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Differential *in vitro* inhibition of feline enteric coronavirus and feline infectious peritonitis virus by actinomycin D

Evelyn L. Lewis,[1] D. A. Harbour,[1]* J. E. Beringer[2] and J. Grinsted[3]

[1] *Department of Veterinary Medicine, Langford House, Bristol BS18 7DU,* [2] *Department of Botany and Zoology, Woodland Road, Bristol BS8 1UG and* [3] *Department of Pathology and Microbiology, Medical School, University of Bristol, University Walk, Bristol BS8 1TD, U.K.*

The growth of feline enteric coronavirus strain 79-1683 in whole feline embryo cells was inhibited by the presence of 1 μg/ml of actinomycin D in the culture fluid. No virus-specific mRNAs could be detected in such cultures and yields of infectious virus were depressed by >99%. By contrast, the antigenically related feline infectious peritonitis virus strain 79-1146 was unaffected by the presence of actinomycin D, indicating a fundamental difference between the two feline coronavirus strains in their requirements for host-encoded function(s).

Feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) are two coronaviruses which, although antigenically similar (Sanchez *et al.*, 1990), show markedly different tissue tropisms and virulence. FECV causes a mild or inapparent enteritis in kittens whereas FIPV induces a fatal, immune-mediated peritonitis (Pedersen *et al.*, 1981; Lutz *et al.*, 1986; Evermann *et al.*, 1991). The critical event in the pathogenesis of FIPV-mediated disease appears to be the ability of the virus to infect macrophages productively (Stoddart & Scott, 1989). FECV is unable to do so efficiently and remains localized in the epithelial cells of the gut (Pedersen *et al.*, 1981). The mechanism underlying this difference in pathogenicity is not known. Currently we are sequencing the genome of FECV strain 79-1683 for comparison with that of FIPV strain 79-1146.

For the purpose of preparing viral RNA template during cDNA cloning, FECV was grown in whole feline embryo (WFE) cells (Harbour *et al.*, 1991) in the presence of 1 μg/ml of actinomycin D. This concentration of actinomycin D has been reported to have a minimal effect on the replication of other coronaviruses [FIPV, de Groot *et al.* (1987); bovine coronavirus, Keck *et al.* (1988); rat coronavirus, Parker *et al.* (1970); mouse hepatitis virus (MHV), Keck *et al.* (1989), Spaan *et al.* (1981) and Malluci (1965); infectious bronchitis virus (IBV), Lomniczi & Kennedy (1977); human coronavirus (HCV) 229E, Schreiber *et al.* (1989)]. However FECV appeared to be strongly inhibited since no viral intracellular RNAs were detected. We therefore studied this phenomenon in more detail. Monolayers of WFE cells grown in 25 cm^2 flasks were inoculated with 10 p.f.u./cell of either FECV 79-1683 or FIPV 79-1146. After 1 h incubation at 37 °C, growth medium with or without 1 μg/ml actinomycin D was added and the cultures were reincubated at 37 °C. Mock-infected WFE cells were processed simultaneously. After 6 h, total cellular RNA was prepared by extraction with guanidinium isothiocyanate, and pelleted through a caesium chloride step gradient (Maniatis *et al.*, 1982). Viral intracellular RNAs were visualized by Northern blot hybridizations using a cDNA probe representing the 3′ end of the FIPV genome. RNA preparations were electrophoresed in formaldehyde–formamide 1% agarose gels at 70 V for approximately 4 h, transferred to nitrocellulose filters (Meinkoth & Wahl, 1984) and cross-hybridized to the ^{32}P-radiolabelled randomly primed FIPV *Pst*I fragments of plasmid pB12 (de Groot *et al.*, 1988) as described by de Groot *et al.* (1987). Six different virus-specific RNA species were detected in cells infected with either FIPV or FECV in the absence of actinomycin D (Fig. 1). The FECV hybridization pattern shows the 3′-coterminal nested set arrangement typical of coronaviruses (Siddell *et al.*, 1982). Using rRNA and denatured λ and pGEM DNA markers, the lengths of FECV RNAs were calculated to be comparable to those of FIPV (de Groot *et al.*, 1987) with the exception of RNAs 3 and 6, which are both smaller. These differences have now been confirmed by sequence data (E. L. Lewis, unpublished observations; Vennema, 1991). However, in the presence of actinomycin D, no viral intracellular RNAs were seen in FECV-infected cells, whereas in FIPV-infected cultures viral intracellular RNA synthesis

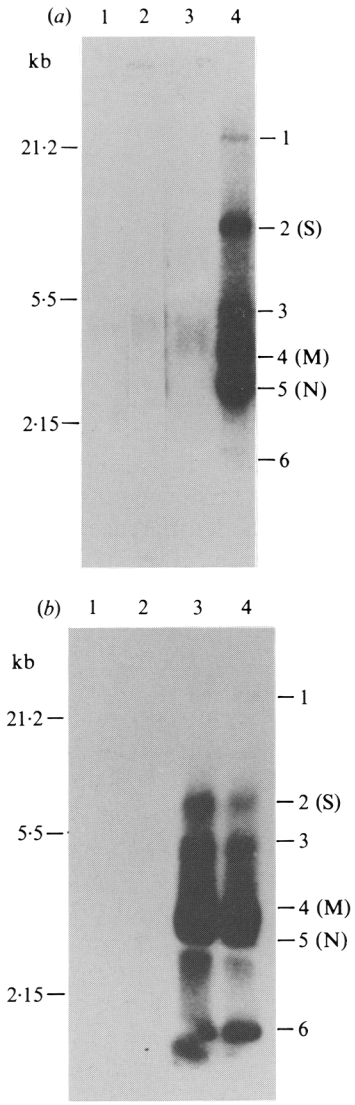

Fig. 1. Northern blot hybridizations of FIPV probe, pB12 (representing the 3′ end of the genome), to electrophoretically separated intracellular RNAs from FECV-, FIPV- or mock-infected WFE cells with (Act D$^+$) or without (Act D$^-$) actinomycin D (1 μg/ml). Ribosomal RNA size markers in kb are indicated on the left of each filter. Feline coronavirus RNAs are labelled according to the recommended nomenclature (Cavanagh *et al.*, 1990) on the right-hand side of each filter. (*a*) Lanes 1 and 2, mock-infected Act D$^+$ and Act D$^-$ WFE cells, respectively. Lane 3, FECV 79-1683-infected and Act D$^+$ WFE cells. Lane 4, FECV 79-1683-infected and Act D$^-$ WFE cells. (*b*) Lanes 1 and 2, mock-infected ActD$^+$ and ActD$^-$ WFE cells, respectively. Lane 3, FIPV 79-1146-infected and Act D$^+$ WFE cells. Lane 4, FIPV 79-1146-infected and ActD$^-$ WFE cells.

was comparable to that in cells without the drug (Fig. 1).

Further monolayers of WFE cells or feline embryo lung (FEL) fibroblasts (O'Reilly *et al.*, 1979) were infected with FECV 79-1683 or FIPV 79-1146 at an m.o.i. of 5 p.f.u./cell. After 1 h for adsorption at 37 °C,

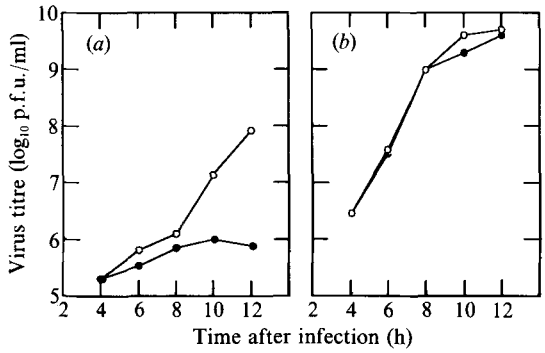

Fig. 2. Feline coronavirus yields from confluent monolayers of WFE cells with (●) or without (○) 1 μg/ml actinomycin D. (*a*) WFE cells (passage 25) infected with FECV 79-1683 (passage 10). (*b*) WFE cells (passage 28) infected with FIPV 79-1146 (passage 9).

growth medium [Eagle's MEM supplemented with 10% foetal calf serum (FCS)] with or without 1 μg/ml actinomycin D was added. Virus was harvested at intervals by subjecting replicate cultures to three cycles of freezing and thawing. Cell debris was pelleted by centrifugation at 2000 ***g*** for 10 min and the supernatant fluid was titrated for infectious virus by plaque assay as follows. Confluent monolayers of WFE cells grown in 60 mm tissue culture dishes were inoculated in quadruplicate with 0·1 ml volumes of virus suspensions serially diluted in serum-free Eagle's MEM. After 1 h adsorption at 37 °C 1 ml of overlay medium (Eagle's MEM with 2% FCS and 0·75% w/v methyl cellulose) was added and the cultures were incubated for 24 h. Neutral red in maintenance medium (0·004%) was then added (0·5 ml), and the cultures were incubated at 37 °C for a further 24 h, fixed with 10% formol saline and the plaques counted. The yield of FECV in WFE cells was similar in cultures with or without actinomycin D up to 8 h post-infection (p.i.). Thereafter virus yield increased a further 100-fold by 12 h p.i. in the absence of actinomycin D; the yield of virus in cultures with actinomycin D did not increase (Fig. 2*a*). A different growth curve was observed in WFE cultures infected with FIPV (Fig. 2*b*) where titres rose more rapidly and to higher levels than seen with FECV. No differences were seen between cultures with or without actinomycin D. Similar results were seen in FEL cells (data not shown). The inhibitory effect of actinomycin D on FECV replication was more pronounced after 8 h of antibiotic treatment, a time by which the FIPV replication cycle was more or less complete (Fig. 2). To determine whether replication of FIPV was affected by longer exposure of the host cells to the antibiotic, confluent monolayers of WFE cells were treated with 1 μg/ml actinomycin D for 6 or 8 h before infection with FIPV 79-1146 at an m.o.i of 5 p.f.u./cell. After adsorption of virus, fresh medium containing the

Table 1. *Yields of FIPV 79-1146 in cells treated with actinomycin D before infection*

Time (h) cells pretreated with actinomycin D before infection	Yield of FIPV at 12 h p.i (log_{10} p.f.u./ml)
6	8·78
8	8·43
None added	9·32

Table 2. *Effect of time of actinomycin D addition on yields of FECV 79-1683*

Time of actinomycin D addition (h p.i.)	Yield of FECV at 12 h p.i. (log_{10} p.f.u./ml)
1	5·60
2	5·54
3	5·48
4	5·65
6	6·18
8	8·04
None added	8·49

antibiotic was added, and the yield of virus was titrated at 12 h p.i. The yield of FIPV in cells pretreated with actinomycin D for 8 h before infection was reduced by eightfold; 6 h pretreatment resulted in a 3·5-fold decrease compared to untreated cells (Table 1).

To investigate the time at which actinomycin D was acting to limit FECV replication, six pairs of confluent monolayers of WFE cells were infected with FECV 79-1683 at an m.o.i. of 5 p.f.u./cell. After 1 h adsorption at 37 °C, growth medium was added. Actinomycin D, to a final concentration of 1 μg/ml, was added to one of each pair of flasks 1, 2, 3, 4, 6 or 8 h p.i. The other flask of each pair received no drug and served as a control. At 12 h p.i. all cultures were harvested as before and the cell lysate was titrated (Table 2). Yield of FECV was markedly inhibited (>99%) when actinomycin D was added up to 6 h p.i. When added at 8 h p.i., yield was 36% of control levels.

It has been accepted generally that coronaviruses do not require any host cell functions for their replication (Siddell *et al.*, 1982). However replication of IBV in BHK-21 cells was blocked when these cells were enucleated or irradiated with u.v. light prior to infection (Evans & Simpson, 1980). Moreover inhibition by actinomycin D of the growth of some coronaviruses (determined by plaque assay) and coronavirus RNA synthesis have been reported [MHV, Dupuy & Lamontagne (1987); HCV 229E, Kennedy & Johnson-Lussenberg (1979); IBV, Lomniczi (1975); transmissible gastroenteritis virus, Clarke (1968) and Mishra & Ryan (1973)].

Using PAGE, Tupper *et al.* (1987) compared the polypeptide profiles of [^{35}S]methionine-labelled FIPV- and FECV-infected Crandell feline kidney (CrFK) cells treated with 5 μg/ml actinomycin D. The structural proteins, nucleocapsid (N), membrane (M) and spike (S) appeared simultaneously at 6 h p.i. (FECV in much lower abundance) and continued to be synthesized throughout the 14 h study period. Yields of infectious virus were only about threefold lower in the presence of actinomycin D than in its absence. This contrasts markedly with our results where yields in the presence of 1 μg/ml actinomycin D were inhibited by >99%. This may reflect a difference in the host cells, perhaps with a critical function being expressed constitutively in the transformed CrFK cells (which also express an endogenous retrovirus) but only following induction in the non-transformed WFE cells. A difference in the sensitivity of coronavirus replication in different cell types has been demonstrated by Lomniczi (1975) who reported that 0·02 μg/ml actinomycin D reduced yields of IBV by 97% compared to controls in chicken lung cells, but a 10-fold greater concentration of the drug was required to produce the same level of inhibition in kidney cells.

The importance of cellular functions in MHV replication has been demonstrated by Dupuy & Lamontagne (1987) who studied the cellular mechanism involved in the host restriction of MHV (Taguchi *et al.*, 1981). Macrophages or thymocytes obtained from resistant A/J or from susceptible C57BL/6 mice were treated with 5 μg/ml actinomycin D. This treatment did not modify the restriction observed in cells from the resistant A/J mice, but prevented the usual replication seen in cells from susceptible C57BL/6 mice as early as 24 h p.i. They postulated that the latter effect may be due to loss of cell control of viral replication resulting in viral protein accumulation without efficient assembly, and subsequent precocious cell lysis as observed in MHV-3-infected YAC lymphoid cells.

The critical cellular function inhibited by actinomycin D in FECV-infected WFE cells appears to operate early in the viral growth cycle since addition of the drug prevents viral RNA synthesis. However addition of the drug up to 6 h p.i. almost completely inhibits the production of infectious virus particles, suggesting that either the function is also required later in the cycle (but before virus maturation, because addition at 8 h p.i. only had a minor effect on virus yields), or more than one function is required. Similarly, Kennedy & Johnson-Lussenberg (1979) reported that yields of HCV 229E grown in L132 cells were most inhibited when actinomycin D was present throughout the growth cycle, and maximal up to 8 to 10 h p.i., after which the effect of the drug rapidly diminished. In our experiments, yield of FIPV was decreased, by less than 10-fold, only when the

cells were treated for 6 to 8 h before infection. It is likely that this decrease is a result of non-specific toxic effects on the cell produced by prolonged exposure to actinomycin D rather than inhibition of a specific cellular function required by the virus.

In conclusion, our results show that FIPV and FECV have a fundamental difference in their requirements for host-encoded functions.

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