AVIAN INFECTIOUS BRONCHITIS VIRUS ENTERS CELLS VIA THE ENDOCYTIC PATHWAY

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1. INTRODUCTION

Avian infectious bronchitis virus (IBV) is a group III coronavirus that has a major economic impact in the poultry industry. Infected layers often have a large drop in egg production due to impaired ovary and oviduct functions. Clinical manifestations of IBV-infected chickens include respiratory, renal, and reproductive diseases and are often coupled with secondary infections such as airsaculitis and oviduct salpingitis.[1] Although the etiology, pathogenesis, and diagnosis have been described since the 1930s, the molecular mechanism of viral entry remains elusive.

Viruses utilize various mechanisms to gain entry into their host cells. Enveloped viruses need to shed the viral envelope in order to release their genomes into target cells and initiate further replication.[2] The process of uncoating typically follows two distinct mechanisms: pH-dependent fusion in the endosome or pH-independent fusion at the cell surface.[3] Many enveloped viruses (e.g., influenza viruses) utilize various endocytic pathways to travel deep into the cell cytoplasm in order to smoothly bypass the cortical cytoskeleton near the cell periphery.[4] The endocytic pathway also provides a suitable acidic environment for the pH-dependent membrane fusion to deliver the viral genome into the cell.[5, 6] In contrast, paramyxoviruses (e.g., Sendai viruses) are well established to enter target cells via pH-independent fusion with the plasma membrane.[7] For coronaviruses, contrasting results have been reported regarding the role of endosomes and low pH activation, and consequently, there is no general consensus regarding the entry mechanism.[8-11]

A major characteristic of IBV infected cells is the formation of large syncytia, which has become a key piece of evidence supporting fusion with the cell plasma membrane at neutral pH during viral entry.[12] In this model, pH-neutral syncytia formation is thought to be a representative model of coronavirus fusion during entry. However, electron microscopy from Patterson *et al.* clearly shows IBV entering chorioallantoic membrane (CAM) and chick kidney cells via "viroplexis" through cellular engulfment into

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cytoplasmic vacuoles.[8] In addition, Li and Cavanagh demonstrated that IBV strain Beaudette (IBV/Bdtt) infection can be reduced by as much as 95% with the lysosomotropic agent ammonium chloride.[11] Recently, we established a tissue culture system using IBV/Bdtt viruses to infect baby hamster kidney (BHK-21) cells, using anti-S1 monoclonal antibody labeling to study coronavirus entry requirements. Our goal was to investigate the molecular mechanism of IBV/Bdtt entry. We found that IBV infection was sensitive to endocytosis inhibitors including monensin and chlorpromazine in a dose-dependent manner. We therefore conclude that despite syncytia formation during late times of infection, IBV strain Beaudette utilizes the endocytic pathway and low pH-induced fusion to gain entry into BHK-21 cells.

2. RESULTS AND DISCUSSION

2.1. Effect of Monensin and Chlorpromazine on IBV Strain Beaudette Infection

To examine the route of entry during IBV/Bdtt virus infection, we made use of a pH neutralizing agent monensin. As an ionophore, monensin neutralizes the pH gradient of endocytic vesicles by exchanging luminal protons (H^+) with potassium ions (K^+),[13] hence it inhibits low pH dependent virus-cell fusion. IBV strain Beaudette was propagated and cultured from the allantoic fluid of 10-day-old specific pathogen free (SPF) embryonated chicken eggs. Sendai virus, strain Cantell, (ATCC) was also propagated as a control virus. A standard IBV/Bdtt or Sendai/Cantell infection was achieved by inoculating a monolayer of BHK-21 cells (ATCC) with 5 MOI of each virus in 200 µl of RPMI1640 binding media (Cellgro) containing 0.2% bovine serum albumin (Cellgro), 10 mM HEPES (Cellgro) at pH 7.3 in a 24-well tissue culture treated plate. Virus was adsorbed in a 37°C and 5% CO_2-free incubator for 60 minutes with gentle rocking. Then viral inoculum was replaced with DMEM infection media (Cellgro) containing 2% fetal bovine serum (Cellgro), 1% penicillin and streptomycin (Cellgro), and 10 mM HEPES (Cellgro), and incubation was resumed in a 37°C and CO_2 incubator for additional 7 hours.

The BHK-21 cell monolayer was fixed with ice-cold methanol (Sigma) for 2 minutes at 8 hours postinoculation. IBV infection was identified using the anti-S1 monoclonal antibody 15:88.[14] A chicken polyclonal anti-Sendai virus antibody (USBiological) was used to identify Sendai virus infection. Secondary antibodies were Alexa 488-conjugated goat anti-mouse or anti-chicken (Molecular Probes). Cells were observed under a Nikon Eclipse E600 fluorescence microscope and images collected using a SPOT RT camera and SPOT 3.5 software.

For monensin treated samples, BHK-21 cell monolayers were pretreated with 15 µM monensin (CalBiochem) at 37°C for 10 minutes prior to virus inoculation. Fifteen µM monensin was added to both RPMI1640 binding media and DMEM infection media to prevent low pH dependent fusion activation during IBV/Bdtt infection. In contrast to Sendai virus infection, which was unaffected by the monensin treatment, Fig. 1A shows that IBV/Bdtt infection in BHK-21 cells was sensitive to monensin treatment at a concentration of 15 µM. The infection could be restored when monensin was added 2 hours post–viral inoculation (data not shown). This indicated that monensin had no indirect effects on viral replication and specifically affected virus entry.

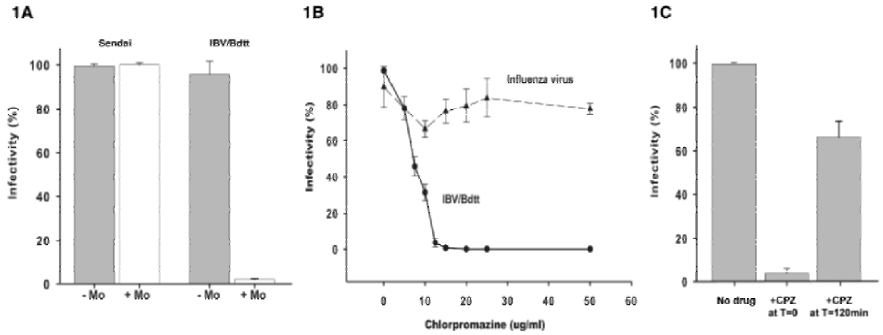

Figure 1. A. Effect of monensin (Mo) treatment during entry of Sendai virus and IBV/Bddt. B. Effect of chlorpromazine treatment during entry of influenza A/WSN/33 (dashed line) or IBV/Bdtt (solid line). C. Chlorpromazine (CPZ) delay treatment assay. Greater than 200 cells were counted in each sample. Each experimental condition was performed in triplicate. Statistical analysis was performed in SigmaPlot 9.0 software, and error bars represent the standard deviation of the mean.

To narrow down the specific route of IBV/Bdtt entry, we studied the effects of chlorpromazine during IBV entry. Chlorpromazine induces clathrin lattice assembly around endosomes while inhibiting adaptin-2 protein (AP-2) binding to the cellular membrane, hence preventing clathrin-coated pit formation and clathrin-mediated endocytosis.[15] Infection of BHK-21 cells by IBV/Bdtt or influenza virus, strain A/WSN/33, (ATCC) was performed essentially as described above. Chlorpromazine (Calbiochem) was diluted in RPMI1640 binding media to a concentration of 0–50 μg/ml, and drug treatment followed by virus infection was performed. Figure 1B shows that IBV/Bdtt infectivity is inhibited by chlorpromazine treatment in a dose-dependent manner. However, the control influenza virus remained unaffected by chlorpromazine treatment since influenza viruses can enter cells through both clathrin-dependent and-independent endocytic pathways.[16] To exclude any nonspecific effect of chlorpromazine on virus replication, chlorpromazine was added 2 hours post–virus inoculation to bypass its effect on IBV/Bdtt entry. IBV/Bdtt infection was restored to as much as 60% of the nontreated control suggesting that chlorpromazine specifically inhibited IBV/Bdtt entry through a clathrin-mediated endocytic pathway (Fig. 1C).

The use of drugs such as monensin and chlorpromazine to screen for IBV entry pathways served as an effective tool during our initial investigation. However, more specialized assays and screening methods targeting individual endocytic compartments or cellular pathway are required to draw a definitive conclusion regarding to the molecular mechanism during IBV entry to its target cell. Co-localization studies between specific cellular compartments (e.g., clathrin coated pits, caveolin, early, late, or recycling endosomes) and infective virions using a panel of antibody markers can help reveal more precise location at each stage of IBV entry. It has also been shown that cholesterol can function as a co-factor for fusion of mouse hepatitis virus (MHV)[17]; therefore, lipid rafts may also play a role in coronavirus entry. Finally, dominant negative proteins and small interfering RNAs may serve as effective tools to investigate the entry requirements for IBV, which will be addressed in future experiments.

3. ACKNOWLEDGMENTS

We would like to thank Dr. Benjamin Lucio-Martinez and Dr. Syed Naqi for their provision of viruses and antibodies and Xiangjie Sun for experimental support. This work was supported by NIH grant R03 AI060946.

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