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Modulation by colostrum-acquired maternal antibodies of systemic and mucosal antibody responses to rotavirus in calves experimentally challenged with bovine rotavirus

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Abstract

The effect of colostral maternal antibodies (Abs), acquired via colostrum, on passive protection and development of systemic and mucosal immune responses against rotavirus was evaluated in neonatal calves. Colostrum-deprived (CD) calves, or calves receiving one dose of pooled control colostrum (CC) or immune colostrum (IC), containing an IgG1 titer to bovine rotavirus (BRV) of 1:16,384 or 1:262,144, respectively, were orally inoculated with $10^{5.5}$ FFU of IND (P[5]G6) BRV at 2 days of age. Calves were monitored daily for diarrhea, virus shedding and anti-BRV Abs in feces by ELISA. Anti-rotavirus Ab titers in serum were evaluated weekly by isotype-specific ELISA and virus neutralization (VN). At 21 days post-inoculation (dpi), all animals were euthanized and the number of anti-BRV antibody secreting cells (ASC) in intestinal and systemic lymphoid tissues were evaluated by ELISPOT. After colostrum intake, IC calves had significantly higher IgG1 serum titers (GMT = 28,526) than CC (GMT = 1195) or CD calves (GMT < 4). After BRV inoculation, all animals became infected with a mean duration of virus shedding between 6 and 10 days. However, IC calves had significantly fewer days of diarrhea (0.8 days) compared to CD and CC calves (11 and 7 days, respectively). In both groups receiving colostrum there was a delay in the onset of diarrhea and virus shedding associated with IgG1 in feces. In serum and feces, CD and CC calves had peak anti-BRV IgM titers at 7 dpi, but IgA and IgG1 responses were significantly lower in CC calves. Antibody titers detected in serum and feces were associated with circulation of ASC of the same isotype in blood. The IC calves had only an IgM response in feces. At 21 dpi, anti-BRV ASC responses were observed in all analyzed tissues of the three groups, except bone marrow. The intestine was the main site of ASC response against BRV and highest IgA ASC numbers. There was an inverse relationship between passive IgG1 titers and magnitude of ASC responses, with fewer IgG1 ASC in CC calves and significantly lower ASC numbers of all isotypes in IC calves. Thus, passive anti-BRV IgG1 negatively affects active immune responses in a dose-dependent manner. In ileal Peyer’s

Abbreviations: ASC, antibody secreting cells; BRV, bovine rotavirus; CD, colostrum deprive; CC, control colostrum; CCIF, cell culture immunofluorescence; IC, immune colostrum; IND, INDIANA, bovine rotavirus reference strain; LP, lamina propria; LIC, large intestinal content; MLN, mesenteric lymph nodes; MNC, mononuclear cells; PP, Peyer’s patches; RV, rotavirus; SIC, small intestinal content; VN, virus neutralization

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patches. IgM ASC predominated in calves receiving colostrum; IgG1 ASC predominated in CD calves. The presence in IC calves of IgG1 in feces in the absence of an IgG1 ASC response is consistent with the transfer of serum IgG1 back into the gut contributing to the protection of the intestinal mucosa.

Keywords: Bovine rotavirus; Maternal antibodies; ELISPOT; Mucosal immunity

1. Introduction

Group A bovine rotavirus (BRV) is a major cause of neonatal diarrhea in calves worldwide (Saif et al., 1994). Most calves are exposed to BRV by the third week of life and remain susceptible up to at least 8 weeks of age (Kohara and Tsunemitsu, 2000; Saif et al., 1994). Bovine rotavirus produces villous atrophy and malabsorptive diarrhea by infecting the apical villous epithelial cells of the intestine.

Given the early susceptibility of calves to BRV infection, local passive Abs (IgG1 and IgA) in the gut lumen acquired through colostrum and milk play an important role in protection against BRV infection and disease (Saif et al., 1983, 1987). In addition, high titers of passive circulating Abs have been shown to have a complementary role in protection against BRV diarrhea due to transfer of serum IgG1 into the intestine of neonatal calves (Besser et al., 1988a,b; Saif et al., 1983, 1987). A direct correlation between virus neutralizing (VN) Ab titers in serum of neonatal beef calves and protection against BRV disease under field conditions, has been described (Kohara and Tsunemitsu, 2000). Serum antibodies are essential in dairy calves which are separated from their dams after colostrum intake and fed subsequently with milk replacers lacking antibodies. Due to the significant economic losses caused by BRV infection in cattle, an inactivated vaccine, administered to pregnant cows has been used extensively since the middle 1980s (Saif and Fernandez, 1996). Immunization of dams greatly increases Abs in colostrum; and using effective vaccines by the intramammary route milk Abs are also increased (Fernandez et al., 1996; Kim et al., 2002; Saif and Fernandez, 1996; Saif et al., 1984). Hence, protection against diarrhea in neonatal calves born to vaccinated cows might be related to passive maternal Abs in serum acquired by intake of colostrum in dairy calves, and both serum (colostral) and intestinal (milk) antibodies for suckling beef calves.

The kinetics of primary antibody responses of different isotypes to BRV, as well as VN Ab responses have been described in colostrum-fed and colostrum-deprived gnotobiotic and conventional calves (Fernandez et al., 1998; Saif et al., 1983, 1987; Saif and Smith, 1985). However, the magnitude and distribution of antibody secreting cells (ASC) involved in the development of systemic and mucosal antibody-mediated immunity in the presence of passive maternal Abs, have not been evaluated in cattle. Studies conducted in mice and CD gnotobiotic pigs indicate that after rotavirus infection, anti-RV ASC are mainly located in the intestinal lamina propria and mesenteric lymph nodes, with very few ASC present in spleen and blood (Blutt et al., 2002; Hodgins et al., 1999; Yuan et al., 1996). Additionally, studies of passive protection in gnotobiotic and conventional pigs show that high titers of passive circulating or colostrally acquired maternal antibodies confer high protection rates against rotavirus infection and disease (Hodgins et al., 1999; Ward et al., 1996). However, high titers of passive maternal Abs also significantly reduce ASC responses to RV infection, in both systemic and gut-associated lymphoid tissues of pigs (Hodgins et al., 1999). In contrast, little is known about the ASC responses against RV infection in systemic and mucosal-associated lymphoid tissues of ruminants and their modulation by the presence of maternal Abs. The ASC responses in blood of gnotobiotic lambs after RV infection have been measured; anti-RV IgA and IgG ASC are present after 7 dpi (van Pinxteren et al., 1998). The aim of the present study was to investigate the development of the active Ab-mediated immune response against BRV in 2-day-old calves experimentally challenged with BRV, in the presence or absence of colostral Abs. The experimental design in giving calves a single oral dose of pooled control colostrum (unvaccinated cows) or immune colostrum (rotavirus vaccinated cows) shortly after birth, followed by milk without Abs, closely mimics the rotavirus vaccine and management
practice currently used in dairy cattle. A better understanding of mucosal immune responses against BRV infection in calves should improve immunization strategies to control gastrointestinal diseases in cattle.

2. Materials and methods

2.1. Experimental design

2.1.1. Colostrum feeding and calf inoculation

Newborn Holstein male CD calves procured from a dairy farm were removed from their mothers at birth prior to suckling and transported to isolation facilities within the first 4 h of life. The animals were located in individual isolation rooms under controlled conditions. Briefly, each animal had its umbilical cord disinfected with iodine, received an intramuscular dose of vitamins (Vitamin A, 500 U; Vitamin D3, 75 000 U; Vitamin E, 50 mg, BAGOAD3E, Bago Laboratories S.A., Bs. As., Argentina) and 80 mg of selenium (Selfos, Agroinsumos, Bs. As., Argentina), at entry. To prevent bacterial infections, the calves were maintained under antibiotic therapy consisting of 0.3 g per day of Gentamicin (Equi systems SRL, Martinez, Bs. As., Argentina) administered intravenously, and 1 g per day of Cefalosporin (Cefalomicina-BAGO Laboratories S.A., Bs. As., Argentina) administered intramuscularly for the first 10 days.

Five to eight calves were randomly assigned to the following colostrum feeding groups: colostrum-deprived (CD); control colostrum (CC) and immune colostrum (IC). Colostrum-fed calves received 1 l of control or immune colostrum pool within the first 6 h of life. After the initial colostrum intake, all the calves were fed 2 l of milk without antibodies (commercial sterilized bovine milk for human consumption; Mastellone Hnos, Bs. As., Argentina) twice a day. All calves were orally inoculated between the third and fourth feeding (approximately 36 h after colostrum intake) with 20 ml of inoculum containing 10^{5.5} FFU of virulent IND BRV, previously verified to cause diarrhea and virus shedding in 100% of inoculated CD calves.

2.1.2. Clinical observations and sample collection

Calves were observed for the onset of diarrhea and other associated clinical signs such as anorexia and hyperthermia (rectal temperature was registered daily), after BRV inoculation. Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semi-liquid; 3, liquid. A score equal to or greater than 2 was considered diarrhea. Fecal samples were collected before and after BRV inoculation daily for assessment of virus shedding. Serum samples were collected before the initiation of colostrum feeding (within 2 h after birth), at inoculation, and then weekly (7, 14, and 21 days post-inoculation, dpi). Antibodies in serum against BRV were detected by ELISA and VN. Coproantibodies were evaluated by ELISA. Peripheral blood lymphocytes were obtained at 0 dpi and then weekly to follow active immune responses by an enzyme-liked immunospot (ELISPOT) assay. At 21 dpi the animals were euthanized and the number of anti-RV ASC were quantified by ELISPOT in the mucosal-associated lymphoid tissues: duodenum, jejunum, and ileal lamina propria (LP); jejunum and ileal Peyer’s patches (PP); jejunum and cecal mesenteric lymph nodes (MLN) and in systemic lymphoid tissues: spleen and bone marrow. Large (LIC) and small (SIC) intestinal contents from all the calves were collected at 21 dpi for Ab detection.

2.1.3. Virus

Virulent IND BRV (P[5]G6) used for calf inoculation consisted of the intestinal contents of a CD calf orally inoculated with a fecal suspension containing virulent BRV from a gnotobiotic calf (kindly supplied by Dr L. Saif, The Ohio State University, USA). Briefly, a 2-day-old CD calf was orally inoculated with a fecal suspension containing 10^{5.3} FFU of IND BRV. The animal was euthanized 2 h after onset of diarrhea, and the entire intestinal contents were collected, pooled, aliquotted and stored at −70 °C. The pool had an infectious titer of 10^{6.5} FFU/ml and had the same double-stranded RNA electropherotype as the inoculated strain. No other viruses were detected in the intestinal contents by electron microscopy and no bacterial pathogens were identified by standard bacteriologic isolation techniques. The cell-culture adapted IND BRV was propagated in monkey kidney (MA-104) cells for use in ELISPOT, ELISA and virus neutralization (VN) assays.

2.1.4. Preparation of colostrum pools

For preparation of an immune colostrum pool, nine pregnant Holstein cows were vaccinated intramuscularly with three doses of 5 ml each of an attenuated
IND BRV vaccine at 45, 30, and 15 days before calving. The vaccine was formulated using the tissue culture supernatant of IND BRV (10^{7.33} FFU/ml) emulsified with an equal volume of incomplete Freund’s adjuvant. The entire first milking of each cow was collected. Whey samples were obtained from each milking as described previously (Fernandez et al., 1996) and the VN and ELISA Ab titers were determined. The immune colostrum pool was prepared from colostrum from the six cows with the highest Ab titers (anti-BRV IgG1 ranged from 65,536 to 1,048,560). The first milking of five non-vaccinated dairy cows from the same herd was collected to prepare the control colostrum pool (the milkings were selected to have an anti-BRV IgG1 titer of 16,384 to resemble the average IgG1 titers detected in a survey previously conducted in non-vaccinated dairy cows, data not shown). In both cases, the selected colostrums were pooled, mixed thoroughly, aliquoted and stored at −20 °C until use. Whey samples were prepared from each pool to determine VN and ELISA Ab titers.

2.1.5. Rotavirus antigen detection

Rotavirus shedding was detected in fecal samples using an Ag capture ELISA as previously described (Cornaglia et al., 1989). Polyacrylamide gel electrophoresis (PAGE) was performed on the fecal sample corresponding to the peak of virus shedding to confirm that the virus shed for each calf was the same as the challenge strain (BRV IND) (Laemmli, 1970).

2.1.6. Cell culture immunofluorescence assay

Virus infectious titer was assessed by a cell culture immunofluorescence (CCIF) assay (Hodgins et al., 1999). Briefly calf fecal samples were diluted 1:10 in MEM and clarified by centrifugation for 20 min at 1500 × g. Ten-fold serial dilutions of each sample were assayed in duplicate on 96-well plates containing MA-104 cell monolayers. After 48-h incubation at 37 °C, the cells were fixed with 70% acetone. Fluorescent foci were visualized using a FITC-conjugated hyperimmune bovine antiserum to IND BRV and fluorescent foci-forming cells were counted using a fluorescence microscope. Titters were expressed as the number of fluorescence forming units per ml (FFU/ml).

2.1.7. Isotype-specific antibody ELISA

IgM, IgA and IgG Abs to IND BRV were detected in colostrum pools, calf sera, feces, LIC and SIC. IgG1 and IgG2 were detected by an indirect ELISA using the same reagents as a protocol previously described (Fernandez et al., 1996, 1998). IgM and IgA were measured using capture ELISAs, adapted from previously described assays using an anti-bovine IgM mAb (kindly supplied by Dr. L.J. Saif, The Ohio State University, USA) or an affinity purified sheep antiovine IgA polyclonal antibody (Bethyl Laboratories Inc., Montgomery, Tex) as capture (Naslund et al., 2000; Van Zaane et al., 1986). The cross-reactivity of the latter commercial Ab was checked by immunodiffusion and non-specific reactions were not observed. The assays were standardized according to the Ab titers of known BRV positive serum and colostrum samples (Fernandez et al., 1996, 1998). Negative samples at a dilution 1:4 were assigned a titer of 1:2 for the calculation of geometric mean titers (GMTs).

2.1.8. Fluorescent focus reduction virus neutralization test

Virus neutralizing Ab titers to IND BRV in colostrum pools were determined by a fluorescent focus neutralization (FFN) test as described (To et al., 1998). The VN titer was expressed as the reciprocal of the highest sample dilution that resulted in a >80% reduction in the number of fluorescent foci.

2.1.9. Isolation of mononuclear cells (MNC)

Approximately 15 cm tissue samples of duodenum, jejunum, and ileum were collected for isolation of cells from the lamina propria. Discrete Peyer’s patches (n = 3) were identified at different points along the mucosal surface of the jejunum. Surrounding intestinal tissue was carefully separated and PPs were collected. A segment of intestine corresponding to a portion of the distal ileum continuous Peyer’s patch (10 cm cranial to the ileo-caecal junction) was also obtained.

To evaluate the anti-BRV ASC responses in MLN draining the small and large intestine, MLN corresponding to the jejunum and ileal-cecal regions were collected and processed separately. Peyer’s patches and MLN localization and sampling was supervised by an histo-pathologist.
Intestinal tissues were initially digested with dithiothreitol (GIBCO BRL, Grand Island, NY, USA) and collagenase type II (Sigma Chemical Co., St Louis, MO, USA). MNC suspensions were obtained by centrifugation through 30% Percoll (Amersham Pharmacia Biotech, Upsala, Sweden) followed by 43 and 70% density gradients of Percoll as previously described for pig tissues (Yuan et al., 1996).

Mononuclear cells from blood, spleen, and bone marrow were extracted to evaluate ASC responses in systemic lymphoid tissues. Bone marrow was collected from the proximal one-third of the femur and suspended in Ca$^{2+}$ and Mg$^{2+}$ free Hank’s balanced salt solution (GIBCO BRL, Grand Island, NY, USA). The tissue was passed through stainless steel 80-mesh screens of a cell collector (Cellector; E-C Apparatus Corp., Petersburg, FL, USA). Mononuclear cells from bone marrow and blood were isolated by Lymphoprep (NYCOMED PHARMA AS diagnostic, Oslo, Norway) density gradient centrifugation following the recommendations of the manufacturer. Purified MNC from all tissues were resuspended at a final concentration of $5 \times 10^6$ MNC/ml in RPMI-1640 (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% FCS, 20 mM HEPES, 2 mM Glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential aminoacids, 100 IU/ml penicillin, 67 μg/ml streptomycin and 50 μg/ml gentamycin (E-RPMI). The cell viability of each MNC suspension was assessed by Trypan blue exclusion (in all cases it was >95%).

2.1.10. ELISPOT assay for BRV-specific ASC

An ELISPOT assay for quantification of anti-BRV IgM, IgA, IgG1 and IgG2 ASC was used to evaluate effector B-cell responses in the inoculated calves. The assay was adapted from that conducted in pigs (Hodgins et al., 1999; Yuan et al., 1996). Briefly, IND BRV infected MA-104 cells in 96-well plates were fixed with 70% acetone, air dried, and stored at $-20\,^\circ C$ until used. The plates were used only if they showed $\geq 80\%$ infection by CCIF. The quality of plates was also confirmed by ELISPOT assay using RV-specific hybridoma cells (MA-49 clone, secreting VP7, G6 BRV specific mAb, kindly supplied by Dr. L.J. Saif, The Ohio State University, USA). Single-cell suspensions of MNC from each tissue were added to duplicate wells ($5 \times 10^3, 5 \times 10^4, 5 \times 10^5$ cells/well). After centrifugation at 500 $x$ g for 5 min, plates were incubated for 12–14 h at 37 $^\circ C$ in 5% CO$_2$. The plates were washed with PBS/0.05% Tween-20 to remove adherent cells and the spots were developed using two different protocols based on either mAb or polyclonal Abs. The assays using commercial polyclonal Abs were developed as less expensive and less labor intensive alternatives to the mAb antibody assays. All samples were evaluated using both ELISPOT systems.

2.1.10.1. Monoclonal antibody protocol. Plates were incubated for 2 h with mAb (from ascites) to bovine IgM (1:8000), IgG1 (1:20,000) and IgG2 (1:4000) (provided by Dr. L. Saif, The Ohio State University, USA) or a commercial monoclonal Ab to bovine IgA (1:1000) (clone: K84 2F9, from tissue culture supernatant SEROTEC, UK), followed by the addition of a 1:4000 dilution of goat biotin-labeled affinity purified Ab to mouse IgA + IgG + IgM (H + L) (KPL, Gaithersburg, MD, USA) for 2 h at room temperature. Plates were washed and then incubated with horseradish peroxidase-conjugated streptavidin (1:8000) (Boehringer-Mannheim, Indianapolis, IN, USA) for 45 min at room temperature. The spots were developed with a tetramethylbenzidine peroxidase substrate system (KPL, Gaithersburg, MD, USA).

2.1.10.2. Polyclonal antibody protocol. In order to standardize a less expensive and less time consuming procedure for the ELISPOT assay, duplicate plates were incubated in parallel with a set of commercial peroxidase-labeled sheep polyclonal Ab to bovine IgM (1:4000); IgG1 (1:10,000); IgG2 and IgA (1:1000) (Bethyl Laboratories Inc., Montgomery, Tex) for 2 h at room temperature and the spots developed as described above.

As a negative control in the CD calves used for technique standardization, samples were seeded in RV-infected and mock-infected cell monolayers. Non-specific spots were not detected in the latter. In addition, ASC were not detected in MNC collected prior to BRV inoculation in any of the experimental calves. ELISPOT plates were all enumerated by a single operator experienced in ELISPOT technique, in order to provide consistent interpretation.

The statistical concordance between ELISPOT assays developed using monoclonal and polyclonal
Abs to bovine Igs was determined using the Bland-Altman graphic method (data not shown) (Bland and Altman, 1995, 1999; Parreno, 2002). After analysis, the acceptable difference in spot counts between the two assays was: 9 spots for IgM, 6 spots for IgA and 4 spots for IgG1. The percentage of samples with discordant spot counts (higher that the described limits) was 4% for IgM, 4.4% for IgA and 6.7% for IgG1, over a total of 393 samples processed. The analysis indicated a good concordance between the assays. The final number of anti-RV ASC/5 × 10⁵ MNC for each tissue was determined as the mean number of blue spots obtained by both development systems (at least four replicates of each cell dilution were considered in the calculation).

2.2. Statistical analysis

Fisher’s exact test was used to compare proportions of calves with diarrhea and virus shedding among groups. The Kruskal Wallis rank sum (non-parametric) test was used to compare days to onset and duration of diarrhea and virus shedding, cumulative diarrhea scores, cumulative titers of virus shed and days with hyperthermia, among treatment groups. Neutralizing and isotype-specific Ab titers were log_{10}-transformed prior to statistical analysis. Differences in Ab titers among groups were evaluated by general analysis of variance (ANOVA) under a model of repeated measures throughout time, followed by the general contrast post-ANOVA test. At 21 dpi, the ASC numbers were compared among groups using the Kruskal-Wallis rank sum test. Statistical significance was assessed at \( P < 0.05 \) for all comparisons. All the statistical analyses were conducted using STATISTIX 4.1 (Analytical Software, Tallahasee, USA).

3. Results

3.1. Virus neutralization and isotype-specific ELISA antibody titers against IND BRV in colostrum pools

Titers of VN and IgG1 Abs to IND BRV in the bovine colostrum pools are summarized in Table 1. The immune colostrum pool VN and IgG1 titers were 16-fold higher than those of the control colostrum pool. The immune colostrum pool IgA titer was four-fold higher than that of the control colostrum pool. Both pools were negative for IgG2 to BRV; only the immune pool had very low IgM titers.

3.2. Passive antibodies in neonatal calves after colostrum feeding

After colostrum intake, IC calves (receiving immune colostrum) had significantly higher anti-BRV Abs, GMTs (IgG1 = 28,526; VN = 9410) in serum than CC calves (given control colostrum) (IgG1 = 1024; VN = 431) or CD calves (IgG1 and VN < 4) (Table 2; Fig. 1). Additionally, IC calves had significantly higher titers of IgG1 (IgG1 = 1024) in feces than CC calves (IgG1 = 117), at the time of oral)...
### Table 2

Fecal virus shedding and clinical disease after oral challenge of calves with BRV IND

| Treatment groups | n | IgG1 Ab GMT against BRV IND at inoculation | Virus shedding<sup>a</sup> | Diarrhea | Days with fever (>39°C) |
|------------------|---|------------------------------------------|-----------------------------|-----------|------------------------|
|                  |   | Serum | Feces | % animals shedding | Mean onset (dpi) | Mean duration (days) | Mean Peak shed virus (CCIF) | % affected animals | Mean onset (dpi) | Mean duration (days)<sup>b</sup> | Mean cumulative score<sup>c</sup> |
| Colostrum-deprived (CD), IgG1 < 4, VN < 4 | 5 | <4 C<sup>d</sup> | <4 C | 100% A<sup>e</sup> | 1.4 B (0.5) | 6.4 A (0.9) | 10<sup>3.4</sup> A | 100% A<sup>e</sup> | 1.2 B (0.4) | 11 A (3) | 29 A | 14 A (3.3) |
| Control colostrum (CC), IgG1 = 16384, VN = 65536 | 8 | 1024 B (1577) | 117 B (1394) | 100% A | 2 AB (0.9) | 10 A (5.7) | 10<sup>3.4</sup> A | 100% A | 2.4 AB (2.1) | 7 A (4.1) | 19 A | 9 A (5.5) |
| Immune colostrum (IC), IgG1 = 262144, VN = 1048576 | 5 | 28526 A (26922) | 1024 A (1988) | 100% A | 3.4 A (1.7) | 7.2 A (2.3) | 10<sup>3.4</sup> A | 60% A | 6 A (0.0) | 0.8 B (0.8) | 3.2 B | 7 A (3) |

Numbers in parentheses indicate standard deviation (S.D.). Unsuckled newborn calves received one dose of 1 l of pooled colostrum within the first 6 h of life. After colostrum intake, animals were fed 2 l of milk without Ab, twice a day. At 2 days of age calves were orally inoculated with BRV IND.

<sup>a</sup> Determined by ELISA and CCIF.

<sup>b</sup> Duration of diarrhea determined by number of days with fecal scores ≥2. Stool consistency was scored daily (0, normal; 1, pasty; 2, semi-liquid; 3, liquid).

<sup>c</sup> Mean cumulative scores from dpi 0 to 21 calculated as a measure of severity of diarrhea: (sum daily fecal score)/n.

<sup>d</sup> Means in the same column with different upper case letters differ significantly (Kruskal Wallis rank sum test; P < 0.05).

<sup>e</sup> Proportions in the same column with different upper case letters differs significantly (Fisher’s exact test, P < 0.05).
Fig. 1. Left axis: number of RV-specific ASC/5 x 10^5 MNC in peripheral blood (bars); right axis: geometric mean isotype and neutralizing Ab titer (GMT) against IND BRV in serum collected weekly (lines) from: (a) colostrum-deprived calves (CD); (b) calves receiving control colostrum (CC) or (c) immune colostrum (IC) and orally inoculated with IND BRV. No statistical differences were observed in the numbers of ASC detected in blood for all isotypes, among groups in the different time points (Kruskal Wallis non-parametric sum rank test, P < 0.05). Antibody titers with a (*) indicate a significant difference compared to CD calves at that time. Antibody titers with a (#) indicate a significant difference between CC and IC calves for that time point (repeated measures ANOVA model, P < 0.05).
inoculation (Table 2; Fig. 2). These IgG1 coproantibodies decreased to very low titers (GMT range: 4–16) in IC calves and were undetectable in CC calves by 3 dpi (Fig. 2).

3.3. Protection against diarrhea and virus shedding

3.3.1. High titers of passive IgG1 antibodies conferred partial protection against BRV diarrhea but failed to prevent infection

After inoculation with BRV, 40% (2/5) of IC calves were fully protected against disease, whereas the other three animals developed a delayed, less severe diarrhea of shorter duration (1–2 days) compared to CD and CC calves (means days: 11 and 7, respectively) (Table 2).

All animals in the three groups became infected. The mean duration of virus shedding (6–10 days) was not significantly different among groups (Kruskal Wallis, \(P = 0.643\)). The amount of virus shed was not significantly different either (mean peak titers: \(10^{5.4}\) to \(10^{4.8}\) FFU/ml, \(P = 0.451\); mean cumulative titers: \(10^{6.2}\) to \(10^{5.7}\) FFU/ml, \(P = 0.443\)). However, colostrum-deprived calves had the shortest mean duration of virus shedding and the group was homogenous, while calves receiving control colostrum had a high intra-group variability in the amount and duration of virus shedding (Fig. 3).

All calves receiving colostrum showed a delay in the onset of virus shedding, which was significantly delayed for IC calves (3.4 days) compared to CD
calves (1.4 days) (Table 2, $P = 0.02$; Figs. 2 and 3). All CD calves began to shed virus within 1–2 dpi. The delayed onset of virus shedding observed in some of the CC calves (3 dpi) and all the IC calves (3–6 dpi) was associated with the progressive decrease to titers of <1:16 in IgG1 Ab detected in feces (Fig. 2). All animals showed hyperthermia after the onset of infection, with a longer duration in CD calves compared to calves receiving colostrum (CC and IC), but not significantly (Table 2, $P = 0.06$).

3.4. Isotype-specific antibodies to IND BRV detected in feces and serum by ELISA and the active ASC responses in PBL

Isotype-specific and VN Ab responses to IND BRV in serum and the number of anti-RV ASC in PBL (detected weekly) are depicted in Fig. 1. Isotype-specific Ab responses to IND BRV in feces (evaluated daily), and in LIC and SIC (determined on 21 dpi) are shown in Fig. 2.
3.4.1. The presence of passive IgG1 circulating antibodies significantly suppressed active antibody responses in serum

The CD calves had a peak IgM response in serum at 7 dpi, concomitant with a strong IgA response (peak at 14 dpi), followed by an IgG1 response (Fig. 1). IgG1 was the predominant isotype by 21 dpi. Virus neutralizing Ab responses were also detected from 7 dpi on. The CC and IC calves maintained the initial serum levels of passively acquired IgG1 and neutralizing Abs. The CC calves developed an IgM response at 7 dpi and a delayed IgA response at 14 dpi, both of which were significantly lower than those in CD calves. No IgM or IgA responses were detected in sera of IC calves. IgG2 Ab responses were not detected in any animal during the 21 days of the experimental period (Fig. 1).

3.4.2. An active ASC response was detected in peripheral blood

No anti-BRV ASC were detected in blood of any of the calves at the onset of the experiment (0 dpi) (Fig. 1). An IgM ASC peak response was detected in PBL of all animals at 7 dpi, simultaneously with IgA ASC from 7 dpi on. Lower numbers of ASC were associated with increased titers of passive antibodies (CC and IC calves), although not significantly so, at any dpi. The highest numbers of IgM and IgA ASC were detected in the CD calves at 7 dpi. CD and CC calves, but not IC calves had low numbers of IgG1 ASC at 14 and 21 dpi (Fig. 1).

3.4.3. Clearance of infection was associated with fecal IgM and IgA antibody responses in CD and CC calves, but with an IgM antibody response and passive IgG1 in feces of IC calves

The CD calves developed high IgM, IgA and IgG1 coproantibody responses. IgM was detected from 3 to 18 dpi with a peak at 7 dpi; IgA was also detected early (4–5 dpi) and remained at high levels through 21 dpi; an IgG1 response was detected from 15 to 17 dpi through 21 dpi. The CC calves showed a similar profile of Ab responses for IgM, but their IgA responses were of lower magnitude and shorter duration compared to those of the CD calves. Low IgG1 titers were detected intermittently in feces of 3 out of 8 animals from this group. One of these three calves from the CC group had a longer period of BRV shedding (19 days) and diarrhea (13 days). This calf had bloody feces from day 10 to day 15; IgG1 Ab was detected in feces during that period and also from 18 dpi on. It is unknown whether other enteric pathogens besides BRV contributed to the severity of disease in this calf. The other two animals had mild diarrhea and IgG1 Ab was detected in feces from 14 to 21 dpi. In contrast, IC calves had high titers of passive IgG1 (ranging from 256 to 4096) in feces at the time of inoculation; these titers decreased sharply (ranging from 4 to 64) by 3 dpi, simultaneous with the onset of virus shedding. The IgG1 titers in feces stayed low during virus shedding, but increased slightly 1 day before the clearance of the infection, remaining present throughout the entire experiment (up to 21 dpi).

An active Ab response induced by BRV infection in IC calves was evident by an IgM peak in fecal samples at 7 dpi. However, no IgA Ab was detected in this group of calves, except for one calf which had a brief IgA response in feces during 6–12 dpi. In both CD and CC calves the resolution of BRV infection was associated with the presence of fecal IgM and IgA responses. In contrast, in IC calves, the cessation of virus shedding was associated with only an IgM response and low levels of passive IgG1 (Fig. 2).

3.4.4. At 21 dpi IgG1 antibodies predominated in the SIC of the three groups of calves and titers were higher than those in LIC or feces

At 21 dpi, all calves had IgG1 in their intestinal contents; only CD and CC calves had IgA, whereas IgM Ab were undetectable in the three groups of calves. In addition, in calves with both IgG1 and IgA coproantibodies, IgG1 titers were higher than IgA in both SIC and LIC (Fig. 2).

When comparing the Ab titers detected in different portions of the intestine of the same animal, IgG1 in SIC was always higher than in LIC or feces, in the three groups of calves, suggesting some degradation of intestinal antibodies during transit through the intestines.

When comparing the Ab titers in the corresponding intestinal contents among groups, IC calves had the highest geometric mean IgG1 titers in SIC (GMT CD = 111, CC = 128 and IC = 446; Kruskal Wallis, \( P = 0.198 \)). In contrast, both CD and CC calves had IgA in SIC, as an indicator of active response, while IC calves did not; IgA in CD was significantly higher than
in IC (GMT CD = 28, CC = 10, IC =< 4; Kruskal Wallis, \( P = 0.0017 \)).

3.5. Active ASC responses in systemic and intestinal lymphoid tissues at 21 dpi

3.5.1. Rotavirus-specific ASC responses in neonatal calves were mainly localized in the small intestine lamina propria and Peyer’s patches, followed by MNL and spleen, but not in bone marrow

The number of IgM, IgA, IgG1, and IgG2 anti-BRV ASC determined by ELISPOT are depicted in Fig. 4. Rotavirus ASC responses were detected in all the lymphoid tissues tested, except for bone marrow (data not shown), suggesting that in cattle, bone marrow is not a site of ASC colonization during the primary immune response to BRV. The majority of ASC were detected in the intestinal lamina propria (duodenum, jejunum and ileum), jejunal and ileal Peyer’s patches, and MLN draining both the small and large intestine, with fewer ASC in spleen and PBL.

3.5.2. IgM ASC response

At 21 dpi, CD calves showed reduced number of IgM ASC in all the tissues analyzed, suggesting that these calves were already in the convalescent period after infection. In contrast, higher numbers of IgM ASC were detected in most of the tissues of CC calves.

![Fig. 4. Mean numbers of anti-BRV ASC per 5 x 10^5/MNC obtained from mucosal-associated lymphoid tissues (MALT): duodenum LP, Jejunum LP and PP, Ileum LP and PP and MLN draining the small and large intestine and systemic lymphoid tissues: spleen, at 21 dpi. (a) Colostrum derived calves (CD), (b) calves receiving control colostrum (CC) and (c) calves receiving immune colostrum (IC). For each tissue, when comparing mean ASC numbers of the same isotype, among treatment groups: bars with (*) indicate a significant difference compared to CD calves. Bars with (#) indicate a significant difference between CC and IC calves (Kruskal Wallis rank sum test, \( P < 0.05 \)). No anti-BRV ASC were detected in bone marrow (data not shown). \( n = \) number of calves in each group.](image-url)
compared to those of CD calves, with significantly higher numbers in the duodenum and spleen. Immune colostrum calves showed fewer IgM ASC than CD calves for all the analyzed tissues, except for the ileal Peyer’s patches. High mean numbers of IgM ASC were detected in the ileal Peyer’s patches from the three groups of calves (CD = 25; CC = 22.5; IC = 46.5 IgM ASC/5 × 10⁵ MNC).

It is noteworthy that in most of the CD calves (4/5), IgG1 was the predominant isotype in the ileal Peyer’s patches, followed by IgM and IgA. The high mean IgM count in this group was due to one animal with an especially high response for this isotype (115 IgM ASC/5 × 10⁵ MNC). In contrast, in the calves receiving passive colostral IgG1, the IgM ASC predominated over IgG1 in an average ratio of 13:1 and 46:0 for CC and IC calves, respectively. For jejunal Peyer’s patches, IgM was also the predominant isotype of ASC in calves receiving colostrum, but not in CD calves.

3.5.4. IgG1 ASC responses

In CD calves, the active immune response against BRV was characterized by high numbers of IgG1 ASC in all tissues analyzed at 21 dpi, especially in the duodenum and ileum lamina propria, suggesting that IgG1 also plays an important role in calf mucosal immune responses, in the absence of passive IgG1. In contrast, fewer number of IgG1 ASC were detected in tissues of CC calves compared to CD calves, with significantly fewer numbers in the ileal lamina propria, ileal Peyer’s patches and spleen. Almost no IgG1 ASC were detected in IC calves.

3.5.5. IgG2 ASC response

Consistent with the lack of detectable titers of IgG2 in serum and feces, no IgG2 ASC were detected in blood at 7, 14, or 21 dpi, or in the systemic and intestinal lymphoid tissues of the CD and IC calves at 21 dpi. However, in the CC group two animals showed IgG2 ASC responses. One CC calf had IgG2 ASC in the spleen detected by both monoclonal and polyclonal reagents, while the other calf showed IgG2 ASC in the duodenum and jejunum detected only by the polyclonal reagent.

4. Discussion

In the present study we evaluated the development of active serum and fecal Ab responses to BRV infection and the numbers of anti-BRV ASC in systemic and mucosal-associated lymphoid tissues in conventional calves under the influence of high or low maternal Abs. Colostral IgG1 ingested by the neonate during the first hours of life are mainly transferred intact across the small intestinal absorptive epithelium into the calf circulation (and any compartments in equilibrium with the circulation). The amount of transferred immunoglobulin has been reported to increase from the cranial to caudal small intestine, with the ileum being the region of major absorption in calves (Jochims et al., 1994). Passive circulating Abs, although associated with protection against RV infection, are also known to interfere with development of active immune responses in neonates (Aldridge et al., 1998; Hodgins et al., 1999; Parren et al., 1999). Hence, in this study, these two properties (protection and immune interference) were evaluated. In this study Ab titers in control and immune colos- strum were selected to resemble those present in colostrum from non-vaccinated and vaccinated cows, respectively, in order to evaluate their impact based on currently used parenteral inactivated maternal vaccines.

At oral inoculation (0 dpi), approximately 36 h after colostrum intake, IC calves had significantly higher Ab titers in both serum and feces than CC calves. Passively acquired Ab titers (IgG1, VN) initially detected in serum remained high throughout the experiment. In contrast, fecal anti-BRV IgG1 detected after colostrum feeding decreased sharply by 3 dpi
(5 days of age), suggesting a progressive elimination of non-absorbed colostral antibodies in feces within the subsequent 4 days. It has been reported previously that calves receiving 8 l of colostrum with low titers of anti-BRV Abs within the first 48 h of life, followed by milk without Abs, have fecal IgG1 up to 6 days of age (Van Zaane et al., 1986).

The IC calves had detectable IgG1 Ab in feces throughout the experiment, and had the highest IgG1 titers in their SIC at 21 dpi. The fecal IgG1 titers were reduced during virus shedding, but immediately increased (2–3-fold) thereafter to remain approximately constant. This observation, together with the absence of IgG1 ASC in peripheral blood (analyzed weekly) or intestinal and systemic lymphoid tissues (tested at 21 dpi) in this group of calves, strongly suggests that the IgG1 detected in feces is due to transfer of passive circulating IgG1 from the serum to the gut. This transport took place at least up to 22 days after colostrum intake. The intestinal elimination of IgG1 is proposed to be the main route of passive IgG clearance in calves; the amount of IgG1 excreted into the intestine is proportional to its serum concentration (Besser et al., 1988a,b). The transfer of IgG from the serum to the gut has also been reported in gnotobiotic pigs injected intraperitoneally with maternal Abs and in conventional pigs fed colostrum with high anti-RV Ab titers (Hodgins et al., 1999; Ward et al., 1996). However, the transfer mechanism remains unknown for both species. The recent detection of a FcRn receptor capable of bi-directional and pH dependent IgG transport in the gut epithelium of rodents, humans and lambs suggests that a similar receptor could be involved in this process in calves (Antohe et al., 2001; Borvak et al., 1998; Dickinson et al., 1999; Israel et al., 1997; Mayer et al., 2002; Simister and Rees, 1985).

After BRV inoculation, all calves in the three groups became infected. The onset and duration of virus shedding and diarrhea in CD calves were similar to those reported in previous studies (Fernandez et al., 1998; Saif et al., 1987). However, in both groups of calves receiving colostrum, the onset of virus shedding was delayed until fecal IgG1 titers decreased to 1:16 or lower. As described in previous studies in which calves were fed sterilized milk supplemented with 1% immune bovine colostrum daily through 5–7 days of age (Fernandez et al., 1998; Saif et al., 1987), the presence of local IgG1 plays a major role in protection against BRV infection and diarrhea. In the present study although calves receiving one dose of control colostrum within 6 h after birth were not protected against BRV infection, they showed a slightly delayed diarrhea of shorter duration compared to CD calves. Higher titers of passive colostral IgG1 failed to prevent BRV infection, but significantly reduced BRV diarrhea in IC calves. The protection observed in these calves might be due to neutralization of BRV by a combination of residual colostral Abs present in the intestine at the time of inoculation and IgG1 transferred into the gut from the circulation (Besser et al., 1988a; Kohara and Tsunemitsu, 2000). However, it is important to note that clearance of virus shedding in IC calves was observed only after the development of an active IgM response in the gut.

The ELISA and VN data showed that CD calves developed strong primary immune responses in both serum and feces, which allowed a rapid clearance of the BRV infection. The CC calves had significantly lower IgM and IgA Ab responses in serum and delayed responses in feces compared to CD calves, whereas the antibody responses of IC calves were even more strongly suppressed. The short duration of virus shedding in CD calves, associated with a strong local IgM and IgA response and the clearance of infection in colostrum-fed calves only after an IgM response, highlight the importance of local active immunity in resolving BRV infection.

The results obtained in calves are consistent with previous findings in gnotobiotic pigs receiving different titers of passive circulating Abs (Parreño et al., 1999). In the present study it was found that low passive IgG1 Ab titers (CC calves) impaired the development of active immune responses and were not enough to protect against infection and disease. This observation together with the high incidence of BRV in cattle, clearly indicates that calves born to non-vaccinated dams are highly susceptible to BRV diarrhea. On the other hand, the protective effect of high colostral IgG1 titers, which significantly reduced BRV diarrhea but not virus shedding, help to explain why the current commercial vaccines are not effective in preventing BRV infection, although they significantly reduce morbidity, severity of diarrhea, and mortality in field trials (Bellinzoni et al., 1989; Cornaglia et al., 1992). The design of improved parenteral vaccines capable of inducing high titers of Ab directed
to the neutralizing antigens of BRV in colostrums and milk would be the best option to control BRV disease in beef herds.

The absence of detectable titers of IgG2 Ab to BRV in serum following inoculation and the detection of IgG2 ASC to BRV in only two calves suggests that antibodies of this isotype play a minor role in immune responses of neonatal calves to this virus, and are not essential for recovery from BRV infection.

A noteworthy finding in this study was the strong IgA response detected in serum of CD and CC calves. These data agree with the high titers of serum IgA described in gnotobiotic pigs (monogastric) and lambs (ruminants) after RV infection (Parreño et al., 1999; To et al., 1998; van Pinxteren et al., 1998), but disagree with the very low IgA responses detected in both CD and colostrum-fed calves, in previous reports (Fernandez et al., 1998; Saif et al., 1987; Van Zaane et al., 1986). This discrepancy could be explained by the higher analytical sensitivity of the capture ELISA used here. This assay avoided inter-isotype competition that can impair the detection of isotypes present in lower titers (as is the case with IgA in the presence of high titers of passive IgG1). High IgM and IgA responses against bovine coronavirus have been detected in serum, colostrum and milk using a similar approach (Naslund et al., 2000).

In the three groups of calves, anti-RV ASC were detected in peripheral blood from 7 dpi on. The ASC response profile observed in blood of CD calves was similar to that reported in gnotobiotic pigs (Yuan et al., 1996), but differed from the profile described in gnotobiotic lambs, in which high numbers of both anti-RV IgA and IgG ASC were detected after 7 dpi (van Pinxteren et al., 1998). The inverse relationship observed between the numbers of ASC detected in blood and the amount of colostral maternal antibodies present in serum agree with results reported in pigs (Hodgins et al., 1999).

Study of the distribution of RV-specific ASC in different lymphoid tissues at 21 dpi showed that anti-RV ASC were present in all the tissues analyzed, except bone marrow, as reported previously for piglets (Yuan et al., 2001). In contrast, studies conducted in mice after systemic viral infections show the bone marrow to be one of the major sites of ASC localization (Ochsenbein et al., 2000; Sangster et al., 2000). These differences highlight the compartmentalization between the mucosal-associated and the systemic immune systems.

The ASC responses in CD calves (in the absence of maternal Abs) were characterized by high numbers of IgG1 ASC in all tissues analyzed. The intestinal lamina propria (especially duodenum) was the main site of IgA ASC localization; both IgA and IgG1 ASC counts were high. In contrast, in Peyer’s patches, MLN and spleen, the number of IgG1 ASC clearly predominated over IgA. These observations are in agreement with those in a recent study conducted in mice, where B cells activated in the gut lamina propria or in the Peyer’s patches switched preferentially to IgA, but B cells activated in the gut lamina propria or in the Peyer’s patches but homing into Peyer’s patches switched to IgG with higher efficiency (Fagarasan et al., 2001). This differential isotype switching might be related to differential microenvironments among different lymphoid tissues. Immunological studies have indicated that transforming growth factor β (TGF-β) together with IL-4, IL-5 and IL-6 (in mice and humans, but not in cattle) or TGF-β with IL-2 and IL-10 and the recently identified factor IgA-inducing protein (IGIP) (in cattle) are the factors related to IgA switching and production (Austin et al., 2003; Brandtzaeg et al., 1997; Griebel and Hein, 1996; Stavnezer, 1995).

At 21 dpi, CC calves had lower IgG1 ASC, along with higher IgM ASC numbers in most analyzed tissues compared to CD calves, except for the duodenum and jejunum lamina propria. The reduction of IgG1 ASC was especially noticeable in MLN, spleen and blood (all tissues in close contact with circulating Ab). In contrast, although higher numbers of IgM ASC were detected in the lamina propria of the duodenum and jejunum of CC calves, the numbers of IgG1 ASC were unaffected by low levels of passive IgG1 Ab. The observed shifts in ASC response profiles suggest that low titers of IgG1 passive Abs may impair or delay isotype switching. The strongly suppressed ASC responses observed in IC calves, clearly indicate that passive colostral IgG1 suppress B cell responses in a dose-dependent manner.

The discovery of a T-cell independent pathway of induction of secretory IgA in the gastrointestinal mucosa (Litinskii et al., 2002; Macpherson et al., 2000), opens new questions about intestinal immune responses to rotaviruses. It is postulated that T-cell
independent pathway(s) are involved in production of secretory IgA antibodies (destined for the gut lumen), predominantly by B1-cells located in the lamina propria. In contrast serum IgA antibodies are considered to be products of T-dependent pathway(s) with B2-cells in organized lymphoid tissues being responsible. Both serum and fecal IgA antibodies to BRV were documented in the present study, and similar results have been reported for human infants (Ward et al., 1997; Bishop et al., 1996) and gnotobiotic pigs (Parreño et al., 1999), suggesting multiple pathways for generating IgA Ab responses to rotaviruses.

The ASC responses in ileal Peyer’s patches were noteworthy. In CD calves, IgG1 ASC were predominant over IgA and IgM in most of the calves. In contrast, in calves receiving colostrum an increased number of IgM ASC were detected with a reduction (CC calves) or complete absence (IC calves) of IgA and IgG1 ASC. In calves, as in sheep, the B-cell/T-cell composition of jejunal Peyer’s patches corresponds to that of a secondary lymphoid organ. This was also evident in this study for B cells where the ASC responses in the jejunal PP of all groups mirrored the corresponding ASC in spleen (Fig. 4). In contrast, ileal Peyer’s patches contain 95% B cells (Parsons et al., 1989) and are postulated to mainly function as primary lymphoid organs. In a recent study conducted in a neonatal lamb model of mucosal immunity, no ASC were detected after direct antigen inoculation into intestinal loops containing ileal Peyer’s patches, suggesting their non-participation in the immune response (Mutwiri et al., 1999). In contrast, the results of the present study show an active participation of the calf ileal Peyer’s patches in the antibody response against infection with BRV. Finally, the accumulation of high numbers of IgM ASC in the ileal Peyer’s patches of IC calves suggests that passive IgG1 might inhibit isotype switching from IgM to IgG1 and IgA at early stages of the mucosal immune response. Further studies are needed to better define the function of the different bovine Peyer’s patches in mucosal immunity.

5. Conclusions

To our knowledge, this is the first description of the magnitude and distribution of ASC responses against BRV infection and their modulation by passive colostral Abs in calves. The effector B-cells responsible for the antibody-mediated responses against BRV infection in calves were mainly localized in the intestinal lamina propria and Peyer’s patches, followed by MLN and spleen. The bone marrow was not involved in the effector Ab responses against BRV. The intestinal lamina propria, especially of the duodenum, was the major site of anti-BRV IgA ASC localization. We further demonstrated that passive colostral Abs negatively affected the development of the mucosal and systemic Ab-mediated immune responses against BRV, in a dose-dependent manner. The presence of IgG1 in feces and intestinal contents of the calves receiving high titers of IgG1 (immune colostrum), in the absence of active IgG1 ASCs in the intestine, supports the theory of IgG1 transfer from serum to gut, as previously reported in calves.

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