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Cell Receptor-Independent Infection by a Neurotropic Murine Coronavirus

THOMAS M. GALLAGHER,*,[1] MICHAEL J. BUCHMEIER,* AND STANLEY PERLMAN†,[2]

*Division of Virology, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037; and †Departments of Pediatrics and Microbiology, University of Iowa, Iowa City, Iowa 52242

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The cellular receptors for a coronavirus, mouse hepatitis virus (MHV), have been recently identified as one or more members of the carcinoembryonic antigen (CEA) family. The neurotropic JHM strain of MHV (MHV-JHM) possesses a highly fusogenic surface (S) glycoprotein. This protein is now shown to promote the spread of MHV into cells lacking the specific CEA-related MHV receptor. Resistant cells are recruited into MHV-induced syncytium with consequent production of progeny virus. Cell-to-cell spread of virus via membrane fusion without the requirement for specific cell surface receptor offers a novel way for virus to spread within infected hosts. © 1992 Academic Press, Inc.

Attachment of virus to a host cell is the first step in any viral infection. This process has been shown for many viruses to be mediated by specific cell receptor molecules, although in some cases, receptors for a given virus vary on different cells (*3, 4*). The cellular receptors for several viruses, including poliovirus, rhinovirus, echovirus 1, Epstein–Barr virus, human immunodeficiency virus (HIV), reovirus, rabies virus, and three coronaviruses, have been identified and partially characterized (*1–7*). In specific, one or more members of the carcinoembryonic antigen (CEA) family serve as cellular receptors for the A59 and JHM strains of mouse hepatitis virus (MHV) (MHV-A59 and MHV-JHM) and expression of CEA on the surface of some resistant cells renders them susceptible to MHV ((*2*), T.G., S.P., unpublished observations).

The viral protein involved in binding to the host cell has been identified for many viruses and in some cases this attachment protein or a second viral protein has been shown to mediate a subsequent plasma membrane fusion event (*2, 8*). This latter function leads to syncytium formation and rapid viral spread in tissue culture cells and, most likely, in animals as well. In the case of MHV, the spike (S) glycoprotein has dual functions. S both binds to cellular receptor and induces cell-to-cell fusion (*9*). Fusion may originate from either external virus (fusion from without) or infected cells (fusion from within) and is believed to require the presence of receptor on uninfected cells.

MHV-JHM, like some other coronaviruses, encodes a highly fusogenic S glycoprotein. Fusion is evident following infection with MHV or with either recombinant vaccinia virus (VV) or recombinant baculovirus expressing the coronavirus S protein (*10, 11*). Furthermore, the recombinant constructs cause fusion of cells lacking the MHV cellular receptor. These experiments indicate that only the S protein is required for cell fusion.

The ability of a virus to infect a cell not encoding its cellular receptor would greatly increase its potential host and tissue range. In this report, we show that MHV-JHM is capable of spreading to cells resistant to infection by virions. Spread only occurs when the resistant cells are exposed to cell-associated MHV and is a consequence of the strong receptor-independent fusion activity of the S protein.

Unlike cells of murine origin, hamster and human fibroblasts are resistant to infection with MHV and in some cases, resistance occurs at the level of the virus receptor (*2*). To demonstrate that the JHM strain was similarly capable of infecting only murine cells, DBT (mouse astrocytoma) (*12*), BHK (baby hamster kidney), and RK13 (rabbit kidney clone 13) cells were infected at a multiplicity of 0.2 plaque-forming units/cell (PFU/cell). Virus infection was measured qualitatively by an indirect immunofluorescence assay (IFA). At 22 hr postinoculation (p.i.), only the DBT cell monolayers expressed detectable levels of virus antigen (data not shown) confirming the species specificity of MHV-JHM. The JHM strain used in these experiments encoded a full-length (4131 nucleotides) S protein (*13*).

In sharp contrast, both BHK and RK13 cells were susceptible to infection with cell-associated MHV-JHM (Fig. 1). In these experiments, DBT cells in suspension

[1] Present Address: Department of Microbiology and Immunology, Loyola University Medical Center, Maywood, IL 60153.

[2] To whom correspondence and reprint requests should be addressed at Department of Pediatrics, University of Iowa, Iowa City, IA 52242. Fax: 319-356-4855.

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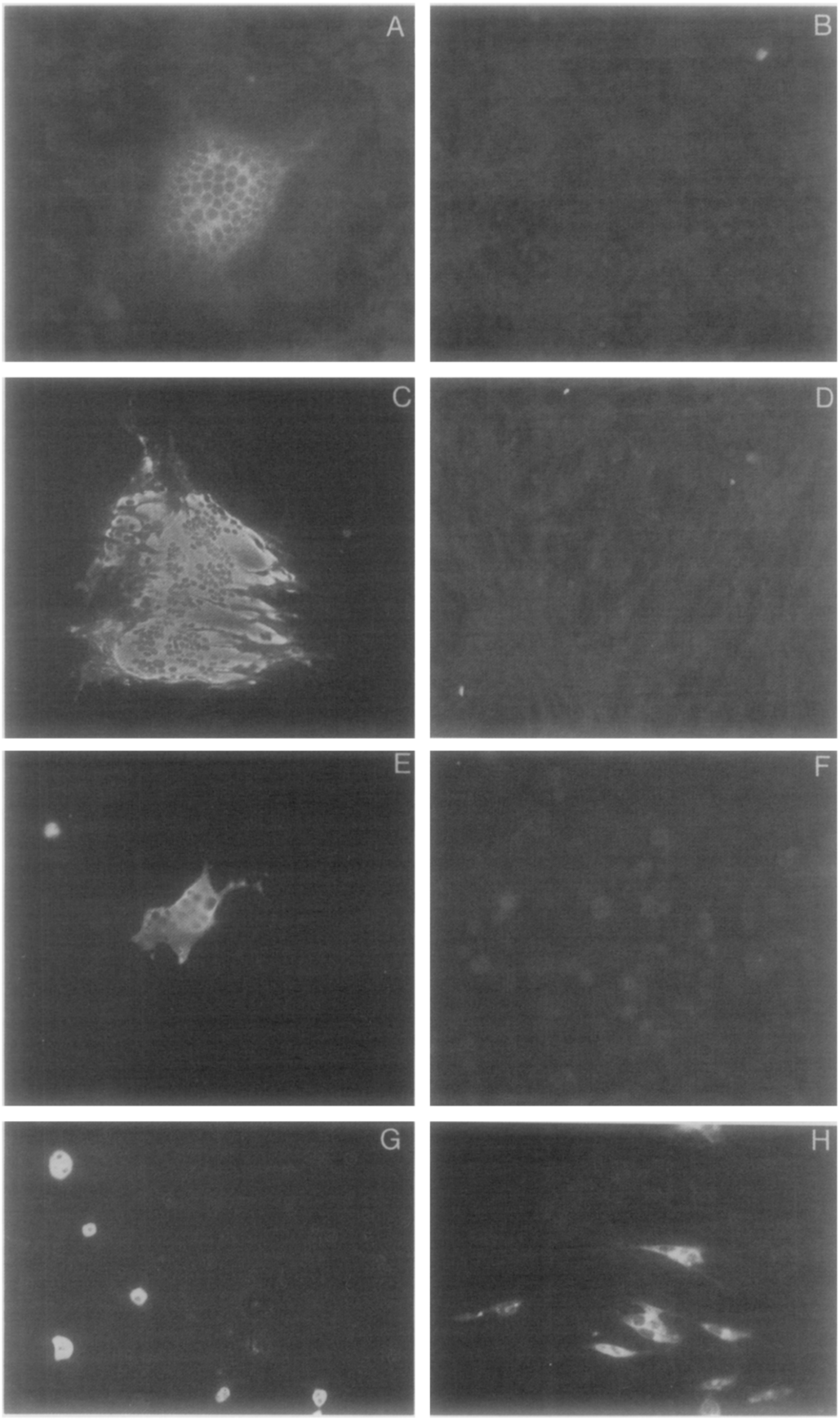
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were infected with MHV-JHM (0.5 PFU/cell for 1 hr), washed extensively, and seeded onto empty plates or onto lawns of DBT, BHK, or RK13 cells. An immunofluorescence assay performed 22 hr later revealed that, in the absence of an underlying cell sheet, these infected cells remained mononuclear, unable to recruit the widely dispersed uninfected population into syncytia (Fig. 1G). Only 2–10% of the suspended DBT cells were infected, rather than 40–50% expected at an m.o.i. of 0.5. This discrepancy may reflect either inefficient adsorption in suspension (S. Stohlman, personal communication) or loss of receptor after trypsinization since the cells were removed from tissue culture plates immediately prior to infection.

When similar numbers of infected DBT cells were seeded onto a lawn of uninfected DBT cells, large syncytia were apparent by 11 hr p.i. (Fig. 1A), with involvement of the entire cell layer by 22 hr p.i. (data not shown). Comparing the number of syncytia at 11 hr with the number of infected cells observed in the sample without an underlying lawn indicated that nearly every infected cell was able to form a syncytium. These results showed that virus transmission was efficient by this route and suggested that virus infected the underlying monolayer via cell-to-cell spread and not via free virus. When BHK or RK13 cells were similarly overlaid with infected DBT cells, small syncytia were apparent by 11 hr p.i. (data not shown) and were more substantial by 22 hr p.i. (Figs. 1C and 1E). BHK cells were more susceptible to syncytium formation than were RK13 cells as indicated both by the larger size of the individual syncytium and by the higher ratio of syncytia to single cells. The average syncytium on BHK cells contained approximately 20–30 nuclei, whereas the RK13 syncytia averaged two nuclei. The latter number reflected the relatively high number of infected cells that remained unfused at 22 hr p.i.

Previous results have shown that BHK cells transfected with the MHV cellular receptor will support virion production, indicating that the cells were fully permissive for all other functions required for viral replication ((*2*) T.G., S.P., unpublished observations). To determine if BHK or RK13 cells recruited into syncytia would similarly support MHV-JHM production, a series of infected cultures were assayed for the presence of infectious progeny virus (Table 1). On average, BHK and RK13 cell monolayers overlaid with infected DBT cells produced 250 and 21 times more virus compared to that of the sample lacking a monolayer, in agreement with the IFA results. When DBT cells were titered in this assay, an increase of approximately 7800-fold was observed, consistent with involvement of the entire monolayer at 22 hr p.i.

To demonstrate by another method that the underlying BHK cells were recruited into MHV-induced syncytia, BHK cells were transiently transfected with a plasmid (pCMV-β) encoding *Escherichia coli* β galactosidase. One day later, MHV-infected DBT cells were seeded onto the transfected lawn and syncytia allowed to develop for 22 hr. Cells were then fixed and assayed both histochemically for β galactosidase and by IFA for viral antigen. As shown in Fig. 2, some syncytia were uniformly positive for β galactosidase activity (B) and viral antigen (A), confirming the recruitment of the BHK cells.

Some strains of MHV encode a second surface glycoprotein, HE, which could potentially serve as a viral attachment protein (*14, 15*). The MHV-JHM which we used synthesizes little or no HE protein ((*14*) T.G., unpublished observation), making it unlikely that this protein was responsible for our results. However, two approaches were taken to prove directly that receptor-independent spread was S-mediated. First, syncytium formation was inhibited in DBT, BHK, and RK13 cells if either of two different anti-S monoclonal antibodies (5B19.2 and 5A13.5 (*9*)) was added to the cell supernatant (data not shown). Second, others have shown that the S protein was capable of initiating syncytium formation in the absence of expression of other MHV proteins (*10, 11*). This was confirmed by dually infecting DBT cells with recombinant VV expressing T7 RNA polymerase (vTF7.3) (*16*) and a second VV encoding the MHV-JHM S glycoprotein (vTF7.3/S) (*17, 18*). These cells were extensively washed and seeded onto uninfected lawns of BHK and RK13 cells. As before, the number of infected cells observed in a sample without underlying cells was very similar to the number of syncytia noted on the BHK and RK13 lawns, suggesting spread from the DBT-infected cells. Syncytia were larger and formed earlier when the seeded cells were infected with vTF7.3/S than when these cells were infected with MHV. These results probably reflected a

FIG. 1. Demonstration of spread of MHV infection to resistant BHK and RK13 cells. DBT cells were suspended to 10^6 cells/ml by treatment with trypsin and EDTA and were mock-infected or infected with MHV-JHM (0.5 PFU/cell) or MHV-A59 (4 PFU/cell). After 1 hr at 37°C, cells were washed extensively to remove unbound virus. Aliquots (1.0×10^6 cell) of the JHM-infected (A, C, E, G), mock-infected (B, D, F), and A59-infected (H) cells were then seeded onto 10-cm^2 wells containing confluent lawns of either DBT (A, B), BHK (C, D, H), or RK13 (E, F) cells or onto empty wells (G). After 11 hr (A, B) or 22 hr (C–H), cells were rinsed with PBS, fixed with 10% formalin, permeabilized with 2% NP40, and incubated with a mixture of murine monoclonal antibodies specific for the nucleocapsid (N), matrix (M), and spike (S) MHV proteins. Antiviral antibody was detected with fluoroscein-conjugated goat anti-mouse antibody (Fab fragment).

TABLE 1

MHV-JHM TITERS[a] IN BHK, RK13 AND DBT CELLS

Exp. no.	DBT	BHK	RK13	None
1	NT	4.76	NT	2.59
2	6.11	5.10	NT	2.62
3	6.23	4.41	4.18	1.85
4	NT	4.25	3.54	2.60
5	6.74	5.82	3.66	2.69
Mean	6.36	4.87	3.79	2.47

Note. Plates (10 cm^2) containing no cells ("none") as well as those containing confluent DBT, BHK, and RK13 cells monolayers were overlaid with MHV-JHM-infected DBT cells as described in Fig. 1. After 22 hr, cells were disrupted by repeated cycles of freezing and thawing. Cellular debris was removed by centrifugation and virus titered on DBT indicator cells. In each experiment, two to six individual wells were harvested and each sample was titered in duplicate. NT, not tested.

[a] Geometric mean titers per milliliter are shown.

higher surface density of the S protein, since the S protein is greatly overproduced in this VV system (*18, 19*) (Figure 3).

To determine whether there was a correlation between the neurovirulence of MHV-JHM and its fusogenic properties, the ability of MHV-JHM to cause fusion was compared to that of the minimally pathogenic MHV-A59 (*20*). The S proteins of these two viruses share greater than 90% homology at the nucleic acid and protein level, with the major difference being a 156-nucleotide deletion in the MHV-A59 sequence (*13, 21*). To determine whether these differences affected the ability of the virus to cause syncytium formation, MHV-A59-infected DBT cells were seeded onto a lawn of BHK cells. As shown in Fig. 1H, few of these cells were incorporated into syncytia and these syncytia were in general much smaller than what was observed after infection with the pathogenic MHV-JHM strain.

Together these results indicate that the extent of receptor-independent virus spread is determined by both virus strain and host cell type. Thus, neurovirulent MHV-JHM disseminates more extensively in this assay than the less virulent A59 strain. This variability in syncytium formation most likely reflects structural differences between the two S proteins, but other factors, such as transport to the cell surface or efficiency of cleavage of S (*22*) could also affect this process. The increased fusion activity may be an important factor in the greater virulence of MHV-JHM in animals, although the two viruses are sufficiently different so that other viral genes could also have a role in this process.

Infection spreads more rapidly in BHK cells relative to RK13 cells. This may reflect differences in the composition of the host cell plasma membrane. Indeed, MHV-induced fusion is known to be affected by lipid content (*23, 24*). Another cell line, OBL-21A, a retrovirus-transformed cell of neural derivation, is susceptible to infection with MHV-JHM but essentially refractory to MHV-induced fusion (*17*).

Cell type may be important in another way as well, since MHV binds to some cells, but is unable to initiate infection efficiently (*25, 26*). Virus replication proceeds normally in the small number of productively infected cells, suggesting that the block to infection with MHV occurs at an early stage of viral replication such as internalization. Whether cell-associated virus would abrogate such a block is not known at present but is

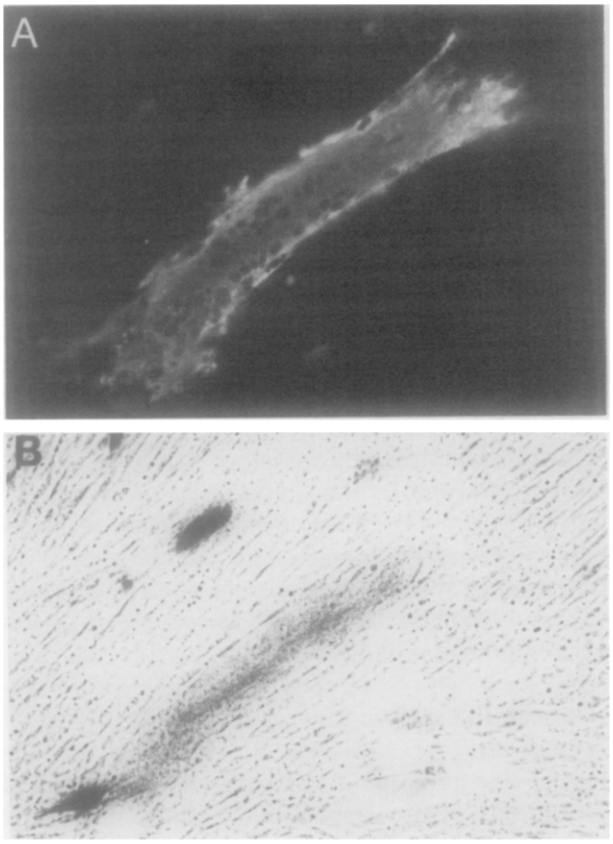

FIG. 2. Recruitment of BHK cells into MHV-induced syncytia. BHK cells (50–60% confluent) in 10-cm^2 wells were transfected with CMV-β (β galactosidase under the control of the human CMV major immediate-early promoter and enhancer (Clontech Laboratories, Inc.)) using lipofectin (Bethesda Research Laboratories) according to the manufacturer's specifications. After 24 hr, these cells were overlaid with MHV-JHM-infected DBT cells as described in Fig. 1. After an additional 22 hr, cells were fixed as above with 10% formalin, washed, and treated with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (*33*). After 6–8 hr, cells were assayed for viral antigen as described in Fig. 1. Cells dually stained for β galactosidase and viral antigen were detected by sequential light and fluorescent microscopy.

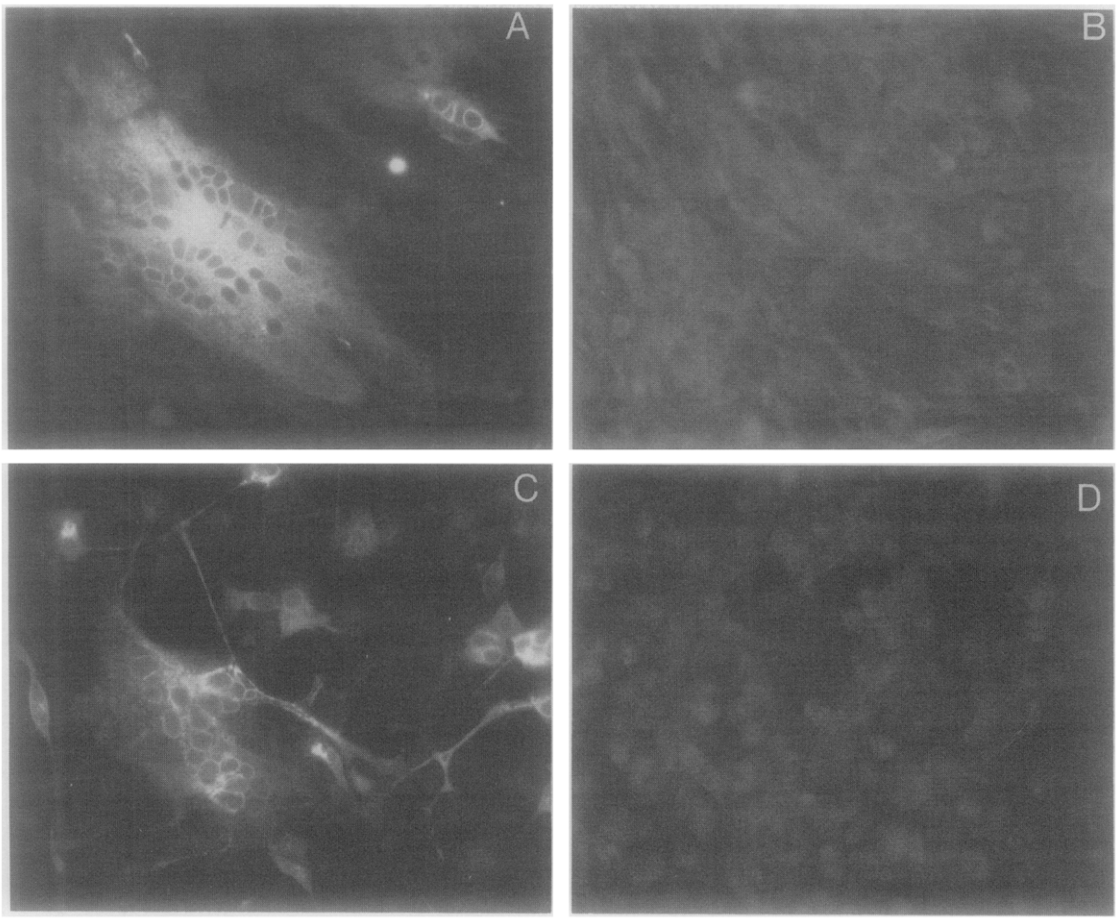

FIG. 3. Demonstration of syncytium-forming capacity of MHV-JHM S glycoprotein. The full-length S sequence (4131 nucleotides) (*17*) was subcloned into the VV shuttle vector pTM1 (*34*) and then recombined into VV as previously described (*18*). DBT cells were trypsinized and dually infected in suspension with this virus and a VV recombinant expressing T7 RNA polymerase (vTF7.3) (*16*). Infection multiplicity for each virus was 4 PFU/cell. Dually infected (A, C) and mock-treated (B, D) cells were seeded onto coverslips containing confluent lawns of either BHK (A, B) or RK13 (C, D) cells. Cells were fixed at 11 hr p.i. and processed as in Fig. 1.

important to determine since the result would affect the ultimate host range of the virus.

These results show that virus can spread to cultured cells lacking specific viral receptors and suggest that virus may also spread within infected animals by a receptor-independent mechanism. One case where this might be prominent is in MHV infections of relatively resistant hosts such as rats and monkeys. Both can be infected with MHV if large amounts are used and if virus is administered in the form of cellular lysate or brain tissue homogenate (*27–29*).

Receptor-independent spread would require the presence of a surface protein with fusion activity, such as is present in paramyxoviridae, some herpesviridae, and in lentivirinae. Recently, it has been shown that cells expressing no HIV proteins but the fusogenic gp41 transmembrane protein could initiate syncytium formation in cells normally resistant to HIV, consistent with this model (*30*). Although this mechanism does not depend on the presence of a high-affinity cellular receptor, it may still require cell–cell contact via adhesion molecules or binding to a specific, ubiquitous cell surface protein or sugar epitope.

This mechanism may have a role in human and animal diseases, such as subacute sclerosing panencephalitis (SSPE), in which virus spreads by cell-to-cell contact in the presence of a strong host immunological response. Many SSPE variants have alterations or deletions in the cytoplasmic domain of the fusion (F) protein (*31*). This part of the F protein is believed to interact with the matrix (M) protein and its loss may facilitate lateral movement of the protein, with increased fusogenicity. Similarly, some variants of HIV-2 with increased ability to fuse uninfected tissue culture cells have env proteins deleted in their cytoplasmic domains (*32*). The role of these changes which increase fusogenicity in facilitating receptor-independent spread is not known at present, although by analogy with our

results, such changes would make it more likely that this mechanism would occur.

The assay described in this report will be useful in studies of viral entry, since it will now be possible to separate the receptor binding event from fusion. Variant S proteins expressed in native MHV or in VV recombinants are both amenable to study by this technique and it should be possible to determine the effects on fusogenicity of individual mutations in the S protein.

In summary, MHV-JHM has the potential to infect nominally resistant cells by a receptor-independent mechanism. The role of such spread in infected animals is not proven at present, but receptor-independent spread potentially provides an additional way for virus to increase its host and tissue range. In addition, the possible contribution of receptor-independent spread must be considered in studies of the trafficking of MHV in infected animals.

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