In Vivo and in Vitro Models of Demyelinating Disease

Factors Influencing the Disease Process Caused by Coronavirus Infection of Rats

O. Sorensen, S. Beushausen, M. Coulter-Mackie, R. Adler, and S. Dales

1. INTRODUCTION

Murine coronaviruses, including MHV-A59, MHV-3, and JHM, have a neurotropic potential in rodents causing either an acute fatal encephalitis or a paralytic disease (Bailey *et al.*, 1949; Lampert *et al.*, 1973; Le Provost *et al.*, 1975; Hirano *et al.*, 1980; Knobler *et al.*, 1981b). Central nervous system (CNS) infection of mice may result from intracerebral (IC) or intraperitoneal (IP) inoculation of a number of these coronaviruses, which have tropisms for various CNS cells including glia and neurons (Virelizier *et al.*, 1975; Fleury *et al.*, 1980; Knobler *et al.*, 1981a). By contrast, in rats overt CNS symptoms are associated exclusively with JHM virus (JHMV) (Hirano *et al.*, 1980; Sorensen *et al.*, 1980). An IC inoculation of MHV-3 into rats may result in virus replication without overt symptoms of disease (Hirano *et al.*, 1980; our unpublished data), implying that this strain replicates in cells that are not critical for CNS function, at least as judged by clinical criteria. Although MHV-3 infection of the rat CNS appears to be transitory, JHMV RNA can be identified in the CNS of some asymptomatic animals several months post-inoculation (Sorensen *et al.*, 1984). It remains to be established whether, under these circumstances, JHMV persists at low titers as an infectious entity or enters into a truly latent state.

Thus, a number of factors can determine the coronavirus-induced disease of the rodent CNS. We describe and discuss several of these factors, including the coronavirus strain employed, the age at inoculation, the genetic constitution of the host, the tropisms

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of the virus for specific glial cells, and the influence of the state of differentiation of these cells and the host immune responses, each of which may affect the disease process.

2. INFECTION OF RATS

Rat strains of varying sensitivity to IC challenge with JHMV have been identified (Sorensen *et al.*, 1980, 1982). Wistar Furth (WF), an inbred strain sensitive to JHMV inoculation, is infectable for 15–21 days after birth, whereas other inbred and outbred strains become resistant to challenge within 10 days post-partum (Table 1). Data from cross-breeding experiments between WF and Wistar Lewis (WL) rats indicate that resistance is a heritable factor. Analysis of the susceptibility to JHMV infection in the (WF × WL) F_1 and F_2 generations as well as among rats derived from backcrosses to WF and WL

Table 1. The Effect of Intracerebral Inoculation of JHMV into Various Rat Strains

Strain of rat	Infected at age (days)	Number infected	Number of deaths[a]	Number paralyzed/deaths[a] at days post-inoculation			
				1–7	8–14	15–21	>21
Outbred							
Wistar	2	30	16	0/4	1/12	0	0
	5	29	9	0	0	1/2	7/7
	10	12	0	0	0	0	0
	15	10	0	0	0	0	0
	30	6	0	0	0	0	0
Long–Evans	2	16	10	0/1	1/5	2/3	1/2
	15	10	1	0	0	0	1/1
	21	10	0	0	0	0	0
	30	7	0	0	0	0	0
Sprague–Dawley	2	33	23	0/8	2/9	4/4	2/2
Inbred							
Fischer 344	2	19	10	0/1	0/7	2/2	0
	5	15	9	0/1	0	2/2	6/6
	10	6	0	0	0	0	0
	15	11	0	0	0	0	0
Wistar–Lewis	2	19	8	0	0/3	1/2	2/3
	5	10	1	0	0	0	1/1
	10	25	4	0	1/1	1/1	2/2
	15	20	0	0	0	0	0
	21	14	0	0	0	0	0
	30	15	0	0	0	0	0
Wistar–Furth	2	54	50	0/36	2/13	0	1/1
	5	24	17	0/5	1/12	0	0
	10	88	58	0/1	18/29	18/21	7/7
	15	27	7	0	0	3/3	4/4
	21	8	1	0	0	1/1	0
	30	18	0	0	0	0	0

[a] Rats that died or were killed *in extremis*.

shows that the gene conferring resistance to JHMV infection is expressed in WF rats as homozygous recessive (*rr*) trait, whereas in the WL strain it is in the heterozygous (*RR, Rr, rr*) form (Fig. 1; Sorensen *et al.*, 1984).

The disease resulting from JHMV inoculation is influenced by the age at which the animal is challenged (Sorensen *et al.*, 1980, 1982; Wege *et al.*, 1984). In general, the infection of neonatal rats rapidly causes an acute infection (Table 1) with cytopathology of both neurons and glia. Challenge of somewhat older animals (Table 1) leads to a paralytic disease after longer incubations (Sorensen *et al.*, 1980; Wege *et al.*, 1983), involving primarily glia of the rhombencephalon and spinal cord (Sorensen *et al.*, 1980).

A fraction of the infectable WF rats injected IC with JHMV fail to develop any symptoms of disease. However, following immunosuppression with cyclophosphamide, as described by Sorensen *et al.* (1982), an exacerbation of latent or inapparent infections becomes evident in the population of asymptomatic WF rats (Table 2). Concomitantly, viral RNA can be detected in CNS tissue for at least 5 months from the time of inoculation in some asymptomatic rats (Sorensen *et al.*, 1984). By contrast, in WL rats, immuno-suppression by cyclophosphamide fails to abrogate the naturally acquired age-related resistance (Table 2). However, immunosuppression by cyclosporin A (CsA), which is presumed to act primarily on cell-mediated immunity (Huegin *et al.*, 1985), does prolong susceptibility to JHMV infection in the resistant WL strain (Table 2). Since CsA has
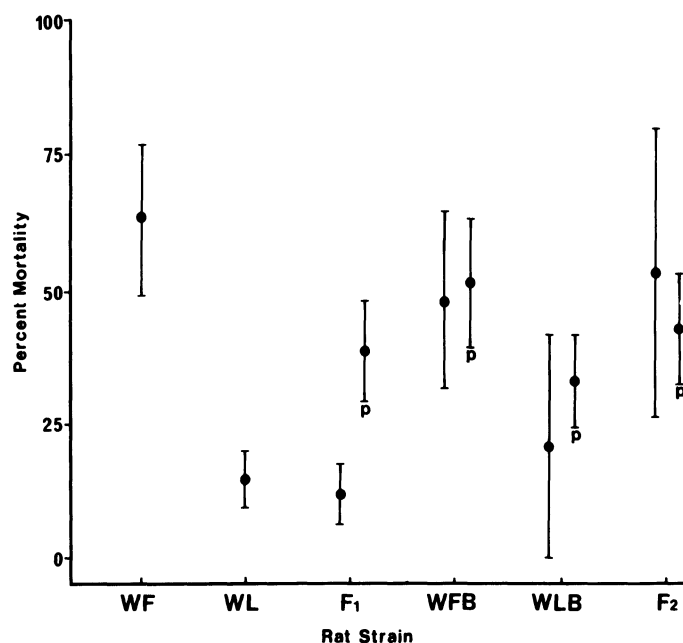

Figure 1. The average percentage mortalities (with standard errors) of 10-day-old WF or WL rats and various cross matings of these strains following intracerebral inoculation with JHMV. Bars designated "p" indicate values predicted on the assumption that a single gene controls the acquisition of resistance, that WF rats were homozygous recessive (*rr*), and that the WL strain was heterozygous (*RR,Rr,rr*). WL, Wistar–Lewis, WF, Wistar–Furth, F_1 = WF × WF, WFB = F_1 × WF (Wistar–Furth backcross), WLB = F_1 × WL (Wistar–Lewis backcross), and F_2 = F_1 × F_1.

Table 2. The Effect of Immunosuppression on the Infection of Rats by JHMV

	Immunosuppressive agent		
	Cyclophosphamide[a]		Cyclosporin A[b]
Rat strain	Wistar–Furth	Wistar–Lewis	Wistar–Lewis
Age at inoculation with JHMV (days)	5	17	16
Age immunosuppression begun (days)	33	10	15
Number immunosuppressed	9	4	13
Number paralyzed	8	0	4
Number of deaths	6	0	4
Number recovering	2	0	0

[a] Cyclophosphamide administered i.p. at 10 mg/kg body weight daily for 14 days.
[b] Cyclosporin A administered i.p. at 25 mg/kg body weight daily for 10 days.

been shown to inhibit T-helper cell functions and perhaps to stimulate the activity of T-suppressor cells (Morris *et al.*, 1983), it is likely that one factor in the age-relative resistance to JHMV infection involves T-cell responses.

This evidence is consistent with our published studies on adult athymic nude mice, which are highly sensitive to neurological disease caused by infection with JHMV (Sebesteny and Hill, 1974; Sorensen *et al.*, 1982), and is also consistent with our recent preliminary findings on the susceptibility of athymic nude (*rnu/rnu*) rats described by Festing *et al.* (1978). These rats continue to be susceptible beyond 9 weeks of age (Table 3). Although nude rats do not develop B-cell responses to T-cell-dependent antigens, T-cell-independent antigens can stimulate a B-cell response in these animals (Vos *et al.*, 1980). However, the induction of antiviral antibodies is probably irrelevant to the disease process, since their presence, whether in the serum or cerebrospinal fluid (CSF) of infected rats, does not appear to confer protection against JHMV infection (Sorensen *et al.*, 1984). Furthermore, since a fraction of 3- to 10-week-old nude rats inoculated with JHMV do

Table 3. The Effect of JHMV Infection on Nude (rnu/rnu) Rats

Route of inoculation	Infected at age (days)	Number infected	Number of deaths[a]	Number paralyzed/deaths at days post-inoculation			
				1–7	8–14	15–21	>21
Intraperitoneal	5	11	0	—	—	—	—
Intracerebral	5	1	1	0	0	0/1	0
	15	3	3	0	0	0	3/3
	21–28	15	10	0	0	0	9/10
	42–49	6	3	0	0	0	3/3
	63–70	4	2	0	0	0	0/2

[a] Rats that died or were killed *in extremis*.

become paralyzed and die (Table 3), it is reasonable to assume that the age-related resistance of other rat strains to JHMV is, in part, regulated by T-cell functions. It should be noted, however, that an age-related resistance to JHMV is also intrinsic to nude rats. This resistance is manifested in older nude rats by the long incubation periods that elapse from the time of injection to the initial appearance of disease symptoms. The duration of these intervals can range from 35 days for animals inoculated 3–4 weeks after birth to 69 days for animals inoculated when 9–10 weeks of age. Our observations on nude rats, therefore, indicate that despite an absence of thymocytes, there is an age-related check on the spread and severity of the infection, although virus clearance is apparently incomplete.

3. INFECTION OF RAT CELLS IN VITRO

We have sought to develop in vitro correlates with the in vivo infection in rats. In this regard, both continuous rat cell lines, such as the Schwannoma RN2-2, and primary explants of glial cells, including oligodendrocytes and astrocytes, have been investigated.
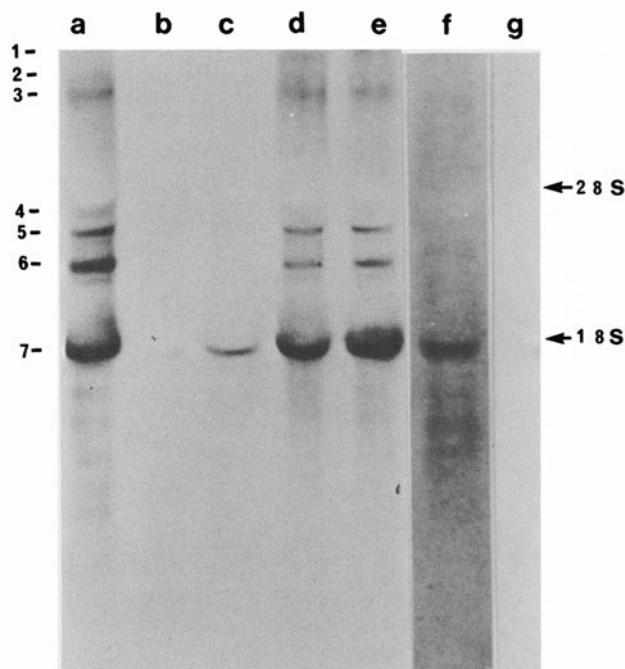

Figure 2. "Northern" transfer and cDNA hybridization to RNA extracted from latently or persistently JHMV-infected RN2-2 cells: (a) after incubation at 32.5°C for 4 days postinoculation; (b–f) after infection at 39.5°C for 4, 3, 2, 1, or 11 days, respectively (f was from a separate experiment and involved twice as long an exposure of the autoradiogram); (g) RNA extracted from uninfected cells. On the left are indicated the positions of JHMV mRNAs 1 to 7, which have molecular weights of 3.7×10^7, 3.4×10^6, 3.1×10^6, 1.6×10^6, 1.45×10^6, 1.15×10^6, and 6.3×10^5, respectively. Locations of the 28S and 18S ribosomal RNAs are shown by arrows.

3.1. RN2-2 and EJ Cells

In RN2-2 cells JHMV readily establishes a persistent infection at the permissive temperature of 32.5°C, but viral replication is suppressed at 39.5°C, presumably by the host cell (Lucas *et al.*, 1977, 1978). Virus replication resumes after the culture is returned to the permissive temperature, suggesting that viral RNA continues to be present and functional in at least some of the cells during the latent infection. Indeed, viral RNA is detectable by Northern blot analysis, where JHMV-specific cDNA probes can identify genomic and mRNA after separation in agarose gels. Such Northern blots reveal significant differences between the RNAs present during permissive and latent infections. Under permissive conditions, all seven mRNAs (Cheley *et al.*, 1981) of JHMV are detected (Fig. 2). During latency, the intensity of the mRNA bands decreases as the duration of the infection at 39.5°C is prolonged, until by the eighth day only mRNA 7, the lowest-molecular-weight mRNA that codes for the nucleocapsid protein, is detectable (Coulter-Mackie *et al.*, 1985). These results suggest that either the amount of RNA per infected cell decreases or the number of infected RN2-2 cells in the culture declines. Infectious center assays and other analyses indicate that both factors may play a role in the decrease of JHMV RNA (Coulter-Mackie *et al.*, 1985).

A feature not previously reported is our finding that the molecular weight of at least one JHMV mRNA species is altered during transcription in RN2-2 cells. Specifically, mRNA 6 (and perhaps mRNA 5) migrates to different positions in agarose gels than that associated with RNA extracted from infected murine L-2 cells (Fig. 3). In RN2-2 extracts, the prominent mRNA 6 band was accompanied by a faint band of slightly lower molecular
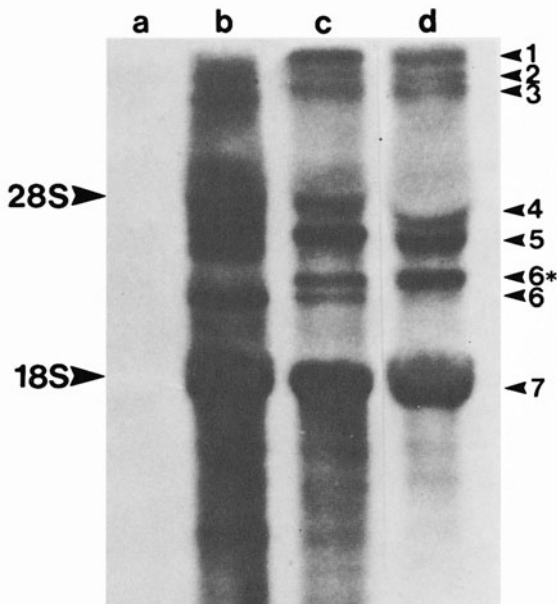

Figure 3. "Northern" transfer followed by cDNA hybridization to RNA extracted from (a) uninfected L cells and from JHMV infections of (b) L cells, (c) EJ cells, a somatic cell hybrid between RN2-2 and L cells, and (d) RN2-2 cells. The positions of mRNAs 1–7 are noted on the right, and the positions of 28S and 18S ribosomal RNAs are noted on the left.

weight, which matched the mRNA 6 band from L-2 cells (Fig. 3). Northern blots of RNA from EJ cells, a persistently infected hybrid cell line created by fusion of RN2-2 and L-2 cells (Coulter-Mackie *et al.*, 1984), revealed two prominent species of mRNA 6 matching the positions noted in extracts from L-2 and RN2-2 cells (Fig. 3). It remains to be determined whether the changes in molecular weight of some JHMV RNA transcripts are caused by host species-specific control over transcription or are related to the persistent infection. It should be noted in this regard that recently Taguchi *et al.* (1985) demonstrated changes in mRNA 2 and 3 of JHMV reisolated from the CNS of rats and grown in murine Sac($-$) cells. Leibowitz *et al.* (1984), studying JHMV infections of the persistently infected Neuro 2A clone of C1300 murine neuroblastoma cells, likewise noted changes in mRNA 2 and 3 as well as mRNA 1.

The functional state of JHMV mRNA in RN2-2 cells after 9 days at 39.5°C was established by the identification of viral mRNA in polysomes. Despite the absence of infectious virus production, active translation occurs at 39.5°C, and JHMV 56K nucleocapsid protein can be detected by Western blot analysis (Coulter-Mackie *et al.*, 1985). However, formation of the glycoproteins E_1 and E_2 of the virion envelope cannot be detected under the restrictive conditions. To test whether interferon (IFN) or IFN-like effects contribute to the persistence or latency of JHMV, infected RN2-2 cultures kept at either 32.5°C or 39.5°C were challenged with vesicular stomatitis virus (VSV). Although only a small percentage of the cells in these RN2-2 cultures are infected by JHMV, the cultures as a whole are resistant to challenge with VSV, indicating that an antiviral state exists, perhaps mediated by IFN (Coulter-Mackie *et al.*, 1985). However, when "conditioned" media from 39.5°C or 32.5°C JHMV-infected RN2-2 cultures are employed, even after applying procedures to concentrate rat IFN in them (Schellekins *et al.*, 1980), they fail to confer resistance to VSV on RN2-2 cells, nor is any cell-associated IFN demonstrable (Coulter-Mackie *et al.*, 1985). These findings indicate that, within the limits of detection of our assay, persistently or latently infected RN2-2 cells, although inducible for IFN, do not elaborate this protein or IFN-like material in response to JHMV infection. Thus, IFN does not appear to be a controlling factor in JHMV persistence in the rat schwannoma RN2-2 cells.

3.2. Primary Rat Glial Cells

In primary cultures of rat CNS cells several factors controlling coronavirus replication have been identified. Mixed cultures from rat CNS, which consist of a subjacent layer of tightly adherent astrocytes over which loosely attached oligodendrocytes are positioned, are able to support the replication of both the neurotropic JHMV as well as the viscerotropic coronavirus MHV-3. Because of the differential adhesion of the oligodendrocytes and astrocytes, oligodendrocytes can be displaced mechanically and isolated in essentially pure culture, and the remaining attached cells are predominantly astrocytic (McCarthy and de Vellis, 1980). In rat cultures separated according to cell type, MHV-3 replicates exclusively in astrocytes, and JHMV in oligodendrocytes (Fig. 4). As with RN2-2 cells, our observations on the tropism of JHMV and MHV-3 for specific glial cells *in vitro* emphasize that control is exerted by the host cell over virus expression (Beushausen and
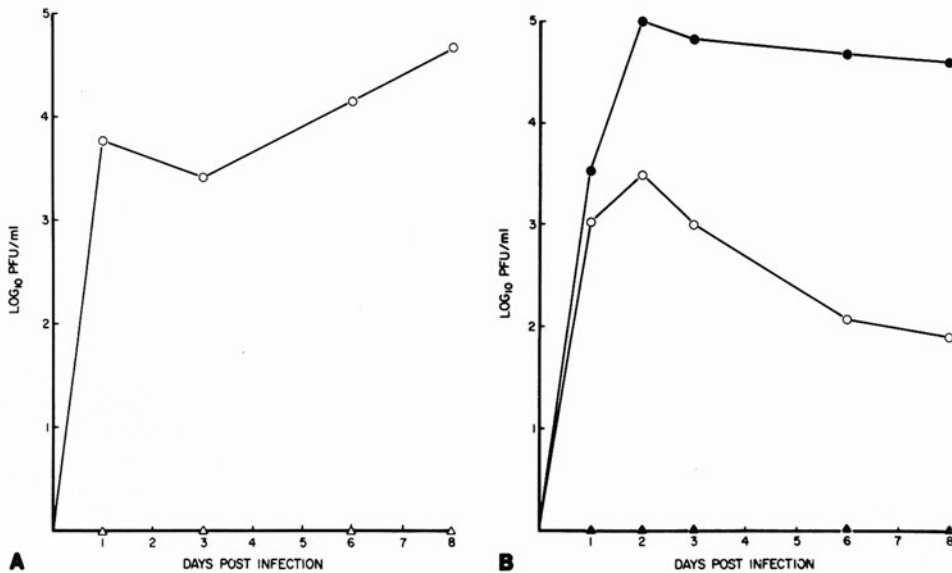
Figure 4. The replication of MHV-3 and JHMV in primary glial cultures. (A) Replication of MHV-3 (○) and JHMV (△) in astrocytes. (B) Replication of JHMV at oligodendrocyte densities of 2×10^5 cells/cm^2 (●) and 10^5 cells/cm^2 (○) and of MHV-3 (▲) in oligodendrocytes at a density of 2×10^5 cells/cm^2.

Dales, 1985). It also demonstrates that the tropisms of the two coronaviruses as defined *in vitro* mirror accurately those evident in the CNS of inoculated rats.

The *in vitro* replication of JHMV in oligodendrocytes is highly sensitive to the cell density of the culture. Thus, with a standard inoculation (multiplicity of infection, MOI) of 1 pfu/cell in cultures where cells are at the low density of 10^5 cells/cm^2, JHMV replicates only transiently and to very low titers (Table 4), and virus is undetectable by10 days post-inoculation. By contrast, at a twofold higher cell density of 2×10^5 cells/cm^2, JHMV is produced at 100- to 1000-fold greater titers and for extended periods (Table 4) (Beushausen and Dales, 1985).

As was found with the RN2-2 schwannoma line (Lucas *et al.*, 1977, 1978), the replication of JHMV in oligodendrocytes and MHV-3 in astrocytes is highly temperature sensitive. Virus yields in the primary cultures of rat glial cells are profoundly reduced at the restrictive temperature of 39.5°C (Beushausen and Dales, 1985).

Coronavirus replication is also controlled by the state of glial cell differentiation. The differentiation process from a precursor or embryonic state into the adult cell in explanted CNS cultures follows a "time clock" inherent in these cells. Thus, an age-related differentiation of oligodendrocytes has been correlated both *in vivo* (Sprinkle *et al.*, 1978) and *in vitro* (McMorris, 1983) with increases of intracellular cAMP and an elevation in the specific activity of the myelin-specific enzyme 2′:3′-cyclic nucleotide 3′-phosphohydrolase (CNPase). The appearance *in vitro* of differentiation-specific markers such as CNPase is coincident with increased resistance of oligodendrocytes to infection

Table 4. The Effect of Cell Density on the Replication of JHMV
in Primarily Rat Oligodendrocytes

Cell density	Multiplicity of infection[a]	JHMV titer ($\times 10^2$ pfu/ml) at days post-inoculation[b]						
		1	2	3	6	8	10	15
Medium[c]	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[d]	12.5	28.8	80	10	2.6	0	0	0
	2.5	10.4	30.8	10	1.2	0.8	0.18	0
	0.25	0.6	3	0.64	0.08	0.08	0	0

[a] Cultures were exposed to JHMV for 1 hr, and extracellular virus was neutralized by treatment with a 1 : 124 dilution of anti-JHMV antibody 4 hr later.
[b] Virus titers were determined for the culture medium on L-2 monolayers as described by Lucas et al. (1978).
[c] Approximately 2.5×10^5 cells/cm^2.
[d] Approximately 10^5 cells/cm^2.

by JHMV (Table 5; Beushausen and Dales, 1985). Virus suppression is also correlated with the induction of differentiation *in vitro* by 1 mM N^6,O$^{2'}$-dibutyryladenosine 3':5'-cyclic monophosphate (dbcAMP) or compounds that increase intracellular levels of cAMP such as 7 μM papaverine, 50 μM forskolin, or 50 μM isoproterenol (Beushausen and Dales, 1985; S. Beushausen and S. Dales, unpublished data). By contrast, modulation of cAMP levels in astrocytes has little or no short-term effect on MHV-3 replication (Beushausen and Dales, 1985). Although pretreatment of oligodendrocytes with 1 mM dbcAMP 48 hr before JHMV challenge totally inhibits replication, similar treatment once

Table 5. The Effect of N^6, O$^{2'}$-Dibutyryl Adenosine 3':5'-Cyclic Monophosphate on the Replication of JHMV in Primary Rat Oligodendrocytes[a]

Treatment of culture with 1 mM dbcAMP[b]	JHMV titer[c] ($\times 10^2$ pfu/ml) at days post-inoculation						
	2	3	4	6	8	12	14
No treatment	13	46.8	55	38.8	720	31	20
Added post-inoculation	33.4	100	15.5	100	500	26	30
Added 48 hr before inoculation	0	0	0	0	0	0	0
Added 48 hr before inoculation, removed post-inoculation	0	0	0	0	0	0	0

[a] MOI 0.5–1.0/cell in each case.
[b] Cultures were exposed to 1 mM dbcAMP in basal minimal essential medium.
[c] Cultures were exposed to JHMV for 1 hr, and extracellular virus was neutralized by treatment with a 1 : 124 dilution of anti-JHMV antibody 4 hr later. Virus titers were determined for the culture medium on L-2 monolayers as described by Lucas et al. (1978).

infection is underway fails to suppress virus expression (Table 4), indicating that the state of oligodendrocyte differentiation is critical at the time of JHMV infection. The observation that oligodendrocytes differentiate *in vitro* in accord with their *in vivo* "time clock" (McMorris, 1983; Sprinkle *et al.,* 1978), coupled with our observation that infection is inhibited following the establishment of the differentiated state, further emphasizes the close parallel between the *in vitro* events in JHMV replication and those that occur after infection of the rat CNS.

4. CONCLUSION

In summary, JHMV infection of rat neural and glial cells *in vitro* and *in vivo* is controlled by several host-determined factors. In addition to the neuron in the CNS (Sorensen and Dales, 1985; Sorensen *et al.,* 1980, 1984), the prime target cell for JHMV is the less differentiated, or immature, oligodendrocyte. The relationship between oligodendrocyte density in culture and maintenance of JHMV replication indicates that close cell–cell contact must occur for the successful dissemination of the infection. Recent observations from *in situ* hybridization with JHMV-specific cDNA probes of CNS tissue sections have led us to postulate that neurons may function as long-term reservoirs of this virus (Sorensen *et al.,* 1985). The state of oligodendrocyte differentiation, characterized in terms of CNPase activity and intracellular cAMP levels, appears to determine the ability of this glial cell type to support or resist infection by JHMV. Host-imposed thermosensitivity, observed with both continuous cell lines and primary explants from the rat CNS, is likewise a determinant of the infectious process. This process may be manifested as virus persistence at the permissive temperature of 32.5°C or viral latency at the restrictive 39.5°C. Studies of the latent state in RN2-2 cells reveal that cells that do not produce infectious virus may, nevertheless, be engaged in virus-specified transcription and at least partial translation. Although the presence of IFN cannot be detected in persistently or latently JHMV-infected RN2-2 cultures, an unidentified antiviral activity, which may be modulating the infectious process, appears to be induced and might play a vital role in maintaining the long-term infections associated with the rat CNS (Sorensen *et al.,* 1984). *In vivo* infections by JHMV are regulated by the genetic background and age of the rats challenged. These infections are also dependent on the activity of the immune system's T-cell compartment. Thus, CsA treatment prolongs the susceptibility to JHMV in WL rats. However, the ability of athymic nude rats, when inoculated as young adults, to restrict the infectious process to a slowly developing CNS disease suggests that T-cell suppression of the spread of JHMV is not the only mechanism protecting the animal. Nevertheless, the antiviral activity of T cells is most probably essential for clearing JHMV from the rat CNS.

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