EVOLUTION OF A CORONAVIRUS DURING PERSISTENT INFECTION IN VITRO

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INTRODUCTION

Coronaviruses usually infect epithelial cells of the gastro-enteric or respiratory tracts. They frequently cause local infections which may be fatal in young animals, but which are usually mild or asymptomatic in adults. In vitro, coronaviruses may induce virulent, cytolytic and cell fusing effects in some host cells but cause a moderate, persistent infection in other cell types. Thus the host or the host cell appears to be of critical importance in determining the outcome of coronavirus infection. This report will focus on the role of the host cell in coronavirus replication and on the mutually dependent changes induced in the host cell and the virus during the establishment of a persistent infection in vitro.

Strong selective pressures may act on coronavirus populations in vitro and in vivo. The local immune response of the host probably selects for strains of virus with altered viral attachment proteins. Indeed, a large number of strains of each of the animal coronaviruses has been isolated. This observation suggests that a variety of mutants of at least one of the viral glycoproteins (probably the peplomeric glycoprotein E2) can be functional.

The host cell appears to be of critical importance for the replication of coronaviruses. Many coronaviruses show stringent species and tissue specificity during primary isolation. During adaptation to tissue culture selection of viruses with altered antigenicity or virulence often occurs. Such selection may operate at several different stages in the intracellular replication of the virus. Three of the many examples of the dependence of coronaviruses on host cell functions will be mentioned here. Sturman and Takemoto

(1972) showed that the yield of infectious mouse hepatitis virus (MHV) was much greater from transformed BALB/c 3T3 cells than from non-transformed cells. Lucas et al (1978) demonstrated that the replication of MHV3 is temperature sensitive in neuronal cells but not in nonneuronal cells. Evans and Simpson (1980) recently observed that a host transcriptional function may be required for replication of infectious bronchitis virus (IBV) in BHK21 cells. Very little is known about the cellular functions which are responsible for such host dependent differences in coronavirus replication.

To analyze the interactions between a coronavirus and its host cell, we have studied the development of persistent coronavirus infection in vitro using the well-characterized A59 strain of MHV in the normally permissive 17 clone 1 (17C1 1) line of spontaneously transformed BALB/c 3T3 cells. A59 virus replicates efficiently in these cells and it produces large plaques in 17C1 1 cells at both 32°C and 39.5°C. Yields of 10^8 to 10^9 PFU/ml of released infectious virus are obtained within 24 hours, yet a persistent infection can readily be established in this host cell system.

In this report, we will describe the normal maturation of coronaviruses and discuss the establishment and characterization of a persistent infection in vitro with MHV. We will analyze the evolution of the virus during the establishment of the persistent infection and suggest one possible mechanism for coronavirus persistence.

MATURATION AND RELEASE OF CORONAVIRUSES

To study the sequence of events in coronavirus maturation, 17 C1 1 cells infected with A59 were examined by TEM at intervals after virus inoculation. The events shown here for the acute infection were later found to be similar in the cells producing virus in persistently infected cultures. After the latent period virions were seen within the rough endoplasmic reticulum (RER) and the Golgi apparatus (Fig. 1). Virions in the RER were spherical and had electron lucent centers. Helical nucleocapsids could be observed within these "immature" virus forms, and shift to a lower temperature could apparently block virus release from the RER (Fig. 2). As the acute infection at 37°C progressed the virions moved through the Golgi into smooth walled vesicles (SWV). During this process virions became flattened, disc shaped and electron dense. These SWV containing "mature" virus particles migrated to the plasma membrane (PM) and fused with it to release virions into the medium. Released virions frequently reabsorbed to the PM, but virions were never observed to bud from the PM. Late in the infectious cycle, large inclusions of viral nucleocapsid were observed in the cytoplasmic matrix.

A schematic drawing of the sequence of events which we believe occurs during coronavirus maturation is shown in Figure 3. Viral

Fig. 1. Maturation of coronaviruses. Virions in the RER and Golgi apparatus 9 hrs. after virus inoculation. Virions budding into the RER are shown in b and c.

glycoproteins as well as virions migrate from RER through the Golgi to the PM where they can be detected by immunoelectronmicroscopy (data not shown). Several host cell functions are required for assembly and release of coronaviruses. We have suggested at this meeting that the glycosylation of the E1 and E2 glycoproteins may utilize two different cellular pathways. Also Doller and Holmes

(1980) showed that these two viral glycoproteins migrate differently within the infected cell, presumably using different host cell glycoprotein transport systems. To be released from the infected cell, coronaviruses apparently utilize the cellular secretory apparatus. Thus cellular mechanisms for glycosylation, glycoprotein transport and secretion all appear to be required for efficient release of coronaviruses.

ESTABLISHMENT OF PERSISTENTLY INFECTED CULTURES

A persistent infection was established by inoculation of 17 Cl 1 cells with plaque purified MHV at a multiplicity of 3 PFU/cell. Cultures were held at 37°C. Cytopathology (CPE) was extensive during the first 24 hours, but approximately 5% of the cell survived

Fig. 2. Effect of temperature shift on virus release. A cell infected with A59 was grown at 37°C for 8 hr then shifted to 32°C for 16 hr. Numerous spherical virions accumulated in the RER and few smooth walled vesicles (arrows) full of mature virions were observed. Inset shows helical nucleocapsids in virions (arrows).

Fig. 3. Sequence of events in coronavirus maturation. The following symbols have been used: N, nucleus; R, rough endoplasmic reticulum; G, Golgi apparatus; SWV, smooth walled vesicles; PM, plasma membrane. Helical nucleocapsid can be seen within spherical "immature" virions in the RER, whereas "mature" virions in SWV and on PM are flattened and electron opaque.

and grew out to form a confluent monolayer. Cultures were passaged twice a week. The supernatant fluid containing released virus was harvested at every passage for plaque assay and a portion of the cells were stored in liquid nitrogen at frequent intervals. Viral structural proteins in the cells were detected by labelling with fluorescent antibody. Cell fusion occurred on the day following trypsinization for about the first six passages, but little or no CPE was observed in later passages. Persistently infected (PI) cultures exhibited a normal growth pattern except for a crisis period at approximately the fourteenth passage when they grew very slowly for several weeks. After recovery, the characteristics of the PI cultures have not changed. The cultures have been maintained for more than 100 passages. The PI cultures showed no CPE (Fig. 4). All of the PI cells were resistant to superinfection with the original wild type (WT) large plaque A59. WT A59 did not grow or induce cytopathic effects in these cultures (Fig. 4), and did not produce plaques on PI cultures. However, unrelated viruses such as VSV or Semliki Forest virus formed plaques on PI cultures as well as on uninfected cells. At every passage 10 to 20% of the cells were positive for MHV viral antigens. TEM showed normal development of virions in 10 to 20% of the cells and no evidence of virus replication in the remaining cells.

CHARACTERIZATION OF VIRUS FROM PI CULTURES

The PI cultures continually shed virus throughout these experiments. In the first two passages the virus produced was predominantly large plaque virus like the WT A59. By the 7th passage however, a wide variety of plaque morphology virus mutants had developed (Fig. 5). Plaque phenotypes including large, medium and small, turbid or clear, ragged or smooth were observed. The proportion of small plaque virus in the population increased irregularly (Fig. 6) until, by the 22nd passage only small plaque virus was detected.

Initially we believe that all of the small plaque viruses from the 22nd passage were temperature sensitive (ts), since no plaques were observed at 39.5°C under conditions in which WT A59 plaqued normally. However, we discovered that supplementation of the medium with additional fetal calf serum permitted detection at 39.5°C of small plaques caused by non-ts mutants which developed more slowly than WT A59. Thus the conditions necessary for plaquing small plaque non-ts mutant virus at 39.5°C were different from those used for WT A59. The proportion of small plaque viruses which was temperature sensitive varied during passage of the chronically infected cells (Fig. 7). No regular pattern was evident (Fig. 8) and PI cultures always contained both ts and non-ts small plaque virus.

Several ts mutants have been plaque purified and characterized.

Fig. 4. Effects of A59 infection on normal and persistently infected cells. Normal 17 clone 1 cells (a) showed cell fusion and rounding 24 hr. after infection (b). Persistently infected cultures (c) were indistinguishable from normal cells and showed no CPE 24 hr. after superinfection with A59(d).

Fig. 5. Development of plaque morphology mutants during persistent infection. Large plaques from WT A59 are shown in A and a variety of plaque morphology mutants from the 7th passage of PI cultures are shown in B.

Fig. 6. Selection of small plaque mutants during persistent infection.

One mutant from the 22nd passage called 22B will not replicate at 39.5°C. Growth curves of WT A59 and 22B are shown in Fig. 9. This mutant appears to be a multiple sten mutant. At the non-permissive temperature, synthesis of viral specific RNA by 22B was not detectable (Fig. 10; Behnke and Holmes, 1978). Thus 22B appears to have an early defect in viral RNA synthesis. In addition, 22B virions are less stable than WT A59 and SDS PAGE shows altered migration of the E2 glycoprotein.

MECHANISMS OF CORONAVIRUS PERSISTENCE

To study the interactions between ts and non-ts viruses, mixed infections of WT A59 and 22B were done at permissive and non-per-

Fig. 7. Development of temperature sensitive virus during persistent infection. % ts virus was calculated using the titer at 32°C as total virus and the titer at 39.5°C as non-ts virus.

Fig. 8. Yields of released virus from persistently infected cultures. Virus from passages of PI cultures was assayed at 32°C and 39.5°C .

missive temperatures (Table 1). Both viruses replicated after co-infection at 32°C , but at the non-permissive temperature the mutant virus could not replicate and interfered with the replication of the wild type virus. This conditional interference with the replication of wild type virus may be an important reason for modulation of viral virulence in persistently infected cell cultures. Studies are continuing on the mechanism of inhibition of virus replication at the non-permissive temperature.

Fig. 9. Growth of WT A59 and the ts mutant 22B at different temperatures. The yield of virus in the supernatant fluid was assayed at 32°. □ yield from cultures treated with 5 µg/ml Actinomycin D; ■ yield without Actinomycin D.

Three mechanisms have been demonstrated for the establishment of persistent infections in a variety of virus-cell systems (Friedman and Ramseur, 1979). The development of ts mutants and their interference with the replication of wild type virus has been shown in rhabdoviruses, togaviruses, and paramyxoviruses. Development of defective interfering particles which interfere with the replication of wild type virus is another factor in persistent infections. Generation of DI virus particles of VSV has been shown to depend upon the host cell (Kang and Allen, 1978). Generation of ts mutants may similarly depend upon a host cell mechanism. The third mechanism for the maintenance of persistent infection *in vitro* is production of interferon (IF). From our persistently infected cell cultures little IF could be detected and VSV could plaque efficiently on the PI cultures. This suggests that IF may not be an important factor in the persistence of A59 virus in these cultures. However,

Fig. 10. Synthesis of viral RNA by WT A59 and mutant 22B at different temperatures. ● Incorporation of ^3H uridine into acid insoluble label 4 hr. after the addition of $5\text{ }\mu\text{g/ml}$ Actinomycin D. Cells were labeled for 1 hr. at the times indicated. ▲ Incorporation of ^3H uridine into similarly treated uninfected control cells.

Table 1

<u>Inoculum</u>	<u>24 Hour Virus Yield ($\times 10^4$ PFU/ml)*</u>		
	<u>32⁰</u>	<u>37⁰</u>	<u>39.5⁰</u>
WT A59	8L	105L	73L
ts mutant 22B	56s	2s	0s
Mixed infection (WT A59 - 22B)	6L 43s	1L 1s	1L 0s

*All virus was titered at 32⁰
 L represents large plaque virus
 s represents small plaque virus

Table 1. Conditional interference with the replication of wild type A59 by a small plaque, ts mutant derived from a persistently infected culture.

if A59 were sensitive to much lower levels of IF than VSV, IF could still be an important factor in this system. Therefore we cultured PI cells in the presence of anti-IF antibodies and looked for changes in the yield of infectious virus and development of CPE in PI cultures (Sekellick and Marcus, 1979). The persistent infection was not cured by anti-IF antibody and only a modest increase in the yield of infectious virus was detected in anti-IF antibody treated cultures in comparison with those treated with normal rabbit serum. Even passage of the PI culture in the presence of anti-IF antibody did not abrogate the persistent infection nor lead to the development of CPE. Of the three mechanisms for persistent infection shown to operate in other persistent virus infections, we have found only one which may be involved here. That is the development of ts mutants which cause conditional interference with the replication of non-ts virus.

To summarize these experiments, the cells in the PI culture were selected for resistance to viral CPE and became selective for small plaque virus and for ts mutants that could interfere with the growth of non-ts virus. This development of virus resistant cells and coronavirus mutants is like the process of evolution in vitro. Similar observations have been made on persistent infections with VSV (Holland et al., 1979). Some unknown factor(s) in the host cell limits replication of wild type virulent virus. This then becomes a selective system for virus variants. Such variants may be preexisting spontaneous mutants or may be the result of host cell action on viral replication. Simultaneously in this process host cells resistant to viral CPE have been selected by the cytolytic effects of virus replication. The virus-cell system has been stabilized to give no apparent CPE and to yield only one plaque morphology of virus. However detailed study has shown that

this apparently simple in vitro system is very complex.

Similar mechanisms may operate during persistent infections of cells in the respiratory or enteric epithelium in vivo, and may in part be responsible for some of the problems associated with the isolation of coronaviruses from persistently infected animals. Additional factors in persistent coronavirus infections in vivo are the effects of the host's immune response. Coronaviruses, unlike viruses which bud from the plasma membrane, mature intracellularly before viral antigens are present on the surface of the infected cell. Thus the immune system may not be able to identify and destroy infected cells prior to the maturation of infectious virus. This intracellular maturation, the ability of coronaviruses to replicate in macrophages and the superficial location of virus-infected cells may be additional important mechanisms for coronavirus persistence in vivo.

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