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ANTIBODY RESPONSES IN SPIRAL COLON, ILEUM, AND JEJUNUM OF BOVINE CORONAVIRUS-INFECTED NEONATAL CALVES

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Abstract—A preliminary study was conducted to compare the regional intestinal immune responses of neonatal calves inoculated with virulent or attenuated bovine coronavirus (BCV) to determine the cause of reported vaccine failures. A group of 9 newborn, colostrum-deprived calves was used; two calves were inoculated with attenuated virus, four calves were infected with virulent virus (including one naturally infected calf), and three calves were uninfected controls. Calves inoculated with virulent virus produced higher titers of BCV antibodies in the intestines than those inoculated with the attenuated virus. The failure of the calves to respond to the attenuated virus was apparently due to the inability of the virus to replicate to high titers. Spiral colon, ileum, and jejunum were found to be immunologically distinct; the highest anti-BCV antibody responses were detected in spiral colon, the primary site of infection, and involved all four isotypes of bovine immunoglobulins. The antibody response in ileum was lower than in spiral colon. The immune responses developed slowly in jejunum and were associated primarily with the IgG subtypes.

Key words: Bovine coronavirus, enteric disease, mucosal immunity, calf scours, regional immunity.

Résumé—Une étude préliminaire a été réalisée pour comparer locale la réponse immunitaire au niveau intestinal de veaux nouveau-nés infectés par un coronavirus bovin virulent ou atténué et pour déterminer les causes d'échec des vaccinations. Un groupe de neuf veaux nouveau-nés, privés de colostrum, a été utilisé: deux veaux ont été infectés par un virus atténué, quatre par un virus virulent (incluant un veau naturellement infecté), et trois non infectés servent de témoins. Les veaux inoculés par le virus virulent ont produit un titre en anticorps BCV plus important au niveau de l'intestin que ceux inoculés par le virus atténué. La différence de réponse chez les veaux inoculés par le virus atténué semble due à l'impossibilité du virus de se répliquer à des hauts titres. Le colon spiral, l'ileum et le jejunum ont présentés une réponse immunologique différente; les plus hauts titres d'anticorps étaient trouvées dans le colon spiral, site primaire de l'infection et impliquaient quatre isotypes d'immunoglobulines bovines. La réponse immunitaire était plus faible dans l'ileum que dans le colon spiral. Elle s'est développée lentement dans le jejunum et était associée primitivement à des sous types d'immunoglobulines G.

Mots-clefs: Coronavirus bovin, maladie enterifique, immunité des muqueuses, maladies du veau, immunité locale.

INTRODUCTION

Bovine coronavirus (BCV) is an important cause of scours in neonatal calves of up to 1 month of age [1] and may also cause pneumoenteritis in some cases [2]. The use of oral attenuated virus vaccine has been reported to be ineffective under both field [3] and

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experimental situations [4]. The reasons of vaccine failure are not known but may be attributed to an unsuitable vaccine or to the lack of immune responsiveness of newborn calves. Since the mechanisms responsible for immune protection in BCV infection are not known, the evaluation of vaccine response is difficult. Intestinal immune responses appear to be important because passive oral transfer of monoclonal antibodies has been found to offer some protection against BCV infection [5]. In other intestinal infections, such as rotavirus infection, protection correlates well with the intestinal immunity but not with serum immunity [6].

Different regions of the gut may differ in immune responsiveness to BCV infection. This virus primarily infects the spiral colon but has also been detected intermittently in other areas of the gut e.g. ileum and jejunum [7]. Saif *et al.* [8] have reported different levels of the virus in spiral colon, ileum, and jejunum during acute BCV infection. Since different regions of the intestine have variable levels of involvement in BCV infection, it is necessary to evaluate regional immune responses against BCV. The isotype-specific immune responses against BCV and its structural proteins have been studied [9]. The present study was undertaken to evaluate the anti-BCV immune responsiveness of different regions of the intestine in 0–4 week old calves.

MATERIALS AND METHODS

Experimental infection

The details of experimental animals and viruses used in this study have been described earlier [2, 7]. Briefly, nine colostrum-deprived, unvaccinated, male, Holstein calves were divided into three groups and were inoculated orally at the age of 5 days with a tissue culture-adapted, attenuated BCV (Mebus strain; calves 1 and 9) or a virulent isolate of BCV (calves 2, 3, 6 and 7; calf 3 being a naturally infected calf) or virus-free diluent (calves 4, 5 and 8).

Table 1. Levels of immunoglobulins in the intestines of neonatal calves as detected by single radial immunodiffusion

Calf inoculation	Amount of various immunoglobulins ($\mu\text{g/ml}$) at indicated age in days				
	2†	7	12	19	26
	<i>Immunoglobulin M</i>				
Attenuated virus	—	93	103	68	293
Virulent virus	69	1270	1366	1130	105
Uninfected control	318	ND††	420	1407	1377
	<i>Immunoglobulin A</i>				
Attenuated virus	—	—	—	—	12
Virulent virus	—	420	485	1553	100
Uninfected control	—	ND	83	343	223
	<i>Immunoglobulin G₁</i>				
Attenuated virus	—	37	54	34	106
Virulent virus	270	1143	359	491	137
Uninfected control	91	ND	218	534	1012
	<i>Immunoglobulin G₂</i>				
Attenuated virus	—	—	—	—	—
Virulent virus	—	20	13	9	12
Uninfected control	—	ND	—	—	—

†Prior to infection.

††ND = not done.

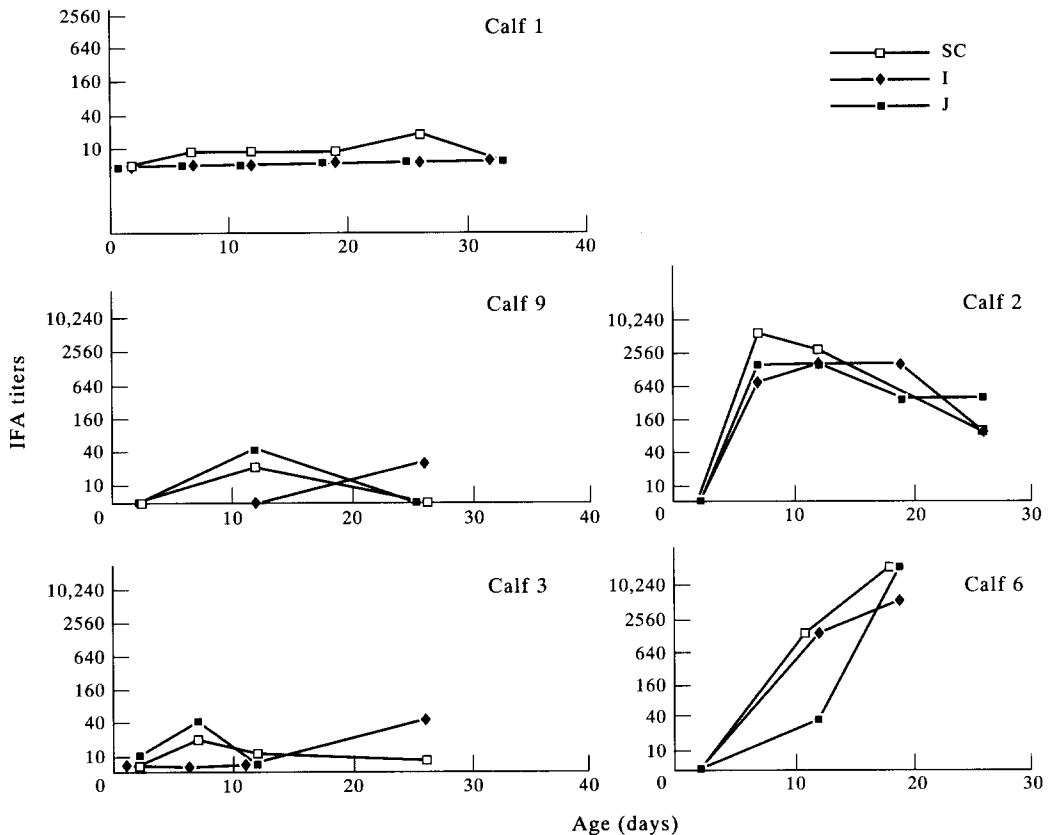


Fig. 1. Anti-BCV antibody responses as measured by indirect fluorescent antibody test in calves infected with attenuated virus (calves 1 and 9), naturally infected calf (calf 3), and calves inoculated with the virulent virus (calves 2 and 6). IFA = reciprocal of indirect immunofluorescence titers; SC = spiral colon; I = ileum; and J = jejunum.

Laparotomy

Right flank laparotomies were performed prior to infection and at weekly intervals thereafter to collect intestinal contents from spiral colon, ileum and jejunum. Each calf was operated on twice with a third sample being obtained at euthanasia. The schedules were staggered to cover all needed times between calves in a group. The calves were sedated with xylazine hydrochloride (Rompun; 0.02–0.05 mg/kg intravenously and 0.04–0.1 mg/kg intramuscularly) and were restrained in left lateral recumbency. The right flank was clipped and the paralumbar fossa prepared for aseptic surgery. A regional nerve block was done with 20–30 ml of 2% Lidocaine in an inverted L pattern. A 10–15 cm vertical incision was made in mid-flank. The muscle layers were separated in a grid pattern and peritoneum was sharply incised. The spiral colon, ileum, and jejunum were exteriorized for sample collection, the order of collection being the same for all calves on all occasions. A 2-inch section of the bowel in each region was isolated by digital compression. 2 ml of sterile saline was infused into the isolated segment and immediately aspirated via enterocentesis using a 12 ml syringe with a 20 or 22 gauge needle. The flank incision was closed in a routine

manner. After collection, the samples were transported on ice to the laboratory and centrifuged at 4000 *g* for 20 min. The supernatants were stored at -70°C .

Single radial immunodiffusion (SRID)

Polyclonal antisera with specificity to bovine immunoglobulin classes (IgA, IgM) and subclasses (IgG1 and IgG2), and reference standards (44₁ colostral and serum) with predetermined amounts of immunoglobulins for preparing standard curves were obtained from Dr Hans Fey, University of Bern, Switzerland. The highest dilutions of antisera that produced clearly visible precipitation rings in 96 h were considered optimum for incorporation in agarose; these were 0.8, 1.0, 2.0 and 3.0% (v/v) for IgA, IgM, IgG2 and IgG1, respectively. 20 ml of agarose (containing antiserum) was poured on glass slides (8.3 × 10.2 cm, Eastman Kodak, Rochester, N.Y.). The sample wells (4 mm) were punched and 20 μl of the sample was loaded in each well. A battery of reference standards was run simultaneously on each plate. The plates were incubated in humid conditions for 96 h and the diameters of precipitation rings were measured using a hand held comparator with a measuring scale. For confirmation, the precipitation rings were stained with amido black [10].

Indirect fluorescent antibody (IFA) test

Madin Darby bovine kidney (MDBK) cells infected with attenuated, Mebus strain of BCV were placed on 12-well slides (Cell Line Associates, Newfield, N.J.) using 1000–1500 cells per well in 20 μl of Eagle's MEM containing 4% fetal bovine serum. After incubation at 37°C for 2–3 days, the slides were washed with PBS (pH 7.2), fixed in cold acetone for 10 min, dried, and stored at -70°C until used. 2-fold dilutions (20 μl) of intestinal contents were placed on infected fixed cells followed by incubation at 37°C for 1 h under humid conditions. After washing with PBS (pH 7.2), 20 μl of rabbit anti-bovine FITC conjugate was added. The slides were incubated again for 1 h, washed with PBS (pH 8.5), counterstained with Evans blue, and were examined under a fluorescent microscope. The antibody titer of the sample was considered to be the reciprocal of the highest dilution showing detectable fluorescence.

Isotype specific ELISA

Microtiter, 96-well plates (Immunolon I; Dynatech lab., Alexandria, Va) were coated with ammonium sulfate precipitated monoclonal antibodies against bovine IgM (DAS 6, 50 ng), IgA (DAS 7, 1 μg), IgG1 (DAS 17, 100 ng) and IgG2 (DAS 2, 2 μg), dissolved in coating buffer (carbonate–bicarbonate, pH 9.6). After coating overnight at 10°C , the plates were washed 3 times with ELISA buffer (PBS, pH 7.4). Unoccupied sites were blocked with 2% casein enzymatic hydrolysate (CEH) for 2 h at 37°C under humid conditions. The ELISA procedure had several consecutive steps with 1 h of incubation at 37°C under humid conditions at each step. Washing at each step was done with PBS containing 0.05% Tween 20 and 0.1% CEH. Sample dilutions (1:5–1:50) prepared in PBS–CEH–Tween 20 were added to appropriate wells followed by the addition of BCV. A second site antibody to BCV (1:100 dilution of polyvalent porcine anti-BCV) was then added, followed by the addition of a 1:250 dilution of goat anti-porcine IgG labelled with horseradish peroxidase. The color development was done with substrate azinobis–ethyl–benzthiazolinesulfonic acid (ABTS) for 15 min and the reaction was stopped with hydrofluoric acid (1:400). The OD readings were taken at λ 405. The samples were considered positive if the ratio of sample

to negative OD (S/N) was ≥ 2.0 . In all isotype-specific ELISAs, strong positive, weak positive, and negative controls were included. Optimum working concentrations of various reagents (capture monoclonal antibodies, bovine coronavirus, porcine anti-BCV antiserum, antiporcine IgG conjugate) were determined by checker-board titrations and dilutions that gave the highest difference between positive and negative controls were used. The specificity of each reactant was verified by eliminating it from one of the columns of the plate, thus preventing the reaction of the positive controls. Each sample dilution was run in triplicate.

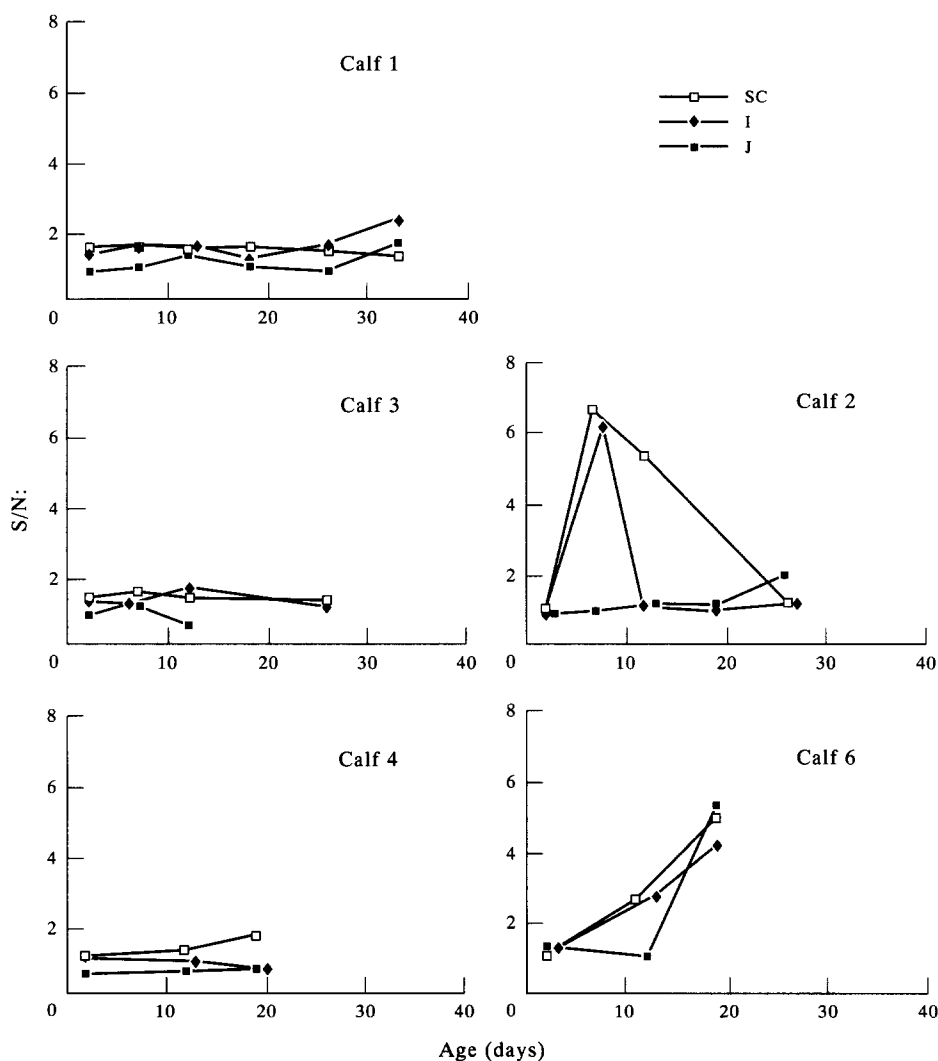


Fig. 2. Anti-BCV IgM responses as measured by antibody capture ELISA assay in calves after infection with attenuated (calf 1) and virulent viruses (calves 2 and 6) and in naturally infected (calf 3) and uninfected calves (calf 4). S/N = sample to negative ratio; SC = spiral colon; I = ileum; and J = jejunum.

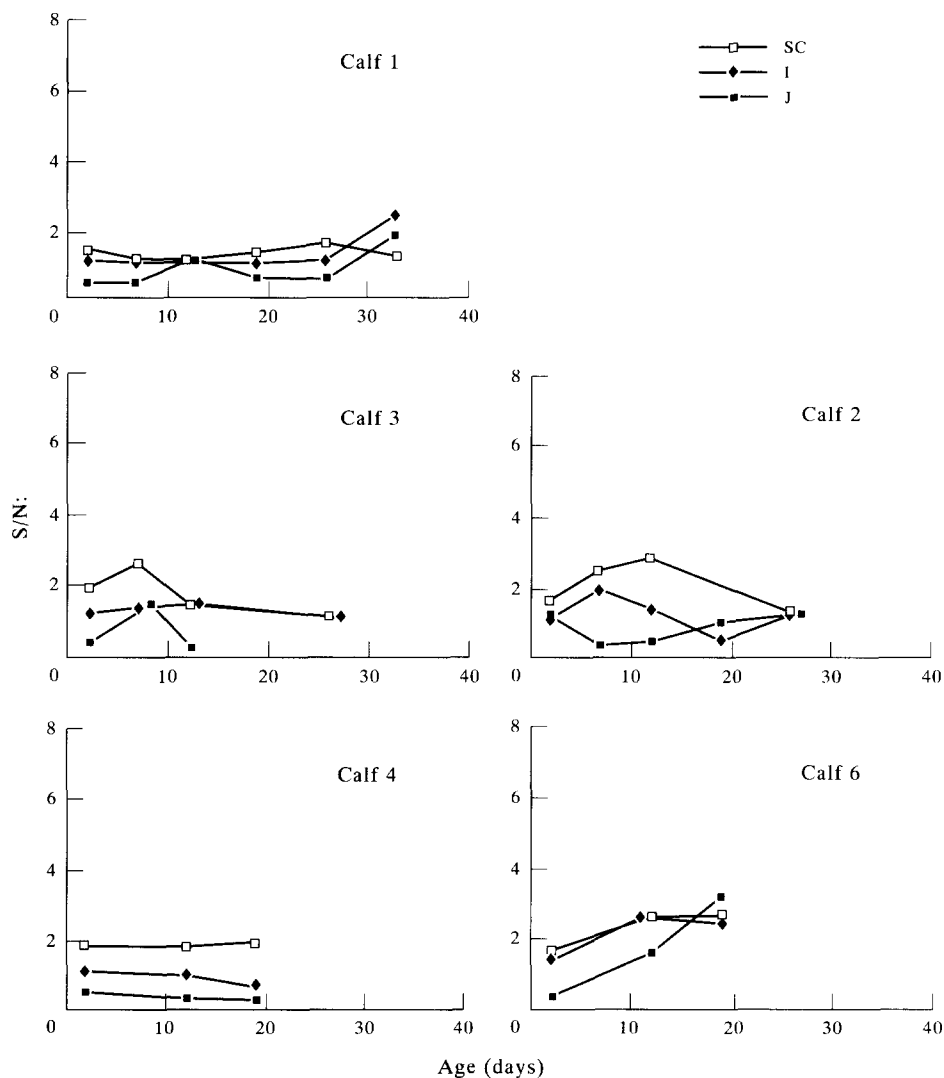


Fig. 3. Anti-BCV IgA responses as measured by antibody capture ELISA assay in calves after infection with attenuated (calf 1) and virulent viruses (calves 2 and 6) and in naturally infected (calf 3) and uninfected calves (calf 4). S/N = sample to negative ratio; SC = spiral colon; I = ileum; and J = jejunum.

RESULTS

To evaluate regional anti-BCV immune responses in the intestines of neonatal calves, quantitative (SRID) and qualitative (IFA and ELISA) approaches were used. On the basis of SRID test, IgM was found to be the most predominant immunoglobulin followed by IgG1, IgA and IgG2 (Table 1). The levels of IgG2 were below detection limits of SRID in all but virulent virus-infected calves.

Total anti-BCV activity of intestinal secretions was determined by IFA. All calves were negative at 2 days of age (before virus inoculation) except for calf 3 (naturally infected calf)

which had a low titer of 1:5. Calves 1 and 9, following inoculation with attenuated virus, had peak titers of only 1:20–1:40. In calf 1, antibody activity could be detected only in the spiral colon (Fig. 1) whereas calf 9 had detectable antibody activity in all three regions of the gut. The IFA titers were much higher in calves inoculated with the virulent virus; peak titers in calves 2 and 6 being 5120 and 20,480, respectively. However, calf 7, which was also inoculated with the virulent virus, had no detectable antibody activity (<1:10) by IFA in any region of the intestine. Uninfected calves were always negative at 1:10 by IFA. Calf 3 that developed a natural BCV infection also did not produce high IFA titers.

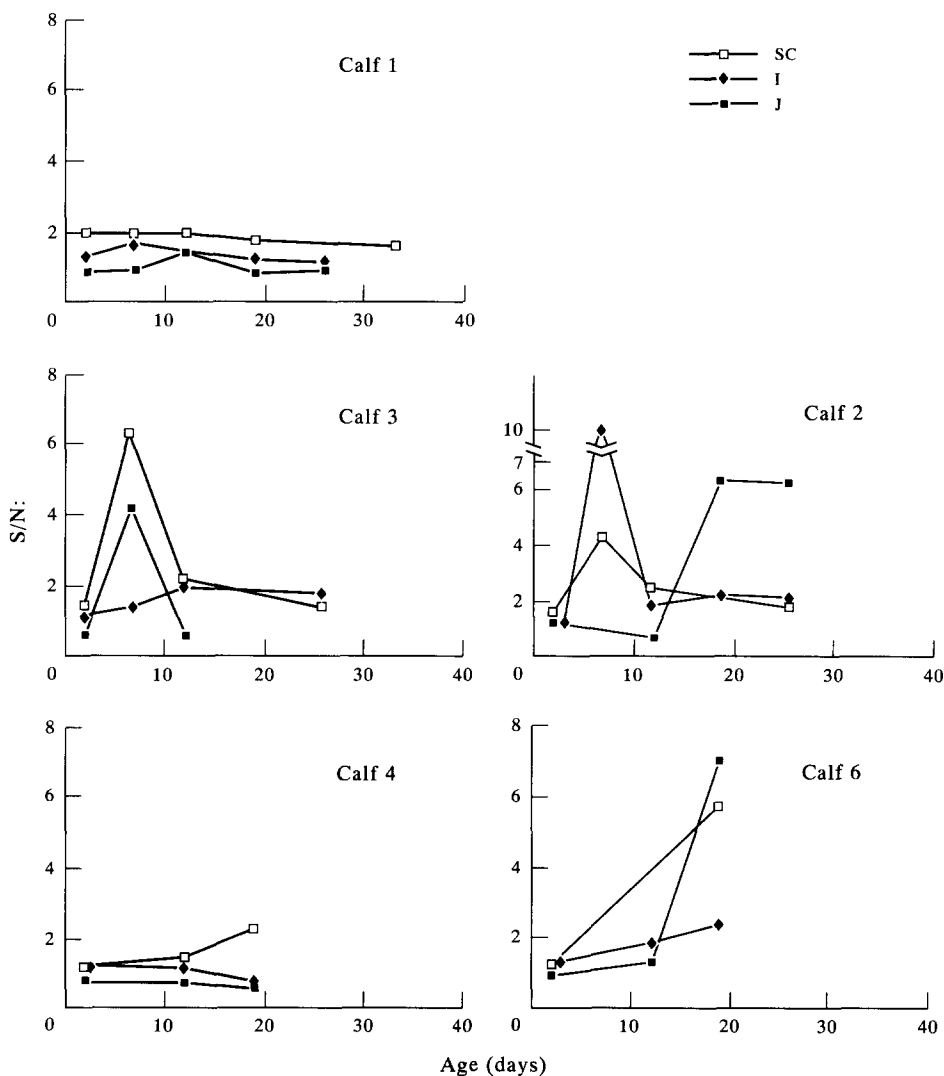


Fig. 4. Anti-BCV IgG1 responses as measured by antibody capture ELISA assay in calves after infection with attenuated (calf 1) and virulent viruses (calves 2 and 6) and in naturally infected (calf 3) and uninfected calves (calf 4). S/N = sample to negative ratio; SC = spiral colon; I = ileum; and J = jejunum.

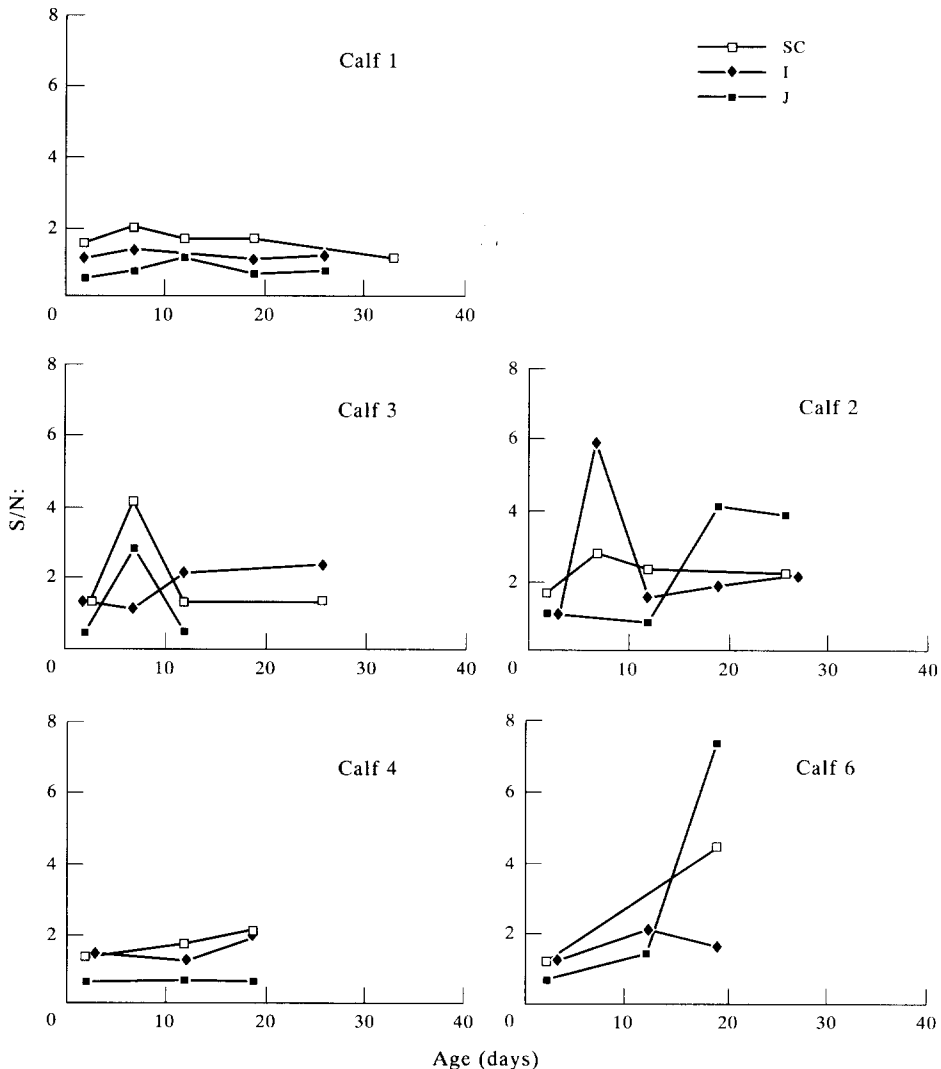


Fig. 5. Anti-BCV IgG2 responses as measured by antibody capture ELISA assay in calves after infection with attenuated (calf 1) and virulent viruses (calves 2 and 6) and in naturally infected (calf 3) and uninfected calves (calf 4). S/N = sample to negative ratio; SC = spiral colon; I = ileum; and J = jejunum.

Antibody capture ELISAs were done to determine anti-BCV activity associated with different isotypes of bovine immunoglobulins. Calves 1 and 9, inoculated with attenuated virus, had no IgM or IgA response immediately after infection but developed low levels of IgM and IgA-associated anti-BCV responses at 4 and 1 weeks after infection, respectively (Figs 2 and 3). No IgG1 and IgG2 responses could be detected in these calves (Figs 4 and 5).

Calves inoculated with the virulent virus (calves 2, 6 and 7) had high IgM, IgA, IgG1 and IgG2 responses (Figs 2 and 5) but the profile of development of antibody responses

was different in each calf. In the spiral colon of calf 2, peak IgM and IgA titers were seen at 4 and 10 days postinfection, respectively. The IgM response in ileum developed rapidly but did not persist for long. No IgM and IgA responses were seen in the jejunum of this calf. High levels of IgG1 and IgG2 developed rapidly in the spiral colon of calf 2 (Figs 4 and 5) but appeared transiently in the ileum. In jejunum, IgG1 and IgG2 responses developed at 2 weeks postinfection.

In calf 6, the immune responses developed slowly in all three regions of the gut (Figs 3 and 4); peak titers were seen at 14 days after infection. The naturally infected calf (calf 3) had no IgM-associated response but developed IgA-associated response on day 2 postinfection in the spiral colon. The IgG1 associated anti-BCV activity in this calf was seen early in infection in the spiral colon and jejunum but not in the ileum. Only IgG2 response developed in the ileum later in this calf. All control calves were negative. There was a direct positive correlation between ELISA and IFA titers and most of the profiles had a similar trend in both tests.

DISCUSSION

Different regions of the intestine are anatomically distinct and their associated lymphoid component also vary considerably. All regions of the gut have isolated lymphocytes in the lamina propria and between the enterocytes (intraepithelial lymphocytes) whereas ileum is endowed with distinct lymphoid nodules called Peyer's patches [11]. The gastrointestinal tract is very responsive to foreign antigens (including viruses) probably because of a large population of macrophages in the intestine which present these antigens to lymphocytes. The levels of bovine immunoglobulins (IgM, IgA, IgG1 and IgG2) in different regions of gut were estimated by single radial immunodiffusion. The most predominant immunoglobulin was IgM followed by IgG1 and IgA. The level of IgG2 was very low in intestinal contents of all calves. The lower levels of immunoglobulins in the intestine 3 weeks after infection with virulent BCV (Table 1) may have been due to generalized immune suppression by BCV.

The IFA titers in calves inoculated with the virulent virus were higher than in those inoculated with the attenuated virus (Fig. 1) probably because virulent pneumoenteric virus replicates to much higher titers than the attenuated virus [7]. However, calf 7, which was inoculated with the virulent virus, failed to develop antibodies to BCV, developed a peracute infection, and died. The level of intestinal immune response has been found to be directly proportional to the rate of virus multiplication and the amount of viral antigen in BCV-infected calves [8]. The presence of higher IFA titers in spiral colon than in jejunum and ileum is not surprising as spiral colon is known to be the primary site of BCV multiplication.

Anti-BCV IgM and IgA were higher in spiral colon than in jejunum and ileum (Figs 2 and 3). The IgA-associated responses developed in the spiral colon of calves inoculated with the virulent virus and of the calf that developed the natural infection. In jejunum, the immune response was associated primarily with IgG1 and IgG2 isotypes and developed late in infection. The profiles of IgG1 and IgG2 associated anti-BCV responses were similar. Since the monoclonal antibodies used for capturing antibodies were of IgG subtype specific and do not cross react [12], the close association of IgG1 and IgG2 responses may indicate that these intestinal antibodies originate from serum.

One of the control calves (calf 3) was found to be naturally infected with BCV later in

the study and was grouped with the virulent virus-infected group. Since this calf had low levels of anti-BCV IFA antibody (titer 1:5) at 2 days of age, we speculate that this calf either acquired infection from dam at parturition or it acquired maternal antibodies due to a leak in the placental barrier. Under normal husbandry conditions, the development of active anti-BCV immunity in feces is influenced by passively derived colostral antibodies [9]. Also, the mucosal immune responses may be directed against the whole virus or the subunits of BCV [9].

Our data suggest that newborn calves are capable of mounting an intestinal immune response and that vaccine failures probably are the result of overattenuation of the virus. Also, different regions of the gut are immunologically distinct. The levels and profiles of anti-coronavirus antibody responses and their association with immunoglobulin isotypes were different at different intervals after infection. The cyclic behavior of BCV in later stages of infection [7] in the 3 regions of the gut may be related to the intestinal immune responses; the virus may be present in a particular region of the intestine when immunity is low in that part of the gut. The development of the intestinal immune response and the immunoglobulin class switchover was more rapid than the systemic responses in calves inoculated with the virulent virus. Similar results have been reported by Kimman and Van Zaane [13] in respiratory tract of calves inoculated with bovine respiratory syncytial virus. It should be recognized, however, that it was a preliminary study employing a relatively small number of animals. More extensive studies using a large number of animals are needed to confirm and extend the results of this study.

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