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In Vivo and in Vitro Models of Demyelinating Disease

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

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The coronaviruses, ubiquitous in mammals, including man, manifest serotype-related predilection 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, demyelinating 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 sero-related 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-
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 differentiation 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 L929 line (Sanford et al., 1948), were routinely propagated 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 a humidified atmosphere containing 5% CO₂.

*Viruses and preparation of stocks.* Mouse hepatitis virus strains MHV3 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 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 (BME₁₀), 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 BME₁₀ 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 BME₁₀, and pelleted at 500 rpm at 4° to remove any residual Ficoll. Batches of cells in these pellets were resuspended in BME₁₀ and plated in a 175-cm² tissue culture flask (Nunc, 156502) at a density of 10 hemispheres/flask, then maintained in a humidified CO₂/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 mM 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(C10, plated at a density of 2 to 3 × 10⁷ per 25-cm² flask (Nunc, 163371), and incubated overnight at 37°. Further purification of oligodendrocytes was achieved by changing the BME(C10 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 × 10⁶ cells/well; (b) medium density: 4 to 6 × 10⁶ cells/well; and (c) high density: 1 × 10⁷ 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(C10. 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 × 10⁷ 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 × 10⁸ 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 BME10, the cultures were overlayed with rabbit Lo Tox complement (Cedarlane, Hornby, Ontario, Canada), diluted 1:14 in NM, and placed in an humidified CO2 incubator for 30 min. The unfixed complement was removed with three rinses of warm BME10. The cultures were reconstituted with BME10 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 N6,O2-di-butyryladenosine 3':5'-cyclic monophosphate (dbcAMP), (Sigma) was carried out as follows: primary cultures were exposed to 1 mM dbcAMP in BME10 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
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 $5 \times 10^5$ cells/cm², progeny were detected within 12 hr, virus yields became maximal at $10^6$ to $10^7$ 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 $1 \times 10^5$ cells/cm² 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...
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 $2 \times 10^5$ cells/cm$^2$. With sparser cultures, at or below $1 \times 10^5$ 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 $5 \times 10^5$ cells/cm$^2$ produced a maximum of approximately $10^6$ PFU/ml, those seeded at $2 \times 10^5$ cells/cm$^2$ approximately $10^5$ 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 $\times 10^6$ PFU/ml.*
**TABLE 2**

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

| Cell density | m.o.i. | 1 | 2 | 3 | 6 | 8 | 10 | 15 |
|--------------|-------|---|---|---|---|---|----|----|
| Medium<sup>a</sup> | 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<sup>b</sup> | 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 ×10⁶ PFU/ml.

<sup>a</sup> Approximately 2.5 × 10⁵ cells/cm².

<sup>b</sup> Approximately 1 × 10⁵ cells/cm².

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
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.

Figure 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.

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 X10^2 PFU/ml |
|---------------------------------------|----------------------------|
| Oligodendrocytes infected with JHMV   | 80                         |
| Complement only                       | 60                         |
| Anti-GC antibody only                 | 60                         |
| Complement + anti-GC antibody         | 0*                         |
| 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.
* 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 mM dbcAMP and 7 µM papaverine (the latter being an inhibitor of cAMP phosphodiesterase which indirectly causes the accumulation of intracellular cAMP), were effective in suppressing 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 mM dbcAMP but were influenced by 5 and 10 mM 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 |
|---------------------------------|-----------------------|
| CNPase activity (nmol/min/mg protein) | 10 15 21 |
| JHMV (PFU/ml)                   | 679 1820 1790        |
| VSV (PFU/ml)                    | 11.1 14.5 0.3        |

Note. All titers expressed X10^2 PFU/ml. Titers monitored 24 hr p.i. m.o.i. 0.5–1.0/cell in each case.
TABLE 5

EFFECT OF N6,O2-DIBUTYRYL 3',5' CYCLIC AMP ON THE REPLICATION OF JHMV
IN PRIMARY OLIGODENDROCYTES

| Days postinfection | 2   | 3   | 4   | 6   | 8   | 12  | 14  |
|--------------------|-----|-----|-----|-----|-----|-----|-----|
| Control            | 13  | 46.8| 55  | 38.8| 720 | 31  | 20  |
| 1 mM postinfection | 33.4| 100 | 15.5| 100 | 500 | 26  | 30  |
| 1 mM 48 hr before infection | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| 1 mM 48 hr before infection | 0  | 0  | 0  | 0  | 0  | 0  | 0  |

Note. All titers expressed x10^2 PFU/ml. m.o.i. 0.5-1.0/cell in each case.

TABLE 6

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

| Conditions of culture | Control | Treated |
|-----------------------|---------|---------|
| Medium density        |         |         |
| 1 mM dbcAMP          | 50      | 0.6     |
| 7 μM Papaverine       | 50      | 8.4     |
| 1 mM dbcAMP infected with VSV | 30,000 | 35,500 |
| High density          |         |         |
| 0.1 mM dbcAMP        | 1,000   | 1,000   |
| 0.5 mM dbcAMP        | 1,000   | 1,000   |
| 1.0 mM dbcAMP        | 1,000   | 1,000   |
| 5.0 mM dbcAMP        | 1,000   | 18      |
| 10.0 mM dbcAMP       | 1,000   | 1       |
| LMTK− mouse fibroblasts | 8,400  | 5,300   |

Note. All titers expressed x10^2 PFU/ml.

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 MHV3 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.

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 logs 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).

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