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Murine Norovirus-1 entry into permissive macrophages and dendritic cells is pH-independent

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Summary

Murine norovirus (MNV) is a recently discovered mouse pathogen. Unlike the fastidious human noroviruses that cause the overwhelming majority of non-bacterial gastroenteritis worldwide, MNV readily infects cells in culture. Its replication in primary murine macrophages and dendritic cells and their derived cell lines allows the study of norovirus cell entry for the first time. In this study we determined the role of pH during MNV-1 infection since the low pH environment of endosomes often triggers uncoating of viruses. We demonstrated that MNV-1 viral titers by plaque assay and expression of the non-structural protein VPg by immunofluorescence were not affected by pH in cultured and primary macrophages and dendritic cells in the presence of two known endosome acidification inhibitors, bafilomycin A1 and chloroquine. These data indicate that MNV-1 enters permissive cells in a pH-independent manner.

Keywords

RNA virus; norovirus; pH-independent entry; macrophages; dendritic cells; enteric virus

Noroviruses are an understudied group of non-enveloped positive strand RNA viruses that belong to the *Caliciviridae* family (Green, 2007). Despite the significant impact of human noroviruses (HuNoV) on public health worldwide as the major agent of non-bacterial gastroenteritis (Green, 2007), no drug or vaccine exists to treat norovirus infections. This is partially due to the absence of a robust tissue culture system (Duizer et al., 2004; Straub et al., 2007). In contrast, murine norovirus (MNV), a highly prevalent agent in research mouse colonies (Hsu et al., 2005; Muller et al., 2007), readily infects murine macrophages and dendritic cells (DC) in culture and in vivo (Ward et al., 2006; Wobus et al., 2004; Wobus et al., 2006). Similar to HuNoV, MNV replicates in the gastrointestinal tract of its wild type or immunocompromised host, is shed in the feces, and is transmitted by the fecal-oral route (reviewed in: (Wobus et al., 2006)). The ability to culture a norovirus has already led to insights into norovirus biology (Chaudhry et al., 2006; Daughenbaugh et al., 2006; Simmonds et al., 2008; Sosnovtsev et al., 2006) and inactivation (for example: (Baert et al., 2008; Belliot et al., 2008)). However, no studies have yet addressed requirements for norovirus entry into cells.

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To gain access into host cells, viruses hijack cellular processes. The most commonly used endocytic pathway for virus entry is clathrin-mediated endocytosis. Viral entry can also occur via caveolin-mediated endocytosis, clathrin/caveolin-independent endocytosis, macropinocytosis, or phagocytosis (reviewed in: (Marsh and Helenius, 2006)). Clathrin-mediated endocytosis delivers viruses into the acidic environment of early endosomes while caveolin-mediated endocytosis can traffic virus into neutral caveosomes or acidic endosomes (Cantin et al., 2007; Liebl et al., 2006; Pelkmans et al., 2001). Feline calicivirus (FCV) is the only calicivirus whose entry has been studied to date. FCV (F9 strain) enters cells by clathrin-mediated endocytosis in a pH-dependent manner (Kreutz and Seal, 1995; Stuart and Brown, 2006). As a part of entry, viruses must deliver their viral genome into the host cytoplasm. This critical event during the virus life cycle, termed uncoating, is often triggered by the acidic environment of endosomes and/or by binding to cellular receptors (reviewed in: (Tsai, 2007)).

To begin to elucidate how a norovirus enters cells, we studied the role of pH during MNV-1 entry into permissive macrophages and DCs. MNV is routinely propagated in RAW 264.7 cells, a murine macrophage cell line. Therefore, we first focused on the role of pH during MNV-1 infection in cultured and primary murine macrophages. Primary bone marrow-derived macrophages (BMM ϕ) were prepared from seronegative male Swiss Webster mice (Charles River) as previously described (Wobus et al., 2004). RAW 264.7 cells and BMM ϕ were pretreated for thirty minutes with chloroquine (200 μ M or 100 μ M), a lysosomotropic agent that raises intracellular pH, or bafilomycin A1 (250 μ M), a specific inhibitor of vacuolar ATPases. For all experiments, concentrations were chosen after performing dose-response studies that maintained at least 80% cell viability compared to untreated control cells while at the same time showing a significant effect on Vesicular stomatitis virus (VSV), our positive control for a pH-dependent virus (Superti et al., 1987). Cells were infected with MNV-1 or VSV in the presence or absence of these inhibitors at a multiplicity of infection (MOI) of 5 for one hour on ice and then washed three times in phosphate buffered saline (PBS). To maintain cell viability, media containing inhibitor was added for four hours and then replaced with fresh media without inhibitor. At 0, 8, 10 and 12 hours post infection (hpi), cells and media were frozen together at -80°C . After two freeze/thaw cycles, MNV-1 and VSV viral titers were determined by plaque assay on RAW 264.7 cells as previously described (Wobus et al., 2004) (Fig. 1 and data not shown). Experiments repeated with each virus at an MOI of 0.5 and 0.05 showed similar results (data not shown). For all experiments, cellular respiration, specifically mitochondrial dehydrogenase activity, as a measure of cell viability was monitored by WST-1 reagent (Roche) following the manufacturer's recommendations. Viability throughout the experiment remained above 80% for all conditions (Fig. 1E, F). VSV viral titers were significantly reduced 8, 10, 12 hpi in both bafilomycin A1- and chloroquine-treated murine macrophages as expected for a pH-dependent entry of VSV (Fig. 1A-D, data not shown). However, no changes in MNV-1 titers between untreated and treated cells were seen at these timepoints (Fig. 1A-D, data not shown). This indicated that MNV-1 infection may be pH-independent in cultured and primary murine macrophages.

These data suggested that endosome acidification is not required for MNV-1 entry. Since toxicity of the inhibitors required their removal after four hours, it is unclear whether the ablation of endosome acidification lasted throughout the timeframe of the experiment. To ensure that removal of the drugs did not alter the experimental outcome, viral gene expression was examined at 6 hpi by immunofluorescence assay in the continued presence of the drugs. Infections were performed as described above with the following modifications. To maintain cell viability above 80% as determined by WST-1 (Fig. 2C, 2D), the concentration of chloroquine was reduced to 50 μ M. RAW 264.7 cells and BMM ϕ were infected at an MOI of 10 to increase the percentage of infected cells per field of view. The immunofluorescence assay was performed as previously described (Straight et al., 2000) with the following modifications. Macrophages were seeded on coverslips at 1×10^6 cells/ml and allowed to attach overnight.

After fixing and permeabilization, cells were blocked with 10% bovine serum in PBS. A solution of 1% bovine serum and 1% goat serum in PBS was used for diluting primary (1:10,000) and secondary (1:5,000) antibodies and was also used for washes. To stain nuclei, 4',6-diamidino-2-phenylindole (DAPI) (1 µg/ml final concentration) was added to the secondary antibody dilution. Fluorescently labeled cells were examined using the Olympus IX70 inverted microscope at the Center for Live Cell Imaging at the University of Michigan. To identify infected cells, MNV-1 expression of genes encoding non-structural proteins was followed using a monoclonal antibody to MNV-1 VPg (viral protein, genome-linked) (Ward et al., 2007). VSV viral gene expression was scored using the monoclonal antibody 23H12 against matrix protein (Lyles et al., 1988). 700 cells as indicated by DAPI staining were counted per condition and scored for MNV-1 or VSV gene expression. No VPg signal was observed at 0 hpi in untreated or treated macrophages (Fig. 2E, I, data not shown). Approximately 10% of cells stained positive for VPg at 6 hpi and increasing over time. A cell was scored as infected if the average fluorescent intensity was three times that of the uninfected controls using the Metamorph Premier v6.3 image analysis software (Molecular Devices, Downingtown, PA) (Fig. 2F-L). Their percentage was normalized to the no treatment control. No significant differences were observed in the number of cells expressing MNV-1 VPg in the presence or absence of inhibitor in either primary or cultured murine macrophages (Fig. 2A, B). Interestingly, the level of fluorescence was increased in the majority of bafilomycin A1-treated macrophages. In contrast to MNV-1 infected cells, the diffuse cytoplasmic staining of the VSV matrix protein indicative of VSV viral gene expression was significantly decreased by both inhibitors (Fig. 2A, B and data not shown). These results are consistent with viral growth data (see Fig. 1A-D) and demonstrated that in murine macrophages MNV-1 entry is pH-independent while VSV entry is pH-dependent.

In addition to murine macrophages, MNV-1 also shows a tropism for murine DCs (Ward et al., 2006; Wobus et al., 2004). To test whether the pH-independent entry mechanism observed in macrophages also occurred in DCs, we performed immunofluorescence assays as described above with the following modifications. As a source of DCs, we used both primary bone marrow-derived DCs and a DC-like cell line. Ruiz *et al.* generated an immortalized DC cell line, termed SRDCs, that have a phenotype, morphology and activity similar to CD4-CD8α+ CD205+ CD11b- DCs purified *ex vivo* (Ruiz et al., 2005). SRDCs were cultivated as described (Ruiz et al., 2005). MNV-1 infection of SRDCs resulted in titers similar to MNV-1 infection of RAW 264.7 cells (Fig. 3A) (Thackray et al., 2007). Primary bone marrow-derived murine DCs were generated from seronegative Swiss Webster mice (Charles River) as previously described (Wobus et al., 2004). The adherent SRDCs were plated at 1×10^6 /ml on coverslips overnight. To promote adherence of primary DCs to coverslips, sterile coverslips were first coated overnight at 4°C with rat tail collagen type 1 (2 mg/ml in 60% ethanol) before seeding primary DCs (5×10^5 /ml) onto coated coverslips overnight. Immunofluorescence assays were performed as described above. For primary DCs, inhibitors and virus were added directly to cells without media change, and viral gene expression was scored only in cells that co-stained with CD11c (1:2000 dilution, BD Pharmingen), a DC marker. SRDC and primary DC cell viability remained above 80% throughout the experiment as determined by WST-1 (Fig. 3D). No VPg signal was observed at 0 hpi in untreated or treated DCs (Fig. 3E, I, data not shown). Infected cells were scored as described above. No significant difference was observed in the number of MNV-1-infected DCs expressing VPg with or without inhibitors (Fig. 3B, C, F-L). In contrast, the number of cells expressing VSV matrix protein was significantly decreased by both inhibitors (Fig. 3B, C). These data demonstrated that MNV-1 viral gene expression is also independent of pH in primary and cultured DCs, suggesting MNV-1 entry into DCs does not require low pH.

Taken together, our data demonstrate that MNV-1 infection and viral gene expression in murine macrophages and DCs occur in a pH-independent manner. This is consistent with a pH-independent entry mechanism for MNV-1 into productively infected cell types.

Due to the absence of an efficient cell culture system for HuNoV and the recent discovery of a MNV-1 culture system, no studies have addressed the cell biology of norovirus entry in tissue culture. Here, we have used MNV-1 to elucidate the role of pH during entry into murine macrophages and DCs. We have shown that MNV-1 infection at 12 hpi and viral gene expression at 6 hpi were not inhibited by bafilomycin A1 or chloroquine, two known endosome acidification inhibitors. These findings suggest that a low intracellular pH does not trigger MNV-1 uncoating.

Feline calicivirus (FCV) has been used extensively as a surrogate for noroviruses, because it is a member of the calicivirus family and grows in tissue culture. A recent study using the FCV-F9 strain showed that treatment of Crandell-Rees feline kidney cells with either bafilomycin A1 or chloroquine during the first hour of infection dramatically decreases the number of FCV-infected cells as determined by immunofluorescence analysis (Stuart and Brown, 2006). The sensitivity of FCV to low pH is explained by its uptake mechanism via clathrin-mediated endocytosis and entry into Rab-5 positive early endosomes (Stuart and Brown, 2006). The different effect of low pH on MNV-1 and FCV may suggest a clathrin-independent entry route for MNV-1 infection of macrophages and DCs or cell type specific differences.

MNV-1 is an enteric virus that infects its host by the oral route and replicates in lamina propria cells of the small intestine (Mumphrey et al., 2007). Thus to reach the site of replication MNV must travel through the acidic pH of the stomach. A previous study demonstrated that extracellular low pH, including a pH of 2, does not affect MNV-1 infectivity, while the same treatments significantly decreased infectivity of FCV-F9 (Cannon et al., 2006). Together with our data indicating no role for intracellular pH during MNV-1 infection, this suggests that pH does not trigger conformational changes in the capsid that are required for uncoating. Similarly, other enteric viruses that infect the host via the gastrointestinal tract also enter cells by pH-independent mechanisms (Brandenburg et al., 2007; Golden et al., 2004; Golden and Schiff, 2005; Lopez and Arias, 2006; Pietiainen et al., 2005; Regan et al., 2008). This insensitivity to extracellular low pH among enteric viruses may be a feature of their route of infection. Encountering the low pH of the stomach would needlessly activate a pH-sensitive virus, making it fusogenic in a location of the host that lacks cells used for virus replication. Consistent with this idea is the observation that FCV-F9, a respiratory virus, is pH-sensitive (Cannon et al., 2006).

Further investigations into the cellular mechanisms of MNV-1 entry and uncoating are important to understand MNV biology. In addition, the findings reported here also contribute to the discovery of common themes in enteric virus infections and may lead to the elucidation of potential antiviral targets.

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Figure 1. MNV-1 infection is pH-independent in murine macrophages

RAW 264.7 cells (A and B) and primary bone marrow-derived (BMM Φ) macrophages (C and D) were infected with murine norovirus 1 (MNV-1) or vesicular stomatitis virus (VSV) in the absence (No Treat.) or presence of chloroquine (Chl.) or bafilomycin A1 (Baf. A) at a multiplicity of infection (MOI) of 5 for one hour on ice. Viral titers were determined by plaque assay. (E and F) Viability of uninfected cells undergoing drug treatment for 12 hours using WST-1 (Roche). A-D) Results represent means $\pm$ SE from three independent experiments. Statistical analysis was performed using the paired t-test (GraphPad Prism). * $P < 0.05$.

Figure 2. Expression of MNV-1 VPg is pH-independent in murine macrophages

RAW 264.7 cells (A) and primary bone marrow-derived macrophages (B) were infected with MNV-1 and VSV at a MOI of 10 for one hour on ice in the absence (no treat) or presence of 250 μ M bafilomycin A1 (Baf A.) or 50 μ M chloroquine (Chl.). The percentage of cells showing viral gene expression 6 hpi was determined by immunofluorescence assay and normalized to a no treatment control set at 100%. (C and D) Viability of uninfected cells undergoing drug treatment for 6 hours using WST-1 (Roche). A-D) Results represent means $\pm$ SE from three independent experiments. Statistical analysis was performed using the paired t-test (GraphPad Prism). Representative immunofluorescence images are shown for RAW 264.7 cells (E, F, G, H) and primary bone marrow-derived macrophages (I, J, K, L) infected with MNV-1 and stained with DAPI (blue) and a monoclonal antibody against VPg (red) at 0 (E, I) or 6 hpi (F, G, H, J, K, L). Images were collected with Metamorph Premier v6.3 image analysis software (Molecular Devices, Downingtown, PA) and compiled using Adobe Photoshop v9.0 (Adobe Systems, Mountain View, CA). *** $P < 0.001$; VPg = viral protein, genome-linked

Figure 3. Expression of MNV-1 VPg is pH-independent in murine dendritic cells

(A) Splenic derived-dendritic cells (SRDC) were infected with MNV-1 at MOI of 0.05 and 2 and viral titers determined at various time points after infection via plaque assay.

Immunofluorescence analysis of VPg gene expression 6 hpi in SRDC (B) and primary bone marrow-derived dendritic cells (C) infected with MNV-1 or VSV at a MOI of 10 in the absence (no treat) or presence of 250 μ M bafilomycin A1 (Baf. A.) or 50 μ M chloroquine (Chl.). The no treatment control was set at 100%. (D) Viability of uninfected cells undergoing drug treatment for 6 hours using WST-1 (Roche). A-D) Results represent means $\pm$ SE from three independent experiments. Statistical analysis was performed using the paired t-test (GraphPad Prism). Representative immunofluorescence images are shown for SRDCs (E, F, G, H) and primary bone marrow-derived DCs (I, J, K, L) infected with MNV-1 and stained with DAPI (blue) and a monoclonal antibody against VPg (red) at 0 (E, I) or 6 hpi (F, G, H, J, K, L).

Primary Dendritic cells were also stained with a CD11c (green). Images were collected with Metamorph Premier v6.3 image analysis software (Molecular Devices, Downingtown, PA) and compiled using Adobe Photoshop v9.0 (Adobe Systems, Mountain View, CA). ***

$P < 0.001$; VPg = viral protein, genome-linked