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Pathogenesis and Immune Responses in Gnotobiotic Calves after Infection with the Genogroup II.4-HS66 Strain of Human Norovirus[▽]

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We previously characterized the pathogenesis of two host-specific bovine enteric caliciviruses (BEC), the GIII.2 norovirus (NoV) strain CV186-OH and the phylogenetically unassigned NB strain, in gnotobiotic (Gn) calves. In this study we evaluated the Gn calf as an alternative animal model to study the pathogenesis and host immune responses to the human norovirus (HuNoV) strain GII.4-HS66. The HuNoV HS66 strain caused diarrhea (five/five calves) and intestinal lesions (one/two calves tested) in the proximal small intestine (duodenum and jejunum) of Gn calves, with lesions similar to, but less severe than, those described for the Newbury agent 2 (NA-2) and NB BEC. Viral capsid antigen was also detected in the jejunum of the proximal small intestine of one of two calves tested by immunohistochemistry. All inoculated calves shed virus in feces (five/five calves), and one/five had viremia. Antibodies and cytokine (proinflammatory, tumor necrosis factor alpha [TNF-α]; Th1, interleukin-12 [IL-12] and gamma interferon [IFN-γ]; Th2, IL-4; Th2/T-regulatory, IL-10) profiles were determined in serum, feces, and intestinal contents (IC) of the HuNoV-HS66-inoculated calves ($n = 5$) and controls ($n = 4$) by enzyme-linked immunosorbent assay in the acute (postinoculation day 3 [PID 3]) and convalescent (PID 28) stages of infection. The HuNoV-HS66-specific antibody and cytokine-secreting cells (CSCs) were quantitated by ELISPOT in mononuclear cells of local and systemic tissues at PID 28. Sixty-seven percent of the HuNoV-HS66-inoculated calves seroconverted, and 100% coproconverted with immunoglobulin A (IgA) and/or IgG antibodies to HuNoV-HS66, at low titers. The highest numbers of antibody-secreting cells (ASC), both IgA and IgG, were detected locally in intestine, but systemic IgA and IgG ASC responses also occurred in the HuNoV-HS66-inoculated calves. In serum, HuNoV-HS66 induced higher peaks of TNF-α and IFN-γ at PIDs 2, 7, and 10; of IL-4 and IL-10 at PID 4; and of IL-12 at PIDs 7 and 10, compared to controls. In feces, cytokines increased earlier (PID 1) than in serum and TNF-α and IL-10 were elevated acutely in the IC of the HS66-inoculated calves. Compared to controls, at PID 28 higher numbers of IFN-γ and TNF-α CSCs were detected in mesenteric lymph nodes (MLN) or spleen and Th2 (IL-4) CSCs were elevated in intestine; IL-10 CSCs were highest in spleen. Our study provides new data confirming HuNoV-HS66 replication and enteropathogenicity in Gn calves and reveals important and comprehensive aspects of the host's local (intestine and MLN) and systemic (spleen and blood) immune responses to HuNoV-HS66.

Caliciviruses infect a variety of animal hosts, causing a wide range of diseases from gastroenteritis to fatal hemorrhagic disease (52). Human noroviruses (HuNoV), members of the *Caliciviridae* family, are the leading cause of epidemic food- and waterborne nonbacterial gastroenteritis worldwide (16). Bovine NoVs have also been detected in cattle from England and Germany (13, 18) and the United States (45, 46). Two strains from Europe, Jena and Newbury agent 2, and the U.S. strain CV186-OH are genetically similar to GI HuNoV (13, 30, 39, 46) and constitute a third NoV genogroup (GIII.1 and GIII.2) (39). The pathogenesis and antibody (Ab) responses of gnotobiotic (Gn) calves to the bovine NoV GIII.2 strain CV186-OH and to the unassigned NB strain have been previously characterized in our lab (20, 45). Both strains infected the villous epithelial cells of the small intestine, especially in duodenum and jejunum but less so in ileum, causing their destruction and resulting in severe diarrhea.

The HuNoV are fastidious viruses, and only recently were GI and GII HuNoV strains cultured in vitro in a complex organoid model of human small intestinal epithelium (48). Therefore, because of the lack of routine in vitro cell culture assays for these viruses, animals such as Gn pigs (10) and Gn calves are important as infectivity models to understand the pathogenesis and host immune responses to HuNoV in comparison with the host-specific NoV strains. Newborn calves are delivered and maintained under sterile conditions, and because they are raised on a milk diet, their rumen does not develop and their gut physiology and immune responses (presence of secretory immunoglobulin A [sIgA]) remain similar to those of infants, providing an alternative animal model for the study of gastrointestinal viruses.

Immune responses differ according to the infectious agent and the cytokine secretion patterns induced. Evidence of a polarized T-cell response to certain pathogens has been found in humans (7) and in mice (5). Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that is produced by cells of the innate immune system, including monocytes/macrophages, natural killer (NK) cells, mast cells, and neutrophils, and it is an important inflammation mediator, having a broad spectrum of action including pathogen control and induction

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of apoptosis (42). The Th1 cytokines (gamma interferon [IFN-γ] and interleukin-2 [IL-2]) support macrophage activation, generation of cytotoxic T cells, and the production of opsonizing Abs, whereas Th2/T-regulatory (T-reg) (IL-4, IL-5, IL-10, and IL-13) cytokines support B-cell activation, the production of nonopsonizing Abs, the control of extracellular parasites, and the elicitation of allergic reactions (36). The Th1 responses occur during intracellular bacterial and viral infections, whereas Th2 cytokines predominate during parasitic infections, although this dichotomy is complex and in some infections both types of responses occur (14). IL-12 is mainly produced by macrophages, dendritic cells (DC), and other antigen-presenting cells. It is a key cytokine in the innate immune system but is also produced in a T-cell-dependent manner during the development of adaptive immune responses. It also stimulates NK cells and $CD8^+$ T cells to produce IFN-γ (22). Type II IFN (IFN-γ) is produced mainly by T cells, NK cells, and cytotoxic T cells but also by DC in response to cytokines such as IL-12. The IFN-γ production by $CD4^+$ Th1 cells is primarily induced by IL-12, especially during infections with intracellular pathogens (32).

The Th2 cytokine IL-4 is produced in response to antigen activation by $CD4^+$ Th2 cells and some $CD8^+$, $NK1^+$, and γδ T cells. Mast cells also express IL-4, representing an early source of IL-4 for naïve T and B cells during initial antigen encounter. IL-4 also induces isotype switching to IgG and IgE (33). IL-10 is produced by T cells with regulatory activity and also by DC, macrophages, and B cells. It inhibits cytokine production by Th1 cells and the induction of activities initiated by other cytokines such as IFN-γ, IL-2, and TNF-α (40), exerting strong anti-inflammatory effects (26).

In cattle the Th1/Th2 paradigm is less well defined, and antigen-specific $CD4^+$ T cells coexpress IL-4, IFN-γ, and IL-10 in response to parasite pathogens such as *Babesia bovis* and *Fasciola hepatica* (50), but in vivo responses to certain pathogens can be biased toward a Th1 or Th2 response (8). The bovine IL-12 molecule is structurally similar to that of humans and mice, and it increases IFN-γ secretion in vitro by cattle peripheral blood mononuclear cells in response to viral antigens (57). IL-4 plays a role in protective immunity in cattle against helminth parasites (2). During persistent viral infection, IL-10 was produced mainly by bovine monocytes/macrophages, and during *Trypanosoma congolense* infection, the increase in IL-10 synthesis coincided with decreased IFN-γ synthesis (49). TNF-α is also a pleiotropic cytokine in cattle, and it may induce apoptosis in B cells infected with the bovine leukemia virus, therefore playing an important role in the pathogenesis of this viral infection (24).

We previously confirmed that the HuNoV-HS66 strain infects Gn pigs and described its pathogenesis in Gn pigs (9, 10). We also have recently delineated the immune responses to infection by the HuNoV-HS66 strain in Gn pigs (47). In this study we report the enteropathogenicity, viral shedding, and Ab and cytokine immune responses, both locally and systemically, of Gn calves experimentally infected with the HuNoV-HS66 strain and document the replication of HuNoV not only in Gn pigs as previously reported but also in Gn calves.

MATERIALS AND METHODS

Virus inoculum. A single aliquoted pool of the original human fecal sample identified as NoV/GII/4/HS66/2001/US (HS66 strain) (10), GenBank accession no. EU105469, containing approximately 5.4×10^6 genomic equivalents (GE)/ml, was used for oral inoculation of Gn calves. Each Gn calf received one oral dose of the HuNoV inoculum consisting of 3 ml of the original HuNoV-HS66 strain diluted 1:10 in minimal essential medium (MEM) (Gibco Invitrogen, Carlsbad, CA), which was further processed by vortexing, centrifugation at $3,000 \times g$ for 20 min, and filtration through an 0.8-μm-pore-size filter followed by 0.2-μm filters. The mock inoculum was MEM.

Inoculation of the experimental calves. Near-term calves were derived aseptically by Caesarean section and maintained in individual sterile isolator units (21). Five-day-old Gn calves were inoculated as follows: one oral dose (1.6×10^7 GE) of the original HuNoV-HS66 inoculum (diluted and processed as described earlier to a final volume of 30 ml) ($n = 5$ calves) or equal volumes of MEM as controls ($n = 4$). The HuNoV-HS66-inoculated and control calves were euthanized acutely at postinoculation day 3 (PID 3), 1 day after diarrhea was first observed (HuNoV, $n = 2$; controls, $n = 2$) for histopathology and viral antigen detection by immunohistochemistry (IHC) and at the convalescent stage at PID 28 (HuNoV, $n = 3$; controls, $n = 2$).

Assessment of diarrhea. Daily fecal samples were collected by digital anal massage using sterile gloves directly into sterile specimen cups. Diarrhea scores of feces were noted and recorded (0, normal; 1, semisolid; 2, pasty; 3, semiliquid; 4, liquid) as previously described (21). Fecal samples with scores of 2 to 4 were considered diarrheic. The cumulative scores of each calf were calculated based on the sum of daily fecal sample scores from PIDs 1 to 6, and the mean cumulative score of each group is the sum of each calf's diarrhea cumulative score divided by the number of calves in that group.

Histopathology. Tissues of major organs (kidney, liver, spleen, and lung) and 5-cm-long intestinal sections of the duodenum (~5 cm from the pylorus), jejunum (~5 cm into the intermediate jejunal zone), ileum (~10 cm from the ileocecal junction), midcecum, and midcolon were collected from controls and HuNoV-HS66-infected calves and fixed in 10% neutral-buffered formalin for 2 days. A total of three sections were obtained from each part of the intestine (duodenum, jejunum, ileum, and colon). The sections were dehydrated in a graded ethanol series and embedded in paraffin, and 3-μm sections were cut for histopathological examination followed by hematoxylin and eosin staining. Tissue sections from both HS66-infected and age-matched control calves were evaluated, with blinding, i.e., no information on the infection status. Villous height-to-crypt depth (VH/CD) ratios were measured as previously reported (23), and intestinal tissues were classified according to the VH/CD ratios as follows: normal intestinal villi ranging from 6 to 7 in VH/CD ratio, mild intestinal villous atrophy ranging from 5 to 6 in VH/CD ratio, moderate intestinal villous atrophy ranging from 4 to 5 in VH/CD ration, severe intestinal villous atrophy ranging from 2 to 4 in VH/CD ration, and very severe intestinal villous atrophy ranging from 1 to 2 in VH/CD ratio.

Viral antigen detection by IHC. For detection of HuNoV-HS66 viral capsid antigens, the tissue sections were prepared and cut as described above and collected on positively charged microscope slides (Fisher Scientific, Pittsburgh, PA). Slides were kept at 60°C for 20 min, deparaffinized in xylene twice for 5 min, and rehydrated through a graded ethanol series (100% to 50%). Antigen retrieval was performed using 100 μg/ml of proteinase K (Invitrogen Corp.), and sections were immersed in 0.3% H_2O_2 in methanol for endogenous peroxidase removal. The slides were washed three times in phosphate-buffered saline (PBS), pH 7.4, and blocked with 1% normal goat serum for 30 min at room temperature (Rt). The tissues were immersed in either a 1:50 dilution of guinea pig hyperimmune antiserum against HuNoV-HS66 virus-like particles (VLPs) (10) or a 1:250 dilution of monoclonal Ab (MAb) NS14 (10), kindly provided by Mary Estes (Baylor College of Medicine, TX), which mapped to the P1 domain of the capsid protein of all GII NoVs tested (25). Tissues were incubated overnight at 4°C. After two washes in PBS, tissues were incubated with the secondary Abs, either horseradish peroxidase (HRP)-labeled rabbit anti-guinea pig IgG (1:50) (Dako, CA) (for the primary guinea pig hyperimmune antiserum) or an alkaline phosphatase-labeled goat anti-mouse IgG (1:200) (Dako, CA) (for the primary NS14 MAb). The tissues were incubated for 1 h at 37°C, followed by immersion of the sections in the substrate solutions: 3′-diaminobenzidine (BD Biosciences, CA) for 10 min at Rt for the HRP-labeled secondary Ab or a solution of red substrate (1 tablet of fast red in 2 ml of 0.1 M Tris-HCl, pH 8.2; Roche Applied Science) for 20 min at Rt for the alkaline phosphatase-labeled secondary Ab. Sections were counterstained with Mayer's hematoxylin and examined using light microscopy.

Detection of viral shedding by RT-PCR. Viral shedding was determined using rectal swab fluids and 1:20 dilutions of intestinal contents (IC) by reverse transcription-PCR (RT-PCR), using the primer pair Mon 431/433 (43) targeting the RNA-dependent RNA polymerase region of HuNoV GII, using the same conditions as previously described (10). Samples that were inhibited in RT-PCR, as revealed by the use of an internal control (10), were retested after being reextracted using the RNeasy Mini kit (Qiagen Inc., Valencia, CA). Negative controls (rectal swabs from mock-inoculated calves and RNase-free water) for RNA extraction and RT-PCR were included in each assay. A microplate hybridization assay (53) was performed to confirm the product specificity using a probe specific for HuNoV-HS66 (10).

Quantitation of viral RNA by real-time PCR. RNA was extracted using the RNeasy Mini kit (Qiagen Inc., Valencia, CA). A real-time PCR assay was standardized using serial dilutions of known concentrations of the internal control to generate a noncompetitive standard curve with Mon 431/433 primers using the Sybr green I kit (Roche Diagnostics, Mannheim, Germany) as previously described (10) with modifications. Briefly, for synthesis of the cDNA, 2 μl of the RNA (5 ng/μl) was added to a total reaction volume of 20 μl with 1× reverse transcription buffer (50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol [pH 8.3]), 0.5 mM (each) deoxynucleoside triphosphates, 2.5 μM random hexanucleotide mixture (Promega, Madison, WI), 20 U RNasin (Promega, Madison, WI), and 50 U avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). After incubation for 45 min at 42°C, the mixture was heated for 5 min at 99°C to denature the products and then stabilized at 4°C. The RNA extracted from the processed HuNoV-HS66 original sample was used as a positive control, and negative controls (RNA extracted from rectal swabs from mock-inoculated calves and RNase-free water) were also included in each assay.

Real-time PCR was performed using the LightCycler DNA Master Sybr green I kit (Roche Diagnostics, Mannheim, Germany). The cDNA (4 μl) was added to a total reaction volume of 25 μl with 2.5 μl 10× Sybr green I mix, 1.25 μl 25 mM $MgCl_2$, and 0.5 μl of each primer (25 pmol stock concentration). The PCR was conducted under the following conditions in a SmartCycler real-time machine (Cepheid, CA): one cycle of 2 min at 95°C and 40 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. Melting-curve analysis was performed immediately after amplification, under the following conditions: 0 s (hold time) at 95°C, 15 s at 65°C, and 0 s (hold time) at 95°C. Relative fluorescent units (RFU) for incorporated Sybr dyes were monitored during each cycle at 540 nm. The cycle threshold (C_T), which is the cycle number at which a positive amplification reaction was identified, was defined when the RFU exceeded 10 times the standard deviation of baseline RFU values of all samples. The C_T values were plotted against virus RNA concentration in $\log_{10}$ ng/μl. The standard curve was used to convert C_T values for each specimen to ng/μl equivalents and expressed as estimated GE/ml (10).

Detection of viral shedding by antigen ELISA. The antigen enzyme-linked immunosorbent assay (ELISA) was performed as previously described (10). Samples were considered positive when the mean absorbance (450 nm) of the positive coating wells minus the mean absorbance of the negative coating wells was higher than the mean absorbance of the negative control wells plus three times the standard deviation.

Viremia. Sera from blood collected from calves on PID 2 were analyzed by RT-PCR and microwell hybridization for detection of HuNoV-HS66 RNA or amplicon, respectively, as previously described (10). As described for the fecal samples, serum samples that were inhibited in RT-PCR were reextracted using the RNeasy Mini kit and retested by RT-PCR.

Ab detection. An immunocytochemistry assay was performed to detect HuNoV-HS66-specific Abs in the serum and IC of Gn calves, as previously described (54). For this assay a recombinant baculovirus expressing HS66 capsid was used to infect *Spodoptera frugiperda* (Sf9) cells as the HuNoV antigen source and the recombinant baculovirus-infected cells or mock-infected cells were subsequently fixed using 10% formalin in PBS. Ab titers were defined as the reciprocal of the highest serum dilution at which brown-stained cells representing NoV Ab complexed to HS66 capsid antigen could be detected.

Isolation of MNC for ELISPOT assays to detect Ab-secreting cells (ASC) and cytokine-secreting cells (CSCs). Segments of the small intestine (jejunum and ileum), mesenteric lymph nodes (MLN), a section of the spleen, and blood were collected at euthanasia and processed for the isolation of mononuclear cell (MNC) populations, as previously described (51, 55). Single MNC suspensions from each tissue and blood were prepared at concentrations of 5×10^6 and 5×10^5 MNC/ml in complete medium prepared with Roswell Park Memorial Institute (RPMI) 1640 (GIBCO) enriched with 8% fetal bovine serum, 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 μg of gentamicin/ml, 100 μg of ampicillin/ml, and 50 μg of 2-mercaptoethanol (E-RPMI).

ELISPOT assay for HuNoV-HS66-specific ASC. An ELISPOT assay for detection of isotype-specific (IgM, IgA, and IgG) ASC was conducted using previously published methods (11, 56). Briefly, Sf9 cell plates infected with the HS66 recombinant baculovirus and noninfected Sf9 cell plates (mock plates) were prepared and fixed as described under "Ab detection" and washed with deionized water prior to use. Single MNC suspensions from each tissue were added to duplicate wells (5×10^5 and 5×10^4 MNCs/well). Plates were then incubated at 37°C for 12 h in 5% CO_2 and then washed three times with PBS buffer and incubated at 37°C for 2 h with 100 μl/well of HRP-labeled Abs: goat anti-bovine IgM (μ) (Serotec) (0.25 μg/ml), sheep anti-bovine IgA (Serotec) (0.3 μg/ml), and goat anti-bovine IgG (KPL Inc.) (0.25 μg/ml). Plates were then washed three times in PBS buffer and developed with tetramethylbenzidine (TMB) (KPL Inc.) for 2 h at Rt. The numbers of virus-specific ASC were determined by counting blue spots in the wells, using a light microscope, and were reported as the number of virus-specific ASC per 5×10^5 MNCs, after any background spots (<3), evident on the mock plates, were subtracted.

CSC ELISPOT assay. A cytokine ELISPOT assay for detection of proinflammatory (TNF-α), Th1 (IL-12 and IFN-γ), and Th2/T-reg (IL-4 and IL-10) CSCs was developed. Multiscreen-IP sterile 96-well plates (Millipore, Bedford, MA) were coated with MAbs to bovine TNF-α (5 μg/ml) (bovine TNF-α screening set; Endogen; Pierce Biotechnology, Inc., Rockford, IL), bovine IL-12 (2 μg/ml) and bovine IFN-γ (1.3 μg/ml) (bovine IFN-γ screening set; Endogen; Pierce Biotechnology), bovine IL-4 (7.5 μg/ml) (bovine IL-4 screening set; Endogen; Pierce Biotechnology), or bovine IL-10 (2 μg/ml) (Serotec) diluted in coating buffer (0.05 M carbonate buffer, pH 9.6) and incubated at Rt overnight. The plates were blocked with E-RPMI before MNCs, stimulated with 50 μg/ml of CsCl-purified HS66 VLPs (10) or 10 μg/ml of phytohemagglutinin (positive control) or RPMI (negative control), were added to the plates at concentrations of 5×10^5 and 5×10^4 MNCs/well. Plates were then incubated at 37°C in 5% CO_2 for 48 h. After the plates were washed with PBS-0.05% Tween, biotin-labeled detection MAbs to bovine TNF-α (2 μg/ml) (bovine TNF-α screening set; Endogen; Pierce Biotechnology), bovine IL-12 (2 μg/ml) (Serotec), bovine IFN-γ (0.3 μg/ml) (bovine IFN-γ screening set; Endogen; Pierce Biotechnology), bovine IL-4 (5 μg/ml) (bovine IL-4 screening set; Endogen; Pierce Biotechnology), or bovine IL-10 (2 μg/ml) (Serotec) were added and the plates were incubated at 4°C overnight. Plates were washed, HRP-conjugated streptavidin (Biosource, Camarillo, CA) was added at a concentration of 0.3 μg/ml, and the plates were incubated at Rt for 2 h. The spots were developed with AEC substrate (Sigma-Aldrich, St. Louis, MO), and the numbers of CSCs were counted using an ImmunoSpot series 3A analyzer (Cellular Technology Ltd., Cleveland, OH) and expressed as CSCs per 5×10^5 MNCs. The HuNoV-HS66-specific CSC numbers were computed after the numbers of CSCs in the controls (RPMI-stimulated cells) were subtracted from the HS66 VLP-stimulated cells.

Cytokine ELISA. Fecal samples collected at PIDs 0, 1, 2, 4, 6, 10, 14, and 21; blood samples collected from calves at PIDs 0, 2, 4, 7, 10, 14, 21, and 28; and IC collected at euthanasia (PIDs 3 and 28) were tested. Serum samples were processed and stored at −20°C (4). The fecal samples and IC samples were diluted 1:2 in MEM with a protease inhibitor cocktail to prevent cytokine degradation (3) and were immediately frozen at −20°C until further testing. An ELISA was performed to detect TNF-α, IL-12, IFN-γ, IL-4, and IL-10. Briefly, 96-well microtiter plates (Nalgene Nunc, Rochester, NY) were coated using the same Abs used in the ELISPOT assay but with different concentrations only for the MAb to bovine IL-12 (4 μg/ml) and the MAb to bovine IL-10 (4 μg/ml) (Serotec). Plates were incubated at Rt overnight and blocked with PBS-4% bovine serum albumin-5% sucrose for 2 h at Rt before the samples were added. After 2 h at Rt, the plates were washed and the same detection Abs were added at the same concentrations used in the ELISPOT assay, except for the MAb to bovine IL-12 (4 μg/ml) and the MAb to bovine IL-10 (4 μl/ml) (Serotec). After 1.5 h at Rt, the plates were washed and HRP-conjugated streptavidin (Biosource, Camarillo, CA) was added at a concentration of 0.1 μg/ml. The plates were incubated at Rt for 1 h and developed with TMB (KPL Inc.). Standard curves were generated using recombinant bovine TNF-α, IL-4, and IFN-γ (bovine screening set; Endogen; Pierce Biotechnology) and recombinant human IL-12 and IL-10 (Biosource). A computer-generated four-parameter curve-fit was used to calculate the concentration of each cytokine. The detection sensitivity limits for the reactions were as follows: 7 pg/ml for IFN-γ, TNF-α, and IL-4 and 15 pg/ml for IL-10 and IL-12.

Statistical analysis. Due to the small number of animals used in the study, statistical analysis was precluded. However, clear trends were observed as shown graphically and reported in the text.

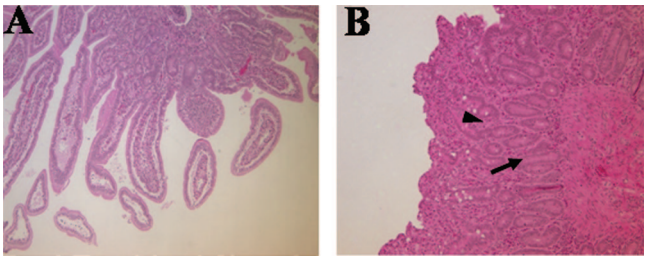

FIG. 1. Histopathology. (A) Jejunum of a mock-inoculated calf at PID 3. No abnormalities were evident (VH/CD ratio = 7). Original magnification, ×150. (B) Jejunum of a HuNoV-HS66-inoculated calf showing moderate to severe atrophic enteritis (moderate = VH/CD ratio of 4 to 5; severe = VH/CD ratio of 2 to 4) characterized by loss (50 to 70%) of villi and mild to moderate proliferation of crypt cell layers (arrow) and increase of cell populations in the lamina propria. Original magnification, ×200. Hematoxylin and eosin stain was used.

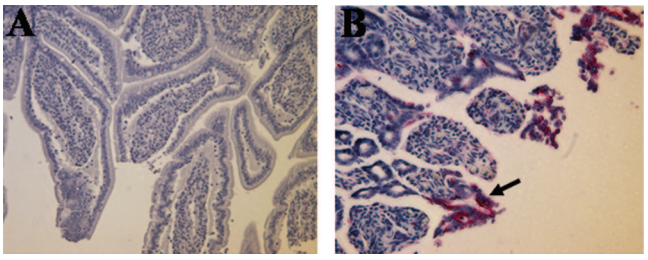

FIG. 2. IHC for detection of GII HuNoV capsid antigens. (A) Jejunum of mock-inoculated calf at PID 3. No positive cells were observed (magnification, ×150). (B) Jejunum of HuNoV-HS66-inoculated calf at PID 3. Viral capsid antigens (stained red) were observed in the cytoplasm of a few intestinal epithelial cells attached to the villi or exfoliated enterocytes (arrow) (original magnification, ×300). IHC, 3,3′-diaminobenzidine, and Mayer's hematoxylin counterstain were used.

RESULTS

The HuNoV-HS66 strain caused intestinal lesions in the jejunum of a HuNoV-HS66-inoculated Gn calf. Intestinal tissue sections of two HuNoV-HS66-inoculated Gn calves and two mock-inoculated calves euthanized at PID 3 were analyzed. No lesions were observed in the intestinal tissues of mock-inoculated calves (Fig. 1A). One of the two HuNoV-HS66-inoculated calves had mild villous atrophy (VH/CD ratio = 5 to 6) with mild to moderate enterocyte vacuolization in duodenum and midjejunum. The duodenum and midjejunum of the other calf showed moderate to severe (moderate, VH/CD ratio = 4 to 5; severe, VH/CD ratio = 2 to 4) diffuse atrophic enteritis characterized by severe loss (50 to 70%) of villi (Fig. 1B). Mild lesions were observed in ileum, and no lesions were detected in colon. Mild to moderate proliferation of crypt cell layers, increased numbers of MNCs, and a few necrotic cells were also observed in the lamina propria of this calf (Fig. 1B), compared to the mock-inoculated calf. No microscopic lesions were observed in the lungs, spleen, kidneys, or liver of the two HuNoV-HS66-inoculated or the two mock-inoculated calves (data not shown), demonstrating the localization of the lesions to only the small intestine.

Viral antigen was detected by IHC in the jejunum of a HuNoV-HS66-inoculated Gn calf. No positive cells were present in any tissues of the mock-inoculated calves (Fig. 2A). Viral capsid antigen, detected as IHC-positive cells (stained red), was present in enterocytes of the jejunum (Fig. 2B) and in a small number of enterocytes of the ileum of one HuNoV-HS66 inoculated calf but not detected in the duodenum. Positive signals were also detected in macrophage-like cells present in the lamina propria.

HuNoV-HS66 caused diarrhea, viral shedding, and viremia in Gn calves. After inoculation with HuNoV-HS66, five of five (100%) of the inoculated calves developed diarrhea and shed virus as detected by RT-PCR and microwell hybridization assay (Table 1). Fecal samples from three HuNoV-HS66-inoculated calves and fecal samples from a mock-inoculated calf were analyzed by real-time PCR. The processed HuNoV-HS66 positive control had an estimated 4×10^5 GE/ml, and the negative control (extracted from a fecal sample of a mock-inoculated calf) was negative. The calf samples at PID 1 had an average of approximately 7.7×10^3 GE/ml, at PID 2 had 4×10^4 GE/ml, peaked at PID 3 or at PID 4 with 6×10^4 to 2×10^5 GE/ml, and thereafter at PIDs 5 and 6 had 5×10^4 to 7×10^4 GE/ml. Diarrhea was detected from PIDs 2 to 6, and viral shedding was detected in the fecal samples of the calves by RT-PCR and microwell hybridization (PIDs 1 to 6) and antigen-ELISA (PIDs 2 to 5), with a mean duration of shedding of 3 days by RT-PCR, 4 days by microwell hybridization, and 2 days by ELISA. One of the two calves euthanized at PID 3 had viral RNA detectable in the IC, and one of five HuNoV-HS66-inoculated calves (20%) had viral RNA detectable in the serum by RT-PCR (data not shown).

HuNoV-HS66 elicited 67% seroconversion and 100% coproconversion rates in Gn calves. Low IgM, IgA, and IgG Ab titers were detected by immunocytochemistry after inoculation with HuNoV-HS66 (geometric mean titer [GMT] = 20 to 80) (Fig. 3). The IgM Ab was initially detected in serum at PID 4 (data not shown) and peaked at PID 14 (Fig. 3). The IgA Ab was initially detected in serum at PID 7 and peaked at PIDs 21 and 28 (GMT = 63). Elevated IgG Ab titers in serum were first detected at PID 21 (GMT = 40) and peaked at PID 28 (GMT = 50). Two of three (67%) HuNoV-HS66-inoculated calves euthanized at PID 28 seroconverted by PIDs 21 to 28 with both IgA (titers from 20 to 320) and IgG (titers from 40 to 160) Abs. Low IgA Ab titers (GMT = 20 to 40) were also detected in the IC of two of the three calves inoculated with HuNoV-HS66 and euthanized at PID 28. All three calves had IgG Abs in their IC, although one of them did not have serum Abs (data not shown). No HuNoV-HS66-specific Ab responses were detected in the serum or IC of the control calves.

HuNoV-HS66 induced higher numbers of IgA and IgG ASC locally (intestine) than systemically in the inoculated Gn calves. The numbers of IgA and IgG HS66-specific ASC detected by ELISPOT assay on PID 28 are shown in Fig. 3. Low numbers of ASC were elicited in the HuNoV-HS66-inoculated calves, and no ASC were detected in the control calves. Low to moderate numbers of IgA ASC were detected locally in the intestine (27 ASC per 5×10^5 MNCs) and MLN (11 ASC per 5×10^5 MNCs) and systemically in spleen (17 ASC per 5×10^5 MNCs) and blood (10 ASC per 5×10^5 MNCs). Higher numbers of IgG ASC were elicited in the intestine (77 ASC per 5×10^5 MNCs) and also in spleen (21 ASC per 5×10^5 MNCs),

TABLE 1. Diarrhea, fecal virus shedding, and viremia detected by RT-PCR and seroconversion detected by immunocytochemistry in Gn calves inoculated with HuNoV-HS66 or mock-inoculated controls[a]

Inoculum (no. of calves)	Mean no. of days of virus shedding (range)	Diarrhea[b]			No. of calves positive/total no. of calves (%)		
		No. of calves positive/total no. of calves (%)	Mean no. of days (range)	Mean cumulative score[c] (range)	Viremia[d]	Seroconversion[e]	Coproconversion[e]
HS66 (n = 5)	3 (1–6)	5/5 (100)	3 (2–6)	10 (2–14)	1/5 (20)	2/3 (67)	3/3 (100)
Control (n = 4)	0	1/5 (20)	0	2 (1–4)	0/5 (0)	0/2 (0)	0/2 (0)

[a] Due to the small number of animals used in the study, statistical significance was omitted.
[b] Diarrhea was deemed to be present if fecal swab scores were ≥2 after inoculation.
[c] Represents the sum of daily rectal swab scores from PIDs 1 to 6 of each calf divided by the number of calves in that group.
[d] Viremia was determined by RT-PCR on PID 2.
[e] Seroconversion and Ab titers in IC were determined by immunocytochemistry (HS66 recombinant baculovirus-infected Sf9 cell staining assay) at PIDs 21 and/or 28.

with lower numbers in MLN (7 ASC per 5×10^5), but none were detectable in blood MNCs, compared to IgA ASC.

HuNoV-HS66 induced a higher early increase (PID 2) of IFN-γ in serum of the inoculated Gn calves, compared to mock controls. The cytokine ELISA results are depicted in Fig. 4. Overall, the HuNoV-inoculated Gn calves developed higher proinflammatory (TNF-α), Th1 (IL-12 and IFN-γ), and Th2/T-reg (IL-4 and IL-10) cytokine levels than did control calves, whose cytokine concentrations remained at or below the detection limits, except for IL-10. The proinflammatory cytokine, TNF-α, and also IFN-γ increased early in infection at PID 2 (20- and 5.5-fold above controls, respectively) and again at lower concentrations at PIDs 7 and 10 (10- and 7-fold and 14- and 6-fold above controls, respectively). The Th1 cytokine IL-12 was initially detected at PID 4 and increased at PID 10 (25-fold above controls). Both IFN-γ and TNF-α early increases in serum in the HuNoV-HS66-inoculated calves coincided with the onset of diarrhea (PIDs 2 to 6), viral shedding (PIDs 1 to 6), and viremia (PID 2).

The Th2 cytokine (IL-4) was detected only in serum of the HS66-inoculated calves and reached its highest at PID 4 (24-fold above controls). The Th2/T-reg cytokine (IL-10) was detected constitutively in serum of both HuNoV-HS66- and mock-inoculated calves from PIDs 0 to 28 but with elevated concentrations detected at PIDs 4 and 14 in the HuNoV-HS66-inoculated calves (eight- and fourfold above controls, respectively). The increase in the anti-inflammatory cytokine IL-10 concentration at PID 4 occurred shortly after the early (PID 2) and later (PID 10) increases in the concentrations of the proinflammatory cytokines TNF-α and IFN-γ.

The Th2 (IL-4) and Th2/T-reg (IL-10) cytokines increased earlier (PID 1) in fecal samples than in serum of HuNoV-HS66-inoculated Gn calves. The concentrations of proinflammatory (TNF-α), Th1 (IL-12 and IFN-γ), and Th2/T-reg (IL-4 and IL-10) cytokines were generally higher in the fecal samples of the HS66-inoculated calves than in the control calves at all PIDs tested (Fig. 5) but usually lower than the concentrations of the corresponding cytokines in serum (Fig. 4). In feces of the HuNoV-HS66-inoculated calves, in comparison to the serum cytokine profiles and kinetics, we found a similar pattern of TNF-α secretion with the highest concentrations detected at PID 2 and PID 6 (3.5- to 4-fold above controls), corresponding to the viral shedding period (PIDs 1 to 6). However, later (PID 21), increased TNF-α concentrations were detected only in feces. For Th1 cytokines, a low IL-12 increase (11-fold above controls) was detected earlier in infection (PID 2), compared to the later increase in serum (PID 10). In feces, increased concentrations of IFN-γ were initially detected at PID 1 and were highest at PIDs 2 and 6 (2.7- to 3.6-fold above controls); similarly, the highest concentration of IFN-γ in serum was detected at PID 2. For Th2 (IL-4) and Th2/T-reg (IL-10) cytokine concentrations, earlier increases (PID 1, 11- to 13-fold above controls) were observed in fecal samples than in

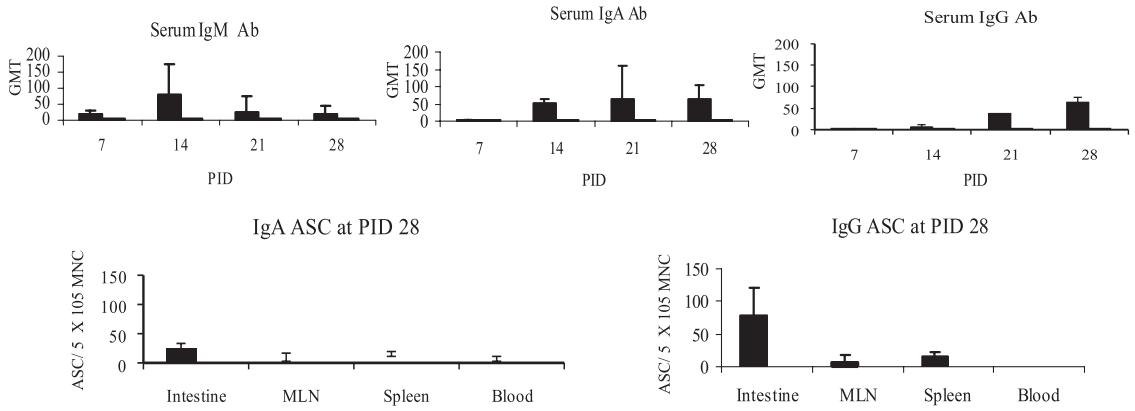

FIG. 3. Isotype-specific (IgM, IgA, and IgG) GMTs in the serum and mean numbers of ASC in intestine, MLN, spleen, and blood MNCs of Gn calves inoculated with HuNoV-HS66 (solid bars) or controls (open bars, baseline values).

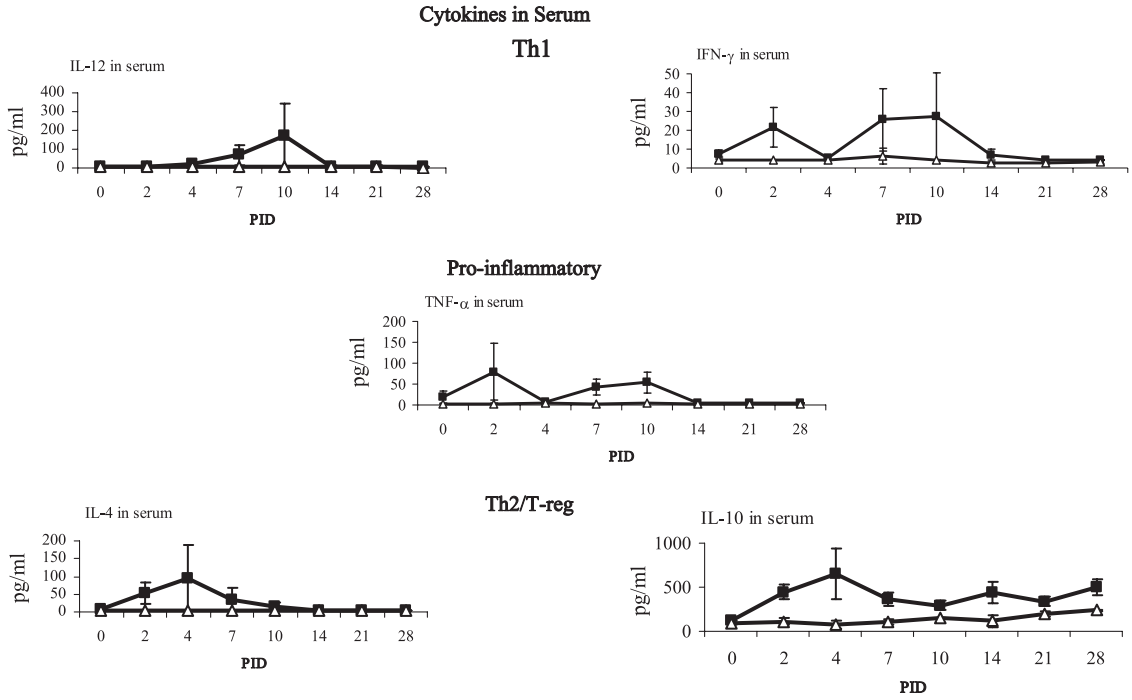

FIG. 4. Th1 (IFN-γ and IL-12), proinflammatory (TNF-α), Th2 (IL-4), and Th2/T-regulatory (IL-10) cytokine concentrations in serum of Gn calves inoculated with HuNoV-HS66 (solid squares) or mock controls (open triangles).

serum (PID 4, four- to ninefold above controls) (Fig. 4 and 5), with later increases at PID 6 or 10 and 21.

The cytokine concentrations were measured in the IC of infected calves and controls, after euthanasia at PIDs 3 ($n = 4$) and 28 ($n = 5$). All cytokine concentrations tested were higher in the IC of the HuNoV-HS66-inoculated calves than in the IC of control calves, but because of potential degradation of some cytokines in IC, the data were not shown. The highest mean

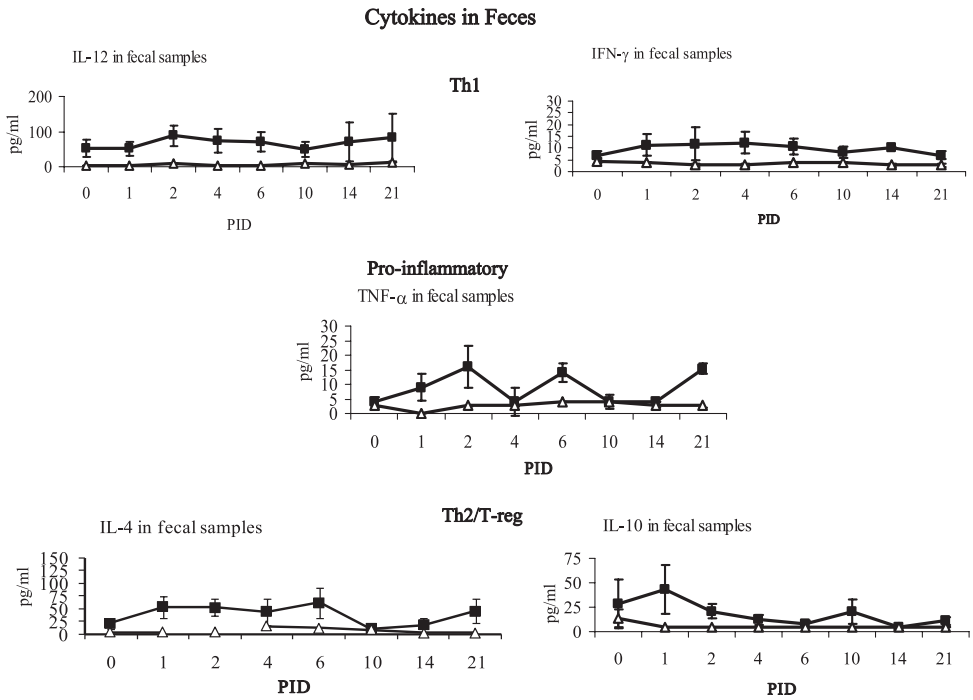

FIG. 5. Th1 (IFN-γ and IL-12), proinflammatory (TNF-α), Th2 (IL-4) and Th2/T-regulatory (IL-10) cytokine concentrations in fecal samples of Gn calves inoculated with HuNoV-HS66 (solid squares) or mock controls (open triangles).

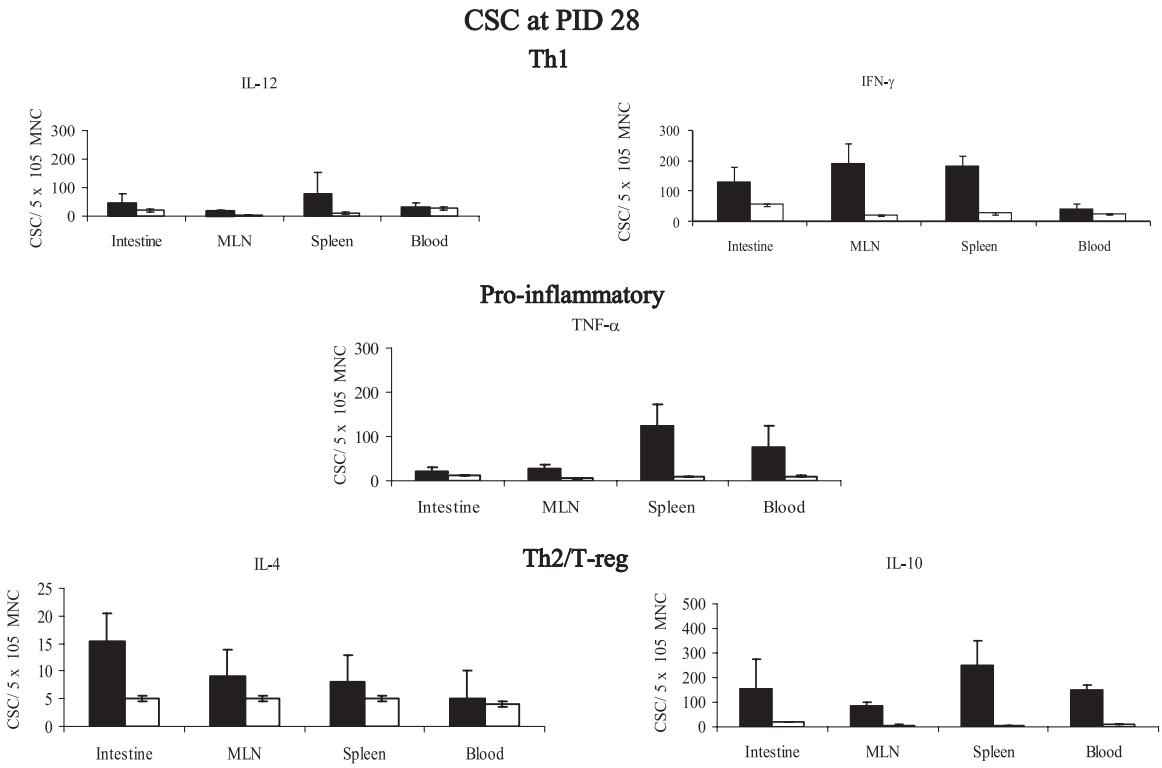

FIG. 6. Th1 (IFN-γ and IL-12), proinflammatory (TNF-α), Th2 (IL-4), and Th2/T-regulatory (IL-10) mean CSC numbers in intestine, MLN, spleen, and blood of Gn calves inoculated with HuNoV-HS66 (solid bars) or mock controls (open bars).

concentrations of TNF-α and IFN-γ were detected in the IC of the HuNoV-HS66-inoculated calves euthanized at PID 28 (6.5- and 41-fold above controls, respectively), compared to PID 3 (8- and 1.5-fold above controls, respectively). IL-12 and IL-4 were each detected in similar concentrations at both PID 3 and PID 28. Only IL-10 was detected at the highest mean concentration (4.4-fold above controls) early at PID 3.

In Gn calves HuNoV-HS66 induced high numbers of proinflammatory (TNF-α) CSCs systemically (spleen and blood) and high numbers of Th1 (IFN-γ) and Th2/T-reg (IL-10) CSCs both locally (MLN or intestine) and systemically (spleen). The numbers of CSCs were higher both locally (intestine and MLN) and systemically (spleen and blood) in the HuNoV-HS66-inoculated calves than in control calves (Fig. 6). The proinflammatory (TNF-α) CSC numbers were highest systemically in spleen and blood (12.5- and 8-fold above controls, respectively) and lowest in intestine and MLN (2- to 5.4-fold above controls) of the HuNoV-HS66-inoculated calves. Similarly, the Th1 IL-12 CSC numbers were highest overall in the spleen (sixfold above controls) and lowest in the MLN (4.5-fold above controls). In comparison, the highest IFN-γ CSC numbers were detected in spleen (sevenfold above controls), MLN (ninefold above controls), and intestine (2.6-fold above controls) with the lowest numbers in blood (1.8-fold above controls) of the HuNoV-HS66-inoculated calves compared to controls. Although the Th2 (IL-4) CSCs were detected in the lowest numbers, they were highest in the intestine compared to the other tissues of the HuNoV-HS66-inoculated calves. The numbers of Th2/T-reg (IL-10) CSCs both locally (intestine) and systemically (spleen) in the HS66-inoculated calves were the highest compared to the other cytokines.

DISCUSSION

Animal models are important to study the pathogenesis and immune responses to enteric caliciviruses, and we previously described the detailed pathogenesis (10) and host immune responses of the HuNoV-HS66 strain in Gn pigs (47).

In this study, we present new evidence that the preruminant Gn calf also constitutes an alternative experimental animal model to study HuNoV pathogenesis and host immune responses. Importantly, the occurrence of GIII NoVs in cattle also permits comparative studies of host-specific versus NoV adoptive host strains. In the present study, loss of epithelial cells in the villi, moderate to severe diffuse atrophic enteritis, and moderate to severe diffuse villous atrophy were detected mainly in the jejunum of a HuNoV-HS66-inoculated calf at PID 3. Increased MNC numbers and a few necrotic cells were observed in the lamina propria of this infected calf. In comparison, experimental infection of Gn calves with a GIII NoV strain (Newbury agent 2) showed more severe lesions also in the jejunum (19). The Gn calves experimentally infected with an unrelated bovine enteric calicivirus (unassigned genogroup), strain NB, also had intestinal lesions that were more severe in the duodenum and jejunum and milder lesions in the ileum (45). We further detected HS66 capsid antigens in enterocytes of the jejunum and in smaller numbers in the ileum, with positive signals also being detected in macrophage-like

cells in the lamina propria. No lesions or positive cells were present in any tissues of the mock-inoculated calves. In Gn pigs, only mild pathological changes were observed in the intestine after infection with the HuNoV-HS66 strain. Those changes included multifocal villous atrophy, virus enterocytes with low columnar morphology, and subtle edema of the lamina propria of the duodenum; no changes were observed in the jejunum and ileum. Viral antigen was distributed in a patchy manner in the villi of the duodenum and jejunum and only in a few cells of the ileum. Furthermore, all the HuNoV-HS66-infected pigs showed a higher number of apoptotic cells than did mock-inoculated pigs (10). The target cells for NoV replication in humans also appear to be the villous enterocytes of the proximal small intestine. Biopsies of the jejunum of adult volunteers who were infected with the GI NoV Norwalk virus (NV) or GII Hawaii virus showed broadening and blunting of the proximal intestinal villi with MNC infiltration and cytoplasmic vacuolization (1).

Viral shedding evaluated by RT-PCR, hybridization, and ELISA was also detected in fecal samples from all infected calves with a mean duration of shedding of 4 days by the hybridization assay. Both calves euthanized at PID 3 shed virus in feces from PIDs 1 to 3, and by real-time PCR, the peak viral shedding was observed in the feces of HuNoV-HS66-inoculated calves from PID 3 to PID 4. The IC of only one calf with intestinal lesions was positive for viral RNA. Diarrhea was observed in five/five (100%) infected calves, and viremia was detected in the serum of one calf, one/five (20%), at PID 2.

Sixty-seven percent of the HS66-inoculated calves seroconverted with low IgA and IgG Ab titers and with typical progression of primary Ab responses in serum (IgM and then IgA and IgG); similarly, 65% of the Gn pigs seroconverted by PID 21 or 28, with either IgA or IgG Abs to HuNoV-HS66 (47). Low titers of IgA Ab to NoV were also detected in the IC of two calves (67%), and low titers of IgA and IgG were detected in the IC of one of three calves euthanized at PID 28.

Experimental NV infection of nonhuman primates showed that common marmosets and cotton top tamarins shed virus for only 2 days, and only one of four NV-inoculated rhesus macaques, which shed NV for a long period in the feces, developed serum NV-specific IgM and IgG Ab responses. No IgA NV-specific Abs were detected in the plasma or saliva of this animal. Clinical signs were not observed in any of the animals (44). Data on the role of sIgA in HuNoV infections are conflicting; however, a correlation between salivary sIgA and protection against NV infection has been established recently, although not all susceptible individuals who did not become infected had strong salivary sIgA responses (29).

The numbers of ASC induced systemically (spleen and blood) in HuNoV-HS66-infected calves were generally low and similar to those in the Gn pigs infected with the HuNoV-HS66 strain (47), reflecting localized intestinal viral replication and transient viremia detectable in only one of the five HuNoV-HS66-inoculated calves but in 56% of the inoculated pigs. However, higher mean numbers of IgG ASC (77 ASC/5 $\times$ 10^5 MNCs) were detected in the intestine of the HS66-inoculated calves at PID 28 than in the HuNoV-HS66-inoculated Gn pigs (4 ASC/5 $\times$ 10^5 MNCs). The IgA ASC were detected in the highest numbers (27 ASC/5 $\times$ 10^5 MNCs) in the intestine of the HS66-inoculated calves and were higher than the mean intestinal numbers (8 ASC/5 $\times$ 10^5 MNCs) detected in the infected Gn pigs (47). The presence of the higher numbers of IgG and IgA ASC in the intestine of HS66-infected Gn calves corroborates the more extensive intestinal lesions observed in the calves than in the similarly inoculated Gn pigs (10).

In humans, seroconversion rates vary (50 to 90%) (34), local and secretory immune responses to HuNoV infections have been poorly documented (6, 38), and the association between local jejunal Ab titers and resistance to NV infection has not been established in adult volunteers orally exposed to this virus (17). Because most adult volunteers have preexisting Abs to HuNoV upon challenge, paired serum samples are needed for interpretation of the Ab responses to viral challenge. Primary immune responses cannot be easily assessed because adult humans are frequently exposed to these viruses during their lifetime. Thus, the Gn pig, together with the Gn calf model, may provide important information on the primary immune responses to HuNoV, and especially the local intestinal immune responses.

There is little information on the cytokine responses to HuNoV. The concentrations of the proinflammatory cytokine (TNF-α) increased in the serum of HuNoV-HS66-inoculated calves at PIDs 2, 7, and 10, compared to control calves, coinciding with the peaks of IFN-γ and the presence of diarrhea at PIDs 2 to 6. In the fecal samples, increased levels of TNF-α were observed acutely and later in infection, and higher levels were detected in the IC of HuNoV-HS66-inoculated calves at PID 3, compared to controls. At convalescence (PID 28), higher numbers of effector memory (HuNoV-HS66-VLP-stimulated) TNF-α CSCs in spleen and of IFN-γ CSCs in MLN, intestine, and spleen of HuNoV-HS66-inoculated calves were also detected.

A similar synergy between IFN-γ and TNF-α has been reported during *Toxoplasma* infections in mice (27), and increased levels of both cytokines were also detected in the serum of Gn pigs infected with virulent human rotavirus (4). Low to moderate levels of IL-6, another proinflammatory cytokine, were also detected in Snow Mountain virus (SMV)-challenged humans by Lindesmith et al. (28), and low to moderate levels of this same cytokine were also detected in the serum of Gn pigs infected with the HuNoV-HS66 strain at PID 4 (47), but IL-6 was not assayed in the Gn calves (due to a lack of available reagents).

Significant increases in IFN-γ secretion were also detected in the serum of volunteers 2 days after SMV challenge (28), coinciding with increased IFN-γ concentrations in the serum and fecal samples of HuNoV-HS66-inoculated Gn calves at PIDs 2 and 4, respectively, and corroborating the early increase of IFN-γ also seen in the serum of HuNoV-HS66-infected pigs (47). These findings confirm the early induction of IFN-γ responses during viral infection (41), including after HuNoV challenge of both humans and animal models. This early IFN-γ peak (PID 2) is likely due to an early innate immune response to viral replication (31), and the later peaks (PID 7 and PID 10 in serum and PID 4 in the fecal samples) were most likely elicited in response to the increased levels of the Th1 inducer (IL-12) at PIDs 2, 4, 6, and 10 in the feces and at PIDs 4, 7, and 10 in serum (12, 22, 50). The early increases of IL-12 in the serum and fecal samples (starting at PID 1) may represent the early innate responses of macrophages and DC to NoV infec-

tion (8). The prolonged elevation of IL-12 cytokine concentrations in feces through PID 21 and the presence of IL-12 CSCs mainly in spleen and intestine likely reflect induction of both intestinal and systemic Th1 responses. The induction of IFN-γ and TNF-α or their CSCs locally (fecal samples, IC, intestine, and MLN) and systemically (serum and spleen) may reflect the host response to virus replication and intestinal pathology observed, resulting in gut inflammation with induction of proinflammatory cytokines and also driving the later Th1 (IFN-γ and IL-12) responses observed.

The Th2 (IL-4) and Th-2/T-reg (IL-10) cytokines were also elicited early in infection both locally (fecal samples and IC) and systemically (serum) in HuNoV-HS66-infected calves. Significantly higher levels of IL-4 were also observed early in HuNoV-inoculated Gn pigs than in controls (47). Enhanced levels of IL-10 were also detected in the serum of HuNoV-HS66-infected Gn pigs at PIDs similar to those for the Gn calves but, however, at much lower concentrations (maximum of 15 pg/ml) than those in the serum of Gn calves (maximum of 650 pg/ml). The IL-10 level was significantly elevated early and detected at moderate to low levels at most PIDs in the serum of HuNoV-HS66-infected pigs (47).

Higher numbers of both IL-4 and IL-10 CSCs were also detected in the HuNoV-HS66-inoculated calves locally in intestine and also systemically for IL-10 (spleen) at convalescence (PID 28), compared to controls. However, the numbers of IL-10 CSCs in the intestine and spleen were dramatically higher than those of IL-4 (154 and 251 CSCs and 15 and 8 CSCs, respectively), corroborating the regulatory and anti-inflammatory actions of IL-10, both locally and systemically, during viral infections. When adult volunteers were challenged with SMV, no significant changes were detected between pre- and postchallenge concentrations of IL-10 in their serum samples, and significant changes were not detected between prechallenge and postchallenge peripheral blood mononuclear cell secretion of IL-4 or IL-10 after vitro stimulation with SMV (28). However, significantly higher levels of IL-5, another Th2 cytokine, were observed, confirming that the GII.2 HuNoV induced a Th1 immune response, but not exclusively. The differences in the IL-4 and IL-10 serum responses between calves and humans may reflect differences due to previous NoV exposure history in adult humans versus seronegative neonatal calves, requiring similar cytokine studies in NoV-infected seronegative infants to clarify these differences.

IL-10 is a regulatory cytokine in pigs and humans (15, 35, 37), with a role in the control of inflammation (15). Human IL-10 inhibits IFN-γ and TNF-α synthesis, and during parasitic infections in cattle, an increase in IL-10 with a decrease in IFN-γ synthesis was also detected (49). Increased levels of IL-10 were detected early in the fecal samples and again later, with higher levels also observed acutely in the IC of the HuNoV-HS66-inoculated calves. The higher IL-10 concentrations at PID 4 detected in the serum of the HuNoV-HS66-inoculated calves coincided with the decreased IFN-γ and TNF-α concentrations, both of which increased again at PID 7 after the concentrations of IL-10 diminished.

In conclusion, the HuNoV-HS66 strain induced low levels of Abs and low to moderate numbers of ASC both systemically and in the intestine with 67% seroconversion in calves. The HuNoV-HS66 induced both local (feces) and systemic (serum) low to moderate Th1 (IFN-γ and IL-12) and Th2 (IL-4) responses, corroborating our previous results for the induction of both Th1 and Th2 responses in Gn pigs by the same HuNoV strain. The early local and systemic peaks of the proinflammatory cytokine TNF-α and its increased concentrations in the IC of the HuNoV-HS66-inoculated calves during viral shedding and diarrhea, in concert with the intestinal lesions shown by histopathology, suggest that the HuNoV-HS66 strain induced more pronounced intestinal lesions in Gn calves than in the Gn pigs (10). Also the elevated early proinflammatory TNF-α response likely prompted the subsequent high anti-inflammatory IL-10 serum responses. Our results may reflect the wider prevalence of NoVs (GIII) observed in association with diarrhea in young calves, reflecting their more pronounced susceptibility to NoV infection (45). However, both species of Gn animals (calves and pigs) appear to be suitable models for the study of HuNoV pathogenesis and host immune responses.

This study provides new information on the enteropathogenicity, Ab levels, and cytokine secretion kinetics, both locally and systemically, in response to HuNoV-HS66 infection of Gn calves and supports the use of Gn calves as an experimental animal model for GII HuNoV infection and disease.

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