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Biological and Macromolecular Properties of Murine Cells Persistently Infected with MHV-JHM

By

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With 5 Figures

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Summary

A persistently-infected neuroblastoma culture [Neuro-2A(JHMV)] was established with the murine hepatitis virus JHM [MHV-JHM]. After 100 days of passage, the endogenous virus [Neuro-2A(JHMV)end] released by this culture was unable to induce the syncytia typical of MHV-JHM and the endogenous virus was not temperature-sensitive. The Neuro-2A(JHMV) culture was cured of virus production by passage under neutralizing antibody [Neuro-2A(JHMV)Ab]. The Neuro-2A(JHMV) and the Neuro-2A (JHMV)Ab cultures were as susceptible to heterologous infection with mengovirus and vesicular stomatitis virus as the uninfected Neuro-2A culture. However, the Neuro-2A(JHMV) and Neuro-2A(JHMV)Ab cultures were partially resistant to homologous superinfection by MHV-JHM and the closely related MHV-A59. Virus related to MHV-JHM was rescued from the antibody-cured cells by cell fusion. The synthesis of MHV-JHM specific antigens by Neuro-2A(JHMV) cells, Neuro-2A(JHMV)Ab cells and 17Cl-1 cells infected by Neuro-2A(JHMV)end was studied by SDS-PAGE. The genomic RNAs of MHV-JHM and Neuro-2A(JHMV)end were compared by oligonucleotide mapping. The results of the protein and RNA studies indicated that the genome of Neuro-2A(JHMV)end was substantially modified from the genome of MHV-JHM, but the modifications did not significantly alter the molecular size of the viral-specific proteins.

Introduction

The Coronaviridae are a family of pathogenic, enveloped viruses that infect and cause disease in many species of animals (20, 30). The murine hepatitis group (MHV) experimentally produce several diseases in mice, dependent upon the serotype used, the age and strain of mouse, and the route of inoculation (20).

The JHM strain of MHV (MHV-JHM) is a strongly neurotropic agent and produces an acute encephalomyelitis in mice a few days after intracerebral inoculation (11, 20, 21, 32). Those animals which survive the acute encephalomyelitis have marked demyelinating lesions. The mechanism of demyelination appears to be the cytocidal effect of virus multiplication in oligodendrocytes (11, 19, 32). Herndon *et al.* (5) demonstrated that some mice show evidence of active demyelination as late as 16 months after intracerebral inoculation of MHV-JHM. Persistent infections of mice with other strains of MHV such as MHV-3 have also been reported (15, 29, 31).

Persistently-infected cell cultures allow the study of virus persistence in a system that is somewhat simpler than the intact animal. Cell cultures persistently-infected with MHV have been established and characterized biologically (7, 8, 16, 26).

We established a neuroblastoma cell culture persistently-infected with MHV-JHM. This culture was cured of virus production by passage under neutralizing antibody, and virus was rescued from the antibody cured cells by cell fusion. In this study, we describe the biological and macromolecular properties of these persistently-infected cultures.

Materials and Methods

Cells and Virus

The 17Cl-1, L2 and DBT cell lines have been previously described (2, 6, 28). The Neuro-2A clone of the C1300 mouse neuroblastoma cell line (9) was obtained from Dr. Carol Miller. The BHK-21 cell lines wa obtained from Dr. John Holland. The cells were grown at 37° C as monolayer cultures in prescription bottles using Dulbecco's modified Eagle's medium (DME) supplemented with 10 percent newborn or fetal bovine serum as previously described (2, 21).

The origin and growth of the MHV-JHM and MHV-A59 have been described (21). Vesicular stomatitis virus (VSV), Indiana serotype, was supplied by Dr. John Holland. Mengovirus has been previously described (17). VSV and mengovirus stock were grown in BHK-21 cells at a multiplicity of infection (MOI) of 0.1. Cells were infected with virus in suspension as previously described (2) or monlayers of cells were infected and incubated at 37° C.

The titers of MHV-JHM and MHV-A59 were determined by an endpoint dilution assay (21) or by plaque assay on 17Cl-1 cells. The titers of VSV and mengovirus were determined by plaque assay on BHK-21 cells. The results of the plaque assay were quantitatively equivalent to the end-point dilution assay.

Infectious Center Assay

To determine the number of cells releasing infectious virus, an infectious center assay was done. The cells to be tested were suspended at 1×10^6 cells per ml in DME containing 10 percent heat-inactivated mouse-anti-MHV-JHM serum and incubated at 37° C for 30 minutes. Ten-fold dilutions of these cells were prepared in DME 2 and added to a suspension of indicator cells (17 Cl-1) and seeded into Falcon Microtest plates. The number of infectious centers per ml (IC/ml) was calculated on the basis of cytopathic effect from the Poisson distribution. An alternative method of determining infectious centers was also used. Cells to be tested were suspended at 1×10^6 cells per ml in DME containing 10 percent heat-inactivated mouse-anti-MHV-JHM serum and incubated at 37° C for 30 minutes. Ten-fold dilutions of these cells were prepared in DME 2 and 0.5 ml volumes were added to confluent monolayers of 17 Cl-1 cells. The monolayers were incubated at 37° C to allow attachement of the test cells, aspirated and overlaid with DME 2 containing one percent agarose. The dishes were incubated at 37° C for 72 hours and the plaques were counted.

Preparation of Antiserum and Immunofluorescence

Antisera were prepared against MHV-JHM in BALB/c mice (21). The antisera were adsorbed against methanol fixed 17 Cl-1 cells to remove nonspecific antibodies except where otherwise indicated. The antisera were polyspecific and reacted with all of the MHV-JHM specific proteins detectable by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (1, 2). Immunofluorescence was done by the indirect immunofluorescence technique previously described (21).

Passage of Cells Under Neutralizing Antibody

The same antiserum used for immunofluorescence (unadsorbed) was used in these studies. The antiserum had a 50 percent plaque reduction titer of 1:1280 and was added to DME 10 to a final concentration of 5 percent (final antibody titer of the medium was 1:64). Cells were resuspended in medium containing antiserum at the time of passage. The medium was replenished after 3 days and the cells were passaged weekly.

Cell Fusion

Cells were fused by treatment with polyethylene glycol (18, 24, 25). Cells to be fused (1.5×10^6 cells of each line in DME 10) were cocultivated in 6 cm dishes for 24 hours at 37° C. The cells were washed twice with serum-free DME (DME 0) and one ml of 50 percent polyethylene glycol 6000 (Sigma) in DME 0 (pH 7.8) was added to each culture and the dishes were incubated at room temperature for 3 minutes. The medium was aspirated and at one minute intervals, the following volumes of DME 0 were added, 0.2 ml, 0.5 ml, 0.5 ml, 1.0 ml and 2.0 ml. The DME 0 was aspirated one minute later and the monolayers were washed once with DME 10 and fed with 3 ml of DME 10. The fused cultures were incubated at 37° C for 18 hours. The medium was replaced with 3 ml of DME 2, and incubated at 37° C for an additional 78 hours. The dishes were monitored for CPE at 12 hour intervals.

Radiolabeling of Intracellular Proteins

Monolayers of 17 Cl-1 cells were infected with virus at an MOI of 0.3 PFU/cell and incubated at 37° C. The infected cells [MHV-JHM at 6.5 hours PI, Neuro-2A (JHMV) end at 8.5 hours PI] were washed twice with methionine-free DME 2, and radiolabeled with ^{35}S-methionine (300 μCi/ml) in methionine-free medium for 1 hour at 37° C. Persistently-infected cells (2×10^6 cells in DEM 10) were seeded into 35 mm dishes and

incubated at 37° C for 24 hours. The cell monolayers were washed twice with methionine-free DME 2 and radiolabeled with ^{35}S-methionine (300 μCi/ml) in methionine-free medium for 3 hours at 37° C. At the end of the labeling period, the monolayers were lysed *in situ* at 0° C with buffer B 10 (1) and the cytoplasmic lysates were immunoprecipitated as described previously (1). SDS-PAGE was done as described by Laemmli and Favre (10) except the resolving gel was supplemented with 0.5 percent linear polyacrylamide. Volumes representing equal cell numbers were loaded in each lane of the slab gel. Fluorographs were prepared by the method of Laskey and Mills (12).

Ribonuclease T1 Oligonucleotide Fingerprints

A stock of Neuro-2A (JHMV) end was prepared from virus isolated from the Neuro-2A (JHMV) cell line at 178 days PI. Cells (17Cl-1) were infected with the Neuro-2A (JHMV) end stock or MHV-JHM at an MOI of 0.1 PFU/cell in phosphate-free medium and radiolabeled with ^{32}P-orthophosphate (150 μCi/ml) throughout the multiplication cycle. Virions were purified, the genomic RNA was extracted and fingerprinted as previously described (14).

Results

Establishment and Characteristics of the Persistently-Infected Cell Culture

A persistent infection of Neuro-2A cells was initiated by infecting cells with MHV-JHM at an MOI of 0.1 IU/cell. After the acute phase of the multiplication cycle was completed, characterized by minimal cell fusion and cytolysis, a resistant population of cells repopulated the flask in about 3 days. The culture was designated Neuro-2A(JHMV) and was passaged at weekly intervals at 37° C for over 280 days.

The titers of endogenous virus in the form of cell-free virus or cell-associated virus were determined at weekly intervals (Fig. 1). The amount of endogenous virus produced varied in a cyclical manner between 1.1×10^5 and 2×10^7 IU/10^6 cells. The proportion of virus that remained cell-associated varied between 2 and 56 percent. The fluctuations in the amount of virus produced did not correlate well with either the fluctuations in the fraction of cell-associated virus or the fraction of cells stained for MHV-JHM specific antigen by immunofluorescence. The percentage of cells capable of producing infectious centers was 1.2 to 2.5 fold greater than the percentage of cells containing virus-specific antigen (data not shown).

After establishment, no cytopathic effect (CPE), including syncytia formation, was ever observed in the Neuro-2A(JHMV) persistent culture. It was not possible to distinguish the Neuro-2A(JHMV) cells from the Neuro-2A parent cells by phase-contrast microscopy (Fig. 2). Both cell lines expressed dendritic processes to a similar extent. An approximate generation time of 30—36 hours was estimated for both lines over the first 21 passages. At 91 and 105 days, generation times of 30 hours were measured for both cell lines.

Effect of Neutralizing Antibody on Persistently-Infected Cultures

The Neuro-2A(JHMV) culture was passaged in the presence of MHV-JHM specific antibody for 4 weeks. Before treatment at 190 days post-establishment, the culture released 4.7×10^5 infectious units (IU) of endogenous virus per ml of culture fluid. Viral antigens were detected by immunofluorescence in 9 percent of the cells of the culture and 15 percent of the cells produced infectious centers. After treatment with neutralizing antiserum, no endogenous virus or viral antigens were detected and infectious centers were not produced. The antibody-cured culture was passaged without neutralizing antiserum for an additional 10 weeks. The properties of the antibody-cured cells did not change during this period. The antibody-cured cell line was designated Neuro-2A(JHMV)Ab.

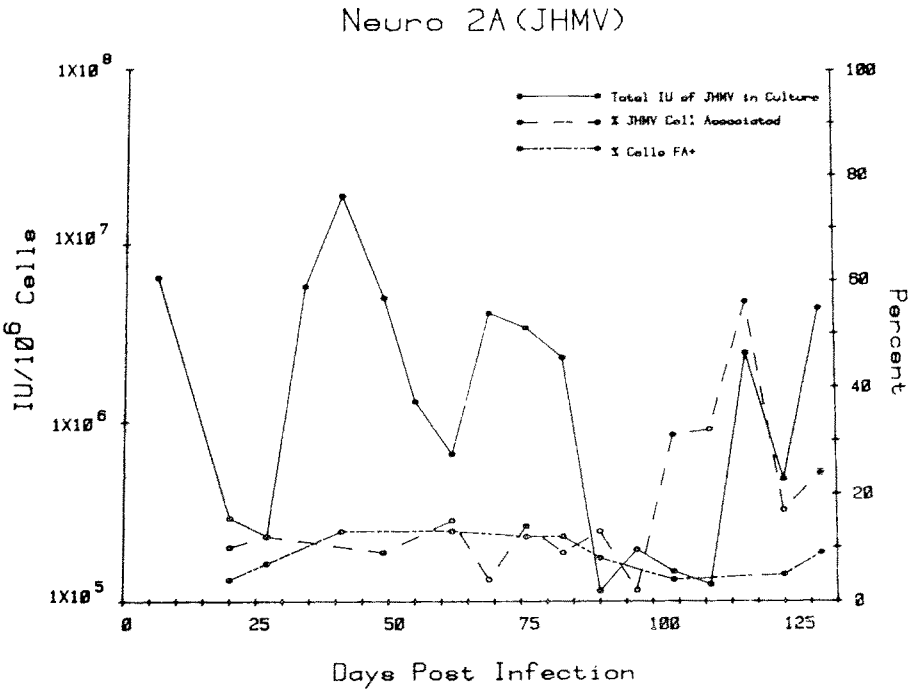

Fig. 1. Endogenous virus titer and the proportion of cells containing MHV-JHM specific antigens as a function of the time after establishment. The amount of endogenous virus was determined at the time of subculture by the end-point dilution method. The proportion of cells containing MHV-JHM specific antigen was determined by the indirect immunofluorescence method. Four hundred cells were counted and the percentage of positive-staining cells was calculated

MHV-JHM Specific Immunofluorescence of Persistently-Infected Cultures

The persistently-infected culture [Neuro-2A(JHMV)] and the antibody-cured culture [Neuro-2A(JHMV)Ab] were examined for MHV-JHM spe-

cific antigen by the indirect immunofluorescent method (Fig. 3). Viral-specific antigen was not detectable in the Neuro-2A or Neuro-2A(JHMV) Ab cells. Approximately 9 percent of the Neuro-2A(JHMV) cells contained viral-specific antigen on the basis of immunofluorescence.

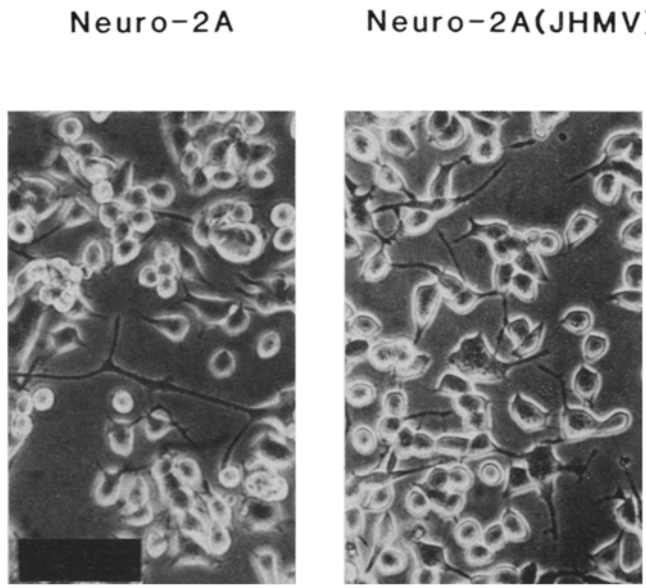

Fig. 2. Phase-contrast microscopy of Neuro-2A and Neuro-2A (JHMV) cells. Cells were seeded into 35 mm dishes and incubated for 48 hours at 37° C. Phase-contrast photomicrography was done without fixation. The scale bar represents 25 μm

Temperature-Sensitivity of Endogenous Virus

The endogenous virus [Neuro-2A(JHMV)end] released by the persistent culture at 118 days postestablishment was titrated at 33° and 39° C to

Fig. 3. Indirect immunfluorescence assay of Neuro-2A, Neuro-2A (JHMV) (210 days postestablishment) and Neuro-2A (JHMV)Ab cells (209 days after antibody curing) (top frame). Cells (10 μl containing 600 cells) were seeded into 60 well Microtiter dishes and incubated for 48 hours at 37° C. Center frame: Indirect immunofluorescence of 17Cl-1 cells acutely infected by MHV-JHM or Neuro-2A (JHMV)end (stock isolated at 196 days postestablishment) or mock-infected. Cells (17Cl-1) were acutely infected in suspension at an MOI of 0.3. The cells (10 μl containing 1000 cells) were seeded into 60 well Microtiter dishes and incubated for 12 hours at 37° C. Lower frame: Indirect immunofluorescence of 17Cl-1 cells infected by Neuro-2A (JHMV res) or mock-infected. Cells (17Cl-1) were acutely infected in suspension with undiluted Neuro-2A (JHMV) res (MOI approximately 0.05). The cells (10 μl containing 1000 cells) were seeded into 60 well Microtiter dishes and incubated for 12 hours at 37° C. The dishes were fixed and stained for MHV-JHM specific antigens by the indirect immunofluorescent method. The first column shows phase-contrast micrographs of representative fields of cells. The second column shows immunofluorescent micrographs of the same fields. The scale bars in the top frame represent 100 μm

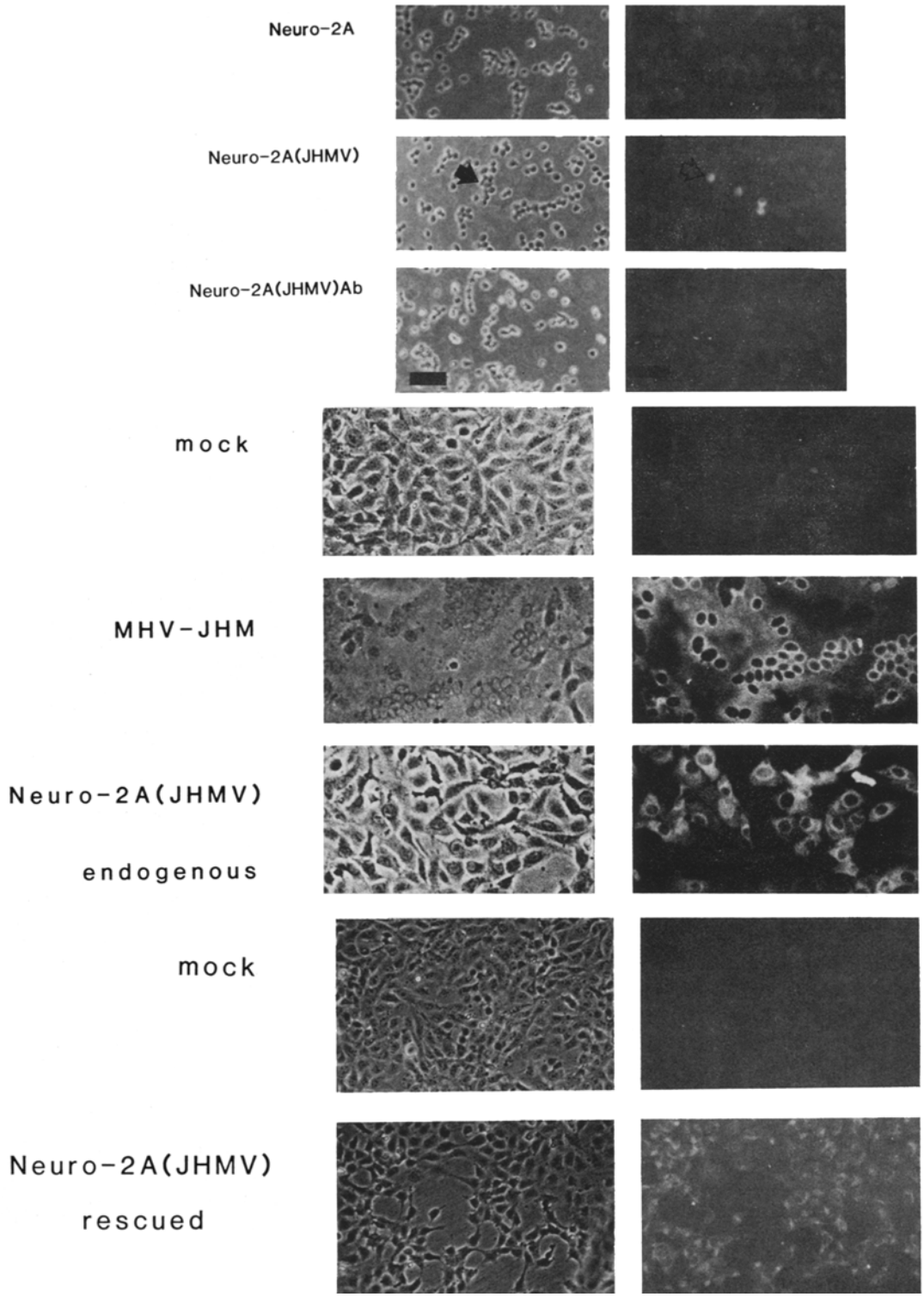
Neuro-2A
Neuro-2A(JHMV)
Neuro-2A(JHMV)Ab
mock
MHV-JHM
Neuro-2A(JHMV)
endogenous
mock
Neuro-2A(JHMV)
rescued

Fig. 3

determine if it was temperature-sensitive *(ts)*. The efficiency of plating (EOP), the ratio of the virus titer at 39° C to the virus titer at 33° C, of Neuro-2A(JHMV)end was 1.06 compared to 0.93 for MHV-JHM. To determine if multiplication was *ts*, 17Cl-1 cells were acutely infected with the endogenous virus or MHV-JHM and incubated at 33° or 39° C for 16 hours. The virus yields were determined by plaque assay at 33° C. The ratio of the yield of the acute infection at 39° C to the yield at 33° C was 0.86 for Neuro-2A(JHMV)end as compared to 0.39 for MHV-JHM. To further confirm these results, 25 clones of this stock of endogenous virus were selected by plaque assay and tested for temperature sensitivity at 39° and 33° C. All of the clones had efficiencies of plating between 0.2 and 5.0. MHV-JHM had an EOP of 0.93. These data indicate that the endogenous virus was not significantly *ts*.

Cytopathic Effect of Endogenous Virus on 17Cl-1 Cells

At approximately 100 days postestablishment, a change was observed in the acute CPE produced in 17Cl-1 cells by endogenous virus [Neuro-2A(JHMV)end] from the persistently-infected culture. Endogenous virus was isolated from the Neuro-2A(JHMV) culture at 196 days postestablishment and the cytopathic effect in an acute infection of 17Cl-1 cells was determined (Fig. 3). The CPE characteristic of MHV-JHM in 17Cl-1 cells at 37° C was syncytia formation followed by lysis with a fraction of the cells surviving. Syncytia were not observed in the cells infected by the endogenous virus at any time PI, but extensive lysis was evident in all cultures by 24 hours PI. An examination of the clones used to test for temperature-sensitivity [cloned from Neuro-2A(JHMV)end isolated at 118 days postestablishment] indicated that none of the clones were able to induce syncytia formation [syn(−)]. The plaques obtained in 3 days with MHV-JHM on 17Cl-1 cells were uniform in size and had a mean plaque diameter (n = 10) of 2.23 mm with a standard deviation of 0.13 mm. In the same experiment, Neuro-2A(JHMV)end plaques (n = 10) had a mean plaque diameter of 1.67 mm with a standard deviation of 0.30 mm.

Superinfection of Persistently-Infected Cultures

The persistently-infected and antibody-cured cultures were tested for their susceptibility to heterologous (mengovirus or VSV) infection (Table 1). The persistently-infected and the antibody-cured cultures were as susceptible to heterologous infection by mengovirus or VSV as the 17Cl-1 and Neuro-2A parent cultures. The MOI of the mengovirus and VSV was varied from 0.1 to 10 PFU/ml. The yields obtained did not vary as a function of MOI (data not shown). To further test for the presence of interferon in the Neuro-2A(JHMV) culture, supernatant Neuro-2A(JHMV) culture fluid

obtained at 190 days postestablishment was layered onto monolayers of DBT cells overnight. The monolayers were then infected with 20 PFU of VSV per dish in duplicate and overlaid with medium containing agarose. The VSV plaques were counted at 32 hours PI. There was no difference in yield between the control cultures (no supernatant cell culture fluid) and the experimental cultures. No plaques due to endogenous virus were evident at the time that the VSV plaques were read.

The susceptibility of the cultures Neuro-2A(JHMV) and Neuro-2A (JHMV)Ab to homologous infection by MHV-JHM and MHV-A59 was determined. Limited syncytia and lysis typical of an acute infection by MHV-A59 and MHV-JHM in 17Cl-1 cells were observed in the Neuro-2A parent culture. However, CPE was not observed at any time in either the Neuro-2A(JHMV) or Neuro-2A(JHMV)Ab cultures. Paradoxically, the yield of virus obtained from superinfected cultures (Table 1) suggest that these cultures may not be resistant to superinfection by MHV-JHM or MHV-A59. To further investigate this observation, virus released by the Neuro-2A(JHMV) cells infected by MHV-JHM or MHV-A59 was cloned by plaque assay. Fifteen clones were tested from each infection. Cells

Table 1. *Yield of virus obtained from the infection of 17Cl-1, Neuro-2A, Neuro-2A (JHMV) and Neuro-2A (JHMV)Ab cells with MHV-JHM, MHV-A59, VSV or mengovirus*[a]

Cell line	Endogenous[b] ($\times 10^{-5}$)	MHV-JHM ($\times 10^{-5}$)	MHV-A59 ($\times 10^{-5}$)	VSV ($\times 10^{-5}$)	Mengovirus ($\times 10^{-5}$)
Neuro-2A	0	3.2 ± 2.9	4.8 ± 3.7	1.3 ± 0.3	4.8 ± 0.4
Neuro-2A(JHMV)	4.9 ± 0.6	9.5 ± 0.4[c]	1.1 ± 0.3[c]	1.1 ± 0.7	3.8 ± 0.9
Neuro-2A(JHMV)Ab	0	3.2 ± 1.6	3.7 ± 1.7	4.2 ± 2.1	8.1 ± 0.5

[a] The Neuro-2A (JHMV) culture was assayed at 210 and 224 days postestablishment. The Neuro-2A (JHMV) Ab culture was assayed at 132, 146 and 157 days after antibody curing. Cells (1 to 1.5×10^6) were seeded into 6-well cluster dishes and incubated at 37° C. The monolayers were washed once with DME-2 and infected with 0.2 ml of the indicated virus. The MOI of MHV-JHM and MHV-A59 was 0.1 PFU/cell, and of VSV and mengovirus was 1.0 PFU/cell. The dishes were incubated at 37° C for 24 hours and the virus was titrated by plaque assay. The results are expressed as the mean and standard deviation of independent determinations

[b] The endogenous virus titer is representative of the amount of virus released by the cells when mock-infected. The sensitivity of the assay is 10 PFU/10^6 cells.

[c] The yield of virus obtained in these infections was similar to the amount of endogenous virus released by the cells when mock infected. It was not possible to reliably differentiate between the yield due to endogenous virus and the yield due to superinfection by plaque size. Fifteen clones were isolated from each infection and tested for the ability to form syncytia. All were negative suggesting that the released virus was endogenous virus

(17Cl-1) were acutely infected by each clone and the CPE monitored by phase-contrast microscopy. No syncytia were observed in any of the clonal infections at any time. These results suggest that the virus yielded was endogenous virus, not progeny virus due to the superinfecting MHV-JHM or MHV-A59.

Interference with MHV Multiplication

Supernatant cell culture fluids were collected from the persistently-infected culture Neuro-2A(JHMV) at 190 days postestablishment. Dilutions of the supernatant fluids were tested for the ability to interfere with the multiplication of MHV-JHM or MHV-A59 in 17Cl-1 cells (data not shown). No interference in the multiplication of standard virus was detected. In addition, 2 different pools of Neuro-2A(JHMV)end (isolated at 196 days and 220 days postestablishment) were serially passaged 5 times in L2 cells without dilution. The MOI of the first passage was 0.3 PFU per cell. Aliquots of each serial passage were saved and subsequently assayed for the ability to interfere with the multiplication of standard MHV-JHM in L2 cells and 17Cl-1 cells. Although the titers of the virus yields declined approximately 50 fold during serial passage, no interference with the multiplication of MHV-JHM was detected by plaque assay. These results indicate that defective-interfering particles were not present in the persistently-infected culture or selected for during multiplication in L2 cells. The mechanism for the decrease in titer of the Neuro-2A(JHMV)end during serial undiluted passage is currently under investigation.

Rescue of Virus from Antibody-Cured Cells

On the basis of the results from the homologous superinfection experiments, it was suspected that the viral genome was maintained in a covert state in the antibody-cured cells. Several attempts were made to rescue virus from the antibody-cured cells by cocultivation with 17Cl-1 cells. At various times after initiation, the cocultures were tested for viral antigen by immunofluorescence and for virus multiplication by plaque assay and end-point dilution assay. No viral antigens or infectious viruses were detected. When the Neuro-2A(JHMV)Ab cells were fused to 17Cl-1 cells with polyethylene glycol, focal lysis was observed in 3 to 4 days in 3 trials (fusions were done at 174, 195 and 209 days after the Neuro-2A(JHMV)Ab line was established). Infectious rescued virus was detected by the end-point dilution assay. The yield of virus obtained from the 3 trials was in the range of 10^3 to 10^4 IU per 10^6 cells. Several plaque assays were attempted at 33°, 37° and 39° C to detect infectious rescued virus. The assays were observed daily for 7 days. No plaques were observed at any of the temperatures tested. The reason for this is not known. No infectious virus was detected in parallel unfused cultures. Two clones of rescued virus obtained

were recloned by the end-point dilution method. The rescued virus was designated Neuro-2A(JHMV)res.

Since it was possible that a contaminating virus such as a paramyxovirus may have been rescued in the cell fusion experiment, each rescued clone was tested for its ability to synthesize MHV-JHM specific viral antigen, and induce a CPE typical of MHV. Cells were infected in suspension with 10-fold serial dilutions of each rescued clone. Ten replicas of 10 μl cultures were seeded in 60 well Microtest dishes and incubated at 37° C for 48 hours. The cells were stained for MHV-JHM antigens by the immunofluorescent method. Mock cultures were prepared in the same manner using lysates from fused 17Cl-1 cells for the infection. Typical results are shown in Fig. 3. No CPE and no viral antigen were detectable in the mock cultures. A significant amount of MHV-JHM specific antigen detected by immunofluorescence and CPE was evident in the cultures infected with rescued virus. At higher virus dilutions, no CPE or viral antigens were evident. The MHV-JHM specific fluorescence observed in the rescued cultures was considerably weaker than observed in the MHV-JHM cultures (Fig. 3). No syncytia were observed in any of the rescued cultures. The results indicate that the rescued virus clones were related to MHV-JHM.

MHV-JHM Specific Protein Synthesis in Persistently-Infected and Antibody-Cured Cultures

Viral-specific antigens were detected by immunofluorescence in the persistently-infected culture Neuro-2A(JHMV) (Fig. 3). However, no MHV-JHM specific antigens were detectable in the antibody-cured culture Neuro-2A(JHMV)Ab. To further these studies, each of the cell lines were radiolabeled with ^{35}S-methionine, cell lysates were prepared and immunoprecipitated with the adsorbed antisera used in the immunofluorescence studies. The immunoprecipitates were analyzed by SDS-PAGE. Seven viral-specific proteins have been identified in 17Cl-1 cells infected by MHV-JHM (E2-150K, N-63K, p61, p56, p35, p23, p22 and p14; references 1 and 23). A reference immunoprecipitate from a lysate of 17Cl-1 cells acutely infected by MHV-JHM was used to identify viral-specific proteins in the immunoprecipitates of the persistently-infected cells and of 17Cl-1 cells acutely infected by Neuro-2A(JHMV)end. The results shown in Fig. 4 indicate that the MHV-JHM specific proteins were present in the Neuro-2A(JHMV) culture, but absent in the Neuro-2A(JHMV)Ab or Neuro-2A cultures. The profile of MHV-JHM specific antigens in the Neuro-2A(JHMV) culture was slightly modified from the profile of the MHV-JHM acute infection. The 61K and 56K antigens were not detectable, and the 23K antigen appeared to migrate slightly faster. The MHV-JHM specific antigens synthesized in the acute infection of 17Cl-1 cells by MHV-JHM and Neuro-2A(JHMV)end appeared similar. A band with an apparent molecular size

of 120 kilodaltons was apparent in the Neuro-2A(JHMV)end immunoprecipitate (lane F), but not in the MHV-JHM (lane E). The identity of this band is not known, but a 120 kilodalton protein has been demonstrated to be the precursor to E2-150K (13). A 45 kilodalton protein was observed in the lysates of the Neuro-2A(JHMV) and lysates of cells infected by MHV-JHM or Neuro-2A(JHMV)end. This protein is not a unique protein and is probably an aggregate of E1 (27). In other experiments (data not shown) the intensity of the 45 kilodalton protein varied inversely with the intensity of the E1 protein. Other differences were due to intensity not

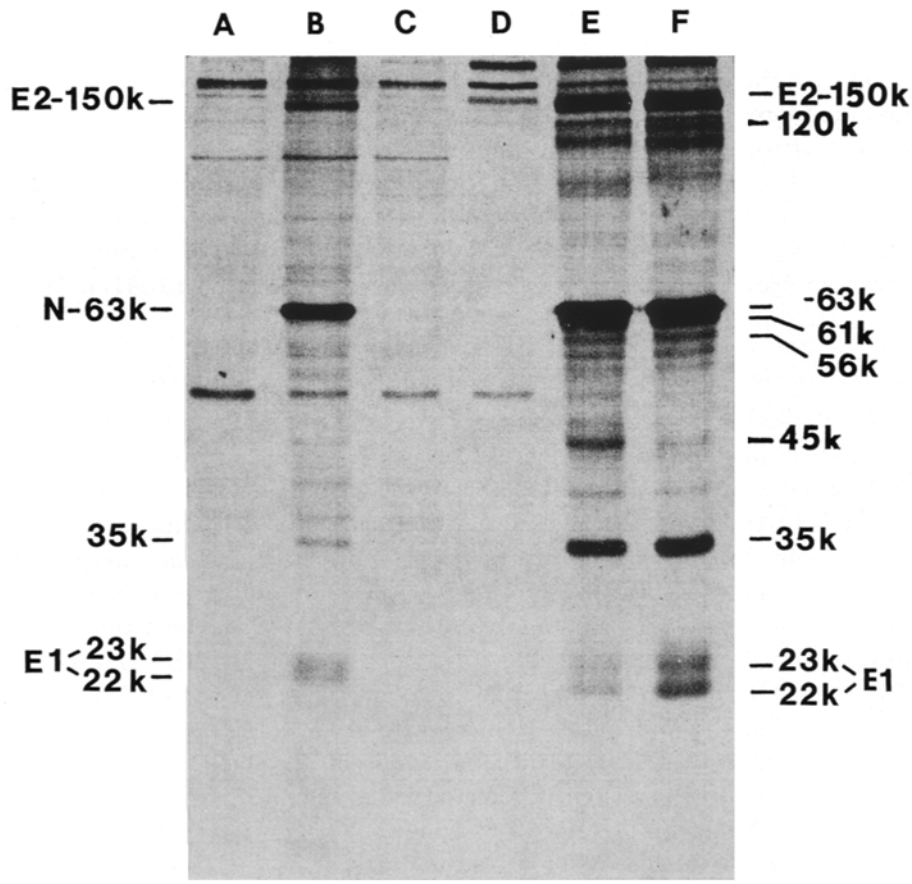

Fig. 4. MHV-JHM specific proteins in the persistently-infected culture [Neuro-2A (JHMV)] (23 days postestablishment), antibody-cured culture [Neuro-2A (JHMV) Ab] (153 days after antibody curing), and 17Cl-1 cells acutely infected with MHV-JHM or Neuro-2A (JHMV)end (stock isolated at 196 days postestablishment). Lysates of Neuro-2A cells (lane A), Neuro-2A (JHMV) (lane B), Neuro-2A (JHMV) Ab (lane C), and 17Cl-1 cells acutely infected by MHV-JHM (lane E) or Neuro-2A (JHMV) end (lane F) or mock-infected (lane D) were prepared, immunoprecipitated and analyzed by SDS-PAGE

the presence or absence of a virus-specific band. The 14 kilodalton protein was not detectable under these experimental conditions.

Oligonucleotide Fingerprints of Genomes

The oligonucleotide fingerprints of the genomes of MHV-JHM and Neuro-2A(JHMV)end were compared in an effort to detect specific modifications in the persistent viral genome (Fig. 5). The stock of Neuro-2A (JHMV)end used was the same as the stock used for the generation of clonal isolates for the temperature-sensitivity study (isolated at 196 days postestablishment). The molecular sizes of the 2 genomes were identical on the basis of sedimentation analysis and agarose gel electrophoresis (data not shown). Several differences between the Neuro-2A(JHMV)end and MHV-JHM genomes were evident. Arrowhead 1 indicates an oligonucleotide present in Neuro-2A(JHMV)end, but absent in MHV-JHM. Arrowheads 2—6 represent oligonucleotides present in MHV-JHM, but absent in Neuro-2A(JHMV)end. Several differences in intensity of other oligonucleotides, not pointed out in Fig. 5, were evident on the original autoradiograms.

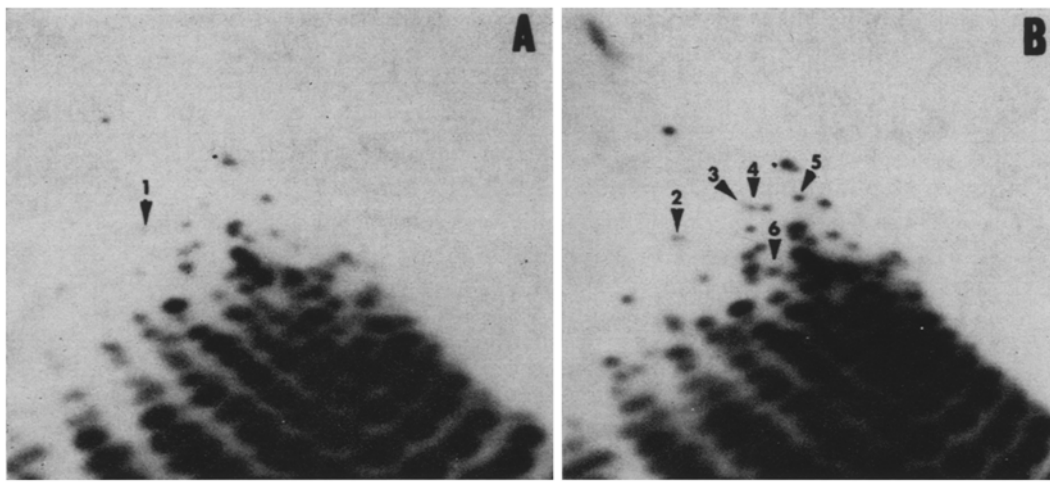

Fig. 5. Oligonucleotide fingerprints of the genomes of MHV-JHM and Neuro-2A (JHMV)end (isolated at 196 days postestablishment). Panel A is the fingerprint of the Neuro-2A (JHMV)end genome and panel B is the fingerprint of the MHV-JHM genome. Arrowhead 1 represents an oligonucleotide present in the Neuro-2A (JHMV) end genome, but absent in the MHV-JHM genome. Arrowheads 2—6 represent oligonucleotides present in the MHV-JHM genome, but absent in the Neuro-2A (JHMV) end genome

Discussion

Friedman and Ramseur (4) have proposed mechanisms for the establishment and maintenance of persistent infections: the induction of

interferon; the development of defective-interfering particles; the development of *ts* mutants; and the integration of DNA copies of RNA genomes into cellular DNA.

The persistently-infected culture Neuro-2A(JHMV) and the antibody-cured culture Neuro-2A(JHMV)Ab were as susceptible to infection by mengovirus or VSV as the parent culture Neuro-2A (Table 1). There was no effect on the yield obtained when the MOI was varied from 0.1 to 10 PFU per cell. In addition, culture supernatant fluid from Neuro-2A (JHMV) did not reduce the titer of VSV assayed on DBT cells. These experiments suggest that interferon did not play a role in the maintenance of the persistent infection. These results are in general agreement with others (7, 16, 26).

Supernatant cell culture fluids from the persistent culture Neuro-2A (JHMV) did not interfere with the multiplication of MHV-JHM or MHV-A59 in 17Cl-1 cells. No interference in the multiplication of MHV-JHM or MHV-A59 was observed during serial undiluted passage (20). Further, Neuro-2A(JHMV)end was passaged serially in L2 cells and no virus was produced that interfered with the multiplication of standard MHV-JHM. These results suggest that the presence of defective-interfering particles in our cultures was unlikely.

Of the 25 clones of endogenous virus tested for temperature-sensitivity, none could be considered to be significantly *ts*. Holmes and Behnke (8) found *ts* endogenous virus in their persistently-infected cultures. However, others have found the endogenous virus to be non-*ts* (7, 25, 26).

There was an evolving relationship between the virus and the host cell during the first 100 days of establishment. After this period of time, the endogenous virus released by the culture was unable to induce syncytia formation during an acute infection (Fig. 3). This was also true for the 25 clones established from the released virus.

The plaques of the syn(−) endogenous virus released by the persistent cultures were smaller than the plaques typical of MHV-JHM. Others have reported that the mean plaque size of endogenous virus released by cultures persistently infected with MHV-JHM or MHV-A59 was reduced (7, 8, 26, 33). However, only Yoshikura and Tejima (33) reported the loss of ability to induce syncytia during an acute infection.

The Neuro-2A(JHMV) culture was cured of endogenous virus production by passage under neutralizing antibody. MHV-JHM specific antigen was not detectable in the antibody-cured culture by immunofluorescence or by immunoprecipitation of radiolabeled proteins (Figs. 3, 4). Virus was rescued from these cultures and was found to be antigenically related to MHV-JHM (Fig. 3). Stohlman and Weiner (26) antibody cured a culture similar to our Neuro-2A(JHMV) by passage under antibody. They were unable to detect endogenous virus in their antibody-cured culture. How-

ever, they were able to detect virus-specific antigen by immunofluorescence. Cold-sensitive mutants were isolated from antigen positive and antigen negative clones of their antibody-cured cells (25). Since the persistent infections could be cured by passage under neutralizing antibody, it seems reasonable that the persistent infections were maintained by cell to cell transfer of virus. However, MHV-JHM related virus was rescued from the antibody-cured cells by cell fusion. The results of these workers and our results indicate that the viral genome was capable of persisting in the absence of infectious virus. The mechanism of genome persistence is under investigation.

The persistently-infected culture Neuro-2A(JHMV) and the antibody-cured culture Neuro-2A(JHMV)Ab were resistant to superinfection by either MHV-JHM or MHV-A59 on the basis that no typical CPE was observed during the infection. These results are similar to those obtained by others (8, 26). However, the Neuro-2A(JHMV) and Neuro-2A(JHMV) Ab cultures yielded about the same amount of virus as the acutely infected Neuro-2A control culture (Table 1). Clones of the virus released by the superinfected Neuro-2A(JHMV) were syn(—) suggesting that the progeny virus was endogenous virus. An alternative explanation is that superinfecting virus multiplied, but the CPE typical of MHV was altered by a mechanism such as phenotypic masking (34). However, this is unlikely because the clones of released virus did not produce syncytia during an acute infection of 17Cl-1 cells.

The MHV-JHM specific proteins synthesized by cells acutely infected by MHV-JHM or by Neuro-2A(JHMV)end were compared on the basis of molecular size (Fig. 4). The gel profiles were almost identical suggesting that there were no modifications in the genome of Neuro-2A(JHMV)end resulting in changes in the molecular size of the proteins. These data are in general agreement with the data reported by YOSHIKURA and TEJIMA (33) for MHV-S. However, the profile of viral-specific proteins synthesized by the Neuro-2A(JHMV) cells was somewhat altered from the acute infections. There appeared to be minor modifications in the E1-23K protein and the 61K and 56K proteins were not detectable in the Neuro-2A (JHMV) lysate. The change in migration of the E1-23K protein was probably due to the differences in cell-specific glycosylation of the E1-22K protein between the 2 cell lines. No viral-specific proteins were detected in the Neuro-2A(JHMV)Ab culture. An additional protein band with an apparent molecular weight of 120 kilodaltons was observed in the immunoprecipitate of the lysate of 17Cl-1 cells infected by Neuro-2A(JHMV)end. A protein with a similar size was shown to be the precursor to E2-150 in *in vitro* translation studies (13).

We previously identified 7 virus specific RNAs in cells infected by MHV-JHM (14). These RNAs formed a nested set of 6 subgenomic sized

RNAs in addition to the genomic sized RNA as determined by oligonucleotide mapping. The genomic sized RNA was designated RNA-1. The subgenomic species were designated RNA-2 to RNA-6 in order of decreasing size. At least 6 differences were observed in the oligonucleotide patterns of MHV-JHM and Neuro-2A(JHMV)end (Fig. 5). One spot (arrowhead 1) was present in the Neuro-2A(JHMV)end genome, but not in the MHV-JHM genome. This probably represents an alteration in the oligonucleotide indicated by arrowhead 2 in the MHV-JHM genome. Arrowheads 2—6 indicate oligonucleotides detected in the MHV-JHM genome, but not in the Neuro-2A(JHMV)end genome. We have determined that oligonucleotide 2 was from the 5′ region of RNA-3, oligonucleotides 3 and 4 were from the 5′ region of RNA-1, and oligonucleotides 5 and 6 were from the 5′ region of RNA-2. Siddell *et al.* (22) demonstrated that RNA-3 codes for the precursor to the glycoprotein E2. Others have implicated the E2 protein in MHV-induced cell fusion (3, 8). Thus, the modification in the RNA-3 portion of the genome which codes for E2 may result in the syn(−) phenotype.

The results described in this paper suggest that it is unlikely that the induction of interferon, the development of defective-interfering particles or the development of *ts* mutants play a role in the persistent infection of Neuro-2A cells with MHV-JHM. However, a particular phenotype, syn(−), developed during the course of the infection. The syn(−) phenotype was presumably selected by the host cell on the basis of its decreased cytopathogenicity.

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References

1. Bond, C. W., Anderson, K., Goss, S. H., Sardinia, L.: Relatedness of virion and intracellular species of the murine coronaviruses JHMV and A59V. In: ter Meulen, V., Siddell, S., Wege, H. (eds.), Biochemistry and Biology of Coronaviruses (Adv. Exp. Biol. and Med., Vol. 142), 103—110. New York: Plenum Press 1981.
2. Bond, C. W., Leibowitz, J. L., Robb, J. A.: Pathogenic murine coronaviruses II. Characterization of virus-specific proteins of murine coronaviruses JHMV and A59V. Virology **94**, 371—384 (1979).
3. Collins, A. R., Knobler, R. L., Powell, H., Buchmeier, M. J.: Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. Virology **119**, 358—371 (1982).
4. Friedman, R. M., Ramseur, J. M.: Mechanisms of persistent infections by cythopathic viruses in tissue culture. Arch. Virol. **60**, 83—103 (1979).

5. Herndon, R. M., Griffin, D. E., McCormick, U., Weiner, L. P.: Mouse hepatitis virus recurrent demyelination. Arch. Neurol. **32**, 32—35 (1975).
6. Hirano, N., Fujiwara, K., Hino, S., Matumoto, M.: Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. Arch. ges. Virusforsch. **44**, 298—302 (1974).
7. Hirano, N., Goto, N., Makino, S., Fujiwara, K.: Persistent infection with mouse hepatitis virus strain JHM in DBT cell culture. In: ter Meulen, V., Siddell, S., Wege, H. (eds.), Biochemistry and Biology of Coronaviruses (Adv. Exp. Biol. and Med., Vol. 142), 301—308. New York: Plenum Press 1981.
8. Holmes, K. V., Behnke, J. N.: Evolution of a coronavirus during persistent infection *in vitro*. In: ter Meulen, V., Siddel, S., Wege, H. (eds.), Biochemistry and Biology of Coronaviruses (Adv. Exp. Biol. and Med., Vol. 142), 287—299. New York: Plenum Press 1981.
9. Kleebe, R. J., Ruddle, F. H.: Neuroblastoma: Cell culture analysis of a differentiating stem cell system. J. Cell Biol. **43**, 69a (1969).
10. Laemmli, U. K., Favre, M.: Maturation of the head protein of bacteriophage T4: I. DNA packaging events. J. Mol. Biol. **80**, 575—599 (1973).
11. Lampert, P. W., Sims, J. K., Kniazeff, A. J.: Mechanism of demyelination in JHM virus encephalomyelitis. Electron microscope studies. Acta Neuropathol. **24**, 76—85 (1973).
12. Laskey, R. A., Mills, A. D.: Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**, 335—341 (1975).
13. Leibowitz, J. L., Weiss, S. R., Paavola, E., Bond, C. W.: Cell-free translation of murine coronavirus RNA. J. Virol. **43**, 905—913 (1982).
14. Leibowitz, J. L., Wilhelmsen, K. C., Bond, C. W.: The virus-specific intracellular RNA species of two murine coronaviruses: MHV-A59 and MHV-JHM. Virology **114**, 39—51 (1981).
15. LePrevost, C., Virelizier, J. L., Dupuy, J. M.: Immunopathology of mouse hepatitis virus type 3 infection. III. Clinical and virologic observation of a persistent viral infection. J. Immunol. **115**, 640—643 (1975).
16. Lucas, A., Flintoff, W., Anderson, R., Percy, D., Coulter, M., Dales, S.: *In vivo* and *in vitro* models of demyelinating diseases: Tropism of JHM strain of mouse hepatitis virus for cells of glial origin. Cell. **12**, 553—560 (1977).
17. Plagemann, P. G. W., Swim, H. E.: Replication of mengovirus. I. Effect on synthesis of macromolecules by host cell. J. Bacteriol. **91**, 2317—2326 (1966).
18. Pontecorvo, G.: Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. Somatic Cell Genetics **1**, 397—400 (1975).
19. Powell, H. C., Lampert, P. W.: Oligodendrocytes and their myelin-plasma membrane connections in JHM mouse hepatitis virus encephalomyelitis. Lab. Invest. **33**, 440—445 (1975).
20. Robb, J. A., Bond, C. W.: Coronaviridae. In: Fraenkel-Conrat, H., Wagner, R. R. (eds.), Comprehensive Virology, Vol. 14, 193—247. New York: Plenum Press 1979.
21. Robb, J. A., Bond, C. W.: Pathogenic murine coronaviruses. I. Characterization of biological behavior *in vitro* and virus-specific intracellular RNA of strongly neurotropic JHMV and weakly neurotropic A59V viruses. Virology **94**, 352—370 (1979).
22. Siddell, S., Wege, H., Barthel, A., ter Meulen, V.: Intracellular protein synthesis and the *in vitro* translation of coronavirus JHM RNA. In: ter Meulen, V., Siddell, S., Wege, H. (eds.), Biochemistry and Biology of Coronaviruses (Adv. Exp. Biol. and Med., Vol. 142), 193—207. New York: Plenum Press 1981.

23. Sorenson, O., Coulter-Mackie, M., Percy, D., Dales, S.: *In vivo* and *in vitro* models of demyelinating diseases. In: ter Meulen, V., Siddell, S. Wege, H., (eds.), Biochemistry and Biology of Coronaviruses (Adv. Exp. Biol. and Med., Vol. 142), 271—286. New York: Plenum Press 1981.
24. Stohlman, S. A., Sakaguchi, A., Weiner, L. P.: Rescue of a positive stranded RNA virus from antigen negative neuroblastoma cells. Life Science **24**, 1029—1036 (1979).
25. Stohlman, S. A., Sakaguchi, A., Weiner, L. P.: Characterization of the cold-sensitive murine hepatitis virus mutants rescued from latently infected cells by cell fusion. Virology **98**, 448—455 (1979).
26. Stohlman, S. A., Weiner, L. P.: Stability of neurotropic mouse hepatitis virus (JHM strain) during chronic infection of neuroblastoma cells. Arch. Virol. **57**, 53—61 (1978).
27. Sturman, L. S.: The structure and behavior of coronavirus A59 glycoproteins. In: ter Meulen, V., Siddell, S., Wege, H. (eds.), Biochemistry and Biology of Coronaviruses (Adv. Exp. Biol. and Med., Vol. 142), 1—17. New York: Plenum Press 1981.
28. Sturman, L. S., Takemoto, K.: Enhanced growth of a murine coronavirus in transformed mouse cells. Infect. Immun. **6**, 501—507 (1972).
29. Tamura, T., Taguchi, F., Veda, K., Fujiwara, K.: Persistent infection with mouse hepatitis virus of low virulence in nude mice. Microbiol. Immunol. **21**, 683—691 (1977).
30. Tyrrell, D. A. J.: Biology of coronaviruses 1980. In: ter Meulen, V., Siddell, S. A., Wege, H. (eds.), Biochemistry and Biology of Coronaviruses (Adv. Exp. Biol. and Med., Vol. 142), 419—425. New York: Plenum Press 1981.
31. Virelizier, J. L.: Pathogenicity and persistence of mouse hepatitis virus in inbred strains of mice. In: ter Meulen, V., Siddell, S., Wege, H. (eds.), Biochemistry and Biology of Coronaviruses (Adv. Exp. Biol. and Med., Vol. 142), 349—358. New York: Plenum Press 1981.
32. Weiner, L. P.: Pathogenesis of demyelination induced by a mouse hepatitis virus. Arch. Neurol. **28**, 298—303 (1973).
33. Yoshikura, H., Tejima, S.: Role of protease in mouse hepatitis virus-induced cell fusion. Studies with a cold-sensitive mutant isolated from a persistent infection. Virology **113**, 503—511 (1981).
34. Yoshikura, H., Taguchi, F.: Mouse hepatitis virus strain MHV-S: Formation of pseudotypes with a murine leukemia virus envelope. Intervirology **10**, 132—136 (1981).

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