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In vivo and in vitro models of demyelinating diseases

XII. Persistence and expression of corona JHM virus functions in RN2-2 Schwannoma cells during latency

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Summary

The coronavirus JHMV persistently infects rat Schwannoma cells RN2-2 at 32.5°C and enters a host-imposed reversible, latent state at 39.5°C. JHMV can remain up to 20 days in the latent state and about 14 days before the cultures lose the capacity to resume virus production upon return to 32.5°C.

Although persistently and latently infected RN2-2 cells display resistance to superinfection by a heterologous agent VSV, these cells do not release detectable soluble mediators (e.g., interferon) of the antiviral state. Nevertheless, RN2-2 cells are competent to synthesize and release interferon when treated with the appropriate inducers. These observations suggest that interferon does not play any role or may not be the major factor in the control of latency in the Schwannoma cell.

Hybridization with virus-specific cDNAs shows that all viral mRNAs are present during latency and that viral mRNAs are present in the polysomes of infected cells at 39.5°C. Western immunoblotting with hybridoma antibodies demonstrates that viral specific proteins are produced at the restrictive temperature. These results

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suggest that despite the absence of production of infectious virus at 39.5°C, there is active transcription and translation into virus-specified products.

latent persistent infection, coronavirus, rat, Schwannoma cell

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Introduction

Viruses of many types are associated with the etiology of neurological diseases in animals and man. Among the RNA agents the coronaviruses have sparked considerable interest in connection with demyelinating diseases of the CNS. The murine agents of the mouse hepatitis virus (MHV) group, constituting several serotypes, have in particular been studied extensively in persistent infections in vitro (Holmes and Behnke, 1981; Stohlman and Weiner, 1978; Lucas et al., 1977) and in vivo (Knobler et al., 1981; Hirano et al., 1980; Nagashima et al., 1979; Sorensen et al., 1980, 1982, 1984). The epidemiological surveys which revealed that in some countries essentially 100% of the human population is seropositive (Hovi et al., 1979; Hasony and MacNaughton, 1982; MacNaughton, 1982) attest to the ubiquity of these agents.

In various studies we have described a model involving the persistent infection of RN2-2 rat Schwannoma cells by the neurotropic coronavirus JHMV (Lucas et al., 1977, 1978). With this agent, virus production, measured by the quantity of extracellular infectious particles, occurs in a cyclical fashion when cultures are maintained at 32.5°C. Elevation of temperature to a restrictive 39.5°C interrupts JHMV production. However, infectious particle formation may be resumed when the cultures are returned to the permissive 32.5°C. In these cell–virus systems the control on replication, related to alterations in the temperature, appears to be imposed by the host cell because thermosensitivity of either virus is insufficient to account for the observed temperature-related restriction of virus production, termed hereafter latency.

On the assumption that during prolonged restriction imposed by the host RN2-2 cell, latent JHMV are somehow perpetuated, we focussed our attention on the nature of the incomplete virus expression, particularly the detection of viral genomes, mRNA and proteins. Data from studies presented here reveal that both viral mRNA and protein are expressed during latency.

Materials and Methods

Cell cultures and viruses

The source and characterization of the rat Schwannoma cell line, RN2-2, and mouse L-2 cells has been previously reported (Lucas et al., 1977, 1978).

Primary rat embryo cells (RE) were derived from 15-day embryos of the Wistar rat. Cells were released from tissues by mincing and dispersal into phosphate-buffered

saline, containing 22 mM glucose and 0.25% trypsin, employing agitation at 37°C for 15 min. The monodisperse RE cells and continuous lines were grown in nutrient medium (NM) consisting of Eagle's minimal essential medium supplemented with 5 or 10% fetal bovine serum (FBS).

The origin and propagation methodology for JHMV has been published (Lucas et al., 1978). Virus titres, expressed as plaque-forming units per millilitre (pfd/ml) were measured by assay on L-2 cells for JHMV, according to Lucas et al. (978).

Infectious centre assays

In order to determine the fraction of cells yielding pfu in the persistently or latently infected RN2-2 cultures, the monolayers were trypsinized, free cells counted and then plated at 2-fold serial dilutions onto monolayers of the indicator L-2 cells, in 96-well microtitre plates. The co-cultures were incubated at 32.5°C for 4–6 days, then fixed with formaldehyde and stained with crystal violet. The minimum number of test cells added that were required to cause a cytopathic effect (CPE), i.e. the end point, became a measure of the frequency of infectious centers in the population of the culture.

Persistent and latent infections

The initiation of persistent or latent infections by JHMV in RN2-2 cells has been described (Lucas et al., 1978). Inocula are Millipore-filtered before adding to cells (Coulter-Mackie et al., 1984).

VSV interference assay

Interference with vesicular stomatitis virus (VSV) production by JHMV was tested in persistently or latently infected RN-2 cells using as the controls, uninfected RN2-2 cells. For this purpose test monolayers, in 35 mm dishes, were inoculated with 10^2 pfu of VSV, incubated for 24 h, then fixed and stained for plaque counting.

Induction of an antiviral state

The efficacy of induction of an antiviral state was measured as interference with VSV replication. To produce an antiviral state, some uninfected cells were pre-incubated with NM containing 0–20 μg/ml poly(I):poly(C) (Sigma) for 14 h. The medium was removed, cells were washed and then infected with VSV, as above. The amount of VSV inhibition became a measure of the cell-associated antiviral state. To ascertain whether a soluble mediator of the antiviral state, e.g. interferon, was being released, the 'conditioned' culture fluid taken at least 24 h after medium change from well-established persistently infected cultures, was treated with 1 μg/ml pancreatic RNase (Worthington) at 37°C for 30 min, then added for 24 h to fresh cultures of either RN2-2 or RE cells, prior to challenge with VSV. Alternatively, induction of the antiviral state was performed with UV-inactivated reovirus (Henderson and Joklik, 1978). Monolayers of either RN2-2 or RE cells were inoculated employing a range of multiplicities of infection (m.o.i.) equivalent to about 1–1000 pfu of the unirradiated virus. After adsorption, the cultures were incubated at 37°C and the medium sampled at intervals to measure interferon.

Specifically the sampled medium was adjusted to pH 2 with 1 N HCl, kept for 48 h at 4°C, then neutralized and assayed for content of interferon. Two-fold serial dilutions of the test medium were added to RN2-2 or RE cells in 96-well microtitre plates. After 24 h exposure at 37°C the medium was removed, cells were washed with PBS, then challenged with VSV.

RNA extractions and 'Northern' transfers

Seven 600 cm^2 culture plates (Gibco) of RN2-2 cells were infected with JHMV at a m.o.i. of 1. Five plates were maintained at 39.5°C and two at 32.5°C. At 24 h intervals titrations were made for virus production. Cultures sampled at intervals were extracted for total RNA, using guanidine-HCl (Strohman et al., 1977). Purified RNAs were denatured with glyoxal and electrophoresed on agarose gels (McMaster and Carmichael, 1977), using 20 μg/ml RNA/lane. Transfers were made by blotting to Nylon-66 (Fisher), using 40 mM Tris-acetate/20 mM sodium acetate/1 mM EDTA, pH 7.4 as buffer. Hybridization with JHMV-specific cDNA was carried out using [^{35}S]dCMP-labelled cDNA. Preparation of the JHMV cDNA and the conditions for hybridization have been described (Sorensen et al., 1984; Coulter-Mackie et al., 1980).

Polyribosome preparations

Six 150-cm^2 culture flasks of RN2-2 cells were infected with Millipore-filtered JHMV. Three of these were maintained at 32.5°C and the other three continuously at 39.5°C. 9 days post-infection (PI) cells at 32.5°C were yielding virus at ca. 10^3 pfu/ml and those at 39.5°C at 0 pfu/ml. [^{3}H]Uridine (30 Ci/mmol, New England Nuclear) was added to a concentration of 2 μCi/ml and cells were incubated a further 16 h. Cell extracts containing polyribosomes (polysomes) were prepared according to Gielkens et al. (1971). Cells which had been treated with 50 μg/ml cycloheximide for 20 min were trypsinized, then lysed in NP40. The extract was layered on 20–39.6% isokinetic gradients and centrifuged $10^5 \times g$ for 70 min. Then the gradient was divided into aliquots and the labelled RNA measured in a scintillation counter. The polysome-rich fractions were pooled, concentrated by ethanol precipitation and the pellets formed were taken up in a small volume of water. In preparation for dot-blotting analysis (White and Bancroft, 1982), the solution was made 7% with respect to formaldehyde and 10% with respect to SSC giving a total volume of 0.5 ml. Samples were heated 60 min at 50°C and then applied to nitrocellulose (BioRad), using a Schleicher and Schuell 'Minifold' filtration apparatus.

Nitrocellulose blots were hybridized with [^{32}P]cDNA (JHMV), washed and subjected to autoradiography. Preparation of the cDNA and hybridization conditions have all been described (Sorensen et al., 1984; Coulter-Mackie et al., 1980).

Western immunoblotting

Extracts of both uninfected and infected RNA-2 cell cultures were prepared by washing twice with ice-cold phosphate-buffered saline (PBS), followed by solubilization for 10 min at 37°C in lysis buffer: 0.15 M Tris-HCl, pH 8.8/10% glycerol/5%

2-mercaptoethanol/2% sodium dodecyl sulfate (SDS). Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 10.5% acrylamide gels (Laemmli, 1970). Replicas were prepared by electrophoretic transfer for 18 h at 6 V/cm onto 0.2 μm nitrocellulose filters (Schleicher and Schuell).

The replicas were cut into strips, placed in PBS + 0.05% Tween 20 for 1 h at 25°C and incubated for 2 h at 25°C with murine monoclonal antibodies (kindly supplied by Dr. Michael Buchmeier, Scripps Clinic and Research Foundation, La Jolla), according to Towbin et al. (1979). The blots were washed six times in PBS–Tween 20, and rabbit anti-mouse kappa chains (Miles Laboratories) were added to serve as links between JHMV polypeptides and Protein A. Subsequently the strips were washed six times in PBS–Tween 20, reacted for 2 h at 25°C with ^{125}I-labelled Protein A, specific activity 5 μCi/μg (New England Nuclear) and finally washed six times in PBS–Tween 20, dried, and exposed to X-ray film to detect radioactive bands by autoradiography.

Results

Restriction of virus production at elevated temperature

The phenomenon of coronavirus persistence at 32.5°C and profound suppression of virus production at 39.5°C in RN2-2 cells, and other continuous rat cell lines, is quite striking (Lucas et al., 1977; 1978). Similar cell–virus interactions were shown, more recently, to occur in primary rat neural (Sorensen et al., 1984; Beushausen and Dales, 1985) and embryonic RE cells (M. Coulter-Mackie, unpublished), indicating that in appropriately selected cells, temperature restriction on virus replication is under host control.

To ascertain the extent to which JHMV can be maintained latently at 39.5°C, a long-term study of events prevailing at the elevated temperature was undertaken. At intervals of 3–4 days, prior to change of NM, the extracellular virus titre was determined. At weekly intervals cells in monolayers were released by trypsinization, transferred into new culture flasks and perpetuated at 39.5°C. The fraction of cells which could function as virus yielders was determined by an infectious centre assay. The duration for which the latently infected cells maintained capacity to resume virus production at 32.5°C was also determined. Resumption of virus production after temperature shift down from 39.5°C was usually detectable within 4–5 days at 32.5°C. Under coculture conditions with indicator L-2 cells, cytopathic effects (CPE) due to JHMV became evident within 1–3 days after return to 32.5°C.

The results of the infectious centre assays, summarized in Fig. 1, revealed that there was a progressive decrease, with time, in the fraction of infectious centres. RN2-2 cultures persistently infected with JHMV at 32.5°C usually contained 0.1–10% virus yielding cells during the course of a well-established infection. Since upon infection of RN2-2 cultures with JHMV at 39.5°C and maintenance in a state of latency at that temperature for several days, the cells commenced yielding pfu upon shift down to 32.5°C (Lucas et al., 1978), these data suggest that penetration and eclipse of the virus inoculum must have occurred normally at 39.5°C and restriction most probably developed at a subsequent stage.

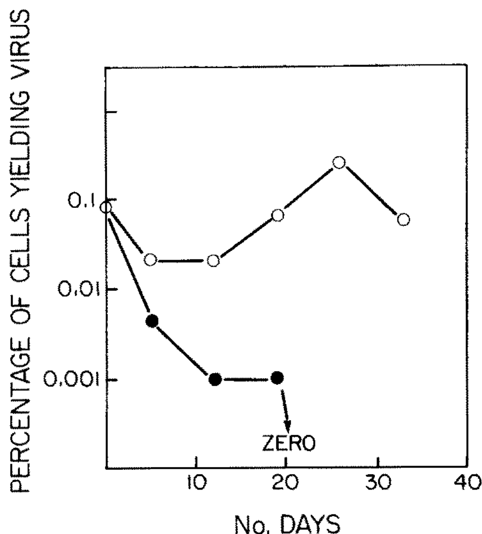

Fig. 1. Effect of persistence or latency on maintenance of JHMV in RN2-2 cells. Infectious centres assays, as described in Materials and Methods, were carried out at weekly intervals on samples from cultures at 32.5°C (○) or 39.5°C (●). The data, plotted semilogarithmically, indicate a decline to zero (arrow), at the time infectious centres were no longer detectable. In the data shown, the experiment was initiated 28 days PI, i.e. with a well-established persistent infection.

Although infectious centres were detectable for about 20 days at 39.5°C the capacity of latently infected RN2-2 cultures to resume virus production, upon temperature shift down to 32.5°C, was retained for only about 14 days. Apparently the infectious centres assay is the more sensitive measure of potential infectiousness.

Incubation of RN2-2 cells at 32.5°C, 37°C, or 39.5°C for 24–48 h prior to infection had no effect on the outcome of the infection, indicating that the observed inhibition of JHMV replication does not result from induction of factors in the uninfected cells when they are cultured at the elevated temperature.

Co-culture of latently infected cells with L-2 cells

One approach towards rescue of JHMV from latently infected RN2-2 cells was by means of co-culture with the indicator line L-2, host cells which do not restrict virus production at 39.5°C. Upon co-cultivation for 1–3 days cell–cell fusion became evident, typical of that seen during lytic infection of L-2 cells. These observations revealed the presence of latent JHMV which could be transferred to indicator cells upon close cell–cell contact and therein established a lytic infection.

Attempt to demonstrate an antiviral state during persistent or latent infections

To test whether some factor such as interferon (IFN), or an interferon-like effect, prevailed during persistence or latency in RN2-2 cells, JHMV-infected cultures were superinfected with VSV at either 32.5 or 39.5°C. Such cells were, indeed, resistant to challenge with VSV showing 100% inhibition of VSV inoculated at the m.o.i. of 1.

TABLE 1

POLY(I): POLY(C) INDUCTION OF THE ANTIVIRAL STATE

Treated cells [a]	Expt. No.	Percent reduction of VSV titer		
q		Cell-associated effect	Induction of other cells by soluble factors [b]	
			RN2-2	RE
RE	1	44	1	32
	2	100	86	100
RN2-2	3	44	0	10
	4	37	0	11

The procedure is described in Materials and Methods. Cells were inoculated with 2×10^2 pfu of VSV and checked for plaque counts 24 h later. Results are given as the percent reduction in VSV titer compared to untreated controls. Control cells gave plaque counts in the range of 180–200 on RE cells and 150–175 on RN2-2 cells.

[a] Uninfected cells were treated with 10 μg/ml poly(I): poly(C).

[b] Poly(I): poly(C)-containing medium, collected from cells being tested for the cell-associated effect, was RNase treated and tested on fresh RN2-2 and RE cells for its IFN inductive effects.

These results revealed that although only a small fraction of the cells in a population were scored as positive for JHMV, the ongoing infectious state of the culture was sufficient to inhibit totally the replication of VSV. This finding suggests that persistent or latent infections of only a few cells were adequate to protect the entire culture against VSV.

Evidence showing that RN2-2 and other rat cells are inducible for Interferon

The above results led us to initiate tests for the presence of antiviral material i.e. IFN. For this purpose, 'conditioned' NM from latently or persistently infected RN2-2 cells was used to treat uninfected RN2-2 cells prior to inoculation with VSV, as described in Materials and Methods. There was no significant reduction in the titer of VSV produced. Nor was evidence found for presence of extracellular mediators in 'conditioned' NM from which the protein had been concentrated by procedures used to concentrate rat IFN, described by Schellekens et al. (1980). These findings indicate that, within the limits of detection of our assay, persistently or latently infected Schwannoma cells were not producing any soluble interferon or IFN-like material.

Failure to detect IFN during persistence and latency led us to investigate whether the Schwannoma line, like primate Vero cells (Desmyter et al., 1968), is deficient for IFN inducibility, as defined by Lockart (1973). The analyses involved both the ability of test cells to become resistant to VSV and to produce a transferable, soluble factor which conferred resistance against VSV. Our results, summarized in Table 1, showed that primary rat embryo (RE) cells were highly sensitive to the interferon inducer poly(I): poly(C), both with respect to acquisition of resistance to VSV and production of a soluble antiviral material. By comparison, RN2-2 cells were affected to a lesser extent by this inducer. However, exposure of either RE or RN2-2 cells to

UV-inactivated reovirus type 3 produced maximal responses within 24 h in both cell types and some IFN was already detectable by 6–7 h after treatment. Following addition of inactivated reovirus, equivalent to 10–1000 pfu/cell of live virus, the same amount of IFN in the NM was produced by either RE or RN2-2 cells, about 1500 units/ml by comparison with a standard IFN preparation. This demonstrated that both the rat cell types tested had equal responsiveness to induction of IFN by reovirus. From this one can conclude that RN2-2 cells are not lacking in capacity to make IFN.

Effects of Interferon addition during persistence or latency

Despite absence of detectable IFN in JHMV-infected culture medium, the RN2-2 cells were resistant to superinfection with VSV. This led us to test the influence of exogenously added IFN by exposing persistently or latently infected cultures to purified rat IFN, characterized as the α type (Lee Biomolecular). Preliminary tests revealed that addition of 50 units/ml of this IFN reduced by over 75% the titer of VSV in previously uninfected RN2-2 cells. On the other hand, treatment of this line, with 20–200 units/ml of the rat IFN, at the time the cells were already undergoing persistent infections with JHMV, failed to decrease significantly the yields of JHMV. Therefore, JHMV persistence in this system does not appear to be sensitive to or under the control of IFN.

Detection of viral messenger RNA during persistence and latency

'Northern' transfer hybridization with isotopically labelled cDNA probes, specific for JHMV was conducted using extracts of RNA from cultures maintained after infection at either 32.5 or 39.5°C. Bands were evident in the autoradiograms at positions corresponding generally to those associated with mRNAs of JHMV (Fig. 2). The number and approximate M_r's of the mRNA bands (1–7) agreed with findings from lytic infection of L cells, as reported by Cheley et al. (1981b), except that the band related to the 19S nucleocapsid mRNA (mRNA 7) was evident at a position of slightly lower M_r than anticipated. This was most probably due to a displacement by the presence of relatively much more abundant 18S rRNA in the vicinity, which could have produced a distortion of the migration of the viral mRNA in the agarose gels. It was noted that mRNA 6, which has an approximate molecular weight of 1.15×10^6, showed a secondary faint band of slightly lower molecular weight. The lower molecular weight band predominated when JHMV was grown lytically in L-2 cells (data not shown). These findings are of interest in light of those of Taguchi et al. (1985), who observed alterations in the molecular weights of mRNAs 2 and 3 in brain-derived vs. culture cell-derived material.

Throughout persistent infection of RN2-2 cells, at 32.5°C, all species of JHMV mRNA were detected, as illustrated with a 4-day PI sample in channel a of Fig. 2. Judging by the unchanged intensity of the bands with time of sampling, the quantity of mRNA did not vary appreciably. By contrast, during latency at 39.5°C, the intensity of the bands decreased with time elapsing PI as evident in channels b–e. Thus, by day 4 PI at the elevated temperature, the bands became faint. An extract of RNA from a culture, kept in a state of latency for 11 days, apparently contained

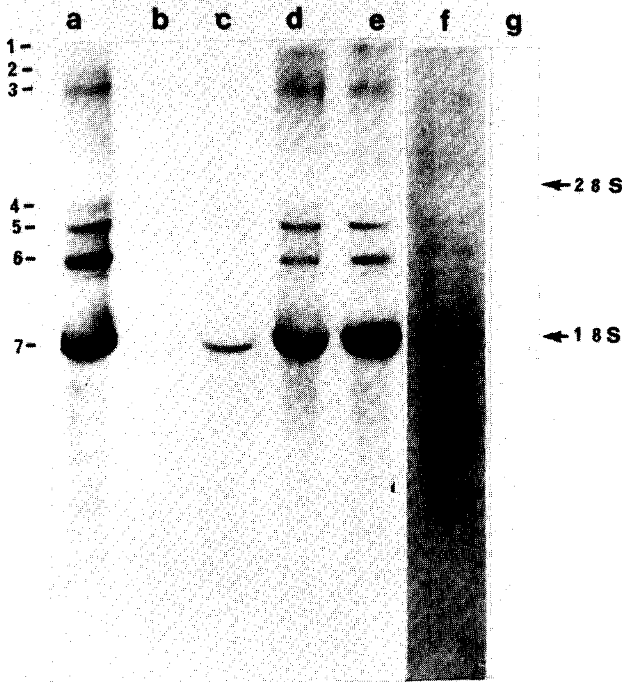

Fig. 2. 'Northern' transfer and cDNA hybridization to RNA extracted from latently or persistently JHMV infected RN2-2 cells. Data after incubation at 32.5°C for 4 days PI are in channel a; data from infection at 39.5°C for 4, 3, 2, 1 or 11 days PI in channels b–f, respectively. Material in f was from a separate experiment and involved a 2× longer exposure time for the autoradiogram. RNA extracted from uninfected cells in channel g. On the left-hand side are indicated the M_r's of JHMV mRNAs 1 to 7, which are respectively $3.7 \times 10 \times 10^6$, 3.4×10^6, 3.1×10^6, 1.6×10^6, 1.45×10^6, 1.15×10^6 and 6.3×10^5. Location of the 28s and 18s ribosomal RNAs is shown by arrows on the right side of the figure.

mRNA corresponding to the nucleocapsid gene. These results suggested that early during latency all or most of the JHMV mRNAs were being expressed but later than 4 days PI the nucleocapsid mRNA, the most abundant viral mRNA (Stern and Kennedy, 1980), was the only mRNA detectable by these means.

The data suggest that transcription at the restrictive temperature continued but the abundance of the mRNAs decreased rapidly within a few days. The results from cDNA probing of transcripts are consistent with the observed decline in the number of infectious centers which became evident as the duration of latency was prolonged (see Fig. 1).

To ascertain whether active translation was underway at 39.5°C, polysomes from infected RN2 cells were isolated, fractionated and subjected to hybridization with JHMV-specific [^{32}P]cDNA and autoradiography, as described in Materials and Methods. The autoradiograms, shown in Fig. 3, indicated that mRNA specified by

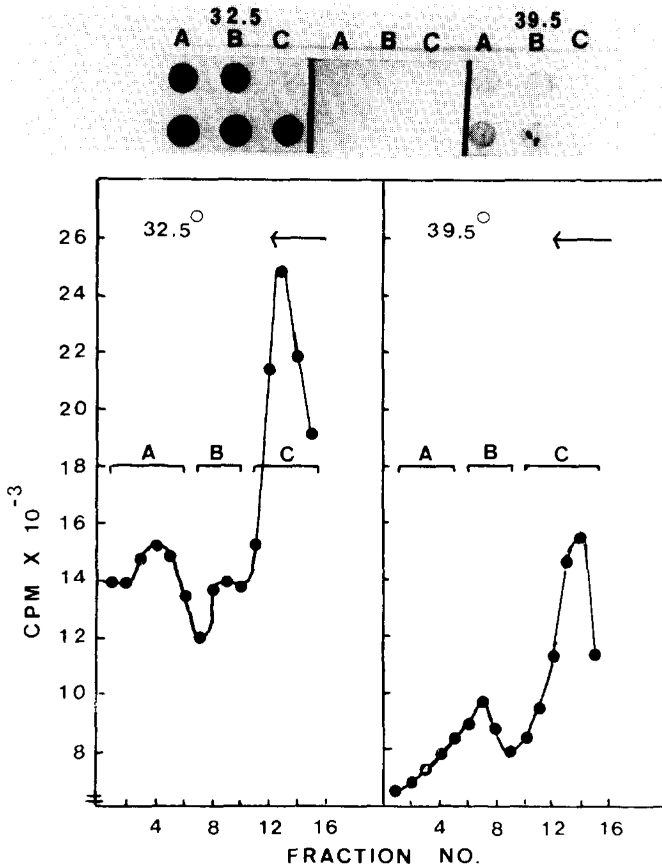

Fig. 3. Correlation between polyribosome sedimentation profiles and presence of JHMV mRNA from persistently and latently infected RN2-2 cells. Polysome extracts, prepared as described in Materials and Methods, were layered onto 20–39.6% sucrose isokinetic gradients and centrifuged at $10^5 \times g$ for 70 min. The gradients were fractionated and acid insoluble cpm determined. Fractions from 3 major areas of each gradient were pooled (A, B and C), precipitated with ethanol and used for 'dot-blotting', as described in the Materials and Methods. The incubation temperatures for the persistently or latently infected RN2-2 cells, from which the extracts were made, are indicated. The 'dot-blot' hybridization autoradiograms of the pooled gradient fractions are shown above the gradient profiles. The columns designated A, B and C correspond to similarly identified pooled fractions, shown below. The lower dot in each panel represents 1/10 of the total material in the pooled fractions. The upper dot is a 1/10 dilution of material present in the lower dot. The centre panel of the autoradiogram shows hybridization results with polysome extracts from uninfected RN2-2 cells.

JHMV was present in the gradient fractions enriched for polysomes, isolated from cells kept at either 32.5 or 39.5°C for 9 days PI. This finding suggests that active translation of coronavirus messengers was also taking place at the restrictive temperature, in the absence of infectious virion formation.

Expression of JHMV nucleocapsid protein at permissive and restrictive temperatures

Since it was apparent from work with polyribosomes that some JHMV transcrip-

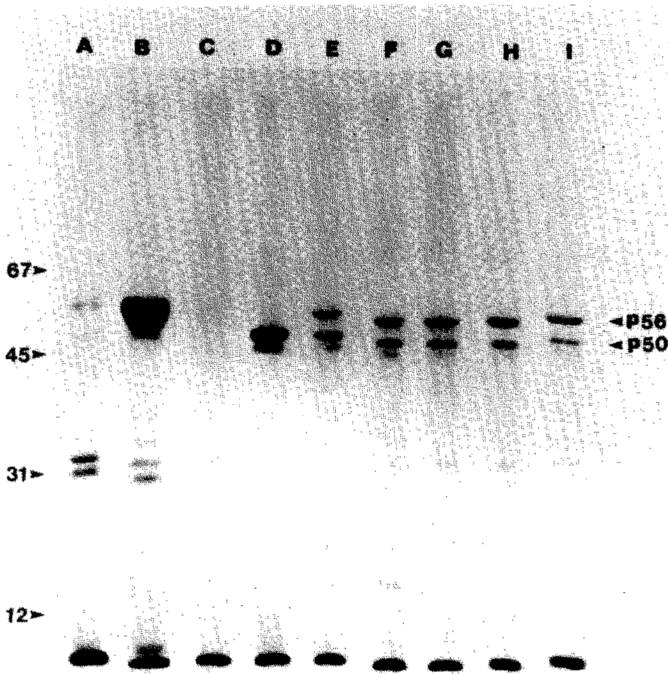

Fig. 4. Immunoblot analysis, with anti-nucleocapsid hybridoma antibody, of persistently (32.5°C) infected RN2-2 cells. Inoculation, preparation of extracts and blotting were carried out as in Materials and Methods. Channel A: uninfected L cells; B: L cells productively infected for 12 h; C–I: RN2-2 cells; C: infected controls; D: sampled immediately after adsorption; E–I: sampled respectively 12 h, 48 h, 72 h and 7 days post-inoculation.

tion occurred during latency at 39.5°C, analyses were made to ascertain whether presence of viral mRNA could be related to translation into viral polypeptide(s). As already mentioned, incubation of infected RN2-2 cells at the permissive 32.5°C was associated with only minimal cytopathology, implying that detection of viral polypeptide(s) might be different, especially during latency at 39.5°C. To maximize the sensitivity of detection, virus related antigens were identified by immunoblotting and autoradiography. For this purpose, infected RN2-2 cells, used as controls, were cultured at the permissive or restrictive temperatures. Samples were removed at intervals and extracts prepared from them, then subjected to SDS–PAGE, immunoblotting with hybridoma antibodies and autoradiography. The results from the permissive conditions, after tagging with anti-nucleocapsid antibodies, are presented in Fig. 4. It is evident from channel B, representing material sampled 12 h after inoculation, that there was a significant quantity of material in a band at the 56 000 Da (56K) nucleocapsid position, corresponding to the M_r of primary product of viral nucleocapsid (Cheley and Anderson, 1981a). The other bands with apparent M_r's 31K and 33 K, evident in these gels, were presumably irrelevant and artifactual,

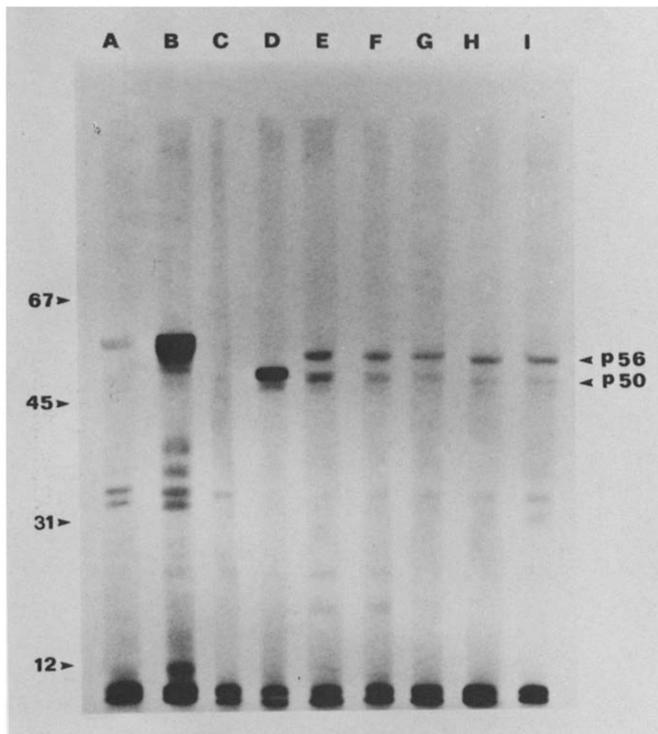

Fig. 5. Immunoblot analysis of latently (39.5°C) infected RN2-2 cells. Experimental conditions as in Fig. 2.

since they occurred in other channels, including those representing uninfected controls whenever the anti-mouse kappa chain antibody was utilized as a linker.

Lanes C–I of Fig. 4 represent immunoblots of extracts from, respectively, uninfected RN2-2 cells kept at 32.5°C, those sampled 60 min after infection (0 h) at 32.5°C or respectively at 12, 24, 48, 72 h and 7 days post-inoculation. In samples prepared 60 min after inoculation the antibodies could identify a predominant polypeptide band of 50K and a minor one of 48K. In samples taken 12 h after inoculation the antigen(s) recognized by the hybridoma was 56K and 50K, as evident in Lane E. Previous work by Cheley and Anderson (1981a), confirmed by our own studies, established by means of pulse-chase labelling experiments that a precursor–product relationship exists between the 56K and 50K polypeptides. Prominent 56K and 50K antigens were detected also in cell extracts for 7 days or longer after inoculation, revealing that production of nucleocapsid protein was continuous throughout the course of the persistent infection. There was therefore, good correspondence between data on JHMV transcription (Figs. 2 and 3), translation and formation of infectious JHMV.

By contrast, identical immunoblotting utilizing extracts of latently infected RN2-2 cells, maintained at 39.5°C, gave somewhat different results. As evident in channel B

of Fig. 5, in the infected L-2 cells, used as controls, the predominating antigen was 56K. With RN2-2 cells sampled at 0 h (60 min after inoculation), the predominant form of nucleocapsid species was the 50K antigen (Fig. 5, channel D). Similar antigenic bands were identified in the 12 h samples (Fig. 5, channel E). It is, however, not clear from these data alone whether the 50K band material is nascent antigen or the residual antigen introduced with the inoculum.

Judging by the bands corresponding to the nucleocapsid, the antigen which accumulated at 39.5°C, when infection was extended beyond 12 h, was the 56K precursor polypeptide (channels F–I of Fig. 5). It is, therefore, most probable that the 50K polypeptide evident in channel D was an intracellular cleavage product of nascent 56K since, with time, the 50K component decreased at a relatively faster rate than the 56K antigen. It is very significant that during incubation at the restrictive temperature for as long as 7 days and beyond, when RN2-2 cells had multiplied through 6–7 generations, the 56K antigen was readily detectable (channel I), implying that during latency, in the absence of infectious virus production, translation into JHMV nucleocapsid protein continued. This finding is consistent with the presence of JHMV-specified mRNA in the polyribosomes isolated from RN2-2 cells, latently infected for 9 days (Fig. 3).

Probing for the expression of the other structural components, the E_1 and E_2 glycoproteins, of apparent M_r 21K and 180K, respectively, was also undertaken. Employing anti-E_1 and anti-E_2 hybridoma antibodies of Collins et al. (1982) for immunoblotting, it was found that at the permissive temperature both antigens were expressed in RN2-2 cells, but during latency at 39.5°C neither glycopeptide was detectable (data not shown). These findings suggest that the temperature-related inhibition of infectious virus formation might be due either to the selective suppression of the synthesis or rapid turnover of JHMV envelope components, while production of the nucleocapsid polypeptide is much less affected. On the other hand, the normally greater abundance of the nucleocapsid polypeptide than of the other structural components could account for our inability to detect E_1 or E_2 in RN2-2 cells at 39.5°C.

Discussion

The present study was feasible because it is possible upon infection of rat Schwannoma cells RN2-2 with JHMV, to establish rapid and reproducible persistence or latency, influenced by elevated temperature, as demonstrated in an earlier report by Lucas et al. (1978). Thus, when infections are initiated at 39.5°C the virus is able to establish itself without formation of infectious virions. The temperature restriction on the replication process is most probably controlled by a host-imposed function and depends on the continued presence of the virus in state of latency, since susceptibility to reinfection is restored at 32.5°C, after RN2-2 cells become 'cured' of the infection.

The prolonged latency of JHMV under restrictive conditions is remarkable. In comparison, measles virus which also establishes a persistent infection in RN2-2 cells (Lucas et al., 1978) has shown a latency period of up to 70 days (Coulter-Mackie and

Dales, unpublished observations). The duration of latency with JHMV during which reactivation remains possible is shorter, perhaps only 14–20 days. The difference between the two viruses is probably related to the proportion of cells in a culture which carry viral information, the fraction involved with JHMV persistence being much lower than with measles virus persistence.

The conversion of JHMV infection from the persistent into latent states upon temperature elevation to 39.5°C, and the prolonged latency which can occur in the in vitro cultures, could have direct bearing on understanding the infectious process during JHMV infection of the rat central nervous system (CNS). Since symptom-free rats, surviving an initial intracerebral infection with JHMV, can be caused, following treatment with the immunosuppressive agent, cyclophosphamide, to develop a chronic, demyelinating disease (Sorensen et al., 1982), it is quite possible that this virus can be perpetuated in a covert form within the CNS. Consistent with this view is the finding that rats which remain asymptomatic for as long as 150 days after inoculation may harbour JHMV RNA in the CNS (Sorensen et al., 1984).

In the present study several approaches were taken towards an understanding of the nature of the block in virus production at the elevated temperature. As previously discussed by Lucas et al. (1978), involvement of defective-interfering particles or development of thermosensitive virus variants was not demonstrable and, therefore, is unlikely to account for the inhibition observed. Host factors might be responsible for the observed virus suppression. One possible candidate for the host factor in question is IFN. It is well known that during chronic in vitro infections with neurotropic agents, such as rabies virus, there is an inverse correlation between the cycling virus titre and IFN levels in the medium (Wiktor and Clark, 1972). It may also be significant in the present context that IFN activity can be enhanced at elevated temperature (Heron and Berg, 1978). Thus, in our system it was postulated that IFN or interferon-like substance might influence the control of the persistence at 32.5°C and suppression of infectious particle formation at 39.5°C. Although in the present study the development of resistance to infection of RN2-2 cells by a heterologous virus, VSV, could be demonstrated during persistent and latent infections with JHMV and likewise with measles virus (Coulter-Mackie and Dales, unpublished observations), no evidence was obtained to demonstrate that such persistently or latently infected cells produce IFN or an IFN-like substance. These two viruses differ in that measles virus is known to be an IFN inducer (DeMaeyer and Enders, 1961; McKimm-Breschkin and Rapp, 1981) while JHMV probably does not induce IFN in cultured cells, although replication of this coronavirus is sensitive to exogenous IFN (Garlinghouse et al., 1984). However, since these Schwannoma cells are inducible for IFN with UV-inactivated reovirus and can respond to exogenously added IFN, yet continue to produce JHMV at a normal rate after the addition of purified rat IFN to persistently infected RN2-2 cultures, it is questionable whether IFN exercises any control in this system or that involving rat neural cells in general. Therefore, the interference with VSV replication during latency or persistence remains unexplained.

Probing for JHMV-specific RNA by means of 'Northern' transfer indicated that transcription can occur at both 32.5 or 39.5°C. While at 39.5°C the mRNA specifying the nucleocapsid was the predominant species and was still evident on the

11th day, mRNAs corresponding to the other viral functions could be detected only during the first few days post-inoculation. However, the corresponding immunoblots made on extracts from latently infected cells could detect only the 56K nucleocapsid protein, suggesting that preferential translation of the nucleocapsid mRNA occurs in the repressed state. The situation regarding the other major JHMV structural polypeptides E_1 and E_2 remains unresolved. While these glycoproteins were expressed normally at the permissive temperature, we were unable to detect their presence during latency, despite the presence of mRNA species corresponding to E_1 and E_2 during the initial 2–3 days of latency. These data suggest that infection at the elevated temperature may lead to either a differential inhibition of the synthesis of envelope polypeptides, or their rapid turnover, or both.

The Western blot analysis also revealed a processing step connected with the nucleocapsid protein, which is blocked during latency. The nucleocapsid is initially produced as the 56K form and integrated into the virus. Soon after infection, however, the nucleocapsid material of the inoculum exists as a 50K molecular weight polypeptide (Figs. 4 and 5). This precursor–product relationship has been documented by Cheley and Anderson (1981a). Both sizes of this antigen were found to be phosphorylated (our data, not shown, and Siddel et al., 1983). The 50K proteins found in infected RN2-2 cells incubated at 32.5°C are likely, therefore, to represent the product of processing which may occur during cell-to-cell spread of progeny virus. Upon incubation of RN2-2 cells at 39.5°C assembly and/or spread of the virus progeny are probably inhibited. This is consistent with our preliminary observations (Adler et al., unpublished), which reveal that compounds interfering with JHMV entry into L-2 cells also affect processing of 56K nucleocapsid of inoculum virions.

From the above it is clearly evident that in latently infected RN2-2 cells the viral genomes are not dormant and merely segregated to daughter cells but are instead, active in transcription and translation. It remains to be shown, first of all, whether genome duplication is reduced or stopped, secondly, whether the expression of some functions is specifically reduced during latency and thirdly, why the production of infectious progeny ceases.

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