Comparison of pathological changes and viral antigen distribution in tissues of calves with and without preexisting bovine viral diarrhea virus infection following challenge with bovine herpesvirus-1

María A. Risalde, DVM, PhD; Verónica Molina, BMSc; Pedro J. Sánchez-Cordón, DVM, PhD; Miriam Pedrera, DVM, PhD; Fernando Romero-Palomo, DVM; María J. Bautista, DVM, PhD; Alberto Moreno, BMSc, PhD; José C. Gómez-Villamandos, DVM, PhD

Objective—To compare pathological changes and viral antigen distribution in tissues of calves with and without preexisting subclinical bovine viral diarrhea virus (BVDV) infection following challenge with bovine herpesvirus-1 (BHV-1).

Animals—24 Friesian calves.

Procedures—12 calves were inoculated intranasally with noncytopathic BVDV-1a; 12 days later, 10 of these calves were challenged intranasally with BHV-1 subtype 1. Two calves were euthanized before and 1, 2, 4, 7, or 14 days after BHV-1 inoculation. Another 10 calves were inoculated intranasally with BHV-1 only and euthanized 1, 2, 4, 7, or 14 days later. Two calves were inoculated intranasally with virus-free tissue culture fluid and euthanized as negative controls. Pathological changes and viral antigen distribution in various tissue samples from calves with and without BVDV infection (all of which had been experimentally inoculated with BHV-1) were compared.

Results—Following BHV-1 challenge, calves with preexisting subclinical BVDV infection had earlier development of more severe inflammatory processes and, consequently, more severe tissue lesions (limited to lymphoid tissues and respiratory and digestive tracts) and greater dissemination of BHV-1, compared with calves without preexisting BVDV infection. Moreover, coinfected calves had an intense lymphoid depletion in the Peyer patches of the ileum as well as the persistence of BVDV in target organs and the reappearance of digestive tract changes during disease progression.

Conclusions and Clinical Relevance—In calves, preexisting infection with BVDV facilitated the establishment of BHV-1 infection, just as the presence of BHV-1 favors BVDV persistence, thereby synergistically potentiating effects of both viruses and increasing the severity of the resultant clinical signs. (Am J Vet Res 2013;74:598–610)

Viral-bacterial coinfections in cattle are considered key factors in the pathogenesis of respiratory tract disease.1 Bovine viral diarrhea virus is one of the pathogens involved in the bovine respiratory disease complex; not only does the virus induce disease on its own, but also, and perhaps more importantly, it can predispose calves to infections with other microorganisms.2–4 Results of experimental inoculation of calves with low-virulence BVDV have indicated that even infections that have a subclinical course (and would not be noticed under field conditions) can cause a marked, although transient, immunosuppression.5–8 This explains why coinfections involving BVDV and other pathogens, such as rotavirus, coronavirus,7,8 BHV-1, or bovine respiratory syncytial virus,9,10 have a synergistic effect, thereby worsening the severity of the resultant clinical signs. Furthermore, sequential inoculation of calves...
with BVDV and Mannheimia haemolytica also increases the severity of lung lesions.11,12

On the basis of antigenic and genetic differences, cattle pestivirus isolates can be classified into 2 genotypes, BVDV-1 and BVDV-2, which are each further characterized as noncytopathic and cytopathic biotypes, depending on their effect on cell cultures.13,14 Bovine viral diarrhea virus has a special tropism for the mucosa-associated lymphoid tissue (tonsils and intestines) and lymphoid organs, with monocytes and macrophages, dendritic cells, and lymphocytes as the main virus target cells.13,16

Bovine herpesvirus-1 is an important pathogen of cattle with worldwide distribution. Infections with BHV-1 result in major economic losses in the cattle industry. This virus is a member of the Alphaherpesvirinae subfamily and is classified as 1 of 3 subtypes (BHV-1.1, BHV-1.2a, and BHV-1.2b) on the basis of results of antigenic and genomic analysis. Bovine herpesvirus-1 isolates are the causative agents of infectious bovine rhinotracheitis and are frequently found in the respiratory tract as well as in aborted fetuses.17,18 Moreover, this pathogen can establish latent or persistent infections in ganglionic neurons and in nonneural sites, such as tonsils and lymph nodes.19-21

To the authors’ knowledge, a detailed and longitudinal study of the tissue effects of preexisting BVDV infection in calves that are subsequently experimentally inoculated with a respiratory viral pathogen has not been performed. Therefore, the purpose of the study reported here was to compare lesions and viral antigen distribution in tissues of calves with preexisting subclinical BVDV infection and healthy calves without preexisting BVDV infection following challenge with BHV-1. Our aim was to characterize and evaluate the type and severity of the pathological changes that develop in calves with subclinical BVDV infection following challenge with BHV-1 and compare those findings with observations for calves infected with BHV-1 only and to determine whether BHV-1 antigen distribution in tissues is affected by preexisting infection with BVDV.

Materials and Methods

Animals and experimental design—The experimental procedure used was based on that described in detail by Risalde et al.4 In brief, 24 male Friesian calves (8 to 9 months old) were obtained from a herd that was free of tuberculosis, brucellosis, and bovine leucosis virus infection and housed in the Animal Experimental Centre of Cordoba University (Spain). The calves were tested via ELISA to confirm their BVDV and BHV-1 antigen-free status and anti-BVDV and anti-BHV-1 antibody-free status. The calves were allocated to 1 of 3 groups for study purposes. The entire experimental procedure was performed in accordance with the Code of Practice for Housing and Care of Animals used in Scientific Procedures, approved by the European Economic Community (86/609/EEC amended by the directive 2003/65/EC).

The calves were allowed to adapt for 1 week before starting the study. Clinical examination (including assessment of rectal temperature, appetite, and presence of diarrhea and examination of accessible mucosal surfaces) as well as clinopathologic analyses were performed on all animals; calves were included in the study if they were considered clinically normal on the basis of those findings and results. After viral inoculation, any calf with a clinical condition indicative of an irreversible stage of disease would be withdrawn from the study and euthanized according to welfare regulations.

BVDV–BHV-1 group—Twelve calves were inoculated in each nostril with 5 mL of a suspension of noncytopathic BVDV-1a strain 7443a (10⁵ TCID₅₀/mL). Twelve days later, when the calves had no clinical signs or evidence of BVDV-related viremia, 10 of them were challenged via each nostril with a 1-mL suspension of BHV-1 subtype 1 (BHV-1.1) strain Iowa4 (containing 10⁵ TCID₅₀/mL). At 1, 2, 4, 7, or 14 days after inoculation with BHV-1.1, 2 calves were sedated with xylazine hydrochloride and euthanized via IV injection with thiopental sodium.4 The 2 calves that were not inoculated with BHV-1.1 were similarly sedated and euthanized on the day that other calves were inoculated with BHV-1.1 and used as BVDV-infected, BHV-1-free controls.

BHV-1 group—At the time that calves in the BVDV–BHV-1 group were inoculated with BHV-1, another 10 calves were inoculated in each nostril with 2 mL of BHV-1.1 (10⁷ TCID₅₀/mL). At 1, 2, 4, 7, or 14 days after BHV-1.1 inoculation, 2 calves were sedated and euthanized, as described.

Negative control group—At the time that calves in the other 2 groups were inoculated with BHV-1, 2 additional calves were inoculated in each nostril with 2 mL of virus-free tissue culture fluid. At the end of the study period (ie, 14 days after calves in the other 2 groups were inoculated with BHV-1), these 2 calves were sedated and euthanized, as described.

Assessments—After virus inoculation, calves were assessed (rectal temperature and clinical signs) twice daily (at 8:00 AM and 8:00 PM) during the study period. Signs of depression, salivation, lacrimation, nasal discharge, cough, dyspnea, nasal lesions, and diarrhea were each evaluated as absent, mild, moderate, or severe. The mean grade of each clinical evaluation was calculated by representing increasing severity with a numeric score from 0 to 3. Comparison of the overall sums of clinical sign scores for calves inoculated only with BHV-1.1 versus calves inoculated with BVDV and BHV-1.1, as well as the significant differences in rectal temperature and clinical signs between calves with single and dual infections, has been described by Risalde et al.4 In that previous study,4 the viral infections in calves were confirmed by results of blood sample and nasal swab PCR testing; the systemic inflammatory and immune responses were determined through assessment of serum concentrations of cytokines (IL-1β, TNFα, IFNγ, IL-12, IL-4, and IL-10) and acute-phase proteins (haptoglobin, serum amyloid A, and fibrinogen) via ELISA and colorimetric methods.

Assessment of pathological changes in tissue samples—All euthanized calves underwent postmortem examination. From each calf, samples of lymphoid tissues (retropharyngeal, tracheobronchial, mesenteric
and ileocecal lymph nodes, pharyngeal and lingual tonsils, thymus, spleen, and bone marrow), respiratory tract (turbinates of the nasal mucosa, trachea, cranial and caudal lobes of the lung), digestive tract (liver, esophagus, duodenum, jejunum, distal portion of the ileum, and ileocecal valve), and nervous system (anterior and posterior cerebrum, cerebellum, trigeminal ganglia, medulla oblongata, and spinal cord) were collected. The collected tissue samples were fixed in neutral-buffered 10% formalin and in 2.5% glutaraldehyde in 0.1M PBS solution.

Formalin-fixed samples were processed routinely, embedded in paraffin wax, sectioned (3 μm), and stained with H&E stain for ultrastructural evaluation. The type and location of gross and histopathologic lesions were graded as absent, mild, moderate, and severe.

Presence of apoptosis in formalin-fixed samples was investigated via DNA fragmentation with a TUNEL detection method, according to the manufacturer’s instructions. Internal positive control specimens consisted of sections of the distal portion of the ileum with apoptotic lymphocytes from calves experimentally inoculated with BVDV-1 in a previous study.

Immunohistochemical analysis—Tissue sections from formalin-fixed samples were dewaxed and rehydrated. Endogenous peroxidase activity was exhausted by incubation with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature (approx 25°C). The sections were subjected to different methods for antigen retrieval: incubation with 0.2% protease K in Tris buffer for 20 minutes at 37°C for BVDV detection or with 0.1M citric acid (pH, 6) in a microwave for 5 minutes at sub-boiling temperature for BHV-1 detection. After pretreatment, sections were rinsed 3 times in PBS solution (pH, 7.2) for 10 minutes each and then covered with 1% normal horse serum in 0.05M Tris buffer (pH, 7.6) for 30 minutes at room temperature. After this blocking stage, sections were incubated with primary monoclonal antibodies at 4°C overnight (approx 18 hours). After primary incubation, the sections were washed in PBS solution (3 times; 5 min/rinse) and then incubated with biotinylated horse anti-mouse IgG secondary antibody, diluted in 0.05M Tris buffer (pH, 7.6) containing 1% normal horse serum, for 30 minutes at room temperature. After 3 further 5-minute washes in PBS solution, samples were incubated with the avidin-biotin peroxidase complex for 1 hour at room temperature. All tissue sections were finally rinsed in PBS solution and incubated with chromogen solution. Sections were counterstained with Mayer hematoxylin stain.

Identification of BVDV and BHV-1.1 surface glycoproteins Erns (BVDV) and gC (BHV-1.1) was performed with the primary monoclonal antibodies 15c5 (1:75 diluted in normal horse serum) and F2 (1:1,000 diluted in normal horse serum), respectively. As part of the immunohistochemical detection of Erns, tissues from calves persistently infected with BVDV were analyzed as a positive control. As part of the immunohistochemical detection of gC, tissues from several aborted fetuses that were positive for BHV-1.1 and tissues from specific pathogen-free calves that were not exposed to either BVDV or BHV-1 were analyzed as a positive and negative control, respectively. Mouse nonimmune sera were substituted for specific primary antibodies as additional negative controls.

Antigen distribution was evaluated as absent, scarce, moderate, and intense. The identification of the target cells for both viruses was based on morphological features, location, and size of the cells.

Transmission electron microscopy—Tissue samples fixed in glutaraldehyde for ultrastructural examination were postfixed in 2% osmium tetroxide, dehydrated in acetone, and embedded in resin. Sections (50 nm) were counterstained with uranyl acetate and lead citrate and examined by use of a transmission electron microscope.

PCR analysis of BHV-1 DNA and BVDV RNA in lung tissue samples—Bovine herpesvirus-1 DNA was extracted from lung tissue samples with a DNA extraction kit, following the manufacturer’s protocol. The real-time PCR analysis of the extracted DNA template was performed as described. Data were analyzed in a thermocycler, and any sample that had a cycle threshold value ≤ 45 was considered a positive finding. The positive control sample was the BHV-1.1 strain Iowa (10^8 TCID₅₀/mL).

Bovine viral diarrhea virus RNA was extracted from lung tissue samples with an RNA extraction kit, according to the manufacturer’s instructions. A 1-step PCR assay of the RNA was performed with the primers and probes based on conserved regions of the 5’-untranslated region of BVDV-1 described by Letellier and Kerkhofs and a kit for real-time 1-step reverse transcriptase PCR analysis of viral RNA used according to the manufacturer’s instructions. The reactions were performed in a thermal cycler instrument. Any sample that had a cycle threshold value ≤ 45 was considered a positive finding. The positive control was the noncytopathic BVDV-1a strain 7443 (10^1 TCID₅₀/mL).

Results

Clinical signs, virus detection in blood samples via PCR assay, and determination of serum concentrations of cytokines after experimental infection with BHV-1.1 of these calves have been described by Risalde et al. In brief, only calves inoculated with BVDV followed by BHV-1 had systemic spread of BHV-1 as well as the development of more intense clinical signs, mainly associated with the respiratory and digestive tracts, and an exacerbation of the inflammatory response, compared with findings in calves inoculated with BHV-1 only. Calves in the BHV-1 group developed slight lacrimation and serous nasal discharge following inoculation, together with signs of mild depression and sialorrhea 8 to 10 days after virus inoculation. For the BVDV-1 group calves, mean rectal temperature 5 through 11 days after inoculation (39.6°C) was significantly (P < 0.05) increased, compared with values before inoculation with BHV-1.1, and was associated with the greater severity of clinical signs 7 to 9 days after inoculation. On the other hand, calves in the BVDV–BHV-1 group had characteristic clinical signs of disease, including mild sialorrhea, lacrimation, and serous nasal discharge.
as well as intense diarrhea 4 to 9 days after inoculation with BVDV. At the time of BHV-1 inoculation (12 days after inoculation with BVDV), the BVDV–BHV-1 group calves were healthy; however, rectal temperature increased (>40°C) at 4 and 5 days after inoculation with BHV-1, and the number and severity of clinical signs that developed in these calves were greater than the corresponding findings in calves inoculated with BHV1.1 only. These BVDV–BHV-1 group calves had moderate to severe clinical signs 4 to 11 days after inoculation with BHV-1, including signs of depression, salorrhrea, lacrimation, mucopurulent nasal discharge, cough, dyspnea, open-mouth breathing, and nasal lesions as well as the appearance of moderate recurrent diarrhea at 4 to 7 days after inoculation with BHV-1.1 (Table 1).

Gross and histologic evaluations—Following euthanasia, each calf in each experimental group underwent postmortem examination to identify gross lesions. Tissue samples (lymphoid tissues [retropharyngeal, tracheobronchial, mesenteric and ileocecal lymph nodes, pharyngeal and lingual tonsils, thymus, spleen, and bone marrow], respiratory tract [turbinate of the nasal mucosa, trachea, and cranial and caudal lobes of the lungs], digestive tract [liver, esophagus, duodenum, jejunum, distal portion of the ileum, and ileocecal valve], and nervous system [rostral and caudal portions of the cerebrum, cerebellum, trigeminal ganglia, medulla oblongata, and spinal cord]) from calves in each experimental group were processed and examined. For the calves in the negative control group, there were no remarkable gross lesions or histopathologic lesions in the various tissue samples collected. Type, location, and changes in lesions with time in both inoculated groups were summarized (Tables 2 and 3).

Lymphoid tissues—Calves in both the BVDV–BHV-1 and BHV-1 groups had large lymph nodes with petechial hemorrhages that became more evident during the course of the study; the most intense lesions were associated with the retropharyngeal lymph nodes. Additionally, the BVDV–BHV-1 group calves had petechial hemorrhages in the lingual tonsils, splenic congestion and splenomegaly, and atrophic bone marrow; these findings were also observed in the BVDV-infected control calves.

Microscopically, the tonsils from BHV-1 group calves and BVDV-infected control calves did not have histologic lesions, whereas the tonsils from the calves in the BVDV–BHV-1 group had hyperemia, petechial hemorrhages within lymphoid follicles, and dense accumulations of leukocytes in epithelium with occasional ulceration. From 2 days after inoculation onward, the BVDV–BHV-1 group calves developed progressively depleted lymphoid follicles in which there was evidence of pyknosis, cellular fragmentation, and macrophages with phagocytized cell debris (tingible body macrophages), characteristic of apoptosis as confirmed by a TUNEL detection method (Figure 1). Moreover, 4 through 7 days after inoculation with BHV-1, we observed focal necrosis in tonsil epithelium, crypts, and lymphoid follicles, along with an inflammatory response of infiltrated macrophages, lymphocytes, and...
hyperplasia that was more evident with time, mainly BHV-1.1 inoculation, lymph nodes developed intense evident in lymph nodes of all inoculated calves. After techial hemorrhages within lymphoid follicles, were calves. However, these splenic changes were not apparent in the remainder of the aforementioned splenic lesions. Differences between these inoculated groups with regard to the increased number and size of the follicles, together with an intense scattering of lymphocytes. Sinus hyperplasia resulted from migration of lymphocytes and macrophages, which occluded the lumen (sinus catarrh; Figure 2). Retropharyngeal lymph nodes had the most notable alterations.

**Respiratory tract**—There were no gross lesions in the respiratory tract of BVDV-infected control calves. Gross respiratory tract lesions were almost nonapparent in the BHV-1 group calves and mild in the coinfect ed calves, and included congestion of the nasal mucosa after BHV-1,1 inoculation and occasional tracheitis that developed in later stages of the study. Ulcerated lesions of the nases were detected only in the BVDV–BHV-1 group calves.

Histologically, the epithelial lesions in coinfection ed calves were described as necrotic ulcerations, accompanied by connective tissue proliferation and mononucle ar infiltrate in the dermis. Eosinophilic IIBs were identified in epithelial cells of the margins of necrotic lesions.

The upper respiratory tract of all BHV–1–infected calves had mild hyperemia of the lamina propria as well as diffuse and periglandular infiltrates composed of macrophages, lymphocytes, and plasmatic cells. Occasional epithelial synctia with slightly basophilic IIBs were observed in the nasal mucosa of the BVDV–BHV–1 group calves (Figure 2).

The histopathologic lesions in lung tissues were almost nonapparent in the BVDV–infected control calves. Inflammatory changes were also observed microscopi cally in lung tissue after inoculation with BHV-1,1. In calves in both the BVDV–BHV–1 and BHV–1 groups, the pulmonary parenchyma had evidence of interstitial pneumonia with alveolar septal thickening as a result of

Table 2—Evaluation of gross lesions in healthy calves that received intranasal inoculation with noncytopathic BVDV–1a followed 12 days later by intranasal inoculation with BHV–1 subtype 1 and calves that received only intranasal inoculation with BHV–1 subtype 1 at that same time point, as well as in calves that received inoculation with BVDV–1a only (BVDV–infected control group) or that received no inoculation (negative control group).

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Calves in the BVDV–BH1 group and the BHV–1 group were euthanized in pairs at each time point. Two calves were euthanized 12 days after BVDV inoculation and were used as BVDV-infected, BHV–1–free controls. At the end of the study period (ie, 14 days after calves in the other 2 groups were inoculated with BHV–1), these 2 calves were euthanized and used as negative controls. Data are reported as mean grades of lesion severity for 2 calves in each group at each time point. 

See Table 1 for key.
interstitial aggregations of lymphocytes, macrophages, and, to a lesser extent, neutrophils. This alteration appeared earlier and in a more severe form in the BVDV–BHV-1 group calves and was associated with occasional alveolar edema and hemorrhages (Figure 2). Moreover, sporadic epithelial syncytia in pulmonary alveoli were observed in the BVDV–BHV-1 group calves 2 through 7 days after BHV-1.1 inoculation.

Digestive tract—Calves in the BHV-1 group did not develop gross lesions in the alimentary tract, whereas BVDV-infected calves (BVDV-infected control and BVDV–BHV-1 group calves) had congestion and edema in the mucosa of the small intestine from just after the start of the study. These changes increased after BHV-1.1 inoculation in the BVDV–BHV-1 group calves and were accompanied by a congested and edematous gallbladder mucosa and petechial hemorrhages in the colon.

Microscopically, the ileum of the BVDV-infected control and BVDV–BHV-1 group calves had hyperemia, sporadic hemorrhages in interfollicular areas, and mononuclear infiltrate in lamina propria at all evaluated time points throughout the study. The ileal submucosa had intense dilation of lymphatic vessels with migration of lymphocytes, together with severe depleted lymphoid follicles in the Peyer patches. This lymphoid depletion was characterized by the reduction in the number of lymphocytes as well as infiltration of macrophages and visibly enlarged stellate cells. Moreover, we observed pyknosis, cellular fragmentation, and TUNEL-positive tingible body macrophages in the interfollicular lymphoid tissue. By contrast, there were no histologic lesions in the ileum of the BHV-1 group calves.

The most characteristic lesion found in the ileocecal valve of the BVDV-infected control and BVDV–BHV-1 group calves was a proliferation of Lieberkühn crypts resulting in crypt hyperplasia. Affected crypts were dilated and filled with mucus, epithelial debris, and inflammatory cells. Some of these crypts appeared herniated into the submucosal space previously occupied by...
involuted lymphoid follicles. This kind of change was not observed in the BHV-1 group.

Nervous system—For any of the calves in the experimental groups, there were no gross nervous system lesions. Similarly, there were no histologic lesions in the sections of nervous tissue analyzed in the present study.

Immunohistochemical analyses—In the appropriate positive control tissue samples, Erns- and gC-specific labeling appeared as evenly distributed dark red granules or diffuse cytoplasmic staining. There was no Erns- or gC-specific labeling in any control tissue samples when anti–BHV-1 or anti-BVDV antibodies were replaced by murine nonimmune serum in the immunohistochemical analyses.

Neither BVDV nor BHV-1 antigens were detected in tissue sections from the negative control calves. The distributions of both viral antigens, principally associated with lymphoid tissues and intestinal mucosa of inoculated animals, were summarized (Table 4).

Bovine viral diarrhea virus antigen was only detectable in BVDV-infected control and BVDV–BHV-1 group calves, being present in lymphoid tissues (specifically in the thymus and ileocecal lymph node) as well as in mucosa-associated lymphoid tissue of the ileum and ileocecal valve. The amount of BVDV antigen in tissues from the BVDV–BHV-1 group calves decreased during the study period until complete disappearance and was never more abundant than the amount of BHV antigen in BVDV-infected control calves. Erns protein was not detected in tissue samples from the respiratory tract, CNS, upper portion of the digestive tract, liver, spleen, or bone marrow of any of the BVDV-infected control and BVDV–BHV-1 group calves.

In sections of the thymus of the BVDV-infected control and BVDV–BHV-1 group calves, viral Erns was detected throughout the study period, being scarce at 14 days after inoculation with BHV-1.1 in the BVDV–BHV-1 group calves. Positive staining was associated with reticular epithelial cells, macrophages, and occasional lymphocytes of the thymic cortex as well as with fibroblasts of the adjacent stroma (Figure 3).

A large amount of BVDV antigen was detected in sections of the ileocecal lymph nodes from BVDV-infected control calves as well as from BVDV–BHV-1 group calves until 4 days after inoculation with BHV-1.1. The most extensive viral antigen distribution was seen in the medullary sinuses, where the antigen was confined to the cytoplasm of macrophages, stellate-like cells, and some lymphocytes. In 1 calf, viral Erns was detected in the tunica media of muscular arteries of the retropharyngeal lymph node, in association with lymphocytic arteritis.

Bovine viral diarrhea virus antigen was present in great amounts within depleted lymphoid follicles and interfollicular areas in ileal Peyer patches of BVDV-infected control calves and BVDV–BHV-1 group calves until 4 days after inoculation with BHV-1.1. In this period, macrophages and stellate cells as well as some lymphocytes, fibroblasts, and apoptotic bodies were positive for BVDV.
antigen (Figure 3). Thereafter, we observed a gradual decrease in the number of the cells with Erns-specific labeling toward the end of the study period, when viral Erns was only detected in macrophages.

Bovine herpesvirus-1 antigen distribution was mainly confined to ulcerations in the nares and both pharyngeal and lingual tonsils of calves in the BVDV–BHV-1 group at 4 and 7 days after inoculation. Viral gC
Transmission electron microscopy—Ultrastructural examination of tissue samples revealed BHV-1–like particles and virus-like replication sites in epithelial cells. The BHV-1 target cells were epithelial with the areas where the 2 types of IIBs in epithelial cells were observed. The BHV-1–target cells were epithelial cells and, to a lesser extent, lymphocytes and macrophages (Figure 3).

PCR analysis of BHV-1 DNA and BVDV RNA in lung tissue samples—The presence of viral agents in lung tissue samples obtained from the BVDV–BHV-1 group calves (including the BVDV-infected control calves) and BHV-1 group calves was confirmed via molecular techniques to detect BHV-1 DNA and BVDV RNA. Bovine viral diarrhea virus was detected via PCR assay of BHV-1 from its entry site. It has been suggested that changes in resident CD8+ T lymphocytes with regard to their cytotoxic capacity against infected cells may contribute to a delayed local immune response to BHV-1 and to inefficient elimination of the pathogenic agent.

Discussion

In the present study, the lesions and viral antigen distribution in calves with and without preexisting subclinical bovine viral diarrhea that were subsequently inoculated with BHV-1.1 were assessed. Compared with their healthy counterparts, calves with subclinical bovine viral diarrhea had more severe tissue lesions, particularly inflammatory lesions, and more abundance of BHV-1 antigen following BHV-1.1 inoculation. Moreover, during later stages of the study and disease progression, BVDV was detected via immunohistochemical techniques in the BVDV–BHV-1 group calves; interestingly, BVDV has not been detected at similar time points in calves inoculated with BVDV alone.

The greater intensity of the clinical signs and severity of lesions observed in the BVDV–BHV-1 group calves may have been a result of greater dissemination of the agent with direct action on target cells or triggering of a more intense host inflammatory response or, as the findings of the present study suggested, a combination of the 2 mechanisms.

In the calves in the BHV-1 group, only a small amount of viral antigen was found in tonsil samples at 4 days after inoculation. By contrast, in the calves in the BVDV–BHV-1 group, an abundance of viral antigen together with IIBs was observed in tonsil samples at 4 and 7 days after BHV-1.1 inoculation; the BHV-1.1 antigen was associated with necrotic lesions because of the cytopathic effect of BHV-1. The greater abundance of the secondary agent in calves inoculated with BVDV might be linked to an inadequate local cell-mediated response and the consequent failure to stop spread of BHV-1 from its entry site. It has been suggested that changes in resident CD8+ T lymphocytes with regard to their cytotoxic capacity against infected cells may contribute to a delayed local immune response to BHV-1 and to inefficient elimination of the pathogenic agent.
Moreover, in the BVDV–BHV-1 group calves in the present study, BVDV was detected in association with macrophages in the thymus and Peyer patches throughout the study period (up to 26 days after inoculation with BVDV), a finding not reported for calves in single-infection studies in which elimination of this virus from different tissues begins 12 or 14 days after inoculation. Thus, just as BVDV facilitates the dissemination of BHV-1, the presence of BHV-1 favors the persistence of BVDV in target organs and the reappearance of digestive-tract changes attributed to BVDV infection, thereby potentiating the pathogenic action of both agents and increasing host susceptibility to other infections.

With regard to inflammatory lesions, calves in the BHV-1 group had only moderate changes such as hyperemia and mononuclear cell infiltrates in the upper airways and lungs; similar cell recruitment has been reported in other studies of BHV-1 infection. However, lesions in the liver of BHV-1 and BVDV–BHV-1 group calves were especially evident (moderate or severe). In calves in the BVDV–BHV-1 group, these inflammatory changes occurred earlier and were more severe, particularly at 4 days after BHV-1 inoculation, which coincided with the appearance of eosinophilic IIBs in epithelial cells at the necrotic margins (arrowheads). Expression of BHV-1 antigen is evident in stellate cells, macrophages, and fibroblasts. B—Erns-positive cells are visible within the medulla of depleted Peyer patches follicles at 2 days after BHV-1 inoculation (14 days after BVDV inoculation). Erns-specific immunohistochemical stain; bar = 72 µm. Inset—Higher magnification of the area outlined. Expression of BVDV antigen is evident in stellate cells, macrophages, and fibroblasts. Erns-specific immunohistochemical stain; bar = 5 µm. C—Within a tonsil crypt of the pharyngeal tonsil, focal necrosis with infiltrated macrophages, lymphocytes, and neutrophils is evident at 4 days after BHV-1 inoculation (16 days after BVDV inoculation). H&E stain; bar = 72 µm. Inset—Higher magnification of the area outlined to illustrate the presence of eosinophilic IIBs in epithelial cells at the necrotic margins (arrowheads). H&E stain; bar = 5 µm. D—Bovine herpesvirus-1 surface glycoprotein gc is also associated with the necrotic lesion of the pharyngeal tonsil crypt. gc-specific immunohistochemical stain; bar = 72 µm. Inset—Higher magnification of the area outlined. Epithelial cells and necrotic cell debris are immunopositive for BHV-1. gc-specific immunohistochemical stain; bar = 5 µm.
ability and tissue damage. Retropharyngeal lymph nodes were the most affected in the BHV-1 and BVDV–BHV-1 group calves because of the nodes’ proximity to the inoculation route, where they were subjected to a greater antigen stimulation, thereby increasing the chemotactic effect for leukocytes.25,26

Massive recruitment of mononuclear cells in the lungs of the BVDV–BHV-1 group calves prompted development of interstitial pneumonia, with a marked thickening of the lung parenchyma 4 to 7 days after BHV-1 inoculation, coinciding with the onset of respiratory signs. This finding may be attributable to a direct action of both viruses, which were detected in lung tissues via PCR assays. Accordingly, it could be suggested that preinfection with BVDV worsens the lesions attributed to secondary infections because of a synergistic action between both pathogens.1,25 Despite the identification of both agents in the lungs via molecular techniques, the occasional presence of syncytiotrophoblastic cells in alveoli (associated with BHV-1 replication9), and ultrastructural detection of herpesvirus virions in alveolar macrophages in BVDV–BHV-1 group calves in the present study, no BHV-1 antigen was detected via immunohistochemical analysis; a similar lack of antigen detection via immunohistochemical analysis has been reported by other authors1,12,13,25,42 and is likely due to the lack of virus and the low sensitivity of the technique.

Calves in the BVDV–BHV-1 group had other lesions not directly related to the inflammatory response prior to inoculation with BHV-1.1 However, these included numerous TUNEL-positive apoptotic bodies phagocytized by macrophages in B-dependent areas of lymph nodes, thymus, and ileal Peyer patches, along with marked lymphoid depletion associated with BVDV infection. Similar lesions have been identified in other in vivo studies5,9,25,26 of this virus. In the present study, the findings attributed to BVDV infection were more intense after BHV-1 inoculation, a pathogen that can induce apoptosis both in vivo and in vitro. The enhanced severity of such apoptotic lesions in infected calves would also be related to a prolonged release of tumor necrosis factor-α (TNF-α), a cytokine with proven ability to induce apoptosis.46,47 These data suggest that the presence of both BVDV and BHV-1 has a synergic effect, thereby potentiating the immunosuppressive action of BVDV.70

Another lesion observed in BVDV-infected control calves and BVDV–BHV-1 group calves was intense bone marrow hypoplasia, which persisted in the coinfected animals until the end of the study. To our knowledge, this lack of hematopoietic nests has not been reported hitherto for infections with low virulence BVDV-1 or BVDV-2 strains, although it has been observed following inoculations with more virulent BVDV-2 strains.48,49 The severe lack of myeloid progenitor cells observed in these calves coincided with the presence of severe lymphopenia; this suggests a possible virulence mechanism of BVDV through which the proliferative capacity of such cells is compromised. However, BVDV antigen was not detected via immunohistochemical analysis in bone marrow samples, indicating that the changes observed may have been prompted by indirect virus actions such as depletion of T lymphocytes, which are involved in modulating hematopoiesis, or the altered cytokine expression in stromal cells, leading to a modification of the hematopoietic microenvironment.31,32 This finding has been reported for other pestiviruses, such as classical swine fever virus, and future studies on the mechanisms triggered by BVDV to alter the environment regulating these cells are warranted.

The results obtained in the present study in calves indicated that BVDV infection does not prevent the development of a cell-mediated response, but in fact favors the earlier development of more severe inflammatory processes following subsequent BHV-1 inoculation, leading to a worsening of clinical signs and lesions limited to lymphoid and airway tissues and also promoting development of lesions of the digestive tract. Moreover, a synergism exists wherein BVDV facilitates the multiplication of BHV-1 and the presence of BHV-1 favors the persistence of BVDV in target organs, especially digestive tract, in coinfected calves.

References
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