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CELLULAR IMMUNE STATUS OF CORONAVIRUS-INFECTED NEONATAL CALVES

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Abstract—A preliminary study was conducted to determine the cellular immune status of neonatal, colostrum-deprived calves following inoculation with either attenuated or virulent bovine coronavirus (BCV). Uninfected calves served as controls. To determine the intestinal and systemic cellular immune status, we performed MHC-restricted cytotoxic lymphocyte (CTL) assay on mesenteric lymphocytes, enumerated T cell subsets in peripheral blood lymphocytes, and examined histopathological alterations in mesenteric lymph nodes and gut-associated lymphoid tissue. Target cells for the CTL assay were autologous testicular cells and effector cells were mesenteric lymphocytes from calves infected with BCV. No appreciable specific lysis was observed in any group of calves indicating the absence of demonstrable CTL responses. The T_C/T_S population was severely depressed in the calf inoculated with the virulent virus but not in those inoculated with either the attenuated virus or placebo. The mesenteric lymph nodes and Peyer's patches of calves inoculated with the virulent virus showed severe depletion of lymphocytes. These calves developed intestinal antibody responses in the acute phase of infection (1 week after infection) but were immunosuppressed in the later stage of infection.

Key words: Bovine coronavirus, cellular immunity, T lymphocytes, calf scours, immunosuppression.

Résumé—Une étude préliminaire a été conduite pour déterminer le statut immunitaire de veaux nouveau-nés privés de colostrum, et subissant une inoculation d'un coronavirus bovin (BVC) soit virulent, soit atténué. Les veaux non infectés ont servi de témoins. Pour déterminer le statut immunitaire cellulaire, systémique et intestinal, on a mis au point un test de lymphotoxicité (CTL) efficace sur les lymphocytes mésentériques. On a aussi dénombré la sous-population cellulaire T parmi les lymphocytes du sang périphérique, et on a examiné les altérations histopathologiques touchant les noeuds lymphatiques mésentériques et le tissu lymphoïde de drainage associé. Les cellules cibles pour le test CTL étaient des cellules autologiques de testicules et les cellules effectrices étaient les lymphocytes mésentériques des veaux infectés par le BCV. Quelque soit le groupe de veaux, aucune lyse spécifique significative n'a été observée, indiquant l'absence de réponse au CTL. Alors que veaux inoculés avec le virus virulent présentaient une dépression sévère des populations Tc/Ts, ce n'était pas le cas chez ceux inoculés avec le virus atténué ou le placebo. Les noeuds lymphatiques mésentériques et les plaques de Peyer des veaux ayant reçu le virus virulent montraient une déplétion marquée en lymphocytes. Ces veaux ont développé des réponses en anticorps intestinaux lors de la phase aigüe de l'infection, une semaine après l'inoculation, mais étaient immunodéprimés par la suite.

Mots-clefs: Coronavirus bovin, immunité cellulaire, lymphocytes T, veau, immunodépression.

INTRODUCTION

Bovine coronavirus (BCV) is an important cause of scours in calves up to 1 month of age [1]. After infection with BCV, the calves become weak and dehydrated, lose weight, and

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may die. The mortality rate in BCV-infected calves is approx. 5–10% in uncomplicated cases [2]. Secondary infections in calves infected with BCV have not been commonly documented in the literature but coronavirus enteritis is often associated with rotavirus and other diarrheal agents and, in many cases, calf scours is accompanied by pneumonia. No suitable vaccine is available for the control of scours caused by BCV although one or more have been marketed. A few reports on mucosal humoral immunity in BCV-infected calves are available [3, 4] but the role of cell mediated immune (CMI) responses in BCV infection is not known. This study was undertaken to evaluate the cellular immune status of calves infected with either virulent or attenuated BCV by examining the cytotoxic lymphocyte (CTL) activity in mesenteric lymphocytes and by assessing the T cell subset alterations in the peripheral blood leukocytes of these calves. Histopathological alterations in gut-associated lymphoid tissue (GALT) of these calves were also examined.

MATERIALS AND METHODS

Experimental calves

Male, colostrum-deprived, newborn calves used in these experiments were obtained from the dairy barn of the University of Minnesota and were transferred to the isolation facility immediately after birth. They were housed individually and strict quarantine procedures were followed. A daily dose of trimethoprim and sulfadiazine (Tribrissen 48%, Coopers Animal Health, Kansas City, Kan.) and ceftiofur sodium (Naxcel, Upjohn Company, Kalamazoo, Mich.) was given to all calves. All calves used in these experiments were colostrum-deprived and unvaccinated so that the immune status of these calves could be studied independent of maternal or vaccinal contribution.

Bovine coronaviruses

The attenuated BCV (Mebus strain) had undergone 39 passages in a pig kidney (PK_{15}) cell line and was given 3 additional passages in A549, a human lung carcinoma cell line. The virulent pneumoenteric isolate of BCV (Minnesota isolate) was derived from a bovine fecal sample submitted to the Minnesota Veterinary Diagnostic Laboratories, University of Minnesota, St Paul, Minn. The sample contained BCV only as determined by electron microscopy and was free of bovine virus diarrhea (BVD) virus as determined by inoculation in cell culture and staining with fluorescein conjugated anti-BVD IgG. When inoculated in calves, this isolate consistently produced severe diarrhea, malabsorption, and pneumonia [5]. The calves were infected orally at 5 days of age; two calves (calves 1 and 9) were inoculated with attenuated BCV, three calves (calves 2, 6 and 7) with virulent BCV, one calf (calf 3) was naturally infected with BCV, and three calves (calves 4, 5 and 8) were uninfected controls. Calves 7, 8 and 9 (attenuated virus-inoculated calf, uninfected control calf, and virulent virus-inoculated calf, respectively) were further studied for T subset alterations in their peripheral blood.

Laparotomy

The laparotomy procedure has been described previously [6]. For studies on cell mediated immunity, mesenteric lymph nodes (effector cells) and testicle cells (target cells) were collected from each calf at the time of surgery.

Mesenteric lymph node biopsies

Peritoneum overlying the mesenteric lymph node was incised and the node was isolated from surrounding fat by blunt dissection. A piece of the node (0.5 × 0.5 cm) was sharply excised with scissors and was placed in RPMI 1640 containing 5×10^{-5} M mercaptoethanol, 10% fetal bovine serum (FBS), and penicillin-streptomycin-fungizone (150 IU, 150 μg and 1 μg/ml, respectively). The node was cut into small pieces and was triturated on metal mesh with glass pestle. The lymphocytes were washed twice with RPMI 1640 and then maintained in RPMI 1640 with different concentrations of concanavalin A (2.5, 5.0, 10.0 and 20.0 μg/ml). The mesenteric lymphocytes were used within 24 h after collection.

Collection of testicle

One testicle was surgically removed from each calf to establish an autologous calf testicle cell culture. Briefly, the testicle was washed in Hanks balanced salt solution (HBSS, pH 7.3), the tunica albuginea was incised and the mass of seminiferous tubules was removed and cut into small pieces. After trypsinization for 1 h at room temperature, the seminiferous tubules and isolated cells were centrifuged and washed twice in HBSS with 2 × antibiotics. The cells were suspended in Dulbecco's minimum essential medium with 8% FBS and incubated for 5 days at 37°C. An autologous system was used for the CTL assay, the testicle cells and the lymphocytes were from the same calf in each experiment.

Cytotoxic lymphocyte test (CTL assay)

To perform MHC-restricted CTL assay, autologous testicular cells were infected with attenuated BCV at different multiplicities of infection (m.o.i. 0.15–0.001) and at different times postinfection (2, 6, 12, 24, 48 and 72 h). These target cells were labelled with ^{51}Cr (Amersham, Arlington Heights, Ill.) at 100 μCi per 1×10^{6} cells for 90 min in a water bath at 37°C. The cells were washed twice with RPMI 1640 containing FBS and 2-mercaptoethanol and reincubated for 30 min. The effector (E) cells were mixed with target cells (T) at various ratios (E:T: 100:1 to 25:1) followed by incubation at 37°C for 6 h. The test was done in quadruplicate in 96-well, flat bottom, tissue culture plates (Nunclon, Nunc, Denmark). At the end of the incubation, the plates were centrifuged at 1000 g and 100 μl of supernatant was pipetted out. Spontaneous lysis (target cells only) and maximum lysis (target cells incubated with Triton X-100) were simultaneously measured in all cases. Spontaneous lysis was 10–20% of maximum lysis. Percentage specific lysis was calculated according to the following formula.

$$\text{Percent specific lysis} = \frac{\text{maximum release} - \text{spontaneous release}}{\text{experimental release} - \text{spontaneous release}} \times 100$$

Enumeration of T subsets in peripheral blood

The blood was mixed with an equal volume of HBSS, carefully layered on Ficoll-hypaque (sp. gr. = 1.077), and then centrifuged at 4000 g for 30 min at room temperature. The peripheral blood leukocytes were resuspended in normal goat serum and were placed on ice for 2 h to block non-specific binding sites. The cells were centrifuged at 800 g for

5 min and then resuspended in 200 μl of phosphate buffer saline (pH 7.2). Monoclonal antibodies against bovine T lymphocyte surface markers were obtained from Dr Cindy Baldwin, International Laboratory for Research on Animal Disease, Nairobi, Kenya [7, 8]. These were: IL-A 12 (BoT4) for T_H/T_D, and IL-A 17 (BoT8) for T_C/T_S. The cells were incubated with monoclonal antibodies on ice for 1 h and centrifuged at 800 g for 5 min. To the pellet was added fluorescein conjugated goat antimouse IgG followed by incubation on ice for 30 min. After washing, the cells were uniformly suspended in 50% glycerol-PBS (pH 8.5) and were examined by fluorescent microscopy. Cells with fine, green, surface fluorescence were counted. The total number of cells were also counted to calculate the cells belonging to a particular T cell subset. At least 500 cells were counted in each experiment to calculate the % T subset population.

RESULTS

In the MHC-restricted cytotoxic lymphocyte test using autologous testicular cells as targets and mesenteric lymphocytes as effectors, the percent specific lysis in all calves was less than 10% which is considered as negative or non-significant [9]. Lymphocytes of calf 9 inoculated with attenuated BCV showed no specific lysis at 1 week after infection but showed a 9.9% specific lysis at 3 weeks after infection. Of the calves inoculated with virulent BCV, calf 2 showed specific lysis of 4.2 and 0.9%, at 1 and 2 weeks postinfection, respectively; calf 6 showed no specific lysis at any interval studied; and calf 7 showed a specific lysis of 2.3% at 1 week after infection. Uninfected control calves showed no specific lysis at any of the intervals studied. The differences in specific lysis among various groups of calves were not significant at any time interval. Even the incorporation of 50 IU/ml of recombinant bovine IL-2 (rIL-2, American Cyanamid, Princeton, N.J.) did not increase specific lysis. Although IL-2 caused an increase in counts per minute (cpm), a parallel increase was observed in spontaneous lysis indicating that the percent specific lysis remained the same.

The T cell subsets (T_C/T_S and T_H/T_D) were enumerated in an indirect fluorescent antibody test using antibodies against T lymphocyte surface markers. In calf 7, that was infected with virulent BCV, the number of T_C/T_S population decreased from 10.5% before infection to 2% at 6 days postinfection (Fig. 1). In addition, this calf experienced a decrease of T_H/T_D population as compared to the control and attenuated virus-inoculated calves (Fig. 2). On histopathological examination of mesenteric lymph nodes of the calves inoculated with the virulent virus, severe depletion of lymphocytes was noticed in Peyer's patches and mesenteric lymph nodes when compared to uninfected controls.

DISCUSSION

No virus-specific, MHC-restricted CTL activity was demonstrable in calves inoculated with either virulent or attenuated BCV indicating that either these calves did not have CTL activity or it was too low to be detected by the technique used in this study. The low amount of CTL activity observed may have been due to a low number of virus-specific CTLs or to low lytic activity of these CTLs. The administration of cephalosporins, as was done in this study, has been shown to suppress the generation of virus-specific CTLs in mice [10]. In *Theileria parva*, a protozoan infection of cattle, the frequency of precursors is only one out of 60,000. No attempts were made to clone CTLs or to increase their
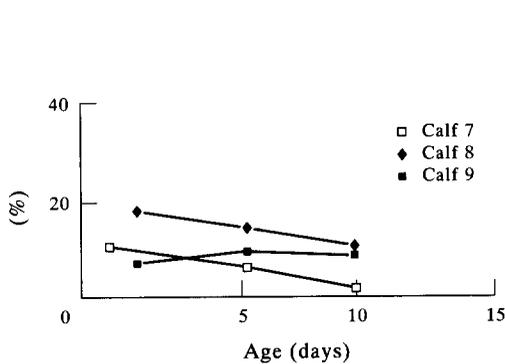
Fig. 1. Levels of T_C/T_S subpopulations in the peripheral blood of calves inoculated with virulent (calf 7) or attenuated virus (calf 9), and in an uninfected control calf (calf 8).
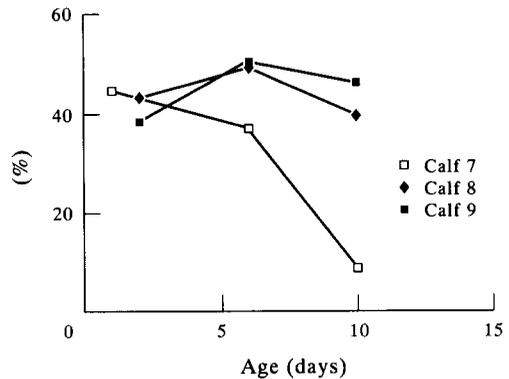
Fig. 2. Levels of T_H/T_D subpopulations in the peripheral blood of calves inoculated with virulent virus (calf 7) or attenuated virus (calf 9), and in an uninfected control calf (calf 8).

number by treatment with IL-2 since we were interested only in the *in vivo* status of CTL activity in these calves. Moreover, severe depression of T_C/T_S was seen in calf 7 (virulent virus-inoculated calf) which might have resulted in decreased CTL activity.

The autologous testicular cells, which were used as target cells in this study, may not express MHC. However, no attempt was made to demonstrate the lack of this MHC. In a previous study, bovine testicular cells have been reported to be less sensitive as target cells [11]. It is possible that intraepithelial lymphocytes (IEL) may develop CTL activity because they are directly exposed to BCV in the intestinal lumen. In a recent study, non-MHC restricted cytotoxic activity against BCV was found in IEL but not in peripheral blood lymphocytes of BCV-infected calves [12]. More studies are needed to prove or disprove these hypotheses.

The negative CTL results obtained in this study are in agreement with published reports on other animal coronaviruses. For example, CTL activity in transmissible gastroenteritis, a coronavirus of pigs, is caused by a non-T cell [13]. Also, in mouse hepatitis virus (MHV), a coronavirus of mice, CTL activity is not seen in mesenteric lymphocytes but is associated with an NK-like cell present in the IEL [14]. The true CTL activity is, however, associated with the T_C/T_S subpopulation.

Paradoxically, BCV-infected calves develop anti-BCV antibodies in the acute phase of infection [4]. The bulk of anti-BCV activity is associated with IgM and may be directed against T independent antigens such as glycoproteins of BCV. Thus, in acute phase, BCV-infected calves develop high levels of antibodies and in chronic phase, they are immunosuppressed. In calves infected with virulent BCV, mesenteric lymph nodes and Peyer's patches showed depletion of lymphocytes which is in agreement with previous studies [15]. The mechanism(s) by which BCV causes depletion of lymphocytes is not known but appears to be due to the replication of BCV in these cells. Similar findings have been reported for MHV [16].

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