Effects of a single intranasal dose of modified-live bovine respiratory syncytial virus vaccine on resistance to subsequent viral challenge in calves

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Objective—To determine whether a single intranasal dose of modified-live bovine respiratory syncytial virus (BRSV) vaccine protects calves from BRSV challenge and characterize cell-mediated immune responses in calves following BRSV challenge.

Animals—13 conventionally reared 4- to 6-week-old Holstein calves.

Procedures—Calves received intranasal vaccination with modified live BRSV vaccine (VC-group calves; n = 4) or mock vaccine (MC-group calves; 6) 1 month before BRSV challenge; unvaccinated control-group calves (n = 3) underwent mock challenge. Serum virus neutralizing (VN) antibodies were measured on days -30, -14, 0, and 7 relative to BRSV challenge; nasal swab specimens were collected for virus isolation on days 0 to 7. At necropsy examination on day 7, tissue specimens were collected for measurement of BRSV-specific interferon gamma (IFN-γ) production. Tissue distribution of CD3+ T and BLA.36+ B cells was evaluated by use of immunohistochemistry.

Results—The MC-group calves had significantly higher rectal temperatures, respiratory rates, and clinical scores on days 5 to 7 after BRSV challenge than VC-group calves. No difference was seen between distributions of BRSV in lung tissue of VC- and MC-group calves. Production of BRSV-specific IFN-γ was increased in tissue specimens from VC-group calves, compared with MC- and control-group calves. Virus-specific IFN-γ production was highest in the mediastinal lymph node of VC-group calves. Increased numbers of T cells were found in expanded bronchial-associated lymphoid tissue and airway epithelium of VC-group calves.


Bovine respiratory syncytial virus (BRSV) is a widespread cause of respiratory disease in cattle. Infection with BRSV contributes to enzootic calf pneumonia and bovine respiratory disease complex (ie, shipping fever) of feedlot cattle. The virus can also cause outbreaks of acute fatal respiratory disease in calves and adult cattle. Vaccination against BRSV has been problematic; results of some field studies on commercially available vaccines have indicated lack of efficacy, and others have reported apparent disease enhancement in vaccinated calves. Results of studies on vaccine-enhanced disease indicate that vaccination against BRSV can lead to enhanced disease. Although recent evidence indicates that some commercially available vaccines are able to protect calves against experimental BRSV challenge, it is clear that more information is needed to ensure the consistent development of BRSV vaccines that are safe and also effective.

Intranasal vaccination against BRSV has promise as an improved means of protecting cattle against BRSV. Intranasally administered bovine herpesvirus type 1 and parainfluenza virus vaccines have been effective. Moreover, intranasally administered vaccines against human respiratory syncytial virus, which is closely related to BRSV, have been tested in clinical trials. In older research, European investigators compared the efficacy of BRSV vaccines given by the intranasal and IM routes. Intranasal vaccination with modified-live BRSV gave improved protection against experimental BRSV challenge. Importantly, a single intranasally administered vaccine was able to prime calves in the face of maternal antibodies. Despite this information, little has been done in recent years to investigate the value of intranasal vaccination against BRSV. It has been suggested that intranasally administered BRSV vaccines could be superior to existing vaccines, all of which are labeled for IM or SC use. The purpose of the study reported here was to confirm...
whether a single dose of intranasally administered BRSV vaccine could protect against virulent BRSV challenge and characterize the cell-mediated immune response as measured by production of BRSV-specific IFN-γ by mononuclear cells isolated from various tissue specimens in vaccinated calves following BRSV challenge. The distribution of T and B cells in these tissue specimens was also characterized by use of immunohistochemistry (IHC).

Materials and Methods

Calves—Conventionally reared 4- to 6-week-old Holstein calves were allocated randomly to receive a modified-live BRSV vaccine or mock (spent tissue culture media) vaccine intranasally. Because of the time required on the day of necropsy for collection of cells from tissue specimens for an IFN-γ assay, calves were studied in pairs, with 1 calf receiving modified-live BRSV vaccine and 1 calf receiving mock vaccine intranasally. Challenge to BRSV occurred 1 month after vaccination. Prior to BRSV challenge, vaccinated calves were isolated so that the vaccine strain of the virus would not spread to the mock-vaccinated calves. The number of calves in each group was initially balanced, but some calves developed unrelated diseases between the time of vaccination and BRSV challenge. These calves were removed from the study, and the final groups consisted of 6 mock-vaccinated BRSV-challenged calves (MC-group calves) and 4 BRSV-vaccinated BRSV-challenged calves (VC-group calves). Three additional unvaccinated calves were allocated to receive a mock challenge with spent tissue culture media and served as the unvaccinated negative controls (control-group calves). The control-group calves were the same age and had the same management background as that of the MC- and VC-group calves; the control-group calves were housed under identical conditions and mock-challenged several weeks after the last pair of BRSV-challenged calves was studied to prevent unintended exposure to BRSV. The University of Georgia Institutional Animal Care and Use Committee approved the research described.

Experimental vaccination and BRSV challenge—The experimental, intranasally administered vaccine consisted of a high-passage, low-virulence isolate of BRSV. One milliliter of the virus (1 × 10^3 median tissue culture infective doses [TCID₅₀/mL]) was instilled into each nostril of the VC-group calves through a 4-cm-long plastic cannula. Calves of the MC group received 1 mL of spent tissue culture media (taken from noninfected bovine kidney cells) in an identical fashion. Experimental BRSV challenge was provided 30 days after vaccination, as previously described. Briefly, 6 mL (5 × 10^7 to 1 × 10^8 TCID₅₀/mL) of a low-passage, high-virulence isolate⁶ was administered through a snugly fitting mask via aerosol by a nebulizer. The 3 negative control-group (mock-challenged) calves were treated with 6 mL of spent tissue culture media (taken from noninfected bovine turbinate cells) in an identical manner.

Evaluation of clinical signs—Calves were examined on the day of vaccination, daily for 7 days following vaccination, and then once a week until the day of challenge. Calves were also examined daily for 7 days following challenge. Clinical signs were evaluated as previously described,⁷ with a clinical score determined on the basis of a system by Collie et al.⁸ On each day the calf was evaluated, the rectal temperature and respiratory rate were measured and the presence or absence of nasal and ocular discharge, spontaneity or induced cough, adventitious lung sounds, anorexia, or depression was noted.

Serologic evaluation and virus isolation—Nasal swab specimens were collected for virus isolation daily for the first 7 days after vaccination (experimental days –29 through –23) and for 7 days after challenge (experimental days 1 to 7). Sterile cotton swabs were inserted into the caudal nasal passage and then placed into 2 mL of transport media (Eagle minimum essential medium [EMEM] with 10% fetal calf serum [FCS], penicillin [100 U/mL], and streptomycin [100 µg/mL]); these were transported on ice to the laboratory and inoculated onto cells in chamber slides within 2 hours of collection. Between 0.6 to 0.8 mL of media from each swab specimen was filtered through a 0.2-µm-diameter filter into each of the 2 wells containing bovine turbinate cells that had been seeded 24 hours before inoculation. In addition to the 2 sample wells, each slide contained a negative and positive (BRSV-inoculated) control well. When a cytopathic effect was present (after 7 days), the slides were collected and stored at –20°C until they were acetone-fixed and stained with anti-BRSV fluorescein isothiocyanate-labeled antibody,⁹ according to the manufacturer's instructions. Slides were scored as positive or negative for virus on the basis of positive fluorescent antibody staining in 1 or both of the sample wells.

Serum samples were obtained for all calves on the day of vaccination (day –30), 16 days later (day –14), on the day of BRSV challenge (day 0), and day 7 after BRSV challenge and submitted to the Athens Veterinary Diagnostic Laboratory for assaying of virus neutralizing (VN) antibodies to BRSV by standard techniques. At the beginning of the experiment, all calves had low-to-moderate concentrations of antibody that were attributed to passive colostral transfer.

Necropsy examination—On day 7 after BRSV challenge, calves were euthanatized with an overdose of barbiturate and a necropsy examination was performed. Prior to euthanasia, blood was collected into tubes containing acid citrate dextrose for isolation of peripheral blood mononuclear cells (PBMCs) and tubes containing no additive for serologic evaluation. Gross abnormalities in the respiratory tract and other major systems were recorded. Tissue specimens collected for isolation of mononuclear cells for ex vivo evaluation of BRSV-specific IFN-γ production included the cranial and caudal lung lobes, palatine tonsils, pharyngeal tonsil, mediastinal lymph node, tracheobronchial lymph node, and spleen. Lung tissue specimens were collected from areas of obvious gross abnormality in the right cranial and right caudal lung lobe; if no abnormalities were present, lung tissue specimens were collected from a standard region (caudolateral margin) of the right cranial lung lobe and dorsal aspect of the right caudal lung lobe. Tissue specimens for mononuclear cell isolation were collected into 50-mL conical tubes containing 25 mL of EMEM without FCS and with penicillin (100 U/mL) and streptomycin (100 µg/mL) and kept on ice until processing. Sections of lung and spleen tissue that were approximately 2 cm² in area were collected; adjacent areas were obtained for histologic examination and IHC analysis. Whole tonsils and lymph nodes were collected and divided evenly for mononuclear cell isolation, histologic evaluation, and IHC. Specimens of lung tissue from all calves were submitted to the Athens Veterinary Diagnostic Laboratory for examination by use of an immunofluorescent assay (IFA) for the detection of BRSV, bovine herpesvirus type 1, parainfluenza virus type 3, and bovine viral diarrhea virus as well as for aerobic bacterial culture.

Ex vivo production of BRSV-specific IFN-γ by tissue and blood mononuclear cells—Mononuclear cells were isolated from lung and tonsil tissue specimens by incubation of minced tissue in tissue digestion media (RPMI with 5% FCS, penicillin [100 U/mL], streptomycin [100 µg/mL], collagenase [200 U/mL], and DNase [50 U/mL]). Tissues were incu-
bated on a rotator for 2 hours at 37°C. The tissue was then pressed through sterile stainless steel mesh while it was washed with media. The tissue suspension was allowed to settle briefly, and the supernatant was then decanted and filtered through small gauge stainless steel mesh. The cell suspension was pelleted at 200 × g for 10 minutes. The pellet was resuspended in 20 mL of RPMI without FCS, layered over sodium diatrizoate, and centrifuged at 400 × g for 30 minutes. Mononuclear cells were washed twice with RPMI without FCS and then resuspended in DMEM with 10% FCS. At the end of processing, cells were in a single cell suspension with > 95% viability as demonstrated by Trypan blue exclusion.

Primary antibody was used for the detection of BRSV antigen was a murine monoclonal antibody (1:600 dilution). Primary antibody used for the detection of CD3+ T cells was a rabbit polyclonal antibody (1:250 dilution of biotinylated antibody was added (anti-BRSV, B cell); anti-rabbit for T cells) and incubated with either avidin-biotin-peroxidase (BRSV, B cell) or avidin-biotin-alkaline phosphatase (T cell). Substrates consisted of microwaving in citrate buffer for 10 minutes.

Antigen retrieval consisted of diaminobenzidine (for peroxidase) and an alkaline phosphatase substrate. Development was stopped by rinsing with running tap water. Antigen retrieval consisted of microwaving in citrate buffer for 10 minutes. The pellet was resuspended in 200 × g for 10 minutes and supernatants were stored at –80°C until analysis by an ELISA for bovine IFN-γ. Supernatants were run in triplicate with mean values calculated. Results were expressed as a percentage of that of the positive control by use of the following formula:

\[ \text{sample OD} = \frac{\text{positive control OD} - \text{kit negative control OD}}{100} \]

where OD stands for optical density.

Detection of BRSV and B and T cells by use of IHC—The section of right cranial lung tissue from each calf was subjected to immunohistochemical staining for detection of the following: BRSV antigen, CD3+ T cells, and B cells. Briefly, the protocol was as follows: sections were cut onto charged glass slides and heated at 65°C for 15 minutes. Deparaffinization was accomplished with 3 changes of a commercial solvent. For those protocols using peroxidase, endogenous peroxidase was quenched by incubating with 3% H2O2 for 10 minutes at room temperature (or 10 minutes at room temperature, followed by washing in running tap water. Antigen retrieval consisted of microwaving in citrate buffer for 10 minutes. Primary antibody was suspended in PBS solution with 0.005% Tween 20 and incubated over the sections for either 2 hours at 37°C or overnight at 4°C. Primary antibody used for the detection of BRSV antigen was a murine monoclonal antibody (1:600 dilution). Primary antibody used for the detection of CD3+ T cells was a rabbit polyclonal antibody (1:1000 dilution). Primary antibody used for the detection of BRSV antigen was a murine monoclonal antibody (1:600 dilution). Primary antibody used for the detection of CD3+ T cells was a rabbit polyclonal antibody (1:1000 dilution). Primary antibody used for the detection of BRSV antigen was a murine monoclonal antibody (1:600 dilution). Primary antibody used for the detection of CD3+ T cells was a rabbit polyclonal antibody (1:1000 dilution). Primary antibody used for the detection of BRSV antigen was a murine monoclonal antibody (1:600 dilution). Primary antibody used for the detection of CD3+ T cells was a rabbit polyclonal antibody (1:1000 dilution).

Statistical analysis—Data on clinical signs were analyzed with a linear mixed repeated measures model by use of a computer software program. The mixed model evaluated the association between the treatment group, day, and baseline value for each outcome, controlling for random variation within a calf over time. Respiratory rates and clinical scores were log-transformed before analysis. For IFN-γ results, values were arcsin square root-transformed to normalize variance, and for each stimulus in each tissue, a 1-way ANOVA was used to evaluate the difference between treatment groups. For values of P < 0.05, a Bonferroni posthoc test was used to compare groups. For virus neutralization, data were entered into an electronic database and analyzed by use of a commercial software package. Differences in titers between vaccinated and unvaccinated calves were evaluated by use of a Mann Whitney U test. To evaluate the changes in titers within calves in an experimental group over the course of the study, a Wilcoxon matched-pairs test was done when data were sufficient. Values of P < 0.05 were considered significant.

Results

Clinical signs—Following mock vaccination, no clinical changes were detected within 7 days in MC-group calves (Fig 1). In VC-group calves, 2 calves had a rectal temperature > 40°C on day –23,
leading to a significant increase in rectal temperature in VC-group calves, compared with MC-group calves. One VC-group calf also had a high clinical score (ie, 407) on day –23 as a result of a fever and harsh lung sounds; this led to an increase in the mean clinical score for VC-group calves on day –23, although the difference in clinical scores between MC- and VC-group calves on day –23 was not significant. The 2 VC-group calves that were febrile on day –23 were also febrile the next day but had lower rectal temperatures; they had no further clinical abnormalities.

Following challenge, MC-group calves had significantly increased rectal temperatures, respiratory rates, and clinical scores on days 5 to 7, compared with VC- and control-group calves (Fig 2). One MC-group calf died unexpectedly during the night of day 5. Another MC-group calf was euthanatized because of severe disease on day 6. Following challenge, no significant differences were found in clinical signs between VC-group calves and control-group calves.

Development of peak clinical signs on day 7 after BRSV challenge was consistent with previously published results based on this challenge model. Clinical signs were not followed past day 7 in our study because euthanasia and necropsy examination on day 7 were done to allow measurement of tissue mononuclear cell IFN-γ production and T and B cell distribution at the peak of disease.

Serologic evaluation and virus isolation—Virus neutralizing antibodies were detectable at low-to-moderate concentrations at the beginning of the study and were attributed to colostral transfer. Initially, concentrations of antibody were comparable in MC- and VC-group calves, but as a result of the loss of calves from each group, VC-group calves ultimately had significantly ($P = 0.04$) higher titers than MC-group calves on day –30 (Fig 3). This difference was not significant on day –14 ($P = 0.09$) and day 0 ($P = 0.34$). Calves in the VC group maintained higher titers following BRSV challenge than MC-group calves, although this difference was not significant ($P = 0.08$) at the 95% confidence level. Calves in the VC group had lower titers on the day of BRSV challenge than on either previous test day, but this difference was not significant ($P = 0.07$). Although titers in MC-group calves seemed to decrease following BRSV challenge, whereas titers in VC-group calves remained high, no detectable difference in titers was found in response to the BRSV challenge on day 7 in either group.

Virus isolation from nasal swab specimens indicated that calves began shedding virus on day 4 after the BRSV challenge. In MC-group calves, 4 of 6 calves had positive results for viral shedding on 1 or more days between days 4 and 7 after BRSV challenge; these results were consistent with those previously reported for calves challenged with this BRSV isolate. In VC-group calves, only 1 of 4 calves had positive results for viral shedding between days 4 and 7, indicating that vaccinated calves were less likely to shed virus following BRSV challenge. Because of technical problems, viral shedding following vaccination could not be determined.

Necropsy examination—In the lungs, gross pathologic findings typically paralleled clinical signs, with more striking changes present in calves with more severe clinical signs. As a result, gross findings were...
generally more extensive in MC-group calves than in VC- or control-group calves. Findings were comparable to those previously described. In all MC-group calves, consolidation was present in cranial lung lobes and ranged from a variegated multilobular pattern to extensive and sometimes total consolidation of cranial lung lobes. Consolidation was also present in the caudal lung lobes of most calves, ranging from multilobular consolidation within the parenchyma to total consolidation of the ventral region of the caudal lung lobes. Grossly apparent interstitial or bullous emphysema was present in 2 of 6 MC-group calves. In 1 MC-group calf, small foci of purulent material could be identified within lung parenchyma. Mediastinal and tracheobronchial lymph nodes were often large and edematous, and the pharyngeal tonsil was sometimes reddened.

In VC-group calves, lobular consolidation was mild-to-moderate in cranial lung lobes and present to a lesser degree in the caudal lung lobes. Grossly apparent emphysema was not present in any calf. In 1 of 4 VC-group calves, small foci of purulent material could be identified in the lung parenchyma. In another VC-group calf, abundant mucus was present in the respiratory tract, and in a third VC-group calf, bronchioles in the affected lung lobe contained purulent material. The tracheobronchial lymph node was large in 2 VC-group calves; no remarkable changes were observed in the tonsils.

Of the 3 control-group calves, 1 had small multifocal consolidated lobules throughout the lung and the tracheobronchial lymph node was edematous. Two small areas of consolidation were present in 1 lobe of the second control-group calf, and in the third control-group calf, no lung abnormalities were observed. Outside of the respiratory tract, no gross abnormalities were found in calves from any group.

The following tissue specimens were examined histologically: right cranial lung lobe, palatine tonsil, pharyngeal tonsil, tracheobronchial lymph node, and mediastinal lymph node. No abnormal findings were found in tissue specimens from any control-group calves. Calves of the MC group had the most foci of consolidation, congestion, edema, syncytial cells, and neutrophils in selected lung lobules. These lesions were typical of BRSV infection and consistent with those previously reported in calves challenged with this BRSV isolate. In some MC-group calves, the damage was more extensive than in others. Calves of the VC group had many similar changes to those of MC-group calves in the right cranial lung lobe; however, in all of these VC-group calves, an increased number of lymphocytes were found around the respiratory tract, compared with that found in MC- or control-group calves. In MC- and VC-group calves, palatine tonsils had an increase in the prominence of lymphoid follicles and numerous germinal centers. Similarly, in these 2 groups, substantial follicular development was found in the pharyngeal tonsil, compared with that found in control-group calves. Tracheobronchial lymph nodes were similar in appearance in VC- and MC-group calves with an increase in germinal centers, edema, and variable numbers of acute inflammatory cells. In the mediastinal lymph node of MC-group calves, acute inflammatory cells and early development of germinal centers were found. In VC-group calves, mediastinal lymph node germinal centers were more developed than in MC-group calves.

Bovine respiratory syncytial virus was detected in lung tissues from all MC- and VC-group calves by use of an IFA. No BRSV was detected in lung tissues from the 3 control-group calves. Lung tissues from all calves in all groups had negative IFA results for the detection of bovine herpesvirus type 1, parainfluenza virus type 3, and bovine viral diarrhea virus. Aerobic bacterial culture of lung tissues revealed no growth in 3 of 6 MC-group calves, light growth of a Bacillus sp (attributed to contamination) in 1 MC-group calf, a single colony each of a Pasteurella sp and a coagulase-negative Staphylococcus organism in 1 MC-group calf, and light growth of Pasteurella multocida in 1 MC-group calf. No bacterial growth was obtained from lung tissue of 3 VC-group calves, and a light growth of a coagulase-negative Staphylococcus organism (attributed to contamination) was found in lung tissue from 1 VC-group calf. No bacterial growth was obtained from the lung tissue of any control-group calves.

Ex vivo BRSV-specific IFN-γ production—Interferon gamma production by mononuclear cells

![Graph](Image)

Figure 4—Mean (± SEM) BRSV-specific interferon gamma (IFN-γ) production by mononuclear cells isolated from mediastinal lymph nodes (MLN), tracheobronchial lymph nodes (TBLN), and pharyngeal tonsil of VC-, MC-, and control-group calves on day 7 after challenge. Cells were stimulated with spent media (SM, negative control), con A (positive control), challenge strain BRSV (Ch BRSV), or vaccine strain BRSV (Vc BRSV). Production of IFN-γ in response to challenge strain BRSV was significantly (P < 0.05) higher in the MLN of VC-group calves, compared with that of MC- and control-group calves.
stimulated with the challenge BRSV isolate was increased for VC-group calves, compared with MC- and control-group calves, in the pharyngeal tonsil, tracheobronchial lymph node, mediastinal lymph node, and spleen, although the difference was significantly higher only for the mediastinal lymph node (Fig 4 and 5). In contrast, IFN-γ production by PBMC stimulated with the challenge BRSV isolate was greatest for MC-group calves, although this finding was not significantly different from that of VC- and control-group calves. In the cranial lung lobe, IFN-γ production in response to the challenge BRSV isolate was low for all groups.

For VC-group calves, IFN-γ production by tissues in response to the vaccine BRSV isolate was generally lower than the response to the challenge BRSV isolate; the exception to this was IFN-γ production by PBMC. In the lung, IFN-γ production was low in response to the vaccine BRSV isolate as it was for the challenge BRSV isolate. Tissues of MC- and control-group calves had little or no response to the vaccine BRSV isolate. Because 1 MC-group calf died unexpectedly, tissue specimens were available from 5 MC-group calves. In all calves, it was difficult to isolate an adequate number of cells from the palatine tonsil and caudal lung lobe; because of this, mononuclear cell IFN-γ production could only be evaluated for 1 or 2 calves in each group and these data are not presented. Isolation of an adequate number of cells from the pharyngeal tonsil of MC-group calves was also difficult; enough cells to culture were isolated from only 2 of 5 MC-group calves. However, an adequate number of cells were isolated from the pharyngeal tonsil of all of VC- and control-group calves and, in general, from all other tissue specimens from all calves.

Figure 5—Mean (± SEM) BRSV-specific IFN-γ production by peripheral blood mononuclear cells (PBMC) and mononuclear cells isolated from the cranial lung lobe or spleen of VC-, MC-, and control-group calves on day 7 after challenge. Cells were treated as described for Figure 4.

Figure 6—Results of immunohistochemistry (IHC) analysis for detection of CD3+ T cells in lung tissue sections from control-group calves (top), MC-group calves (middle), and VC-group calves (bottom). In lung tissue from control-group calves, few CD3+ cells can be seen in the submucosa (arrow); cells are similarly distributed (arrow) in lung tissue of MC-group calves. In lung tissue of VC calves, increased staining in expanded bronchial-associated lymphoid tissue (BALT) can be seen (lower arrow) as well as CD3+ cells in the airway epithelium (upper arrow). Hematoxylin stain and an alkaline phosphate substrate; bars = 40 µm.
Detection of BRSV and B and T cells by use of IHC—Distribution of BRSV antigen was examined in the cranial lung lobe. By use of IHC, BRSV antigen was found in the lung tissues of all VC- and MC-group calves. Viral antigen distribution was found to follow the distribution of histologic lesions, and no difference was found in this relationship between VC- and MC-group calves. Staining was strongest in areas of consolidation. The epithelium of small airways stained intensely, and this staining often spread into alveoli. No positive stain result for BRSV antigen was found for control-group calves.

Distribution of B and T cells was examined in the cranial lung lobe, pharyngeal and palatine tonsils, and tracheobronchial and mediastinal lymph nodes. In lung tissue of control-group calves, staining for CD3 (T cells) was consistently present in expanded bronchial-associated lymphoid tissue (BALT) and scattered throughout interlobular septa, the subpleural space, and the submucosa (Fig 6). Staining was similar in lung tissues of MC-group calves. In VC-group calves, an increase in staining for CD3 was observed in expanded BALT. Also in VC-group calves, positively stained cells were often seen migrating through the epithelium into the lumen. In the lymphoid tissue of control-group calves, positive CD3 staining was evident and distinct in interfollicular areas of palatine and pharyngeal tonsils and tracheobronchial and mediastinal lymph nodes. In MC-group calves, interfollicular staining was similar but less prominent because of the expansion of the follicular areas. Scattered follicular staining was apparent in the pharyngeal tonsil and subcapsular and medullary sinuses of the tracheobronchial lymph node. In VC-group calves, findings were similar to those of MC-group calves.

In lung tissues of control-group calves, BLA.36 (B cell) staining was present in scattered submucosal cells in bronchi and large bronchioles. In comparison, MC-group calves had a similar distribution of staining, but it was more intense and cells were often clustered and closer to the epithelial surface. In VC-group calves, findings were similar to those of control-group calves. In tonsils of control-group calves, both follicular and interfollicular staining were evident (Fig 7). This pattern was the same in MC-group calves. However, in VC-group calves, lymphoid follicles in the tonsils were large and staining was often intense. In lymph nodes of control-group calves, infrequent positively stained cells were found in the cortex and also in medullary cords. In contrast, in MC-group calves, an increase in the numbers of positively staining cells was found in the medullary cords. Similar staining was present for VC-group calves.

Overall, major differences in VC-group calves, compared with MC- and control-group calves, were seen in the distribution of CD3+ cells in lung tissues, with increased staining in expanded BALT and infiltration of CD3+ cells into the bronchiolar epithelium. Also, VC-calves had an increase in the distribution of BLA.36+ cells in the tonsils, with increased, more intense staining in the follicles, compared with MC- and control-group calves.

**Discussion**

The purpose of our study was to determine whether a single intranasal dose of modified-live BRSV could induce protection against a virulent BRSV challenge 1 month after vaccination. To characterize possible mechanisms of vaccine-induced protection, cell-mediated immune responses were measured in various tissue specimens of vaccinated and control calves. Distribution of T and B cells in these tissue specimens...
was also evaluated. Vaccinated calves were protected from BRSV challenge as indicated by rectal temperatures, respiratory rates, and clinical scores after BRSV challenge and were not significantly different from that of the mock-challenged control calves. In contrast, mock-vaccinated calves had obvious signs of disease after BRSV challenge. Nasal virus shedding after BRSV challenge also decreased in vaccinated calves, compared with mock-vaccinated calves. The vaccine, which was a high-passage, low-virulence isolate, did induce a fever on day –23 (day 7 after vaccination) in 2 of 4 calves and harsh lung sounds in 1 calf. The calves that were febrile on day –23 were also febrile the next day (day –22) but had lower rectal temperatures; they had no further clinical abnormalities.

Although vaccinated calves had no clinical signs of disease after BRSV challenge, vaccination did not completely prevent gross lesion development or virus replication in the lung. As measured by use of IFA and IHC, BRSV antigen was found in the lung tissues of all calves in both the VC and MC groups. Distribution of viral antigen followed the distribution of histologic lesions. It could thus be inferred that total virus load was decreased in calves with less extensive lesions, which was typical of calves in the VC group, but formal efforts to quantify viral antigen in each group were not undertaken. If the findings of our study are extrapolated to the field situation, it is possible that some intranasal vaccinated calves would be capable of transmitting the virus to in-contact calves; they might also be at risk for developing secondary infections following primary BRSV infection.

Because it was of interest to determine whether intranasal vaccination could be effective in calves with low-to-moderate concentrations of maternal antibodies, we did not wait until the VN antibody titers of the calves had completely deteriorated before beginning the study. Importantly, a single intranasal vaccination was able to protect calves from clinical signs of disease following virulent BRSV challenge even though calves had measurable VN antibody titers at the time of vaccination. A weakness of our study was that VC-group calves had higher VN antibody titers on the day of vaccination than did MC-group calves. The VN antibody titers were initially balanced in both groups, but loss of antibodies was more severe in MC-group calves even though no significant difference was found in VN antibody titers between VC- and MC-group calves on the day of BRSV challenge. These results have clinical relevance because they suggest that calves with low to moderate concentrations of VN antibody are susceptible to severe disease, which can be mitigated by a single intranasal dose of modified-live BRSV.

Although VC-group calves were protected against BRSV challenge at 1 month after vaccination, they had no evidence of seroconversion as measured by VN antibody titers. Results of Kimman et al.18 likewise indicated that calves receiving an intranasally administered BRSV vaccine in the face of maternal antibodies did not seroconvert following vaccination as measured by BRSV-specific IgG titers. In the study by Kimman et al.,18 intranasally vaccinated calves did seroconvert by day 6 after BRSV challenge. In contrast, the calves in our study did not seroconvert by day 7 after BRSV challenge when they were euthanatized. The difference between our results and those of Kimman et al.18 may be related to the fact that those authors measured total BRSV-specific IgG, whereas VN antibody titers were measured in our study.

It would have been of interest to know whether mucosal concentrations of BRSV-specific IgA were significantly different between VC- and MC-group calves in our study. Results of the previous study18 indicate that rapidity of onset of production of nasal mucosal IgA was associated with protection in intranasally vaccinated calves, but magnitude of titer was not. Because the objective of our study was to evaluate the cell-mediated immune responses to intranasal vaccination, which has not been previously studied, we elected to focus budget and personnel on measurement of cell-mediated immunity in this set of experiments.

Unfortunately, it was not possible to include a group of calves vaccinated with a conventional BRSV vaccine delivered by the IM route. Although recent work has confirmed the efficacy of a single dose of some conventional IM administered vaccines in protecting calves against virulent BRSV challenge,12 studies comparing the responses of calves vaccinated by the intranasal or IM routes would be of value. We speculate that intranasal vaccination would improve responses in respiratory-associated lymphoid tissue and that such responses could be related to improved protection in the face of maternal antibodies, possibly with fewer doses of vaccine.

Virus-specific IFN-γ production by mononuclear cells isolated from tissue was measured as a marker of a cell-mediated immune response in vaccinated calves. Interferon gamma is produced primarily by CD4+ and CD8+ T cells and natural killer cells, so any of these may have contributed to the IFN-γ production mea-
sured in our study. The fact that strong responses to con A stimulation were present in all tissues indicates that T cells were present in all cell preparations. Measurement of virus-specific IFN-γ has recently been shown to be an important correlate of vaccine-induced protection against BRSV challenge in calves vaccinated with commercially available BRSV vaccines and in calves vaccinated with commercially available vaccines containing bovine herpesvirus type 1. As it is not known which of the possible contributing cell populations was most important in the vaccine-induced protection seen in our study, future research focused on identification of IFN-γ production by specific cell subsets would be a logical next step. Determination of the relative number of T cells present in each tissue would also be informative. Clearly, differences in IFN-γ production measured in different tissues may have been related either to increased migration of cells producing the cytokine into the tissues, increased IFN-γ production by cells already present in tissues, or both. Additional research to characterize the relative contribution of T cell influx into tissues, versus IFN-γ upregulation by cells already present in tissues, would clarify the effect of intranasal vaccination against BRSV on T cell responses.

The use of IHC to characterize T and B cell distribution helped to identify the effect of vaccination on lymphocyte kinetics in our study. Vaccinated calves had an increased number of T cells in the expanded BALT in the lung, and T cells were often seen moving into the bronchioalveolar epithelium. This finding indicates that vaccination primed calves for increased movement of T cells into the respiratory tract, the site of BRSV infection. Given this finding, it was surprising that BRSV-specific IFN-γ production by mononuclear cells isolated from the lung was low. It may be that for some reason, relatively few T cells survived isolation from the lung, although the response to con A by the lung mononuclear cells indicated that T cells were present in the cell preparations. It may also be that most of the T cells responsive to BRSV were in the respiratory tract and airway cells were not well represented in the total lung cell preparations. Measurement of BRSV-specific IFN-γ production by T cells isolated by bronchoalveolar lavage might be more representative of the pulmonary T cell response to BRSV infection.

Identification of CD3+ and BLA36+ cells via IHC in our study was noteworthy because cells could be identified in formalin-fixed tissue specimens. Identification of lymphocyte subsets in tissue specimens has previously been most often accomplished in frozen tissue. We were also able to identify CD8+ cells (data not shown); identification of CD4+ cells in formalin-fixed tissue has been attempted, but at this point, the technique has not worked well.

To our knowledge, our study is the first to evaluate any aspect of the immune response to BRSV infection in the pharyngeal and palatine tonsils. The distribution of lymphocytes in the bovine pharyngeal and palatine tonsils has been evaluated, and the distribution of T and B cells seen in our study agreed with findings in the previous work. T cells predominated in the parafollicular region, with few T cells seen in the follicles or germinal centers and epithelium, and B cells predominated in the follicles and germinal centers. The pharyngeal tonsil is considered to be an important site of induction of mucosal B cell responses. In our study, the results of IHC evaluation of B cell distribution suggested that protection of VC-group calves against BRSV challenge was related at least in part to the expansion of B cells in the pharyngeal tonsil.

In contrast to B cells, the role of T cells in the tonsil is poorly characterized. Tonsillar T cells have been considered to be of primary importance in providing help to B cell activation. This is logical because most of the T cells in the pharyngeal tonsil are CD4+ (T helper cells). However, T cells are also present in the follicular epithelium and may exert effector activity at the mucosal surface. If this is the case, the tonsil may also participate in induction of mucosal T cell responses. The fact that increased IFN-γ production by mononuclear cells from the pharyngeal tonsil could be identified in VC-group calves indicates that a relative T helper cell type-1 cytokine bias in the tonsil may be associated with the development of a protective response to BRSV.

References