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Establishment and Maintenance of a Persistent Infection of L 132 Cells by Human Coronavirus Strain 229 E

By

GILLIAN CHALONER-LARSSON and C. MARGARET JOHNSON-LUSSENBURG

Department of Microbiology and Immunology, School of Medicine,
University of Ottawa,
Ottawa, Ontario, Canada

With 5 Figures

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Summary

A persistent infection by human coronavirus 229 E (HCV/229 E) was established in a human continuous cell line (L 132). Following the initial infection with stock HCV/229 E, several cultures were established of which two (HV 1 and HV 4) have been maintained by continous passage for two years. These cultures have shed high titres of infectious virus continuously into the supernatant fluid since their initiation. The persistently infected cells were resistant to homologous superinfection but supported polio virus replication to normal titres. Preliminary tests indicated that 50—100 percent of the cells contain virus. Neither interferon nor reverse transcriptase could be detected in these cultures and the presence of defective interfering particles could not be demonstrated. VH 1 and VH 4 coronaviruses, isolated from these persistently infected cultures (HV) and identified by 229 E antiserum neutralization, were more cytocidal than the parent virus as judged by plaque characteristics and CPE however, they were indistinguishable on the basis of density, EM morphology, and genome size. Present evidence indicates that temperature plays an important but as yet undetermined role in the establishment and maintenance of stable 229 E persistently infected cell cultures.

Introduction

Coronaviruses are important pathogens of humans and animals causing a variety of respiratory and enteric infections of worldwide distribution (17). In addition to the well known acute infections of man, swine, dogs, cats, calves and chickens, long term coronaviral infections have been described in chickens, mice and swine. Also, it has been reported that human coronavirus (HCV), which causes a mild upper respiratory infection, can be isolated from nasal washings of humans for up to 18 days (11) and human enteric coronavirus (HECV) has been isolated

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from faeces for several months (12). Thus it seems evident that closer attention should be given to the existence of chronic inapparent coronaviral infections of humans. Increasingly, interest is being focussed on human coronavirus because of its suggested involvement in persistent infection (Balkan endemic nephropathy) (1) and its possible etiological role in multiple sclerosis (MS) (3, 20). But, the inability to temporally assay human tissues for HCV or HECV after acute infection has made the investigation of persistent human coronavirus infection very difficult. This difficulty is further compounded because coronaviruses are notoriously fastidious, their host range is usually restricted to the natural host, and there are no suitable human coronavirus models of *in vitro* persistent infection reported to date.

On the other hand, *in vivo* and *in vitro*, persistent or chronic infection of mouse cells by strains of mouse hepatitis virus, the murine coronavirus, are well documented (5, 10, 19). In the absence of a suitable human host coronavirus model, these murine systems have been proposed as suitable models for the study of human disease (10).

This paper describes the succesful establishment of an *in vitro* persistent infection of a human cell line (L132) with human coronavirus, strain 229E (HCV/229E), which has to date been maintained for more than 300 passages over a period of 24 months and continues to shed high titres of infectious virus. This system has great potential for further studies on virus replication, pathogenesis of coronavirus disease and coronavirus genetics.

Materials and Methods

Cells and Cell Culture

Human fetal lung cells L132, a continuous cell line which supports human coronavirus replication were used throughout. They were routinely propagated as described previously (8), using Eagle's minimal essential medium (Flow Laboratories, Inc., Mississauga, Ontario, Canada) supplemented with 10 percent fetal bovine serum (FBS, Flow Labs., Inc.), sodium bicarbonate (20 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), neomycin (50 μg/ml) and glutamine (2 mM) at 37° C. The cells were passaged every two days at a split ratio of 1:3.

Viruses

Human coronavirus strain 229E used in these experiments was stock virus grown in L132 cells and maintained in this laboratory (8). Poliovirus Type 1, Sabin strain, used for the superinfection study was obtained from Dr. S. A. Sattar of this department.

Nomenclature

Normal cells — L132 (H); Standard virus — 229E (V); Persistently infected cells — L132/229E (HV); Virus derived from persistently infected cells — 229E/L132 (VH).

Growth of Virus

L132 monolayers were infected with 229E at a multiplicity of 3—5 PFU/cell and, following an adsorption period of 1 hour at room temperature, were incubated at 33° C for 24 to 40 hours in medium 199 (M199, Flow Labs. Inc.) without serum, supplemented with glutamine, antibiotics and 1 per cent sodium bicarbonate. The infected cultures were subjected to three freeze-thaw cycles at −20° C and then stored in aliquots at −70° C for use as virus inoculum as required. The titre of stock virus was $0.7—1.0 \times 10^8$ PFU/ml.

For the preparation of radioisotope labelled virus, the cells were infected as above. At the end of the adsorption period, either [5-^{3}H]-uridine (10 μCi/ml: specific activity 28 Ci/mmol) or ^{14}C-amino acid mixture (1 μCi/ml) were included in the maintenance medium (M 199). Radiochemicals were from New England Nuclear.

Virus Purification and Concentration

All steps were carried out at 0° to 4° C. Following three freeze-thaw cycles to release virus, 229 E-infected cell lysates were clarified by two cycles of centrifugation, first at low speed (IEC/PRJ, 1800 rpm × 15 minutes) and then at medium speed (IEC/B-20 A, 15,000 × g × 30 minutes). The virus was then cushioned onto 7 ml of 65 percent (w/v) sucrose in phosphate buffer (0.001 M, pH 7.2) in a Beckman SW 25.2 rotor at 20,000 rpm (48,000 × g) for 60 minutes. The concentrated virus was collected from the interface and was pelleted in a Beckman FA 50 rotor at 40,000 rpm (97,000 × g) for 2 hours. The pellet was resuspended in 0.001 M sodium phosphate buffer (pH 7.2), overlaid onto a linear 25—65 percent (w/w) sucrose gradient in 0.001 M sodium phosphate and centrifuged to equilibrium in a Beckman SW 41 rotor at 22,000 rpm (63,000 × g) for 16 to 24 hours. Fractions were collected and samples up to 200 μl were counted in a cocktail composed of BBS-3, 60 ml; butyl-PBD, 15 g (both from Beckman Instruments); water, 120 ml; and toluene to 3 liters, using a Beckman LS-250 liquid scintillation counter. The peak fractions were pooled and the virus pelleted for two hours in a Beckman FA 50 rotor at 40,000 rpm (97,000 × g). The virus pellets were resuspended in a few drops of 0.001 M sodium phosphate buffer, pooled and either used immediately or stored at −70° C.

Plaque Assay

All virus titrations were performed by the standard plaque assay in monolayers of L 132 cells in 75 cm^2 disposable culture flasks (Lux Scientific Corporation) as described previously (8). For plaque development, 229 E incubation was at 33° C for 6 days and polio at 37° C for two days. Virus titres are expressed as plaque-forming units per ml (PFU/ml).

Infectious Center Assay

To determine the percentage of persistently infected cells releasing infectious virus, monolayers of L 132/229 E cells (HV) were washed, trypsinized and appropriate dilutions of the cells in 5 to 10 ml of maintenance medium were added to confluent uninfected L 132 monolayers, with or without pretreatment with anti-229 E guinea pig serum. After two to three hours at 37° C to allow the cells to settle and attach to the layer, the monolayers were covered with overlay medium (8) and plaques allowed to develop for 5—6 days at 33° C.

Preparation of Anti-229 E Serum

Antiserum against purified and concentrated 229 E virus antigen was prepared in male guinea pigs (Hartley/Albino outbreed weighing 400—500 g) according to the following schedule: 1st day, 0.2 ml antigen in the footpad; 4th day, 1 ml of antigen with complete Freund's adjuvant (GIBCO) 1:1 subcutaneously; 19th and 32nd day, 1 ml antigen/adjuvant 1:1 intramuscularly. The animals were bled by heart puncture prior to immunization (control) and weekly throughout the procedure. Virus neutralization titres of $10^{-3.7}$ were obtained by standard plaque reduction assay.

Neutralization of Persistent Virus (VH)

Ten-fold serial dilutions of guinea pig anti-229 E serum were prepared in saline. 0.5 ml of each were mixed in equal amounts with suspensions of 229 E or 229 E/L 132 (VH) viruses, diluted appropriately to give between 20 and 50 PFU/ml. 0.5 ml saline without antiserum was added in parallel to serve as virus controls. After one hour at room temperature, 0.33 ml of each sample was seeded in duplicate onto L 132 monolayers, allowed to adsorb for one hour, covered with overlay medium and the plaques allowed to develop for 5—6 days at 33° C. Endpoints were calculated on the basis of 50 per cent reduction in plaque formation.

Electron Microscopy

All preparations were negatively stained with sodium or potassium phosphotungstate (2 per cent) following standard procedures as described previously (8). Grids were made directly from gradient fractions in sucrose or, more commonly, the fractions were diluted in 0.001 M sodium phosphate buffer and centrifuged at $97{,}000 \times g$ for 1 to 2 hours. Resulting pellets were resuspended in distilled water and grids prepared immediately. All grids were examined in a Philips EM 300 electron microscope.

RNA Dependent DNA Polymerase Assay (Reverse Transcriptase)

The assay for reverse transcriptase (RT) activity was carried out through the courtesy of Dr. A. Greig (Animal Disease Research Institute, Agriculture Canada, Ottawa) using the Kit for Mammalian Viral Reverse Transcriptase from Collaborative Research Inc., Waltham, Mass. and following the recommended procedures. This kit is designed to distinguish the RT enzyme from other DNA polymerase activities, and *E. coli* polymerase I, active on the provided templates, is included as an internal control for test performance. Supernatant fluid from uninfected and persistently infected L 132 cells was pelleted, resuspended in 25 µl and tested for viral RT activity using the primer template provided (Oligo dT . Poly rA). Positive and negative controls were supernatant fluid from Bovine Leukemia Virus infected fetal lamb kidney cells and uninfected fetal lamb spleen cells respectively. The cation requirements (Mg^{++} or Mn^{++}) of the enzyme were assessed in the tests.

Interferon Assay

Samples of supernatant fluid were collected from uninfected L 132 cells and from the persistently infected cell cultures at 36 hours, and from L 132 cells infected with 229 E or VH virus 35 hours post infection. After acidification, the samples were assayed for interferon activity at three fold dilutions from 1:4 to 1:324 using a plaque reduction technique on both human foreskin and PBS-1 cells against VSV in comparison with interferon reference controls. These assays were carried out through the courtesy of Dr. S. H. S. Lee (Department of Microbiology, Dalhousie University, Halifax, Nova Scotia, Canada).

Results

Establishment of Persistently Infected Cell Lines

Monolayers of L 132 cells were infected with stock 229 E virus at multiplicities (MOI) of 0.03, or 3.0 in 75 cm^2 tissue culture flasks. After adsorption at room temperature for one hour, maintenance medium (M 199) or growth medium (MEM) was added and the flasks incubated at either 33° or 37° C. After 30 to 36 hours the medium was decanted, the layers rinsed with phosphate buffered saline (PBS) and, depending on the integrity of the monolayer, the cells were either trypsinized and reseeded into fresh flasks or overlaid with fresh growth medium. The cell layers were monitored daily, the growth medium was changed daily and the cells were passaged when the layer approached confluence. Once it became clear that a monolayer was growing satisfactorily (approximately 2 weeks), aliquots of the medium were collected and assayed for infectious virus by plaque titration.

Maintenance of Persistently Infected Cell Lines

The successful maintenance of the persistently infected cell cultures was directly related to the incubation temperature of the cultures subsequent to the initial infection with 229 E virus. No distinct visual difference in the cell monolayers could be correlated with the virus input multiplicity but the infected

cultures kept at 33° C did not fare as well as those at 37° C. Therefore, the effect of temperature on maintenance was examined, the temperatures chosen being 33° C which is optimal for 229 E replication and 37° C the optimum for L 132 cell growth. Only those cultures kept at 37° C (supraoptimal for 229 E virus replication) during the initial incubation with virus or changed to 37° C shortly after the 30 to 36 hour incubation at 33° C survived the infection and formed a stable population of cells which appeared normal and grew at rates characteristic of L 132 cell cultures. In contrast, those cells infected and maintained at 33° C throughout or changed from 37° to 33° C during the course of our experiments grew slowly and irregularly, never reaching a confluent monolayer. During 6—8 weeks of maintenance at 33° C these cells continued to shed virus into the medium but in progressively decreasing amounts (up to 3 logs less before termination). Furthermore, after several weeks, these cells did not recover when returned to an incubation temperature of 37° C. By comparison, control uninfected L 132 cultures grew more slowly at 33° C than at 37° C, but they appeared normal and resumed normal growth rates when returned to their optimal incubation temperature (37° C).

The stable persistently infected cells maintained at 37° C have been stored at −80° C for up to 15 months and have been revived with no adverse effects—still growing characteristically and shedding virus. To distinguish these cells and their virus from the standard system, the cells were termed HV (L 132/229 E) and the virus derived from these cultures VH (229 E/L 132).

Several subsequent attempts have been made to establish new 229 E persistently infected cell lines. New long term stable virus-shedding populations have been obtained, but, the successful outcome of each attempt could not be predicted. Further work aimed at defining the critical procedure(s) for consistent production of such persistent infections is in progress.

Characteristics of Persistently Infected Cultures

Since the time of the initial infection with 229 E virus, strict precautions have been taken to ensure that virus has not been reintroduced. At the time of writing, the persistently infected cells have been passaged over 300 times on the same splitting schedule as uninfected L 132 cells (1 : 3 every two days) for over 24 months. They have continued to shed high titres of virus (10^5 to 10^6 PFU/ml representing 5—10 virions/cell) and show no apparent deleterious effects.

A visual difference between L 132 and HV cell layers was apparent on the first day after passage before a confluent monolayer was reached. Consistently, the HV cultures showed a pattern of cell growth which was distinguishable from L 132 cell cultures. During the initial growth, the individual cells of the persistently infected HV cultures did not show the characteristic elongation and spreading seen in the uninfected L 132 cells (Figs. 1 A and 1 B). Once confluent however, the monolayers were visually identical (Figs. 1 C and 1 D). Since the number of cells in 48 hour confluent monolayers of both uninfected and persistently infected cells were equivalent (Fig. 2), this characteristic pattern probably reflects the presence of replicating virus. Infectious center assays to determine the number of infected cells in the HV cultures tentatively indicate that 50—100 percent were producing virus. When HV cells were cloned, all resulting cultures (15/15) were virus produc-

ing and/or resistant to superinfection by 229E virus. These results need to be confirmed by immunofluorescent methods.

Throughout this study, the quantity of infectious virus shed into the culture medium (25 ml per 75 cm^2 flask) was routinely determined at weekly intervals by

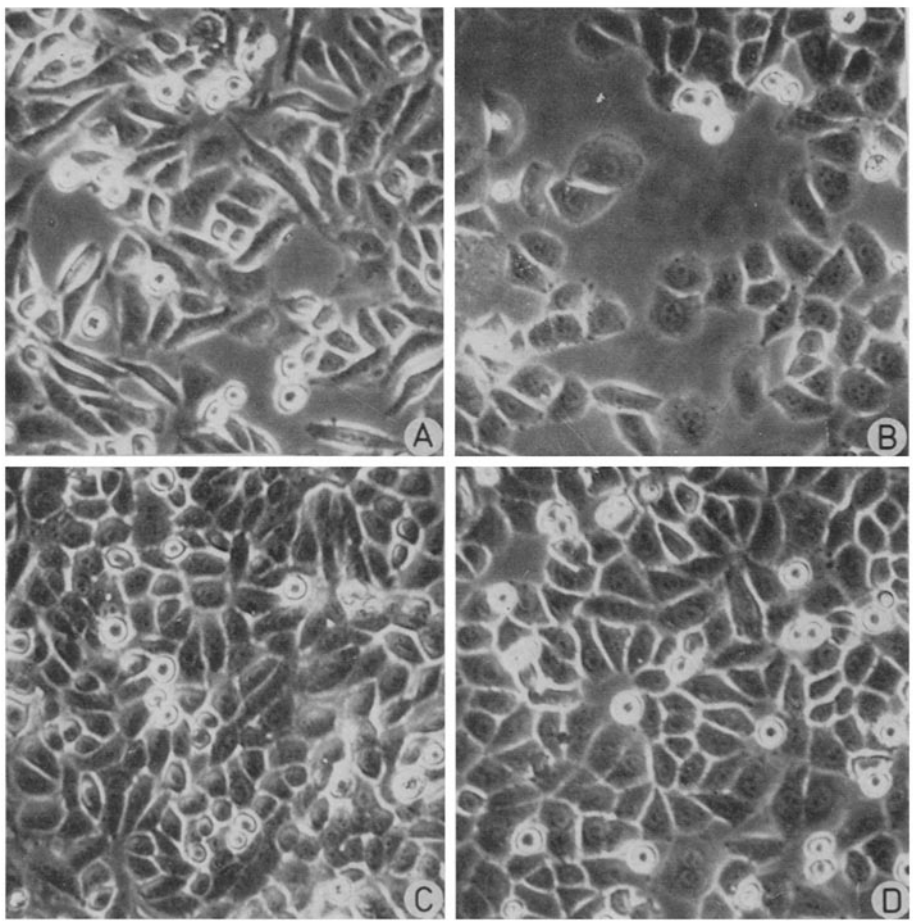

Fig. 1. Photomicrograph of cell monolayers of uninfected L132 cells (*A* and *C*) and persistently infected L132 cells (*B* and *D*). Photos were taken 24 hours (*A* and *B*) and 48 hours (*C* and *D*) after passaging

plaque assay. The titres of virus found in the supernatant fluid of two different HV cultures at 48 hours were 1.2×10^6 PFU/ml (HV1) and 5.0×10^5 PFU/ml (HV4) each with a range of $\pm \frac{1}{2}$ log and showing no evidence of cyclic variation. At intervals the amount of cell associated virus was also measured and consistently revealed five to ten times greater amounts of virus. On the basis of the total yield of virus/cell during a two day growth period, i.e. one cell passage, the production of virus correlated directly with the increase in the number of cells (Fig. 2A and 2B). Both showed a 3 to 4 fold increase during the 48 hour period. When the cells

were left for longer periods (up to 7 days) at 37° C, the infectious virus in the supernatant fluid progressively diminished by several logs, but when cells were passaged, the virus shedding recovered to normal levels and the cells showed no deleterious effects. These finding suggest that active cell division is a requirement for virus production i n this persistent system.

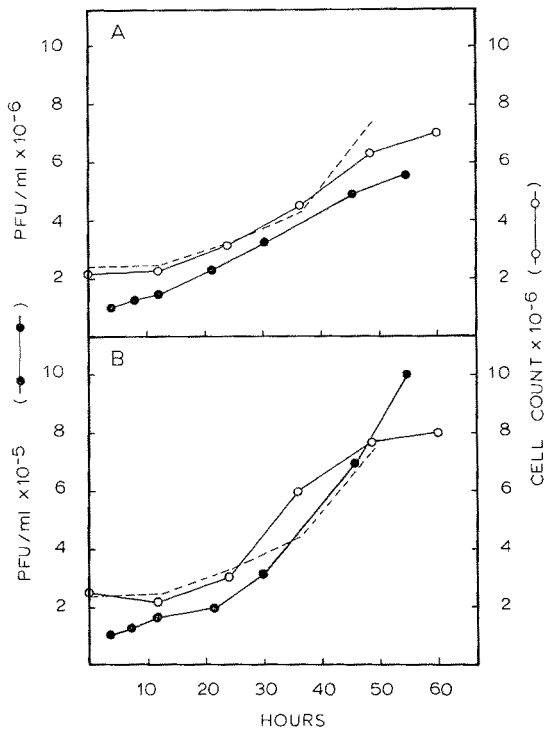

Fig. 2. Growth of HV1 *(A)* and HV2 *(B)* (two lines of L132 cells persistently infected with 229E virus) and the corresponding titre of virus shed into the medium (8 ml) from these cells during one passage. Cell growth is given as cells per 25 cm² layer (o) and virus titre as plaque forming units per ml (•). The dotted line on each graph represents the growth of uninfected L123 cells

Viral Challenge

To determine whether the persistently infected cultures were resistant to superinfection by 229E or by an unrelated virus, plaque assays were carried out with stock 229E and polio I (Sabin) viruses on HV and L132 cells. Cells at both low and high passage levels were tested (Table 1). None of the persistently infected HV cells supported replication of 229E at either 37° or 33° C but they did support replication of polio I (Sabin) equally as well as the L132 cells at 37° C. Control experiments included HV cells and L132 cells under agar without added virus. Both of these monolayers retained their integrity under agar at 37° C and at 33° C for up to seven days.

Table 1. *Number of plaques obtained following superinfection of HV cultures with homologous and heterologous virus*

	Cells					
	Low passage[a]			High passage[b]		
Virus	L132	HV1	HV4	L132	HV1	HV4
229E[c]	*24*	0	0	*19*	0	0
VH1[c]	74	*0*	0	95	*0*	0
VH4[c]	68	0	*0*	49	0	*0*
Polio (Sabin)[d]	19	28	20	50	56	54

[a] L132 cells were at our passage 20; HV1 and HV4 were at passage 78
[b] L132 cells were at our passage 40; HV1 and HV4 were at passage 178
[c] Six days incubation at 33° C. Persistently infected HV monolayers remained intact during six days under agar at 33° and 37° C
[d] Two days incubation at 37° C

Properties of Virus Released from Persistently Infected Cells

The results of neutralization tests performed with anti-229E serum indicated that the persistent virus was in fact a coronavirus. The serum was equally effective in neutralizing both VH and 229E viruses, the 50 percent plaque reduction end-points being between $10^{-3.4}$ and $10^{-3.8}$. The kinetics of neutralization were not evaluated.

The morphology of both types of coronaviruses was compared by examining negatively stained preparations of each in the electron microscope. No relevant difference could be seen between the persistently shed virions and the 229E virions.

Despite the lack of cytopathic effects in the persistently infected culture, the isolated VH virus seemed to be more cytocidal than stock 229E virus. When acute VH virus infections in L132 cells were carried out under liquid medium, generalized

Table 2. *Assay for RNA dependent DNA polymerase activity*[a]

	Mg^{++}	Mn^{++}
	cpm	cpm
Negative control[b]	605	464
Positive control[c]	202,500	50,000
L132 cells	707	793
HV1 cells	514	927
HV4 cells	554	1,232

[a] In addition to the 25 µl sample, the reaction mixtures contained: 50 mM Tris. HCl (pH 8.0); 50 mM $MnCl_2$ or $MgCl_2$; 5 mM Dithiothreitol (DTT); 20 µM thymidine 5-triphosphate [methyl-^{3}H], (Sp. Act. 40—60 Ci/mmole, New England Nuclear); 20.5 µg/ml Oligo dT.poly rA. Reactions were incubated at 37° C for 30 minutes before counting. Results are negative if the cpm are less than 3x the negative control
[b] Fetal lamb spleen cell culture (FLS)
[c] Fetal lamb kidney cell culture infected with bovine leukemia virus (FLK/BLV)

cell deterioration (CPE) was evident 12—15 hours earlier than with standard 229 E virus infections under similar conditions. Furthermore, a one-step growth curve experiment with both of these viruses showed that replication of VH virus was consistently earlier and reached higher titres than the standard 229 E virus (Fig. 3). This increase in replication efficiency of the VH virus was further indicated by the earlier development (by 1—2 days) of clearer and slightly larger plaques as compared to those of 229 E virus (Fig. 4).

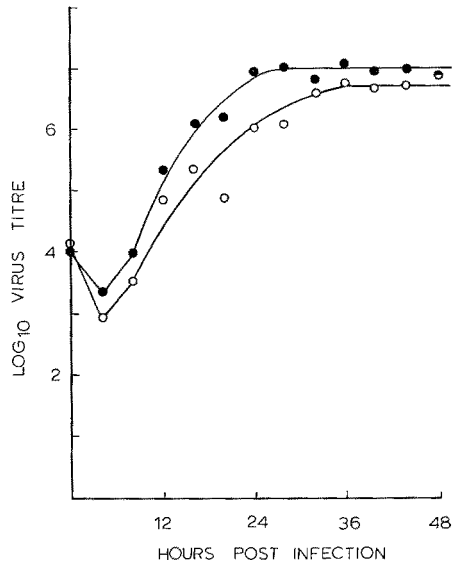

Fig. 3. Growth curves of 229 E (o) and VH 1 (•) viruses. Virus at 3—5 MOI was adsorbed in replicate onto L 132 cells for 1 hour at room temperature. The inoculum was replaced with M 199 and cultures incubated at 33° C. At 4 hour intervals, sample cultures were removed, subjected to 3 freeze-thaw cycles and stored at −20° C. Samples were titrated in duplicate by plaque assay. Titres are expressed as log PFU/ml

VH virus, labelled *in situ* with [5-^{3}H] uridine or ^{14}C-amino acids, and VH virus isolated and labelled also with [5-^{3}H] uridine or ^{14}C-amino acids during an acute infection of L 132 cells, were harvested, purified and compared with the standard 229 E virus similarly prepared. The density of the virus as determined by isopycnic sucrose gradient analysis was the same for 229 E and VH virus (1.18—1.19 gm/cc) (Fig. 5). RNA isolated from these viruses in isokinetic sucrose gradients gave the same profile of a single large molecular weight species of RNA (data not shown). Viral yields from lytic infections carried out at 33° C were always ½—1 log higher for the VH than 229 E viruses (Table 3).

Factors Involved in Maintenance of Persistence

The mechanism of HCV/229 E persistence in L 132 cells does not appear to involve either interferon production, integration or defective interfering particles. Assays for reverse transcriptase in L 132 cells and in persistently infected cells measured in the presence of Mg^{++} or Mn^{++} were negative (Table 2). Interferon, as assayed

by the plaque reduction method, was not detected in normal, acutely infected, or persistently infected cells (data not shown). Interference by defective interfering particles (DI) could not be completely ruled out but all efforts to detect such particles have been unsuccessful to date. The VH virus could not be distinguished from standard stock 229E virus by any of the methods tried i.e. density (Fig. 5),

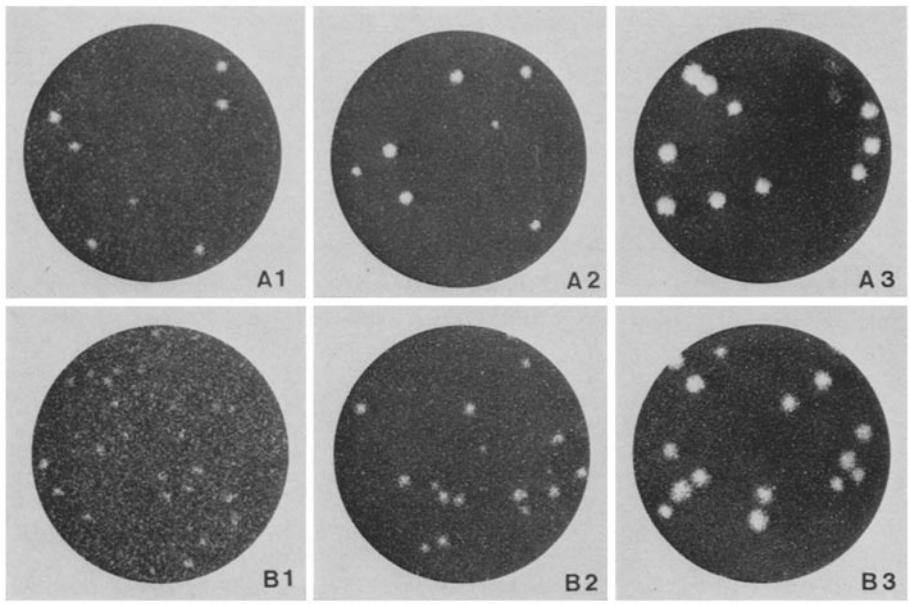

Fig. 4. Plaque morphology of VH virus *(A)* and 229E *(B)*. Appropriate dilutions of virus were seeded onto L132 monolayers and plaques allowed to develop for 4, 5, or 6 days at 33° C. Persistent VH virus plaques *(A 1, 2, 3)* developed earlier and were clearer than 229E virus plaques *(B 1, 2, 3)*. Plaques were visualized by crystal violet staining of the cells

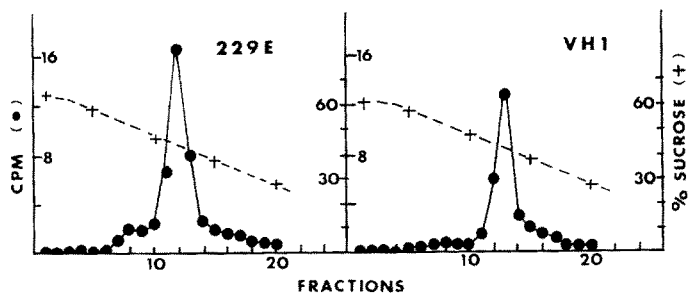

Fig. 5. Buoyant densities of 229E virus *(A)* and VH virus isolated from persistently infected cells *(B)* in isopycnic sucrose gradients. L132 cell monolayers were infected with 229E and ^{3}H-uridine was present in the medium M199 throughout the infection (36 hours). The persistently infected cells (HV) were grown to confluence in MEM, the medium was replaced with M199 containing ^{3}H-uridine, and incubation continued for 36 hours. Virus was harvested and purified as described in Methods. Both viruses equilibrate at 43 per cent sucrose which corresponds to a density of 1.185 g/cm^3

Table 3. *Total virus yields (30 hours p.i.) following mixed and serial infections at 33° C*

Experiment	Virus		MOI (PFU/cell)	Yield (PFU/ml) $\times 10^8$
Mixed	229E[a]		1.8	0.61
	VH1[a]		8.7	2.51
	VH4[a]		2.3	1.50
	229E+VH1		1.8+8.7	3.25[b]
	229E+VH4		1.8+2.3	1.91[b]
Serial	229E	1	~2	0.60
		2	2	0.40
		3	1.5	0.35
	VH1	1	~10	6.40
		2	20	6.80
		3	20	7.00
	VH4	1	~2	3.10
		2	10	3.00
		3	10	2.98

[a] Control for mixed infection

[b] Differences in plaque morphology were distinguishable in the mixed infections and occurred in the expected proportions

EM morphology, or physical characteristics of the isolated genome. In addition, serial infections with two strains of VH virus (MOI > 1) showed no interference effect and double infections showed no reduction in expected titres (Table 3). Also, the fact that the virus titre of the HV cells has not varied over the course of 2 years suggests that DI particles are unlikely to be involved.

Temperature sensitivity was therefore, the more promising avenue for investigation, since the persistent state showed some temperature dependence. The persistently infected cell cultures had been successfully maintained at 37° C, but not at 33° C which is the optimal temperature for 229E virus replication. At 33° C, the initial persistent infection had aborted after several weeks and several attempts to change HV cultures from 37° to 33° C met with a gradual deterioration and eventual halt in growth and loss of integrity of the layer. This was however not accompanied by an increase in virus shedding. Furthermore plaquing efficiencies of each of these viruses was 90 percent lower at 37° C as compared to 33° C. These facts do not indicate the emergence of temperature sensitive mutants per se, but rather imply a contribution by the host cell which is temperature dependent.

Discussion

The *in vitro* virus/host system described here appears to be the first reported instance of human coronavirus HCV 229E giving rise to a persistent infection in cell culture. After infection with 229E and an initial adjustment period, persistently infected L132 cells (HV cells) have been continuously subcultured and have shed virus into the medium at high titres for more than two years. Preliminary experiments suggest that the majority of these HV cells are infected.

In other *in vitro* persistent infections, several different mechanisms have been proposed. These include suppression of virus production by interferon (4), interference by defective interfering (DI) particles (6, 7, 13, 16), integration of the viral genome (23), and genetic mutations of the virus (15).

In the L132/229E system reported here, the first three mechanisms do not seem to play a role. Neither interferon nor reverse transcriptase activity has been detected. DI particles did not seem to be involved since there was no significant change in the virus shed over 300 passages of the persistently infected cell cultures and there was no biochemical or morphological evidence for two types of particles. Also, no interference could be demonstrated in mixed infections of 229E and VH virus. However, further experiments are necessary to provide sufficient evidence to exclude the involvement of DI particles.

A common factor in several other *in vitro* persistent virus/host systems is the production of temperature sensitive virus progeny. For example, such mutants have been isolated in persistent infections of measles (2), Newcastle disease virus (14), vesicular stomatitis virus (22), and mumps virus (21). Among the Coronaviruses, only mouse hepatitis virus has been reported to develop a persistent infection *in vitro* (17). Both temperature sensitive mutants of MHV (K. HOLMES, abstract, Int'l Virol. IV: 453, 1978) and cold sensitive mutants of JHM (a neurotropic strain of MHV) (18) have been isolated. LUCAS *et al.* (9,10) have described a temperature association with *in vitro* persistence of mouse hepatitis virus. They found that viral replication was thermosensitive in cell cultures persistently infected with strain JHM, although the virus progeny from the persistent infection were not thermolabile.

In our HV system, some temperature effect is associated with both the establishment and maintenance of persistence, however, further experiments are required to determine the role of temperature in this virus/host system. Since the virus isolated from the persistently infected cells (HV) caused more pronounced CPE than 229E virus in L132 cells, it seems probable that changes have occurred in both the virus and the host cells during the early stages of the establishment of persistence. Experiments are in progress to elucidate the mechanism or mechanisms involved in the establishment and maintenance of persistence of human coronavirus *in vitro*.

Acknowledgments

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Authors' address: Dr. C. M. Johnson-Lussenburg, Department of Microbiology and Immunology, School of Medicine, University of Ottawa, 275 Nicholas St., Ottawa, Ontario K1N 9A9, Canada.

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