In Vivo and In Vitro Models of Demyelinating Diseases

II. Persistence and Host-Regulated Thermosensitivity in Cells of Neural Derivation Infected with Mouse Hepatitis and Measles Viruses

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Following inoculation of continuous cell lines of neural and other derivations, persistent infections are established with facility by mouse hepatitis and measles viruses. This occurs equally with the prototype MHV₃ and its neurotropic variant JHM as well as with the Edmonston vaccine and SSPE Hallé measles variants. In almost every instance that the infection becomes persistent at 32.5°, virus replication is found to be thermosensitive at 39.5°; however, progeny virus derived from such infections at 32.5° is itself thermostable when replicating in the indicator, fully permissive cell lines. The new data, therefore, reveal the existence of a host-conferred interrelationship between persistence and virus restriction at elevated temperature. They indicate that the two agents with neurotropic potential, when they become established as pathogens in the nervous system, could be under close host cell regulation involving as yet unknown mechanisms.

INTRODUCTION

There is abundant evidence implicating viruses in the etiology of neurological diseases in both animals and man. Studies *in vitro* and *in vivo* using spontaneous and selected virus mutants have shown that when the infection becomes defective, persistent, or latent, the agent sometimes spreads along neuronal tracts, causes extensive cytopathology including demyelination, or may establish slow and chronic infections. The mechanisms underlying such virus–cell interactions in the nervous system have been elucidated in part for the herpes viruses (Cook *et al.*, 1974; Price *et al.*, 1975) but remain obscure in the case of others; however, several animal model and *in vitro* cell–virus carrier systems suitable for investigating such problems have been developed.

Two neuropathogens of current interest in this laboratory are a derivative of mea-

sles associated with a progressive and fatal human disease, subacute sclerosing panencephalitis (SSPE) (Horta-Barbosa *et al.*, 1971), and the JHM strain of murine hepatitis virus pathogenic for rodents (Cheever *et al.*, 1949; Bailey *et al.*, 1949; Weiner, 1973). The SSPE agent known to prevail persistently in the brain of afflicted individuals has been evoked by growing brain explants in continuity with permissive cells (Horta-Barbosa *et al.*, 1969; Burnstein *et al.*, 1974). The emergent virus has close serological identity with measles virus (Baublis and Payne, 1968) and can simulate symptoms of the human disease, including demyelination and neuronal vacuolation, when inoculated intracerebrally or intranasally into susceptible rodents (Zlotnik and Grant, 1975). Pathogenesis with the JHM agent after inoculation into mice is manifested as demyelinating lesions in the central nervous system presumably associated with infection of the oligodendroglia (Weiner, 1973; Lambert *et al.*, 1973). Demyelination may occur intermittently, particu-

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larly when older mice are inoculated with this agent (Herndon *et al.,* 1975), suggesting this as a useful model for certain human demyelinating diseases.

In a recent publication we described an *in vitro* correlate to this *in vivo* model (Lucas *et al.,* 1977), involving a rat Schwannoma RN2-2 cell line, which functions as a discriminating host for the JHM virus. JHM readily established a persistent infection in RN2-2 cells during which there occurred cyclical variations in the titer of virus released into the medium and self-limiting syncytiogenesis. In contrast, infection of the RN2-2 cells with a prototype murine hepatitis virus, MHV$_3$, was aborted immediately.

In this report we describe further experiments on the two virus models in which we have compared replication of JHM versus MHV$_3$ and the Hallé versus Edmonston strains of measles virus in continuous cell lines of neural and other origin.

MATERIALS AND METHODS

Cells and cell culture. The rat astrocytoma C6 cells were obtained from Dr. A. Marks (Banting and Best Institute, Toronto), the rat hepatoma HTC cells from Dr. K. Yamamoto (University of California, San Francisco), and the rat myoblast L6 cell line from Dr. D. B. Sanwal (University of Western Ontario, London). Dr. S. E. Pfeiffer (University of Connecticut, Farmington) kindly supplied the murine glioblastoma cell lines, G26-20 and G26-24. The murine neuroblastoma C1300 cells were obtained in 1971 from Dr. J. P. Broome (New York University Medical School) and the African green monkey kidney Vero cells from Dr. L. Hatch (St. Joseph's Hospital, London). The sources of the rat Schwannoma RN2-2 cell line and the murine fibroblast L-2 line were previously described (Lucas *et al.,* 1977).

Prior to use, the cells were cloned by limit dilution in 96-well Microtest II trays (Falcon Plastics), and a representative clone of each cell line was used in this particular study.

The cells were routinely propagated as monolayers in Eagle's minimal essential medium (MEM) supplemented with 10%

heat-inactivated fetal bovine serum (FBS) (Microbiological Associates), sodium bicarbonate (2 g/liter), penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37° in a humidified atmosphere containing 5% CO$_2$.

Viruses. The source and routine propagation of the MHV$_3$ and JHM strains of mouse hepatitis virus have been previously described (Lucas *et al.,* 1977).

The Hallé strain of SSPE and the Edmonston strain of measles virus were obtained from the American Type Culture Collection at passage levels of HeLa/2–Vero/3 and HK/24–HAM 141, respectively. Both viruses were routinely maintained by passage on Vero cell monolayers. Diluted virus was allowed to adsorb at a multiplicity of infection (m.o.i.) of 0.01 at 32.5 or 37° for 1 hr before medium was added.

Plaque assay. MHV$_3$ and JHM virus production was determined by a plaque assay on L-2 cell monolayers as previously described (Lucas *et al.,* 1977). Measles virus production was ascertained using a similar plaque assay on Vero cell monolayers. For plaque assays after overlaying with MEM containing 5% FBS and 0.5% methyl cellulose, 4000 cps, the infected monolayers were incubated at 32.5° for 4 days in the case of Hallé virus and 5 days when assaying the Edmonston strain, then were fixed and stained. The titer is expressed as plaque-forming units per milliliter (PFU/ml).

Infectious center assay. To determine the fraction of cells able to release PFU during the state of persistence, the monolayers were washed, and the cells were released from them by trypsinization and then plated at serial dilutions on either L-2 monolayers in the case of JHM and MHV$_3$ infections or on Vero monolayers when Hallé and Edmonston infections were involved. After 3 to 5 hr attachment period at 32.5°, the plates were overlaid with the methyl cellulose–MEM mixture and incubated until plaques developed as above.

Infection of cell lines. Various cell lines grown into monolayers were inoculated at a m.o.i. of 0.01 at 32.5 or 39.5° with one of MHV$_3$, JHM, Hallé, or Edmonston viruses. Following adsorption for 1 hr, unadsorbed virus was removed, and the cultures were

overlaid with nutrient medium. The infected monolayers were examined for development of virus-induced cytopathology and monitored daily for production of free plaque-forming units per milliliter necessitating the complete replacement of the medium each day.

Determination of cerebroside sulfate. Confluent monolayers of the various cell lines were incubated for 48 hr at 37° in MEM supplemented with 2.5% FBS and containing 40 μCi/ml of [^{35}S]sulfate (43 Ci/mg; New England Nuclear). Cells were harvested by scraping and washed three times with phosphate-buffered saline. Aliquots were kept for protein determination by the method of Lowry *et al.* (1951) using bovine serum albumin as standard, but the bulk of material was extracted with chloroform:methanol according to the method of Bligh and Dyer (1959). The lower phase lipids were subjected to mild alkaline hydrolysis (Kates, 1972) and chromatographed in chloroform:methanol:water (65:25:4, v/v/v), on silica gel H thin-layer plates using cerebroside sulfate as a standard. After preparative isolation of the cerebroside sulfate band and elution with chloroform:methanol:water (95:95:10, v/v/v), the counts per minute of [^{35}S]sulfate incorporated were determined by liquid scintillation counting in a Beckman LS-350 instrument.

RESULTS

Properties of Cell Lines

The origin and nature of continuous cell lines used in the present study are shown in Table 1. L-2 cells were found to be satisfactory as a fully permissive host in which MHV$_3$ and JHM can be grown to high titer and assayed. In the case of measles, Vero cells originating from monkey kidney served as the host for virus production and quantification.

Cells of neural origin are identified according to the type of tumor from which they originated and by salient biochemical properties which they have retained. Thus, lines C6 (Benda *et al.*, 1968), RN2-2 (Pfeiffer and Wechsler, 1972; Lucas *et al.*, 1977), G26-20, and G26-24 (Sundarraj *et al.*, 1975) continue to synthesize *in vitro* the nervous-

TABLE 1

CELL LINES

Designation	Species	Type	Cerebroside sulfate (determined by [^{35}S]sulfate incorporated)[a] (dpm/mg of protein)
RN2-2	Rat	Schwannoma	5650
C6	Rat	Astrocytoma	<300
HTC	Rat	Hepatoma	3800
L6	Rat	Myoblast/myocyte	ND[b]
L-2	Murine	Connective tissue fibroblastic	<300
C1300	Murine	Neuroblastoma "axonal"	<300
G26-20	Murine	Oligodendroglioma	ND[b]
G26-24	Murine	Oligodendroglioma	8400
Vero	Monkey	Kidney epithelial	4200

[a] Determined as described in Materials and Methods.

[b] Not done.

specific S100 protein and contain high concentrations of the 2':3'-cyclic nucleotide 3'-phosphohydrolase. Furthermore, G26-20, G26-24 (Dawson *et al.*, 1977), and RN2-2 cells (Lucas *et al.*, 1977) contain cerebroside sulfate, a lipid characteristically present in myelin. Absence of this sulfated glycolipid from C6 astrocytoma and mouse neuroblastoma C1300 cells is not surprising since neither originated from myelin-producing cells; however, the presence of detectable levels of this sulfolipid in Vero cells from monkey kidney and the HTC line of rat hepatoma shows that occurrence of cerebroside sulfate is not per se an adequate diagnostic marker for cells of glial origin.

Persistence of MHV$_3$ and JHM in Neural Cell Lines

The previously established tropism of the JHM agent for RN2-2 cells (Lucas *et al.*, 1977) led us to investigate in detail whether a similar interaction occurs with other neural lines. Following inoculation of monolayers with either JHM or MHV$_3$ viruses, persistent infections could be readily established. In the case of the mouse hepatitis viruses, infection in RN2-2 cells was rapidly aborted with MHV$_3$ but became cyclical in

the case of JHM, as shown in Fig. 1A and previously described (Lucas *et al.,* 1977). Thus the RN2-2 cell line is able to discriminate between the two coronaviruses. Associated with the JHM virus release, one could observe development of syncytia which were self-limiting in area, disappearing and reappearing continually, without ever completely destroying the monolayer. Infectious center assays demonstrated that during persistence there was also a cyclical increase in the fraction of virus-producing cells, ranging between 0.01 and 1%.
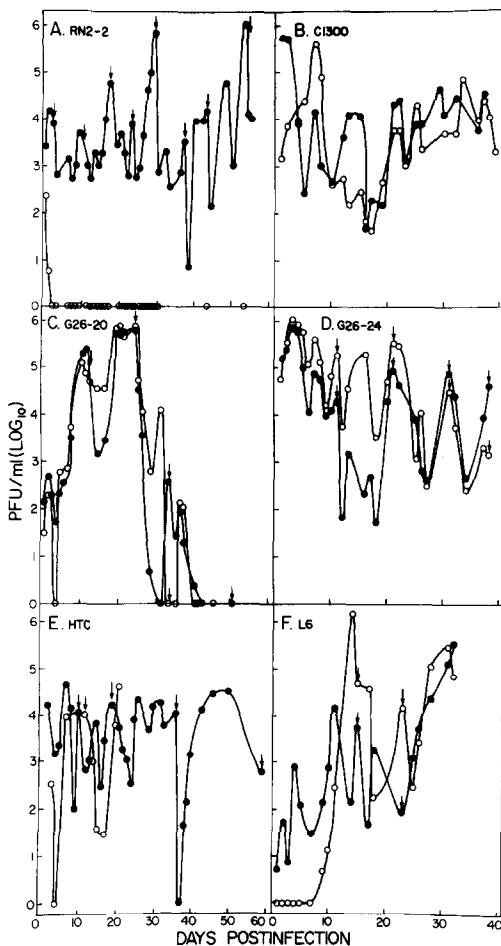

FIG. 1. Persistent infections with JHM and MHV₃ murine hepatitis viruses. Monolayer cultures of the various cell lines were infected at 32.5° with either the JHM or MHV₃ virus as described in Materials and Methods. Virus present in the medium was assayed using the plaque assay on L-2 cells. The arrows indicate when the cells were subcultured. ●———●, JHM infection; ○———○, MHV₃ infection.

Inoculation of the C6 rat astrocytoma failed to elicit any evidence for virus-induced cytopathology (CPE) or virus replication. Within 1 day after inoculation, the titer commenced to decline and become nil within 3 to 5 days. By contrast, infection of murine oligodendroglioma lines, G26-20 and G26-24, and neuroblastoma C1300 cell lines with MHV₃ and JHM readily initiated persistent infections, characterized also in each case by a cyclical rise and fall in titer (Figs. 1B, C, and D). Unlike the CPE present with JHM in RN2-2 cells, the infection of G26-20 or G26-24 cells did not elicit any syncytiogenesis, despite the fact that high titers comparable to those found in lytic infections of L-2 cells were recorded. Throughout the 50-day duration of the experiments, the fraction of cells scored as infectious centers also fluctuated between 0.1 and 100% in the case of JHM and between 0.06 and 100% for MHV₃ infecting either G26-20 or G26-24 cells.

Inoculation of the C1300 neuroblastoma with either coronavirus produced a more extensive and destructive CPE than observed when JHM infects RN2-2 cells. Most of the cells in C1300 monolayers were eventually involved. The remaining cells not recruited into the massive syncytia could repopulate the cultures but thereafter the CPE was again manifested. Over long term, cultures of C1300 continue to generate PFU for 3 or more months indicating that the infection is continually held in balance between a lytic and persistent state. Duration of infection could also be prolonged deliberately by reseeding almost completely destroyed cultures with uninfected C1300 cells. The number of infectious centers could not be determined because the cells released by trypsinization that remained as intact units and could be plated on indicator L-2 monolayers were not virus producers. Perhaps use of trypsin in this case eliminated all C1300-infected cells.

Persistence of MHV₃ and JHM in Cell Lines not Originating in the Nervous System

It is apparent that both the JHM and MHV₃ strains readily establish persistent infections in several neural cell lines, al-

though the ability to discriminate in favor of JHM, with complete suppression of MHV_3, was an unique property of the RN2-2 rat Schwannoma. It remained possible that this type of host regulation might be a species-related characteristic, although this seemed unlikely in view of the inability of either virus to replicate in the C6 rat astrocytoma. To further investigate species-related control of infectiousness, monolayers of rat HTC hepatoma and L6 myoblast cells were inoculated with JHM and MHV_3. Both corona agents elicited the characteristic CPE within 2 days postinfection. The syncytiogenesis was limited, never resulting in the complete destruction of the monolayers, despite the continuous elaboration of PFU in cyclical waves (Figs. 1E and F). Throughout the period of the experiments, the fraction of cells scored as infectious centers fluctuated between 0.7 and 22.4% in the case of HTC infected with JHM, and between 3 and 45% for the MHV_3 infection. The infectious centers with L6 cells remained relatively constant between 6.3 and 11.5% when JHM was the infecting agent and between 14 and 30% if inoculated with MHV_3. From this it becomes apparent that the capacity for discriminating between JHM and MHV_3 is an unique property of RN2-2 Schwannoma cells and is not species-related. These data also show that establishment of persistence by JHM and MHV_3 is not exclusive to neural cells but may also occur readily in cells originating from a hepatoma and in myoblasts.

Replication of Hallé and Edmonston Measles Viruses in Neural and Non-neural Cell Lines

The relative ease in establishing persistence with the coronaviruses led us to investigate in the same cell lines comparable associations with measles virus. For this purpose, the Hallé and Edmonston strains were used.

When fully permissive, Vero cell monolayers were inoculated with either strain at 32.5; at an m.o.i. of 0.01, large polykaryocytes first became apparent within 24 hr using the Hallé virus and within 36 hr with the Edmonston virus. Titers as high as 10^8 PFU/ml were determined on cell-free medium removed 4 days postinfection with the Hallé strain. At this time the entire monolayers had become virtually one continuous syncytium. Infection with the Edmonston strain produced lower titers, only 10^7 PFU/ml, of cell-free virus and at a slower rate since the peak was not reached until 6 to 7 days postinfection.

To investigate the replication of measles in the other non-neural and neural continuous cell lines, monolayer cultures were inoculated at 32.5° and examined for cytopathology, virus production, and development of infectious centers.

Infection of G26-20 and G26-24 cells proved to be aborted since there was no CPE or infectious virus formation. Instead, commencing 1 day after infection, there was a progressive eclipse of the inoculum to zero titer within 5 days. Restriction on virus production was maintained for longer than several weeks.

By comparison, inoculation of RN2-2, C6, HTC, and L6 rat cell lines with either Hallé or Edmonston virus readily established persistent infections. As illustrated in Figs. 2A–D, in all cases tested infectious particles were produced and released into the medium in a cyclical manner over a period of 1 to 2 months. Initially, the titers were relatively low, but with time higher virus yields were recorded. In none of the above associations was there any evidence of CPE, despite the fact that in some instances virtually all cells became infectious centers. Usually the fraction of cells shown to be infectious centers fluctuated between 5 and 80% with C6 cells, 0.05 and 60% with RN2-2 cells, 0.1 and 0.5% in the case of HTC cells and 1 and 10% with the L6 cells as the host for either virus. From the foregoing it is evident that inoculation of these particular rat cell lines at low m.o.i. with Hallé or Edmonston measles viruses leads directly to the establishment of persistent but inapparent infections.

Temperature-Sensitive Replication of Murine Hepatitis and Measles Viruses

The detailed mechanisms involved in the establishment and maintenance of persistent virus infection in vitro remain obscure. In several systems a general feature is the
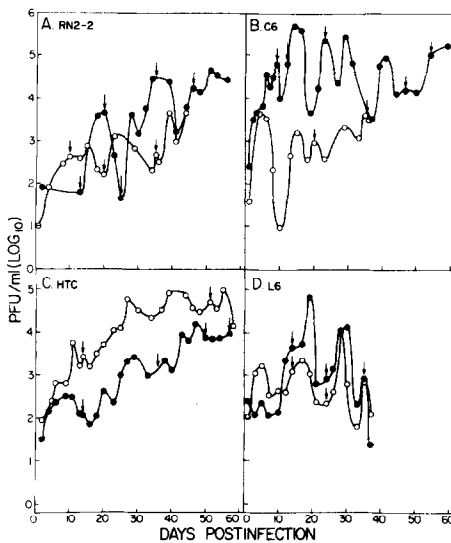
FIG. 2. Persistent infections with Hallé and Edmonston strains of measles virus. Monolayer cultures of the various cell lines were infected at 32.5° with either the Hallé or Edmonston virus as described in Materials and Methods. Virus present in the medium was assayed using the plaque assay on Vero cells. The arrows indicate when the cells were subcultured. ●——●, Hallé infection; ○——○, Edmonston infection.

outgrowth of or selection for a variant progeny virus that is temperature-sensitive for growth (Haspel et al., 1973; Preble and Youngner, 1973; Shenk et al., 1974; Gould and Linton, 1975; Kimura et al., 1975; Youngner and Quagliana, 1975; Youngner et al., 1976; Armen et al., 1977; Truant and Hallum, 1977). To determine whether a similar appearance of thermolabile progeny occurred in the cell–virus systems being examined here, temperature-shift experiments were conducted after initiating persistent infections. In some instances, cultures actively producing virus at 32.5° were moved to incubators set at 39.5°, then the medium was changed daily and assayed at 32.5° for PFU per milliliter.

With the Hallé and Edmonston persistent infections of RN2-2 cells, the elevated temperature caused an abrupt cessation of virus release. As indicated in Figs. 3A and B, the titer declined within 1 day, and no virus at all was present within 2 or 3 days following shift-up of the temperature, while cultures maintained at 32.5° continued to

yield PFU in the characterisic cycling fashion. Overall, very similar effects were observed with other persistently infected cell lines. One exception was L6 myoblasts carrying the Edmonston measles virus which continued to elaborate PFU at 39.5°, although the yield of virus was 1 log lower than at 32.5°.

The production of PFU from the majority of the coronavirus persistent infections was inhibited or profoundly reduced at the elevated temperature. Upon shift to 39.5°, the titer declined rapidly and production ceased completely within 4 or 5 days (data not shown). Coincidentally the cytopathology evident with several of the infections at 32.5° was absent at 39.5°. Only partial reduction of replication of JHM in HTC, L6, and G26-24 cells was demonstrated at 39.5°, whereby the titer was 2 log units lower than at 32.5°. With neuroblastoma C1300 there was no appreciable difference in JHM titer at the two temperatures. It should be remembered that coronavirus infection of C1300 cells borders between the persistent and lytic type.

Restriction of virus replication at 39.5° could also be instituted from the time of inoculation if infection was also made at 39.5° as shown in Figs. 4A–E exemplifying infections of representative cell lines by
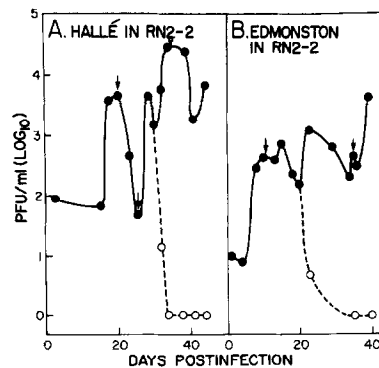
FIG. 3. Temperature-sensitive viral production in measles-infected RN2-2 cells. Cultures of RN2-2 cells persistently infected with either Hallé or Edmonston strains were trypsinized and split in half. One culture was maintained at 32.5° (●——●); the other, at 39.5° (○——○). The culture medium was changed daily and assayed for the presence of virus by the plaque assay at 32.5° on Vero cells. The arrows indicate when the cells were subcultured.
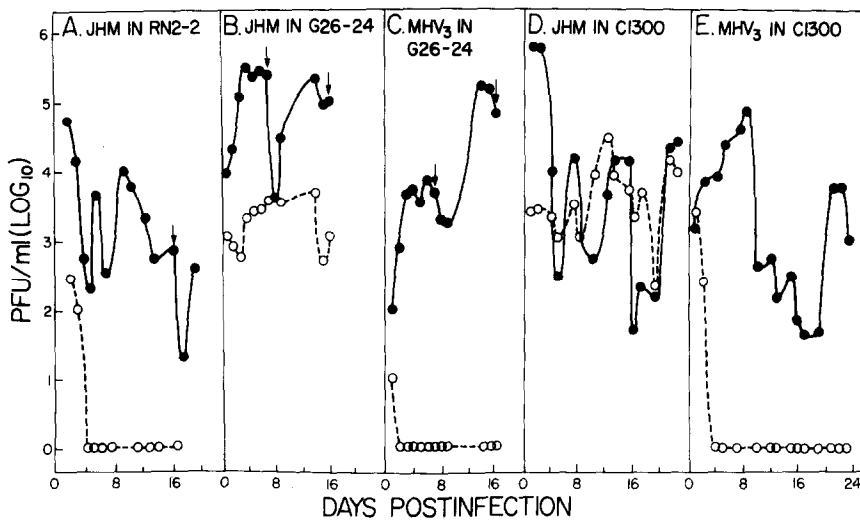
FIG. 4. Temperature-sensitive replication of JHM and MHV3. Monolayers of the various cell lines were infected with either JHM or MHV3 virus at 32.5 or 39.5° as described in Materials and Methods. The culture medium was changed daily and assayed at 32.5° for the presence of infectious virus as described in the legend to Fig. 1. The arrows indicate when the cells were subcultured. ●——●, 32.5° infection and incubation; ○——○, 39.5° infection and incubation.

murine hepatitis viruses. In most cases, for some of which the data are not shown, the results were like those with JHM infection of RN2-2 cells at 39.5° in which CPE was absent and virus production declined sharply until none was being made within 4 to 5 days. As in the exceptions described above for shift-up experiments, JHM infection of the G26-24, HTC, and L6 cells which commenced at 39.5° was incompletely suppressed and that of C1300 cells not at all inhibited.

Parallel infections commenced and maintained at 39.5° with the Hallé or Edmonston measles viruses in RN2-2, C6, HTC, and L6 cells gave results identical to those obtained after shift of carrier cultures from 32.5 to 39.5°. In all cases except that of the Edmonston virus infection of L6 cells, no PFU were formed.

It was possible that thermosensitivity of the replication process described above was due to the selection for and appearance of thermolabile variants of the measles and coronaviruses employed. This idea seemed unlikely, however, because thermosensitivity of infections carried out at 39.5° was immediate. Otherwise all four agents used must themselves have been thermolabile.

An answer to these alternatives was sought by plaque assays of progeny derived from lytic or persistent infections. Titers were compared on indicator cultures incubated at 32.5 or 39.5°. A summary of the data shown in Table 2 revealed that, with two partial exceptions, none of the progeny was thermolabile, whether originating from long-term or newly established cultures, nor was there any evident alteration in the plaque morphology which might have implied a change of the virus during passage in carrier cells.

One exception concerned the Hallé virus which was minimally temperature-sensitive whether it originated from permissive or partially restrictive host cells.

Following long-term MHV3 infection of C1300 cells, it appears that there may have been some selection for somewhat temperature-sensitive progeny viruses as indicated by reduction of 1 log in titer at 39.5 versus 32.5°.

It thus appears that in the majority of the carrier cultures, the host was conferring thermosensitivity upon the replication process without altering the genotype of the progeny. Furthermore, as summarized in Table 3, there was an almost absolute

TABLE 2

THERMOLABILITY OF VIRUS PROGENY RELEASED FROM PERSISTENTLY INFECTED CELL LINES WHEN TESTED IN FULLY PERMISSIVE HOST CELLS[a]

	Coronaviruses													
	L-2		C1300		G26-20		G26-24		RN2-2		HTC		L6	
Temperature (°C)	32.5	39.5	32.5	39.5	32.5	39.5	32.5	39.5	32.5	39.5	32.5	39.5	32.5	39.5
Titer (PFU/ml)	10^5	10^5	10^2	10^2	10^4	10^4	10^4	10^4	10^2	10^2	10^3	10^3	10^4	10^4
JHM	2.6	8.5	5	3	1.4	1.2	20	15	1.1	1.7	20	27	17	19
MHV₃	12	10	4	0.3	55	39	1.3	2.6	R(−)	R(−)	5.9	2.0	2.3	2.3

	Measles viruses									
	Vero		RN2-2		C6		HTC		L6	
Temperature (°C)	32.5	39.5	32.5	39.5	32.5	39.5	32.5	39.5	32.5	39.5
Titer (PFU/ml)	10^6	10^6	10^2	10^2	10^2	10^2	10^2	10^2	10^2	10^2
Hallé	61	25	990	500	4.2	2.2	2.3	1.8	5.2	1.3
Edmonston	2.3	36	2.3	6.3	3.5	9.0	8.2	3.0	1.4	2.0

[a] The virus being produced by cultures at 32.5° was assayed for temperature sensitivity. Aliquots of the medium were removed and assayed for the ability to form plaques on monolayers of either L cells (for JHM and MHV₃) or Vero cells (for Hallé and Edmonston) at both 32.5 and 39.5° as described in Materials and Methods. R(−) indicates replication negative.

correlation between the ability of specific cell types to undergo chronic infection and thermosensitivity of the replication process.

Reversibility of Temperature Restriction on Virus Replication

The host-regulated temperature sensitivity observed in cells persistently infected with the hepatitis and measles viruses raised questions concerning the reversibility of the thermosensitive restriction. To study this, we utilized persistent infections of RN2-2 cells by the JHM or the Hallé viruses. Long-term cultures actively producing virus at 32.5° were shifted to 39.5° and incubated until no PFU were being formed, usually 3 to 5 days. Then such cultures were returned to 32.5°, and the medium was changed and assayed daily for PFU per milliliter. With the JHM infection, if cultures were kept at 39.5° for up to 6 days, virus production recovered in most cases in 2 to 3 days following shift-down to 32.5°. In some instances when cultures were kept at 39.5° for 10 days, production of PFU resumed after shift-down. This recovery, however, required a longer duration at 32.5° before progeny reappeared.

Analogous data were derived from the measles infection. In most instances, virus production was resumed several days after shift-down of cultures that had been kept for as long as 10 days at the elevated temperature.

To discount the possibility that resumption of virus replication after temperature reversal from 39.5 to 32.5° was due to une-clipsed virus adsorbed at the cell surface, cultures were inoculated with JHM or Hallé viruses at 32.5 or 39.5°. After 1 hr to allow for adsorption, extracellular virus remaining was neutralized by adding specific antisera to cultures maintained at their respective temperatures. Some cultures were kept at 39.5° for several days with daily changes of medium, then were moved to an incubator set at 32.5°. Treatment with antiserum did not alter the recovery following shift-down indicating that intracellular genome expression must have been involved in resumption of virus production. These results further imply that both the JHM and Hallé viruses were maintained in a latent form at the restrictive temperature.

Replication of Vaccinia and Vesicular Stomatitis Viruses

The relative ease with which persistent infections with the coronaviruses and measles strains were established and the associated thermosensitivity of the replication

suggested that infections with many virus types may indiscriminately become persistent. To investigate this possibility monolayers of the various cell types were infected at 32.5 and 39.5° with IHD-W vaccinia virus at an m.o.i. of 5 PFU or vesicular stomatitis virus (VSV) at an m.o.i. of 0.01 PFU. Following 1 hr of adsorption, unadsorbed virus was washed away, and cultures were covered with nutrient medium. After 24 hr of incubation at 32.5 or 39.5°, the concentration of VSV in the medium was determined by plaque titration on L-2 cell monolayers. With vaccinia virus, combined cell-associ-

TABLE 3

SUMMARY DATA ON PERSISTENCE AND THERMOLABILITY OF VIRUS REPLICATION IN VARIOUS CELL LINES TESTED

Cell line	Virus	Outcome of infection			Temperature-sensitive restriction of replication
		Lytic	Abortive	Persistent	
L-2	JHM	+			
	MHV₃	+			
Vero	Edmonston	+			
	Hallé	+			
RN2-2	JHM			+	+
	MHV₃		+		
	Edmonston			+	+
	Hallé			+	+
HTC	JHM			+	(+)[a]
	MHV₃			+	+
	Edmonston			+	+
	Hallé			+	+
C6	JHM		+		
	MHV₃		+		
	Edmonston			+	+
	Hallé			+	+
L6	JHM			+	(+)[a]
	MHV₃			+	+
	Edmonston			+	(+)[a]
	Hallé			+	+
G26-20	JHM			+	+
	MHV₃			+	+
	Edmonston		+		
	Hallé		+		
G26-24	JHM			+	(+)[a]
	MHV₃			+	+
	Edmonston		+		
	Hallé		+		
C1300	JHM			(+)[b]	
	MHV₃			(+)[b]	(+)[c]
	Edmonston	ND[d]		ND[d]	
	Hallé	ND[d]		ND[d]	

[a] Restriction partial.
[b] In equilibrium between lytic and persistent.
[c] Virus progeny partially thermolabile.
[d] Not done.

ated and released virus was determined by titration on L-2 cell monolayers. VSV infection of the RN2-2, C6, HTC, and C1300 produced an extensive CPE and yielded high titers of virus at both 32.5 and 39.5°. VSV infection of the G26-20 and G26-24 cell lines, however, appears to be persistent at both temperatures, yielding low titers of virus over a period of at least several days, with no evidence of CPE. Vaccinia virus caused extensive CPE at both temperatures in the RN2-2, C6, HTC, and C1300 cell lines, although the viral production was decreased at 39.5° by 60% in the C1300 and by 90% in the C6 cells. In the vaccinia-inoculated G26-24 cell line kept at either temperature, the infection was apparently aborted since there was no rise in titer between 2 and 24 hr after inoculation; however, a virus induced CPE was observed.

DISCUSSION

Results from these studies focus attention on three salient findings: (1) The strains of measles and mouse hepatitis viruses used can readily become established in a persistent form of infection in cell lines of neural and non-neural origin; (2) almost invariably when the infection is of the persistent type, virus replication becomes thermosensitive due to unknown factors under host control; the virus progeny from persistent infections are themselves not thermolabile; and (3) among the many cell types tested a rat RN2-2 Schwannoma has the unique ability to discriminate between the prototype MHV_3 and the neurotropic variant, JHM, supporting the persistence of only the latter.

The usual outcome of infecting a spectrum of cells originating from neural and non-neural tissue with any one among JHM, MHV_3, Hallé, or Edmonston viruses is the development of persistence as documented in Table 3. The systems under investigation here differ from some of those examined previously by others in the directness, speed, simplicity, and reproducibility in establishment of chronic infections after inoculation at low m.o.i. The latent period may be less than 1 day in duration, and no additional manipulation is required, such as repeated virus passage at high titer

or the addition of neutralizing antibody. Although each type of cell–virus association examined here exhibits some individual peculiarity, one feature characteristic of all is the cyclical rise and fall of low virus titers extending over a period of at least several weeks and perhaps continuing indefinitely. The murine hepatitis viruses can infect chronically both murine and rat cell lines, but a small fraction of the cells in culture become infectious centers. The cytopathology produced may be self-limiting as in the case of rat cells RN2-2, HTC, and L6, or verge on the lytic when C1300 neuroblastoma is the host, or exhibit no evident CPE as with murine oligodendroglioma lines G26-20 and G26-24.

The Hallé and Edmonston strains are incapable of infecting the murine cells tested indicating that in this case there may exist a species-related restriction. A large fraction of measles-infected cells in a culture can become infectious centers without any apparent relationship to the extent of CPE, if any, associated with the infectious process.

Although the molecular events underlying the maintenance of persistence in the systems under study here remain to be elucidated, in other virus carrier states chronic infections have been explained by effects involving the formation of and competition from defective interfering (DI) particles for host synthetic machinery (Holland *et al.*, 1976; Kawai and Matsumoto, 1977), or by inhibition of virus production due to interferon, sometimes in a cyclical fashion (Wiktor and Clark, 1972), or due to selection of genotypically changed variants of the virus (Thacore and Youngner, 1969; Kawai *et al.*, 1975; Youngner *et al.*, 1976; Truant and Hallum, 1977).

Involvement of DI particles in the present studies seems to be unlikely because the inoculation was made at low m.o.i. and medium was changed daily which should have minimized the production or accumulation of DI particles, unless such particles can somehow be maintained in a cell-associated state. Nevertheless, some direct proof for discounting any role for DI particles has not been provided.

Evidence with the JHM persistent infec-

tion of RN2-2 cells for the involvement of an interferon-like mechanism comes from the partial resistance to superinfection with VSV (Lucas *et al.,* 1977). It was not possible, however, to transfer interference against VSV to uninfected RN2-2 cells by material in medium taken from persistently JHM-infected RN2-2 cultures. Clearly additional work is required to ascertain whether interferon or some other factors function in the chronic infectious process under study by us.

As indicated earlier, a common feature of other virus-carrier systems is the outgrowth of or selection for a variant progeny virus that is temperature-sensitive for growth. But, for a majority of chronic infections with mouse hepatitis and measles viruses examined currently, the situation is quite different. Despite a drastic suppression of virus production at the elevated temperature, the progeny with one exception, are not thermolabile when tested in permissive cells. The thermal restriction instead appears to be host-controlled. In fact, there is a close correlation between the capability for establishment of persistence and thermosensitivity of the virus replication process (Table 3). The basis for temperature restriction at 39.5° remains unknown. It should not be forgotten that control leading to persistence and thermosensitivity of the replication process with other agents can be virus- as well as host-specified as indicated in the case of VSV which has a full productive cycle at 32.5 and 39.5° in the RN2-2, C6, C1300, or HTC cells, but is restricted to a chronic infection in G26-20 or G26-24 cells. Similarly, replication of vaccinia is fully productive at 39.5° in the RN2-2 and HTC cells and is only partially depressed in C6 and C1300 cells. The host function(s) controlling the temperature restriction of virus production do not appear to be involved in the growth and viability of host cells as shown by growth rates or plating efficiencies which are similar at 32.5 and 39.5° for several of the lines tested (unpublished observations).

The present experimental data indicate that thermosensitivity is a reversible process. The factors involved in this recovery process or the state of the virus information

during suppression remain unknown. The data already in hand strongly indicate that at elevated temperatures both the corona and measles agents can be maintained in some latent form; however, preliminary results based on infectious center assays with the measles-infected cells suggest that under the nonpermissive conditions, the virus information is segregated unequally among all the daughter cells.

The ability to distinguish specifically between the JHM and MHV$_3$ viruses described previously (Lucas *et al.,* 1977) and in this report appears to be an unique property of the rat RN2-2 cell line. The capability for discrimination between the prototype and neurotropic variant is not species-related because neither virus can replicate in the rat C6 astrocytomas, yet both agents readily establish persistence in the rat L6 myoblasts and HTC hepatoma cell lines. Some preliminary experiments indicate, however, that the restriction on MHV$_3$ multiplication can be circumvented if RN2-2 cells are inoculated at an m.o.i. of 10 or greater. Under such circumstances, the CPE becomes extensive and rapid, resulting in a total destruction of the monolayer culture. Progeny from this infection when injected into mice cause necrotizing meningitis essentially identical to that characterizing infection with MHV$_3$ propagated in the permissive L cells (Lucas *et al.,* 1977). Thus, the restriction on MHV$_3$ appears to involve some quantitative equilibrium controlled by both the virus and its host.

Taken together, the results described in this report suggest that the host cell has a profound influence in regulating the replication process of agents with neurotropic potential. The relative ease with which persistence and thermosensitivity develop in the cell lines examined provides new systems for inquiries into the molecular events and mechanisms by means of which certain viruses operate as slowly acting pathogens in the nervous system.

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