Coronavirus Subversion of Host Cell Membranes

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BRIEF. This study focused on the novel use of dextrans to assess the capacity for a model Coronavirus to induce macropinocytosis through the course of infection.

ABSTRACT. Coronaviruses present a threat to societies' health, as demonstrated by the devastating 2003 SARS outbreak. Studies show that a model Coronavirus, murine hepatitis virus (MHV), modifies host cell membranes throughout the course of infection. The observed membrane changes are similar to macropinocytosis, an endocytic mechanism utilized only by a few viruses; however, the route of Coronavirus entry is unclear. The ability of Coronaviruses to induce macropinocytosis was studied using dextrans, which serve as markers of macropinocytosis. Results suggest that MHV likely induces macropinocytosis to infect cells, and define the time-course over which the process occurs. Studies focused on viruses are important because understanding the determinants of these membrane changes may define novel infection pathways utilized by Coronaviruses. By extension, this research may also result in better prevention methods against a future Severe Acute Respiratory Syndrome (SARS) outbreak and similarly potent viruses.

INTRODUCTION

Viruses are frequent causes of infection. Because of the broad scope and varying severity of viral infections, it is crucial to understand mechanisms by which viruses function. In this study, we describe a novel process that Coronaviruses (CoVs), utilize to augment infection. CoVs are not only the cause of a large percentage of the common cold, but also the causative agent of the SARS outbreak in 2003. Increasingly, studies have shown that CoVs induce dramatic host cell membrane modifications as a way of amplifying infection [1]. These global membrane alterations aid not only in virus entry, but also in replication and exit (Figure 1).

However, the mechanisms by which CoVs subvert viral membranes to support replication are not well understood. Macropinocytosis is a process that is gaining recognition in microbial pathogenesis. Macropinocytosis is a process by which a cell samples its environment by absorbing bulk extracellular material and internalizing large vesicles known as macropinosomes [2,3] (Figure S1). This phenomenon can be examined using dextrans, complex carbohydrates that have been used as indicators of macropinocytosis and bulk fluid uptake [2,3]. Studies suggest that macropinocytosis directly or indirectly contributes to virus entry, replication, immune evasion, and escape through usurping of host cell signaling pathways [3]. Studies focused on viral pathogenesis are important because continuous induction of macropinocytosis has not been described in any microbial infections, and only a few (i.e. HIV and Adenovirus) use this process to initiate infection [3,4]. Thus understanding the determinants, pathways, and relationships to other membrane modifications may define novel replication pathways utilized by CoVs, or infectious agents in general. Here, this project describes the novel use of fluorescent dextrans markers to examine and characterize the possibility of CoV-induced macropinocytosis. Data derived from this study will lead to a greater understanding of the biological mechanisms that CoVs and similar viruses use to modify and traffic host cell membranes and subvert host cell signaling, and provide greater insight into how to prevent future SARS outbreaks.

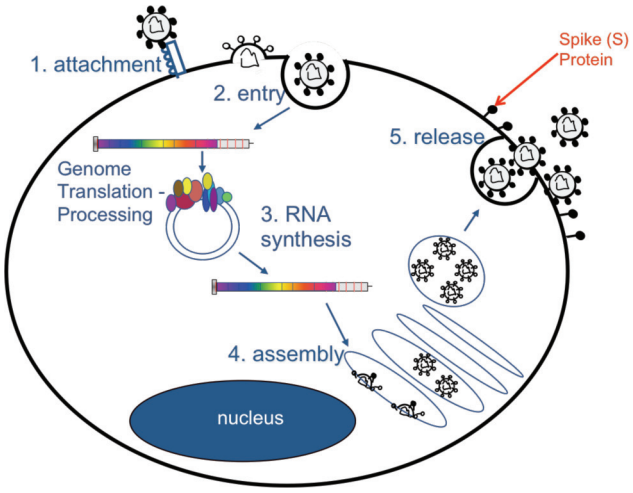

Figure 1. Attachment (1) is mediated by the spike (S) protein (red). Entry (2) can occur by more than one mechanism, and is often pH-dependent. RNA synthesis (3) occurs on virus-induced double-membrane vesicles. Virions are assembled (4) in the Golgi and released (5) at the host cell surface, which allows syncytia (multinucleated cells) to occur.

MATERIALS AND METHODS.

Optimization of Dextran Uptake.

Dextrans have been used in prior virus related macropinocytosis studies, but have not been used in Coronavirus or murine hepatitis virus (MHV)-infected cells [3,4]. Therefore, the first step of this study was to optimize dextran conditions. Soluble 70,000 molecular weight (MW) dextrans, fluorescently labeled with Texas-Red (TR), were used to assess putative macropinocytosis capabilities in MHV-infected cells by monitoring bulk fluid uptake. Murine astrocytoma delayed brain tumor cells (DBT-9) were seeded on glass coverslips at a concentration of 1.2 x 10^4 cells per coverslip and infected with MHV at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell when cells were 80% confluent. Eight hours post-infection (h p.i.), 0, 0.5, 1, 2.5, or 5 μg/ml of dextrans were added to cells for 30 minutes and washed twice with pre-warmed tissue culture media for 5 minutes each [5].

Pulse and Pulse Chase Labeling.

To determine the optimal time of dextran uptake in infected cells, an optimized concentration of 2.5 μg/ml dextrans was added (pulsed) to DBT-9 cells (seeded and infected in the same manner as stated above) for 0, 15, 30, 60, or 90 minutes 8 h p.i., followed by two five minute washes with prewarmed tissue culture media. To determine the ultimate fate of internalized dextrans, DBT-9 (seeded and infected in the same manner as stated above) were exposed to the optimized concentration of 2.5 μg/ml dextrans for 30 minutes (pulse) 8 h p.i., followed by an initial rinse with prewarmed tissue culture media, and then a final rinse (chase) with prewarmed tissue culture media for 15, 30, 60, or 90 minutes.

Role of Spike Protein in MHV-induced Macropinocytosis

To assess whether syncytia formation (mediated by the spike (S) protein) is necessary for CoV-induced macropinocytosis, DBT-9 cells (seeded in the same manner as above) were infected with murine hepatitis virus lacking the fusogenic spike protein (MHV-2S) at an MOI of 10 PFU/cell and exposed to the optimized concentration of 2.5 μg/ml of dextrans 8 h p.i.

Dextran Uptake in HeLa Cells.

To ensure that the putative macropinocytosis in MHV-infected DBT-9 cells was consistent with other cell types, HeLa cells, an established model of macropinocytosis, were used. HeLa cells were serum starved (2% fetal bovine serum (FBS)) for 16 hours and then exposed to 10 ng/ml of epidermal growth factor (EGF) to stimulate macropinocytosis [6,7]. Growth media was replaced with serum-free media, and 2 hours later, the optimized concentration of 2.5 μg/ml dextrans was added.

Imaging and Staining.

Cells were fixed in 100% methanol and frozen at -20°C. Cells used in MHV experiments were first washed with phosphate buffered saline (PBS) for 20 minutes. Cells were then blocked with a 5% bovine serum albumin (BSA) and PBS solution for five minutes. Cells were then prewashed with a PBS, 1% BSA, .05% nonyl phenoxypoly-ethoxylethanol (NP-40) solution for ten minutes. Cells were washed and incubated with the prewash solution plus 2% normal goat serum, and an anti-MHV nsp8-specific rabbit polyclonal antibody for 45 minutes, followed by a 45 minute incubation with the prewash solution, 2% normal goat serum, and a goat anti-rabbit antibody conjugated with AlexaFluor 488 (Molecular Probes). Both the primary and secondary antibodies were added at a dilution of 1:1000. Cells were then washed three times for ten minutes with the prewash solution, followed by one 30 minute wash with PBS. Finally, milli-Q (mq) H_2O was added to cells before mounting onto slides with aquapolymount. All cells were viewed by fluorescence microscopy under a Zeiss Axio 200 microscope.

Image Quantification.

Fluorescently labeled dextrans were used to visualize the bulk fluid uptake in cells. To quantify dextran uptake, ImageJ was used to calculate the number of internalized particles per area by converting the original image into a binary image, and counting the number of internalized particles (dextran internalization), excluding cell edges, surfaces, and processes and then dividing by the calculated area. The internal mean of fluorescence within cells, excluding the cell edges, surfaces, and processes, was also calculated using ImageJ. Thus dextran uptake was defined as the dextran internalization, and the internal mean of fluorescence.

RESULTS.

Optimization of Dextran Uptake.

To optimize dextran concentration, we exposed mock-infected and MHV-infected DBT9 cells to 0, 0.5, 1, 2.5, or 5 μg/ml of dextrans. MHV-infected cells showed significantly increased dextran uptake as assessed by image quantification (Figure 2). The optimal dextran concentration was 2.5 μg/ml, as it showed the most effective uptake in MHV and mock-infected cells with the lowest background (Figure 2). Mock-infected cells displayed a steady increase in both internalized particles and mean of fluorescence, but these values were significantly lower than those of MHV-infected cells (Figure 2).

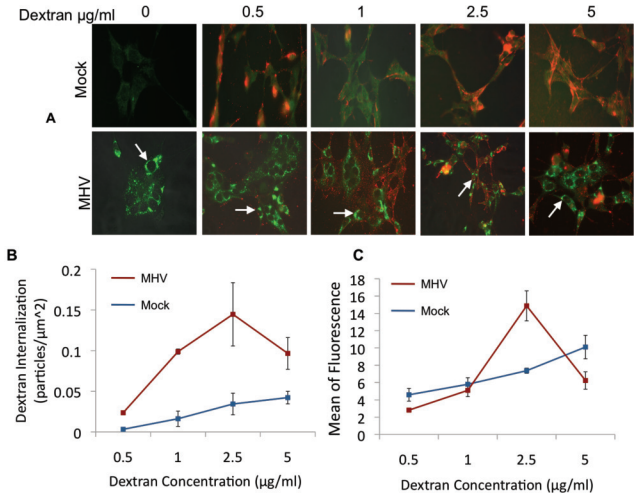

Figure 2. **(A)** Cells were mock infected or infected (white arrows) with MHV, stained for nsp8 (green). Dextrans (red) were added 8 h p.i. at various concentrations. **(B)** Dextran internalization in MHV and mock infected cells, p = 0.0052. **(C)** Average Mean of Fluorescence of MHV and mock infected cells, p = 0.0249.

Pulse Chase Studies.

To assess the optimal time of dextran uptake, DBT-9 cells were exposed (pulse) to 2.5 μg/ml dextrans for 0,15, 30, 60, or 90 minutes. As expected, MHV-infected cells showed significantly greater dextran uptake as compared to mock-infected cells, and exhibited the greatest uptake between 60 and 90 minutes (Figure 3). Mock-infected cells displayed variable dextran internalization and mean of fluorescence (Figure 3). Cells were also exposed to 2.5 μg/ml dextrans for 30 minutes, followed by rinses (chase) of 15, 30, 60, or 90 minutes. Infected cells showed substantial internalization of dextrans initially, but at 60 minutes, dextrans seemed to migrate to the cell edges and periphery, reflected by a decline in dextran internalization and mean of fluorescence for MHV-infected cells (Figure 3). Unexpectedly, dextrans were re-internalized at 90 minutes, as evidenced by an increase in both dextran internalization and mean of fluorescence (Figure 3). Mock-infected cells showed a steady decline in dextran internalization and an slight increase in mean of fluorescence as rinse lengths increased (Figure 3).

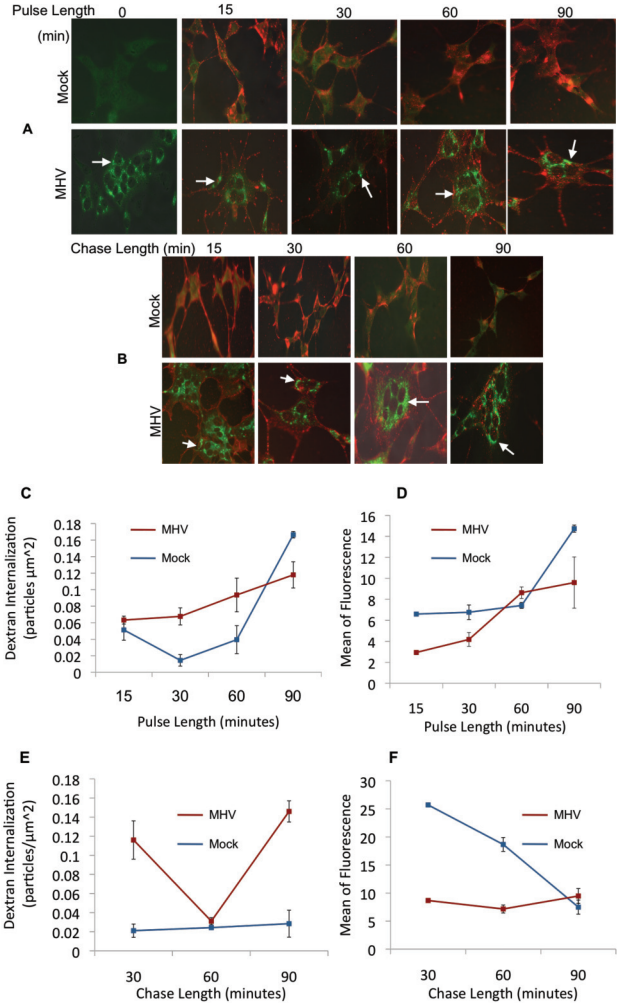

Figure 3. Cells were mock infected or infected (white arrows) with MHV, stained for nsp8 (green) for varying pulse **(A)** and chase **(B)** lengths. **C)** Dextran internalization of MHV and mock infected cells for pulse label, p = 0.0131. **(D)** Mean of Fluorescence of MHV and mock infected cells for pulse label, p = 0.0147. **(E)** Dextran internalization of MHV and mock infected cells for pulse-chase label, p = 0.0121. **(F)** Mean of Fluorescence of MHV and mock infected cells for pulse-chase label, p = 0.0444.

Role of Spike Protein in Dextran Internalization

To assess the role of the spike (S) protein in macropinocytosis, DBT-9 cells were infected with an MHV mutant expressing a non-fusogenic spike protein, known as MHV-2S. Dextrans were added at a concentration of 2.5μg/ml, and MHV-2S-infected cells showed a lower dextran internalization and mean of fluorescence as compared to wild type MHV, but higher dextran internalization and when compared to mock-infected DBT-9 cells (Figure S2).

HeLa Cell Internalization of Dextrans

HeLa cells, an established model of growth factor induced macropinocytosis, were serum starved and exposed to 10ng/ml epidermal growth factor (EGF), followed by the addition of 2.5 μg/ml dextrans [6,7]. Dextran internalization was slightly increased in the HeLa cells exposed to EGF, which was comparable to the dextran internalization of MHV-infected DBT9 cells, indicating similar bulk fluid uptakes (Figure S3). However, there was no significant difference in internalized particles per area or mean of fluorescence between HeLa cells in the presence and absence of EGF.

DISCUSSION.

The overall purpose of this study was to assess the ability of CoVs to induce macropinocytosis, and determine the methodology to do so with dextrans. Consequently, the first step of our study was to optimize the dextran conditions used in MHV infections. Results demonstrated a significant increase in dextran uptake in MHV-infected cells compared to mock-infected cells, which is evidenced by both numbers of internalized particles per area and mean of fluorescence (Figure 2). This indicates that a large increase in bulk fluid uptake occurs in MHV-infected cells, indicating that MHV may be inducing macropinocytosis as a mechanism of cell entry and infection. Because these experiments investigated the novel use of dextrans in MHV-infection, the data also show the optimal dextran concentration for these studies to be 2.5 μg/ml. This optimization is important for future work because dextrans have never been used before in Coronavirus studies, and can now be used as a control when assessing other characteristics of putative CoV induced macropinocytosis in relation to dextran uptake.

Several time-coursed experiments were used to determine the optimal uptake time and ultimate fate of internalized dextrans. Pulse labeling experiments showed that the greatest dextran uptake occurred between 30 and 60 minutes, highlighting the peak of the putative MHV-induced macropinocytosis process (Figure 3). Pulse-chase labeling showed fluctuations in dextran internalization, which indicates a cyclical internalization and recycling of macropinosomes. The data suggest that this pattern may occur every thirty minutes, as dextrans appear to move in and out of the cell during varying chase lengths (Figure 3). This is significant because in the context of viruses that utilize macropinocytosis as a mechanism of infection, very few use this strategy for purposes other than entry and exit. These findings suggest that MHV may use this in the replication process by continually using and reusing the host cell membranes.

When comparing MHV-infected dextran internalization to EGF-treated HeLa cells, we found both the dextran internalization and mean of fluorescence to be very similar, and there was no discernable or statistically significant difference between HeLa cells that had been and had not been treated with EGF (Figure S2). Despite this, there was a slight trend of increased dextran internalization in EGF-treated cells. These results, although not statistically significant, are an essential first step in refining the protocol for combining serum starvation, EGF treatment, and dextran uptake. Future work must focus on refining this protocol.

When assessing the role of the spike protein in MHV infection, the results indicate that a decrease in dextran internalization and mean of fluorescence occurs when compared to cells infected with MHV. This indicates that the spike protein does play a role in MHV-induced macropinocytosis, as there was greater dextran uptake and signal in syncytial cells versus non-syncytial cells (Figure S3). However, loss of this protein does not completely inhibit this process, making it difficult to determine the extent to which it is involved in MHV-induced macropinocytosis.

Although these experiments strongly indicate that MHV induces macropinocytosis, much more work is needed to fully ascertain whether this is the case. Because macropinocytosis is a dynamic process that is defined not only by bulk fluid uptake (as dextrans show), but also by global cellular actin polymerization, signaling pathways involved in the internalization and trafficking of macropinosomes, and dependence on the Na^+/H^+ pump, future studies must look at these factors with respect to CoVs [3-7]. These variables can be tested in a variety of fashions such as fluorescence resonance energy transfer (FRET) analysis, live cell imaging, staining, and the use of inhibitors at various steps involved in the macropinocytotic signaling pathway. Future studies should also focus on refining EGF treatment, and examining various virus constituencies involved in CoV-induced macropinocytosis, such as CoV proteins that localize at replication complexes.

From these experiments, we can conclude that MHV significantly increases bulk fluid uptake in infected cells, which likely reflects macropinocytosis. We also defined the optimal uptake time and fate of the putative macropinosomes. These results are important in the virology field because they indicate a novel shift in the standard dogma of CoV endocytotic entry. Furthermore, this study defines the methodology and quantification for the novel use of dextrans in MHV infection, and tracks the progress of putative macropinosomes in several time-dependent assays. Finally, these experiments will lead to a greater understanding of the biological mechanisms that CoVs and similar viruses use to modify and traffic host cell membranes to subvert host cell signaling.

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SUPPORTING INFORMATION.

Figure S1. Macropinocytosis

Figure S2. Role of Spike Protein, Figure S3 EGF Stimulation of HeLa Cells

REFERENCES.

1. D. M. Knipe, *Fields Virology* (Wilkins, New York, 5, 2007).
2. M. Ulasli, M. H. Verheije, C. M. Hann, F. Reggiori, *Cell. Microb*. **12**, 844 (2010).
3. J. Mercer, A. Helenius, *Nat. Cell. Bio.* **11**, 510 (2009).
4. A. T. Jones, *J. Mol. Med.* **11**, 670 (2007).
5. J. Meier, *et al., J. Cell Bio.* **158**, 1119 (2002).
6. J. L. Zaro, T. E. Rajapaksa, C. T. Okamoto, W. C. Shen, *Mol. Pharm.* **2**, 181 (2006).
7. J. P. Lim, J. T. H. Wang, M. C. Kerr, R. D. Teasdale, P. A. Gleeson, *BMC Cell Bio.* **9**, 58 (2008).

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