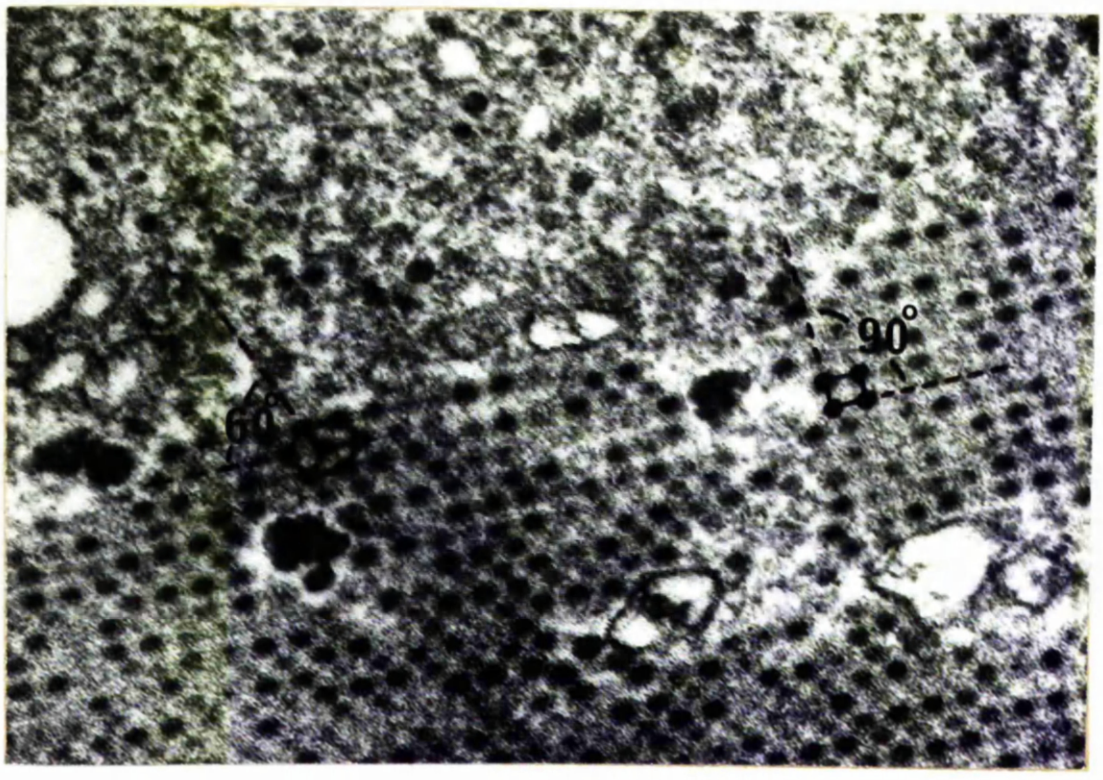


There is a crystalline array of virus particles at the top of the plate (V). At the edge of the crystal a collection of cytoplasmic vesicles (CV) and ribosomes (R) are present. Note the size difference between virus particles and ribosomes. Two grey areas (G) are also present. (X 80,000)

Fig. 7.4: FCV G1 infected cell.

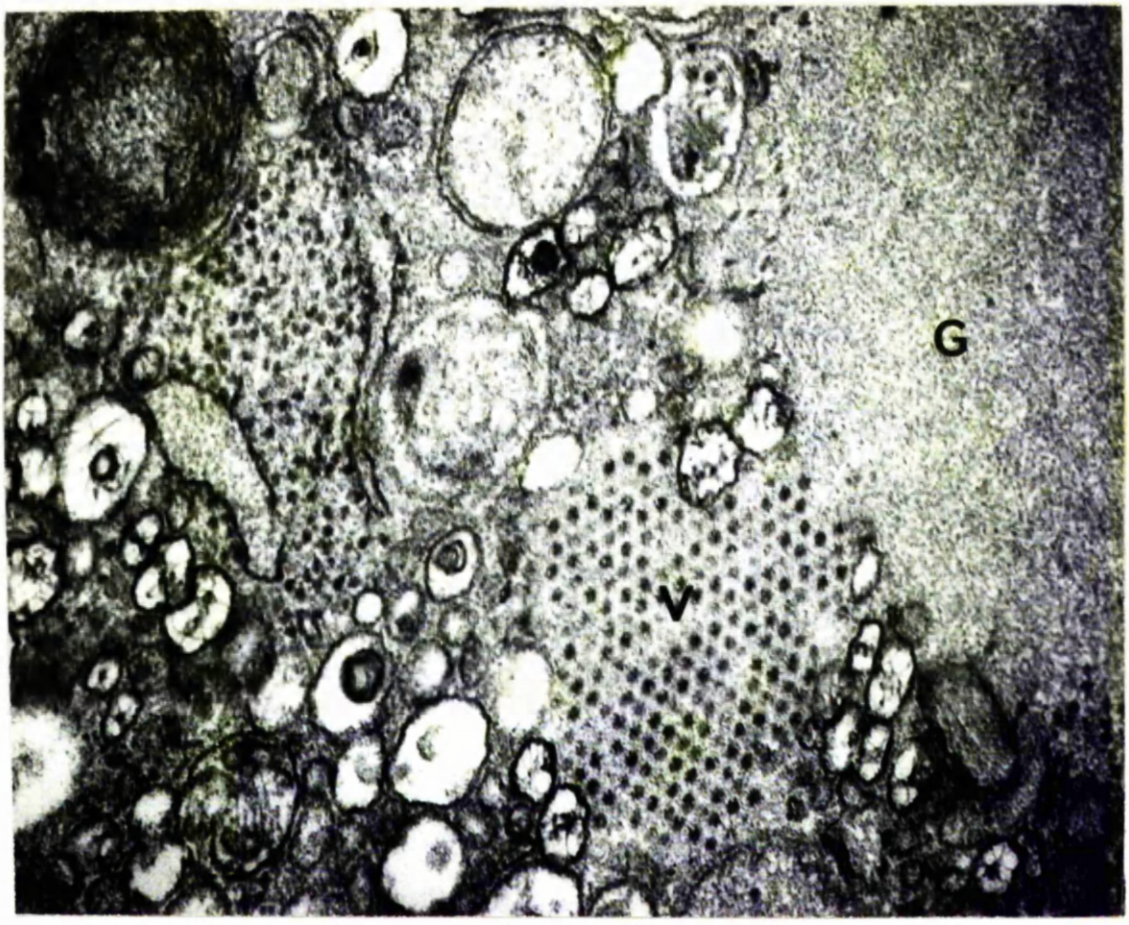
A large multi-faceted crystalline array of virus particles (V) can be seen near the cell periphery. (X 30,000)

Fig. 7.5: FCV G1 infected cell.



Virus crystals are cubic with lattice axes at either 60° (3 fold symmetry) or 90° (2 or 4 fold symmetry). (X 120,000)

Fig. 7.6: FCV G1 infected cell.

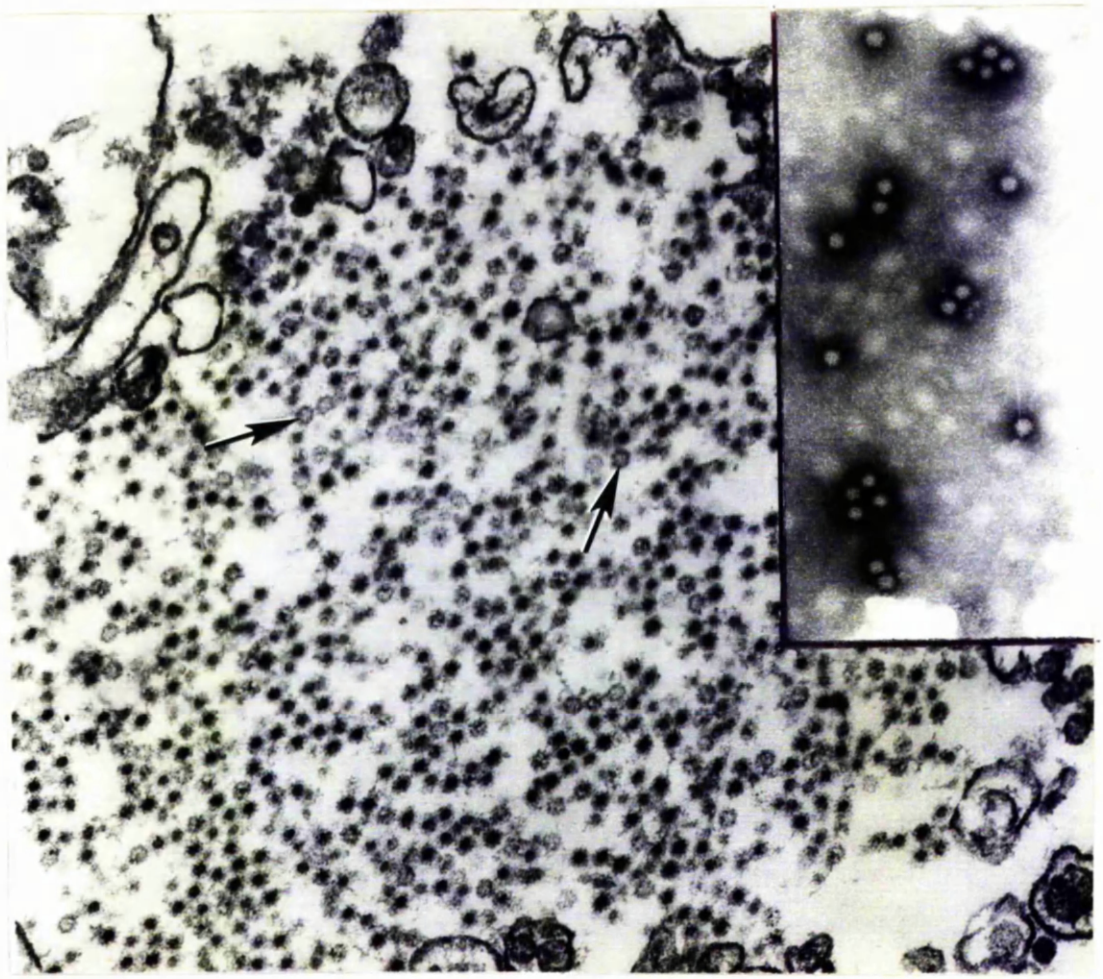


A small virus crystal (V) can be seen at the edge of a grey area (G). Note the apparent continuity of the fine granular material of the grey area with the intercore substance of the virus crystal. (X 80,000)

Fig. 7.7: FCV G1 infected cell.

A collection of circular structures, each of approximately one virus diameter, is present near the cell periphery. Such areas may represent a "self-assembly" of 15S subunits into capsid structures. (X 80,000)

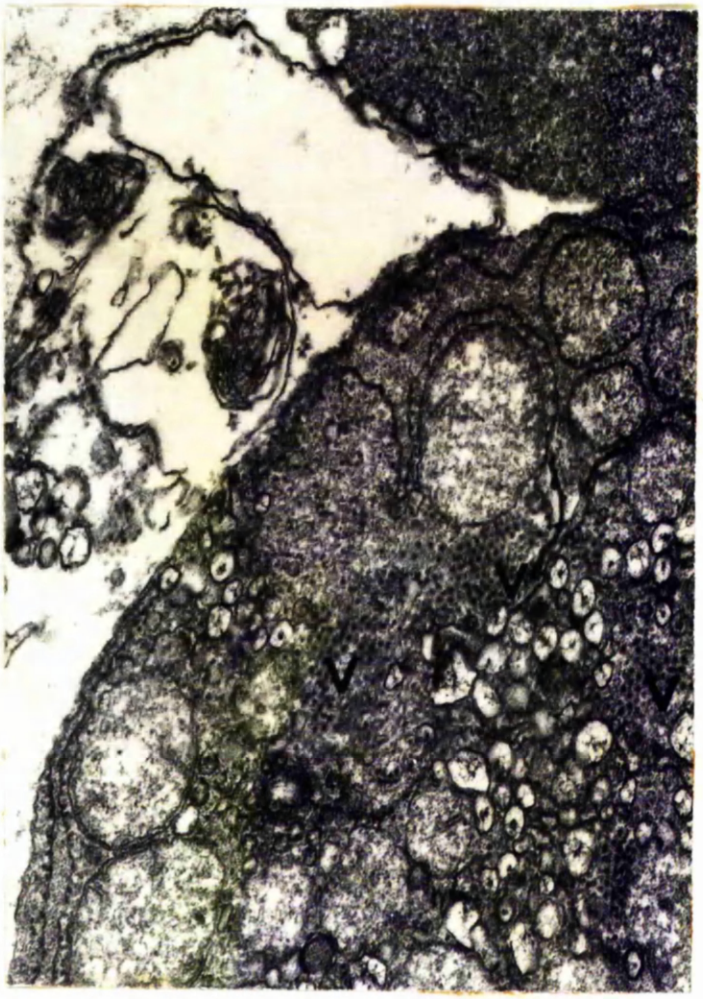
Fig. 7.8: FCV G1 infected cell.



The cell has disintegrated resulting in the disassociation of a virus crystal. Positively stained virus particles have a dark core surrounded by a lighter staining capsid. Empty particles can be seen (arrows) (X 80,000).

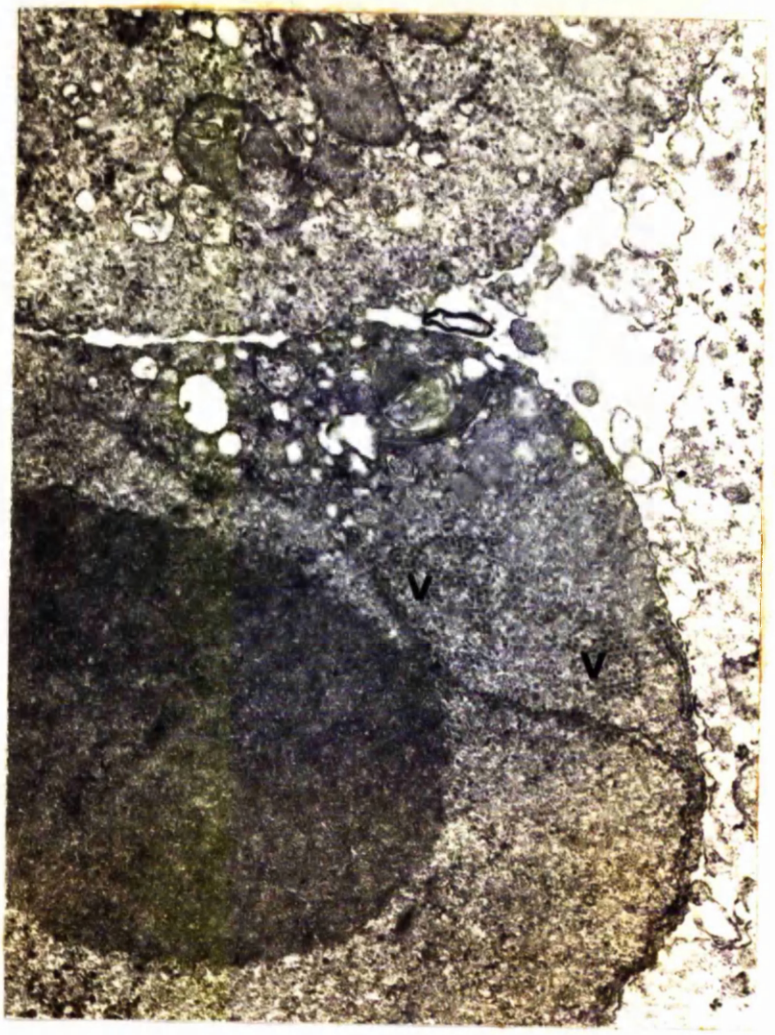
The insert shows negatively stained virions at the same magnification.

Fig. 7.9: FCV G10 infected cell.



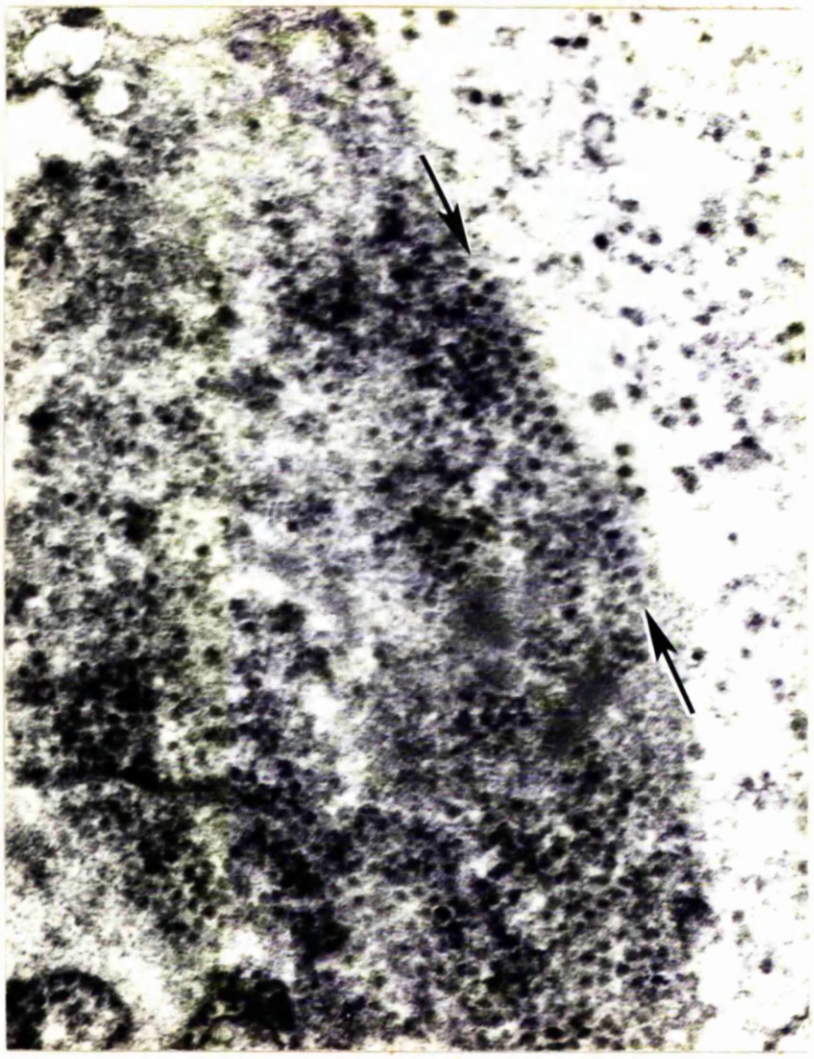
Virus (V) is located in small pockets between cytoplasmic vesicles. (X 40,000)

Fig. 7.10: FCV G10 infected cell.



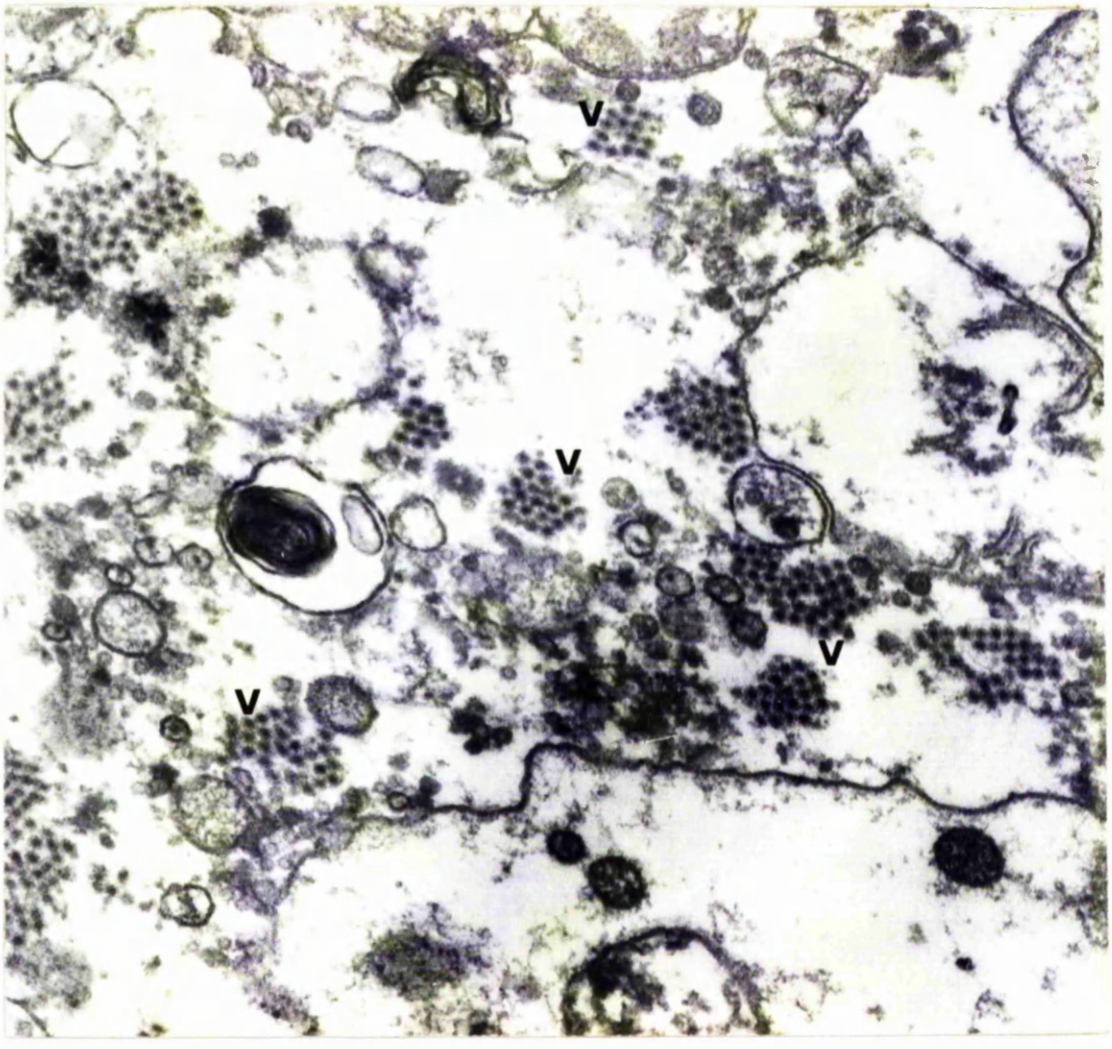
Small accumulations of virus are present near the cell periphery. (X 20,000)

Fig. 7.11: FCV G10 infected cell.



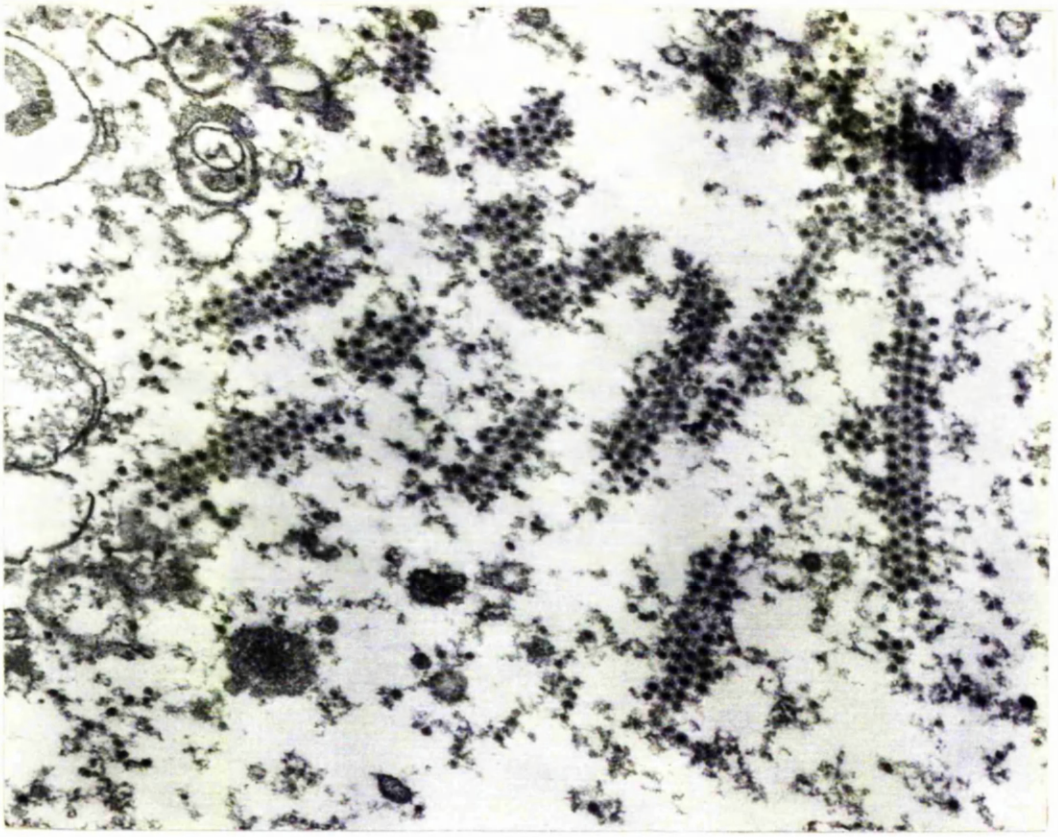
A linear array of virus particles (arrows) is present at the cell periphery (the limiting cell membrane has ruptured). (X 80,000)

Fig. 7.12: FCV G10 infected cell.



The cell has disintegrated and virus (V) can be seen in small semi-crystalline aggregates.(X 60,000)

Fig. 13: FCV G10 infected cell.



The cell has disintegrated and virus can be seen in linear arrays.
(X 60,000)

Cells infected with other FCV isolates In cells infected with isolates F17 and KCD, virus was found in linear arrays bounded by fine filamentous structures (Fig. 7.14). These viruses also formed small crystalline arrays and KCD virus was occasionally present in small linear arrays of 3-5 particles.

Isolates G2, F11, 17FRV and FPV255 produced large, regular particle arrays similar to those described for isolate G1. It was noted that "grey areas" were particularly prominent in FPV255 infected cells. Isolate G2, in addition to the G1-type crystals, also produced elongated and narrow particle arrays (Figs. 7.15 and 7.16).

Isolates M8, FPL and F19 produced changes similar to those described for isolate G10. Fig. 7.17 demonstrates the abundance of cytoplasmic vesicles in a cell infected with strain FPL and Fig. 7.18 a loose non-regular accumulation of FPL virus and cytoplasmic vesicles in a disintegrating cell.

Therefore, as indicated in Table 7.1, isolates could be divided into 3 groups according to their particle aggregation characteristics in infected cells.

Negative staining if isolates G1 and G10 The characteristic calicivirus morphology is shown in Fig. 7.8. The G1 particle diameter was found to be 34 nm $\pm$ 3 nm (from the measurement of 50 particles) and the G10 particle diameter 36.5 nm $\pm$ 3 nm (from the measurement of 20 particles).

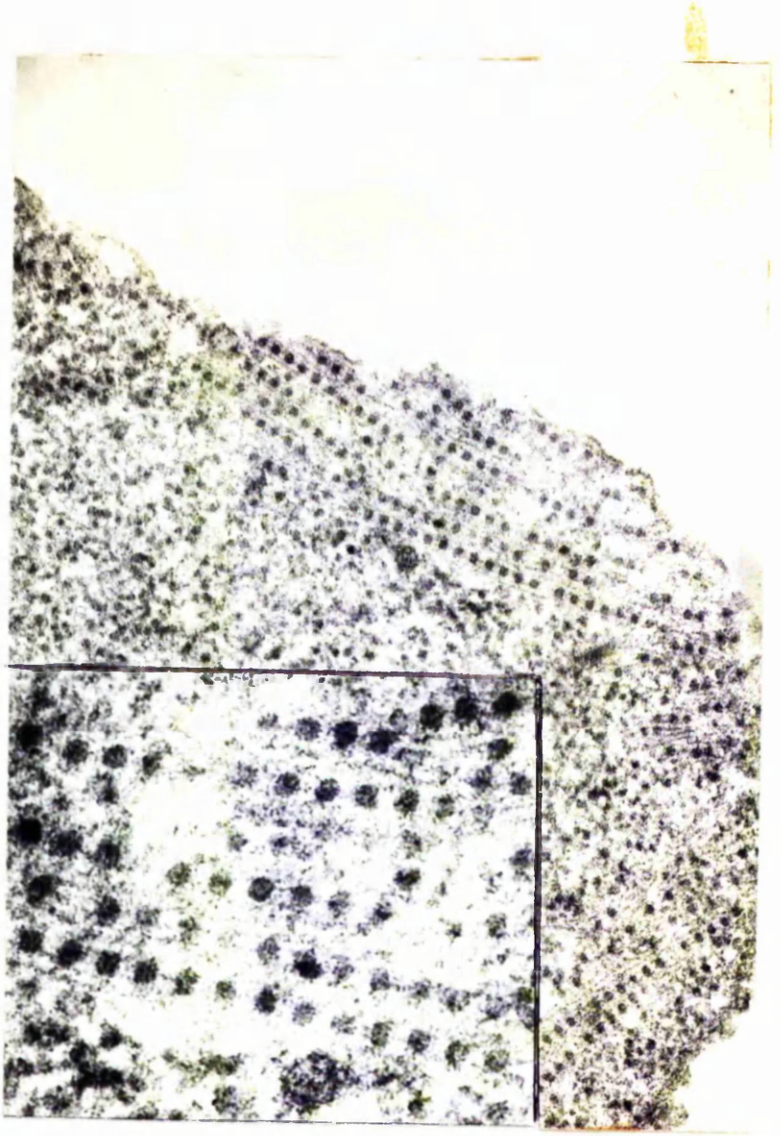
2) Immunofluorescence

FEA cells infected with isolates G1 and G10 showed different patterns of cytoplasmic fluorescence and this is demonstrated in Figs. 7.19 and 7.20. Fig. 7.19 shows cells infected with isolate G1 and stained with rabbit anti-G1 serum followed by conjugated goat anti-rabbit globulin. Fluorescence is limited to the cell cytoplasm and is most obvious in small foci of varying sizes, most of which are apparently situated near the cell periphery. These foci varied in number from cell to cell, although most frequently there were between 5 and 10 foci per cell. There is also a diffuse background cytoplasmic staining.

Fig. 7.20 shows cells infected with isolate G10 and stained as above. Specific fluorescence is again present in the cell cytoplasm but the staining is diffuse throughout the cytoplasm rather than focal.

These two isolates were compared by the same method on several different occasions using different cell cultures and this difference was consistently found. It was noted that a lower dilution of rabbit

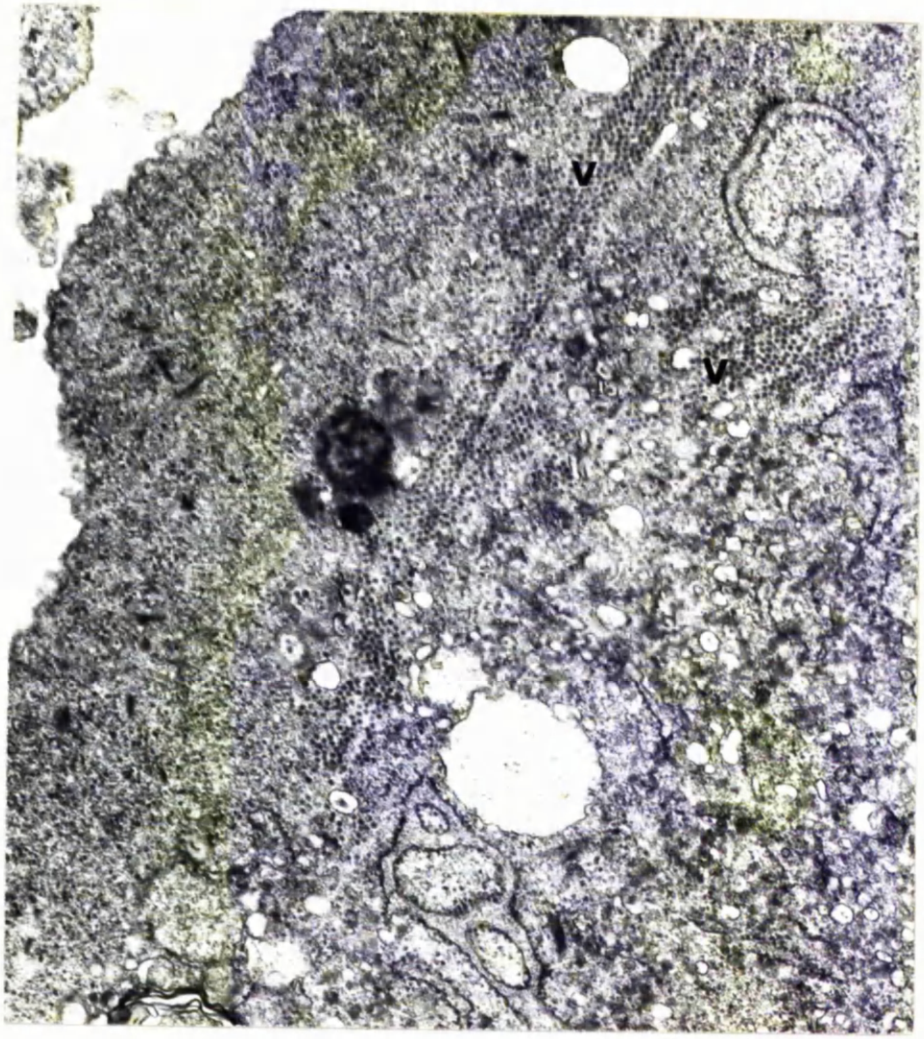
Fig. 7.14: F17 infected cell.



Virus is arranged in linear arrays bounded by fine filamentous structures (X 60,000).

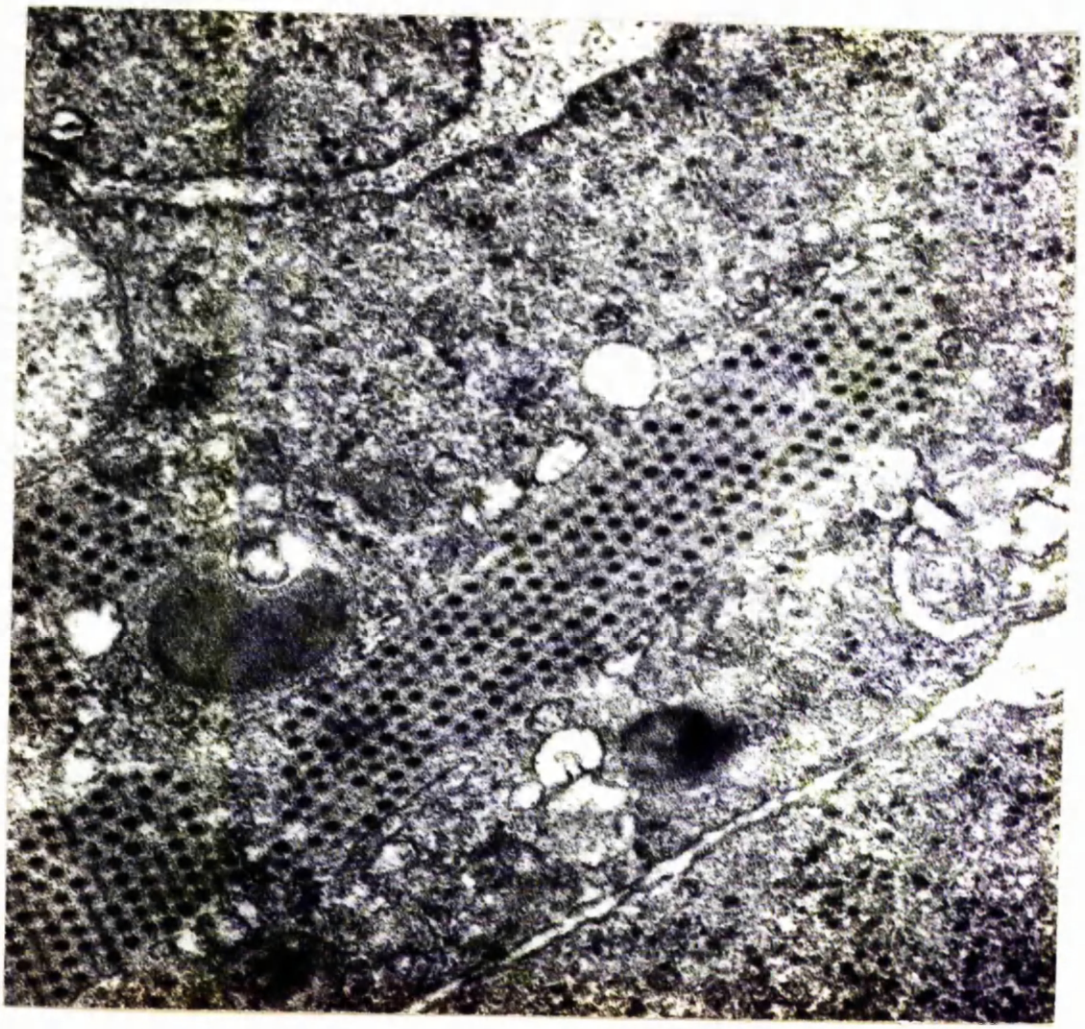
Inset shows detail of arrays (X 120,000).

Fig. 7.15: FCV G2 infected cell.



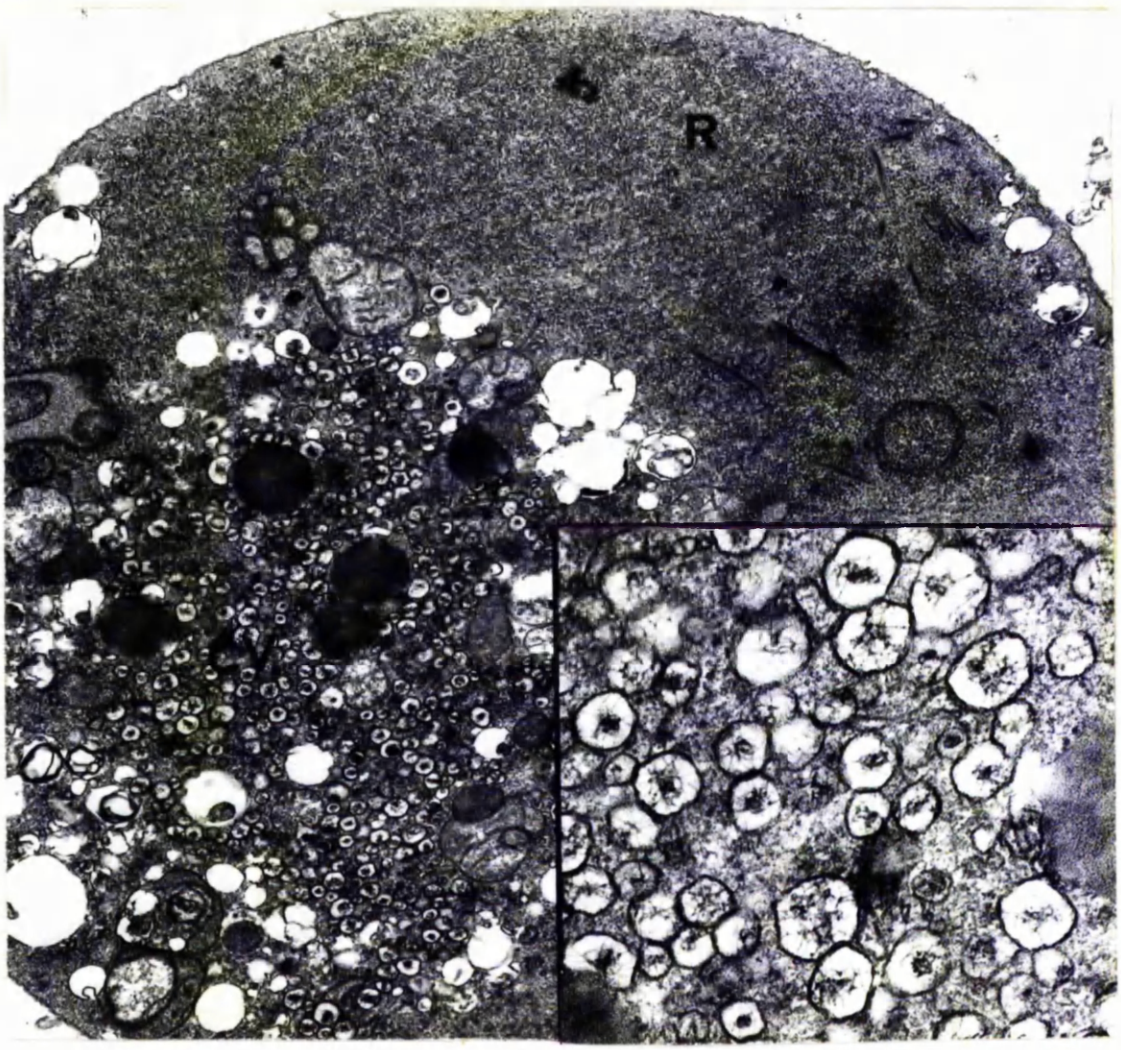
Long, narrow arrays of virus particles (V), as seen here, are a common feature of cells infected with isolate G2. (X 30,000)

Fig. 7.16: FCV G2 infected cell.



A linear virus crystalline array, typical of many observed in cells infected with isolate G2 (X 80,000).

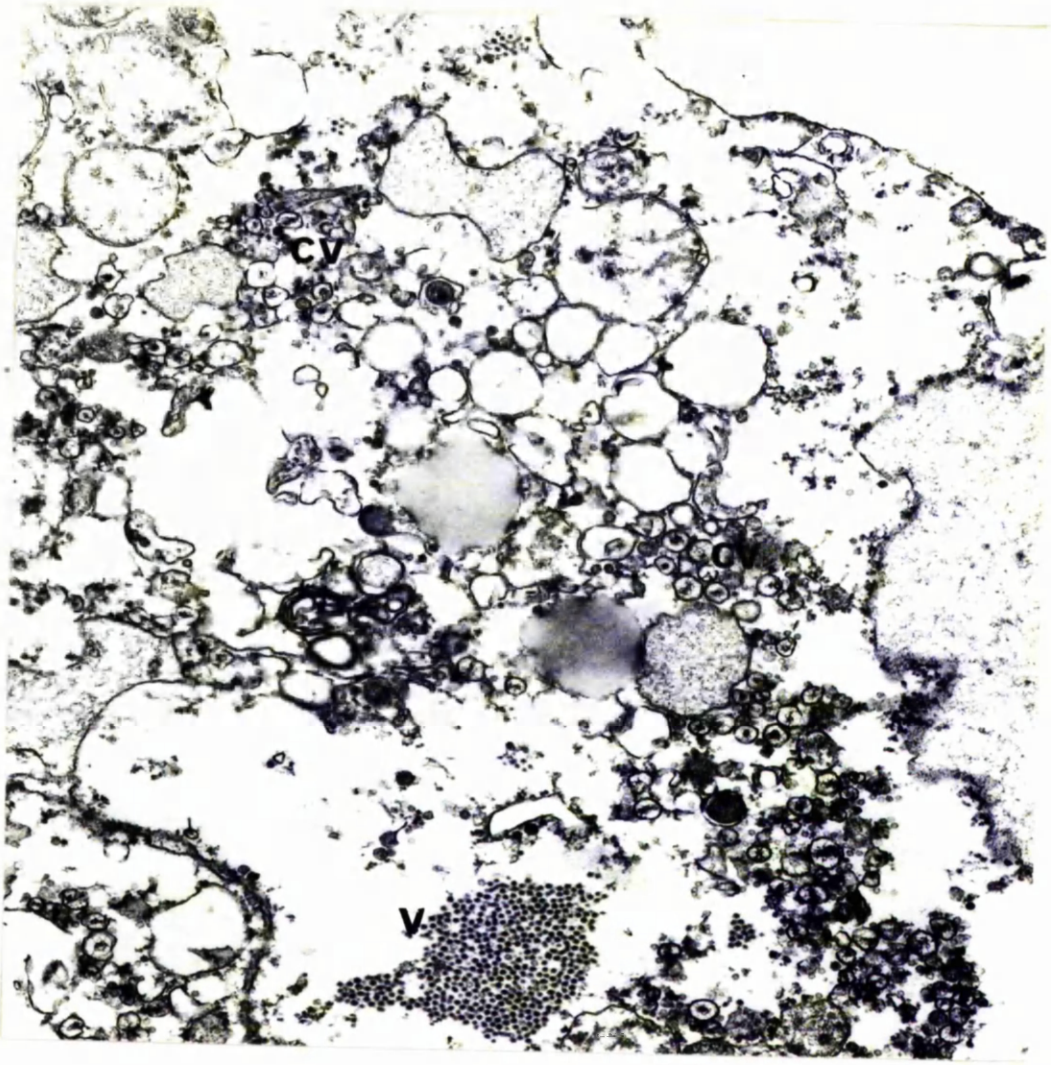
Fig. 7.17: FPL infected cell.



The cytoplasm in the upper right of the cell contains large numbers of ribosomes (R). There are a large number of membrane bound cytoplasmic vesicles (CV) in the lower left of the cell. (X20,000).

The inset shows detail of the cytoplasmic vesicles. (X 80,000).

Fig. 7.18: FPL infected cell.



The cell has disintegrated and accumulations of virus (V) along with cytoplasmic vesicles (CV) can be seen. (X 30,000)

TABLE 7.1 ELECTRON MICROSCOPIC AND IMMUNOFLUORESCENCE OBSERVATIONS OF FEA CELLS INFECTED WITH A NUMBER OF FCV ISOLATES.

ISOLATE	PLAQUE TYPE	ELECTRON MICROSCOPIC OBSERVATION OF THE INTRACELLULAR ACCUMULATION OF VIRUS	IMMUNOFLUORESCENCE TEST: PATTERN OF FLUORESCENCE OBSERVED
G1	ep	Large multi-faceted crystalline arrays.	Multi-focal fluorescence.
FPV255	ep		Cytoplasm homogeneously stained.
G2	lp		Not tested.
F11	sp		Multi-focal fluorescence.
17FRV	mp		" " "
F17	mp	Linear arrays associated with filamentous structures and occasional crystalline arrays.	Cytoplasm homogeneously stained.
KCD	sp		" " "
G10	mp	Loose aggregates and small semi-crystalline arrays	" " "
M8	mp		" " "
F19	mp		" " "
FPL	lp		" " "

anti-G1 virus serum was required to produce good fluorescence in G10-infected cells compared to G1-infected cells (a 1:2 dilution compared to a 1:16 dilution).

Cat anti-G1 and cat anti-G10 virus sera produced the same result, thereby indicating that the different staining patterns observed in G1 and G10 infected cells did not arise as a result of the specificity of rabbit anti-G1 antibody.

Cells infected with G1 and G10 were also stained with rabbit anti-G1 15S subunit serum followed by goat anti-rabbit globulin and it was found that in both cell infections the cytoplasm was diffusely stained (Figs. 7.21 and 7.22). Again, at any dilution of the rabbit serum, G1 infected cells stained more brightly than G10 infected cells.

When cells infected with other FCV strains were examined using rabbit anti-G1 virus serum followed by goat anti-rabbit globulin, it was observed that some (for example F11) produced a multi-focal pattern of fluorescence and others (for example F17) produced a diffusely stained cytoplasm (see Table 7.1). It was also observed that the degree of fluorescent staining varied considerably from strain to strain and FPV255 and FPL infected cells had a particularly low level of fluorescence.

In all experiments the cells contained in the control wells did not stain.

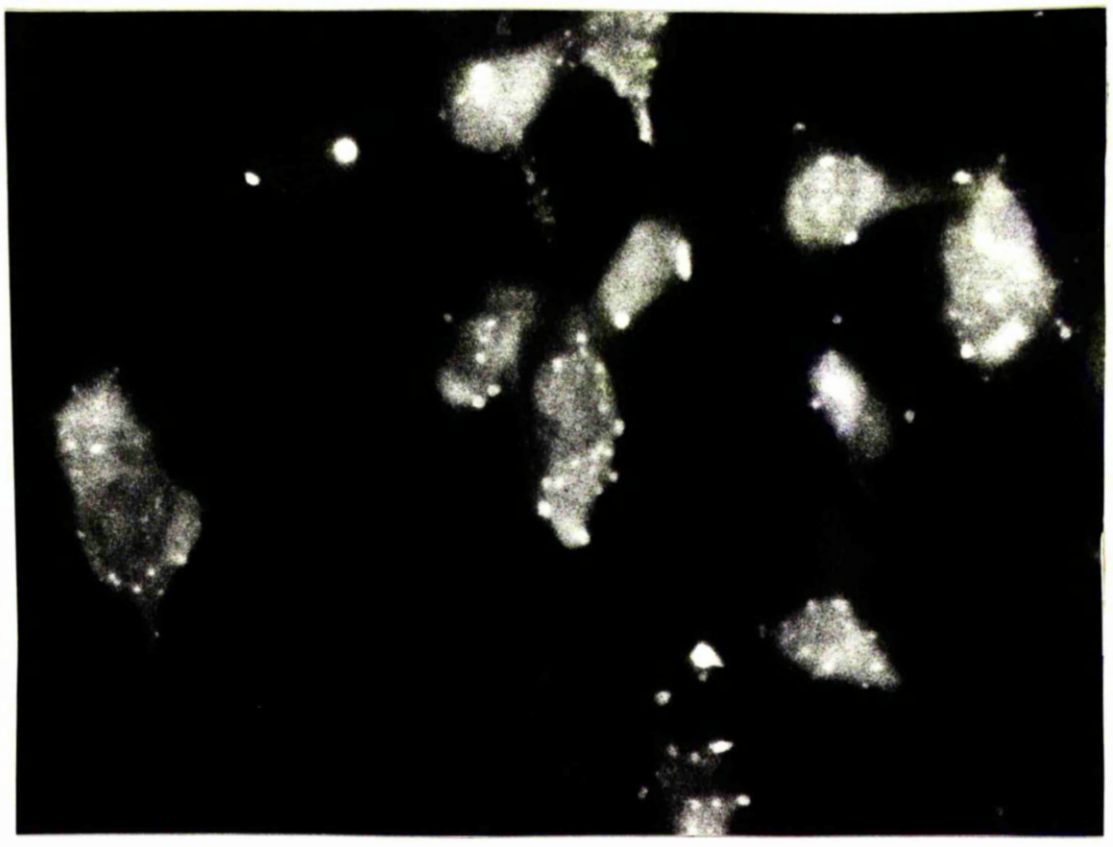


Fig. 7.19: FCV G1 infected cells reacted with rabbit anti-G1 serum followed by FITC-conjugated goat anti-rabbit globulin (800X). A number of foci of fluorescence can be observed in each cell and there is also a diffuse background cytoplasmic staining.

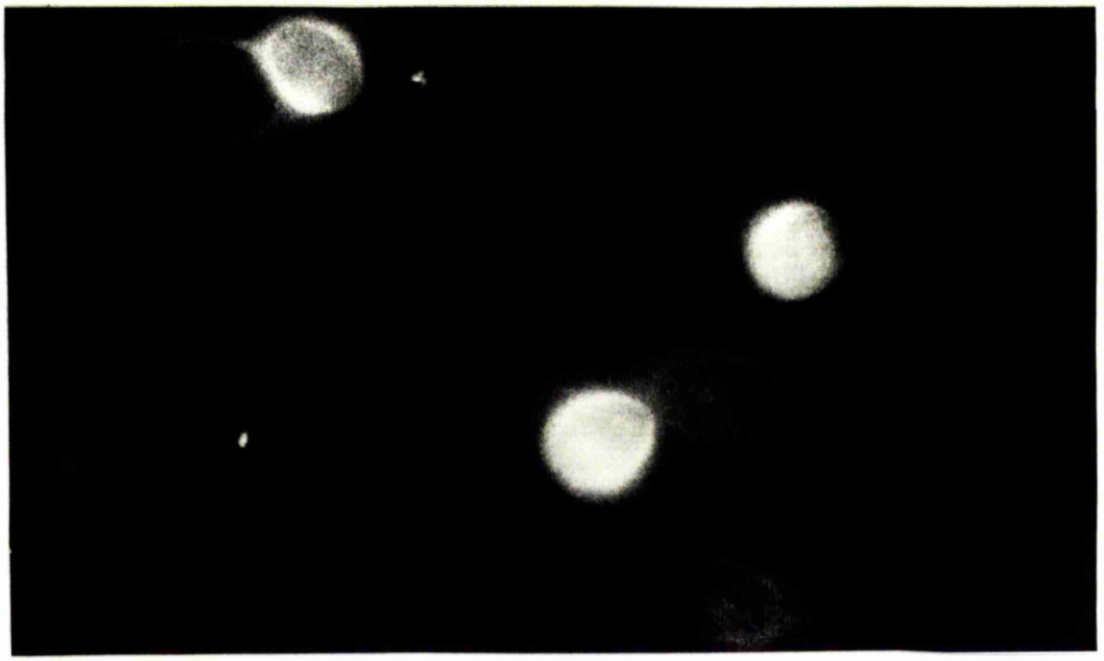


Fig. 7.20: FCV G10 infected cells reacted with rabbit anti-G1 serum followed by FITC-conjugated goat anti-rabbit globulin (800X). Staining is diffuse throughout the cytoplasm.

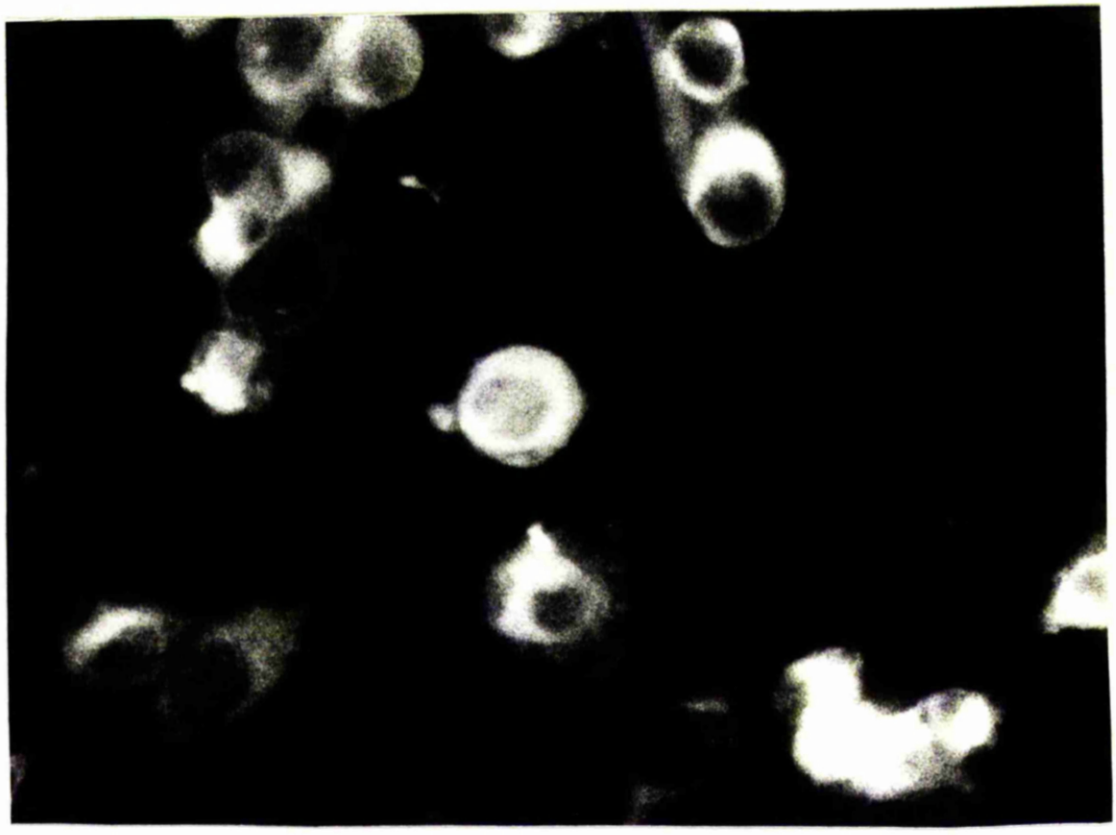


Fig. 7.21: FCV G1 infected cells reacted with rabbit anti-G1 15S subunit serum followed by FITC-conjugated goat anti-rabbit globulin (800X). In contrast to Fig. 7.19 fluorescence is diffuse throughout the cytoplasm rather than focal.

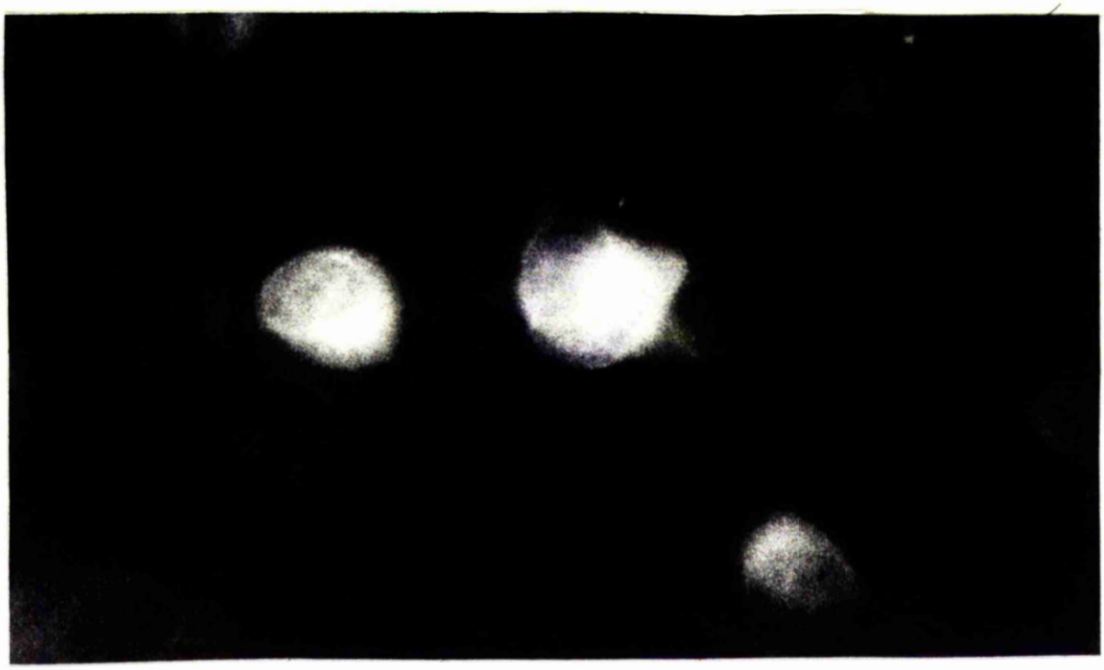


Fig. 7.22: FCV G10 infected cells reacted with rabbit anti-G1 15S subunit serum followed by FITC-conjugated goat anti-rabbit globulin (800X). Staining is diffuse throughout the cytoplasm.

DISCUSSION

The results presented in this section show that different FCV isolates produce different changes in infected cells. The main difference observed between isolates was the way in which virus particles aggregated in the cytoplasm of infected cells. Some isolates aggregated in large, multi-faceted crystals, others aggregated in small, "loose", semi-crystalline arrays or remained in non-regular accumulations and a few isolates were observed in linear arrays associated with microfibrils. It was considered unlikely that these differences arose as a result of experimental variations (e.g., the infection of FEA cells at different passage numbers) since results, at least for isolates G1 and G10, were reproducible and in each experiment several isolates were tested by parallel infection of identical cultures.

The study conducted by Strandberg (1968) produced similar results to those described here. Strandberg examined six FCV strains and found that they produced different virus-cell relationships. Also reported were some specific findings which the present study confirms. Thus, Strandberg noted that KCD virus was associated with filamentous structures in linear particle arrays, that strain 17FRV produced large multi-faceted crystals and that strain FPL virus was found in non-regular accumulations and rarely formed crystals. It is of interest that Studdert and O'Shea (1975) observed all of these types of virus aggregation in a feline embryo cell line infected with a single plaque cloned FCV isolate (strain 10/66).

Strain differences in virus-cell relationships have also been observed in VESV infected tissue culture cells. VESV type A48 produced large cytoplasmic crystalline arrays (Zee, Hackett and Madin, 1968), whereas VESV type H54 was found predominantly associated with filamentous structures in linear arrays (Zee, Hackett and Talens, 1968).

Different calicivirus isolates, therefore, apparently differ in their cytopathology. These differences are evident at a relatively late stage in the virus replication cycle (4-5 hours) and could indicate differences in the mechanisms of virus assembly or release. For FCV the question arises of whether cytopathology is related to any other strain characteristic such as plaque type, virulence or other biological quality. As can be seen from Table 7.1, there appears to be no correlation between plaque type and the nature of virus aggregation in infected cell cytoplasm: isolates classed as mp produced virus aggregates of the three types and

large crystalline arrays were found in cells infected with FCV of all four plaque types. Also there was apparently no correlation between cytopathology and virulence: for example, isolates G1 and FPV255 (both of high virulence) produced the same cytopathology as isolate 17FRV (low virulence), and FPL (high virulence) produced the same cytopathology as isolates G10, M8 and F19 (low virulence).

Electron microscopic observations of cells infected with isolates G1, G10, FPV and F11 suggest underlying causes for the differences observed between them in single step growth curves (see Chapter 6). G10 virus was released earlier than G1 virus from infected cells but at a slower rate and at the burst phase more G10 virus remained cell-associated compared to G1 virus. When cells infected by these isolates were examined by electron microscopy it was observed that G1 virus aggregated in well ordered crystalline arrays which were often situated near to the cell periphery and broke up after the cell membrane became grossly incompetent. By contrast, G10 virus was found in small, semi-crystalline pockets and in loose aggregates throughout the infected cell. After disruption of the limiting cell membrane G10 virus apparently remained aggregated and associated with cellular debris (Fig. 7.12). The relative inefficiency of G10 virus to become extracellular could therefore be explained in terms of this prolonged virus aggregation and possibly also the initial topography of these aggregates, often in a deep cytoplasmic position. The FCV virus release process is probably very similar to that described for picornaviruses (Godman, 1973). For these viruses cytopathic events, in particular the sloughing of pieces of virus-containing peripheral cytoplasm, result in the rapid release of virus in a short 3-hour burst between 3 and 6 hours after initial infection while the larger part of the virus progeny remains cell-associated for up to 12 hours after cell death. The peripheral location of a relatively large amount of G1 virus, in large crystals, together with this sloughing process, could explain the relatively efficient release of G1 virus.

As suggested by the slightly earlier appearance of CPE in G10 infected cultures compared to G1-infected cultures (Chapter 6), the morbid process may be more rapid in G10-infected cells resulting in the earlier loss of portions of virus-containing peripheral cytoplasm and the earlier detection of infectious G10 virus in extracellular fluid. There was, however, no difference between G1 and G10-infected cultures in the time of appearance of virus in the extracellular space as detected by

electron microscopy but samples were examined at hourly intervals and small differences would not have been detected.

A comparison of isolates F11 and FPL revealed a similar situation to that described for G1 and G10. Isolate F11, like G1, formed large crystalline arrays and virus release was relatively efficient (as measured by the ratio of released virus to cell-associated virus). Isolate FPL, like G10, did not form large crystals but remained in loose aggregates in the infected cell cytoplasm and virus was released relatively inefficiently although at an earlier time than F11 virus.

An alternative hypothesis could be made to relate electron microscopic observations on virus aggregation with virus replication characteristics. It might be that with isolates such as G10 and FPL it is the early release of virus which prevents virus crystal formation. Mayor and Jordan (1962) reported that a type 1 poliovirus did not produce crystals in monkey kidney cells maintained during infection at 37°C but when the same cells were maintained at 30°C crystal formation occurred and at this temperature there was a marked decrease in the amount of infectious virus detectable in the extracellular fraction. This suggested that in this particular virus-host system crystal formation took place as a result of increased intracellular virus accumulation which occurred when the rate of virus formation exceeded that of release from the cell by a certain amount. However, the same is probably not true for the FCV isolates examined here: single step growth curves indicated that G1 and G10 virus accumulated at approximately the same rate in the cytoplasm of infected cells and at 5 hours after adsorption, when virus crystals first become a prominent feature in G1 infected cells, the same amount of virus was present in cells infected with both isolates (see Chapter 6). Also, FPL virus, in comparison with F11 virus, accumulated in greater amounts in the cytoplasm of infected cells but did not aggregate into crystalline array form. It would appear more likely that differences in FCV intracellular aggregation indicate differences in the intracellular location of virus synthesis and/or assembly or possible differences in virus structure. It may be significant that the four isolates forming non-regular particle aggregates and semi-crystalline arrays (G10, M8, FPL and F19) were found to be inhibited in their plaque development by agar polyanions. As suggested in Chapter 6, these isolates may have a greater net positive charge on the capsid than agar polyanion-insensitive isolates which possibly hinders close particle to particle contact and therefore prevents crystalline array formation. However, it should be

noted that strain 17FRV is inhibited in its plaque development by agar polyanions but nevertheless can form large virus crystals.

Isolates F17 and KCD were frequently observed in linear arrays associated with filamentous structures similar to those described by Strandberg (1968); Studdert and O'Shea (1975) and Love and Sabine (1975). Linear particle arrays bounded by similar structures have been reported for other viruses, for example echoviruses (Godman, 1973) and VESV type H54 (Zee, Hackett and Talens, 1968). In the latter study, a temporal relationship was noted between the appearance of virus in linear arrays between membranes at the cell surface and an increase in the titre of extracellular virus and it appeared that these structures played a role in the transport and release of virus rather than as sites of virus synthesis. A similar interpretation was made in the present study but based only on the observation that these structures were most frequently found very close to the limiting cell membrane or cytoplasmic boundary (Fig. 7.14).

Many other structures observed in the cytoplasm of FCV-infected cells closely resemble structures described for picornavirus infection of cultured cells (the cytopathology of enteroviruses was reviewed by Godman, 1966). The formation of many membranous structures, as in picornavirus infection, may be related indirectly to viral synthesis in that they are the result of degradation of cellular lipids with the formation of lysolipids and new cell membranes (Amako and Dales, 1967). However, the small cytoplasmic vesicles described in this study (see Fig. 7.17) and by others (Strandberg, 1968; Studdert and O'Shea, 1975; Love and Sabine, 1975) may be more specifically related to virus synthesis since they appear at an early stage (between 2 and 3 hours) of the replication cycle. The synthesis of RNA is apparently initiated in a complex associated with smooth membranes, as has been demonstrated for polioviruses (Caliguiri and Tamm, 1970) and it is possible that the cytoplasmic vesicles associated with FCV infection may represent an RNA replication complex.

Another structure which may be related directly to virus synthesis is the "grey area" (Figs. 7.2, 7.3 and 7.6). Grey areas were particularly abundant in cells infected with isolates G1 and FPV255 and it may be significant that these were the only ep isolates examined. During the multiplication of G1 virus a large excess of 15S subunit is produced (Komolafe, 1978) and the grey areas were interpreted as representing possible sites of subunit accumulation; they had the same staining

affinity and fine-grained texture of virus capsid (that is, the inter-core matrix observed in virus crystals) and they were spatially associated with sites of virus assembly. Grey areas have not been described before, possibly because they are produced only with a minority of FCV isolates. It is not known yet if other FCV isolates produce an equivalent proportion of 15S subunits to virus particles. Grey areas may be analogous to the viroplasm described in some picornavirus-infected cells (Godman, 1973).

Intracellular virus antigen was detected by immunofluorescence and, as in the electron microscopic observations described above, differences were detected between isolates. Cells infected with some isolates, for example G10, exhibited diffuse cytoplasmic staining. In cells infected with other isolates, for example G1, the cytoplasm also stained but bright staining was located in several cytoplasmic foci. The background cytoplasmic staining observed in G1-infected cells was considered to be specific rather than a product of light reflection from the bright staining foci since, as described below, a similar type of fluorescence could be obtained in the absence of foci, and cytoplasmic staining was prevented by using anti-virus serum at higher dilutions although focal fluorescence remained bright. Therefore for certain isolates, for example FCV G1, there were apparently two types of fluorescence; diffuse and focal.

Foci of fluorescence were considered likely to represent virus crystals. Foci were only observed in cells infected with isolates producing virus crystals and foci were situated near the cell periphery, as were virus crystals. There was also a temporal relationship between virus crystals and fluorescent foci. G1-infected cells were fixed and stained at hourly intervals from 1 to 6 hours after exposure to virus: at 3 to 5 hours the cytoplasm of infected cells stained diffusely but only after 6 hours could foci be detected. This corresponds to the time when large virus crystals initially develop.

Rabbit anti-G1 15S subunit serum when reacted with G1-infected cells produced only a diffuse cytoplasmic staining. Komolafe (1978) reported that G1 virus contained two antigenic determinants. Using an immunodiffusion test, 2 precipitation lines were observed with G1 virus against rabbit anti-G1 virus serum and only one line (a line of identity with one of the above lines) was observed with G1 virus against rabbit anti-G1 15S subunit serum. It is likely that in the immunofluorescence test, anti-G1 15S antibody reacted with 15S subunit (and presumably also virus polypeptide) present throughout the infected cell cytoplasm and

hence a diffuse staining pattern was obtained. The anti-G1 virus serum, however, apparently contained two types of antibody against different antigenic determinants and while one type of antibody reacted with the 15S subunit determinant, both types reacted with whole virus capsid and so areas of virus aggregation were highlighted.

The FCV immunofluorescence test gives broader cross-reactivity than the neutralisation test. Thus anti-G1 serum did not neutralise G10 virus (see Table 5.7) but did react with G10-infected cells (see Fig. 7.20). Gillespie, Judkins and Kahn (1971) reported a similar broad cross-reactivity using fluorescein conjugated anti-17FRV serum and this led them to suggest that the immunofluorescence test offered a means by which group-specific identification of FCV could be made. It may be that one of the two antigenic determinants present on the viral capsid is group-specific and the other type-specific. If so, the failure of a virus crystal-forming isolate such as FPV255 to fluoresce focally might be explained by the absence of type-specificity. It may be that both group and type specificity is required to produce the staining pattern observed in G1, F11 and 17FRV infected cells. If immunofluorescence were used further as a method of examining FCV virus-cell relationships it might be advisable to use homologous sera.

CHAPTER 8

GENERAL DISCUSSION

Differences in pathogenicity of FCV isolates.

FCV mutation and attenuation.

Calicivirus antigenic variation.

FCV vaccines.

The main aims of this study were to characterise the variation in plaque morphology between FCV isolates, to investigate a possible correlation between plaque morphology and virulence and to attempt to define the basis for differences between FCV isolates in both plaque morphology and virulence.

These aims have to a large extent been achieved. The study has provided a means of classifying FCV on plaque morphology and also an explanation for the observed differences in plaque phenotype. A correlation between plaque morphology and virulence has been found and an explanation for this relationship suggested. In addition, evidence has been presented which indicates that FCV are highly mutable, at least with regard to their plaque phenotype and that FCV isolates differ in the cytopathology they induce in tissue culture cells.

In this discussion these findings are considered in relation to several important features of the natural history of feline caliciviruses. These include the pathogenesis of FCV and the basis for differences in virulence between FCV isolates; FCV plaque mutation and attenuation after cell culture passage of virus; FCV antigenic variation and the potential usefulness of the mp marker for low virulence in vaccine formulation.

Differences in pathogenicity of FCV isolates

In considering the pathogenesis of FCV infection and the variation in virulence observed between isolates experiments on SPF cats as described in Chapter 5 provide a comparison of the clinical and pathological diseases produced by two isolates of different virulence, FCV G1 (an ep forming isolate) was found to be highly virulent and FCV G10 (an mp forming isolate) was found to be of low virulence. Both isolates produced similar lesions but G1 infection invariably produced more extensive lesions than G10 infection by the same route of inoculation. With either isolate cats infected by virus aerosol were more severely affected than cats exposed to intranasal instillation of virus thus confirming previous suspicions (Love, 1975).

Experimental infection of cats has demonstrated the epitheliotropic nature of FCV and two lesions in particular, oral/nasal vesicle formation and ulceration, and pneumonia are most consistently found. In the present study vesicles and ulcers were observed on the dorsum of the tongue, hard palate and rhinarium. These lesions were produced with strains of high or low virulence although it may be that low virulence strains produce smaller vesicles and ulcers. This was true in this study and for strain KCD as reported previously by Hoover and Kahn (1973). The histological progression of FCV-induced ulcer formation has been described by Hoover and Kahn (1973) and appears to result from an initial degeneration of a number of epithelial cells in the non-keratinising stratum corneum or superficial stratum spinosum followed by neutrophil infiltration. Virus antigen has

been demonstrated in epithelial cells at the periphery of ulcers by immunofluorescence (Holzinger and Kahn, 1970). Vesicle and ulcer formation is a feature of infection with the other two members of the calicivirus genus. Thus, in pigs VESV causes vesicular and ulcerative lesions identical to those of foot-and-mouth disease virus (FMDV) (Traum, 1936) and in northern fur seals and Californian sea lions, San Miguel sea-lion virus (SMSV) apparently causes vesicular lesions on flippers (Sawyer, 1976).

Electron microscopy and immunofluorescent microscopy have indicated that FCV has also a marked tropism for alveolar pneumocytes (Langloss, Hoover and Kahn, 1977; 1978). Earlier reports describe the detection of viral antigen in alveolar macrophages by immunofluorescence (Holzinger and Kahn, 1970; Kahn and Gillespie, 1971) but there is recent evidence that infection of these cells may be non-productive (Langloss *et al.*, 1978). Langloss, Hoover and Kahn (1978) noted that the ensuing inflammatory reaction paralleled the degree of alveolar pneumocyte damage. The later interstitial pneumonia characterised by hypertrophy and hyperplasia of Type II pneumocytes as described in Chapter 5 is thought to be a regenerative or reparative process which is not specific for FCV infection; Langloss, Hoover and Kahn (1977) observed a similar reaction in the lungs of cats exposed to a high concentration of nitrogen dioxide. Virus was not detectable by immunofluorescence or electron microscopy during this phase of FCV-induced pneumonia (Langloss, Hoover and Kahn, 1978). It appears, therefore, that the pneumonic lesion associated with FCV infection results from a short period of virus replication in Type 1 pneumocytes and that the more pneumocytes infected (and destroyed) the more extensive the pulmonary lesion. It follows that the basis for differences in virulence between FCV isolates could be related to the efficiency of replication and/or cell killing ability of an isolate in respiratory or oral epithelium. It was concluded in Chapter 5 that the replication of FCV G10 compared to FCV G1 was less efficient in trubinate and alveolar epithelium based on the amount of virus recovered from these tissues at necropsy.

It was demonstrated in Chapter 5 that a correlation apparently exists between plaque size and virulence in that isolates of the ep type (G1 and FPV 255) are of high virulence and isolates of the mp type (G10, 17 FRV, F17, M8, FJ and F10) are of low virulence. Some of the biological characteristics of these two plaque types were investigated in an

attempt to explain the differences in plaque size and virulence and isolates G1 and G10 were used to represent each type. There appeared to be two reasons for the discrepancy in plaque size. The main one was inhibition of the mp forming isolate by polyanions. All mp forming isolates used in this study were sensitive to agar polyanions. The other reason was that the ep forming isolate replicated more efficiently in FEA cells: higher yields of virus per infected cell were obtained and ep forming virus was released more completely from cells than mp forming virus. The comparison of the growth curves of strains F11 and FPL also provided evidence that growth characteristics are important in determining plaque size in the absence of polyanions. FPL plaques were larger than F11 plaques under an agarose overlay and it was found that FPL virus was released earlier and in greater amounts than F11 virus. It was suggested that the same reasons for plaque size differences might be responsible for the difference in virulence between mp and ep forming isolates.

Electron microscopic observations of FEA infected cells suggested a morphogenetic basis for differences in growth cycles as described in Chapter 7. These studies also illustrated another characteristic in which FCV isolates vary from each other: that is, in cytopathology and particularly the way in which virus particles aggregated in the cytoplasm of infected cells. The recognition of this variability is important since it has been suggested that the pattern of arrays of virus particles is a characteristic that can be used in the classification of caliciviruses (Breese and Dardiri, 1977). Variation between isolates in antigen distribution in infected FEA cell cytoplasm was shown by immunofluorescence. Some isolates (for example G10) produced diffuse staining in cell cytoplasm and others (for example G1) produced focal staining along with diffuse staining. Such variation has not been described previously for FCV. It was suggested that foci of fluorescence represented virus crystals but it was found that not all strains known to produce crystals produced focal fluorescence (e.g. FPV 255). As discussed in Chapter 7 this might be because FPV 255 virus is lacking in the G1 type specific antigenic determinant. Strain F11 which produces foci of fluorescence with G1 antiserum presumably has this type specific determinant or at least a closely related determinant. It might be expected that strain F11 would be more fully neutralised by G1 antiserum than strain FPV 255. This, however, was not tested here. No obvious correlation was found between the cytopathology induced by an isolate and the plaque type or virulence of that isolate.

FCV mutation and attenuation

Feline calicivirus isolates directly plated from mouth swabs onto FEA monolayers and overlayen with the standard agar overlay produced large plaques in over 80% of isolations. Compared with this, FCV strains passaged several or many times in cell culture were predominantly sp and mp type (67%). Smaller plaque types were seen to emerge within several lp forming isolates after passages at high multiplicities of infection in cell culture. Whether these plaque variants were present in the original swab sample or arose by mutation from wild type lp forming virus during propogation in tissue culture is not known. The appearance of smaller plaque virus in three times plaque purified stocks indicated that mutation had taken place. Better evidence for *in vitro* mutation was obtained by studying the reverse mutation, mp to lp as described in Chapter 4. Whether *in vitro* mutation had or had not occurred, selection of the smaller plaque variants must have taken place to allow them to predominate eventually. The earlier release of G10 virus (representing mp forming isolates) compared to G1 virus (representing ep forming isolates) and G2 virus (representing lp forming isolates) might be a selection factor.

In other virus-host systems numerous plaque variants have been derived by passage in tissue culture and there are several examples where wild type virus is found frequently as a particular plaque type but after cell culture passage new plaque types appear (reviewed by Takemoto, 1966). This is true of several picornaviruses. For example, Suto *et al.* (1965) working with echovirus-6 compared six strains before and after cell culture passage. The wild type virus in all six strains was designated M^+ (large plaque). Three strains were altered in plaque character after 1-11 passages when a small plaque type, designated M, was found to predominate. The other three strains did not change in plaque character and these were regarded as genetically stable. Corresponding "stable" strains may occur among FCV isolates; of 19 isolates passaged 6 times in cell culture, 10 remained as the original plaque type. Alternatively this might indicate that these 10 isolates were originally of a single plaque population and the other 9 isolates were of a mixed plaque population. It is of interest that the M mutant of echovirus-6 was sensitive to agar inhibition (Barron and Karzon, 1965) and the selection factor responsible for the emergence of M mutants was thought to be a greater yield of virus per cell. Other picornaviruses in which plaque variants, not commonly found in the natural state, were

produced by passage in cell culture include echovirus-9 (Wigand and Sabin, 1962) and FMDV (Dinter, Philipson and Wesslen, 1959). The plaque types of VESV described by McClain, Hackett and Madin (1958) probably also reflect the effect of cell culture passage. Although passage history was not reported the plaque character of seven antigenic types of VESV was determined and in each, except possibly one, a mixture of minute and large plaques was present.

The difference in the incidence of mp forming virus obtained from field isolates and cell culture passaged isolates may be of importance when considering virulence in natural infection. Marked variation in FCV virulence has been recognised by infecting cats experimentally with a number of FCV strains (Hoover and Kahn, 1973; 1975; Povey and Hale, 1974; Wardley and Povey, 1977a). It may be that strains found to be of low virulence, such as FJ and F10, are those which have been attenuated by cell culture passage, their mp plaque type being an indicator that attenuation (that is, selection of a variant) had taken place. Thus it could be argued that the degree of variation in virulence produced in experiments using laboratory cultivated FCV strains is not representative of the field situation. It would be necessary to infect cats with a number of different field isolates (non-passaged or low passage in cell culture) to determine what variation (if any) was present in the natural infection.

Plaque type heterogeneity within a single isolate has not been described previously, for FCV. In this study several isolates were found to contain a mixture of two plaque types when swab medium was assayed by the standard method. It was considered likely that a number of other isolates were inapparently heterogeneous (Walen, 1963). Mixed plaque populations may have arisen from dual infection or mutation *in vivo* with selection. The persistent carrier state which is a common sequel to acute FCV infection (Povey, Wardley and Jessen, 1973) might provide conditions favourable for mutant selection. Wardley (1976) detected antigenic change in virus excreted by carriers over a period of time. It would be of interest to examine the plaque character of sequential isolations obtained from carrier animals to investigate *in vivo* plaque mutation and its relationship to what may be antigenic drift.

Genetic studies described in Chapter 4 indicated that FCV is highly mutable. The apparently high frequency of mutation presumably relates to a highly mutable region (or regions) in the gene coding for the single capsid polypeptide. This is probably true at least for lp or

ep to mp mutation where there may be a change in the ionic atmosphere of the capsid (see Chapter 6). The marked antigenic heterogeneity observed among isolates also presumably relates to mutation in the same gene; however, it is not known if the same mutational event causes a change in plaque type and antigenicity. This obviously could be investigated by a serological comparison of cloned variants obtained when progeny testing single plaques of cloned virus stock with the original virus. There are some indications however that plaque mutation may be independent of antigenic change. Kalunda et al. (1975) found no correlation between plaque type and any antigenic grouping. Also, FCV remains antigenically stable through a large number of passages in cell culture and yet plaque type changes are common: Crandell and Madin (1960) reported that strain CFI, after 56 serial passages in cell culture was antigenically identical to the original virus although virulence was lost between the twelfth and thirteenth passages; Bürki, Starustka and Ruttner (1976) commented that 5 Swiss strains maintained their original cross-neutralisation pattern after 10 years of laboratory passage. Unfortunately the original plaque type of these isolates is not known but after numerous passages in culture strain CFI was found to be lp forming and the Swiss strains mp forming (3 strains), sp forming (1 strain) and lp forming (1 strain) (see Table 4.4a).

Calicivirus antigenic variation

Antigenic variation is a feature of all caliciviruses: there are at least 13 distinct antigenic types of VESV (Smith and Akers, 1976) and 4 antigenic types of SMSV (Smith, Prato and Skilling, 1977). It appears that plaque type heterogeneity is also a common characteristic of caliciviruses. Plaque variants have been described for VESV (McClain, Hackett and Madin, 1958; Walen, 1963) and SMSV plaque formation and apparent heterogeneity has been reported by Smith et al. (1973). In the latter study the authors reported that the isolate could not be purified into a homogeneous plaque population by cloning. The above information suggests that all caliciviruses isolated to date are highly mutable.

It is becoming increasingly evident that the three caliciviruses, FCV, VESV and SMSV are closely related. Burroughs, Doel and Brown (1978) found that structurally SMSV and VESV were more closely related to each other than to FCV. Recently antigenic relatedness has been examined by immunodiffusion (ID) (Burroughs, Doel and Brown, 1978), immunoelectron microscopy (IEM) (Smith, Skilling and Ritchie, 1978) and radio-immunoprecipitation (RIP) (Soergel et al. 1978). By ID and IEM, VESV and

SMSV were again found to be more closely related to each other than to FCV. SMSV and FCV were apparently more distant from one another than VESV. However, by RIP, FCV F9 antibody reacted with two antigenic types of SMSV. There is strong evidence to suggest that VESV and SMSV are one and the same virus (reviewed by Smith and Akers, 1976; Sawyer, 1976) and that a calicivirus transmitted from pinnipeds to pigs was responsible for outbreaks of vesicular exanthema of swine. The recent isolation of a calicivirus from a fish (Smith, Skilling and Ritchie, 1978) has added more credulity to the concept of there being a common marine source for all three caliciviruses.

FCV vaccines

An obvious extension to the work reported here would be to use the mp marker to select FCV strains which may prove suitable for immunising cats against virulent FCV infection. Strains exhibiting the mp marker and known to elicit a broad spectrum immune response, as measured by cross neutralisation, would be candidates for vaccination studies. Povey and Ingersol (1975) have shown that the presence of homotypic and heterotypic neutralising antibody correlates with clinical protection and the need for a broad antigenic cover has previously been recognised (Bittle and Rubic, 1975). It may be possible to combine two or more mp forming strains to provide complete antigenic cover. A live vaccine of this type may be of use if the following points concerning its safety were found to be valid: first, that a vaccine virus stock could be obtained which was phenotypically homogeneous and genetically stable. This appears to be a major problem since as described in Chapter 4 it was not possible to obtain 100% pure mp forming virus stock and it was assumed that lp variants which were detected at a ratio of 0.1-0.2% had arisen by mutation. It is possible that these lp variants are virulent and it was considered significant that from the cat which was most severely affected by mp virus aerosol, lp forming virus was isolated from pneumonic lung tissue and tongue (see Chapter 5). Secondly, no ill effects should ensue from vaccination. Some mp forming strains when administered by intranasal instillation produce a mild clinical disease with ulceration of the tongue, hard palate and nares. This was found for strains F17 and 17FRV (Povey and Ingersol, 1975) and such a reaction may be unacceptable. However, other strains such as M8 are apparently completely avirulent. Thirdly, there should be no reversion to virulence (that is, in vivo mutation and/or selection should not take

place after vaccination). Intranasal instillation of virus may initiate a persistent carrier infection in some cats and the possibility of mutation and selection of variants may be high in such cases as discussed above. While a vaccinated animal, persistently excreting mp virus of low virulence and thereby infecting and immunising in-contact animals would obviously be beneficial in a cattery, the risk of a reversion to virulence may be high and certainly would require investigation.

Recently two types of commercial FCV vaccines were made available. One was obtained by attenuating strain F9 by passage in cell culture at low temperature (30-32°C) (Bittle and Rubic, 1975). It is interesting that when the vaccine virus was tested in this laboratory it was found to be sp forming and sensitive to agar inhibition. The vaccine is administered by intramuscular injection. Transmission to in-contact cats does not occur and virus cannot be re-isolated from respiratory tissue but remains local to the site of injection (D. Kahn; personal communication). The other vaccine contains a "naturally occurring" FCV strain of low virulence and is administered by direct intranasal or intra-conjunctival instillation (Davis and Beckenhauer, 1976). This virus has been passaged in cell culture an unspecified number of times and the claims that it is a naturally occurring strain of low virulence may therefore be inaccurate. Both types of vaccine are administered in conjunction with live, attenuated FHV. Both vaccines have been associated with a number of untoward reactions and the safety and, to a lesser extent, the efficacy of each has been called into question (reviewed by Povey, 1977). The effect of vaccination on carrier animals has not been investigated nor has the possibility that exposure of a vaccinated animal to the natural infection could result in the establishment of a persistent carrier infection in the absence of any clinical signs; a situation which is known to occur, for example, with FMDV where an asymptomatic carrier state is frequently established after infection of immune cattle (Sutmoller, McVicar and Cottral, 1968). For the live intranasal vaccine there is insufficient published data on the period of virus shedding after vaccination and on the possibility of reversion to virulence.

The above information suggests that FCV immunoprophylaxis is now at least feasible but that better methods of vaccination are required. Also, that vaccination alone may not be sufficient to eradicate FCV from any group of cats. This may require the identification of carrier animals by virological examination of mouth swabs and their removal from the group. The mp marker offers a method for selecting strains of low virulence, for monitoring the phenotypic purity of vaccine virus stocks and for monitoring genetic variation which may follow the administration of a live vaccine.

REFERENCES

Agol, V.I. and Chumakova, M.Y. (1962)
An agar polysaccharide and d marker of poliovirus.
Virology 17, 221-223.

Agol, V.I. and Chumakova, M.Y. (1963)
Effect of polyanions on the multiplication of two variants of poliovirus.
Acta Virology 7, 97-106.

Almeida, J.D., Waterson, A.P., Prydie, J., and Fletcher, E.W.L. (1968)
The structure of a feline picornavirus and its relevance to cubic viruses in general.
Arch. Ges. Virusforsch 25, 105-114.

Amako, K. and Dales, S. (1967)
Cytopathology of mengovirus infection. 1. Relationship between cellular disintegration and virulence. 11. Proliferation of membranous cisternae.
Virology 32, 184-200.

Archetti, I. and Horsfall, F.L. (1950)
Persistent antigenic variation of influenza A virus after incomplete neutralisation in vivo with heterologous immune serum.
J. Exp. Med. 92, 441-462.

Baker, J.A. (1944)
A virus causing pneumonia in cats producing elementary bodies.
J. Exp. Med. 79, 159-172.

Balayan, M.S., Voroshilova, M.K., Sinyak, L.I. and Chumakova, A.B. (1970)
Behaviour of poliovirus strains of different virulence under solid overlays.
Arch. Ges. Virusforsch 32, 299-310.

Barron, A.L. and Karzon, D.T. (1965)
Studies of mutants of ECHO 6 virus. 1. Biological and serological characteristics.
Amer. J. Epidem. 81, 323-331.

Bartholomew, P.T. and Gillespie, J.H. (1968)
Feline viruses. 1. Characterisation of four isolates and their effects on young kittens.
Cornell Vet. 58, 248-265.

Bittle, J.L. and Rubic, W.J. (1975)
A feline calicivirus vaccine combined with feline viral rhinotracheitis and feline panleukopaenia vaccine.
Feline Practice 5, 13-15.

Bittle, J.L., York, C.J., Newberne, J.W. and Martin, M. (1960)
Serological relationship of new feline cytopathogenic viruses.
Amer. J. Vet. Res. 21, 547-550.

Black, D. and Brown, F. (1975/76)
A major difference in the strategy of the calici and picornaviruses and its significance in classification.
Intervirology 6, 57-60.

Black, D. and Brown, F. (1977)
Proteins induced by infection with caliciviruses.
J. Gen. Virol. 38, 75-82.

Blackmore, D.K. and Hill, A. (1973)
The experimental transmission of various mycoplasmas of feline origin to domestic cats (Felis catus).
J. Small Anim. Pract. 14, 7-13.

Bolin, V.S. (1957)
The cultivation of panleukopaenia virus in tissue culture.
Virology 4, 389-390.

Breese, S.S. and Dardiri, A.H. (1977)
Electron microscopic observations on a virus transmissible from pinnipeds to swine.
J. Gen. Virol. 36, 221-225.

Breeze, D.C. (1964)
Studies on encephalomyocarditis virus.
Thesis. University of Glasgow.

Breeze, D.C. and Subak-Sharpe, H. (1967)
The mutability of small-plaque-forming encephalomyocarditis virus.
J. Gen. Virol. 1, 81-88.

Bürki, F. (1963)
Viren des respirationsapparates bei katzen.
Proceedings of the 17th World Veterinary Congress, 559-564.

Bürki, F. (1965)
Picornaviruses of cats.
Arch. Ges. Virusforsch. 15, 690-696.

Bürki, F. (1971)
Virological and immunological aspects of feline picornaviruses.
J. Amer. Vet. Med. Assoc. 158, 916-919.

Burki, F., Starustka, B. and Ruttner, O. (1976)
Attempts to serologically classify feline caliciviruses on a national and an international basis.
Infect. and Immun. 14, 876-881.

Burness, A.T.H. (1967)
Separation of plaque-type variants of encephalomyocarditis virus by chromatography on calcium phosphate.
J. Virol. 1, 308-316.

Burroughs, J.N., Doel, T. and Brown, F. (1978)
Relationship of San Miguel sea lion virus to other members of the calicivirus group.
Intervirology 10, 51-59.

Caliguiri, L. and Tamm, I. (1970)
The role of cytoplasmic membranes in poliovirus biosynthesis.
Virology 42, 100-111.

Campbell, L.H., Snyder, S.B., Reed, C. and Fox, J.G. (1973)
Mycoplasma felis - associated conjunctivitis in cats.
J. Amer. Vet. Med. Assoc. 163, 991-995.

Choppin, P.W. and Eggers, H.J. (1962)
Heterogeneity of Coxsackie B4 virus: two kinds of particles which differ in antibody sensitivity, growth rate and plaque size.
Virology 18, 470-476.

Colter, J.S., Davies, M.A. and Campbell, J.B. (1964)
Studies of three variants of Mengo encephalomyelitis virus. 11. Inhibition of interaction with L cells by an agar inhibitor and by protamine.
Virology 24, 578-585.

Cooper, P.D. (1961)
The plaque assay of animal viruses.
Advances in Virus Research 8, 319-378.

Cooper, P.D. (1964)
Synchrony and the elimination of chance delays in the growth of poliovirus.
J. Gen. Microbiol. 37, 259-266.

Cooper, P.D. (1967)
The plaque assay of animal viruses. In "Methods in Virology", volume 3, 243-311 Edited by K. Maramorosch and H. Koprowski. Academic Press, New York.

Cowan, S.I. and Steel, K.J. (1974)
"Manual for the Identification of Medical Bacteria", second edition, Cambridge University Press.

Craighead, J.E. (1965)
Pathogenicity of r and r^+ variants of encephalomyocarditis (EMC) virus.
Federation Proceedings 24, 159.

Crandell, R.A. (1967)
A description of eight feline picornaviruses and an attempt to classify them.
Proc. Soc. Exptl. Biol. Med. 126, 240-245.

Crandell, R.A. (1973)
Feline viral rhinotracheitis (FVR)
Adv. Vet. Sci. Comp. Med. 17, 201-224.

Crandell, R.A. and Madin, S.H. (1960)
Experimental studies on a new feline virus.
Amer. J. Vet. Res. 21, 551-556.

Crandell, R.A. and Maurer, F.D. (1958)
Isolation of a feline virus associated with intranuclear inclusion bodies.
Proc. Soc. Exptl. Biol. Med. 97, 487-490.

Crandell, R.A., Rehkemper, J.A., Niemann, W.H., Ganaway, J.R. and Maurer, F.D. (1961).
Experimental feline viral rhinotracheitis in the cat.
J. Amer. Vet. Med. Assoc. 138, 191-196.

Davis, E.V. and Beckenhauer, W.H. (1976)
Studies on the safety and efficacy of an intranasal feline rhinotracheitis-calicivirus vaccine.
Vet. Med/Small Anim. Clin. 71, 1405-1409.

Diamond, L. and Crawford, L.V. (1964)
Some characteristics of large-plaque and small-plaque lines of polyoma virus.
Virology 22, 235-244.

Dinter, Z., Philipson, L. and Wesslen, T. (1959)
Properties of foot and mouth disease virus in tissue culture. 1. Change in plaque morphology and antigenicity following passage in tissue cultures.
Arch. Ges. Virusforsch. 9, 411-427.

Dulbecco, R. and Vogt, M. (1954)
Plaque formation and isolation of pure lines with poliomyelitis viruses.
J. Exp. Med. 99, 167-182.

Ehresmann, W.D. and Schaffer, F.L. (1977)
RNA synthesis in calicivirus infected cells is atypical of picornaviruses.
J. Virol. 22, 572-576.

Fastier, L.B. (1957)
A new feline virus isolated in tissue culture.
Amer. J. Vet. Res. 18, 382-389.

Flamand, A. (1970)
Etude génétique du virus de la stomatite vesiculaire: classement de mutants thermosensible spontanés en groupes de complémentation.
J. Gen. Virol. 8, 187-195.

Gallagher, J.T., Kent, P.W., Passatore, M., Phipps, R.J. and Richardson, P.S. (1975)
The composition of tracheal mucus and the nervous control of its secretion in the cat.
Proc. Roy. Soc. London: B 192, 49-76.

Gemmell, A. and Fenner, F. (1960)
Genetic studies with mammalian poxviruses. 111. White (u) mutants of rabbitpox virus.
Virology 11, 219-235.

Gillespie, J.H., Judkins, A.B. and Kahn, D.E. (1971)
Feline viruses X111. The use of the immunofluorescent test for the detection of feline picornaviruses.
Cornell Vet. 61, 172-179.

Gillespie, J.H. and Scott, F.W. (1973)
Feline viral infections. 1V. Feline reovirus (FVR) infection.
Adv. Vet. Sci. Comp. Med. 17, 188-200.

Godman, G.C. (1966)
The cytopathology of enterovirus infection.
International Review of Experimental Pathology 5, 67-110.

Godman, G.C. (1973)
Picornaviruses. In "Ultrastructure of Animal Viruses and Bacteriophages: An Atlas". Edited by A.J. Dalton and F. Haguenau, Academic Press. New York and London.

Holzinger, E.A. and Kahn, D.E. (1970)
Pathological features of picornavirus infection in cats.
Amer. J. Vet. Res. 31, 1623-1630.

Hoover, E.A. and Kahn, D.E. (1973)
Lesions produced by feline picornaviruses of different virulence in pathogen-free-cats.
Vet. Path. 10, 307-322.

Hoover, E.A. and Kahn, D.E. (1975)
Experimentally induced feline calicivirus infection: clinical signs and lesions.
J. Amer. Vet. Med. Assoc. 166, 463-468.

Hoover, E.A., Kahn, D.E. and Langloss, J.M. (1978)
Experimentally induced feline chlamydial infection (feline pneumonitis).
Amer. J. Vet. Res. 39, 541-547.

Howes, D.W. and Melnick, J.L. (1957)
The growth cycle of poliovirus in monkey kidney cells.
1. Maturation and release of virus in monolayer cultures.
Virology 4, 97-108.

Jarrett, O., Laird, H.M. and Hay, D. (1973)
Determinants of the host range of feline leukaemia viruses.
J. Gen. Virol. 20, 169-175.

Kahn, D.E. and Gillespie, J.H. (1970)
Feline viruses. X. Characterisation of a newly-isolated picornavirus causing interstitial pneumonia and ulcerative stomatitis in the domestic cat.
Cornell Vet. 60, 669-683.

Kahn, D.E. and Gillespie, J.H. (1971)
Feline viruses: Pathogenesis of picornavirus infection in the cat.
Amer. J. Vet. Res. 32, 521-531.

Kahn, D.E., Hoover, E.A. and Bittle, J.L. (1975)
Induction of immunity to feline caliciviral disease.
Infect. and Immun. 11, 1003-1009.

Kalunda, M., Lee, K.M., Holmes, D.F. and Gillespie, J.H. (1975)
Serological classification of feline caliciviruses by plaque-reduction neutralisation and immunodiffusion.
Amer. J. Vet. Res. 36, 353-356.

Kapikian, A.Z. and others (1967)
Rhinoviruses: a numbering system.
Nature (London) 213, 761-763.

Karnovsky, M.J. (1965)
A formaldehyde-gluteraldehyde fixative of high osmolality for use in electron microscopy.
J. Cell. Biol. 27, 137A-138A.

Komolafe, O. (1978)
The structure of feline calicivirus particles. Ph. D. Thesis. University of Glasgow.

Langloss, J.M., Hoover, E.A. and Kahn, D.E. (1977)
Diffuse alveolar damage in cats induced by nitrogen dioxide or feline calicivirus.
Amer. J. Path. 89, 637-644.

Langloss, J.M., Hoover, E.A. and Kahn, D.E. (1978)
Ultrastructural morphogenesis of acute viral pneumonia produced by feline calicivirus.
Amer. J. Vet. Res. 39, 1577-1583.

Langloss, J.M., Hoover, E.A., Kahn, D.E. and Kniazeff, A.J. (1978)
In vitro interaction of alveolar macrophages and pneumocytes with feline respiratory viruses.
Infect. and Immun. 20, 836-841.

Ledinko, N. and Hirst, G.K. (1961)
Mixed infection of cells and poliovirus types 1 and 2.
Virology 14, 207-219.

Liebhaber, H. and Takemoto, K.K. (1961)
Alterations of plaque morphology of EMC virus with polycations.
Virology 14, 502-504.

Liebhaber, H. and Takemoto, K.K. (1963)
The basis for the size differences in plaques produced by variants of encephalomyocarditis (EMC) virus.
Virology 20, 559-566.

Lindenmann, J. and Gifford, G.E. (1963)
Studies on vaccinia virus plaque formation and its inhibition by interferon. 1. Dynamics of plaque formation by vaccinia virus.
Virology 19, 283-293.

Love, D.N. (1973)
The utilization of zwitterionic buffer system in the plaque assay of a feline calicivirus.
Aust. J. Exptl. Biol. Med. Sci. 51, 263-266.

Love, D.N. (1975)
Pathogenicity of a strain of feline calicivirus for domestic kittens.
Aust. Vet. J. 51, 541-546.

Love, D.N. and Baker, K.D. (1972)
Sudden death in kittens associated with a feline picornavirus.
Aust. Vet. J. 48, 643.

Love, D.N. and Sabine, M. (1975)
Electron microscopic observation of feline kidney cells infected with a feline calicivirus.
Arch. Virol. 48, 213-228.

Luria, S. and Delbruck, M. (1943)
Mutations of bacteria from virus sensitivity to virus resistance.
Genetics 28, 491-511.

Macpherson, I. and Stoker, M. (1962)
Polyoma transformation of hamster cell clones. An investigation of genetic factors affecting cell competence.
Virology 16, 147-151.

Mayor, H.D. and Jordan, L.E. (1962)
Formation of poliovirus in monkey kidney tissue culture cells.
Virology 16, 325-333.

McClain, M.E. and Hackett, A.J. (1959)
Biological characteristics of two plaque variants of vesicular exanthema of swine virus: Type E54.
Virology 9, 577-597.

McClain, M.E., Hackett, A.J. and Madin, S.H. (1958)
Plaque morphology and pathogenicity of vesicular exanthema virus.
Science 127, 1391-1392.

Melnick, J.L. and other members of the International Enterovirus Study Group (1963)
Picornavirus group.
Virology 19, 114-116.

Peterson, J.E. and Studdert, M.J. (1970)
Feline picornaviruses. Structure of the virus and electron microscopic observations on infected cell cultures.
Arch. Ges. Virusforsch. 32, 249-260.

Piercy, S.E. and Prydie, J. (1963)
Feline influenza.
Vet. Rec. 75, 86-89.

Povey, R.C. (1969)
Viral respiratory disease.
Vet. Rec. 84, 335-338.

Povey, R.C. (1970)
Studies on viral induced respiratory disease of cats.
Thesis. University of Bristol.

Povey, R.C. (1974)
Serological relationships among feline caliciviruses.
Infect. and Immun. 10, 1307-1314.

Povey, R.C. (1977)
Feline respiratory disease - Which vaccine?
Feline Practice 7, 12-16.

Povey, R.C. and Hale, C.J. (1974)
Experimental infections with feline caliciviruses (picornaviruses) in specific-pathogen-free kittens.
J. Comp. Path. 84, 245-256.

Povey, R.C. and Ingersol, J. (1975)
Cross protection among feline caliciviruses.
Infect. and Immun. 11, 877-885.

Povey, R.C. and Johnson, R.H. (1967)
Further observations on feline viral rhinotracheitis.
Vet. Rec. 81, 686-689.

Povey, R.C. and Johnson, R.H. (1971)
A survey of feline viral rhinotracheitis and feline picornavirus infection in Britain.
J. Small Anim. Pract. 12, 233-247.

Povey, R.C., Wardley, R.C. and Jessen, H. (1973)
Feline picornavirus infection: The in vivo carrier state.
Vet. Rec. 92, 224-229.

Prydie, J. (1966)
Viral diseases of cats.
Vet. Rec. 79, 729-738.

Prydie, J. (1973)
Characterisation of feline picornaviruses.
Thesis. University of Reading.

Reynolds, E.S. (1963)
The use of lead citrate at high pH as an electron-opaque stain in electron microscopy.
J. Cell. Biol. 17, 208-212.

Sabin, A.B. (1957)
Properties of attenuated polioviruses and their behaviour in human beings. In "Cellular Biology, Nucleic Acids and Viruses" volume 5, 115-140.
Academic Science, New York.

Sawyer, J.C. (1976)
Vesicular exanthema of swine and San Miguel sea lion virus.
J. Amer. Vet. Med. Assoc. 169, 707-709.

Scott, F.W., Kahn, D.E. and Gillespie, J.H. (1970)
Feline viruses: Isolation, characterisation and pathogenicity of a feline reovirus.
Amer. J. Vet. Res. 31, 11-20.

Sellers, R.F., Burt, L.M., Cumming, A. and Stewart, D.L. (1959)
The behaviour of strains of the virus of foot-and-mouth disease in pig, calf, ox and lamb kidney tissue culture.
Arch. Ges. Virusforsch. 9, 637-646.

Sinha, S.K. (1958)
Feline viruses.
Calif. Vet. 11, 18.

Smith, A.W. and Akers, T.G. (1976)
Vesicular exanthema of swine.
J. Amer. Vet. Med. Assoc. 169, 700-703.

Smith, A.W., Akers, T.G., Madin, S.H. and Vedros, N.A. (1973)
San Miguel sea lion virus isolation, preliminary characterisation and relationship to vesicular exanthema of swine virus.
Nature (London) 244, 108-110.

Smith, A.W., Prato, C.M. and Skilling, D.E. (1977)
Characterisation of two new serotypes of San Miguel sea lion virus.
Intervirology 8, 30-36.

Smith, A.W., Skilling, D.E. and Ritchie, A.E. (1978)
Immuno-electron microscopic comparisons of caliciviruses.
Amer. J. Vet. Res. 39, 1531-1533.

Soergel, M.E., Schaffer, F.L., Sawyer, J.C. and Prato, C.M. (1978)
Assay of antibodies to caliciviruses by radioimmune precipitation using staphylococcal protein A as IgG adsorbent.
Arch. Virol. 57, 271-282.

Strandberg, J.D. (1968)
Eight feline viruses: An electron microscopic study.
Thesis. Cornell University.

Studdert, M.J. and O'Shea, J.D. (1975)
Ultrastructural studies of the development of feline caliciviruses in a feline embryo cell line.
Arch. Virol. 48, 317-325.

Sutmoller, P., McVicar, J.W. and Cottral, G.E. (1968)
The epizootiological importance of foot-and-mouth disease carriers. 1. Experimentally produced foot-and-mouth disease carriers in susceptible and immune cattle.
Arch. Ges. Virusforsch. 23, 227-235.

Suto, T., Karzon, D.T., Bussell, R.H. and Barron, A.L. (1965)
Studies of mutants of ECHO 6 virus. 11. Isolation from human alimentary tract.
Amer. J. Epid. 81, 333-340.

Takemori, N. and Nomura, S. (1960)
Mutation of polioviruses with respect to size of plaque. 11. Reverse mutation of minute plaque mutant.
Virology 12, 171-184.

Takemoto, K.K. (1966)
Plaque mutants of animal viruses.
Progress in Medical Virology 8, 314-348.

Takemoto, K.K. and Kirschstein, R.L. (1964)
Dextran sulfate plaque variants of attenuated type 1 poliovirus. Relationship to in vitro and in vivo markers.
J. Immun. 92, 329-333.

Takemoto, K.K. and Liebhaber, H. (1961)
Virus-polysaccharide interactions. 1. An agar polysaccharide determining plaque morphology of EMC virus.
Virology 14, 456-462.

Tan, R.J.S. (1970)
Serological comparisons of feline respiratory viruses.
Jap. J. Med. Sci. Biol. 23, 419-424.

Tan, R.J.S. (1974)
The susceptibility of kittens to Mycoplasma felis infection.
Jap. J. Exptl. Med. 44, 235-240.

Tan, R.J.S. and Miles, J.A.R. (1973)
Incidence and significance of mycoplasmas in sick cats.
Res. Vet. Sci. 16, 27-34.

Totsuka, A., Ohtaki, K. and Tagaya, I. (1978)
Aggregation of enterovirus small plaque variants and polioviruses under low ionic strength conditions.
J. Gen. Virol. 38, 519-533.

Traum, J. (1936)
Vesicular exanthema of swine.
J. Amer. Vet. Med. Assoc. 88, 316-327.

Vogt, M., Dulbecco, R. and Wenner, H.A. (1957)
Mutants of poliomyelitis viruses with reduced efficiency of plating in acid medium and reduced neuropathogenicity.
Virology 4, 141-155.

Walen, K.H. (1963)
Demonstration of inapparent heterogeneity in a population of an animal virus by single-burst analyses.
Virology 20, 230-234.

Wallis, C. and Melnick, J.L. (1968)
Mechanism of enhancement of virus plaques by cationic polymers.
J. Virol. 2, 267-274.

Wallis, C., Melnick, J.L. and Bianchi, M. (1962)
Factors influencing enterovirus and reovirus growth and plaque formation.
Texas Reports on Biology and Medicine 20, 693-702.

Walton, T.E. and Gillespie, J.H. (1970a)
Feline viruses V1. Survey of the incidence of feline pathogenic agents in normal and clinically ill cats.
Cornell Vet, 60, 215-232.

Walton, T.E. and Gillespie, J.H. (1970b)
Feline viruses V11. Immunity to the feline herpesvirus in kittens inoculated experimentally by the aerosol method.
Cornell Vet. 60, 232-239.

Wardley, R.C. (1976)
Feline calicivirus carrier state. A study of the host/virus relationship.
Arch. Virol. 52, 243-249.

Wardley, R.C., Gaskell, R.M. and Povey, R.C. (1974)
Feline respiratory viruses, their prevalence in clinically healthy cats.
J. Small Anim. Pract. 15, 579-586.

Wardley, R.C. and Povey, R.C. (1977a)
The pathology and sites of persistence associated with three different strains of feline calicivirus.
Res. Vet. Sci. 23, 15-19.

Wardley, R.C. and Povey, R.C. (1977b)
Aerosol transmission of feline caliciviruses. An assesment of its epidemiological importance.
Brit. Vet. J. 133, 504-508.

Watson, M.L. (1958)
Staining of tissue sections for electron microscopy with heavy metals.
J. Biophys. Biochem. Cytol. 4, 475.

Wigand, R. and Sabin, A.B. (1962)
Properties of epidemic strains of ECHO type 9 virus and observations on the nature of human infection.
Arch. Ges. Virusforsch. 11, 683-707.

Zee, Y.C., Hackett, A.J. and Madin, S.H. (1968)
Electron microscopic studies on vesicular exanthema of swine virus: Intracytoplasmic virus crystal formation in cultured pig kidney cells.
Amer. J. Vet. Res. 29, 1025-1032.

Zee, Y.C., Hackett, A.J. and Talens, L.T. (1968)
Electron microscopic studies on the vesicular exanthema of swine virus. 11. Morphogenesis of VESV type H54 in pig kidney cells.
Virology 34, 596-607.

Zwillenberg, L.O. and Bürki, F. (1966)
On the capsid structure of some small feline and bovine RNA viruses.
Arch. Ges. Virusforsch. 19, 373-384.