VIROLOGY 141, 89-101 (1985)

In Vivo and *in Vitro* Models of Demyelinating Disease

XI. Tropism and Differentiation Regulate the Infectious Process of Coronaviruses in Primary Explants of the Rat CNS

S. BEUSHAUSEN AND S. DALES[1]

Cytobiology Group, Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada

Received August 20, 1984; accepted October 25, 1984

The coronaviruses, ubiquitous in mammals, including man, manifest serotype-related predeliction for different tissues. This presentation deals with specificity of the murine viscerotropic MHV3 and neurotropic JHMV for explanted cells from the CNS of newborn, inbred, Wistar-Furth rats. An unambiguous tropism of MHV3 for astrocytes and JHMV for oligodendrocytes is demonstrated. With the latter cell-virus interaction, relatively small differences in spatial density of oligodendrocytes influence profoundly the duration of persistence and virus yield. The *in vitro* temporal program of oligodendrocyte differentiation, monitored by induction of a myelin-related enzyme, 2′:3′-cyclic nucleotide-3′-phosphohydrolase, corresponds to that occurring *in vivo* (F. A. McMorris, *J. Neurochem.* **41**, 506-515, 1983). It is complete within 15-21 days and is coincident with the onset of insusceptibility to disease caused by JHMV. Experimental elevation of intracellular cyclic-AMP levels, presumed to reflect oligodendrocyte differentiation, likewise suppresses JHMV replication without affecting that of MHV3 in astrocytes. On the basis of these data it is concluded that *in vitro* interaction of JHMV with oligodendrocytes reflects accurately the *in vivo* host control over the tropism and expression of this virus, thereby effecting the progressive, demyelinative disease, process.

INTRODUCTION

The coronaviruses (CV) are a widely disseminated group of agents infecting animals, including man (Tyrrell *et al.*, 1965; Hamre *et al.*, 1966). Capability of some rodent CV types to induce a progressive dymyelination in the central nervous system (CNS) (Cheever *et al.*, 1949; Bailey *et al.*, 1949; Nagashima *et al.*, 1978; Sorensen *et al.*, 1980), has placed the serorelated human strains under consideration as having the potential to trigger diseases such as multiple sclerosis (Tanaka *et al.*, 1976; Burks *et al.*, 1980). In mice, depending on age, strain, route of inoculation and other factors, the viscerotropic CV strain, MHV3, usually causes a rapidly fatal hepatic necrosis while the neurotropic strain, JHMV, a CNS disease (Virelizier *et al.*, 1975). Infection of the rat CNS, before weaning, may result in development of an acute, fatal encephalitis or a slowly progressive, chronic and sometimes remitting, paralytic disease characterized by foci of demyelination (Nagashima *et al.*, 1978; Sorensen *et al.*, 1980). Immunohistopathologic analysis of rat CNS from animals undergoing the chronic type of disease reveals that JHMV infects neurons and oligodendrocytes (Nagashima *et al.*, 1978; Sorensen *et al.*, 1984). Since, however, in the rat intracerebral (IC) inoculation of MHV3 can result in virus replication without overt symptoms of disease (Hirano *et al.*, 1980), there is an implication that this serotype is tropic for cells other than neurons and oligodendrocytes.

It is now possible to establish reprodu-

[1] Author to whom requests for reprints should be addressed.

0042-6822/85 $3.00

cibly, *in vitro* cultures of defined neural cells representative of those present in the CNS of rats (McCarthy *et al.*, 1978, 1980; Barbarese *et al.*, 1981). We, therefore, undertook to study the replication and tropisms of CV, utilizing such a simplified CNS system so as to be able to control more precisely the parameters relevant to cell-virus interactions. This enabled us to define, with greater certainty, the tropisms of the virus serotypes, MHV3 and JHMV, for different neural cells. Furthermore, the age-dependent loss of infectability in young rats could now be examined in relation to the continuing, time-related *in vitro* program of differention occurring in neural cells explanted from newborn animals (Abney *et al.*, 1981; Pfeiffer *et al.*, 1981; McMorris, 1983).

MATERIALS AND METHODS

Continuous cell lines. L-2 (Rothfels *et al.*, 1959) and LMTK$^-$ (Merchant *et al.*, 1962) murine fibroblasts derived from the L_{929} line (Sanford *et al.*, 1948), were routinely propogated as monolayers in Eagle's minimum essential medium (Gibco, Grand Island, N. Y.), supplemented with 5% fetal bovine serum, (FBS) (Whittaker Bioproducts, Walkersville, Md.), penicillin (100 U/ml), and streptomycin (100 μg/ml), termed nutrient medium (NM), at 37° in an humidified atmosphere containing 5% CO_2.

Viruses and preparation of stocks. Mouse hepatitis virus strains MHV_3 and JHMV were grown on monolayers of L-2 cells. Once the infection had progressed to the stage when 50% of the syncytia had lifted off, the remainder of the cells were scraped off with a rubber policeman. The suspension was taken up into a syringe and forced through a No. 30 gauge needle, to cause disruption. The lysates produced were centrifuged at 40 *g* for 5 min at 4° and the supernatant filtered through a millipore Millex-GS, 0.22-μm filter unit to remove larger debris. The filtrate, dispensed in 0.5-ml aliquots, was stored at −70° or used when fresh.

The Indiana strain of vesicular stomatitis virus (VSV) was grown on monolayers of L-2 cells for approximately 24 hr and the virus containing supernatant fluid was used as with CV.

The Hallé strain of measles virus (MV) was grown on Vero cell monolayers at 32.5°. After 48 hr or when syncytia of moderate size had formed, the virus in the culture supernatant was prepared as above.

Primary brain cultures. Primary brain explants were prepared as described by McCarthy *et al.* (1978) with minor but fundamental modifications. Briefly, 1- or 2-day-old Wistar-Furth rat pups were decapitated, the cerebral hemispheres removed, and freed from the contaminating meninges. The tissue was placed at 0° in three washes of Basal minimal essential medium (BME_{10}), Gibco, Grand Island, N. Y.) immediately after excision. The medium was supplemented with 10% heat-inactivated FBS, sodium bicarbonate (1 g/liter), 0.6% dextrose, and garamycin (10 μg/ml), (Shering Pharmaceuticals, Kenilworth, N. J.). The tissue was subsequently triturated through a 10-ml serological pipet until the cells were thoroughly dispersed and the resulting suspension was then filtered through a Nitex 130 mesh (Tetko Inc., Elmsford, N. Y.). The cells that passed across were collected into pellets by centrifugation at 750 rpm for 5 min at 4° in a Daimon CRU/5000 centrifuge. The cell pellets obtained from 10 to 12 hemispheres were resuspended in 2 ml of BME_{10} and layered onto a Ficoll-Paque gradient, then centrifuged at 1250 rpm for 25 min at 4° to remove any contaminating erythrocytes. The cell material formed at the interface was collected, diluted in 5 vol of BME_{10}, and pelleted at 500 rpm at 4° to remove any residual Ficoll. Batches of cells in these pellets were resuspended in BME_{10} and plated in a 175-cm^2 tissue culture flask (Nunc, 156502) at a density of 10 hemispheres/flask, then maintained in a humidified CO_2/air incubator at 37°, prior to infection.

Oligodendrocyte-enriched cultures were derived from the above mixed cultures, using the techniques of McCarthy *et al.*

(1980) as modified by McMorris (1983). Briefly, the medium of 7- to 8-day-old mixed cultures was renewed at least 2 hr prior to manipulation. The sealed flasks were then placed on a rotary shaker (New Brunswick Scientific, No. G-26) at 35° and agitated for 90 min at 350 rpm. Cells that had been freed into suspension were filtered through a Nitex 130 mesh. The process of vigorous shaking with another volume of medium was carried out manually so as to remove any residual adherent oligodendrocytes and filtration through Nitex 130 repeated. The pooled filtrates were then passed through a Nitex 33 mesh and the cells that came through pelleted at 1000 rpm for 10 min at 4°. The pellet was resuspended in 5 ml of Hank's balanced salt solution, pH 7.2, containing 1% bovine serum albumin (BSA), 10 μg/ml DNAse (Sigma, St. Louis, Mo.) and 25 m*M* HEPES (Sigma) buffer as described by Snyder *et al.* (1980). The suspension was agitated for 30 min on the rotary shaker at 60 rpm to disperse cell clumps, then centrifuged at 500 rpm at 4° for 5 min. The pellets formed were again resuspended in 5 ml of BME_{10}, plated at a density of 2 to 3 $\times$ 10^7 per 25-cm^2 flask (Nunc, 163371), and incubated overnight at 37°. Further purification of oligodendrocytes was achieved by changing the BME_{10} after 24 hr, hand agitating the flasks vigorously, and filtering suspended cells through a Nitex 15 mesh to remove any extraneous clumps. The cells were enumerated in a counting chamber and plated at the desired, predetermined, density in Costar 3524, 24-well trays. The three cell concentrations usually employed in these studies were (a) low density: 1 to 2 $\times$ 10^5 cells/well; (b) medium density: 4 to 6 $\times$ 10^5 cells/well; and (c) high density: 1 $\times$ 10^6 cells/well or greater. Cells for examination by immunofluorescence were grown on 12-mm coverslips (Chance, Propper Ltd, Smethwick, Worley, England, No. 1) in 24-well trays.

Methods for inoculation and assaying infectivity. The primary cell cultures, in 24-well plates or Nunc NI-409 tissue culture tubes, were inoculated with virus stocks suspended in 0.2 ml per well or 0.5 ml per tube, respectively, of BME_{10}. Adsorption was allowed to proceed for 60 min at the desired temperature. The multiplicity of infection (m.o.i.) was varied, depending on the experiment, as indicated in the figures and tables presented in Results. Three to four hours after adsorption, surface and extracellular inoculum was neutralized for 30 min, using highly active antiviral antisera at a dilution of 1:124.

CV concentrations were determined as plaque-forming units (PFU) on L-2 cell monolayers, as described by Lucas *et al.* (1978), except that plaques were allowed to develop at 37° for 18 to 24 hr. VSV production was monitored in a similar fashion, except the plaques were allowed to develop for 24 hr at 37°.

Preparation of antisera and immune labeling. CV antisera were raised in Swiss mice. Briefly, neonates were tolerized against once frozen and thawed L-2 cells by intraperitoneal (ip) injections with 1 $\times$ 10^7 cells or more per animal in 0.05 ml PBS at 24 and 48 hr postpartum. Two weeks later the pups were reinjected with the same material to maintain tolerance. Four weeks postpartum the mice were injected ip four times, at weekly intervals, with concentrated virus, inactivated by exposure to uv irradiation, which had an initial titer of approximately 5 $\times$ 10^8 PFU/dose. One week after the final injection, the blood drawn from batches of animals was pooled, the serum separated, diluted 1:4 in PBS, and stored at −70°.

Antisera against the glycolipid galactosyl cerebroside (GC) were raised in rabbits according to the procedure of Raine *et al.* (1981), except that reinjections were made with over 1 mg of GC antigen. Specific antibodies in the sera were enriched by passing the sera sequentially through protein A–Sepharose columns, to select for the IgG, and then the recovered IgG was passed through a BSA–Sepharose column to remove any anti-BSA antibodies. The avidity of the antibodies was assessed using the radioimmunoassay (RIA) of Holmgren *et al.* (1980) for de-

tecting the antigen adsorbed onto plastic wells, as modified for use with GC by Raine *et al.* (1981).

Rabbit antiserum to guinea pig myelin basic protein (Ra > MBP) was kindly provided by Zobeeda Hossein, University of Western Ontario. Rabbit anti-human glial fibrillary acidic protein antibody (Ra > GFAP) (Dahl *et al.*, 1976) was a gift from Dr. Doris Dahl.

The procedures for fixation, indirect immunolabeling, and examination of cells under uv for fluorescence were those developed by Manthorpe *et al.* (1979), as modified and described in Dales and Oldstone (1982). Anti-MHV, anti-MBP, and anti-GFAP sera, diluted in PBS, were used at final concentrations of 1:9, 1:19, and 1:39, respectively. The fluorescein (FITC) and rhodamine (RITC) labeled reagents were purchased from Miles Biochemicals (Elkhart, Ind.) and applied at a final concentration of 1:19 for 30 min at room temperature. The cultures were examined and photographed under uv optics, using a Wild-Leitz, Dialux 20 microscope.

Immune lysis of infected cells. Primary oligodendrocyte or astrocyte cultures and $LMTK^-$ cells were infected, monitored for development of cytopathic effect (cpe), then treated with Ra > GC antibody plus complement to cause lysis. The anti-GC antibodies diluted 1:2 in NM were applied at 4° to infected cultures for 60 min. After rinsing away the unadsorbed antibodies with three washes of cold BME_{10}, the cultures were overlayed with rabbit Lo Tox complement (Cedarlane, Hornby, Ontario, Canada), diluted 1:14 in NM, and placed in an humidified CO_2 incubator for 30 min. The unfixed complement was removed with three rinses of warm BME_{10}. The cultures were reconstituted with BME_{10} and incubated at 37° for 24 hr to determine virus yields in terms of plaque-forming units per milliliter in the medium.

Treatment with dibutyryl cyclic AMP and assay of 2′:3′-cyclic nucleotide-3′-phosphohydrolase. Treatment with N^6,O^2-dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP), (Sigma) was carried out as follows: primary cultures were exposed to 1 m*M* dbcAMP in BME_{10} either 48 hr prior to or following infection. The medium, or medium and metabolite, were replaced daily up to the time of infection and on alternate days following inoculation. The experiments employing papaverine (Sigma), were conducted in a parallel manner.

The procedures used for determining levels of the enzyme 2′:3′-cyclic nucleotide-3′-phosphohydrolase (CNPase), in primary cultures were those developed by Prohaska *et al.* (1973) as modified and described by McMorris (1983). Protein was determined by the method of Lowry *et al.* (1951), using BSA as a standard.

RESULTS

Comparison of MHV Replication and the Influence of Cell Density in Mixed and Selected Cultures from the CNS

The ability of MHV3 and JHMV to produce infectious progeny in freshly explanted mixed and shaken cultures was tested for the duration of each of the experiments described. Under the light microscope, the appearance of living mixed and selected primary brain cultures is illustrated in Figures 1A–C. Judging primarily by morphological criteria, cultures enriched in oligodendrocytes and astrocytes were over 99% pure. Examination of mixed brain cultures under phase-contrast optics revealed the presence of a monolayer of subjacent astrocytic-type cells and a covering layer of scattered, refractile smaller cells with associated extensive processes (Fig. 1A). Oligodendrocytes appeared as small, highly refractile cells with extensive, arborizing processes (Fig. 1B). Astrocytes appeared as tightly adhering, angular, very flat cells of low-phase density. Following staining with specific anti-GFAP antibodies the astrocytes displayed bundles of filaments characteristic of astrocyte GFAP (Fig. 1C).

After inoculating mixed cultures with MHV3 at m.o.i. of 0.1 to 1.0 PFU/cell, progeny virus could be detected within 12 hr. Subsequently, the titer progressively increased and remained at the high level

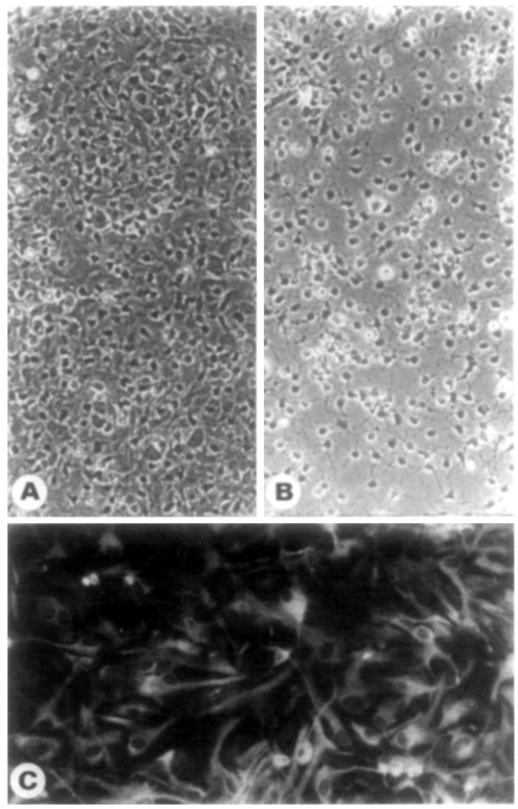

FIG. 1. (A) The appearance of a 10-day-old primary dissociated CNS culture prepared from neonatal rats. (B) Shaken oligodendrocytes plated at low density. (C) The adherent layer left after shaking can be visualized using the indirect immunofluorescence technique with rabbit anti-human GFAP counterstained with goat anti-rabbit conjugated fluorescein. (A, B) ×200. (C) ×360.

for 4 to 5 weeks (Fig. 2A, Table 1). There was a coincidental, slowly developing cpe of the subjacent astrocytic cells which became almost complete at the time virus production ceased. The cpe, in the form of syncytia, was clearly evident under phase-contrast optics within 1 to 2 weeks following inoculation. The covering cells of presumptive oligodendrocytes were, by contrast, unaffected.

Inoculation of mixed cultures with m.o.i. of 0.1 to 1.0 PFU/cell of JHMV gave variable data, depending on the cell density. With dense cultures, plated at 5×10^5 cells/cm^2, progeny were detected within 12 hr, virus yields became maximal at 10^5 to 10^6 PFU/ml within 2 to 3 days and continued for prolonged periods, frequently for 3 to 4 weeks (Table 2, Fig. 2B). Thereafter, production ceased abruptly. When sparsely seeded cultures, containing approximately 1×10^5 cells/cm^2 were infected with JHMV at m.o.i. of 0.1 to 1.0 PFU/cell, there was only a transient burst of virus production, commencing usually at 12 hr, reaching a peak of only 10^2 PFU/ml and then ceasing (Fig. 2A). These observations revealed the close relationship between duration of JHMV replication and the density of primary rat cerebral cell explants, a relationship which did not occur with MHV3. Cell density dependence of JHMV replication in explanted oligodendrocytes will be considered further, below.

Another general feature of the replication process of CV in primary rat brain cultures is thermosensitivity, whereby formation of infectious progeny is arrested at 39–40°, the nonpermissive temperature. However, contrary to the situation with continuous neural and other rat cell lines, in which restriction at 39.5° is complete (Lucas *et al.*, 1978), in the case of primary brain cultures examined here the temperature restriction was found to be incomplete (Fig. 2). As in the case of the continuous rat schwannoma RN-2 line (Lucas *et al.*, 1978), temperature shift down from 39.5 to 32.5° allowed, within the specified period of the experiment, the resumption of replication.

To ascertain the specificity of the tropism of CV serotypes for different cell types from rat brain, separate, relatively pure oligodendrocytic and astrocytic cultures were established as described under Materials and Methods and challenged with JHMV or MHV3. Inoculation with MHV3 of the astrocytes resulted in formation of infectious particles which simulated that described in the case of mixed cultures (Table 1, Fig. 2A). By contrast, infection of oligodendrocytic cultures with MHV3 (data in Table 1) and of astrocyte cultures with JHMV (not shown) at m.o.i. of 0.1 to 1.0 PFU/cell did not lead to any

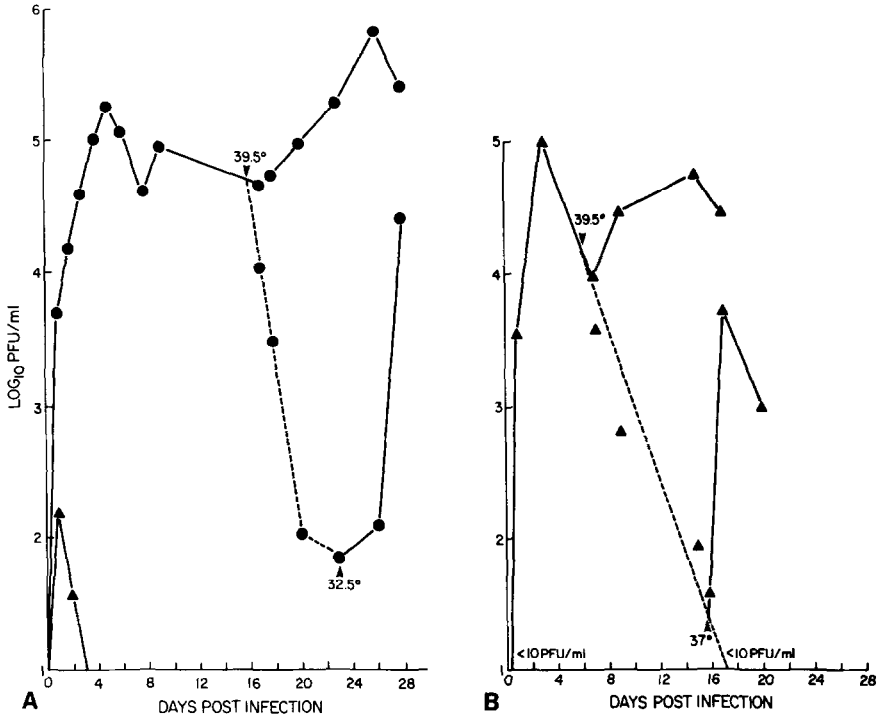

FIG. 2. Replication of JHMV (▲) and MHV3 (●) in low density mixed cultures (A) and high density mixed cultures, (B) at the permissive (32.5° for MHV3, 37° for JHMV) and restrictive (39.5°) temperatures. These data are entirely comparable since unrestricted replication of both viruses can occur at either 32.5 or 37°. Arrows indicate times of temperature shifts. The m.o.i. in each experiment was approximately 0.1 PFU/cell.

virus production. On the other hand, infection of oligodendrocyte cultures with JHMV at the same m.o.i.'s induced replication which, as with the mixed cultures, was either transient or persistent, depending on cell density (Table 2). More specifically, persistent infection could be established routinely in shaken cultures, hereafter termed oligodendrocytes, when seeded at a density greater than 2×10^5 cells/cm^2. With sparser cultures, at or below 1×10^5 cells/cm^2, JHMV was replicated only transiently and to low titer, regardless of the m.o.i. employed (Table 2, Fig. 2A). Thus, cultures seeded at 5×10^5 cells/cm^2 produced a maximum of approximately 10^6 PFU/ml, those seeded at 2×10^5 cells/cm^2 approximately 10^3 to 10^6 PFU/ml and those at 10^5 cells/cm^2 or less only about 10^2 to 10^4 PFU/ml depending on the m.o.i. used to initiate infection. Evidently the yields, in terms of plaque-forming units/cell, were not in direct proportion to the cell number but were related to the space between cells. Furthermore, the rapidity of the CPE manifested was also directly related to cell density, being

TABLE 1

IN VITRO REPLICATION OF CORONAVIRUSES IN LOW DENSITY MIXED OR SHAKEN RAT CORTICAL CELL CULTURES

Days post-inoculation	Mixed cultures		Shaken cultures	
	MHV3	JHMV	MHV3	JHMV
1	10-100	1-10	0.1-1	1-10
2	100	1	0.1	1
4	100-1,000	0-1	0	0-0.1
8	100-10,000	0	0	0

Note. All titers expressed $\times 10^2$ PFU/ml.

TABLE 2

EFFECT OF CELL DENSITY ON THE REPLICATION OF JHMV IN PRIMARY OLIGODENDROCYTES

Cell density	m.o.i.	Days postinfection						
		1	2	3	6	8	10	15
Medium[a]	5	100	1000	500	120	100	10,000	180
	1	33.6	1000	668	488	900	10,000	30
	0.1	2.6	42.6	62	800	944	10,000	30
Low[b]	12.5	28.8	80	10	2.6	0	0	0
	2.5	10.4	30.8	10	1.2	0.8	0.16	0
	0.25	0.6	3	0.64	1.08	0.08	0	0

Note. All titers expressed $\times 10^2$ PFU/ml.

[a] Approximately 2.5×10^5 cells/cm^2.

[b] Approximately 1×10^5 cells/cm^2.

detectable earlier and with greater frequency in the denser cultures.

Identity of the cell type in shaken cultures replicating JHMV. Once it had been established that JHMV could persistently infect shaken cultures it was essential to ascertain whether these cells were, indeed, oligodendrocytes. For this purpose, indirect immunolabeling was carried out to associate the presence of viral antigen(s) with markers for the oligodendrocytes. Characterization of living oligodendrocytes was conducted employing monospecific R > GC antisera (Raff *et al.*, 1978). It was observed that the majority, over 70%, of the cells were GC positive and, therefore, oligodendrocytes (not shown). For correlating presence of oligodendrocyte-specific and viral antigens within the same cells, fixation and permeation with acetone permitted simultaneous labeling with two specific antisera and different fluorochromes. The oligodendrocyte-specific cytoplasmic antigen, myelin basic protein (MBP), was detected with R > MBP monospecific serum. JHMV antigen(s) were detected by polyclonal antibodies raised in mice, as described under Materials and Methods. The data, illustrated in Figs. 3B, C revealed conclusively that MBP and viral antigens coexisted when the oligodendrocyte cultures were established at medium cell density and examined 20 days postexplantation and 10 days after infection. When oligodendrocytes were seeded at low cell density and examined at 10 days postexplantation and 2 days after inoculation, virus antigen occurred with equal frequency within cells that were either MBP positive or negative (Figs. 3E, F). This implies that younger cultures contained fewer cells with MBP, consistent with Barbarese *et al.* (1981) who showed MBP to be a differentiation marker for oligodendrocytes *in vitro.*

Further characterization of virus-producing cell types was carried out at the fine structure level using transmission electron microscopy. Sections of JHMV-infected material from shaken cultures revealed the presence of coronavirus particles in cells with the typical morphology of oligodendrocytes (Mori *et al.*, 1970). By contrast, MHV3 particles were found in cells containing numerous bundles of intermediate filaments, presumably GFAP, diagnostic for the astrocyte (Mori *et al.*, 1969).

An independent approach toward identification of the cell type permissive or restrictive for JHMV was by means of immune lysis, utilizing surface-specific antibody and complement. For this purpose cells in oligodendrocyte cultures were inundated with R > GC antibody plus complement, then monitored 24 hr after

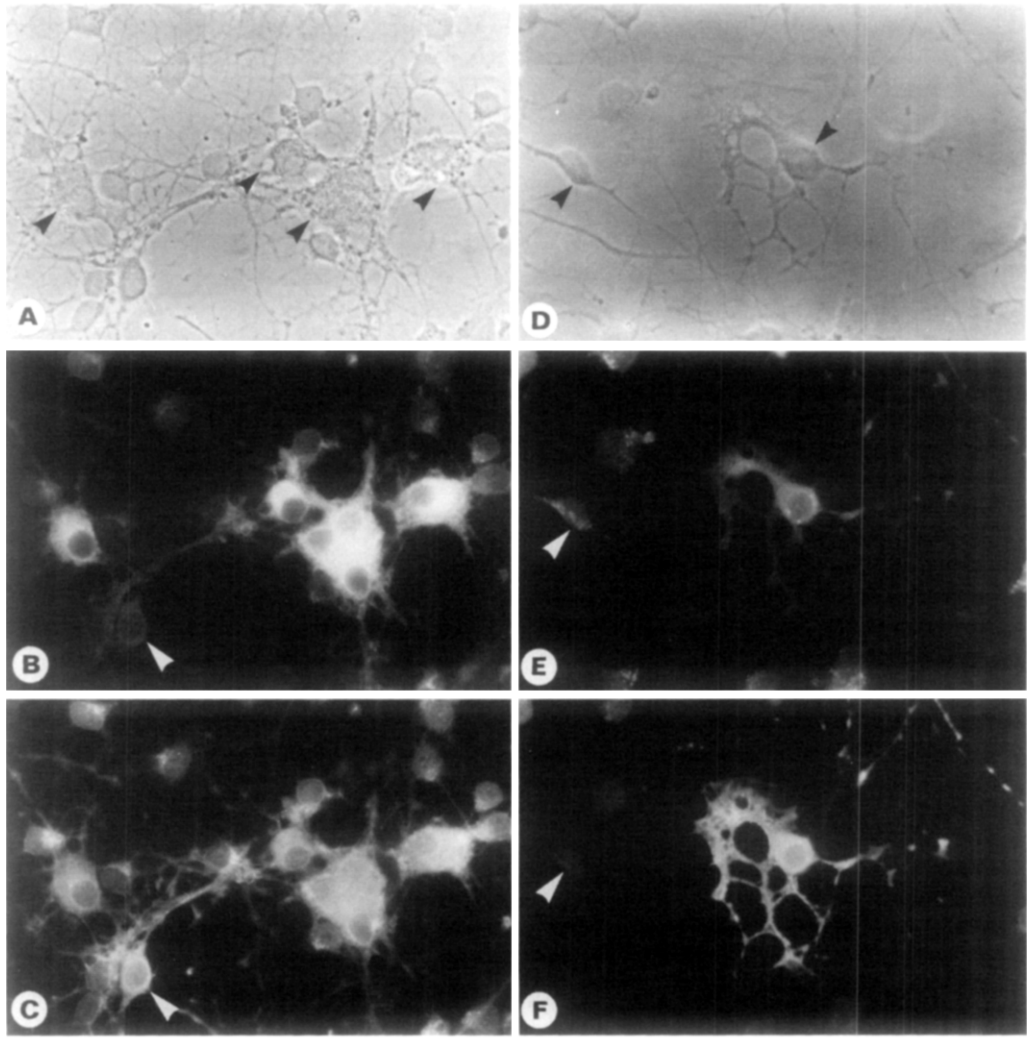

FIG. 3. Images of JHMV infected oligodendrocytes at medium (left) and low density (right). (A, D) Phase image examples of virus-infected cells indicated by arrowheads. (B, E) Immunofluorescent images of cells expressing virus antigen. (C, F) Cells positive for myelin basic protein. The arrowheads in (B) and (C) depict a virus antigen-negative, MBP-positive cell while the arrowheads in (E) and (F) show a cell that stains for virus antigen but not MBP. ×2000.

treatment for production of infectious progeny. A summary of the data, in Table 3, clearly shows that this treatment suppressed virus production, whereas in the appropriate controls virus formation persisted. When checked at 3 and 5 days following immune lysis, the oligodendrocyte cultures did not resume JHMV production. Additional controls, using astrocytes infected with MHV3 and JHMV chronically infected LMTK$^-$ cells (Mizzen *et al.*, 1983), showed that R > GC antibody and complement did not suppress the infections. On the basis of these observations we conclude that *in vitro* tropism of JHMV is exclusive for oligodendrocytes and of MHV3 for astrocytes.

Influence of oligodendrocyte differentiation on JHMV replication. Age-related differentiation of oligodendrocytes has been

TABLE 3

EFFECT OF IMMUNE LYSIS ON THE REPLICATION OF JHMV IN PRIMARY OLIGODENDROCYTES

Treatment of culture	Virus produced $\times 10^2$ PFU/ml
Oligodendrocytes infected with JHMV	80
Complement only	60
Anti-GC antibody only	60
Complement + anti-GC antibody	0[a]
Astrocytes infected with MHV3	1.8
As above complement + anti-GC antibody	1.2
LMTK$^-$ mouse fibroblasts infected with JHMV	1000
As above complement + anti-GC antibody	1000

Note. m.o.i. 0.5–1.0 PFU/cell in each case.
[a] Most of the cells were lysed.

shown to be correlated *in vivo* (Sprinkle *et al.*, 1978) and *in vitro* (McMorris, 1983) with levels of intracellular cAMP and CNPase, the enzyme marker for myelin synthesis. To ascertain whether oligodendrocyte differentiation also influenced virus replication, oligodendrocytes were assayed for CNPase and challenged with JHMV at intervals following explantation. Data, summarized in Table 4 show that enzyme induction was maximal by the 15th day and remained at a high level beyond the 21st day, in general agreement with results reported previously (Sprinkle *et al.*, 1978; McMorris, 1983). JHMV replication was reproducibly suppressed sometime between the 15th and 21st days. With VSV, by contrast, there was no evidence of an age-related inhibition of replication. Another approach for testing the relationship between differentiation and JHMV production was to treat oligodendrocytes with dbcAMP either 48 hr prior to or following inoculation. The data, summarized in Table 5, revealed that cultures treated 48 hr prior to inoculation failed to replicate the virus, whereas cultures treated after inoculation continued to produce progeny. Data, presented in Table 6, reveal that both 1 m*M* dbcAMP and 7 μM papaverine (the latter being an inhibitor of cAMP phosphodiesterase which indirectly causes the accumulation of intracellular cAMP), were effective in repressing JHMV formation in oligodendrocytes seeded at medium density. It may be highly significant that cell density has a profound influence on the efficacy of dbcAMP.Thus, unlike medium density cultures, cells seeded at the high density were unaffected by 1 m*M* dbcAMP but were influenced by 5 and 10 m*M* dbcAMP with respect to suppression of virus replication (Table 6).

To determine whether the effects of dbcAMP were specifically related to the CV, medium density cultures were challenged with VSV and MV. Both agents were replicated with equal efficiency, regardless of the treatment imposed upon the oligodendrocytes (data on VSV in Table 6, on MV not shown).

Virus replication in LMTK$^-$ cells, also used as a control, was affected only marginally by exposure to dbcAMP (Table 6).

DISCUSSION

The rat-coronavirus model under investigation by us has provided significant new information concerning the infectious and disease process in the CNS. The closely parallel, age-related, inducibility

TABLE 4

CORRELATION BETWEEN CNPase ACTIVITY AND VIRUS REPLICATION IN SHAKEN CULTURES

Enzyme activity and virus yield	Days postexplantation		
	10	15	21
CNPase activity (nmol/min/mg protein)	679	1820	1790
JHMV (PFU/ml)	11.1	14.5	0.3
VSV (PFU/ml)	155	735	440

Note. All titers expressed $\times 10^2$ PFU/ml. Titers monitored 24 hr pi. m.o.i. 0.5–1.0/cell in each case.

TABLE 5

EFFECT OF $N^6,O^{2'}$-DIBUTYRYL 3':5' CYCLIC AMP ON THE REPLICATION OF JHMV IN PRIMARY OLIGODENDROCYTES

Treatment of culture	Days postinfection						
	2	3	4	6	8	12	14
Control	13	46.8	55	38.8	720	31	20
1 m*M* postinfection	33.4	100[a]	15.5	100	500	26	30
1 m*M* 48 hr before infection	0	0	0	0	0	0	0
1m*M* 48 hr before infection	0	0[b]	0	0	0	0	0

Note. All titers expressed $\times 10^2$ PFU/ml. m.o.i. 0.5–1.0/cell in each case.
[a] dbcAMP added.
[b] dbcAMP removed.

of disease in animals and infectability of freshly explanted oligodendrocytes by the neurotropic agent JHMV, reported here, is remarkable. This suggests that the *in vitro* tropism of this virus strain reflects accurately its propensity for cells of the CNS in the rat. Furthermore, the idea that JHMV has specific tropism for the oligodendrocytes is supported by data derived from an application of molecular probes and immunopathology to CNS samples from afflicted or asymptomatic animals (Sorensen *et al.*, 1984). Therefore, it is very likely that progress of the chronic, demyelinating disease is related to infection of the oligodendrocyte. One might also presume that tropism of MHV_3 for astrocytes, causing a persistent *in vitro* infection, does not lead to development of an overt CNS disease in the rat because the astrocyte, by virtue of its proliferative capacity, is not eliminated from the CNS as a critical, functional, component.

TABLE 6

EFFECT OF DIFFERENTIATION INDUCERS AND CELL DENSITY ON THE REPLICATION OF JHMV IN PRIMARY OLIGODENDROCYTES

Conditions of culture[a]	Control	Treated
Medium density		
1 m*M* dbcAMP	50	0.6
7 μM Papaverine	50	8.4
1 m*M* dbcAMP infected with VSV	30,000	35,500
High density		
0.1 m*M* dbcAMP	1,000	1,000
0.5 m*M* dbcAMP	1,000	1,000
1.0 m*M* dbcAMP	1,000	1,000
5.0 m*M* dbcAMP	1,000	18
10.0 m*M* dbcAMP	1,000	1
$LMTK^-$ mouse fibroblasts		
1.0 m*M* dbcAMP	8,400	5,300

Note. All titers expressed $\times 10^2$ PFU/ml.
[a] m.o.i. of 1.0 in each case.

The uniqueness of differential tropisms of CV in the rat CNS appears to be species specific, as indicated by comparison of results on rat and murine cells. In the latter, JHMV can replicate equally well in astrocytes, neurons (Knobler *et al.*, 1981a, b; Dubois-Dalcq *et al.*, 1982; Collins *et al.*, 1983), and oligodendrocytes (unpublished results). The *in vitro* infectability of rat neurons remains to be demonstrated, although infection of these cells within the CNS has been documented (Nagashima *et al.*, 1978; Sorensen *et al.*, 1984). In future studies it should be possible to ascertain whether the CV serotypes infecting man are similarly characterized by cell-type-related tropisms within the human CNS.

It should be noted that prompt and reproducible initiation of persistent infection by CV in primary cultures, described

here, is very similar to the establishment of such infections in continuous cell lines of rat cells of neural and other origin (Lucas *et al.*, 1977). Moreover, with both types of cultures production of infectious virus is profoundly inhibited or entirely suppressed at elevated temperatures, approximately 39.5°. Analogous data have been obtained with other neurotropic agents, among them measles and HVJ (Lucas *et al.*, 1978; Ogura *et al.*, 1984). Evidently, similar host cell control over virus production is exerted by primary neural cells, suggesting that the efficiency of virus replication and spread within the CNS may be under close control of the host. It is noteworthy that JHMV replication in explanted oligodendrocytes is influenced more profoundly by cell density than by the m.o.i. employed, implying that cell–cell contacts impart a fundamental influence upon the spread of virus and maintenance of persistence. Thus, in sparsely seeded cultures, where cell contacts are infrequent or absent, virus production is low and transient. By comparison, with greater density, permitting frequent cell–cell contacts, the infection is characterized by development of syncytia and prolonged, persistent release of JHMV, sometimes to titers as much as 4 $\log_{10}$ greater than those produced by cells in sparse cultures.

A primary factor governing JHMV-related demyelinating disease is the age at inoculation. Thus, 3 weeks postpartum, rats become highly resistant (Sorensen *et al.*, 1980). With this in mind it should be noted that rat oligodendrocytes *in vitro* exhibit an age-related control over the infection, perhaps as a consequence of the differentiation process. In this connection, there is evidence to show that in neonatal and embryonic cultures from rat brain, certain characteristics of differentiation, such as the induction of MBP (Barbarese *et al.*, 1981; Pfeiffer *et al.*, 1981) and CNPase (Pfeiffer *et al.*, 1981; McMorris, 1983), appear on schedule during development as regulated by the *in vitro* "time clock" period. The consequence of differentiation on virus replication in neural cells, whether on the positive or negative side, has also been documented for other neurotropic agents, among them MV and rubella virus (Miller *et al.*, 1982; Van Alstyne *et al.*, 1983). It may not be a mere coincidence that the maximal increase in CNPase occurs both *in vivo* and *in vitro* at about 15–20 days postpartum (Sprinkle *et al.*, 1978; McMorris, 1983), closely coincident with the time period at which rats become insusceptible to disease produced by JHMV; the key factor in regulation of infectability of oligodendrocytes could be their state of differentiation with respect to the elaboration of myelin. This notion is supported by our experiments concerned with elevation of intracellular cAMP, either directly or by inducers, since the intracellular concentrations of this metabolite in neural cells is closely related to induction of terminal functions in the differentiation process (Gilman *et al.*, 1971; Ruben *et al.*, 1975; Greengard, 1978; Miller *et al.*, 1982).

ACKNOWLEDGMENTS

The generous advice of Dr. O. Sorensen, Dr. M. Coulter-Mackie, and Dr. W. Flintoff concerning studies with animals and cells is greatly appreciated. Funding for this work was supported by grants from the Canadian MRC and MS Society.

REFERENCES

ABNEY, E. R., BARTLETT, P. P., and RAFF, M. C. (1981). Astrocytes, ependymal cells and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. *Dev. Biol.* **83**, 301–310.

BAILEY, O. T., PAPPENHEIMER, A. M., CHEEVER, F. S., and DANIELS, J. B. (1949). A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin. II. Pathology. *J. Exp. Med.* **90**, 195–231.

BARBARESE, E., and PFEIFFER, S. E. (1981). Developmental regulation of myelin basic protein in dispersed cultures. *Proc. Natl. Acad. Sci. USA* **78**, 1953–1957.

BURKS, J., DEVALD, B., JANKOVSKY, L., and GERDES, J. (1980). Two coronaviruses isolated from central nervous system tissue of two multiple sclerosis patients. *Science (Washington, D. C.)* **209**, 933–934.

CHEEVER, F. S., DANIELS, J. B., PAPPENHEIMER, A. M., and BAILEY, O. T. (1949). A murine virus

(JHM) causing disseminated encephalomyelitis with extensive destruction of myelin. I. Isolation and biological properties of the virus. *J. Exp. Med.* **90,** 181–194.

COLLINS, A. R., TUNISON, L. A., and KNOBLER, R. L. (1983). Mouse hepatitis virus type 4 infection of primary glial cultures from genetically susceptible and resistant mice. *Infect. Immun.* **40,** 1192–1197.

DAHL, D., and BIGNAMI, A. (1976). Immunogenic properties of the glial fibrillary acidic protein. *Brain Res.* **116,** 150–157.

DALES, S., and OLDSTONE, M. B. A. (1982). Localization at high resolution of antibody-induced mobilization of vaccinia virus haemagglutinin and the major histocompatibility antigens on the plasma membrane of infected cells. *J. Exp. Med.* **156,** 1435–1447.

DUBOIS-DALCQ, M. E., DOLLER, E. W., HASPEL, M. V., and HOLMES, K. V. (1982). Cell tropism and expression of mouse hepatitis virus (MHV) in mouse spinal cord cultures. *Virology* **119,** 317–331.

GILMAN, A. G., and NIRENBERG, M. (1971). Effect of catecholamines on the adenosine 3′:5′-cyclic monophosphate concentrations of clonal satellite cells of neurons. *Proc. Natl. Acad. Sci USA* **68,** 2165–2168.

GREENGARD, P. (1978). Phosphorylated proteins as physiological effectors. *Science (Washington, D. C.)* **199,** 146–152.

HAMRE, D., and PROCKNOW, J. J. (1966). A new virus isolated from the human respiratory tract. *Proc. Soc. Exp. Biol.* **121,** 190–193.

HIRANO, N., GOTO, N., OGAWA, T., KATSUKIKO, O., MURAKAMI, T., and FUJINARA, K. (1980). Hydrocephalus in suckling rats infected intracerebrally with mouse hepatitis virus, MHV-A59. *Microbiol. Immunol.* **24,** 825–834.

HOLMGREN, J., ELWING, M., FREDMAN, P., and SVENNERHOLM, L. (1980). Immunoassays based on plastic adsorbed gangliosides. *Adv. Exp. Med. Biol.* **125,** 339–348.

KNOBLER, R. L., DUBOIS-DALCQ, M., HASPEL, M. V., CLAYSMITH, A. P., LAMPERT, P. W., and OLDSTONE, M. B. A. (1981a). Selective localization of wild type and mutant mouse hepatitis virus (JHM strain) antigens in CNS tissue by fluorescence, light and electron microscopy. *J. Neurimmunol.* **1,** 81–92.

KNOBLER, R. L., HASPEL, M. V., and OLDSTONE, M. B. A. (1981b). Mouse hepatitis virus type 4 (JHM strain) induced fatal central nervous system disease I. Genetic control and the murine neuron as the susceptible site of disease. *J. Exp. Med.* **153,** 832–843.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

LUCAS, A., FLINTOFF, W., ANDERSON, R., PERCY, D., COULTER, M., and DALES, S. (1977). In vivo and in vitro models of demyelinating diseases: Tropism of the JHM strain of murine hepatitis virus for cells of glial origin. *Cell* **12,** 553–560.

LUCAS, A., COULTER, M., ANDERSON, R., DALES, S., and FLINTOFF, W. (1978). In vivo and in vitro models of demyelinating diseases II: Persistence and host-regulated thermosensitivity in cells of neural derivation infected with mouse hepatitis and measles viruses. *Virology* **88,** 325–337.

MANTHORPE, M., ADLER, R., and VARON, S. (1979). Development, reactivity and GFA immunofluorescence of astroglia-containing monolayer cultures from rat cerebrum. *J. Neurocyt.* **8,** 605–621.

MCCARTHY, K. D., and DE VELLIS, J. (1978). Alpha-adrenergic receptor modulation of beta-adrenergic adenosine and prostaglandin E_1-increased adenosine 3′:5′-cyclic monophosphate levels in primary cultures of glia. *J. Cell Nucl. Res.* **4,** 15–26.

MCCARTHY, K. D., and DE VELLIS, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell. Biol.* **85,** 890–902.

MCMORRIS, F. A. (1983). Cyclic AMP induction of the myelin enzyme 2′,3′-cyclic nucleotide 3′-phosphohydrolase in rat oligodendrocytes. *J. Neurochem.* **41,** 506–515.

MERCHANT, D. J., and HELLMAN, K. B. (1962). Growth of L-M strain mouse cells in a chemically defined medium. *Proc. Soc. Exp. Biol. Med.* **110,** 194–198.

MILLER, C. A., and CARRIGAN, D. R. (1982). Reversible repression and activation of measles virus infection in neural cells. *Proc. Natl. Acad. Sci. USA* **79,** 1629–1633.

MIZZEN, L., CHELEY, S., RAO, M., WOLF, R., and ANDERSON, R. (1983). Fusion resistance and decreased infectability as major host cell determinants of coronavirus persistence. *Virology* **128,** 407–417.

MORI, S., and LEBLOND, C. P. (1969). Electron microscopic features and proliferation of astrocytes in the corpus callosum of the rat. *J. Comp. Neurol.* **137,** 197–226.

MORI, S., and LEBLOND, C. P. (1970). Electron microscopic identification of three classes of oligodendrocytes and a preliminary study of their proliferative activity in the corpus callosum of young rats. *J. Comp. Neurol.* **139,** 1–30.

NAGASHIMA, K., WEGE, H., and TER MEULEN, V. (1978). Early and late CNS effects of coronavirus infection in rats. *Adv. Exp. Med. Biol.* **100,** 395–409.

OGURA, H., SATO, H., TANAKA, J., KAMIYA, S., YOSHIE, T., and HATANO, M. (1984). Synthesis of M protein of HVJ (Sendai virus) in rat glial cells is selectively

restricted at non-permissive temperature. *J. Gen. Virol.* **65,** 639–643.

PFEIFFER, S. E., BARBARESE, E., and BHAT, S. (1981). Noncoordinate regulation of myelinogenic parameters in primary cultures of dissociated fetal rat brain. *J. Neurosci. Res.* **6,** 369–380.

PROHASKA, J. R., CLARK, D. A., and WELLS, V. W. (1973). Improved rapidity and precision in the determination of brain 2′,3′-cyclic nucleotide 3′ phosphohydrolase. *Anal. Biochem.* **56,** 275–282.

RAFF, M. C., MIRSKY, R., FIELDS, K. L., LISAK, R. P., DORFMAN, S. H., SILBERBERG, D. H., GREGSON, N. A., LEIBOWITZ, S., and KENNEDY, M. C. (1978). Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture. *Nature (London)* **274,** 813–816.

RAINE, C. S., JOHNSON, A. B., MARCUS, D. M., SUZUKI, A., and BORNSTEIN, M. B. (1981). Demyelination in vitro. Absorption studies demonstrate that galactocerebroside is a major target. *J. Neurol. Sci.* **52,** 117–131.

ROTHFELS, K. H., AXELRAD, A. A., SIMINOVITCH, L., MCCULLOCH, E. A., and PARKER, R. C. (1959). The origin of altered cell lines from mouse, monkey and man as indicated by chromosome and transplantation studies. *Canad. Cancer Conf.* **3,** 189–214.

RUBEN, C., and ROSEN, O. (1975). Protein phosphorylation. *Annu. Rev. Biochem.* **44,** 831–887.

SANFORD, K. K., EARLE, W. R., and LIKELY, G. D. (1948). The growth in vitro of single isolated tissue cells. *J. Natl. Cancer Inst.* **9,** 229–246.

SNYDER, D. S., RAINE, C. S., FAROOQ, M., and NORTON, W. T. (1980). The bulk isolation of oligodendroglia from whole rat forebrain: A new procedure using physiologic media. *J. Neurochem.* **34,** 1614–1621.

SORENSEN, O., PERCY, D., and DALES, S. (1980). In vivo and in vitro models of demyelinating diseases III. JHM virus infection of rats. *Arch. Neurol.* **37,** 478–484.

SORENSEN, O., COULTER-MACKIE, M., PUCHALSKI, S., and DALES, S. (1984). *In vivo* and *in vitro* models of demyelinating disease. IX. Progression of JHM virus infection in the central nervous system of the rat during overt and asymptomatic phase. *Virology,* **137,** 347–357.

SPRINKLE, T. J., ZARUBA, M. E., and MCKHANN, G. M. (1978). Activity of 2′,3′-cyclic nucleotide 3′-phosphodiesterase in regions of rat brain during development: Quantitative relationship to myelin basic protein. *J. Neurochem.* **30,** 309–314.

TANAKA, R., IWASAKI, Y., and KOPROWSKI, H. (1976). Intracisternal virus-like particles in brain of a multiple sclerosis patient. *J. Neurol. Sci.* **28,** 121–126.

TYRRELL, D. A. J., and BYNOE, M. L. (1965). Cultivation of a novel type of common-cold virus in organ cultures. *Brit. Med. J.* **1,** 1467–1470.

VAN ALSTYNE, D., and PATY, D. W. (1983). The effect of dibutyryl cyclic AMP on restricted replication of rubella virus in rat glial cells in culture. *Virology* **124,** 173–180.

VIRELIZIER, J., DAYAN, A., and ALLISON, A. (1975). Neuropathological effects of persistent infection of mice by mouse hepatitis virus. *Infect. Immun.* **12,** 1127–1140.