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Immunohistochemical detection of the vomiting-inducing monoamine neurotransmitter serotonin and enterochromaffin cells in the intestines of conventional or gnotobiotic (Gn) pigs infected with porcine epidemic diarrhea virus (PEDV) and serum cytokine responses of Gn pigs to acute PEDV infection

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ABSTRACT

Serotonin is a critical monoamine neurotransmitter molecule stored and released from enterochromaffin (EC) cells into the gut submucosa, transmitting the vomiting signal to the brain. We studied one mechanism by which vomiting is induced in pigs infected with porcine epidemic diarrhea virus (PEDV) by characterization of swine EC cells by immunohistochemistry. Conventional or gnotobiotic (Gn) 9-day-old pigs [PEDV-inoculated (n = 12); Mock (n = 14)] were inoculated orally (8.9–9.2 $\log_{10}$ genomic equivalents/pig) with PEDV PC21A strain or mock. This is the first identification of serotonin-positive EC cells in swine by immunohistochemistry and mainly in intestinal crypts, regardless of infection status. They were morphologically triangular-shaped or round cells with or without apical cytoplasmic extensions, respectively. At post-inoculation hour (PIH) 16 or 24, when vomiting was first or frequently observed, respectively, PEDV infection resulted in significantly reduced numbers of serotonin-positive EC cells in duodenum, mid-jejunum, ileum, or colon. However, two of three PEDV-inoculated Gn pigs that did not yet show vomiting at PIH 16 had numbers of serotonin-positive EC cells in duodenum, ileum and colon similar to those in the negative controls. These findings suggest that serotonin release from EC cells (increased serotonin levels) into the gut submucosa might occur early PEDV post-infection to stimulate the vagal afferent neurons, followed by vomiting. Serotonin might be involved in the mechanisms related to vomiting in PEDV-infected piglets. We also found that mid-jejunum was the primary site of acute PEDV infection, and that systemic innate and pro-inflammatory cytokine responses were induced during the acute stage of PEDV infection.

1. Introduction

Vomiting is a defensive reaction of the body to rapidly remove ingested toxins from the gastrointestinal tract. Vomiting is generally induced when either of the two medullary centers in the brain, integrative vomiting center and chemoreceptor trigger zone (CRTZ), is activated via a variety of their surface chemoreceptors, such as 5-HT_3 (Endo et al., 2000; Sikander et al., 2009; Spiller, 2008). The integrative vomiting center and CRTZ are triggered by activation of vagal afferent neurons in the gastrointestinal tract and circulating toxins in blood, respectively. There are vagal or enteric afferent neurons in the gut submucosa. Vagal afferent neurons are stimulated by a monoamine neurotransmitter molecule, serotonin (5-hydroxytryptamine, 5-HT), released from enterochromaffin (EC) cells in the gastrointestinal tract. Subsequently, vomiting signals are transmitted to the integrative vomiting center and then to the central nervous system (CNS). Emetic efferent signals from the CNS reach the gastrointestinal and abdominal muscles, leading to their expulsive actions by which vomiting is induced (Endo et al., 2000; Sikander et al., 2009; Spiller, 2008).

In humans, approximately 95% and 5% of serotonin is stored in the gut and brain, respectively. In the gut, 90% of serotonin is deposited in the secretory granules of EC cells mainly located in intestinal crypts, and 10% is in neurons in the submucosa (Endo et al., 2000; Spiller, 2008). In humans or rats, by immunohistochemistry (IHC) using

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antibodies against serotonin, EC cells were serotonin-positive and triangular-shaped with apical cytoplasmic extensions (Gustafsson et al., 2006; Spiller, 2008). The EC cells likely originate from intestinal crypt stem cells. A mechanical, biological, or chemical stimulus causes EC cells to secret serotonin into the lamina propria or lumen that acts on serotonin receptors (5-HT_3) at the terminals of the vagal afferent neurons (Endo et al., 2000; Gustafsson et al., 2006; Spiller, 2008). Serotonin also functions to promote immune activation through the receptors expressed on macrophages, dendritic cells and T and B cells (Li et al., 2011; Shajib and Khan, 2015).

Porcine epidemic diarrhea virus (PEDV) (family *Coronaviridae,* genus *Alphacoronavirus*) causes acute diarrhea, vomiting, decreased or loss of appetite, dehydration and high mortality in neonatal piglets (Jung and Saif, 2015). Diarrhea is frequently accompanied by vomiting in PEDV-infected nursing piglets during the acute stage of infection, exacerbating dehydration (Jung and Saif, 2015). However, the mechanisms by which vomiting is induced in PEDV infection are poorly understood. We hypothesized that: i) serotonin is involved in the mechanisms related to vomiting; ii) serotonin release from EC cells into the intestinal lamina propria or lumen occurs early PEDV post-infection to stimulate the vagal afferent neurons, followed by vomiting; and iii) expression levels of serotonin in intestinal EC cells or numbers of serotonin-positive EC cells in the small or large intestine differ between PEDV-infected and uninfected pigs. The *in situ* distribution and characterization of EC cells in swine intestine are also unknown. In our study, therefore, we aimed to develop an IHC to detect and characterize EC cells in swine intestine and to determine whether PEDV infection alters the number of serotonin-positive EC cells in the small and large intestines (primary sites of PEDV infection) of infected gnotobiotic (Gn) or conventional, 9-day-old piglets during disease progression. We also aimed to detail the pathogenesis of acute PEDV infection, including intestinal distribution of PEDV antigen and serum innate and pro-inflammatory cytokine profiles in infected Gn pigs to understand the relationship of acute PEDV infection with the frequency of serotonin-positive EC cells in the small and large intestines.

2. Materials and methods

2.1. Virus

The US virulent (wild-type) PEDV strain PC21A was obtained from intestinal contents of a diarrheic 1-day-old piglet in an Ohio farm in June 2013 (Jung et al., 2014). The original sample was serially passaged 2 times in Gn pigs, and the intestinal contents were negative for other enteric viruses [transmissible gastroenteritis virus, porcine deltacoronavirus (PDCoV), porcine rotavirus groups A-C, etc.] by PCR/RT-PCR and electron microscopic examination. The titer of Gn pig 2nd-passaged PC21A was 11.8 $\log_{10}$ GE/ml and was used as virus inoculum after dilution in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA, USA), as described previously (Jung et al., 2015).

2.2. Gnotobiotic pigs and experimental pig infection

Six Large White × Duroc crossbred Gn pigs were acquired by hysterectomy from a PEDV-seronegative pregnant sow obtained from a PEDV-free, specific-pathogen-free (SPF) (confirmed by history and seronegative sows; lack of qRT-PCR-positive fecal samples) swine herd of The Ohio State University. The SPF herd was seronegative for antibodies to porcine reproductive and respiratory syndrome virus, porcine respiratory coronavirus, transmissible gastroenteritis virus and porcine circovirus type 2. Six 9-day-old Gn piglets were randomly assigned to one of two groups: PEDV-infected (n = 3; pigs 1–3) and Mock (n = 3; pigs 4–6). Pigs were inoculated orally with 2 ml of PEDV strain PC21A [8.9 $\log_{10}$ genomic equivalents (GE)/ml] [9.2 $\log_{10}$ GE (≈3.2 $\log_{10}$ PFU) per pig], a dose similar to that (8.9 $\log_{10}$ GE/pig) used in the previous study (Jung et al., 2015), or mock inoculated with MEM. After PEDV inoculation, the pigs were monitored frequently for clinical signs, such as diarrhea, appetite, activity, etc., especially vomiting. Inoculated and negative control pigs (n = 3/group at each time-point) were euthanized for virological and pathological examination at an acute-stage of infection when or shortly after vomiting was first detected, i.e. approximately post-inoculation hour (PIH) 16 in this study. Diarrhea was assessed by scoring fecal consistency. Fecal consistency was scored as follows: 0 = solid; 1 = pasty; 2 = semi-liquid; 3 = liquid, with scores of 2 or more considered diarrheic. The Institutional Animal Care and Use Committee (IACUC) of The Ohio State University approved all protocols related to the animal experiments in this study.

2.3. Archival intestinal tissues

The other tissue samples tested were archival formalin-fixed, paraffin-embedded tissues acquired from twenty 9-day-old [PEDV-infected (n = 9) and Mock (n = 11)] conventional pigs inoculated orally with 8.9 $\log_{10}$ GE of PEDV strain PC21A or mock (MEM) (Jung et al., 2015). The clinical disease, fecal virus shedding, and gross and histopathology were described in a previous paper (Jung et al., 2015). However, the previous report included only limited information to understand intestinal distribution of serotonin-positive EC cells at the different locations of the intestine and the relationship of the frequency of serotonin-positive cells with vomiting. Therefore, more detailed clinical and histopathological observations relevant to the aims of the current study were described in the Results section. Pigs (n = 3–4/time-point) were euthanized for pathologic examination at post-inoculation days (PIDs) 1, 3, and 5.

2.4. Analysis of PEDV RNA titers in fecal and serum samples

Rectal swabs and serum samples were collected from Gn pigs 1–6 at PIH 16. Two rectal swabs were suspended in 4 ml MEM. The RNA was extracted from 200 µl of centrifuged (2000 ×g for 30 min at 4 °C) fecal suspensions using the Mag-MAX Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. PEDV RNA titers in rectal swabs and serum samples were determined as described previously (Jung et al., 2014).

2.5. Analysis of innate and pro-inflammatory cytokine levels in serum samples

There were increases in serum innate (IFNα) and pro-inflammatory (TNFα and IL-12) cytokine levels in PEDV-infected conventional pigs (10-day-old) at PID 1 (Annamalai et al., 2015) and increased mRNA levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNFα) in IPEC-J2 cells infected with PEDV (Lin et al., 2017). Therefore, serum innate (IFNα and IL-22) cytokines, which are known to play a role in antiviral immune responses (Gimeno Brias et al., 2016; Zhang and Yoo, 2016), and pro-inflammatory (TNFα, IL-6, and IL-12) cytokines were evaluated in Gn pigs 1–6 at PIH 16 to confirm the previous findings as well as to investigate whether acute PEDV infection induces systemic innate and pro-inflammatory immune responses.

Serum IFNα, IL-6, IL-12, IL-22, and TNFα cytokine levels were quantitated by ELISA in the serum samples collected from Gn pigs 1–6 at PIH 16, as described previously (Annamalai et al., 2015; Azevedo et al., 2006; Chattha et al., 2013). Briefly, Nunc Maxisorp 96-well plates were coated with anti-porcine IL-6 (0.75 µg/ml, goat polyclonal antibody), anti-porcine IL-12 (0.75 µg/ml, goat polyclonal antibody), anti-porcine IFNα (2.5 µg/ml, clone K9) (R&D systems, Minneapolis, MN), anti-porcine IL-22 (2.0 µg/ml, rabbit polyclonal antibody), and anti-porcine TNFα (1.5 µg/ml, goat polyclonal antibody) (Kingfisher biotech, Saint Paul, MN) overnight at 4 °C or 37 °C (for IFN-α only). Biotinylated anti-porcine IL-6 (0.1 µg/ml, goat polyclonal antibody), anti-porcine IL-12 (0.2 µg/ml, goat polyclonal antibody), anti-porcine IFNα (3.75 µg/ml, clone F17) (R&D systems, Minneapolis, MN), anti-porcine