IN VIVO AND IN VITRO MODELS OF DEMYELINATING DISEASES, XX: REPLICATION OF CORONAVIRUSES IN PRIMARY NEURAL CULTURES FROM GENETICALLY RESISTANT AND SUSCEPTIBLE MICE

Greame A.R. Wilson and Samuel Dales

INTRODUCTION

It is now well established that several factors may influence infections of rodents by coronavirus (CV), which result in CNS diseases (7,8,10-12). Two among the determinants controlling susceptibility or resistance are the genetic constitution of the host and virus strain used. Regarding the influence of the virus strain, studies from this laboratory demonstrated that when primary cultures from neonatal cortex of rats are challenged JHM virus (JHMV) has a specific tropism for oligodendrocytes and MHV₂ for astrocytes, whereas no such neural cell specificity is evident when the two viruses are used to challenge cells of the mouse (1,15). Concerning the genetic component regulating resistance, studies by Stohlman and his colleagues (12), and Knobler, Collins and their colleagues (3,5,6), demonstrated that in-vivo resistance of SJL mice to JHMV, but not to MHV₂, also occurs with explanted neurons, astrocytes and macrophages from this strain. The present study deals with cell-virus interactions during JHMV and MHV₂ infection of explanted neural cells originating from susceptible and resistant strains of mice.

RESULTS AND DISCUSSION

Replication in neural cells from susceptible and resistant mice

Our previous data showed that JHMV and MHV₂ can replicate with equal facility in oligodendrocytes and astrocytes explanted from neonatal CD.1 mice (15). Data from similar experiments with oligodendrocytes and astrocytes, as well as with mixed cultures from SJL and CD.1 mice, representing respectively strains that are resistant and susceptible to JHMV, are summarized in Tables 1 and 2. It is evident from Tables 1 and 2 that both CV are replicated efficiently within 24 hr in the two neural cell types from CD.1 mice, irrespective of the multiplicity of infection (MOI), ranging from 1 to 10 PFU/cell. By contrast, JHMV replication is restricted in neural cells from SJL mice, as evident by the yields at 24 hr which are one or two orders of magnitude lower. MHV₂ however, is produced in high titers in neural cells from both CD.1 and SJL mice. Data similar to those in Table 1 are obtained when mixed cultures, containing oligodendrocytes and astrocytes, are employed (data not shown). Therefore, since such mixed cultures accurately reflect the

Table 1

Replication of Coronaviruses in Primary Oligodendrocyte and Astrocyte Cultures from Resistant and Susceptible Mice:

Culture derived from	Time after inoculation	MOI ^b	Titer x 10 ² PFU/ml ^a			
			Oligodendrocytes		Astrocytes	
			JHMV	MHV ₃	JHMV	MHV ₃
SJL	24 hr	1	11	3400	5	750
		10	49	2100	-	-
CD.1	24 hr	1	180	180	1950	3000
		10	390	575	-	-

a) values shown are averages from duplicate cultures

b) multiplicity of infection

Table 2

Time Course of Coronaviruses Replication in Primary Neural Cultures^a from Genetically Resistant and Susceptible Mice

Cultures derived from	Time after inoculation	Titer x 10 ² PFU/ml ^b	
		JHMV ^c	MHV ₃ ^c
SJL	12 hr	0.17 ± 0.29 (3)	590 ± 114 (3)
	24 hr	23 ± 12 (14)	435 ± 292 (14)
	48 hr	48 ± 20 (14)	886 ± 62 (10)
	72 hr	22 ± 9 (12)	578 ± 119 (6)
CD.1	12 hr	350 ± 216 (5)	77 ± 12 (4)
	24 hr	3260 ± 2110 (13)	778 ± 30 (11)
	48 hr	9440 ± 500 (9)	3340 ± 2300 (7)

a) neural cultures consist predominately of oligodendrocytes and astrocytes as determined by phase contrast microscopy

b) the values are averages plus standard deviation from the number of cultures shown in parenthesis

c) multiplicity of infection was 1

type of response seen with those enriched for either oligodendrocytes or astrocytes, the remainder of the studies are based on mixed neural cells.

The time-course of virus production shown in Table 2, reveals that MHV₃ replication commences promptly generating maximum titers within 48 hr, whereas infection of SJL cells by JHMV is initiated slowly, producing within 24 hr low titers which do not increase appreciably thereafter. Increasing the MOI from 1 to 10 elevates the yields of JHMV from oligodendrocytes to only a limited extent (Table 1).

Efficiency of replication in neural cells from F₁ hybrid mice

To test whether restriction in SJL cells is under dominant or recessive genetic control, susceptibility to JHMV of neural cultures from F₁ hybrid mice was examined. The F₁ mice are bred from a cross between CD.1 females and SJL males. Data on the yields of PFU, summarized in Table 3, show that neural cells from (SJL x CD.1) F₁ mice are as permissive for JHMV as those of the CD.1 parental strain. As anticipated, MHV₃ replicates to high titers in both cells from hybrid and parental mice. This evidence demonstrates that JHMV restriction in SJL cells is controlled by a recessive trait. These data are in agreement with previous studies, which showed that the factor(s)/or resistance to JHMV in SJL mice and explanted neurons, macrophages and astrocytes is recessive (3,5,6).

Adsorption and uptake by neural cells from susceptible and resistant mice

To determine whether JHMV restriction in SJL cells occurs during initial stages in cell-virus interactions, we examined the efficiency of adsorption and uptake by neural cells. Any difference in either the number or specificity of virus receptors at the cell surface which might account for the SJL restriction should have become evident from these analyses. However, since at 4°C adsorption of [³H]-uridine labelled JHMV and MHV₃ to SJL and CD.1 cultures is equally efficient (Table 4), receptors cannot be the determinant involved in restriction. In the case of JHMV, about 22% of the added inoculum becomes cell-associated, whereas with MHV₃ a smaller fraction, about 11%, becomes bound.

The next stage examined in CV neural cell interactions concerned the uptake of inoculum from the cell surface, as illustrated in Table 5. Following attachment of [³H]-uridine labelled virus at 4°C, the unadsorbed inoculum is removed by washing and the temperature is raised to 37°C for 1 to 3 hr to promote penetration. The residual extracellular inoculum is removed by treatment with proteinase k, whereby only the internalized inoculum remains cell-associated. It is evident from the data in Table 4, that during incubation for 1 and 3 hr at 37°C most of the cpm which become cell-associated at 4°C disappear at 37°C, presumably by virus desorption. Of the remaining cpm complexed with either CD.1 or SJL cells approximately 65 percent become internalized within 1 hr, and most by 3 hr. This evidence also shows that SJL restriction is not due to deficient internalization of JHMV.

JHMV infection of peritoneal macrophages from SJL mice is confined to fewer than 5 percent of cells in culture, regardless of the MOI (5). Once established, the infection does not become disseminated from the initial targets to neighbouring cells, nor is there any appreciable increase in viral titers (5), as evident also in the present studies (Tables 1,2) with neural cells. Estimates of the proportion of glial cells which become infectious centers are made by adsorbing MHV₃ and JHMV, for 1 hr at room temperature, to mixed neural cultures followed by incubation of cell-virus complexes at 37°C for 1½ hr, so as to enhance penetration. Samples are withdrawn at 0, 24, and

Table 3

Replication of Coronaviruses in Primary Neural Cultures from F1 Hybrid Mice:

Cultures derived from	Time after inoculation	Titer x 10 ² PFU/ml ^a	
		JHMV	MHV ₃
CD.1 x SJL ^b	24 hr	961	1120
	48 hr	6260	2400
	72 hr	943	213 ^c
CD.1	24 hr	170	220
	48 hr	3200	1050
	72 hr	4850	880
SJL	24 hr	3	1000
	48 hr	16	900
	72 hr	6	240

a) values are averages and standard deviation from 5 cultures

b) F1 mice were produced by crossing CD.1 females with SJL males

c) decrease in titers attributed to cell destruction

Table 4

Adsorption of [³H]-Uridine Labelled Inoculum Coronavirus to Mixed Cultures

Cultures derived from	CPM/sample ^a			
	JHMV ^b		MHV ₃ ^b	
	Total counts added	% of initial counts adsorbed	Total counts added	% initial counts adsorbed
SJL	2300	22	19100	11
CD.1	2200	22	18000	10

a) Average from duplicate cultures

b) MOI of 1 used for this experiment

48 hr after shift to 37°C, the residual extracellular virus is removed by proteinase K treatment, the cells are released to form a suspension of monodispersed cells, which are diluted in a series of tubes and plated onto freshly prepared monolayers of indicator L cells. The site on the monolayer where an infected neural cell is deposited develops into a plaque. Using this procedure it became evident that during 48 hr after inoculation of SJL cultures with JHMV there is no consistent increase in infectious centers, whereas infection of replicate cultures with MHV₃ generates a many fold increase in the number of infectious centers of the same magnitude as those produced by infecting CD.1 cultures with either JHMV or MHV₃ (Table 6). These data provide suggestive evidence that infection by JHMV of SJL neural cells is limited because cell-to-cell spread of the infection does not occur, as appears to be the case with SJL peritoneal macrophages (5).

Comparison of CV RNA expression in neural cells from permissive and restrictive mice

The above data indicate that restriction on JHMV by SJL neural cells involves a step beyond internalization of the inoculum. To determine whether production of infectious particles corresponds to synthesis of virus components we examined the efficiency of virus RNA expression by means of cDNA probes specific for JHMV and MHV₃. These probes represent information in the entire genome, and can detect both genomic and mRNAs (2). Following inoculation of mixed neural cultures from SJL and CD.1 mice with JHMV and MHV₃ and incubation at 37°C, samples were taken at 24, 48 and 72 hr and extracted for RNA analysis by the dot-blot technique, as described in (9). To enable direct comparisons of the dot-blot data the predominantly cellular total RNA content added per dot in individual samples was standardized. The autoradiogram of a representative dot-blot obtained with ³²P-labelled JHMV cDNA as probe is illustrated in Figure 1, from which it is evident that 24 hr after infection cells from SJL and CD.1 mice synthesize about equal amounts of JHMV and MHV₃ RNA. Although viral RNA increases by 48 hr, the quantity present in the case of JHMV infected SJL cells is about an order of magnitude less than after infection with MHV₃. At 72 hr post-inoculation about the same quantity of MHV₃ and JHMV RNA is present in SJL cells as at 48 hr, consistent with the data on virus titers in Table 2. By comparison, CD.1 mouse neural cells synthesize about equal quantities of MHV₃ and JHMV RNA, as evident from Figure 1. Furthermore, RNA synthesis must be rapid because as much CV RNA is present in the 24 hr as in the 48 hr samples.

Therefore, our data indicate that during the initial 24 hr after inoculation SJL neural cells actively synthesize JHMV RNA as efficiently as MHV₃ RNA. Thereafter there is further amplification of MHV₃ RNA, but much less of JHMV RNA. Unfortunately to date we have no information concerning the functional quality of JHMV RNA from SJL cells. However, since SJL cells generate PFU of JHMV and can be formed into syncytia, albeit to a very limited extent (data not presented), it is unlikely that any defects in biological functions of JHMV RNA can explain the limited virus produced by SJL oligodendrocytes and astrocytes.

Use of proteases in an attempt to amplify production of JHMV in SJL neural cells

Previous work by both Storz and Yoshikura and colleagues on bovine and MHV-S CV demonstrated a requirement for proteolysis to bring about an enhancement of infectious progeny and cell fusion activity (13,16). Recently Sturman, Frana and colleagues showed that proteolytic cleavage of the E2 glycoprotein of A59 activates the cell-to-cell fusing property of this agent (14,4). It is plausible that the defect associated with JHMV replication in SJL mice and explanted cells, which occurs at a stage beyond synthesis of viral RNA, is due to inadequate activation of a viral glycoprotein(s), perhaps

the E2 component. Enhancement of JHMV production and fusion in SJL cultures was attempted by treating the mixed neural cells with several proteases. Following inoculation at 4°C the infection was allowed to proceed for 4 hr at 37°C, then cells were treated with 0.1 ug/ml of trypsin, or chymotrypsin or thermolysin for 10 hr at 37°C. Infectious virus present in the supernatant fluid was determined at 24 and 48 hr by plaque assay. The nutrient medium was replaced each day at the time of sampling. Trypsin increased PFU released by only 25%. There was no evidence of enhanced cell-to-cell fusion throughout the period of study. The increases in JHMV titers due to exposure to chymotrypsin were similar to those obtained with trypsin, but thermolysin treatment did not increase whatsoever the PFU. Therefore, to date we have no credible evidence in favour of the hypothesis that deficient proteolysis at the surface of host cells is at the basis of the SJL restriction.

SUMMARY

Resistance of SJL mice to JHMV could be associated with explanted oligodendrocytes and astrocytes in primary neural cultures from newborn mice. The restriction demonstrated is not at the stage of adsorption, uptake by the host or RNA synthesis and does not occur with the serorelated MHV₃ strain. Experiments with neural cells from F₁ hybrid mice bred from resistant and sensitive parents show that resistance is a recessive trait. It is hypothesized that the defect may involve proteolysis at the cell surface, but to date no clear cut experimental data have been obtained to substantiate this idea. In more general terms our in-vitro observations demonstrate that

Figure 1. Dot-blot analysis of CV RNA present at various times after infection of mixed neural cultures. A-C: CD.1-JHMV RNA 12, 24 and 48 hrs respectively post-infection (P.I.) at 1) 1:1; 2) 1:10; and, 3) 1:100 dilutions. F-H: CD.1-MHV₃ RNA 12, 24 and 48 hrs P.I. at 1) 1:1; 2) 1:10; and, 3) 1:100 dilutions. A-E: SJL-JHMV RNA 12, 24, 48 hrs, 3d and 5d P.I. at 4) 1:1; 5) 1:10; and, 6) 1:100 dilutions. F-J: SJL-MHV₃ RNA 12, 24, 48 hrs, 3d and 5d P.I. at 4) 1:1; 5) 1:10; and, 6) 1:100 dilutions. I-K: Control CD.1, SJL and L cell RNA at 1) 1:1; 2) 1:10; and, 3) 1:100 dilutions.

resistance of SJL mice pertains to both embryonic and newborn animals and involves neural and non neural cell types. Thus, the SJL restriction of JHMV, evident at the cellular level, does not involve an age-related maturation process associated with rat and mouse oligodendrocytes studied previously (1,14).

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