MOUSE HEPATITIS VIRUS RECEPTOR LEVELS INFLUENCE VIRUS-INDUCED CYTOPATHOLOGY

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1. ABSTRACT

We developed human (HeLa) cell lines in which mouse hepatitis virus receptor (MHVR) levels could be regulated by addition of tetracycline. We used these cell lines to determine whether MHVR levels impact the degree of cytopathology induced by infection with the lytic MHV A59 strain. Two cultures were studied; HeLa-MHVR^{lo} (less than 3,000 molecules per cell) and HeLa-MHVR^{hi} (300,000 molecules per cell). Both supported synthesis of infective A59 virus. However, the MHVR^{lo} cells showed no virus-induced cytopathology while the MHVR^{hi} cells uniformly died within 14 hours after infection. This cell death was not related to virus-induced syncytium formation as it occurred even in subconfluent cells overlaid with fusion-blocking antiviral antibodies. MHV A59 spike proteins produced by vaccinia vectors also killed the MHVR^{hi} cells within 12 hours postinfection—MHVR^{lo} cells infected in parallel were intact as judged by trypan blue exclusion. Our current hypothesis is that the accumulation of intracellular complexes composed of spike and MHVR proteins leads to acute single cell lysis.

2. INTRODUCTION

Infection by MHV requires receptor (MHVR) proteins on the cell surface. The receptors, members of the biliary glycoprotein (Bgp) family (Williams *et al.*, 1991), are type I integral membrane glycoproteins whose extracellular structure is comprised of two or four immunoglobulin-like domains (Dveksler *et al.*, 1991). The receptors are functionally important at two temporal stages in the infection cycle. First, the membrane-distal immunoglobulin domain interacts with spike (S) glycoprotein projections on incoming virions, and virion:

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cell membrane fusion ensues (Dveksler *et al.*, 1993a). Second, the same domain interacts with S proteins displayed on the surface of infected cells. During infection by most MHV strains, this latter interaction leads to intercellular fusion and syncytium formation, a hallmark of infection both *in vivo* (Lavi *et al.*, 1986) and *in vitro* (Frana *et al.*, 1985).

Both the virion:cell and the infected cell:cell interactions that ultimately lead to membrane fusion require a critical density of MHVR (Dveksler et al., 1993b). The precise density is not yet known, nor is it known whether the virion:cell fusion requires MHVR levels distinct from those that enhance cell:cell fusion. These questions led us to develop a set of HeLa cell transfectants that vary only in their level of MHVR on the surface. Using these lines we found that the receptor densities required for infection by virions were in fact lower than the densities that promote syncytia. This result is discussed in the context of similar studies focussed on interactions between other enveloped viruses and their receptors. With these cell lines we made the additional observation that virus-induced cytopathology is strongly correlated with receptor levels. This interesting finding is described herein.

3. METHODS AND RESULTS

3.1. Regulated Expression of MHVR cDNA in HeLa-MHVR Cells

In HeLa-MHVR cells (Gallagher, 1996), expression of the MHVR cDNA requires binding of a tetracycline-controlled transactivator (tTA) to tetracycline operator DNA (Figure 1). Addition of tetracycline to the growth medium releases the tTA from operator DNA and consequently reduces MHVR expression (Gössen and Bujard, 1992). Parallel cultures of HeLa-MHVR cells (clone #5) were incubated with doses of the tetracycline-derivative doxycycline (Gossen et al., 1995) ranging from 10^{-3} to 10^{0} μg/ml. Using antireceptor antibody CC1 (kindly provided by Dr. K. Holmes) in FACS analyses, we found that one-week incubation periods were necessary to establish new steady-state surface MHVR levels. By the same FACS methods we also found that the collection of doxycycline-exposed cultures encompassed a 140-fold range of steady-state MHVR densities. Cells with the lowest receptor levels (10^{0} μg/ml doxycycline) were designated HeLa-MHVR^{lo}, intermediate levels (10^{-1} μg/ml doxycycline) were HeLa-MHVR^{int}, and highest levels (no doxycycline) were HeLa-MHVR^{hi}.

The average number of MHVR molecules per HeLa cell was estimated by a western immunoblot procedure in which the MHVR proteins were identified using Mab CC1 (data not shown). The signal intensities in these blots were compared to those generated by known quantities of a recombinant soluble MHVR (Gallagher, 1997). In this way we roughly estimated that actively growing HeLa-MHVR^{hi} cultures contained ~300,000 receptors per cell. Immunoblot signals were not detected among proteins from the corresponding

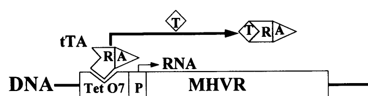

Figure 1. Schematic depiction of the tetracycline-controlled expression system. The tetracycline-controlled transactivator (tTA) is a recombinant protein comprising the carboxy-terminal domain of the herpes simplex virus VP16 (A) and the tetracycline repressor (R). Addition of tetracycline (T) to culture medium prevents binding of tTA to operator DNA (TetO7), thereby preventing transcription from the minimal cytomegalovirus promoter (P).

HeLa-MHVR^{lo} cells; our detection limit in these experiments was ~3,000 receptors per cell. Given that the FACS data indicated that MHVR^{lo} cells had only 0.7% as many surface receptors as MHVR^{hi}, we speculate that the MHVR^{lo} cells contained ~2000 receptors per cell.

3.2. HeLa-MHVR^{lo} Cells Are Resistant to Virus-Induced Syncytium Formation

To directly assess the importance of MHVR levels in virion infection and subsequent syncytium formation, we inoculated the MHVR^{hi} and MHVR^{lo} cultures with MHV strain A59. To identify individual infected cells, indirect immunoflourescence assays were performed using anti-matrix Mab 5A5.2 (Collins *et al.*, 1982). We found that 25% of the MHVR^{lo} cells contained the viral matrix protein. By microscopic examination, there was absolutely no evidence of syncytia in these cultures; this was verified by quantitative fusion assays (Gallagher, 1996; data not shown). In contrast, essentially all MHVR^{hi} cells were matrix-positive and part of multinucleated syncytia (Figure 2).

3.3. High Yields of Progeny Virions from Infected HeLa-MHVR^{lo} Cells

We and others have found that abundant MHVR synthesis inhibits the secretion of MHV-A59 from infected cells (Gallagher, 1995, Chen *et al.*, 1997). A complete mechanis-

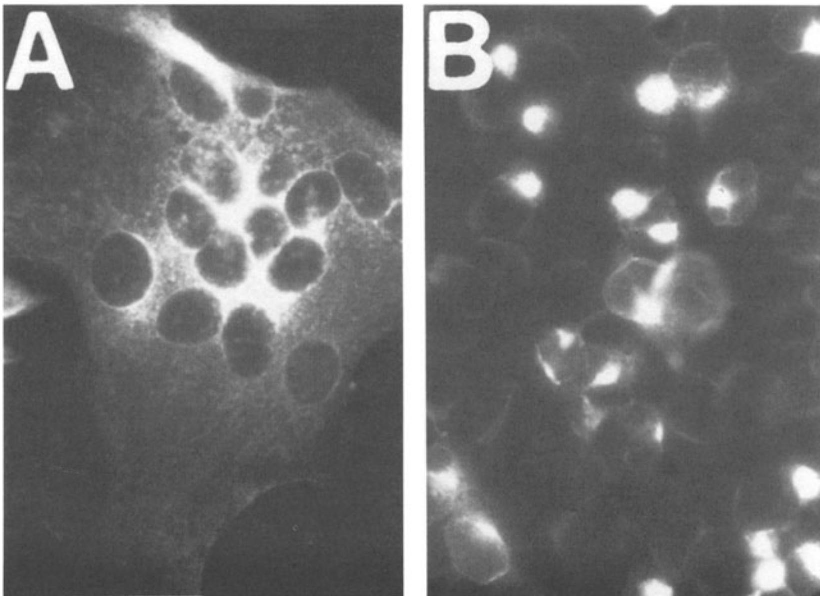

Figure 2. Identification of MHV A59 infection in HeLa-MHVR cells by indirect immunofluorescence. At 8 h post-MHV A59 infection (moi = 100 pfu/cell), cells were washed with PBS, acetone-fixed and incubated with mouse 5A5.2 ascites (1:250 in PBS-2% BSA). After the secondary incubation with FITC-conjugated goat antibody directed against mouse Ig, cells were rinsed, mounted and photographed using a Leitz fluorescence microscope. Panel (A); HeLa-MHVR^{hi}. Panel (B); HeLa-MHVR^{lo}.

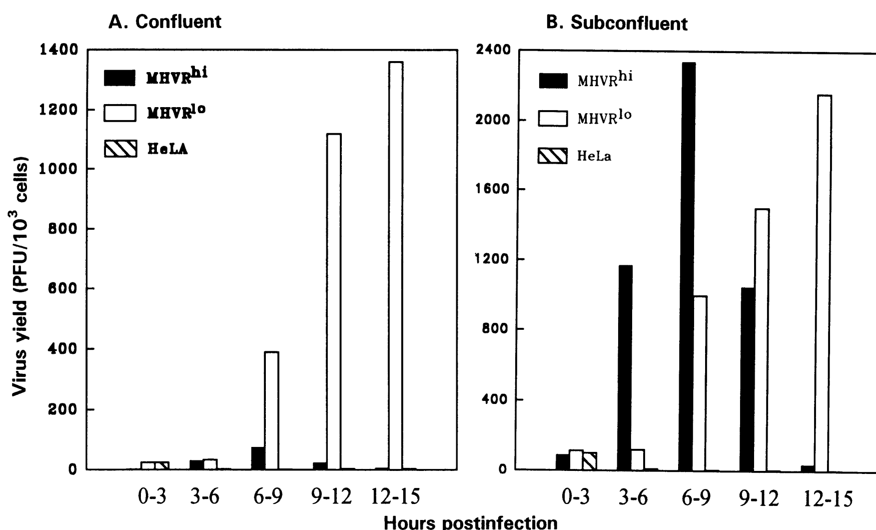

Figure 3. MHV A59 yields from $MHVR^{lo}$ cells exceed those from $MHVR^{hi}$ cells. Following inoculation with MHV A59 (moi = 10 pfu/cell), cultures were incubated at 37°C using 1 ml growth medium per 10^6 cells. At 3 h intervals, growth media were removed and replaced with fresh media. Viral infectivities in the spent media were determined by plaque assays using HeLa-$MHVR^{hi}$ cells as indicators.

tic understanding of this inhibition is not yet available. To further investigate the role of MHVR levels during virus secretion, we monitored the time course of progeny virus development (Figure 3). As expected, our findings indicated that yields from $MHVR^{hi}$ cultures were markedly lower than those from $MHVR^{lo}$ cultures (Figure 3A). These differences were partly due to rapid induction of syncytia in the $MHVR^{hi}$ cultures—this occurred at around 7 hours postinfection and destroyed the capacity to support virus secretion. However, syncytia were not solely responsible for the low yields. Even when $MHVR^{hi}$ cultures were seeded at densities low enough to prevent any intercellular contact, yields at 9 to 15 hours postinfection remained relatively low (Figure 3B). This last finding suggests that the $MHVR^{hi}$ cells—but not the $MHVR^{lo}$ cells—were susceptible to a virus-induced "single cell death" that was not dependent on intercellular fusion.

3.4. A "Single Cell Death" Occurs following Infection of HeLa-$MHVR^{hi}$ Cells

To measure single cell death following virus infection, we seeded cultures at subconfluent densities, infected them with MHV A59, then blocked any possibility of intercellular fusion by adding fusion-inhibiting polyclonal antiserum MK15 (a gift of Dr. Susan Baker). At 14 hours postinfection, viable and non-viable cells were discriminated by trypan blue exclusion.

In $MHVR^{hi}$ cells, we found the proportion of infected cells matched the proportion of non-viable cells (Figure 4). This concordance remained even as the input multiplicity of infection was varied. In the $MHVR^{int}$ and $MHVR^{lo}$ cultures, the proportion of infected

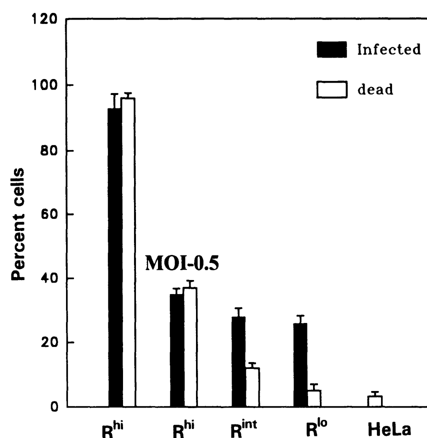

Figure 4. Rapid virus-induced cell death in MHVR^{hi} (but not MHVR^{lo}) cells. Infection of subconfluent cells with MHV A59 (moi = 100 pfu/cell) was followed 3 h later by addition of hyperimmune anti-A59 rabbit serum #MK15, to 1% final concentration. At 14 hours postinfection, cells were suspended using trypsin, and trypan blue was used to monitor cell lysis (white bars). Infected cells (black bars) were scored by indirect immunofluorescence, as described in the legend to Figure 2.

cells significantly exceeded the killed proportion, indicating survival of individual infected cells.

3.5. Spike Proteins Synthesized from Vaccinia Vectors Induce "Single Cell Death" in HeLa-MHVR^{hi} Cells

Mutations in the S gene modulate the pathogenesis of MHV. Additionally, S expression induces extreme cytopathology in tissue culture (Daya *et al.*, 1989). To find out whether S-mediated cytopathology is solely due to its known syncytium-inducing properties, we produced MHV A59 S proteins from vaccinia vectors in subconfluent MHVR^{lo} and MHVR^{hi} cells and monitored the resulting cell death as described above. At 12 hours postinfection, cell death in MHVR^{hi} cells was 25%; in contrast less than 4% death occurred in the MHVR^{lo} cells. We currently speculate that "single cell death" in MHVR^{hi} cells is due to accumulation of MHVR:S complexes within the lumenal cavities of intracellular organelles. Studies addressing this hypothesis are in progress.

4. DISCUSSION

Our results indicate that sensitivity to MHV A59-induced cytopathic effect increases with cellular MHVR levels. This was readily evident by microscopic examination of infected HeLa-MHVR cells. Inoculation of confluent MHVR^{hi} cultures induced complete (100%) syncytia by 9 hours postinfection while the corresponding MHVR^{lo} cells were entirely devoid of syncytia. Given that about 25% of the MHVR^{lo} cells were infected by A59, our results indicate that the MHVR threshold necessary for virion:cell fusion (virus entry) is lower than that required for cell:cell fusion (syncytia development). It is possible that virions fuse with MHVR^{lo} cells due to their closely-spaced spikes—infected cells by contrast may not display spikes at sufficient densities for fusion to occur. This hypothesis implies that a critical density of MHVR must be reached on the cell surface to congregate spikes into proximity close enough to form a "fusion pore". This general view of a fusion pore involving multiple, closely-spaced fusion proteins has support from careful biochemical studies of orthomyxo and retrovirus glycoproteins (White, 1994, Frey *et al.*, 1995).

In this study we observed a second type of cytopathic effect that increased with MHVR levels and was independent of syncytia development. Infected $MHVR^{hi}$ cells died within 14 hours postinfection (as judged by trypan blue exclusion) even when maintained as subconfluent monolayers. $MHVR^{lo}$ cells infected in parallel survived. We speculate that the "single cell death" occurring in $MHVR^{hi}$ cells arises from the formation of MHVR:S complexes within the cell. We have indeed isolated intracellular MHVR:S complexes by co-immunoprecipitation (data not shown) and we suggest that these proteins have properties similar to CD4:gp160 complexes (Cao *et al.*, 1996)—namely that they destabilize intracellular membranous organelles. In this regard, the MHV matrix protein, which is normally localized to the Golgi appratus (Klumperman *et al.*, 1994), is disseminated throughout the cytoplasm in individual A59-infected $MHVR^{hi}$ cells (data not shown).

Infected Hela-$MHVR^{lo}$ cultures immediately supported persistent infection. No acute phase of infection was observed. Infected HeLa-$MHVR^{hi}$ cultures also supported persistent infection, but only after an acute phase that destroyed all but the few cells producing low levels of MHVR, i.e., cells repopulating the culture after infection were $MHVR^{lo}$. Thus the maintanence of persistent infection depends on relatively low MHVR levels, as indicated previously by our colleagues (Chen and Baric, 1996; Sawicki *et al.*, 1995).

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