Stability of Neurotropic Mouse Hepatitis Virus (JHM Strain) During Chronic Infection of Neuroblastoma Cells

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With 3 Figures

Summary

A line of mouse neuroblastoma cells which was chronically infected with the neurotropic strain (JHM) of MHV, a member of the coronavirus group, was established. These cells, designated N_J, exhibited typical MHV cytopathic effects (CPE) at all passage levels along with the continual production of infectious virus. Most cells were positive for viral antigen by immunofluorescence. Viral particles consistent with the morphology of MHV were found by electron microscopy. The uninfected neuroblastoma cell line did not contain a detectable population of cells resistant to JHM, and persistence did not elicit the production of interferon. No plaque morphology or temperature sensitive mutants were selected for in the N_J culture, and we were unable to detect the presence of either a defective or defective interfering virus population. The addition of low concentration antiviral antibody modulated the infection to a carrier culture with viral antigen in the cytoplasm of the cells, but no infectious virus was produced, and the cells lacked both surface viral antigen and CPE. Possible mechanisms of viral persistence *in vitro* are discussed.

Introduction

Mouse hepatitis virus, a member of the coronavirus group, is endogenous to mice and has been shown to occur predominantly as a natural, latent infection *in vivo* (2) and also to be able to establish a latent infection *in vitro* (16). The JHM strain has almost complete neurotropism characterized by encephalomyelitis with both acute and chronic demyelination and an inability to produce apparent hepatitis in its natural host (1, 6, 22). Chronic demyelination following intracerebral inoculation was found in only 60 per cent of surviving BALB/c mice, indicating that a subpopulation of JHM may be responsible for chronic infection. Mechanisms of persistence *in vitro* have been previously analyzed in attempts to

better understand chronic and inapparent infections *in vivo* (14, 15, 23). To approach the study of possible mechanisms of chronic infection *in vivo*, persistence of JHM virus *in vitro* was established and studied.

Materials and Methods

Virus and Cells

Mouse hepatitis virus (MHV), strain JHM (1, 22), used in these experiments was derived from the eighth consecutive suckling mouse brain passage which was prepared as previously described (22). The mouse neuroblastoma C 1300 clone N_2A cells of A strain mice (17) were kindly supplied by Dr. M. Oldstone of Scripps Clinic and Research Foundation, and the DBT cell line (7) was kindly supplied by Dr. A. Hirano of the University of Tokyo. Both cell lines were maintained in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 10 per cent fetal bovine serum (FBS). NCTC 1469 cells (American Type Culture Collection) were maintained in DMEM supplemented with 10 per cent FBS and 5 per cent horse serum. All incubations were 5 per cent CO_2 at 37° C unless otherwise specified.

Plaque and Infectious Center Assays

Supernatant virus was assayed following serial dilution in DMEM plus 5 per cent FBS. Monolayers of either DBT or NCTC cells in 60 mm plastic plates were inoculated with 0.10 ml of virus suspension. Following adsorption at room temperature for 90 minutes, plates were overlaid with DMEM plus 5 per cent FBS containing 0.6 per cent agarose. Plaques were visualized in the DBT monolayers at 72 hours post infection following a second agarose overlay containing $1/10,000$ neutral red. Plaques were visualized in NCTC cell monolayers at 24 hours post infection with 0.5 per cent crystal violet following fixation with 25 per cent formalin for 30 minutes.

For the determination of cell-associated virus, the infected monolayers were washed one time with DMEM and removed with a rubber policeman. The cells were suspended in DMEM plus 5 per cent FBS and sonicated at 4° C for 10 to 15 minutes in a Bransonic 220 ultrasonic cleaner. The resulting suspension containing less than 5 per cent of the original cells intact was then assayed without further treatment as described for supernatant virus.

Infectious centers were determined on monolayers of NCTC 1469 or DBT cells. Monolayers of cells for assay were removed with 0.025 per cent trypsin, washed two times with DMEM, and counted in a hemocytometer. Following incubation at room temperature for 30 minutes in the presence of anti-JHM hyperimmune ascitic fluid (50 per cent plaque reduction neutralization titer $= 1/1400$), serial dilutions were plated on the indicator monolayers and fixed with 0.5 ml of DMEM plus 5 per cent FBS containing 0.6 per cent agarose. After solidification of the primary overlay, the remaining agarose overlay (4.5 ml) was added to the plates. Plaques were visualized as described above.

Preparation and Assay of Interferon

Cell culture supernatants for interferon assay were prepared by centrifugation at $800 \times g$ for 10 minutes and $96,000 \times g$ for 3 hours. The pH was adjusted to 2.0, and following incubation at 4° C for 48 hours, the pH was returned to 7.2. After dialysis against phosphate buffered saline (PBS), pH 7.2, at 4° C for 48 hours and a final centrifugation at $96,000 \times g$ for 3 hours, the preparations were filter sterilized and stored at $-20°$ C until use. In addition to positive control mouse interferon prepared in L 929 cells (American Type Culture Collection) using UV-inactivated Newcastle disease virus (NDV) (24), N.I.H. positive control reference mouse IF (G 002-904-511) was used.

Interferon preparations were assayed by the 50 per cent plaque reduction method using vesicular stomatitis virus (VSV) as the challenge virus. Following serial two-

fold dilution of the interferon preparations in Eagle's minimal essential medium containing 2 per cent newborn calf serum, 2 ml of each dilution was added to 4 confluent L 929 cell monolayers in 60 mm plates. After 18 hours incubation at 37° C, the fluid overlay was aspirated, and the monolayers were challenged with approximately 50 plaque forming units (PFU) of VSV in 0.10 ml.

Immunofluorescence

For immunofluorescence studies, cells were seeded on 12 mm glass coverslips. The coverslips were washed 3 times with PBS, air dried for 15 minutes, fixed at room temperature in acetone for 5 minutes, and stored at $-20°$ C until use. Prior to staining, coverslips were washed once in PBS and drained. Mouse anti-JHM serum prepared as previously described (22) or mouse anti-JHM hyperimmune ascitic fluid prepared in adult male Dub ICR mice (19) were used as a source of antiviral antibody. Following incubation with antiserum for 30 minutes at 37° C, the coverslips were washed 3 times with PBS, and fluorescein isothiocynate conjugated goat anti-mouse IgG (Grand Island Biological Company, Grand Island, New York) containing 0.5 per cent Evan blue was added. After an additional 30 minutes at 37° C the coverslips were washed 3 times in PBS and mounted on 30 per cent glycerol in PBS and examined under a Leitz Ortholux microscope. Fresh, unfixed specimens for surface antigen were stained following the above procedure.

Results

Establishment and Characteristics of the N_J Cell Line

The N_J cell line was established by infecting the mouse neuroblastoma N_2A cells (17) with JHM virus at a multiplicity of infection (MOI) of 0.01. Following the acute phase of JHM growth in N_2A cells, characterized by cell fusion and cytolysis, a small number of cells remained which repopulated the culture vessel in 8 to 10 days. During the first 8 passages, the cells grew at approximately 50 per cent of the rate of uninfected N_2A cells, but by passage 16 and subsequently, the N_J cells grew at the same rate as the N_2A cells.

As the N_J cells approach confluency, the culture begins to exhibit increasing syncytia formation. The N_J cells have shown evidence of viral CPE at all passage levels, and intracellular viral antigen (Fig. 1) was detected by immunofluorescent staining in 80 to 100 per cent of the cells. Viral antigen detected by immunofluorescence did not vary with passage level. Viral antigen in both single cells and syncytia was diffuse throughout the cytoplasm, while only syncytia were found to express viral antigen at the cell surface.

Infectious virus was assayed at 4—5 days after subculturing when the cells became confluent (Fig. 2). Initially there was a decline in infectious virus during the first 8 passages. During this phase, the N_J culture was not resistant to superinfection by JHM virus. Following superinfection, an abbreviated, acute infection took place with increased syncytia formation followed by cellular degeneration. There was little change in the virus titer. By passage 16, infectious virus reached a level (approximately 10^5 PFU/ml) which has remained constant for the remaining 24 passages analyzed.

At passage levels 16, 20, and 28, the N_J cells were refractory to superinfection with JHM. Following superinfection, there was no increase in either CPE or amount of supernatant or cell-associated infectious virus.

Infectious center assays showed a decrease in the number of cells producing infectious virus with continued passage, although no decrease in the percentage of fluorescent cells was found. Cells producing detectable infectious virus decreased from 33 per cent at passage 3 to 5 per cent at passage 20. There was no change in either the levels of infectious virus produced or in the CPE evident following passage 20.
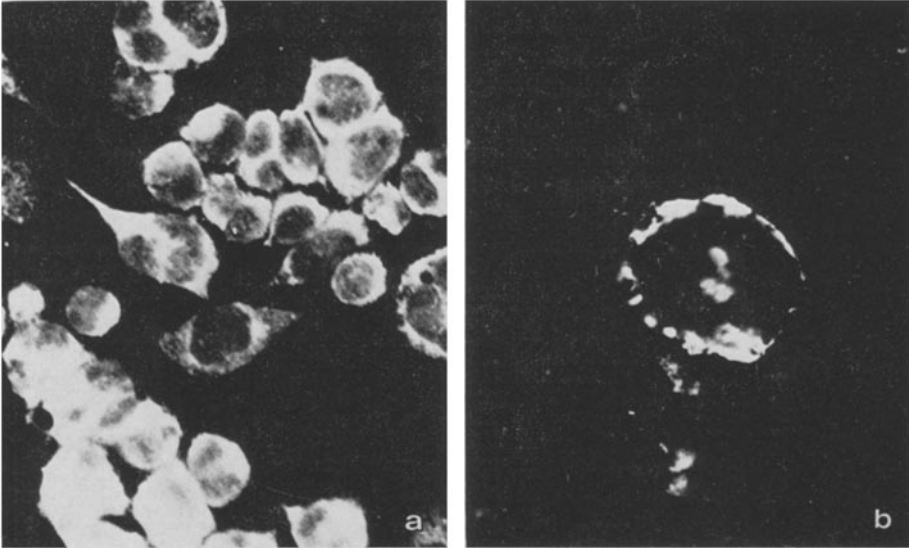

Fig. 1. N_J cells. *a* Demonstration of JHM viral antigen in fixed cells $\times 500$; *b* Surface fluorescence of living cells $\times 350$
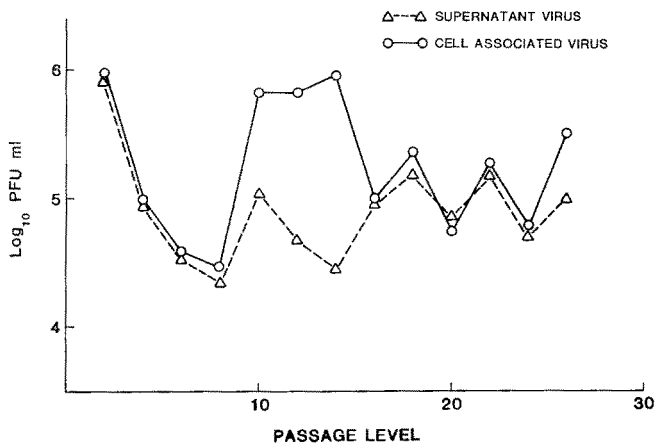

Fig. 2. Titration of even numbered passage levels of N_J cells for supernatant and cell-associated infectious JHM virus assayed in DBT cells. △—△ supernatant virus; ○—○ cell-associated virus

Host Cell Factors

All 167 single cell clones isolated from the N_2A cell line were found to be susceptible to JHM virus, and although there were differences in the viral induced CPE at 18 hours post infection, all clones progressed to massive CPE by 36 hours post infection and yielded essentially identical peak levels of supernatant virus (approximately 5×10^5 PFU/ml).

Interferon activity was not detected in either undiluted or supernatants concentrated 20 times from passage level 7, 18, or 22 of the N_J culture. Although interferon was not detectable, the N_J cultures were tested for resistance to superinfection by heterologous viruses at passages 10 and 20 (12, 21). No decrease in the yield of either vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMC) was detectable when N_J cells were infected at an MOI of 10 or 100. However, at an MOI of 1, the yield of progeny from the N_J cells was significantly reduced compared to that obtained from control N_2A cells (Table 1).

Table 1. *Comparison of the growth of vesicular stomatitis virus and encephalomyocarditis virus in N_J and N_2A cells*

MOI[b]	Vesicular stomatitis virus[a]		Encephalomyocarditis virus[a]	
	N_J	N_2A	N_J	N_2A
100	8.8	8.7	7.5	7.9
10	8.5	8.9	8.6	8.0
1	6.0	9.0	4.9	8.0

[a] Expressed as Log_{10} PFU/ml
[b] Multiplicity of infection

Viral Factors of Persistence

The possible evolution of viral variants in this *in vitro* system was studied by using a suckling mouse brain (SMB) virus pool heterogenous with respect to plaque morphology to initiate the infection. This pool of virus elicits acute encephalomyelitis and demyelination with chronic and recurrent demyelination in mice (6, 22). The N_J virus population was assayed at alternate passage levels (Fig. 2) for virus and the relative numbers of plaque variants. Neither during establishment nor at any passage level up to an including the 40th passage was there a selection for plaque morphology variants. The cell-associated N_J virus, like the cloned and uncloned parental JHM, shows no differential ability to form plaques at 32°, 37°, or 39° C. No differences were found in the ability of the infected cells to form plaques at either 32°, 37°, or 39° C, excluding the possibility that a temperature sensitive *(ts)* defect might prevent maturation of infectious virus at 37° C. In addition, over 200 N_J JHM virus clones were prepared from passage levels 20 and 40 and tested for *ts* characteristics. None of the clones tested showed a temperature defect.

Transmission electron microscopy indicated that most of the cells had intracellular vacuoles containing coronavirus-like particles typical of those seen in N_2A cells acutely infected with JHM and in other coronavirus infected cells (13). All multinucleate cells contained virus particles while only 60—70 per cent of

the uninucleate cells had vacuoles containing viral particles. Intracellular particles which appeared to be devoid of central staining material were found, and negatively stained virions from the N_J culture also appeared to be differentially stained with phosphotungstic acid. Studies were carried out to detect the presence of defective interfering (DI) or defective particles in the N_J culture. To determine if N_J supernatants contained DI particles, monolayers of DBT cells were infected with concentrated N_J supernatants at a MOI of 1 and challenged with JHM at a MOI of 0.05 and 1.0. No difference in supernatant virus yield was found. Similar experiments using the non-neuro-adapted A_{59} strain of MHV, which grows to 100-fold higher titer than JHM as challenge virus, also failed to show any differences in virus yield. Attempts were made using both DBT (7) and 17CL1 (20) cells as hosts to see if DI particles could be generated by undiluted serial passage. No decrease in virus yield or CPE was evident following 20 serial passages in either cell type. Preparations of radiolabeled JHM virus from acutely infected N_2A cells and N_J JHM were compared in CsCl equilibrium density gradients to detect the presence of defective particles. A single peak of radioactivity (density -1.19) was found in both gradients, and there was no difference in the RNA: protein ratios of the two preparations. Although these experiments do not completely rule out the production of DI particles by the N_J cells none are detectable by these techniques.

N_j JHM virus retained the ability to replicate and to produce CPE in cell lines susceptible to the parental JHM. Intracerebral inoculation of mice with N_J JHM from N_J passage levels 3, 20, and 40 produced encephalomyelitis with demyelinating lesions like those seen following infection with the parental virus (1, 22).
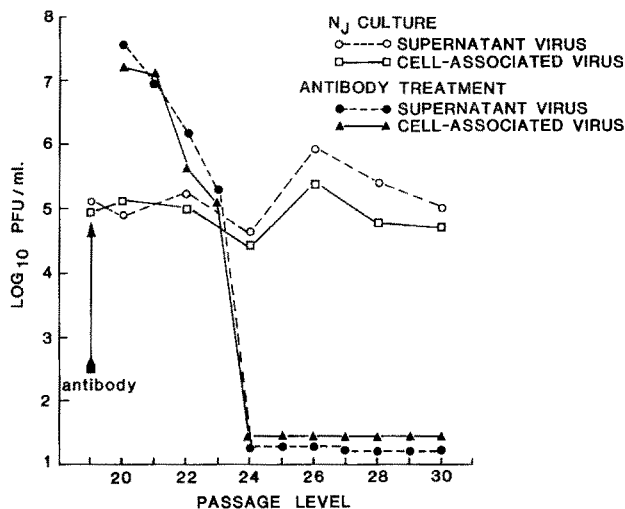

Fig. 3. Effects of antiviral antibody added after each cell passage on the yield of both cell-associated and supernatant infectious JHM virus. Control passages: o—o supernatant, □—□ cell-associated virus; and antibody treated: •—• supernatant, ▲—▲ cell-associated virus. Antibody ($^1/_{500}$ dilution) was added after subculture of the 19th passage and at subsequent passage to 30

Antibody Studies

The effects of antibody addition to the N_J culture were studied using heat-inactivated antiviral antibody at a final dilution of $1/500$ (plaque reduction neutralization 50 titer of $1/1400$). Antibody was added to the culture either immediately after the cells were suspended in fresh medium for passage or after 3—4 hours when the majority of the cells had attached. Following 4 to 5 days incubation, the cultures were examined for virus, CPE, and both surface and cytoplasmic antigen. Figure 3 shows that following the initial passage in the presence of antibody, there was an increase in both the supernatant and cell associated virus titer, which rapidly declined until after 4 serial passages no infectious virus was detectable. Surface antigen and CPE were lost following 2 passages. Intracellular viral antigen was retained for 10 passages in the presence of antibody and 15 subsequent passages in the absence of antibody. No infectious virus could be detected after passage in the absence of antibody. Infectious virus was not released from these cells following incubation for 5 passages at high (39° C) or low (32° C) temperatures nor following co-cultivation with cell lines susceptible to JHM virus at either 32°, 37°, or 39° C.

Discussion

The mechanism of *in vitro* persistence by the JHM strain of MHV, in contrast to most other reported persistent infections by RNA viruses remains unknown. Since JHM virus represents one of the animal models of virus induced demyelination with an ongoing chronic myelin breakdown (1, 3, 6, 22), the mechanisms by which this virus can establish a chronic neurological process is of great interest. The studies described in this paper were carried out to determine if *in vitro* persistence could select for a minor pre-existing or new mutant virus population capable of chronic infection *in vivo*.

The N_J culture was resistant to both homologous virus challenge at passage 16 and beyond and to challenge with heterologous virus infection at low MOI and thus differs from cultures producing DI particle (8) or the agent described by TER MEULEN and MARTIN (11). Interferon, which produces both heterologous and homologous virus resistance was not detectable in N_J culture supernatants. Most cells in the N_J culture showed viral antigen which supports the possibility that resistance of the N_J culture may be the result of intrinsic interference (9, 12); however, no mechanism for homologous virus resistance has been determined.

The selection of temperature sensitive and plaque morphology mutants (14) have also been suggested as possible mechanisms responsible for the establishment or maintenance of persistence. The N_J virus population showed no selection of *ts* or plaque morphology mutants through the 40th passage. In addition to the parameters already discussed, N_J JHM was tested for another biological property unique to JHM, namely its ability to cause demyelination following i.c. inoculation into susceptible mice. The N_J virus retained this property and produced lesions identical to those caused by the parental virus.

Antiviral antibody addition to the N_J culture at passage 19 increased yields of both cell-associated and supernatant virus. Enhancement of Murray Valley

encephalitis virus and rabbit pox virus plaque formation has been described in cells treated with viral specific antisera (5, 10). Furthermore, the addition of non-neutralizing antiviral antibody to leucocytes infected with dengue virus enhances virus growth (4) perhaps by increasing virus adsorption to phagocytic cells, thereby increasing the apparent MOI by facilitating entrance of the infectious virus into the cell cytoplasm while escaping the defense mechanisms of the phagocyte. In N_2A cells no active phagocytosis of colloidal carbon particles was found. In addition, the N_J culture is refractory to superinfection with JHM virus at passage 16 and beyond. Therefore, we must postulate that the effect of the antibody must not be enhanced viral uptake but a modulation of the cell surface that increases viral synthesis. The syncytia are the most likely cells affected since they are the only cell type with detectable cell surface antigen.

Subculturing in the presence of antibody cured the culture of CPE and surface fluorescence and of infectious virus. The cells did not, however, lose internal viral antigen and remained resistant to superinfection by JHM. They did not revert to viral production and were still resistant to JHM infection following 10 passages in the absence of antibody following the "cure". Antibody can, therefore, modulate a JHM infection having infectious virus and CPE to a persistent infection with no infectious virus and no viral antigen at the cell surface. Thus, it is possible that immune modulation, similar to that seen *in vitro* occurs *in vivo* and contributes to establishing chronic infection. A similar process has been postulated as the mechanism for the maintenance of herpes virus in the nervous system (18).

These studies described in this report show that *in vitro* persistence of JHM virus in the N_2A cell line is not mediated by a detectable change in the *in vitro* properties of the virus and in this respect is similar to *in vitro* persistence by other RNA viruses implicated in chronic neuropathology (12, 23). The N_J JHM virus also retained its special neurotropism *in vivo*. The *in vitro* properties of N_J JHM are similar to a JHM virus isolated from the brain of a chronically infected animal which also showed no detectable changes in its *in vitro* or *in vivo* properties (Stohlman, unpublished data). The *in vitro* antibody studies indicate that the host response may be the critical element in the establishment and maintenance of persistence *in vivo*.

Acknowledgments

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