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Adaptation of Coronavirus JHM to Persistent Infection of Murine Sac(−) Cells

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SUMMARY

Coronaviruses can establish persistent infections in the central nervous system of rodents, and these are associated with demyelinating encephalomyelitis. The effects of persistence on the virus are difficult to study *in vivo* but may have a crucial influence on the course of infection. We therefore produced a persistent infection *in vitro* using the neurotropic coronavirus JHM, in order to investigate the events underlying the establishment of such an infection and the adaptation of the virus to persistence. The persistent infection was maintained for over 115 passages and continued to release high levels of infectious virus. During the 18 months of culture the number of cells expressing virus antigen detected by indirect immune fluorescence decreased to 40%. Analysis showed that the carried virus contained a significant proportion of heterogeneous temperature-sensitive mutants. All virus clones isolated possessed the capacity to induce a more productive growth cycle, a less pronounced cytopathic effect and showed a much reduced neurovirulence when inoculated into newborn and weanling rats. Evidence for structural changes involving the surface peplomer protein (E2) was obtained using hybridoma antibodies, which neutralized the parental JHM virus but not the JHM-Pi virus. Defective interfering particles and interferon activities have been excluded as possible agents instrumental in the establishment and maintenance of the chronic infection, and we suggest that the emergence of virus variants of lowered virulence is central to these processes.

INTRODUCTION

Many viruses have been implicated in diseases of the human central nervous system (ter Meulen & Siddell, 1981). Cheever *et al.* (1949) first described a delayed encephalomyelitis associated with demyelination in rats infected with the coronavirus JHM. These early observations have recently been expanded, and infection of rats and mice with JHM virus has become a model for the study of demyelinating diseases (Nagashima *et al.*, 1978, 1979, Sörensen *et al.*, 1980; Stohlman & Weiner, 1981; Knobler *et al.*, 1982; Watanabe *et al.*, 1983). Current evidence suggests that the virus as well as the host animal plays a vital role in determining the outcome of this infection. Host effects include genetic background, age at time of infection, and the possibility of mounting an autoimmune reaction against myelin basic protein (Stohlman & Frelinger, 1978; Knobler *et al.*, 1981; Wege *et al.*, 1983; Watanabe *et al.*, 1983). The viral determinants of virulence are as yet unknown, but temperature-sensitive (*ts*) mutants or plaque variants exist which are of reduced neurovirulence or establish a persistent infection which leads to the development of demyelinating encephalomyelitis (Haspel *et al.*, 1978; Knobler *et al.*, 1982; Stohlman *et al.*, 1982; Wege *et al.*, 1983, 1984). Study of other virus–cell systems has revealed instances in which the virus acquires an alteration in virulence (Simizu & Takayama,

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1971; Thacore & Youngner, 1969) or structure (ter Meulen *et al.*, 1981; Yoshikura & Tejima, 1981; Carter *et al.*, 1983; Makino *et al.*, 1983) during persistence. Such alterations are therefore of direct relevance in chronic coronavirus infection of the animal. Although the structure and biochemistry of coronaviruses is well known (Siddell *et al.*, 1983), relatively little information is available concerning the growth and evolution of these viruses during persistent infections. Virus growth *in vivo* is complicated by the selection pressure applied through the host's immune response; therefore, it was decided to start these studies by establishing a new persistent JHM virus infection *in vitro*. This report describes the production of a long-term persistent infection established in the murine cell line Sac(−). Some biological characteristics of the carried virus have been examined and were found to be altered.

METHODS

Cells and virus. JHM virus was propagated in Sac(−) cells (obtained from Professor M. Mussgay, Tübingen, F.R.G.) as described by Siddell *et al.* (1980) and virus stocks were prepared in the same cell line. Virus was titrated in L cells and $TCID_{50}$ values were calculated according to the method of Reed & Muench (1938).

Infectious centre assay. Infected cell monolayers were harvested with 0.025% trypsin and washed twice with minimal essential medium (MEM) containing 5% foetal calf serum. The cells were then incubated in the presence of mouse anti-JHM serum for 15 min to neutralize extracellular virus, and samples were counted in a haemocytometer. The infected cells were serially diluted and added to microplate wells containing L cells. Infectious centres could then be estimated from the highest dilution of cells that led to c.p.e. in the microtitre plate well in a similar manner to the $TCID_{50}$ assay for infectious virus.

Immunofluorescence. Persistently infected cells were grown on 12 mm glass coverslips or on multispot slides (Dynatech Laboratories) and processed for immunofluorescence using mouse anti-JHM serum (1 in 24 dilution of a neutralizing antibody having a titre of 1:3072). The coverslips were washed extensively and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Miles Laboratories) was added. The coverslips were then mounted on slides with 10% glycerol in phosphate-buffered saline and examined under a Leitz microscope.

Selection of virus clones. Individual clones of the virus shed by the persistently infected cells (JHM-Pi) were obtained by serial dilution of tissue culture medium. This was plated on Sac(−) cells and overlaid with 1% agar; plaques were picked after 3 days. Virus was plaque-purified once, and then grown in Sac(−) cells to produce a virus stock.

Animal inoculation. Weanling (20 to 25 days of age) and newborn rats were inoculated in the left brain hemisphere with 30 to 40 μl of virus using a dispenser syringe. Animals were observed at 48 h intervals for the development of clinical signs. Neuropathological examinations were performed as previously described (Nagashima *et al.*, 1978; Watanabe *et al.*, 1983).

Virus neutralization. The medium from cultured antibody-secreting hybridoma cell lines was used for neutralization tests. These hybridoma cell lines were produced by fusion of spleen cells from immunized BALB/c mice with the myeloma line P3-X63-Ag8 according to standard techniques (Galfré *et al.*, 1977) and will be described elsewhere (H. Wege *et al.*, unpublished method). The antibody solution was mixed in serial dilutions with 150 to 200 p.f.u. of parental JHM virus or JHM-Pi virus (final vol. 200 μl) and incubated for 1 h at 4 °C. Virus controls were incubated with culture medium from non-neutralizing hybridoma cell lines or with normal inactivated rabbit serum dilutions. The virus titre was then determined in Sac(−) cells in 24-well microtitre plates (Costar). The infected cultures were incubated for 20 to 24 h without agar overlay. Microplaques were counted after staining with May–Grünwald and Giemsa stains. The serum dilution resulting in 50% plaque reduction was thus calculated. Rabbits were immunized with gradient-purified JHM virus to raise antiserum, which had a plaque neutralization titre of 1:1860.

RESULTS

Establishment and maintenance of persistently infected cell lines

Sac(−) cell monolayers were initially infected with a stock culture of JHM virus at a multiplicity of infection of 2 $TCID_{50}$/cell. Following adsorption, the infected cells were maintained in MEM containing 5% foetal calf serum at 37 °C. After 24 h more than 98% of the cells had formed syncytia. The medium was then changed regularly and the surviving cells permitted to grow into colonies. The JHM-Pi cells were in sufficient numbers to pass by 14 to 21 days. The infected cell lines were initially maintained by passage at 7 day intervals. At this point the cells grew in a irregular manner, with the occasional emergence of syncytia which almost

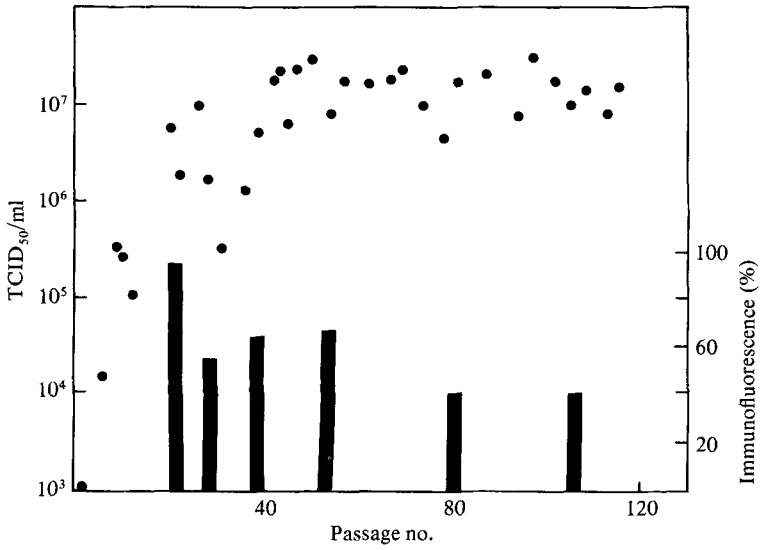

Fig. 1. Virus production and percentage of immunofluorescent cells between passages 1 and 115. Samples of culture medium were obtained immediately before each passage and assayed on L cell monolayers. Virus titres ($TCID_{50}$/ml) are indicated by (●). Cells grown in parallel cultures on coverslips were processed for indirect immunofluorescence. Percentage of positive cells is indicated by height of the columns.

covered the monolayer. After 25 passages the growth of the JHM-Pi cells became more predictable and growth rates were comparable to uninfected Sac(−) cells. From this point the cells were passed twice weekly, diluted 1 in 4. Throughout the whole history of the JHM-Pi cells, areas of syncytia, which were found to be the major sites of JHM virus antigen expression, have been present in the monolayers. These regions constituted 10 to 20% of the total monolayer area and were most prominent when the cells reached confluence. However, they did not lead to complete destruction of the monolayer if the cells were not passed.

Virus was shed throughout the development and continued culture of the JHM-Pi cells (Fig. 1). Initially this was of the order 10^4 $TCID_{50}$/ml and then increased during the first 40 passages to a relatively constant level of 10^7 $TCID_{50}$/ml. During culture of the JHM-Pi cells, the percentage positive by immune fluorescence decreased from 95% to 40% (Fig. 1); consequently, the amount of infectious virus released per fluorescent cell increased 1000-fold, from approximately 0.03 in the early passages, to between 30 and 60 $TCID_{50}$/cell at later times. In contrast to observations in other persistent coronavirus infections (Lucas *et al.*, 1977, 1978) the fluctuations in the level of released virus did not follow a cyclical pattern. The JHM-Pi cells were subcloned under antiserum and in this way cells were obtained which did not express virus antigen. These clones were found to be susceptible to superinfection with JHM virus, confirming that they were cured. It was therefore concluded that the JHM-Pi culture consisted, at late passages, of a dynamic equilibrium between infectible cells and infectious virus.

Mechanisms involved in establishment and maintenance of persistent infection

The survival of the individual cell during establishment of persistent infection is not amenable to direct experimentation. Studies cannot be performed until cultures have undergone the many divisions necessary to produce a monolayer that maintains the persistent infection. Three factors have been implicated for establishment and survival: defective interfering (DI) particle-mediated interference, *ts* mutations and interferon (Rima & Martin, 1976).

DI virus is not readily produced in an asynchronously spreading infection initiated at low multiplicity. We have examined the involvement of DI particles in the establishment of

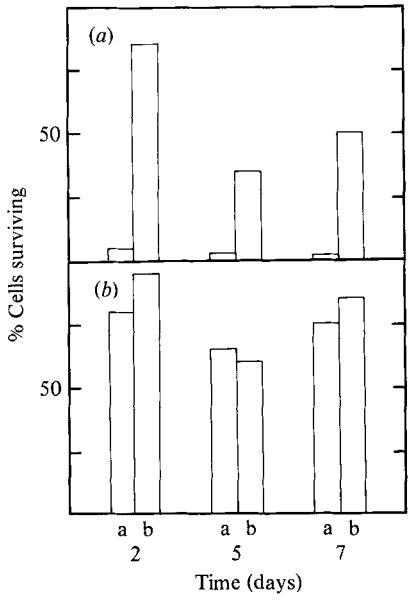

Fig. 2

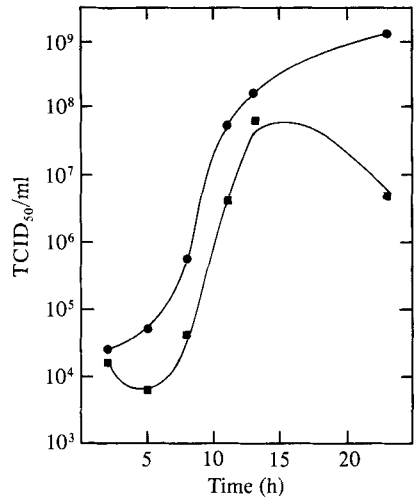

Fig. 3

Fig. 2. Effect of multiplicity of infection on the establishment of persistently infected Sac(−) cells. The cells (2×10^6) were infected with (*a*) 10^{-1} $TCID_{50}$/cell or (*b*) 10^{-5} $TCID_{50}$/cell, using JHM virus (columns a) or JHM-Pi virus (columns b). The number of surviving cells was determined at 2, 5 and 7 days following infection. The figures represent the mean of duplicate samples.

Fig. 3. Growth curves of JHM (■) and JHM-Pi (●) virus, in Sac(−) cells at 37 °C. The cells were infected at 5 $TCID_{50}$/cell, and samples of the media were assayed for progeny virus at the times indicated.

persistent infection indirectly, by infecting equal numbers of Sac(−) cells with different multiplicities of JHM or JHM-Pi carried virus. After 2, 5 and 7 days, surviving cells were harvested, identified by trypan blue exclusion and counted (Fig. 2). At high multiplicity, cells infected with JHM-Pi stood a much greater chance of surviving the infection than cells infected with JHM virus. At the lower multiplicity, this difference was almost abolished, and Sac(−) cells infected with JHM virus showed an increased likelihood of survival. Cells surviving these infections at both high and low multiplicity went on to establish persistent infections characterized by a high percentage of immunofluorescent cells and release of infectious virus. These data are opposite to those expected if DI virus production were important in this process.

It was considered unlikely that DI particles were involved in the maintenance of persistence since the JHM-Pi cells continually produced high levels of infectious virus. This suggested that any interference with virus multiplication could not be extensive. To investigate this possibility, virus shed from the JHM-Pi cells (passage 52) was tested for its capacity to interfere with a normal lytic JHM virus infection of Sac(−) cells, and found to have no effect (Table 1). However, this preparation contained infectious virus as well as putative DI particles and it was possible that the carried JHM-Pi virus could have acquired resistance to DI particle action (Horodyski *et al.*, 1983). This virus could therefore mask the activity of such particles by growing over the interfered parental JHM virus. DI particles are known to be more resistant to u.v. radiation than infectious virus in a number of systems (for review, see Perrault, 1981), and we took advantage of this property to inactivate preferentially the infectious component of the JHM-Pi virus. However, no significant interference could be detected even when infectious virus had been inactivated by this means (Table 1).

Table 1. *Interference of JHM-Pi virus with replication of JHM virus in Sac(−) cells*

Infecting virus (m.o.i.)*		
JHM-Pi	JHM	Virus yield†
0	1	$3{\cdot}2 \times 10^7$
1	0	$5{\cdot}6 \times 10^7$
1	1	$2{\cdot}5 \times 10^8$
5	1	$2{\cdot}9 \times 10^8$
10	1	$2{\cdot}1 \times 10^8$
U.v.-inactivated‡	1	$1{\cdot}8 \times 10^7$

* Virus was harvested from the JHM-Pi cells at passage 52 and used to infect Sac(−) cells for 1 h. This was then removed, the cells were washed twice and then infected with JHM virus for 1 h.

† The virus yield was determined after 24 h at 37 °C and is expressed as $TCID_{50}$/ml.

‡ The JHM-Pi virus was u.v.-inactivated to give a reduction in titre of 4 $\log_{10}$ units. This was then used to infect Sac(−) cells at a multiplicity equivalent to 10 $TCID_{50}$/cell.

Table 2. *Production of virus by JHM-Pi cells, and Sac(−) cells lytically infected with JHM and JHM-Pi virus derived from passage 43*

Infection	No. of cells	Virus yield ($TCID_{50}$/ml)	% Cells infected	Yield/infected cell ($TCID_{50}$)
JHM-Pi cells*	7×10^5	$1{\cdot}8 \times 10^7$	65	38
JHM lytic†	1×10^6	$3{\cdot}2 \times 10^7$	100	32
JHM-Pi lytic†	1×10^6	$2{\cdot}8 \times 10^8$	100	280

* Cells were passed, and the medium was titrated after 24 h: the number of cells, and percentage of infectious centres were then determined.

† Sac(−) cells (10^6) were infected with either JHM or JHM-Pi virus; after 4 h duplicate plates were assayed for infectious centres or titrated 24 h after infection.

Interferon was excluded as a possible factor in the maintenance of the JHM-Pi cultures since these cells were found to be completely susceptible to challenge with a heterologous virus, vesicular stomatitis virus (VSV). JHM-Pi cells as well as uninfected Sac(−) cells developed 100% c.p.e. when infected with VSV at a m.o.i. of 5 $TCID_{50}$/cell. Consequently, no general antiviral state had been induced in the persistent infection.

Properties of the JHM-Pi virus

The total carried virus was tested for temperature sensitivity. Significant differences were noted, although virus plaques were small and indistinct at both temperatures. In order to examine temperature sensitivity more closely, virus clones were isolated by picking plaques under agar which were then grown into stocks. In this way a total of 51 clones was isolated from passages 21, 41 and 110, and analysed for temperature sensitivity by comparing the virus yield at 33 °C with that at 39.5 °C. Approximately 20% of all clones examined showed some temperature sensitivity. The ratio of virus yield of temperature-sensitive clones at 39·5 °C to that at 33 °C varied between $1{\cdot}0 \times 10^{-1}$ and $1{\cdot}5 \times 10^{-5}$. Consequently, a significant proportion of carried virus had acquired a *ts* phenotype although this was heterogeneous in nature, and no single virus variant had become predominant.

Analysis of virus growth

High levels of virus were shed into the medium from the late passage JHM-Pi cells (Fig. 3). This was achieved in the absence of widespread cell destruction. Yields were similar to those of JHM virus in lytic infection, and the amount of virus produced per infectious centre also compared well with that observed in lytic infection (Table 2). When the JHM-Pi virus was used to infect fresh Sac(−) cells, titres were consistently obtained in excess of 10^8 $TCID_{50}$/ml. This was reproducibly at least tenfold greater than the yield from a control JHM virus lytic infection

Table 3. *Reactivity of JHM-Pi virus with neutralizing hybridoma antibodies*

Designation of antibody	Indirect immunofluorescence			Neutralization titre (50% plaque reduction)	
	JHM virus*	JHM-Pi virus*	JHM-Pi cells	JHM virus	JHM-Pi virus
L 99/61	++++	−	−	1:480	<1:10
H 79/62	++++	−	−	1:460	<1:10
B 79/10	++++	−	−	1:4300	<1:10
R 79/12	++++	−	−	1:435	<1:10
H 496/76	++++	−	−	1:363	<1:10
Rabbit anti-JHM virus serum	++++	++++	++++	1:1860	1:2390

* Sac(−) cells were infected with either JHM or JHM-Pi virus (m.o.i. 0·01) and 16 to 20 h post-infection the cells were fixed with acetone for indirect immunofluorescence.

and was observed using all virus clones isolated from the persistent infection, both *ts* and non-*ts*. Analysis of the kinetics of virus growth in Sac(−) cells at 37 °C showed the major difference in yield occurred late in infection, after 13 h post-infection (Fig. 3). After this time the level of JHM virus in the medium of the control infection began to decline and the cell monolayer was completely fused. However, in the case of JHM-Pi virus infection, yet more virus was produced at late times; the cytopathic effect of the virus at this time had not involved the whole of the cell monolayer. Many of the cells had rounded and clumped together but were not fused. No differences were observed between the rates of thermal inactivation of the JHM-Pi carried virus and JHM virus. At pH 6·5 and 37 °C both were reduced in activity by 50% in approximately 2 h.

Biological evidence for structural changes of the peplomer glycoprotein

JHM-Pi cells were grown on multispot slides, fixed with acetone and tested by indirect immunofluorescence for their reactivity with a panel of hybridoma antibodies (H. Wege *et al.*, unpublished results) directed against JHM virus structural proteins. As summarized in Table 3, several hybridoma antibodies which gave brilliant fluorescence with JHM virus did not recognize JHM-Pi cells as an antigen. All of these hybridoma antibodies neutralized JHM virus, but not JHM-Pi virus. However, the structural alteration of the peplomer glycoprotein was probably not extensive, since the majority of hybridoma antibodies raised against this protein of the parental JHM virus also recognized JHM-Pi virus. Furthermore, rabbit anti-JHM sera showed no differences in any test between JHM-Pi and parental JHM virus.

Neurovirulence of the JHM-Pi virus

Virus isolated from JHM-Pi cells was injected intracerebrally into newborn and weanling rats. The results are shown in Table 4. In marked contrast to rats inoculated with the parental JHM virus, animals that received JHM-Pi virus showed no sign of acute clinical disease. In a few instances a subacute demyelinating encephalomyelitis was induced, but only if newborn rats were infected. When 21- to 25-day-old rats were infected, no disease with clinically recognizable symptoms was observed. Virus preparations examined included uncloned virus released from passages 6, 12, 25 and 99, cloned viruses (whether *ts* or not) and JHM-Pi virus which had been passaged ten times in Sac(−) cells; all these were found to be much reduced in virulence for rats. These results demonstrate that the loss of virulence must have occurred early during the persistent infection and was a stable characteristic of JHM-Pi virus.

From each group of infected animals, four rats which had not shown signs of disease during the observation period (30 to 40 days post-infection) were dissected and examined for neuropathological changes. In addition to old lesions which had probably developed within a week of infection (Koga *et al.*, 1984), no significant histological changes were evident. Attempts to isolate infectious virus or to detect viral antigen all proved negative. In parallel experiments to

Table 4. *Neurovirulence of virus derived from JHM-Pi cells in rats (strain CHBB/Thom)*

Virus	Dose ($TCID_{50}$/rat)	Age of rat at infection (days)	Clinical disease	
			Total	Type
JHM	2×10^4	1–4	12/12	AE*
	5×10^4	30	13/13	AE
Uncloned JHM-Pi				
Pass 25	3×10^4	1	1/14	AE
Pass 99	6×10^5	1	1/11	AE
Cloned JHM-Pi†				
Pass 14	4×10^5	1–4	5/30‡	SDE§
	4×10^5	30	0/12	–
Pass 31	8×10^5	1–4	1/28	SDE
	1×10^6	30	0/12	–
JHM-Pi *ts*	4×10^6	1	1/27	SDE

* AE, Acute encephalomyelitis; incubation time 4 to 10 days.
† All cloned viruses had been passaged ten times in Sac(−) cells in the preparation of these virus stocks.
‡ Two rats recovered after being diseased for about 10 and 30 days respectively.
§ SDE, Subacute demyelinating encephalomyelitis; incubation times observed in these experiments, 17 to 44 days.

investigate the kinetics of virus replication *in vivo*, we could re-isolate infectious virus from brain tissue for only 12 to 14 days after infection.

DISCUSSION

Persistent infections with viruses occur both *in vivo* and *in vitro*. The mechanisms by which such infections are induced and maintained can be most readily studied *in vitro*. Here we have started such a study with coronavirus JHM, an agent known to cause long-term demyelinating disease in rats and mice (Nagashima *et al.*, 1979; Knobler *et al.*, 1982; Wege *et al.*, 1984). The virus readily produced a persistently infected culture, a finding similar to that of other authors (Lucas *et al.*, 1977; Stohlman & Weiner, 1978; Makino *et al.*, 1983).

It is not known by what mechanisms the Sac(−) cells survived the original JHM virus infection. The original event permitting cell survival must have occurred at the single cell level and is therefore not amenable to direct examination, but several conclusions may be drawn. Firstly, we have been unable to obtain any evidence for the existence of a subpopulation of cells possessing intrinsic resistance to JHM virus. Such cells were recently found to be involved in the establishment of a persistent murine coronavirus infection in a subline of L cells (Mizzen *et al.*, 1983). Secondly, cell survival and the establishment of persistent infections were both favoured by a low multiplicity in the original infection. This also excludes DI particles as likely participants in these events. Furthermore, DI particles do not always permit survival of the interfered cell (Schnitzlein *et al.*, 1983), or may do so only indirectly through induction of interferon (Sekellick & Marcus, 1979).

Our results have indicated that interferon was not involved in the maintenance of persistence. It is also difficult to understand why cells which could have been stimulated to produce interferon should then cease to do so despite the continued presence of virus. Consequently, it is likely that interferon was not involved in the establishment of the JHM-Pi culture.

DI particles were not involved in the maintenance of the infection, as virus yields were high and no interference activity could be detected. Despite this, yields in the persistent infection were usually tenfold lower than those obtained using the carried virus in lytic infections. We consider that this discrepancy results from comparing a 'one step' synchronous growth cycle in the lytic infection, with continuous virus release in the persistent infection. Heat inactivation would therefore contribute more to the latter case than to the former. A persistent infection arising from the integration of the viral genome into host cell DNA is unlikely. Chaloner-Larsson & Johnson-Lussenburg (1981) could find no RNA-dependent DNA polymerase activity in a human coronavirus persistent infection and this enzyme is also absent from Sac(−) cells.

However, a heterogeneous collection of *ts* mutants was identified, but the *ts* effect was small and such viruses constituted only 20% of virus clones examined. The presence of *ts* mutants in other JHM persistent infections is variable (Stohlman & Weiner, 1978; Lucas *et al.*, 1978; Stohlman *et al.*, 1979).

Holland *et al.* (1980) suggested that a persistently infected cell line favours the evolution of the carried virus genome by the rapid acquisition of mutations. Most of these lead to the production of small plaque, *ts* mutants. The heterogeneous mutants involved in persistent infection by a variety of viruses show an altered plaque phenotype and decreased virulence (for review, see Rima & Martin, 1976), and this is also the case in the JHM-Pi infection described here. Furthermore, *ts* viruses obtained in this way have been observed occasionally to revert to the non-*ts* phenotype but, when this occurred, the reduced virulence was maintained (Haspel *et al.*, 1973; Shenk *et al.*, 1974; Preble & Youngner, 1973; Simizu & Takayama, 1971). These results suggest that the *ts* phenotype itself may therefore have no real significance in the establishment of the persistently infected culture, but arises as an 'effect' rather than a 'cause' of persistence. Low multiplicity infection was found to favour the establishment of persistent infections. This would increase the asynchrony of infection and the number of replication events occurring during the spread of virus through the monolayer. Consequently, the production of virus variants would be favoured. All virus clones examined, regardless of the *ts* phenotype, possessed the capacity to grow to high titres and produce reduced cell fusion, which suggests that these properties were acquired early in the persistent infection. Therefore, we suggest that a change may have occurred in the virus during spread through the monolayer, resulting in a decreased ability to induce c.p.e. and an increased likelihood of survival for the infected cell. It is known that the virus-infected cell is often resistant to superinfection by a closely related virus (Marcus & Carver, 1967). This process is known to be active between two mutants of the same virus (Johnston *et al.*, 1974). Consequently, any cell infected with such a mutant of lowered cytotoxicity might be protected from the action of more virulent viruses spreading through the culture. Only those cells infected with the mutant virus could survive and continue to release virus. The acquisition of further mutations during persistence could refine the balance between cell and virus replication to the equilibrium we have observed.

We have obtained strong evidence for alterations in the peplomer glycoprotein (E2) using hybridoma antibodies. Cell fusion by coronaviruses is probably mediated by this glycoprotein (Collins *et al.*, 1982). This alteration could explain the inefficient c.p.e. production by the JHM-Pi virus which extended the period of virus release and resulted in higher virus titres. Furthermore, the peplomer glycoproteins may exert a strong influence on virus neurovirulence, as demonstrated in the case of reovirus (Weiner *et al.*, 1977). We are currently attempting to define biochemically the nature of the structural alteration in virus glycoproteins and to select virus variants using a panel of monoclonal antibodies.

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