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Human metapneumovirus uses endocytosis pathway for host cell entry

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ABSTRACT

Human metapneumovirus (hMPV) is a prevalent pathogen worldwide and causes various respiratory infections. Although it is a critical pathogen in pediatric patients, it is unclear how it enters host cells. In this study, we focused on hMPV cell entry using two kinds of cell lines (Vero E6 and LLC-MK2), which are most commonly used for isolating and propagating for hMPV, and we used fluorescent dyes to label the virus particles and monitored how they enter the host cell in real time. We found that endocytosis was the predominant pathway by which hMPV entered host cells. When the virus particles were traced inside host cells, we found that a low intracellular pH was needed for intracellular fusion in LLC-MK2 cells.

1. Introduction

Since 2001, when human metapneumovirus (hMPV) was first identified in Holland [1], many research groups have found evidence of hMPV infection worldwide [2–5]. Indeed, hMPV is considered a major pathogen responsible for lower respiratory tract disease in infants and children [6,7]. It also causes acute wheezing in young children [8] and can exacerbate asthma [9]. The importance of hMPV is not related to its prevalence, but rather to the severity of resultant disease. However, how this virus infects cells is still entirely unclear.

There are two primary routes by which enveloped viruses can enter host cells: membrane fusion and endocytosis. The endocytosis can occur via four major kinds of pathways: clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, or clathrin/caveolae-independent endocytosis. Most enveloped viruses are believed to fuse their viral membranes and cells membranes to achieve effective infection [10]. Srinivasakumar et al. [11] showed that ~ 35% of Respiratory Syncytial Virus (RSV) viral particles had fused to the plasma membrane 1 h following infection. Although the fusion mechanism is recognised by many researchers, various endocytic pathways have now been identified. For example, one study showed that RSV enters HeLa cells via macropinocytosis [12], whereas other studies showed that it infects HeLa cells via clathrin-mediated endocytosis [13]. Moreover, another investigation showed that dendritic cells present RSV antigen by caveolae-dependent endocytosis [14]. It seems that RSV mediates different entry pathways at different infection times to enter the same host cells, or RSV via different ways to infect different cells. hMPV, like RSV, represents the subfamily *Pneumovirinae* within the family *Paramyxoviridae* and these viruses cause similar clinical symptoms. So, we speculated that hMPV could choose different avenues to enter different cells, depending on infection conditions, although until now, membrane fusion was regarded to be the major pathway of hMPV cell entry [15,16]. Recently, Cox et al. [17] found that hMPV enters human bronchial epithelial cells via clathrin-mediated endocytosis in a dynamin-dependent manner. This information suggests that hMPV might exploit an endocytosis pathway in different host cells.

Here, we selected Vero E6 and LLC-MK2 as target cells, not only because they are the most common used cells for propagating hMPV, but also owing to their higher efficiency of hMPV infection than other airway epithelial cells [18,19], and used a series of

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fluorescent dyes to label hMPV particles. We have followed the hMPV cell entry process with a confocal laser-scanning microscope. The results indicated that hMPV entered cells primarily via an endocytic pathway. Moreover, a low intracellular pH played a key role in hMPV entry.

2. Materials and methods

2.1. Cell lines

Vero E6 and LLC-MK2 cells were purchased from the China Center for Type Culture Collection and maintained in a humid 5% CO_2 incubator at 37 °C in Dulbecco's Modified Eagle Medium (DMEM), (Gibco, NY, USA) or Minimum Essential Medium (MEM), (Gibco, NY, USA), respectively, supplemented with 10% fetal bovine serum (FBS), (Gibco, NY, USA) and antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml); (Hyclone, UT, USA).

2.2. Virus stocks

hMPV was successfully recovered from full-length cDNA clones of hMPV NL/1/00 by reverse genetics as described previously [20]; hMPV was propagated in Vero E6 cells, and hMPV virions were purified by ultracentrifugation on a modified 35% glucose gradient. The viral supernatant was harvested and gently layered on to the surface of a 35% glucose-HBSS solution (Hyclone, UT, USA). Then, they were centrifuged at 51,500 × *g* for 16 h at 4 °C (Beckman Coulter, FL, USA). Finally, the supernatant was discarded and the virus particles were suspended in medium and maintained as stocks at −80 °C.

2.3. Dyes and antibodies

Octadecyl Rhodamine B Chloride (R18) was obtained from Biotium Inc, USA. DiOC was obtained from Beyotime Biotechnology, China. Cy3 and CypHer5 NHS ester were obtained from GE Healthcare, UK. The monoclonal mouse anti-hMPV fusion protein (F) and monoclonal mouse anti-hMPV nuclear protein (N) antibodies were purchased from Merck Millipore, Germany. The Alexa Fluor® 488/546 donkey anti-mouse IgG antibodies were purchased from Life Technologies, USA.

2.4. Indirect fluorescence assay

hMPV was inoculated on to the cells (MOI = 1). First, the cells were placed at 4 °C for 30 min to synchronize host cell entry. Unbound virus was washed away and the temperature raised to 37 °C to initialize the entry process. After incubation at 37 °C for different times, cells were fixed and permeated by exposure to 0.1% Triton X-100 (Sigma-Aldrich). Albumin bovine V (1%; BSA, Sigma-Aldrich) was used as a blocking agent. The cells were then incubated with anti-hMPV F and N antibodies, followed by fluorescently conjugated anti-IgG antibodies. F-actin was stained by incubating cells with phalloidin. Finally, cell nuclei were labeled by DAPI (Beyotime). Fluorescence was observed under a confocal microscope (Nikon, Tokyo, Japan).

2.5. R18 fusion assay

R18 (1 μl) was added into 1 ml of hMPV (pfu = 10^6/ml) to yield a final R18 concentration of 10 μM. The mixture was shaken gently for 1 h to allow R18 binding. Impurities were removed using a 0.22 μm filter, and unbound dye was removed using CaptoCore 700 (GE Healthcare). R18 alone was also added to medium as a negative control. Both R18-hMPV and R18-control were inoculated on to Vero E6 and LLC-MK2 cells (MOI = 1) for 30 min at 4 °C, and then replaced with fresh medium. Once the cells were placed under the confocal microscope, the temperature was raised to 37 °C, and the fluorescence change was recorded for 4 h. Variations in fluorescence intensity were also measured every 2 min in a microplate reader. The amount of R18 fluorescence dequenching was expressed as the DQ%, calculated using the formula: $DQ\%=(F_d-F_0)/F_t*100\%$, where F_0 is the fluorescence intensity at the beginning, F_d is the fluorescence intensity at each detection time, and F_t is the maximum fluorescence intensity when R18 was infinitely diluted with 1% Triton X-100 [21].

2.6. DiOC/R18 FRET

A mixture of DiOC/R18 was added to 1 ml of hMPV. The final concentration of DiOC was 3.3 μM and that of R18 was 6.7 μM. Gentle shaking was performed to facilitate virus staining and unincorporated dyes were removed in the same way as for the R18 fusion assay. The negative control was treated in the same way. FRET was observed under a confocal microscope. DiOC/R18 labeled hMPV was excited with a laser (488 nm), and fluorescence was detected simultaneously at 500 nm and 575 nm.

2.7. Cy3/CypHer5 dual labeling

Before labeling, hMPV was treated with 0.5 M Na_2CO_3 to increase the pH of the solution to 9.3. Cy3 and CypHer5 were then added to 1 ml hMPV; the final concentration of each was 10 μg/ml. The mixture was then shaken and filtered as described for previous experiments. Dual-labeled virus was then inoculated on to cells at 37 °C and excited with a laser at 550 nm. The fluorescence emitted by the two dyes was detected at 575 nm and 660 nm.

3. Results

3.1. Fusion protein (F) and nucleoprotein (N) co-localize during hMPV entry

Cells were infected with hMPV for different times and then stained with anti-hMPV F and anti-hMPV N antibodies to identify the viral membrane and the nuclear capsid, respectively. At the same time, F-actin was labeled with Alexa Fluor® 647 phalloidin to reveal the cytoskeleton. Since F and N are located on the virus membrane and capsid, respectively, staining by both F and N antibodies indicated intact particles within infected cells. Serial confocal Z-stack images revealed that fluorescent spots of F and N co-localized on the cell surface and in the cytoplasm at 2 h post-hMPV infection (Fig. 1A and B). Vero E6 cells without infection were used as a negative control (Fig. 1C). Krzyzaniak et al. [12] defined capsid-free envelopes (or virus-like particles; VLPs) as particles with F spots but without N spots. Here, we found that the number of VLPs (F only) (Fig. 1D) and intact viral particles (FN both) increased 2 h after infection. We believe that the N spots represent the total number of viral particles (N only), whereas the FN spot represents the number of endocytosed viral particles. Thus, 2 h after infection, both the total number of particles and the number of endocytosed particles increased (Fig. 1E, F). The ratio of FN to N spots in LLC-MK2 cells increased from 0.58 to 0.84 (Fig. 1G, right panel), and that in Vero E6 cells varied from 0.65 to 0.70 (Fig. 1G, left panel) during 2 h of incubation. A possible explanation for this finding is that more hMPV particles were endocytosed by LLC-MK2 during the 37 °C incubation period. In other words, whether incubation at 37 °C is optimal for endocytosis depends on the host cell type. However, in both groups, about two-thirds of hMPV particles entered via the endocytosis pathway.