IN VIVO AND IN VITRO MODELS OF DEMYELINATING DISEASES

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INTRODUCTION

One feature characterizing chronic viral infections of the nervous system (NS) associated with progressive degenerative disease is the capacity of the viral agent to maintain itself in a persistent, and/or latent state for prolonged periods. Ability of DNA viruses of the herpes or papova type to remain covert intermittently or for indefinite periods within nuclei of neurons, particularly in peripheral nerve ganglia, has been clearly documented (1-4). RNA viruses of the retrotype such as Visna and C-type of wild mice which, via a provirus intermediate, can become integrated into the host's genome, likewise possess the potential for maintaining persistent or latent infections in the NS (5, 6). It is, however, puzzling how neurotropic RNA agents, among them coronaviruses with +RNA genomes and paramyxoviruses with -RNA genomes, may be perpetuated in the same manner as the viruses mentioned above. As part of our continuing programme of investigations of mechanisms by which NS diseases are produced by some RNA viruses we have studied on the one hand the pathological process in the central nervous system (CNS) of rodents and on the other cell-virus interactions in selected lines of rodent cells of neural and other derivation.

I CENTRAL NERVOUS SYSTEM DISEASE IN THE RAT PRODUCED BY MURINE CORONAVIRUS

A neurotropic strain of mouse hepatitis virus (MHV) was isolated over 30 years ago and designated as JHM (7). This isolate

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was shown to produce encephalomyelitis and demyelination in mice of various strains (8). Furthermore intracerebral (ic) inoculation of JHMV into white rats produced either fatal or transient neurologic disease, with evidence of demyelinating foci. Demonstration that JHMV could establish persistent or latent infections in a rat cell line of Schwann cell derivation (9) prompted us to study the slowly progressing rat CNS disease caused by JHMV.

(a) Influence of Age and Genetic Constitution of the Host on the Disease Process

Initial experiments by us showed that neurologic disease of rats could be reproducibly initiated by intracerebral (ic) inoculation of JHMV, employing a standard dose of 5×10^4 plaque forming units (pfu). Fewer pfus per inoculum failed to elicit reproducibly either the clinical or histopathological symptoms of disease. By comparison very few pfus are required to initiate disease in mice (8). Intraperitoneal (ip) injection of JHMV or a related viscerotropic MHV_3 at concentrations up to 5×10^5 pfu did not produce any evident disease, nor did the ic injection of MHV_3. Therefore potential host cells in the rat CNS appear to discriminate between the neurotropic and viscerotropic strains of MHV, while those of the mouse do not (10).

When outbred Wistar, Long-Evans or Sprague-Dawley rats are inoculated at two days of age neurologic disease becomes manifested within seven days post inoculation (pi). The symptoms observed include ataxia, tremors and posterior paresis. Deaths in these strains occur during the first 14 days pi and about 3 days after onset of clinical symptoms (Table 1). If animals are injected at 5 days of age, disease is observed later at 15-26 days pi (Table 1). In the latter group posterior paresis or paralysis occurs without apparent effect on forelimb movement. It is highly noteworthy that occasional remissions occur in animals surviving to weaning. Some rats which did not die of acute encephalitic disease by the time of weaning exhibited delayed paralysis and upon autopsy had discrete foci of CNS demyelination. However, some of these rats survived with remissions of neurologic signs and were apparently free of demyelinative lesions.

Inoculation of inbred Wistar-Lewis and Fischer 344 rats caused clinical symptoms which appeared later and the proportion of animals with fatal disease was lower than among the outbred strains (Table 1). By comparison ic inoculation of JHMV into 2 or 5 day old Wistar-Furth rats revealed that this strain is unusually susceptible to rapid encephalitis and death. Paralysis becomes evident only when 10 day old animals are injected (Table 1).

In the resistant strains tested, the longest latent period

Table 1. The Susceptibility of Various Strains of Rat to Paralysis and Death Following JHMV Innoculation

Rat Strain	Age at Inoculation	Number of rats paralysed at death/number of deaths: Time (days pi) 1-7	8-14	15-21	> 21	Number deaths**	Number Inoculated
Wistar	2	0/4	1/12	0	0	16	30
	5	0	0	1/2	7/7	9	29
	10	0	0	0	0	0	12
	15	0	0	0	0	0	10
	30	0	0	0	0	0	6
Long-Evans	2	0/1	1/5	3/2	1/2	10	16
Sprague-Dawley	2	0/8	2/9	4/4	2/2	23	33
Fischer 3-1/4	2	0	0/2	2/2	0	4	12
	5	0	0	0	3/3	3	6
Wistar-Lewis	2	0	0	0/1	2/3	4	15
	5	0	0	0	1/1	1	10
Wistar-Furth	2	0/19	0/9	0	0	28	30
	5	0/5	0/8	0	0	13	14
	10	0	6/8	9/9	5/5	22	24

* each animal was inoculated ic with 5×10^4 pfu JHMV.
** rats died or were killed *in extremis*.
(Data from reference (11)).

recorded was 99 days pi in a rat manifesting posterior paresis. In this particular animal recovery of hind limbs coordination returned within 40 days.

To ascertain the genetic control of sensitivity of rats to JHMV an F_1 cross between Wistar-Lewis and Wistar-Furth was bred and animals inoculated ic at 10 days of age. As shown in Table 2, none of the animals became paralysed within 21 days pi and the mortality rate was approximately as low as that of the resistant Wistar-Lewis strain. Thus high survival rate to JHM appears to be genetically dominant.

Table 2

Rate of paralysis and mortality in the F_1 Wistar-Lewis x Wistar-Furth*

Days after inoculation				No. of deaths	No. inoculated
1-7	8-14	15-21	21		
0	0/5**	0	0/1	6	47

* 10 day old animals were inoculated ic with 5×10^4 pfu JHMV

** ratio animals paralysed/dead

(b) Histopathological Findings

A survey among rats of different strains revealed that the location of histological lesions, identified by means of standard procedures for light and electron microscopy (11), is altered with time elapsing after inoculation (latency period) and the distribution of gray and white matter lesions is also altered (Figure 1). When deaths occur within 1 week pi cerebral gray and white matter lesions are prominent, with those in the gray matter predominating. There was evidence of necrotizing meningioencephalitis with destruction of both gray and white matter, proliferation of endothelial cells and minor infiltration of polymorphonucelar and mononuclear cells into the CNS parenchyma. There was a high incidence of necrotic foci in the cerebral cortex and hippocampal gyrus.

First signs of spiral cord involvement were detected in animals killed or dying 2 weeks pi. These foci were predominantly in the white matter. Animals examined 3 weeks pi exhibited prominent white matter lesions in the rhombencephalon and the cord (Figure 1).

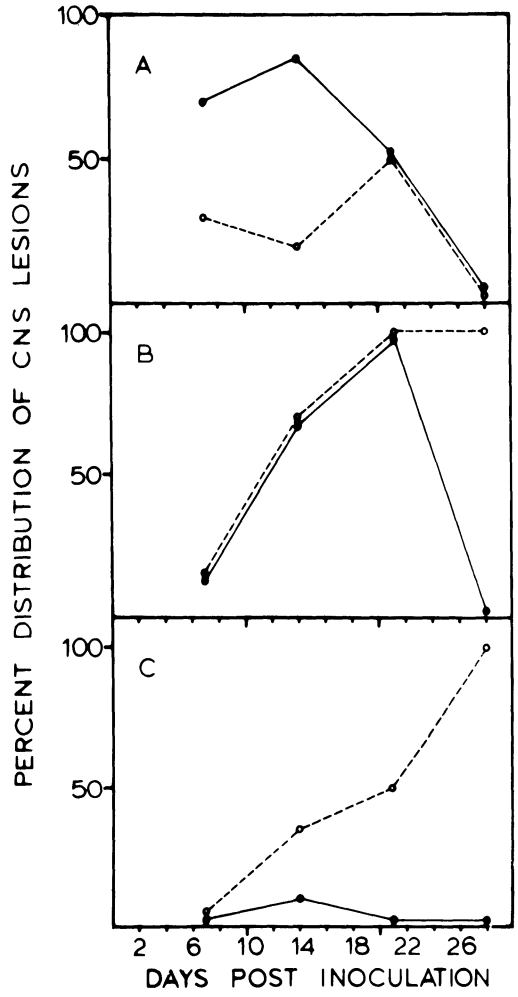


Fig. 1 - Cumulative data on frequency and distribution of white and gray matter lesions in CNS of all rats that died or were killed in extremis after intracerebral inoculation of JHM virus at 2 days of age (A, cerebrum; B, rhombencephalon; C, spinal cord; closed circles, gray matter; and open circles, white matter). (Data from reference (11)).

Specific involvement of the white matter was more evident in rats killed eight days pi or later. Some gray matter destruction was also evident in rats dying eight to 21 days pi (Figure 1). In the CNS of rats in which clinical symptoms developed 15 or more days pi, white matter lesions were more common and more extensive than those in the gray matter. Pathologic changes in the white matter involved various areas of the cerebrospinal axis but were most prominent in the pons, cerebellar folia, myelencephalon, spinal cord, and optic nerve. Characteristically, there was vacuolation of white matter coupled with disruption of normal architecture, absence or scarcity of myelin, and minimal perivascular cuffing with mononuclear cells. Frequently, macrophages with numerous cytoplasmic vacuoles were evident in areas of myelinoclasis implying that breakdown of myelin was followed by phagocytosis of this material. Multinucleated cells, interpreted to be gemistocytic astrocytes, were occasionally observed in demyelinated areas of spinal cord.

It is noteworthy that up to the time of weaning some rats, which did not die of acute encephalitic disease manifested delayed paralytic symptoms and discrete foci of demyelination in the CNS. Some of these paralyzed rats survived with remission of neurologic signs, and were also devoid of lesions on histological and electron microscopic examination, implying that remyelination after paralysis may occur. Similar findings have been reported recently by others (12, 13). Generally, demyelinating foci were absent from rats without clinical signs. To date, exacerbations have not been encountered.

(c) Optic Neuritis

Optic neuritis was prominent in seven of 15 inoculated rats that died or were killed 12 to 25 days pi. Whenever optic nerve involvement was found in a particular rat, there were concurrent lesions in the brain and spinal cord. In optic nerves from rats examined as early as 12 days pi, the lesions were characterized by fragmentation of myelin sheaths, disruption of the normal architecture, and noticeable infiltration by mononuclear cells. In material taken 21 to 25 days pi, areas of demyelination in optic nerves resembled closely demyelinating lesions observed elsewhere in the cerebrospinal axis, including thinning, fragmentation, and dispersal of the myelin covering around individual axons, and infiltration by phagocytic cells.

In view of the tropism of JHMV for oligodendrocytes throughout the CNS, the observed demyelination in the optic nerve might have been due to killing of this cell type. The extent of the lesions observed might reflect the nature of CNS myelination where segments of many axons may be myelinated by a single oligodendrocyte (14).

The optic nerve lesions, when identified, occurred only in those rats that also had lesions elsewhere in the CNS, suggesting great susceptibility of oligodendrocytes in this region to JHMV. Conversely, lesions were not observed in the cerebrospinal axis of rats with optic nerves devoid of lesions. In this context, it is intriguing to note that in man, optic neuritis is frequently associated with multiple sclerosis, and it has been suggested that optic neuritis may be a manifestation of the same disease (15, 16).

(d) JHMV Replication in the Central Nervous System

To assay for the presence of infectious virus, inoculation ic of brain homogenates from JHMV-infected Sprague-Dawley rats was made into suckling mice. Inoculated mice that died displayed typical JHMV histopathologic findings. The data obtained demonstrated the presence of infectious virus in rats during the first 20 days pi. All rats tested both with clinical symptoms, and some without, proved to be positive for JHMV. After injection ic of 3×10^5 LD_{50} JHMV/rat, rats without symptoms killed five or ten days pi yielded in some cases about 3×10^4 LD_{50} JHMV/g of brain. JHMV was also isolated from animals displaying typical symptoms 15 and 20 days pi.

Systematic electron microscopic examination of lesions of the optic nerve, spinal cord, rhombencephalon, or cerebrum, where extensive demyelination and tissue damage occurred failed to provide evidence for the presence of coronavirus. Intracellular viral replication was observed infrequently and only after extensive searching. Invariably the coronavirus particles were confined to oligodendrocytes of the optic nerve and spinal cord of animals sacrificed 12-24 days pi. The appearance of assembling or mature JHMV is indistinguishable from that previously described in rodents and cultured cells as illustrated in our companion article. The process is characterized by virus budding into cytoplasmic vacuoles and the accumulation of intracytoplasmic nucleocapsid material.

(e) Concluding Remarks

Applicability of the rat model for investigating virus-induced progressive demyelinating diseases possesses certain attractive features that deserve further attention. Of particular interest is, first, the delayed onset of neurologic disease with apparent remission after prolonged paralysis; second, the progressive changes in the location and frequency of demyelinating foci in relation to increased length of the latent period; and third, the appearance of demyelinating lesions in the optic nerve.

II INTERACTION OF CORONAVIRUSES IN VITRO WITH CELLS OF NEURAL AND OTHER DERIVATION

(a) Cell Lines

Capability of JHMV and MHV_3 to initiate in rodents chronic and perhaps latent CNS infections raises fundamental questions about the nature of cell-virus interrelationships involving specific neural cell types. With this in mind we undertook a series of studies to examine the infectious process in the less complex in vitro setting and employed established lines of glial, neuronal, and non-nervous system derivation. For comparative purposes interactions with the subsclerosing panencephalitis Hallé and Edmonston vaccine strains of measles, another virus type with neurotropic potential, were examined in parallel with those conducted on the coronaviruses (9, 17). The cell lines employed and their presumed derivation are listed in Table 3. Among them the L-2 murine fibroblasts are fully permissive for JHMV and MHV3 and Vero monkey cells for both the measles isolates. These lines served not only as the prototype permissive host but were also used for propagating virus stocks and plaque assays.

Table 3. 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.

(Data from reference (17))

The rat RN2-2 Schwannoma was selected for the most detailed investigations because it ① can be persistently infected by both JHMV and the measles isolates and ② may have the same stem cell origin as oligodendrocytes in the CNS. In this regard it should be

remembered that JHMV exhibits rather specific tropism for oligodendrocytes of the rat CNS when a chronic disease occurs, as described above (11).

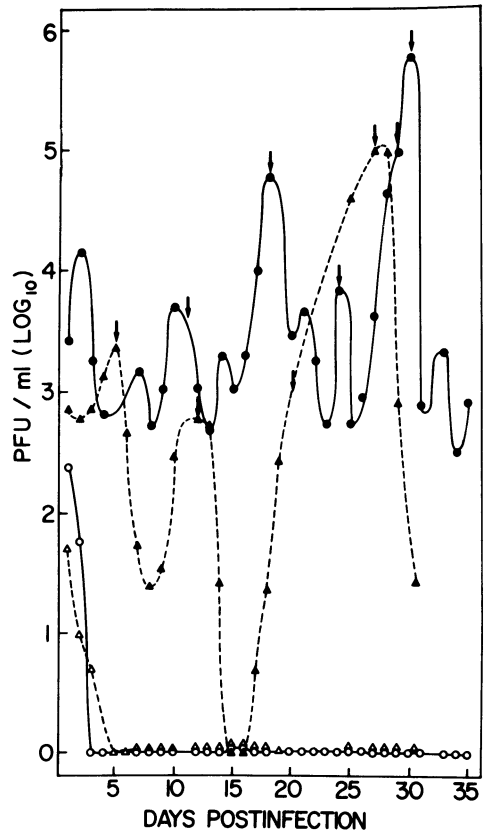
(b) Persistence and Latency

When RN-2 cells are infected with JHMV with a low multiplicity (moi) of 0.1 at 33° persistence is established immediately (9). Cytopathic effects (CPE) in the form of syncytia formation and cell killing are limited to discrete foci and never involve the entire culture. Coincidentally virus is continually shed for indefinite periods into the medium in amounts fluctuating cyclically (Figure 2). Among all cell lines examined the ability of RN-2 cells to discriminate between the neurotropic and viscerotropic subtypes of coronavirus appears to be a unique property of this Schwannoma line. It remains to be shown whether the results obtained in vitro are in some manner, related to suppression of MHV_3 infection in the CNS of the rat, described above.

Concerning the viral and/or cellular regulation of persistence it may be highly significant that measles viruses undergo replication patterns in RN2-2 cells at 33° essentially identical to those recorded for the coronaviruses, without, however, eliciting any overt cytopathology in the host (17).

Upon temperature shift up from 33° to 40° production of JHMV and measles viruses from persistently infected RN2-2 cells ceases rapidly (17). Since, however, the progeny virions assembled at 33° are not themselves thermolabile the thermosensitivity of replication is most probably controlled by host function(s). A comparative analysis with respect to data on infection of RN2-2 cells by JHMV, MHV_3, SSPE-Hallé and Edmonston measles strains was extended to a variety of other rat and mouse lines (17). The data, summarized in table 4, show that at 33° JHMV and MHV_3 were also produced in cyclical waves of the type evident in the Schwannoma cultures (Figure 2), in rat L6 myoblasts, HTC hepatoma cells and murine G26-20 or G26-24 oligodendroglioina lines. However, the coronaviruses failed to replicate in the C6 astrocytoma line (table 4). Murine C1300 neuroblastoma is permissive for the corona (table 4), and measles (18) agents.

A very striking correlation was observed between capacity of these viruses to establish persistence at 33° and become restricted, usually in a complete manner when cultures are kept at 40° (table 4). Intermediate degrees of suppression of virus production occur at 37°. These findings imply that a host function(s) which interferes with the productive virus cycle is activated or enhanced in activity after elevation of the temperatures from 33° to 37° or 40°.



Infection of Rat Glial Cells with Murine Hepatitis Virus

Fig. 2 - 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. (Data from reference (9)).

The relative quantities of cell-associated cerebroside sulfate, a glycolipid species characteristically found in myelin membranes, were ascertained to determine whether persistence and/or latency are correlated with this glycolipid. It may be significant that RN2-2, HTC and G-26-24 cell lines, in which JHMV persistence can be established have cerebroside sulfate levels at least an order of magnitude greater than those of C6 astrocytoma line (table 3), in which infection is aborted (table 4).

(c) Reversibility of Latency

Arrest of virus production at 40° is reversible within certain time limits. The interval elapsing before latently infected RN2-2 cells resume a state of persistence upon temperature shift down to 33° depends upon the duration that cultures are kept at the restrictive temperature. With JHMV, if latency is maintained for 10 days virus production upon shift down, is resumed after a lag of 2-3 days. If the latent state is prolonged beyond 2 weeks the lag period is likewise extended. In the case of Halle measles virus latently infected RN2-2 cells can be maintained for 8 weeks before losing their capacity to resume a state of persistence at 33° (unpublished).

(d) Replication of Other Agents in RN2-2 Cells

The relative ease with which persistent infections with the coronaviruses and measles strains are established and the associated thermosensitivity of the replication process suggested that infections with many virus types may indiscriminately become persistent. To investigate this possibility monolayers of the various cell types are infected at 33° and 40° 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. After 24 hr of incubation, the concentrations of VSV and vaccinia are determined by plaque titration on L-2 cell monolayers. VSV infection of the RN2-2, C6, HTC, and C1300 produces an extensive CPE and yields high titers of virus at both 33° and 40°. 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 causes extensive CPE at both temperatures in the RN2-2, C6, HTC, and C1300 cell lines, although viral production decreases at 40° 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 is apparently aborted since there is no rise in titer between 2 and 24 hr after inoculation; however, a virus induced CPE occurs. These combined observations indicate that with all the cell lines tested, temperature related persistence and latency of the type associated with corona

Table 4. 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	+			
	MHV_3	+			
Vero	Edmonston	+			
	Hallé	+			
RN2-2	JHM			+	+
	MHV_3		+		
	Edmonston			+	+
	Hallé			+	+
HTC	JHM			+	(+)[a]
	MHV_3			+	+
	Edmonston			+	+
	Hallé			+	+
C6	JHM		+		
	MHV_3		+		
	Edmonston			+	+
	Hallé			+	+
L6	JHM			+	(+)[a]
	MHV_3			+	+
	Edmonston			+	(+)[a]
	Hallé			+	+
G26-20	JHM			+	+
	MHV_3			+	+
	Edmonston		+		
	Hallé		+		
G26-24	JHM			+	(+)[a]
	MHV_3			+	+
	Edmonston		+		
	Hallé		+		
C1300	JHM			(+)[b]	
	MHV_3			(+)[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.

(Data from reference (17)).

and measles viruses does not occur after infection with VSV or vaccinia.

(e) Heterologous Virus Challenge

To determine whether the JHM persistently infected RN2-2 cultures are resistant to superinfection by an unrelated virus, VSV was inoculated onto either uninfected or persistently infected RN2-2 cells (passage 5 or 6) at an m.o.i. of 0.1. Following incubation for 24 hr at 33° 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, yields of VSV from the JHMV persistently infected RN2-2 culture, whether determined on L-2 cells or RN2-2 monolayers, are only about 10% of those from uninfected RN2-2 cells (9). RN2-2 cultures kept at 40° latently infected with Hallé-measles when challenged with the heterologous virus produce VSV at a rate only 5% that made in cultures not harboring latent measles (unpublished). After >8 weeks at 40°, when, upon shift down, rescue of measles can no longer be effected, replication of VSV returns to the normal level (unpublished).

Although the molecular events underlying the maintenance of persistence or latency in the systems under study here remain to be elucidated, in other virus carrier states chronic infections have been explained by effects involving defective interfering (DI) particles or by inhibition of virus production due to interferon, sometimes in a cyclical fashion (19), or due to selection of genotypically changed variants of the virus (20).

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 accurmulation of DI particles.

Evidence with the JHM and measles persistent infections of RN2-2 cells for involvement of an interferon-like mechanism comes from the partial resistance to superinfection with VSV (19). It is not possible, however, to transfer interference against VSV to uninfected RN2-2 cells by soluble material in the culture medium taken from persistently 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.

(f) Identification and Rescue of Latent Virus

Attempts have been made to rescue latent virus from RN2-2 cells at 40°. Treatment with low amounts (0.1 μg/ml) of

Actinomycin D to suppress host related transcription or co-cultivation with indicator cells induces appearance of pfus, albeit in low amounts, in the case of measles. Similar attempts are in progress with JHMV.

Fate of the virus in the temperature restricted state is being studied currently using cDNA probes and nucleic acid hybridization. Todate it is evident that a cDNA specific for the measles genome (21), can detect among total RN2-2 cellular extracts genomic RNA in decreasing amounts up to 5-7 days after temperature shift up to 40^{o}. Preliminary observations by immunofluorescence and light microscopy indicate that viral genome RNA can persist beyond the period of active expression into viral antigens. Use of similar probing techniques is being initiated for the coronaviruses.

(g) Concluding Remarks

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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