CHARACTERISTICS OF A LONG TERM IN VITRO PERSISTENT INFECTION
WITH HUMAN CORONAVIRUS 229E

G. Chaloner-Larsson and C.M. Johnson-Lussenburg

Department of Microbiology and Immunology
School of Medicine, University of Ottawa
Ottawa, Ontario, K1N 9A9, CANADA

INTRODUCTION

In October of 1978, we were successful in establishing an in vitro persistent infection of a human cell line (L132) with human coronavirus, strain 229E (229E). These persistently infected cells have been shedding virus since that time (over 300 cell passages) to the same high titer of 10^5 - 10^6 pfu/ml every 48 hours. We have been concerned with studies on both the virus isolated from the cultures (termed VH) and the persistently infected cell cultures per se (termed L132/229E or simply HV). Our preliminary findings have been described at previous meetings and a report has been submitted for publication. A brief summary of these results will be given here to provide the necessary background to our present work.

The persistent VH coronavirus was found to be indistinguishable from our stock 229E strain on the basis of morphology and neutralization by specific antiserum. The L132/229E cultures were resistant to superinfection by 229E virus but supported replication of poliovirus (Sabin). So far, of the treatments tried (short term incubation at 41°C or 33°C, six week incubation at 39°C, overgrowth at room temperature or 37°C and cloning), none have been successful in curing the cells of the persistent virus. Our experimental data have eliminated the involvement of interferon or reverse transcriptase in persistence, and since we could show no interference of replication in mixed infections, we have concluded that defective interfering particles are unlikely to play a role in the maintenance of the persistent state. Evidence has been accumulating which suggests that temperature sensitive (or temperature dependent) step(s) either in host cell metabolism or in virus replication must be involved in this persistent system.

Fig.1. Typical negatively stained virions as found in purified preparations of 229E (A) and supernatant fluids from VH persistently infected cultures (B). Bars represent 100 nm.

In this communication, we will report our recent results which provide further information on the characteristics of both the coronaviruses isolated from the persistent cultures (VH viruses) and the persistently infected cell culture systems (HV cells).

PROPERTIES OF CORONAVIRUS ISOLATED FROM PERSISTENTLY INFECTED CELLS

In several independent attempts, the only successful persistently infected cultures have arisen at 37°C (vide infra) and of these, two, VH1 and VH4 have been studied in detail. The morphology of these viruses was compared with 229E by examining negatively stained preparations of each in the electron microscope. No difference could be seen between the persistently shed virions and the 229E virions as is shown in Figs. 1A and 1B.

Effect of Temperature on Infectivity of VH Viruses

Throughout these studies, temperature has played an unknown but key role in establishing and maintaining the persistently infected cell cultures. To determine whether there was any change in the persistent progeny virus which could be correlated with temperature, the effect of temperature on the stability of the VH and 229E viruses was examined. Suspensions of VH1, VH4 and 229E were incubated at 37°C or 45°C and samples taken at regular intervals were assayed for plaque forming ability on L132 cells. As can be seen in Fig. 2A, there is an almost immediate loss of infectivity of each virus within the first hour at 45°C, while inactivation proceeds more slowly at 37°C being almost complete in 8 hours. There is however, no essential difference in the effect of either temperature on all three viruses.

Fig. 2. Effect of temperature on virus infectivity. Clarified infected cell extracts in medium M199 were incubated at 37°C or 45°C. At intervals aliquots were taken and frozen at -20°C. Samples were thawed and titrated by plaque assay⁵. Infectivity is expressed as log pfu/ml. --- 45°C; — 37°C.

Physical Characteristics of the VH Viruses

The density of the purified VH virions and size of the VH genome was determined. VH virus, labelled in situ with [5-³H]uridine or ¹⁴C-amino acids, and VH virus isolated and similarly labelled during an acute infection of L132 cells, were harvested, purified and compared with the standard 229E virus prepared in parallel. The density of the virus as determined by isopycnic sucrose gradient analysis was the same for 229E and VH virus (1.18-1.19 gm/cc; Figs. 3C and 3D). These results also revealed no differences in the VH virus whether harvested directly from the persistent cultures or after propagation in L132 cells at 33°C, the same conditions optimal for growth of our standard virus (data not shown).

RNA isolated from the VH viruses and 229E by treatment with proteinase K/SDS¹ was examined in isokinetic sucrose gradients. As can be seen in Figs. 3A and 3B, the VH and 229E isolated RNA gave the same profile of a single large molecular weight species.

Studies on the Replication of VH Coronaviruses

While an incubation temperature of 37°C is optimal for cell growth and was required for the establishment of persistence, both the iso-

Fig. 3. Sucrose density gradient profiles of ^3H -uridine labelled virus and viral RNA. Isokinetic gradients of RNA extracted by proteinase K/SDS from 229E (A) and persistent VH virus (B) were spun at 43K g for 10 h through a 5-25% linear gradient containing SDS. Isopycnic gradients of purified 229E virus (C) and VH virus (D) were spun at 63K g for 18 h through a 25-65% linear gradient. Sedimentation in all cases was from right to left.

lated VH virus and the parental 229E replicate in L132 cells most efficiently at 33°C. And despite the lack of cytopathic effects of the VH viruses in the persistent cultures (at 37°C), they are apparently more cytotoxic than the parent 229E virus in L132 cells at 33°C. In fact, from the earliest titrations of the VH viruses, there has been a consistent difference in plaque development and morphology: namely that, when isolated, the VH viruses produce clearer, slightly larger plaques which develop earlier (Figs. 4A and 4B). To date, this has been the only visible difference identified which could be regarded as a marker characteristic.

In further studies the increased cytotoxic property was correlated to the earlier destruction of the L132 cell monolayer seen after infection with VH viruses. A comparison of the growth curves of the VH and

Fig. 4. Plaque morphology of 229E (A) and VH persistent (B) viruses. Dilutions of virus were inoculated onto L132 monolayers and plaques allowed to develop under agar for 6 days at 33°C. Plaques were visualized after fixation with formalin by staining with crystal violet. VH plaques developed earlier and were clearer than 229E virus plaques.

229E viruses revealed that the persistent VH virus did indeed replicate faster and to higher yields than did the stock virus (Fig.5).

The stability of this plaque morphology characteristic was evaluated by examining the yield of a number of serial infections of L132 cells with either stock 229E or the VH viruses at different multiplicities of infection. The results of these experiments are shown in Table 1. All infected cultures were incubated in parallel at 33°C following the initial 1 hour adsorption period at room temperature. Total virus yield was determined by plaque assay of frozen-thawed whole cultures². The characteristics of the plaques produced by all the VH viruses regardless of the number of serial passages was indistinguishable from that originally described (Fig. 4A).

In the series A experiments, there was a 50% decrease in yield of 229E following three serial infections with the usual MOI used for 229E production. This is contrasted in series B by the gradual buildup in the yield of 229E during the six serial passages using at least tenfold lower multiplicities of infection. This result might be taken as an indication of the generation of defective interfering (DI) particles in series A by the stock 229E. However, in both series the VH1 isolate gave remarkably stable yields at both high and low MOI. In fact the yields were consistently one log higher throughout these experiments and appeared to correlate directly with the increase in MOI. The situation with VH4 was similar to VH1, especially at the higher MOI, but yields were somewhat lower in the B series. The reasons for this are unknown, however, in our experience growth characteristics of VH4 usually were found to lie somewhere between the VH1 and 229E results. It was concluded that, within the limits of these experiments, the plaque morphology of the VH viruses was a stable characteristic. Furthermore, the high yields of virus appeared to be stable.

Table 1. Yields of Serial Infection in L132 Cells

Virus	Series A			Series B		
	Pass.	MOI	Yield (pfu/mlx10 ⁷)	Pass.	MOI	Yield (pfu/mlx10 ⁷)
229E	1)	2.0	6.0	1)	0.2	1.29
	2)	2.0	4.0	2)	0.06	3.21
	3)	1.5	3.1	3)	0.16	2.73
				4)	0.13	3.96
				5)	0.2	4.05
				6)	0.2	5.07
VH1	1)	10	64.0	1)	0.2	5.9
	2)	20	68.0	2)	0.3	24.0
	3)	20	70.0	3)	1.2	24.0
				4)	1.2	24.0
				5)	1.2	21.0
				6)	1.0	24.0
VH4	1)	2	31.0	1)	0.2	0.06
	2)	10	30.0	2)	0.003	0.6
	3)	10	29.0	3)	0.03	3.0
				4)	0.15	4.02
				5)	0.2	3.96
				6)	0.2	8.76

We next examined the effect of temperature on the replication of the 229E and VH viruses. As stated earlier, though 37°C was optimal for cell growth and was required for the establishment of persistence both the isolated VH virus and 229E replicated most efficiently at 33°C. Virus replication at three temperatures, 33°C, 37°C and 39°C was evaluated on the basis of plaquing efficiency and virus yields at 40 hours post infection (h.p.i.). A preliminary, one-way temperature shift experiment was then performed to determine if the effect of temperature could be associated with an early event in replication. The results of these experiments are shown in Table 2. It is clear that virus replication is partially inhibited at 37°C and completely inhibited at 37°C and completely inhibited at 39°C for both 229E and the VH viruses.

The results of the temperature shift experiment demonstrate that residual virus inoculum is still detectable in relatively large quantities, a fact also noted in the growth curve experiment (Fig.5) where at 4 h.p.i., 10³-10⁴pfu/ml of residual infectious virus were detected.

Fig. 5. Growth curves of 229E (o) and VH1 (●) viruses. Virus at 3-5 moi was adsorbed in replicate onto L132 cells for 1 h at rm temp. The inoculum was replaced with M199 and cultures incubated at 33°C. At 4 h intervals, sample cultures were removed, subjected to 3 freeze-thaw cycles and stored at -20°C. Samples were titrated in duplicate by plaque assay. Titres are expressed as log pfu/ml.

After 24 hours incubation at 33°C, all three viruses showed two to three log increases in virus titres, the VH viruses having the greatest increase. Again, no virus was detectable after 24 hours incubation at 39°C which confirmed the observations of the previous experiments. When however, infected cells were shifted to 39°C after an initial 4 hour period of incubation at 33°C, there was a marked difference in the yields of the VH viruses compared to the standard 229E. In the latter case, no 229E was detected while in the former, small quantities of both VH viruses were found at concentrations about two logs lower than the residual virus inoculum found after the initial 4 hour, 33°C incubation.

Although these results do not show whether the VH viruses, when inside the cell were less susceptible to temperature inactivation or whether the VH viruses detected represented progeny virus synthesized at reduced rates, they do suggest once again, a temperature related difference between the standard and persistent viruses. Moreover, these results indicate the direction of further experiments necessary to the understanding of temperature involvement in this persistent system.

PROPERTIES OF THE PERSISTENTLY INFECTED CELLS

The establishment and maintenance of five parallel persistently infected cultures was achieved on the second attempt (Table 3). The cultures were numerically identified, two (HV1 and HV4) were chosen

Table 2. Effect of Temperature on Coronavirus Replication

Experiments	Virus	Incubation Temperature			
		33°	37°	39°	
I	229E	4.0x10 ⁷	1.2x10 ⁷	0	
Plaquing Efficiency	VH1	2.1x10 ⁸	8.0x10 ⁷ (30%)	0	
	VH4	5.5x10 ⁷	2.7x10 ⁷ (38%)	0	
			(49%)		
II	229E	3.9x10 ⁷	3.7x10 ⁶	0	
Virus Yields 40 h.p.i.	VH1	2.1x10 ⁸	2.1x10 ⁷ (9.3%)	0	
	VH4	1.2x10 ⁸	1.2x10 ⁷ (10%)	0	
			(10%)		
III	Virus	33° 4 h	33° 24 h	39° 24 h	33°/4 h → 39°/20 h
Virus Yields	229E	1.6x10 ⁵	1.9x10 ⁷	0	0
Temperature	VH1	2.1x10 ⁵	1.47x10 ⁸	0	3.3x10 ³
Shift	VH4	7.8x10 ⁴	7.5x10 ⁷	0	3.0x10 ²

arbitrarily for further detailed study and the others were stored in liquid N₂. Since then, efforts have been made to repeat these experiments to help provide an understanding of the mechanisms involved in their establishment especially the temperature requirement. To date, five independent attempts have been made and the results are summarized in Table 3. In several trials, persistent VH virus was also used and it is of interest to note that with these, stable cultures were also successfully established but only on two occasions. On the basis of this experience, the only conclusions which we can draw are that while it was not difficult, we have not yet found a standard method by which one can predictably or readily establish a persistent infection of L132 cells with 229E except that a temperature of 37°C is a requirement.

Effect of Temperature on Virus Shed by Persistently Infected Cells

Further studies to explore the role of temperature in the persistent cell cultures were concerned with the effect of temperature on

Table 3. Attempts to Establish 229E Persistently Infected Cultures of L132 Cells

Trial	Number of Cultures	Virus	Duration	Result
1	5	229E	1 mo	Unstable
2	5	229E	24 mo	Stable
3	4	229E	2 mo	Unstable
4	2	VH1	1 wk	Acute
	2	VH4	1 wk	Acute
5	3	229E	1 mo	Unstable
	3	VH1	1 wk	Acute
	3	VH4	5 mo*	Stable
6	4	229E	1 mo	Unstable
7	3	229E	2 mo*	Stable
		VH1	2 mo*	Stable
		VH4	1 wk	Acute

* Stored in liquid N₂ for future study.

persistence. Routine maintenance of our two persistent culture (HV1 and HV4) consisted of a regular schedule of passaging at a split ratio of 1:3 every two days with incubation at 37°C. Virus shedding was monitored at intervals over the two day periods and the increase in titres of the persistent VH virus shed into the medium was found to correlate directly with the increase in number of cells in culture. However, when growth of the persistently infected cells was carried out at various temperatures different results were obtained. Stable cell growth and maximum virus shedding occurred at the optimal cell growth temperature of 37°C. But at 33°C cell growth deteriorated, virus shedding decreased and the cultures eventually died out even if returned to 37°C. At 39°C cell growth was aberrant (similar to uninfected cells) and splitting was continued only when the cells reached confluency (4-7 days). Virus shedding was erratic but both cell growth and virus shedding resumed at normal levels when the cultures were returned to 37°C incubation, even after periods as long as six weeks.

The results of these studies correlating the incubation temperature with the quantity of VH virus shed by the persistently infected cells are summarized diagrammatically in Fig.6. Since we have no firm evidence that replication of persistent VH virus occurs at 39°C, our tentative conclusion is that the virus must be protected in situ in the persistently infected cells. Further investigations of this phenomenon are necessary before final conclusions can be made.

HVI SNF Plaque Titration (PFU/ml)					
Day	37°C	39°C	Return to 37°C		
1	2.20x10 ⁶				
7	3.60x10 ⁶				
9					
16	1.56x10 ⁶				
17		6.90x10 ¹			
23	2.10x10 ⁶	2.73x10 ⁵			1.35x10 ⁶
24					
30	0.87x10 ⁶			2.03x10 ⁶	0.63x10 ⁶
31		8.70x10 ³			
37	1.98x10 ⁶			1.76x10 ⁶	1.89x10 ⁶
38					
45	0.96x10 ⁶			2.25x10 ⁶	
46		2.94x10 ⁵	2.88x10 ⁶		
51	0.30x10 ⁶	1.89x10 ⁵	1.41x10 ⁶		
52					
58	1.05x10 ⁶				

Fig. 6. Temperature effect on virus shed by persistently infected cells. Cells passaged regularly at 37°C were split on day 9 and some were incubated at 39°C. After passaging on days 17, 24, and 38, some of these 39°C cultures were returned to 37°C. At intervals samples of supernatant fluid were removed and titrated by plaque assay.

Comparison of Coronavirus Persistently Infected Cells with Uninfected L132 Cells

Comparative studies of the uninfected L132 cells and the persistent cell cultures have been performed to determine if detectable changes have occurred. Most of the characteristics, cell growth, full monolayer morphology and chromosome counts³, were similar. The only consistent difference could be seen when the cells were growing at low density. This visual difference between the uninfected L132 and the persistently infected L132 cells was most apparent on the first day after passage when the pattern of spreading of the persistent cultures could be distinguished from the normal cell cultures. The process of spreading involved much less elongation of the persistent cells. At confluence, the monolayers were virtually identical.

Number of Virus-producing Cells in Persistently Infected Cultures

Infections centres assay was carried out to determine the number

of virus-shedding cells in the cultures. Our results have shown that 50-100% of the cells were infected. Confirmation of this finding was sought by parallel studies involving cloning to determine whether all cells were infected and also whether other growth characteristics had been altered. Both persistently infected and uninfected Ll32 cells have been cloned and in all cases the cells showed essentially the same efficiency of cloning. Of interest however, was the finding that all the clones from the persistently infected cells initially did not shed virus but were resistant to superinfection by coronavirus 229E. After 25 passages of these cultures, virus shedding had resumed. (Data not shown). This indicated that the VH virus could be sequestered in the persistently infected cells.

Morphology of Persistently Infected Cells and of Normal Cells Acutely Infected with Persistent VH Virus

In addition to comparisons of the negatively stained virions in the electron microscope (Fig.1), thin sections of cell monolayers have been prepared for EM characterization. Monolayers of uninfected Ll32 cells, of persistently infected Ll32 cells and of Ll32 cells acutely infected with either 229E or VH viruses were fixed and Epon 812-embedded in situ (I.Dardick, personal communication). Thin sections were prepared, stained with lead citrate and uranyl acetate and examined in a Philips EM 300 electron microscope. In comparisons between the acute and persistently infected cells, though fewer virus particles were seen in the latter, in both cases virions were seen lined up along the surfaces of the cells and within cell vesicles (Figs.7A and 7B). The significance of the rather amorphous material regularly seen in association with the persistent VH virions is not known at this time.

When we compared the appearance of the Ll32 cells following acute infection with either 229E or VH viruses, no differences could be noted (Figs.7C and 7D). In both cases large numbers of virions were seen characteristically lined up along the cell surfaces and in the cell vesicles and intracellular spaces⁴. So far, in these preparations we have not seen the amorphous material referred to above in association with 229E or VH viruses either within vesicles or lined up along the cell surfaces.

We are presently engaged in further morphological studies using immune labelling techniques to locate and identify developing viral components within the cytoplasm and in association with the endoplasmic reticulum of the persistently infected cells. The results of these studies will be of value in further characterization of the persistent system but whether any relevant differences will be identified remains to be seen.

Fig. 7. Thin sections of cell monolayers embedded in situ showing coronavirus particles. Typical coronavirions are seen along the cell surfaces of L132 cells infected with 229E (A) or with VH persistent virus (B). Coronavirions are also seen in vesicles in the cytoplasm of 229E infected L132 cells (C) and HVI persistently infected cultures (D).

CONCLUDING REMARKS

Our studies so far, have been aimed at the elucidation of the mechanisms involved in establishing and maintaining the coronavirus HCV/229E persistent infection of L132 cells. On the basis of the accumulated evidence, it seems clear that this human coronavirus: human cell persistent system belongs to the class of persistent noncytotoxic infections transmitted through cell division⁵. This implies that all the cells contain copies of the viral genome and that expression of the genome requires host permissiveness.

To date our results have shown that the only difference between the acute and persistent infections of the same cell type with the same(?) virus were associated with temperature. Comparisons of the properties of standard 229E with the VH virus derived from the persistently infected L132 cultures have demonstrated no differences beyond an increased efficiency of replication of VH at the optimal temperature (33°C) with greater apparent cytopathic effect. Our experiments on the effect of temperature on virus shedding by persistently infected cultures have demonstrated that the expression of the viral genome along with normal cell replication was dependent on temperature (37°C). At higher temperatures (39°C) virus shedding diminished and the genome was not eliminated because virus shedding resumed normal levels once the cells were shifted back. However, when the persistently infected cells were incubated at the lower temperature (33°C) the cells were ultimately destroyed.

Our preliminary evidence seems to suggest that the effect of temperature is mediated through an alteration in host cell function implying the essential contribution of some host cell product to viral replication. The existence of such a host cell contribution was suggested by the effect of actinomycin D on 229E replication in L132 cells⁶. Further studies are aimed at an understanding of the role of temperature, including the effect of actinomycin D, and the identification of the essential component(s) contributed by the host cell.

REFERENCES

1. M.R. Macnaughton and M. H. Madge, The genome of human coronavirus strain 229E, *J. gen. Virol.* 39:497 (1978).
2. D. A. Kennedy and C. M. Johnson-Lussensburg, Isolation and morphology of the internal component of human coronavirus, strain 229E, *Interviol.* 6:197 (1975/76).
3. R. G. Worton and C. Duff, Karyotyping, in "Methods in Enzymology vol LVIII", S. P. Colowick and N. O. Kaplan, eds., Academic Press, N. Y., (1979).
4. L. S. Oshiro, J. H. Schieble, and E. L. Lennette, Electron microscopic studies of coronavirus, *J. gen. Virol.* 12:161 (1971).

5. W. K. Joklik, Summary of mechanisms involved in persistent viral infections, in "Microbiology 1977", D. Schlessinger, ed. A. S. M. Washington, D. C. (1977).
6. D. A. Kennedy and C. M. Johnson-Lussenburg, Inhibition of coronavirus 229E replication by actinomycin D, J. Virol, 29:401 (1979).

(Supported by the Medical Research Council of Canada and the
Multiple Sclerosis Society of Canada)