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In Vivo and in Vitro Models of Demyelinating Diseases: Tropism of the JHM Strain of Murine Hepatitis Virus for Cells of Glial Origin

Alexandra Lucas, Wayne Flintoff, Robert Anderson, Dean Percy, Marion Coulter and Samuel Dales
Cytobiology Group
Department of Bacteriology and Immunology
University of Western Ontario
London, Ontario N6A 5C1
Canada

Summary

Infection of mice with the neurotropic JHM strain of murine hepatitis virus causes demyelinating lesions resulting from an infection of the oligodendroglia. This was most evident in mice inoculated intraperitoneally with JHM. Such CNS lesions were not observed in mice inoculated intraperitoneally with the MHV_3 strain. An in vitro system is described in which the rat glial RN2 cell line functions as a discriminating host for the JHM virus. Shortly after inoculation, this virus establishes a persistent infection in which there is a cyclical rise and fall in titer with an accompanying cytopathology. Furthermore, this host cell confers a thermal lability which the virus does not demonstrate in the fully permissive host cell, L-2. By comparison, infection of RN2 cells with the prototype MHV_3 is aborted immediately. In the persistent infection of RN2 cells with measles virus, Hallé strain, the cell again confers a temperature sensitivity which the virus does not possess when replicating in Vero cells.

This appears to be the first instance in which a cloned cell line of glial origin determines the outcome of the infectious process, discriminating in favor of a neurotropic variant which possesses a tropism for the glia in vivo. Systems such as the one described here may now offer a specific screening procedure for selecting, identifying and characterizing the nature of neurotropic viruses.

Introduction

One of the most attractive systems available for studying phenomena pertinent to human demyelinating disease such as multiple sclerosis (MS) involves infection of the central nervous system (CNS) by the JHM variant of the murine coronavirus, mouse hepatitis virus, MHV (Bailey et al., 1949; Cheever et al., 1949; Weiner, 1973). This agent causes pathological effects, including zones of demyelination, similar in some aspects to the plaques of human MS. While the JHM virus was initially isolated from a mouse showing paralytic symptoms (Cheever et al., 1949), another variant producing a similar CNS pathology was subsequently derived from mice injected with murine hepatitis virus, MHV_3 (Dick, Niven and Gledhill, 1956). These two independent isolates have maintained their neurotropsim and, after numerous passages, continue to produce CNS demyelination in both mice (Bailey et al., 1949; Weiner, 1973; Herndon et al., 1975) and rats (Bailey et al., 1949). As a further analogy to the human disease, demyelination with JHM may occur intermittently, particularly when older mice are inoculated with JHM (Herndon et al., 1975).

Examination of the CNS by immunofluorescence (Weiner, 1973) and electron microscopy (Lambert, Sims and Kniazeff, 1973) has revealed that JHM-specified products are in cells of the white matter, specifically in the cytoplasm of oligodendrocytes. Since immunosuppression of JHM-infected mice does not alter either the intensity or pattern of CNS lesions (Weiner, 1973), the mechanism of demyelination is presumed to involve a more direct effect of the virus on its target cells, the oligodendroglia.

This murine system acquires an even greater significance as a model for MS because JHM is serologically related to human coronavirus 229E, associated with upper respiratory disease especially in children (McIntosh, 1974). In this regard, it is worth mentioning that electron microscopic examination of a single biopsy from a terminal MS patient by Tanaka, Iwasaki and Koprowski (1976) revealed the presence of numerous intracisternal particles resembling, morphologically, a coronavirus.

In undertaking further studies using the murine model with a view to advancing our understanding of JHM tropism for glial cells, we deemed it logical and desirable to develop an in vitro system that might be a counterpart to the in vivo events. We now report the chance discovery of a selection process demonstrated by a rat glial tumor cell line which discriminates between the parental and neurotropic variants of JHM. In this cell-virus interaction, the host becomes persistently infected, causing cyclical manifestations of cytopathology and virus production.

Results

Growth in Mouse L-2 Cells

Since we wished to compare the growth of the MHV_3 and JHM virus strains in various cell lines, it was necessary to use a cell type that was fully permissive for both agents. The cell selected for this purpose was the mouse L-2, a subline of mouse L-929 (Rothfels et al., 1959).

Following infection of L-2 monolayers with either virus at 32.5°C, using a multiplicity of infection (moi) of 0.01, multinucleate giant cells first became apparent at 16 hr. Maximum titers of 10^6 PFU/ml of cell-free virus in the medium were recorded 30 hr

postinfection, at the time monolayers had become virtually one continuous syncytium.

The capability of MHV_3 and JHM to grow to high titer in L-2 cells allowed the use of these cells as host for routine virus propagation and assay by the plaque technique (see Experimental Procedures).

Growth in Rat Glial Cells

The presence of JHM antigen and virus in the oligodendrocytes of infected mice (Weiner, 1973; Lambert et al., 1973) led us to investigate whether this neurotropic variant and its prototype, MHV_3, were able to replicate in vitro in cells of glial origin. For this purpose, we tested several rodent cell lines, including RN2-2 rat glial cells. Monolayers were inoculated at 32.5°C with MHV_3 or JHM at an moi of 0.01. Following adsorption for 1 hr, unadsorbed virus was removed, and the cultures were overlaid with nutrient medium. We examined the monolayers for development of virus-induced cytopathology, and following daily replacement of the medium, we used aliquots for assay of infectious non-cell-associated virus.

After inoculation of RN2-2 glial cells with MHV_3, there was no evidence whatsoever of any virus-induced cytopathic effects (CPE). Within 1 day after inoculation, the titer declined sharply, and within 3 days, infectious virus disappeared completely, as illustrated in Figure 1. An infectious center assay, summarized in Table 1 revealed that if present at all, MHV_3 must have been harbored by $<1:10^4$–10^5 cells.

By contrast, following inoculation with JHM, a CPE, similar to that formed with fully permissive L-2 cells, occurred within 2 days postinfection. The CPE was characterized by the formation of large flat polykaryocytes, illustrated in Figures 2A and 2B. Unlike the infectious process with L-2 cells, however, where continuous unremitting cell-cell fusion occurs, ultimately involving the entire monolayer, with RN2-2 cells, the syncytia developed only to a limited extent and remained discrete in size—at least during the initial period of 2 weeks after infection—even though virus was being continually shed into the medium (indicated in Figure 1). It thus became apparent that JHM virus could readily establish a persistent infection in RN2-2 glial cells. Judging by PFU/ml in the medium, virus was produced in a cyclical manner over a period of at least 5 weeks and five cell passages. During the time period of the experiments, as indicated in Table 1, the proportion of cells harboring infectious JHM also fluctuated. It should be noted that occasionally after releasing relatively large amounts of infectious JHM, virus production decreased to virtually undetectable levels (see experiment 2 in Figure 1), but upon continued maintenance of the culture, virus titer rose once more.

During prolonged maintenance of individual, persistently infected cultures, the monolayers underwent so-called "crisis" phenomena. These became manifested as dramatic increases in the number and size of syncytia, the quantity of PFU released and the proportion of cells scored as infectious centers. Although on occasion virus titers in RN2-2 cultures reached levels comparable to those in cultures of L-2 cells, the infection never progressed to the stage that RN2-2 monolayers were completely destroyed. Rather, the infectious process apparently became self-regulating, whereby the cultures recovered following the repopulation by daughter cells of the survivors. The number and size of the syncytia were reduced, and the released virus in proportion to cells scoring as infectious centers was drastically curtailed. To date several

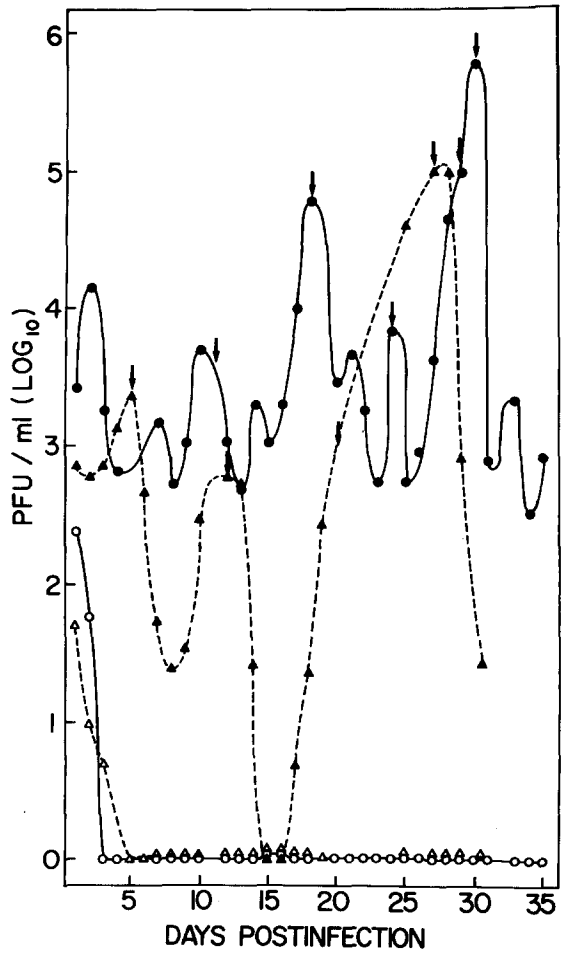

Figure 1. Infection of Rat Glial Cells with Murine Hepatitis Virus

Monolayers of RN2-2 cells were infected as described in the text. Virus present in the medium was assayed using the plaque assay on L-2 cells as described in Experimental Procedures. Circles, solid line-experiment 1; triangles, dashed line-experiment 2. Open symbols-MHV_3 infection; closed symbols-JHM infection. Arrows indicate when the cells were subcultured.

Table 1. Infectious Center Assay of MHV_3- and JHM-Infected RN2-2 Cells

Passage Number	Infectious Centers			
	Experiment 1		Experiment 2	
	MHV_3	JHM	MHV_3	JHM
1	$<5 \times 10^{-5}$	1.3×10^{-3}	$<5 \times 10^{-5}$	2×10^{-4}
2	$<5 \times 10^{-5}$	7.0×10^{-3}	$<1 \times 10^{-4}$	1×10^{-3}
3	$<1 \times 10^{-4}$	3.5×10^{-3}	ND[a]	ND[a]
4	$<1 \times 10^{-4}$	1.0×10^{-1}	$<1 \times 10^{-4}$	1×10^{-2}
5	$<1 \times 10^{-4}$	4.7×10^{-2}	ND[a]	ND[a]

[a] Not done.

independently inoculated RN2-2 cultures conformed to the same pattern and have undergone a number of crises and recovery cycles.

Although to perpetuate each of the above cultures, we passaged them at approximately weekly intervals by releasing the attached cells with trypsin, this manipulation did not affect either the nature of the carrier state or the amount of infectious virus released within 24 hr after subculture.

Properties of Virus Released from the Carrier Culture

To determine whether the JHM virus had been genetically altered in any obvious manner as a consequence of being propagated in the glial cells, virus released from carrier cells was tested for its ability to reestablish a persistent infection in RN2-2 cells, and for its capacity to form plaques on L-2 cells at 32.5 and 39.5°C. JHM released from the carrier culture could once again readily establish a persistent infection in the rat glial cells in the same way as the L-2 cell-grown virus. JHM derived from the carrier culture was not temperature-sensitive for growth on L-2 cells, since the number and size of plaques formed at 39.5 and 32.5°C were the same. Furthermore, the appearance of plaques formed on L-2 cell monolayers by JHM grown on RN2-2 cells is indistinguishable from that of JHM propagated in L-2 cells. It may be of significance, however, that JHM virus, when propagated in RN2-2 cells, is highly temperature-sensitive. This became evident upon shift of RN2-2 carrier cultures from 32.5 to 39.5°C, which resulted in a complete suppression of CPE and the release of PFU into the medium. The fact that freshly inoculated JHM virus failed to replicate but became eclipsed in RN2-2 cells at 39.5°C is consistent with this observation. It is interesting that measles virus, Hallé strain, similarly acquires thermal lability when propagated persistently in RN2-2 cells (manuscript in preparation). These results imply that expression of temperature

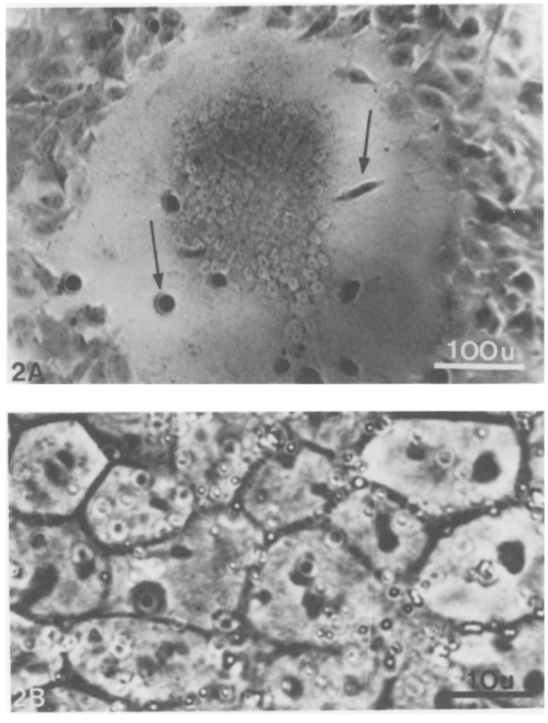

Figure 2. Cytopathology of JHM Infection in RN2-2 Cells

(A) Representative area of a persistently infected RN2-2 culture illustrating the appearance of a polykaryocyte among cells of normal size. Note the presence of a multitude of nuclei at the cell center, and elongated and rounded cells on the surface of the cell syncytium (arrows). (B) Selected area from the center, of the polykaryocyte in (A) illustrates the presence of closely packed nuclei.

restriction on JHM and measles virus was somehow conferred by a property of the host cell rather than by the virus itself.

Heterologous Viral Challenge

To determine whether the JHM persistently infected RN2-2 cultures were resistant to superinfection by an unrelated virus, vesicular stomatis virus (VSV) was inoculated onto either uninfected or persistently infected RN2-2 cells (passage 5 or 6) at an moi of 0.1. Following incubation for 24 hr at 32.5°C, the yield of PFU of VSV in the supernatant of each culture was assayed on monolayers of both L-2 and RN2-2 cells. In two separate experiments, we found that yields of VSV from the JHM persistently infected RN2-2 culture, whether determined on L-2 cells or RN2-2 monolayers, were only about 10% of those from uninfected RN2-2 cells.

Biological Properties of the Viruses

Although the virus produced by the persistently infected cells showed no apparent genetic alteration in vitro, it was of interest to determine whether any of its in vivo biological properties may have

been altered. For testing this possibility, Swiss mice of various ages were injected with graded doses of JHM that had been propagated in L-2 or RN2-2 cells.

Mice inoculated with the JHM strain, grown either on L cells or on RN2-2 cells, exhibited clinical evidence of neurological involvement (ataxia, incoordination and paresis) as early as 36–48 hr post-inoculation. In general, mice that succumbed to the infection died from 2–5 days post-inoculation. Those that showed no clinical evidence of disease during this period usually remained healthy throughout the observation period of 14–28 days.

Histopathological examination of the CNS from mice infected with the JHM strain revealed lesions that were relatively disseminated in the gray and white matter of the prosencephalon and metencephalon. Necrotic and reactive lesions were frequently observed in the hippocampus and brain stem regions. Lesions were frequently destructive in nature, accompanied by mononuclear and polymorphonuclear cell response. Leptomeningitis and ventriculitis were observed in numerous animals. Occasionally, vacuolated areas were observed in the white matter of the areas in the ventricular regions, brain substance and leptomeninges. We interpreted those syncytial cells in the brain substance to be comprised of astroglial cells and inflammatory cells. Multinucleated cells present in the region of the lateral ventricles appeared to be formed from ependymal cells, as indicated in Figure 3.

By contrast, when mice were inoculated intracerebrally with the MHV_3 strain, there was a striking leptomeningitis, with polymorphonuclear and mononuclear cell infiltration. In some animals, histological changes were exclusively confined to the meninges. Destructive and reactive lesions, when evident, occurred within a variety of areas of the brain substance. Cell syncytia, reminiscent of those occurring after in vitro infection, were frequent and indistinguishable in appearance or location from those found in the CNS of mice inoculated with the JHM strain. In the liver, disseminated foci of hepatic necrosis were frequent, emphasizing the viscerotropic properties of MHV_3.

A comparison of the histological findings of the MHV_3- and JHM-inoculated mice reveals the following general differences. First, those mice with CNS lesions due to MHV_3 inoculation had a more striking necrotizing meningitis than animals inoculated with the JHM strain. Second, the involvement of white matter and histological evidence of demyelination were more striking in JHM-infected mice than in MHV_3-inoculated mice. The destructive lesions of the white matter were characterized by vacuolation, disruption of the normal architecture

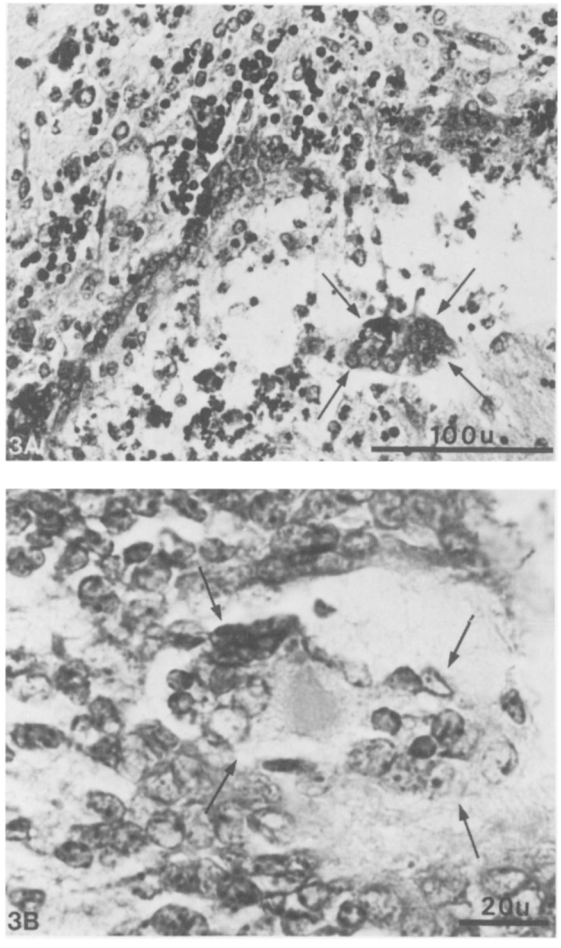

Figure 3. Histopathology of Lesions in the Brain of a Mouse Infected with JHM

(A) Region of lateral ventricle from mouse L5-2, killed at 14 days of age and 2 days post-inoculation with 2.3×10^3 PFU of JHM virus. The multinucleated giant cell (arrows) is comprised of ependymal cells. Inflammatory cells are present in the ventricular lumen and periventricular regions. Haematoxylin and eosin (H and E) staining. (B) Higher magnification of another multinucleated cell in the region of lateral ventricle from animal L5-2. Note the orientation of nuclei at the periphery of the polykaryocyte.

and relative sparing of neurons in affected areas, and were most evident in mice inoculated with JHM at 16 or more days of age. Third, hepatic lesions were observed more frequently in MHV_3-infected animals than in those inoculated with JHM. Fourth, histological evidence of oligodendrocyte damage and demyelination was observed in mice surviving JHM infection. Similar observations have been previously described by Weiner (1973) and Herndon et al. (1975). Conversely, CNS lesions were not observed in mice surviving infection with MHV_3. Fifth, intraperitoneal inoculation of JHM frequently produced striking white matter involvement, while CNS lesions were not observed in mice inoculated intraperitoneally with MHV_3. Death in these MHV_3-

inoculated mice was attributed to the extensive liver damage that occurred.

Discussion

An in vitro system has been described in which a glial cell line supports the growth of a neurotropic virus. Infection of the glial cells with JHM virus readily establishes a persistent cyclical infection in which virus is shed at a low level into the medium and in which a small percentage of the cells is infected. Preliminary immunofluorescence experiments indicate that only a very small fraction of the cells contain viral antigen (unpublished observations). At the time of writing, the persistent infection was maintained for 8 weeks with no apparent alterations in the cyclic pattern of CPE and virus release. The infection appeared to be self-limiting, since at no time was there a complete destruction of the cell monolayer. Infection of the glial cells by the prototype virus MHV_3, on the other hand, was neither productive nor persistent, since there was no detectable virus replication, and the fraction of cells scoring as infectious centers was at best very small indeed. Infection with either JHM or MHV_3 in the glial cells is clearly quite different from that associated with L-2 cells. In the latter case, both viruses undergo fully productive replication cycles, culminating in a complete destruction of the culture.

The tropism of the JHM strain for the rat RN2-2 glial cells uncovered in the present study indicates that a host cell can exert a selective pressure on a virus and determine the outcome of an infection. The ability to discriminate between the JHM and MHV_3 viruses, however, is not a general property of all cultured rodent glial cells examined by us to date. As indicated in Table 2, the C6 line, an astrocytoma-derived line of rat cells (Benda et al., 1968), failed to become infected altogether by JHM or MHV_3. By contrast, inoculation of glial lines G26-20 and G26-24 of murine origin (Sundarraj, Schachner and Pfeiffer, 1975) resulted in the establishment of persistent infections with both JHM and MHV_3. In these latter cases, the carrier state is associated with restricted CPE and cyclical production of low quantities of infectious virus. To date, the ability to discriminate between JHM and MHV_3 therefore appears to be a rather unique property of the RN2-2 cells.

As indicated in Table 2, the Hallé strain of SSPE measles virus (Horta-Barbosa et al., 1971) readily establishes a persistent infection in both the rat RN2-2 cells and C6 cells. The RN2-2 cells are not a strain that is generally nonpermissive or susceptible to the indiscriminate establishment of the carrier state, because, as summarized in Table 2, these cells can fully support the replication of representative agents from several virus groups tested, including VSV, vaccinia virus and mengo virus.

The molecular events by which JHM establishes and maintains its persistent infection and by which MHV_3 is suppressed in the RN2-2 cells remain to be elucidated. In other similar carrier systems, several mechanisms have been proposed. These include competition at the synthetic level by defective interfering (DI) particles (Holland et al., 1976; Kawai and Matsumoto, 1977), cyclical suppression of virus production by interferon (Wiktor and Clark, 1972) and genotypic alterations of the virus (Thacore and Younger, 1969; Kawai, Matsumoto, and Tanabe, 1975; Younger et al., 1976; Truant and Hallum, 1977).

In the JHM persistent infection, the role of DI particles may be improbable since the initial moi used was low, and by changing the medium daily, and DI particles that may have accumulated would have been removed unless they somehow had remained cell-associated. Nevertheless, this argument is based on circumstantial evidence, so that proof against the presence of DI particles must await direct determination.

Partial resistance of RN2-2 cells, persistently infected with JHM, to superinfection with VSV suggests that interferon or an interferon-like mechanism may be functioning. Clearly, additional work is also required to ascertain whether interferon may be a factor in the observed persistence. By analogy to the present system under study, reference should be made to a persistent infection of BHK-21 cells by rabies virus (Wiktor and Clark, 1972). In this system, the virus yield and interferon levels are produced in a cyclical sequence whereby the times when high levels of interferon are present coincide with periods of low yields of virus, and vice versa.

A common feature of other viral carrier systems is the outgrowth of, or selection for, a variant progeny virus that is temperature-sensitive for growth. Thus temperature-sensitive mutants have been isolated in persistent infections of measles virus (Haspel et al., 1973; Gould and Linton, 1975; Armen et al., 1977), Newcastle disease virus (Preble and Younger, 1973; Younger and Quagliana, 1975), VSV (Younger et al., 1976), Sindbis virus (Shenk, Koshelmyk and Stollar, 1974), mumps virus (Truant and Hallum, 1977) and Sendai virus (Kimura et al., 1975). The JHM restriction at elevated temperature is not associated with growth in the fully permissive L-2 cells, however, but only in RN2-2 cells. The JHM strain fails to establish persistence when inoculation is carried out at 39.5°C and is not replicated when carrier cultures are shifted from 32.5 to 39.5°C. Similarly, temperature sensitivity is con-

Table 2. Comparison of Interactions of Several Viruses with Cells of Glial Origin

Cell Strain	Cell Type and Species	Infection with[a]	Outcome of Infection[b]
L-2	Murine fibroblast	MHV_3 (coronavirus)	Fully permissive
		JHM (coronavirus)	Fully permissive
		VSV (rhabdovirus)	Fully permisive
		Vaccinia (poxvirus)	Fully permissive
		Mengo (picornavirus)	Fully permissive
RN2-2	Rat glioblastoma	MHV_3	Abortive
		JHM	Persistent
		VSV	Fully permissive
		Vaccinia	Fully permissive
		Mengo	Fully permissive
		Measles (Hallé SSPE strain, paramyxovirus)	Persistent
C6	Rat astrocytoma	MHV_3	Abortive
		JHM	Abortive
		Measles (Hallé SSPE strain)	Persistent
G26-20 and G26-24	Murine glioblastoma	MHV_3	Persistent
		JHM	Persistent

[a] Monolayers of the cell strains were infected with the various viruses at an moi of <0.1 and usually 0.01. After 1 hr for viral adsorption at 32.5°C, the infected cultures were overlayed with MEM medium.
[b] Fully permissive = release of infectious virus into the medium and destruction of the monolayer; abortive = no infectious virus released, no cell destruction and no detectable cells scoring as infectious centers; persistent = low levels of infectious virus released over a period of weeks, cell monolayer never completely destroyed and varying number of cells scoring as infectious centers.

ferred on replicating measles virus in RN2-2 cells (manuscript in preparation). When taken together, these results imply that the determination of the outcome of the infection, whether fully productive, persistent or aborted, is controlled at least in some measure by the host cell.

Nevertheless, the biological properties of either MHV_3 grown on L-2 cells or JHM grown on L-2 cells or RN2-2 are not host-modified as a result of passaging through the different hosts. Thus the pathological manifestations produced by MHV_3 grown in mice or on L-2 cells are like those previously described for MHV_3 (Piazza, 1969), while the CNS lesions resulting from inoculation by JHM, whether derived from brain passage, L-2 cells or RN2-2 cells, are similar to those previously reported by Bailey et al. (1949), Pappenheimer (1958), Waksman and Adams (1962) and Weiner (1973).

Further investigations currently in progress on the mechanisms of restriction and the persistence of coronaviruses in the rat glial cells may provide significant new information concerning the basis of the tropism for glial cells, and may elucidate the factors involved in the establishment and maintenance of persistence in a system having biological significance.

Experimental Procedures

Cells and Cell Culture

The rat glial RN2 cells were obtained from Dr. S. E. Pfeiffer (University of Connecticut). They are classified as glial because of their origin from a carcinogen-induced glioblastoma, their ability to synthesize the nervous system-specific protein S-100 (recently confirmed for us by A. Marks, Banting and Best Institute, Toronto) and a high specific activity of 2′,3′-cyclic nucleotide -3′-phosphohydrolase (Pfeiffer and Wechsler, 1972). We have subsequently shown that these cells also contain a sulfolipid, presumably cerebroside sulfate, a diagnostic feature of glial cells. The sulfolipid was labeled after growth of the cells in ^{35}S-sulfate-containing medium, was stable to mild alkali and co-chromatographed with authentic cerebroside sulfate used as a standard on silica gel H thin-layer plates developed in chloroform-methanol-water (65:25:4, V/V). The data from repeated experiments showed that ^{35}S was indeed incorporated into sulfolipid at an average of 7200 dpm per 10^7 cells. These observations are at variance with the recent results of Dawson, Sundarraj and Pfeiffer (1977), who were unable to show any incorporation of ^{35}S into sulfolipid, perhaps due to different culture conditions.

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/1), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

Prior to use, the cells were cloned by limit dilution in 96-well Microtest II trays (Falcon Plastics). One clone, RN2-2, was used in this particular study.

Mouse L-2 cells, a subline of L-929 cells (Rothfels et al., 1959),

were maintained either as monolayer cultures in the same medium used for RN2-2 cells, or as suspension cultures in this medium supplemented with methyl cellulose 15 cps (0.1%).

Viruses

The murine hepatitis viruses, MHV_3 and JHM strains, were obtained from the American Type Culture Collection (Rockville, Maryland). The MHV_3 virus was obtained as a 10% mouse liver-spleen suspension with a passage level of SM (VSBS/10–20). The JHM strain was obtained as the third passage in NCTC 1469 cells and had a titer of 10^3 TCD_{50}/0.2 ml. Both viruses were routinely maintained by daily passage on L-2 cell monolayers at an moi of 0.01. The virus was allowed to adsorb at 32.5°C for 1 hr before medium was added. Various L-2 cell passage viruses have been used for both infection of the glial cells and injection of animals.

Plaque Assay

Virus production was monitored by a plaque assay on L-2 cell monolayers. L-2 cells were grown in 6-well multidishes (Costar) at 37°C in a humidified atmosphere of 5% CO_2. Confluent monolayers were inoculated with 0.2 ml of virus dilution in phosphate-buffered saline (PBS). After adsorption at 32.5°C for 1 hr, the infected cells were overlaid with 3 ml of MEM containing 10% FBS and 0.5% methyl cellulose, 4000 cps. The plates were incubated at 32.5°C for 2 days in the humidified atmosphere, after which the medium was removed and the cells were fixed with neutral buffered formalin. The plates were then stained with 0.1% crystal violet. The virus titer is represented as the plaque-forming units per ml (PFU/ml). There is no apparent difference in the morphology or the size of the plaques formed by the two virus strains on L-2 cells.

Infectious Center Assay

To determine the percentage of glial cells releasing infectious virus, infected monolayers of RN2-2 cells were washed and trypsinized. After trypsinization, various dilutions of the cells were plated on L-2 cell monolayers. After 3–5 hr at 32.5°C, the plates were overlaid with the methyl cellulose-MEM mixture, as described for the plaque assay, and processed in a similar manner.

Light Microscopy

Persistently infected RN2-2 cells grown to confluency on coverglass slips were fixed in situ for 30 min in 0.05 M phosphate-buffered 2% glutaraldehyde solution (pH 7.4), washed with PBS, postfixed in 0.05 M phosphate-buffered 1% osmium tetroxide, washed again with PBS and mounted in PBS for examination by phase-contrast microscopy.

Animal Injection and Histological Preparation

Swiss mice (Bio Breeding Laboratories, Ottawa, Ontario), ranging in age from 3 days to 4 weeks, were inoculated intracerebrally or intraperitoneally with either the MHV_3 or JHM viral strain grown in cell culture. Approximately 0.01 ml of virus in culture medium was injected into each animal by means of a syringe. Control littermates received the same volume of culture medium free of virus.

Animals were observed for at least 2–4 weeks post-inoculation for evidence of neurological involvement.

All mice that died or were euthanized in extremis or killed at the termination of the observation period were necropsied for histological and virological evaluation. Tissues collected routinely for microscopic studies included brain and spinal cord. Other tissues examined in selected cases included the liver and the large and small intestine. Tissues destined for histology were fixed by immersion in, or perfusion with, 4% glutaraldehyde or Bouin's fluid. Both sagitally and transversly cut pieces of the CNS were embedded in paraffin, sectioned at 6 $m\mu$, and stained with hematoxylin and eosin (H and E). Randomly selected sections were stained with the Klüver-Barara stain to demonstrate myelin, or with Bodian stain to demonstrate axons.

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