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Growth pattern of various JHM coronavirus isolates in primary rat glial cell cultures correlates with differing neurotropism in vivo

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Summary

The JHM strain of murine hepatitis coronavirus is neurotropic in rats, causing either fatal acute encephalomyelitis or subacute demyelinating encephalomyelitis. We have examined the growth properties of three JHM virus isolates in primary rat glial cultures and found a correlation with their ability to cause disease. Wild type JHM virus has the propensity to cause lytic infections in glial cultures, and a temperature-sensitive mutant designated JHM-ts43 invariably produces persistent infections with reduced cytopathic effects (CPE) as compared to the wild type. Moreover, a non-neurotropic isolate, designated JHM-Pi virus, produces either non-productive persistent infections at low multiplicity of infection (m.o.i.) or productive persistent infections at high m.o.i., with, however, no CPE. The phenotypic expression of persistence is glial cell-dependent, since all three viruses produce similarly lytic infections when grown on various susceptible cell lines. The genetic basis of JHM virus persistence can be explained at the level of direct virus–glial cell interactions.

Murine hepatitis virus; Brain cells; Cytopathic effects; Genetic control

Introduction

Several murine coronavirus strains cause in rodents a broad spectrum of acute to chronic central nervous system diseases which are important in studying the

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mechanisms of persistency and demyelination (Sörensen et al., 1980; Stohlman and Weiner, 1981; Knobler et al., 1981; Sörensen et al., 1982; Lavi et al., 1984). Infection of Lewis rats with the JHM strain of murine coronavirus in particular can lead to a demyelinating encephalomyelitis, which is triggered by a persistent virus infection and is associated with autoimmune reactions against myelin basic protein (Watanabe et al., 1983; Massa et al., 1986a). JHM virus causes in rats both a short term acute encephalomyelitis with involvement of neurons and glial cells and a subacute demyelinating encephalomyelitis (SDE) with predominant involvement of glial cells (Nagashima et al., 1978, 1979; Koga et al., 1984; Wege et al., 1984a). This SDE is caused by a persistent virus infection, and neuropathological changes consist of inflammatory demyelinating lesions. Recovery from the acute disease and progression to viral persistence depends on the type of JHM virus utilized for intracerebral inoculation. Wild type JHM predominantly causes acute fatal encephalomyelitis, while various temperature-sensitive mutants produce a less severe acute phase of disease and establish long-term persistence with high frequency (Wege et al., 1983). Other JHM isolates do not cause productive infections in the brain (Baybutt et al., 1984). The ability of different neurotropic JHM virus isolates to cause fatal acute disease or persistent infections may depend on various factors, including the target cells of the virus, efficiency of virus production, the ability of the virus to spread to other cells and the particular type of immune response to the infection (Sörensen et al., 1982, 1984; Beushausen and Dales, 1985; Taguchi et al., 1985, 1986; Dubois-Dalcq et al., 1982; Massa et al., 1986b). The degree to which direct virus-neural cell interaction or the immunological response to infection controls the frequency of acute versus persistent infection by JHM virus is unknown. We have therefore examined the growth properties of three different JHM isolates in primary rat glial cell cultures. We show that the degree of neurotropism associated with each individual virus in the animal correlates with growth properties of the virus in the glial cell cultures. The differences seen in tissue culture are glial cell-specific, as all three viruses display similar properties when grown on susceptible cell lines.

Materials and Methods

Primary glial cultures

One-day-old Lewis rat pups were anesthetized with ether, washed with a surgical disinfectant, then decapitated. Brains were aseptically removed and brainstems, cerebella and meninges were carefully dissected from cerebral hemispheres and discarded. The pooled cerebral hemispheres were minced with scissors, and repeatedly aspirated through a Pasteur pipet to mechanically dissociate the cells. Dissociated cells were suspended in 4 ml per hemisphere of Dulbecco's Modified Eagle's Medium (DMEM) without antibiotics and containing 0.6% dextrose and 15% non-heat-inactivated fetal bovine serum (complete medium). The suspended cells were sieved through a 130 μm and then a 33 μm polyester screen without

vacuum and plated onto Falcon 24-multiwell plates with or without polyornithine-coated glass coverslips. The cultures were incubated at 37°C with 10% CO₂ at maximum humidity and fed on day 4 and every other day thereafter. Cultures were infected with JHM virus at 3 wk post-plating.

Primary glial cultures from newborn rat brain reach confluency 5 days after plating and consist primarily of three distinct cell populations, as previously described (Massa et al., 1986b). In brief, type I astrocytes positive for glial fibrillary acidic protein (GFAP) and Fc receptor-positive macrophages form a continuous monolayer of cells, occurring in a 1:1 ratio remaining constant for at least 3 wk of culture. The remainder of the cells at five days consist of scattered A2B5⁺ precursors to oligodendrocytes and type II astrocytes, which divide and differentiate into the latter cell types between 1–3 wk after plating (Massa et al., 1986b). At 3 wk post-plating, when the cultures were infected, the cell density was approximately 5×10^5 cells/cm².

Infection of cultures

Cultures grown for 3 wk were infected with either: (1) wild type (wt) JHM virus; (2) a temperature-sensitive neurovirulent mutant designated ts43 (Wege et al., 1983); or (3) a non-neurovirulent isolate of JHM virus designated JHM-Pi (Baybutt et al., 1984). The cultures were infected with different m.o.i. (1.0 p.f.u./cell or 10⁻⁴ p.f.u./cell) for comparison. Prior to infection, cultures were washed twice with DMEM without fetal bovine serum, then inoculated with JHM virus. After adsorption for 2 h, and every other day thereafter, the inoculum was removed and the cultures were fed with fresh complete medium. Supernatants were saved at regular feedings between 2 and 24 days post-infection to determine production of released virus. At 1, 7 and 21 days post infection cultures were processed for immunoperoxidase labeling of intracellular JHM virus antigen or double immunofluorescence of virus antigen and astrocyte-specific glial fibrillary acid protein (GFAP).

Immunoperoxidase labeling of viral antigen

Glial cultures were fixed with 2.0% formaldehyde in 0.12 M phosphate buffer for 1/2 h, treated with 0.25% Triton X-100 in 0.5 M Tris buffer for 20 min to permeabilize the cells, and blocked for non-specific protein binding sites with 20% normal horse serum in 0.5 M Tris buffer, pH 7.6, for 1 h at room temperature. Staining of intracellular viral antigen was performed by incubating the fixed cultures with a mouse monoclonal IgG to the JHM nucleocapsid protein produced in this laboratory (Wege et al., 1984b) (1:50 from hybridoma supernatant) followed by a rabbit anti-mouse IgG antiserum conjugated to horseradish peroxidase (Dakopatts, Denmark). After rinsing, 4-chlor-1-naphthol (0.025%) and H₂O₂ (0.005%) were added, producing an opaque blue-black precipitate in virus antigen-containing cells. The specimens were photographed by bright field microscopy.

Double immunofluorescence

Cells cultivated on polyornithine coated glass coverslips were fixed with 2% formaldehyde in 0.12 M phosphate buffer, pH 7.2, permeabilized with 0.25% Triton X-100, then blocked with 20% normal horse serum in 0.5 M Tris buffer, pH 7.6. The cultures were incubated with a mouse monoclonal antibody to JHM virus nucleocapsid protein (Wege et al., 1984b) and then with a rabbit antiserum to astrocyte-specific glial fibrillary acidic protein (GFAP) (DAKO, Denmark). Co-localization of the virus nucleocapsid and GFAP in individual cells was determined by epifluorescence microscopy after subsequent incubation of preparations with rhodamine conjugated to goat anti-mouse IgG and fluorescein conjugated to goat anti-rabbit IgG (Zymed, South San Francisco, Ca). Normal rabbit serum and mouse monoclonal antibody unrelated to JHM virus antigen or rat antigens were utilized as negative controls in the primary antibody step in parallel specimens.

Virus preparation and titration

The stock viruses were isolated, selected for variants and cloned as previously described (Wege et al., 1983; Baybutt et al., 1984). Virus released into supernatants was titrated as p.f.u./ml on the murine Sac(−) cell line.

Results

The parameters analyzed in this study for the three JHM virus isolates utilizing primary glial cultures from newborn rat brain were (1) differences in cytopathic effect (CPE), (2) differences in rates of spreading via cell fusion, (3) differences in establishment of persistence with high and low m.o.i., and (4) differences in production of infectious virus.

Infection of a susceptible cell line

Infection of the susceptible murine Sac(−) cell line with JHM-wt, JHM-ts43 and JHM-Pi resulted in similar kinetics of replication and cytopathic effects with respect to syncytia formation and cell lysis. All three variants cause a lytic infection both at high (1.0) and low (0.0001) m.o.i. Cytopathic effects (CPE) consisted of plaques formed via cell fusion of infected cells containing abundant virus nucleocapsid protein. The release of infectious virus after infection with a m.o.i. of 1.0 p.f.u./cell, as shown in Fig. 1, starts at 12 h p.i. At 18–24 h p.i. CPE is complete and virus production declines and ceases between 24–30 h for all three viruses.

Infection of three-week glial cultures and virus replication

The target cells of JHM virus in glial cultures are type I astrocytes and brain derived macrophages (microglia) (Massa et al., 1986b) regardless of the various

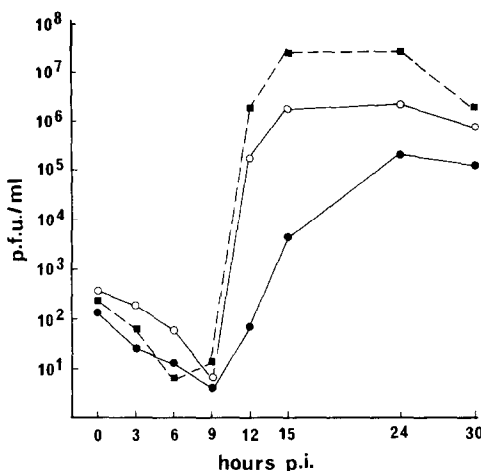


Fig. 1. Virus titers released from lytically infected Sac(−) cells. CPE is complete by 24 h p.i. for all three JHM virus isolates. Peak virus titers obtained are on average 10^7 p.f.u./ml for JHM-Pi, 10^6 p.f.u./ml for JHM wt virus and 10^5 p.f.u./ml for JHM-ts43 virus. CPE is identical for all three viruses. ○—○ JHM-wt, ●—● JHM-ts43, ■—■ JHM-Pi.

JHM virus isolates used. As shown in Fig. 2, at 12–24 h p.i. with high m.o.i., many of the primary target cells of both wild type JHM and ts43 are GFAP⁺ astrocytes. Preferential infection of astrocytes by JHM-Pi virus is also apparent (not shown). As seen at 24–48 h p.i. with high m.o.i. (Fig. 3), spreading of JHM wt and ts43 virus proceeds rapidly via cell fusion of astrocytes and macrophages in the bed layer while JHM-Pi appears to be restricted in its capacity to fuse as described below. At one week p.i. syncytia are still positive for both JHM antigen and GFAP filaments and syncytia are enlarged compared to that seen during the first days p.i. As well, CPE becomes apparent at this time in cultures infected with wt and ts43, but not with JHM-Pi.

Plaques produced by wt, ts43 and JHM-Pi differ in their rates of expansion in the order wt > ts43 \gg JHM-Pi. Immunoperoxidase staining of JHM virus antigen most clearly demonstrates the differences in the extent of plaque formation at 1 day, 1 wk and 3 wk p.i. (Fig. 4). As can be seen, the growth of plaques, produced by JHM-wt and ts43 after infection with low m.o.i., proceeds steadily for at least 3 wk (Fig. 4, a–f). In contrast, JHM-Pi remains restricted mainly to single cells or cell doublets as seen at 1 day p.i. (Fig. 4, g) using a low m.o.i., and the infection appears abortive during the first week of infection. However, at high m.o.i., single or doublet infected cells positive for virus antigen persist for 1 to at least 3 wk p.i. (Fig. 4, h). Whether low multiplicity infection by JHM-Pi is abortive or simply undetectable by immunoperoxidase staining is unclear; however, in some instances, productive infections were apparent after co-cultivation with susceptible cell lines (not shown). This would indicate a propensity of JHM-Pi virus to produce non-productive low level persistent infections after inoculation with low m.o.i. and persistent productive infections using high m.o.i.

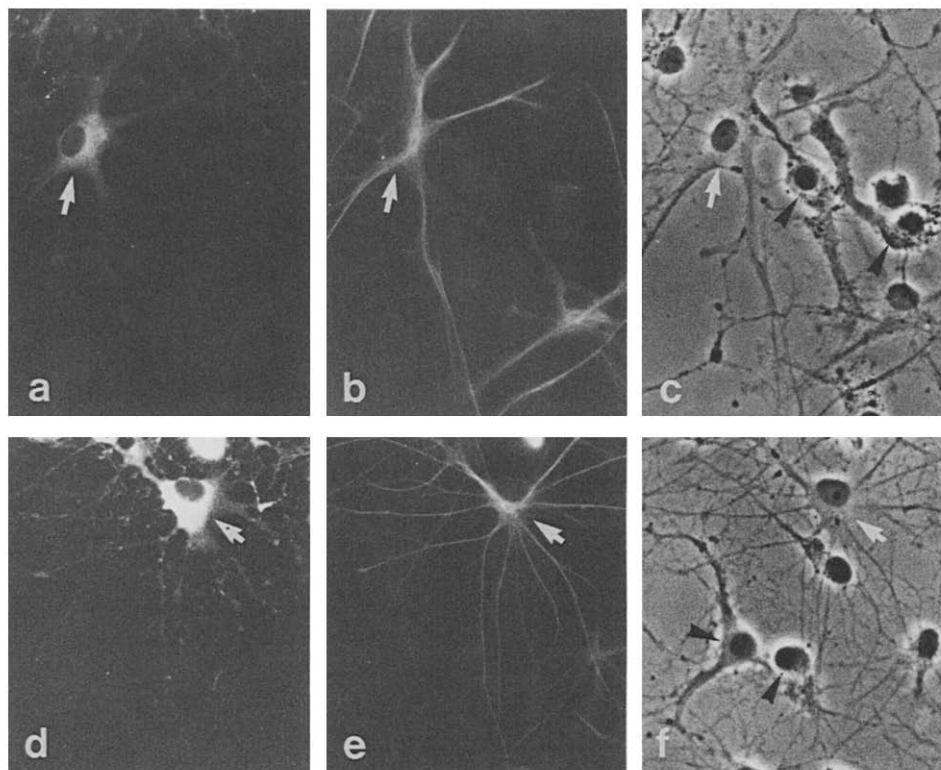


Fig. 2. At 12–24 h p.i. of rat glial cultures, many of the first cells to be infected by JHM virus regardless of the isolate used, are astrocytes, as seen by double immunofluorescence microscopy of JHM virus nucleocapsid protein (a and d) and astrocyte-specific GFAP (b and e). (a–c) JHM-wt virus infection. Double immunofluorescence and phase contrast of one microscopic field showing the nucleocapsid protein and GFAP in the same cell (arrows). (a) JHM virus nucleocapsid protein (FITC); (b) fibrillar staining pattern of intracellular GFAP intermediate filaments; c-phase contrast. Note that some cells, morphologically oligodendrocytes, are negative for both GFAP and virus antigen appearing in the phase contrast image (arrowheads). (d–f) JHM-ts43 virus infection. Double immunofluorescence and phase contrast of one microscopic field showing the nucleocapsid protein and GFAP in the same cell (arrows). (d) JHM virus nucleocapsid protein (FITC); (e) fibrillar staining pattern of GFAP filaments (RITC); (f) phase contrast. Note that some cells, probably oligodendrocytes, are negative for both GFAP and virus antigen appearing in the phase contrast image (arrowheads).

Infection by JHM-wt at high m.o.i. invariably led to a lytic infection that was complete by 1–2 wk p.i., with the exception that fibroblasts and numerous oligodendrocytes remained apparently uninfected, as previously described (Massa et al., 1986b). As described above, at low m.o.i., the cultures usually survived an initial lytic phase and then plaque expansion ceased. This situation was invariably produced by JHM-ts43 regardless of m.o.i. and persisted until at least 8 weeks p.i. (Fig. 4, f).

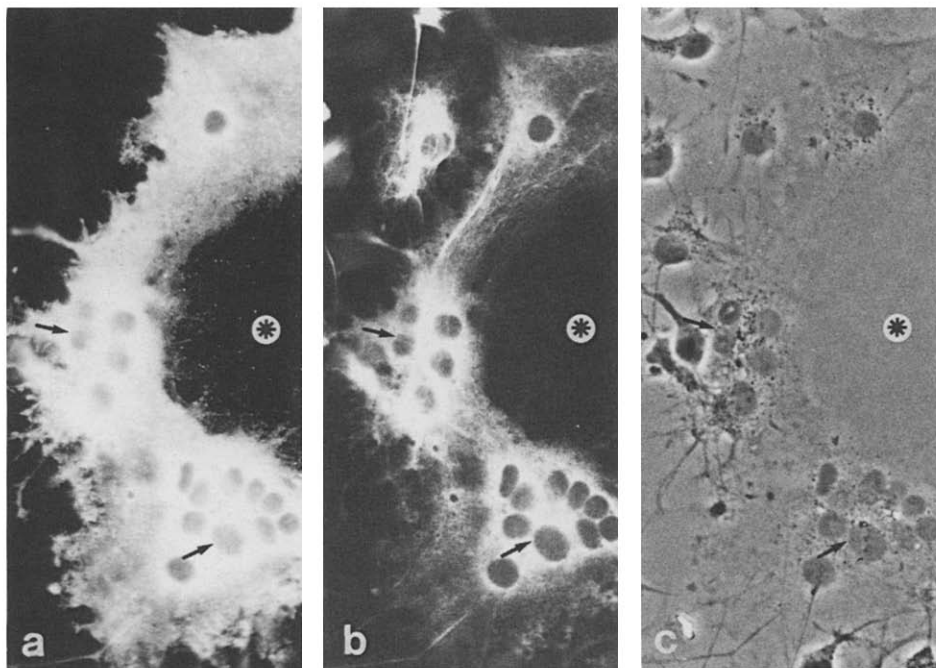


Fig. 3. (a-c). At 48 hours p.i., JHM virus spreads via cell fusion between adjacent GFAP⁺ astrocytes forming small syncytia containing contiguous nuclei (arrows). The center of the syncytium is marked by an asterisk and the outward border is to the left. The border, as viewed by double immunofluorescence, is strongly positive for JHM virus nucleocapsid (a) (FITC) and GFAP filaments (b) (RITC). (c) Phase contrast image of the same field.

In contrast to Sac(−) cells, infection of cultivated brain cells with the three JHM virus variants resulted in different virus growth curves. At high m.o.i. JHM-wt virus release peaked at 4 days p.i. and declined thereafter to undetectable levels by 10 days p.i. (Fig. 5, a). At low m.o.i. JHM-wt was released at constant levels (10^3 – 10^4 p.f.u./ml) between 4 and 24 days p.i. (Fig. 5, b) with a development of restricted focal CPE. After infection with JHM-ts 43 at high m.o.i. (Fig. 5, a) virus release rose at a steady slow rate starting 6–10 days p.i. whereas at low m.o.i. (Fig. 5, b) no evidence for a productive infection was obtained. JHM-Pi infection at high m.o.i. (Fig. 5, a) led to extremely high virus titers over prolonged periods without the development of a detectable CPE. With low m.o.i., infectious JHM-Pi was found on day 2 to day 4 at about 10^2 p.f.u./ml, and then became undetectable thereafter, except in rare instances in some samples (Fig. 5, b).

Discussion

We have examined three different isolates of JHM coronavirus which produce different neuropathological disease patterns after intracranial infection of rats.

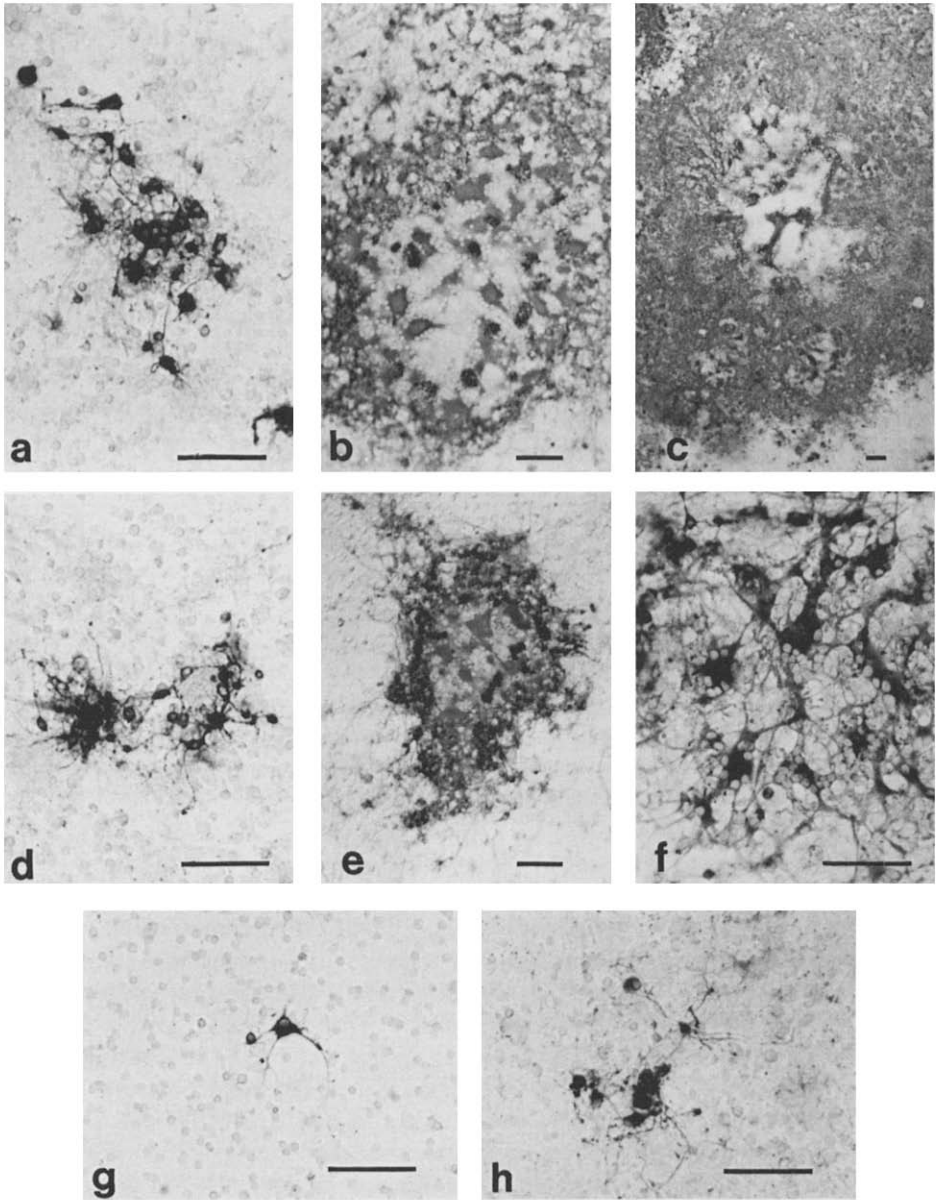


Fig. 4. (a–h). Immunoperoxidase labeling of JHM virus nucleocapsid protein in primary rat glial culture infected with either (1) JHM-wt virus (4a–c), (2) JHM-ts43 virus (4d–f), or (3) JHM-Pi virus (4g and h). Cultures infected at 3 wk post-plating with either of the viruses, were fixed and stained at 1 day (a, d, g) 1 wk (b, e, h) and 3 wk (c, f) p.i. for a comparison of rates of spreading and degree of persistence. The bar appearing on the figures indicates magnification (length = 0.1 mm). (a–c) JHM-wt virus infection (low m.o.i.) at 1 day (4a), 1 wk (4b) and 3 wk (4c) p.i. Note that syncytia occupy a greater area of the field over time and the decreasing magnification from 4a to 4c, indicated by the bars. (d–f) JHM-ts43 virus infection at 1 day (d), 1 wk (e), and 3 wk (f) p.i. Note that syncytia formed by ts43 are smaller than those of wt-JHM at 1 wk p.i., viewed at the same magnification (b = wt; e = ts43). At three weeks p.i., ts43 generally persists in the form of loosely associated small syncytia (f) which remain until at least 8 wk p.i. (g and h) JHM-Pi virus infection at 1 day (g) and 1 week (h). At 1 day p.i. (g), JHM-Pi virus infects single cells that remain solitary and generally does not fuse with neighboring cells, unlike cells infected with either JHM-wt or ts43.

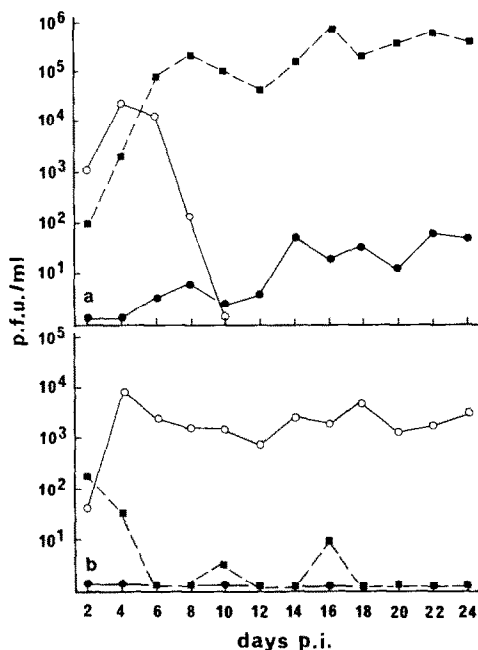


Fig. 5. Infectious virus released from infected primary rat glial cells 2 to 24 days p.i. ○—○ JHM-wt virus, ●—● JHM-ts43 virus, ■—■ JHM-Pi virus. (a) Infection with high m.o.i. leads to a short term lytic infection by JHM-wt and productive persistent infections by both JHM-ts43 and JHM-Pi virus. Note that at high m.o.i., JHM-Pi virus is produced at high titers for prolonged periods without CPE detectable by phase contrast microscopy. Virus release after infection by ts43 is much lower and delayed in onset. Cessation of virus production by JHM-wt virus is concomitant with 100% CPE within 10 days p.i. (b) Infection with low m.o.i. leads to persistent productive infections by JHM-wt and non-productive infections by JHM-ts43 and JHM-Pi. With JHM-Pi, released virus was only detected at a low titer 10 and 16 days p.i.

JHM-wt virus causes a high frequency of acute encephalomyelitis, JHM-ts43 virus produces a high incidence of a demyelinating encephalomyelitis based on a persistent virus infection, and JHM-Pi is non-neurovirulent *in vivo*. The growth properties of these viruses are remarkably different after infection of primary rat glial cultures especially with respect to JHM-Pi and reflect the type of disease pattern resulting in the animal. The observed differences in virus-cell interactions can be largely attributed to properties of glial cells since all three viruses behave similarly on various susceptible cell lines.

The reason for expression of virus-specific phenotypes in glial cells is unknown. With respect to persistence, interferons do not appear to play a role *in vitro* (Massa et al., 1986a). The major virological factor contributing to neurovirulence is probably related to the extent of cell fusion caused by the virus. Resistance against virus-induced cell fusion can be a property of the host cell, as demonstrated for persistent infections of mouse L cell sublines with coronavirus A59 (Mizzen et al., 1983). In contrast, we found that the extent of cell fusion during infection in Sac(—)

cells by the three JHM virus variants described here was similar. However, the fusion capacity of the avirulent JHM-Pi virus was strongly impaired in primary rat glial cultures even at m.o.i. higher than that routinely used in this study (m.o.i. = 10–100).

The viral peplomer glycoprotein E2 (also termed spike protein) is the structural entity responsible for cell fusion, attachment to cells and binding of neutralising antibodies (Collins et al., 1982; Wege et al., 1984b). It has been shown for the murine coronavirus, A59, that cell fusion can only be induced after proteolytic cleavage of this large glycoprotein into two smaller subunits of similar size (Sturman et al., 1985). The extent of cleavage and the degree of fusion differs with the host cell (Frana et al., 1985). The spread of virus by fusion could therefore be influenced by cell-specific factors like glycosylation and protease activities. In our system, such host factors may contribute to differences of spread in glial cells. However, the prerequisite for such different outcomes of infection is probably due to structural differences between E2 proteins of the particular JHM virus population used for infection.

JHM virus variants with enlarged RNA encoding the JHM E2 glycoprotein are selected for both in vivo (Taguchi et al., 1985) and during replication in rat astrocytes in vitro (Taguchi et al., 1986). This probably is an important virus–glial cell interaction involved in the establishment of growth potential and persistence of JHM virus in astrocytes within the brain. A similar phenomenon of spontaneous transition of lytic to persistent infection involving alteration of the E2 glycoprotein has also been observed in cell lines (Baybutt et al., 1984). Antigenic differences between JHM-wt virus and JHM-Pi are detectable by monoclonal antibodies against E2-glycoprotein (Wege et al., 1984b). Furthermore, JHM virus variants selected by escape from neutralisation with monoclonal antibodies revealed distinct changes of antigenic determinants which may be related to reduced neurovirulence (Fleming et al., 1986; Dalziel et al., 1986).

Defective replication within astrocytes by temperature-sensitive mutants of JHM virus and the related A59 coronavirus has been described in mouse CNS cultures (Dubois-Dalcq et al., 1982; van Berlo et al., 1986). Similar observations have been described for human coronavirus OC43 in mouse or human glial cultures (Pearson and Mims, 1985). Therefore, the tropism and cytopathogenicity of coronaviruses for astrocytes may be a major determinant during establishment of persistency in brain tissue. The behavior of different JHM virus isolates during infection of glial cells in vitro will allow the analysis of both virus- and cell-specific properties leading to viral persistence which are independent of factors associated with immunomodulation.

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