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# **Rat Glial C6 Cells are Defective in Murine Coronavirus Internalization**

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#### SUMMARY

Rat C6 glial cells were resistant to infection by several strains of murine coronaviruses. The restriction was not at the adsorption stage, since virus adsorbed to the C6 cells in a similar manner to mouse L cells which supported a lytic infection. The virus could not be internalized by the C6 cells. However, if the virus was introduced into the C6 cells by polyethylene glycol fusion, viral replication occurred and progeny virions were released from the infected cells. These studies indicated that the C6 cells were restrictive to coronavirus replication by preventing the early penetration stage of the viral replicative cycle.

#### INTRODUCTION

The murine hepatitis viruses (MHV), members of the Coronaviridae, cause a wide spectrum of diseases in rodents including hepatitis, enteritis and encephalomyelitis (Wege et al., 1982). The disease pattern is dependent not only upon the strain of virus and the genetic background of the host, but also upon the predilection for certain cells as sites of viral replication. This cellular tropism can be displayed in vivo as pathological lesions in target tissue and in vitro as preferential replication in specific cell types. The hepatotropic strains such as  $MHV_3$  show a preference for non-parenchymal liver cells, whereas the neurotropic strain JHM replicates poorly in endothelial cells (Taguchi et al., 1983; Pereira et al., 1984). The agents that demonstrate neurotropic properties, however, show a preference for glial cells (Herndon et al., 1975; Dubois-Dalcq et al., 1982). The replication is not totally restricted to these neural cells, however, since virus particles have been observed in neurons (Herndon et al., 1975; Knobler et al., 1981 a,b; Collins et al., 1983; Sorensen et al., 1984; Sorensen & Dales, 1985). It has also been suggested that the neuron may play a critical role in the disease state (Knobler et al., 1981b). In primary cultures of differentiated mouse spinal cord certain strains of MHV show tropism for glial cells (Dubois-Dalcq et al., 1982). In dissociated cells from mouse central nervous system tissue, both hepatotropic (Wilson et al., 1986) and neurotropic (Wilson et al., 1986; Van Berlo et al., 1986) strains of MHV can be shown to replicate in glial cells. In one study, using primary explants of rat central nervous system tissue, one JHM strain specifically infects oligodendrocytes and not astrocytes, whereas the MHV<sub>3</sub> strain replicates only in astrocytes and not in primary oligodendrocytes (Beushausen & Dales, 1985). Oligodendrocytes, however, lose their susceptibility to JHM when they are induced to differentiate. In another study, JHM virus was shown to replicate in rat astrocytes but not readily in oligodendrocytes (Massa et al., 1986). The discrepancy between these two studies is not understood but may be due in part to biological variability of JHM strains of different origin or passage history. Nonetheless, at present the mechanism(s) of the displayed tropism and restriction of the various viral strains in the different cell types is (are) unknown.

This preference for replication in certain cell types can be also demonstrated in continuous cell lines. Lines have been identified that can be lytically infected, persistently infected or are non-infectable (Lucas *et al.*, 1978; Siddell *et al.*, 1982; Sturman & Holmes, 1983). At present the mechanisms involved in determining the outcome of the infectious process are not clearly

understood. There is some indication that the involvement of the viral envelope protein E2 and its transport to the cell membrane during the maturation of the virion and the response of the host cell membrane to infection may be determinants in this process (Mizzen *et al.*, 1983; Frana *et al.*, 1985).

In previous work a cell line, the rat C6 glial line, was identified which was resistant to infection by both the  $MHV_3$  and JHM strains of MHV (Lucas *et al.*, 1978). Studies have been initiated to examine the reason for this restriction in order to understand mechanisms whereby cells can be refractory to coronavirus infections and to understand the replication strategy of these agents. In this report we provide evidence that these cells are resistant to infection because of the inability of the viral agent to be internalized within the cell. However, if the virus is introduced by polyethylene glycol (PEG) fusion, viral replication occurs and progeny virions are released from the infected cell indicating that the cell prevents an early stage of the viral replication cycle.

#### METHODS

Cells. The sources and routine propagation of the murine L2 and rat C6 cell lines used in this study were as previously described (Lucas *et al.*, 1977; Flintoff, 1984) except that alpha medium was supplemented with 2% foetal calf serum and 8% Nuserum (Collaborative Research). The C6TK<sup>-</sup> line used in these studies was obtained from Dr B. Schimmer, University of Toronto, Toronto, Canada. For monitoring the early events of virus infection, the cells were grown on glass coverslips coated with 50 µg/ml of poly-L-lysine.

Virus. The sources and routine propagation of the murine hepatitis virus strains  $MHV_3$  and JHM were as previously described (Lucas *et al.*, 1977). MHV strain A59 was a gift of S. Beushausen (Department of Microbiology and Immunology, University of Western Ontario) and was similarly maintained. The Indiana strain of vesicular stomatitis virus (VSV) was propagated in a similar manner using the murine L2 cells as host.

Virus production was monitored by a plaque assay on L2 cell monolayers as previously described (Lucas *et al.*, 1977). Yields are expressed as p.f.u./ml. To determine the fraction of cells able to release virus, an infectious centre assay was performed (Lucas *et al.*, 1978).

For large scale preparations, supernatant virus was harvested when approximately 50% of the fused L2 monolayer had detached from the surface of the tissue culture flask. Cellular debris was removed from the virus-containing medium by centrifugation at 2000 g for 10 min at 4 °C and the resulting supernatant was filtered through a 0.22  $\mu$ m Millex-GV filter unit (Millipore). Virus was concentrated from this virus-containing medium by centrifugation at 4 °C. The virus pellet was resuspended in a small volume of phosphate-buffered saline (PBS) and used to infect the cell monolayers.

Adsorption assay. Virus in PBS was added to cell monolayers and allowed to adsorb at 4 °C for various periods of time. To remove unbound virus, the infected cells were washed three times with PBS containing 0.5% bovine serum albumin and 0.05% Tween 20. One ml of cold medium was added and the cells were removed from the substrate by gentle scraping and disrupted by two passages through a 30 gauge needle. The amount of virus present was titrated as described above.

Virus internalization. Monolayers of either L2 or C6 cells growing on glass coverslips were adsorbed for 60 min with virus at various m.o.i., washed as above to remove unbound virus, and shifted to 37 °C for various periods of time. Subsequently, cultures were washed with cold PBS and treated with 0.5 mg/ml of proteinase K in PBS for 45 min at 4 °C to remove external virus (Helenius *et al.*, 1980; Mizzen *et al.*, 1985). The cell suspension was diluted with an equal volume of 2 mM-phenylmethylsulphonyl fluoride (PMSF), 6% bovine serum albumin and centrifuged for 2 min at 650 g at 4 °C. The cell pellets were washed twice in 2% bovine serum albumin and assayed for internalized virus by an infectious centre assay as described above. To assay for the infectivity of internalized virus was quantified as described above.

*PEG-induced cell fusion.* After virus had been allowed to adsorb to the cell monolayers and unbound virus removed by washing as described above, the infected cells were fused together by exposure to PEG 8000 (BDH) as previously described (Flintoff, 1984). The cultures were incubated at 37 °C and at various times monitored for the number of infected cells by the infectious centre assay described above and for the release of progeny virus into the culture medium by the plaque assay described above.

Immunofluorescence. The procedures for indirect immunolabelling were as described by Beushausen & Dales (1985). The mouse anti-A59 serum was kindly provided by S. Beushausen and was used at a final dilution of 1:8. The rhodamine-conjugated goat anti-mouse IgG was purchased from Miles Laboratories and was used at a final dilution of 1:20. The cultures were examined and photographed under u.v. illumination, using a Wild-Leitz, Dialux 20 microscope.



Fig. 1. Adsorption of coronaviruses to mouse L2 and rat C6TK<sup>-</sup> cells. MHV<sub>3</sub> at an m.o.i. of 3 (a) or JHM at an m.o.i. of 0.5 (b) was adsorbed at 4 °C to monolayers of either L2 cells ( $\bigcirc$ ) or C6TK<sup>-</sup> ( $\bigcirc$ ) cells. Unbound virus was removed by washing with PBS containing 0.5% bovine serum albumin and 0.05% Tween 20. Bound virus was assayed at various times as described in Methods.

### RESULTS

Of a number of cell lines of neural and non-neural origin that were screened for their susceptibility to the murine coronaviruses JHM and  $MHV_3$ , the rat C6 glial line (or its derivative C6TK<sup>-</sup>) was unique in its inability to support the replication of these agents (Lucas *et al.*, 1978). This lack of susceptibility occurred over a wide range of multiplicities (0.01 to 10) and was manifested by the lack of production of viral particles and infectious viral centres. The resistance to infection at the cellular level may be a consequence of a lack of a cellular receptor for virus attachment, lack of internalization of the virus, inappropriate intracellular processing and replication, failure of virus assembly and release, or a combination of several of these events. In order to define the stage or stages that might be defective in the infectious process in the C6 cell, studies were initiated to examine these steps with emphasis on the early events. Comparisons were made with the murine L2 line as the permissive cell.

# Adsorption

To examine the initial phase of the infectious process which involves an interaction between the viral peplomer E2 glycoprotein and the cell receptor (Sturman & Holmes, 1983), MHV (JHM, MHV<sub>3</sub> or A59 strains) was added to monolayers of either L2 or C6TK<sup>-</sup> cells and allowed to interact at 4 °C. At various time intervals, the infected cells were washed with Tween 20 to remove non-specifically bound virus and the bound virus was determined as described in Methods. As shown in Fig. 1, binding as measured by this assay is rapid, time-dependent and saturable. The viruses bound to the C6 cells with similar kinetics and saturation as to the L2 cells. Similar results were obtained over a wide range of m.o.i. from 0.2 to 10 for all three strains of viruses (JHM, MHV<sub>3</sub> and A59). These results indicate that virus adsorption to the C6 cells does not dramatically differ from that of the permissive host L2.



Fig. 2. Internalization of viruses by mouse L2 and rat C6TK<sup>-</sup> cells. Cultures of MHV-adsorbed (a, b) or VSV-adsorbed (c) L2 (()) or C6TK<sup>-</sup> ( $\bullet$ ) cells were warmed at 37 °C for various periods of time, treated with proteinase K to remove external virus, and assayed for internalized virus by the infectious centre assay. (a) MHV<sub>3</sub> at m.o.i. of 5; (b) JHM at m.o.i. of 2; (c) VSV at m.o.i. of 0.5.

### Internalization of virus

The entry of MHV into the host cell appears to be one involving absorptive endocytosis (David-Ferreira & Manaker, 1965). In order to examine whether this process was altered in the C6 cell, MHV-adsorbed cells were warmed to 37 °C for various periods of time, the external virus was removed by treatment with proteinase K and internalized virus was assayed by the infectious centre assay. The results shown in Fig. 2 indicate that virus internalization did not occur in the C6 cells with either JHM or MHV<sub>3</sub> strains. Similar results were obtained with the A59 strain (data not shown). This lack of internalization was demonstrable over a range of m.o.i. from 0.2 to 10. In contrast, the L2 cells internalized the virus in a time-dependent manner until approximately 30% of the cells scored as infectious centres.

This inability of C6 cells to internalize the coronaviruses is a specific phenomenon related to these agents since these cells readily took up VSV (Fig. 2c) and measles virus since this latter agent can replicate persistently in this line (Lucas *et al.*, 1978).

It is conceivable that the coronaviruses may enter these cells but the uncoating process to liberate the virus genome is defective. To examine this possibility,  $MHV_3$  was adsorbed at 4 °C for 60 min at a m.o.i. of 5 to either C6 or L2 monolayers, treated with Tween 20 and shifted to 37 °C to permit virus internalization. At subsequent times the infected cells were treated with proteinase K, disrupted by passage through a needle, and the resulting material was assayed for infectious virus. For the C6 cells, no infectious virus was detected, whereas internalized infectious virus was readily detected from the L cells. In this latter case, the titres peaked at about 60 min post-temperature shift and then began to decline presumably as a result of the uncoating process which would render the virion non-infectious.

Taken together these data demonstrate that the C6 cells are resistant to MHV infection because of their inability to permit virus penetration.

## Internalization of MHV in C6 cells

Because of the various stages that a virus undergoes during its replicative cycle in a host cell, it is possible that several of these may be affected in the C6 cell in addition to that of internalization. Thus attempts were made to introduce MHV into these cells and determine whether viral replication could occur.

# Effect of pH

Recent biochemical and morphological studies have shown that a number of enveloped viruses enter cells through receptor-mediated endocytosis (Marsh, 1984). The internalization

Virus strain	<b>M</b> .o.i.	-PEG	+ PEG
MHV <sub>3</sub>	1	< 0.01	0.12
	3	< 0.01	0.18
	10	<0.01	0.3
	40	0.4	3.0
JHM	4	< 0.01	1.0
A59	10	<0.01	0-4

Table 1. Percentage of infected C6 cells at 120 min after treatment with or without PEG

Table 2. Virus production (p.f.u./ml) from C6 treatment with or without PEG

Virus strain	M.o.i.	Time post-infection (h)	-PEG	+PEG
MHV <sub>3</sub>	1	24	$1 \times 10^{1}$	$1.5 \times 10^{3}$
	3	24	0	$2.5 \times 10^{3}$
	40	24	$2\cdot 2 \times 10^2$	$1.3 \times 10^{4}$
		72	$1 \times 10^{2}$	$2 \times 10^{4}$
A59	10	24	$1.1 \times 10^{2}$	$1.5 \times 10^{3}$
		96	0	$3 \times 10^{2}$

occurs through clathrin-coated pits and in most cases the viral envelope fuses with the endosomal membrane (White *et al.*, 1983). The nucleocapsid is injected directly into the cytoplasm. If this normal infectious route is blocked by various means, infection of cells can be induced by brief treatment at low pH (Helenius *et al.*, 1980).

To examine whether exposure to low pH would permit MHV internalization and replication in the glial cells,  $MHV_3$  was allowed to adsorb to C6 cells and the infected cells were exposed to medium of pHs ranging from 4.5 to 6.0 for periods of 2 to 10 min. After such treatment, internalization of virus was measured as above, or the medium was replaced with normal medium and assayed 24 h later for the presence of infectious virus. In none of the cases was virus shown to be internalized by the low pH treatment nor were any progeny virions detected in the culture medium.

## PEG fusion

PEG has been used to introduce various types of molecules into cells (Klebe et al., 1984); it was therefore reasoned that this compound might be useful to introduce MHV into C6 cells. MHV was adsorbed to monolayers of C6 cells and after removal of non-specifically bound virus, the infected cells were treated with PEG for 1 min. At various times after a shift to 37 °C, the amount of internalized virus was measured by an infectious centre assay. Immediately after the fusion event, infectious centres were detected. The percentage of cells scoring as such varied from 0.01 to 0.1 depending upon the initial m.o.i. This number increased to between 0.12% and 3% over the next 120 min (Table 1). Similar results were obtained for the JHM and A59 strains (Table 1). In cells to which virus had been adsorbed but not treated with PEG, at low m.o.i. the number of infected cells was less than 0.01%. At higher m.o.i. (>40) the non-PEG-treated cells gave variable results in which infected cells could be detected at low numbers in some cases. At these high m.o.i. the PEG-treated cultures consistently yielded at least a 10-fold higher number of infected cells than the non-treated cells. Treatment with PEG had no effect on the infection process in control L2 cells. Similar numbers of infected L2 cells and progeny virus yields were obtained whether the cultures were treated or not with PEG after the adsorption stage (data not shown).

The demonstration that C6 cells can score as infectious centres, provided the virus gains entry to the cell, indicates that MHV is replicating in these cells since the progeny produced must be infecting the neighbouring L2 cells in order to score as foci. To confirm that viral replication was occurring, assays for released virus were carried out on medium from the infected cells. As shown in Table 2, the PEG-treated cultures produced significant amounts of virus which were higher than those from the untreated cultures. The PEG-treated cultures were capable of



Fig. 3. Immunofluorescent labelling of cultures. Cultures of L2 (a, b) or C6TK<sup>-</sup> (c to h) cells were infected with MHV<sub>3</sub> at an m.o.i. of 40, and either treated with PEG (g, h) or not (a, b, e, f); (c) and (d) are uninfected C6TK<sup>-</sup> cells. (a, c, e, g) Viewed by phase-contrast; (b, d, f, h) examined under u.v. illumination after treatment with mouse anti-MHV A59 sera and rhodamine-conjugated goat antimouse IgG. Bar marker represents 10  $\mu$ m.

producing virus over several days. Such cultures did not show the multinucleated giant cells characteristic of an infection in L2 cells, but rather showed discrete foci of two or three cells harbouring viral antigens (Fig. 3). Although these cultures produced virus over several days, there was no evidence that the infection had spread to adjacent cells in the culture, as the infected foci monitored by immunofluorescence remained essentially constant in size. At high initial m.o.i. (>40) the PEG-treated culture ceased to yield virus after about 3 to 5 days in culture.

### DISCUSSION

Resistance to coronavirus infection in rat C6 glial cells is manifested at an early stage in the viral replicative cycle. The coronaviruses can bind to the cell but the stimulus to become phagocytosed is lacking. This conclusion is based on the observation that these agents appear to bind to the C6 cells in a similar manner as to mouse L2 cells which are totally permissive to infection. However, in contrast to the L2 cells, the coronaviruses are unable to become internalized within the C6 cell at m.o.i. below about 40. This is reflected in an inability of the C6 cells to produce progeny virions and develop as infectious centres. This inability to allow coronavirus penetration appears to be the only site of viral restriction since if cells that have coronavirus bound at the cell surface are treated with PEG, the virus becomes internalized, replication occurs and progeny virus are produced and released from the cell. This is supported by the ability to detect infected C6 cells and to detect extracellular viral progeny. In support of this, preliminary pulse-labelling experiments have indicated that the synthesis of at least one viral protein, the nucleocapsid, can be detected in the C6 cells that have been treated with PEG (unpublished observations). The overall infectious process in the PEG-treated C6 cells differs in some aspects with the L2 infection mainly in the extent of the infectious process. Infection in L2 cells results in an extensive cytopathic effect involving the formation of multinucleated giant cells which eventually involve the entire infected monolayer. In contrast, the C6 infection is localized involving only a few cells in the process. This is not unlike other coronavirus infections in which the cytopathic effects are restricted (Lucas et al., 1978; Mizzen et al., 1983; Sturman & Holmes, 1983). Although some multinucleated cells are seen to contain viral antigens, it is not clear whether these foci result from the infectious process or the fusion induced by PEG. Over extended periods of time the infected foci do not appear to increase in size suggesting that the initially infected cells are unable to recruit neighbouring cells. This in part may explain the low yields of virus. In preliminary experiments the addition of trypsin or chymotrypsin to the culture media did not enhance the cytopathic effect nor increase the yields of virus produced. The reasons for this are at present unclear even though the cell fusion capacity of murine coronaviruses requires cleavage of the E2 spike protein for activation (Sturman et al., 1985). Other mechanisms such as the production of interferon may also act to restrict viral infection. However, this may not be the case in this instance since the production of VSV was similar between JHM-infected and uninfected C6 cells when these cultures were challenged with this agent (unpublished observations).

The demonstrations that C6 cells allow the penetration of VSV and support the replication of measles virus (Lucas et al., 1978) suggest that the restriction of coronavirus replication is a unique feature of this virus-cell interaction. It has been demonstrated that viral adsorption, cellcell fusion and spread of infection is a function of the interaction of the cell surface with the viral peplomer glycoprotein E2 (Collins et al., 1982). Proteolytic cleavage of the glycoprotein is necessary to activate its fusion ability (Sturman et al., 1985). It has been suggested that cleavage of the E2 protein may also be required to permit the penetration of the virus into the cell and thus infectivity (Sturman & Holmes, 1985), not unlike the proteolytic activation of viral infectivity in orthomyxo- and paramyxoviruses (Klenk & Rott, 1981). There is host-controlled variation in this coronavirus cleavage event (Frana et al., 1985) and it has not been possible to demonstrate whether cleavage of the E2 glycoprotein is necessary for infectivity as there has not been a source of purified virions in which MHV does not contain totally uncleaved E2. It is intriguing to speculate that C6 cells may be deficient in protease(s) that carry out the cleavage of E2. This would also be consistent with the lack of spread of the virus infection once replication in C6 is initiated (see above). It will be of interest to determine whether treatment with protease will permit viral penetration of the C6 cell. Such studies are currently in progress. It is conceivable that cleavage of E2 may not be the only determining factor. It may also involve other factors such as the ability of the cell membrane to fuse which has been demonstrated for the virusinduced fusion process (Sturman & Holmes, 1983; Mizzen et al., 1983).

The exact type of glial cell from which the C6 glioma arose is uncertain (Benda *et al.*, 1971). This system has been considered a good model for the developing brain because although the cells resemble astrocytes morphologically they express some of the biochemical properties of

oligodendrocytes (Volpe *et al.*, 1975) and under appropriate stimuli can be induced to exhibit properties of either astrocytes or oligodendrocytes (Liao *et al.*, 1978). As indicated earlier, one JHM strain of MHV can replicate in primary rat oligodendrocytes and not in primary astrocytes, whereas the MHV<sub>3</sub> strain can replicate in astrocytes and not in the oligodendrocytes (Beushausen & Dales, 1985). Another strain of JHM, however, appears to replicate in astrocytes and poorly in oligodendrocytes (Massa *et al.*, 1986). Although neither agent replicates in C6 cells unless the viruses are introduced by chemical means, understanding the mechanism of viral restriction in the C6 cell line may be a useful model in delineating mechanisms of tropism that exist and in examining factors required for coronavirus penetration.

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1684

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