Effects of a single intranasal dose of modified-live bovine respiratory syncytial virus vaccine on cytokine messenger RNA expression following viral challenge in calves

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Objective—To characterize cytokine messenger RNA (mRNA) expression in intranasally vaccinated calves after bovine respiratory syncytial virus (BRSV) challenge.

Animals—Twelve 8- to 12-week-old calves.

Procedures—Calves received modified-live BRSV vaccine (vaccinated) or spent tissue culture medium (mock-vaccinated) intranasally, followed by challenge 30 days later with BRSV, or mock challenge with spent tissue culture medium (mock-challenge controls). Interleukin-4 (IL-4) and interferon-γ (IFN-γ) mRNA was measured in lungs, bronchoalveolar lavage (BAL) fluid cells, pharyngeal tonsils, and tracheobronchial lymph nodes, and tumor necrosis factor-α (TNF-α) mRNA was measured in lungs and BAL fluid cells by reverse transcriptase-competitive polymerase chain reaction assay.

Results—Resistance to clinical signs of disease was conferred in vaccinated calves. Expression of TNF-α mRNA in lungs and BAL fluid cells was higher in vaccinated calves than in control or mock-vaccinated calves. In the lung, IL-4 mRNA expression was higher in vaccinated calves than in control or mock-vaccinated calves. In pharyngeal tonsils, expression of mRNA for IL-4 and IFN-γ was higher in mock-vaccinated calves than control calves. In tracheobronchial lymph nodes, IFN-γ mRNA expression was higher in mock-vaccinated calves than vaccinated calves.

Conclusions and Clinical Relevance—Although vaccinated calves had decreased clinical signs of disease after BRSV challenge, compared with mock-vaccinated calves, this difference was not related to a Th helper type 1 bias, as determined by increased expression of interferon-γ mRNA relative to interleukin-4 mRNA in lungs, BAL fluid cells, or tracheobronchial lymph nodes of vaccinated calves. Pulmonary inflammation was decreased in vaccinated calves as determined by decreased expression of TNF-α mRNA. (Am J Vet Res 2004;65:725–733)

Respiratory syncytial virus (RSV), a pneumovirus within the Paramyxoviridae family, is a leading cause of respiratory disease in human infants and calves.1-3 Bovine respiratory syncytial virus (BRSV) and human RSV are antigenically and biochemically closely related and share common epidemiologic, clinical, and pathologic characteristics.2 The pathogenesis of RSV infection is not clear, but direct viral cytopathology may play a minor role, and the majority of injury may result from the host immune-inflammatory response.3

The role of cell-mediated immune responses to RSV infection is not completely defined. The CD8 cells apparently play an important role in protection against disease, possibly through BRSV-specific cytotoxic activity.4 They may also influence the response by producing cytokines such as interferon-gamma (IFN-γ). The role of CD8+ T-helper cells is more complicated and uncertain. A growing body of research indicates that T helper type 1 (Th1) or type 2 (Th2) cytokine imbalances may play a role in the pathogenesis of RSV disease. A hypothesis that RSV bronchiolitis may be the result of increased production of Th2-type cytokines has become popular. Interleukin-4 (IL-4) secreted by Th2 cells is considered to be associated with disease, in part because it induces immunoglobulin production by B cells with isotype switching towards IgE.7 Interleukin-2 and IFN-γ secreted by Th1 cells provide help for generation of cytotoxic T lymphocytes and thus are associated with protection.8 However, results of studies in human infants or mice infected with RSV provide evidence that IFN-γ production may contribute to RSV-induced wheezing. This has not been reported in cattle. Results of previous studies suggest that IFN-γ production is associated with protection against BRSV infection. Additional studies on cytokine responses to BRSV are required to clarify the role of T-helper cell cytokines in the pathogenesis of disease in cattle.

Because cytokine expression can influence the outcome of BRSV infection in cattle, studies of cytokine responses to BRSV vaccination are required to ensure the development of safe and effective vaccines.

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Although many BRSV vaccines are marketed, in rare cases, vaccine-enhanced disease has been reported in association with both killed virus vaccines and a modified-live virus vaccine against BRSV. Results of 1 study indicate that calves vaccinated intranasally with a modified-live BRSV vaccine were better protected than those vaccinated with killed virus vaccines administered intranasally or a modified-live BRSV vaccine administered IM, even in the presence of maternal antibody. Whereas intranasal vaccination may be a safe and effective means of protecting calves against BRSV challenge, little or no research has been performed on the impact of this mode of administration on cytokine responses in cattle after challenge.

The purpose of the study reported here was to characterize expression of key cytokine messenger RNAs (mRNAs) after BRSV challenge at various sites in calves vaccinated intranasally with a modified-live BRSV vaccine. In this study, IL-4 and IFN-γ were specifically targeted because they are known to be important in cross regulation of CD4+ T-helper cell activation. Concentrations of IL-4 and IFN-γ mRNA were measured in the lungs, bronchoalveolar lavage (BAL) fluid cells, pharyngeal tonsils, and tracheobronchial lymph nodes. The pharyngeal tonsil and tracheobronchial lymph nodes were chosen as sites of induction of T-cell responses in the nasopharyngeal region and bronchiolar region, respectively. In addition, tumor necrosis factor-α (TNF-α) was evaluated because it can initiate and modulate inflammatory responses and has been implicated in the pathogenesis of the disease. On the basis of results from previous research in cattle, humans, and mouse models, it was anticipated that a Th2-type response (characterized by increased expression of mRNA for IL-4 or decreased expression of mRNA for IFN-γ) would be identified in the lungs, BAL fluid cells, pharyngeal tonsil, and tracheobronchial lymph nodes of calves following BRSV challenge, and that this response would be diminished or polarized to a Th1 response in calves receiving a modified-live BRSV vaccine intranasally prior to challenge. We further hypothesized that TNF-α mRNA expression would be increased in the lungs and BAL fluid cells of calves after BRSV challenge, reflecting the pulmonary inflammatory response caused by BRSV infection, and that this inflammation would be attenuated in the intranasally vaccinated calves, resulting in decreased expression of TNF-α mRNA in the lung and BAL cells.

Materials and Methods

Animals and sample collection—The research described was performed with the approval of the University of Georgia Institutional Animal Care and Use Committee. Thirty conventionally reared colostrum-fed Holstein calves 8- to 12 weeks old were allocated into 3 groups held in isolation, as previously described. Because of the time required for sample collection, calves were studied in pairs, with 1 calf receiving modified-live BRSV vaccine and 1 calf receiving mock vaccine intranasally. At the time of vaccination, vaccinated calves were isolated from mock-vaccinated calves to prevent spread of the vaccine strain of the virus to unvaccinated calves between the time of vaccination and BRSV challenge. Details of vaccination have been previously described. Briefly, calves were vaccinated intranasally on day 0 with 2 mL of live high-passage, low-virulence BRSV at $1 \times 10^6$ TCID₅₀/mL. Calves were challenged with BRSV 30 days after vaccination via aerosol exposure as previously described. Each calf received 6 mL (5 X $10^7$ to 10$^7$ TCID₅₀/mL) of a low-passage, high-virulence isolate. Four calves were vaccinated and challenged with BRSV (VC-group calves). Six calves received 2 mL of mock vaccine (spent tissue culture medium) intranasally, followed by BRSV challenge (MC-group 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 and were removed from the study. Three unvaccinated calves received only mock challenge (6 mL of spent tissue culture medium) as a negative control (control-group calves). Control-group calves were the same age and had the same management background as the VC- and MC-group calves; they were housed under identical conditions and mock challenged after the last pair of BRSV-challenged calves to prevent unintentional exposure of control-group calves to BRSV. Clinical signs were evaluated as previously described and included measurement of rectal temperatures, respiratory rates, and calculation of clinical scores. Clinical scores were calculated on the basis of a system described by Collie et al. Calves were euthanatized with an IV injection of barbiturate on day 7 after BRSV challenge. A 2 X 2 X 1-cm section of the right cranial lung, a portion of the pharyngeal tonsil, and a portion of a tracheobronchial lymph node were removed promptly and quickly frozen in liquid nitrogen before storage at –80°C. For postmortem BAL, a snugly fitting adapter was inserted into the left cranial bronchus and 500 mL of sterile saline (0.9% NaCl) solution was infused by moderate manual pressure through an IV delivery set attached to the adapter. After infusion, fluid was permitted to run back out through the delivery set into sterile glass bottles. In general, 25% to 50% of the total volume infused was recovered. The fluid was stored on ice and returned to the laboratory within 1 hour, and a manual cell count was performed with a hemocytometer. Recovered cells (2 X $10^7$ to $7 \times 10^7$) were collected by centrifugation at 200 X g, and the entire cell pellet was suspended in 15 mL of a commercial phenol-guanidine isothiocyanate (GIT) preparation and sheared by 4 to 5 passes through an 18-gauge needle. The total cell lysate was stored at –80°C until RNA was isolated.

Total RNA isolation—Frozen tissue was homogenized without thawing in a commercial phenol-GIT preparation. Depending on the amount of tissue available, between 1 to 5 g of tissue was homogenized in 10 mL of phenol-GIT and the RNA extraction was performed in accordance with the manufacturer’s directions, with an amount of homogenate containing the equivalent of 1 g of tissue used in the extraction. Total RNA precipitated from tissue homogenates was washed with 75% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. To prevent contamination by genomic DNA, each RNA sample was treated with RNase-free DNase I for 30 minutes at 37°C, followed by phenol-GIT-chloroform extraction. The RNA was quantified by absorbance at 260 nm, and the purity and integrity were assessed by agarose gel electrophoresis.

Reverse transcriptase-competitive polymerase chain reaction assay—The RNA was incubated at 65°C for 5 minutes. For each sample, 1 µg of total RNA was included in the reverse transcriptase reaction, which was performed by use of oligodeoxynucleotides (oDNA) primers and murine leukemia virus reverse transcriptase at 42°C for 45 minutes and 95°C for 3 minutes. Generated complementary DNA (cDNA) was kept on ice prior to use.
Competitor molecules for bovine TNF-α, IL-4, IFN-γ, and the housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT), were synthesized as previously described. Plasmid DNA was isolated with a commercially available kit. Serial dilutions of competitor molecules were made, and 5 µL of each dilution was added to different tubes. For competitive-polymerase chain reaction (PCR) assays, 5 µL of target cDNA, 0.1 µL of DNA polymerase (0.5 U), and a master mix containing 25 mM of magnesium chloride, 10 X PCR buffer II, and primers were added to each tube for a final volume of 25 µL (Table 1). The mixture was cycled 35 times at 95°C for 15 seconds, 60°C for 20 seconds, and 70°C for 20 seconds, followed by a terminal extension step of 72°C for 7 minutes. Target cDNA of unknown concentration was determined from the concentration of competitor DNA. For TNF-α detection, 0,000 fg and 100 fg of HPRT and TNF-α competitors, respectively, were used (Fig 1). For IL-4 and IFN-γ detection, 46.9 fg of HPRT competitor was used, along with 25 fg and 50 fg of IL-4 and IFN-γ competitors, respectively. All samples were standardized to HPRT concentrations in which the ratio of sample HPRT-to competitor HPRT ratios was adjusted to 0.6 to 1.5.

Gel electrophoresis and analysis of products—The products from each PCR assay were separated on a 1.8% Tris-acetate agarose gel, stained with ethidium bromide, and observed by use of UV light. Analysis of PCR assay products was performed by use of commercially available software. The concentration of cytokine mRNA was expressed as the value standardized to the cytokine competitor, then standardized to HPRT, calculated as (intensity of cytokine cDNA/intensity of cytokine competitor)/(intensity of HPRT cDNA/intensity of HPRT competitor).

Statistical analyses—Standardized values for IFN-γ, IL-4, and the IL-4-to-IFN-γ ratio for each group were compared by use of the Mann-Whitney U test, except for the pharyngeal tonsil data for which groups were compared by use of the Kruskall-Wallis-test and the Dunn test. Values of P < 0.10 were considered significant. The correlation between expression of mRNA for TNF-α in lung and BAL fluid cells was determined by calculation of the Pearson correlation coefficient.

Results

Results of the evaluation of clinical signs have been previously described. Briefly, following intranasal administration of modified-live BRSV vaccine, 2 mL of modified-live BRSV vaccine intranasally followed by aerosol challenge with BRSV (vaccinated); or 2 mL of modified-live BRSV vaccine intranasally followed by aerosol challenge with BRSV (mock-vaccinated) or 2 mL of modified-live BRSV vaccine intranasally followed by aerosol challenge with BRSV (vaccinated).

Table 1—Cytokine competitors, size of the amplicon from target complementary and competitor DNA, and forward (F) and reverse (R) primer sequences used in a reverse transcriptase-competitive polymerase chain reaction assays used to measure tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and interferon-γ (IFN-γ) messenger RNA (mRNA) expression in lung (A) or pharyngeal tonsil (B) from calves receiving an aerosol challenge of 6 mL of spent tissue culture media (control); 2 mL of spent tissue culture medium intranasally followed by aerosol challenge with bovine respiratory syncytial virus (BRSV; mock-vaccinated [MC]); or 2 mL of modified-live BRSV vaccine intranasally, followed by aerosol challenge with BRSV (vaccinated [VC]). Relative ratios were calculated by use of the ratio at the 100 fg concentration of competitor (C) DNA for TNF-α, the ratio at the 50 fg concentration of competitor DNA for IFN-γ, and the ratio at the 25 fg concentration of competitor DNA for IL-4. HPRT = Hypoxanthine phosphoribosyltransferase.

<table>
<thead>
<tr>
<th>Cytokine competitor</th>
<th>Size (bp)</th>
<th>Primer sequence 5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>Target: 230 Competitor: 186</td>
<td>F: GGA GAT GAT CTC ACT TTA ACT GGS R: LAT TAT AGT CAA GGG CAT ATC CCA C</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Target: 410 Competitor: 350</td>
<td>F: CAA GAA TTC AGG TCC TCT CCA GCC TCA AGT AAC R: TTI GGA TCC CCG CAG GGT TAT GAT TTC ACC ACT GAG G</td>
</tr>
<tr>
<td>IL-4</td>
<td>Target: 400 Competitor: 310</td>
<td>F: ATG GGT TTC ACC TAC CAG CTTG R: CAG TTB GAG TAT TTT GCC TCA ATA ATC G</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Target: 440 Competitor: 310</td>
<td>F: TAT GCC CAG GGC CAA TTT TTT AGA GAA ATA GG R: TTA CTT GTC TGC TCC TGG GCC GGC TCG AAA GAG</td>
</tr>
</tbody>
</table>

All samples were standardized to hypoxanthine phosphoribosyltransferase (HPRT).

Figure 1—Examples of agarose gels depicting results of reverse transcriptase-competitive polymerase chain reaction assays used to measure tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and interferon-γ (IFN-γ) messenger RNA (mRNA) expression in lung (A) or pharyngeal tonsil (B) from calves receiving an aerosol challenge of 6 mL of spent tissue culture media (control); 2 mL of spent tissue culture medium intranasally, followed by aerosol challenge with BRSV (mock-vaccinated); or 2 mL of modified-live BRSV vaccine intranasally, followed by aerosol challenge with BRSV (vaccinated).
cine, 2 calves had rectal temperatures > 40°C on day 7 after BRSV challenge and 1 calf had lung sounds that were subjectively considered to be more harsh than normal. No other clinical abnormalities were identified following vaccination or mock vaccination. Following BRSV challenge on day 30 after vaccination, rectal temperatures, respiratory rates, and clinical scores were higher in MC-group calves, compared with VC- and control-group calves, with peak clinical signs of disease observed on day 7 after BRSV challenge. Mean ± SD rectal temperature on day 7 was 40.3 ± 0.4°C for MC-group calves, 39.7 ± 0.8°C for VC-group calves, and 39.4 ± 0.9°C for control group calves. Mean ± SD respiratory rate on day 7 was 85 ± 24 breaths/min for MC-group calves, 53 ± 17 breaths/min for VC-group calves, and 40 ± 11 breaths/min for control-group calves. Mean ± SD clinical scores on day 7 was 464 ± 128 for MC-group calves, 169 ± 106 for VC-group calves, and 94 ± 50 for control group calves. For rectal temperature, respiratory rate, and clinical score on day 7, there was a significant (P < 0.05) difference between MC- and VC-group calves and between MC- and control-group calves; however, there was no significant difference between VC- and control-group calves.

Gross and histologic findings in calves have been previously reported.22 Briefly, gross changes typically paralleled clinical signs, with the most extensive changes observed in MC-group calves, which had more severe clinical signs of disease than VC-group calves. Consolidation was observed in the cranial and sometimes caudal lung lobes of infected calves; interstitial and bullous emphysema was evident in 2 of the MC-group calves. Histologically, consolidation, congestion, edema, syncytial cells, and neutrophil infiltration were seen in lungs from MC-group calves. These lesions were typical of BRSV infection and consistent with those previously reported in calves challenged with BRSV.23 In certain MC-group calves, the lesions were more extensive than in others. Lungs of VC-group calves had many similar changes to those of MC-group calves; however, an increased number of lymphocytes was found around the airways of all VC-group calves,

![Figure 2](#)

**Figure 2**—Standardized ratios of TNF-α mRNA from lungs (A) and bronchoalveolar lavage (BAL) fluid cells (B) from calves receiving an aerosol challenge of 6 mL of spent tissue culture medium (control, white bar); 2 mL of spent tissue culture medium intranasally, followed by aerosol challenge with BRSV (mock-vaccinated, black bar); or 2 mL of modified-live BRSV vaccine intranasally, followed by aerosol challenge with BRSV (vaccinated, shaded bar). In lungs, mean ± SD values for control-, MC-, and VC-group calves were 0.3 ± 0.2, 2.6 ± 2.1, and 0.4 ± 0.1, respectively. In BAL fluid cells, mean ± SD values for control-, MC-, and VC-group calves were 0.6 ± 0.3, 3.8 ± 0.9, and 1.0 ± 0.4, respectively. *Significantly (P ≤ 0.10) different among groups of calves.

![Figure 3](#)

**Figure 3**—Standardized ratios of IL-4 mRNA (A), IFN-γ mRNA (B), and the ratio of the standardized ratios of IL-4-to-IFN-γ (C) from lungs of calves receiving an aerosol challenge of 6 mL of spent tissue culture medium (control); 2 mL of spent tissue culture medium intranasally, followed by challenge with BRSV (mock-vaccinated); or 2 mL of modified-live BRSV vaccine intranasally, followed by challenge with BRSV (vaccinated). For IL-4, mean ± SD values for control, MC-, and VC-group calves were 0.7 ± 0.2, 1.3 ± 0.2, and 2.9 ± 0.2, respectively. For IFN-γ, mean ± SD values for control, MC-, and VC-group calves were 1.3 ± 0.8, 8.9 ± 7.2, and 3.3 ± 1.7. For the ratio of IL-4-to-IFN-γ, mean ± SD values for control, MC-, and VC-group calves were 0.7 ± 0.4, 0.2 ± 0.1, and 1.0 ± 0.4, respectively. *Significantly (P ≤ 0.10) different among groups of calves. See Figure 2 for key.
compared with that in MC- or control-group calves. Eosinophils were not observed in the lungs of any calves. Only minimal nonspecific changes were detected on gross inspection of the lungs of 2 of the 3 control-group calves, and no abnormalities were found in the lungs of the third control-group calf. No histologic abnormalities were found in the lungs of control-group calves. Amounts of BRSV in lung tissue was not quantified, but BRSV was identified in the lungs of all MC- and VC-group calves by immunohistochemistry, and no BRSV was identified in the lungs of control-group calves.

Following BRSV challenge, 1 calf in the MC-group died unexpectedly because of severe BRSV-induced disease during the evening of day 5 after post challenge. Because the calf died unexpectedly, tissue was not processed immediately for RNA isolation; thus results for tissue cytokine analysis are presented for only 5 calves from the MC-group. In the lung, experimental BRSV infection resulted in significantly increased expression of TNF-α mRNA in MC-group calves, compared with that of control-group calves (P = 0.036) and VC-group calves (P = 0.016; Fig 2). Because of ineffective RNA preservation by a buffer originally chosen for sample storage, good-quality RNA could only be isolated from 3 BAL samples from VC-group calves and 4 samples from MC-group calves. As in the lung, expression of TNF-α mRNA in BAL fluid cells was significantly higher in MC-group calves than in control (P = 0.057) and VC-group calves (P = 0.057). The Pearson correlation coefficient for the correlation between mRNA expression for TNF-α in the lung and BAL fluid...
In the lung, expression of IL-4 mRNA was significantly higher in VC-group calves, compared with control (P = 0.057) and MC-group calves (P = 0.016); there was no significant difference between the control and MC groups (Fig 3). Expression of mRNA for IFN-γ was significantly higher in MC-group calves (P = 0.036) and VC-group calves (P = 0.057) than in control-group calves; there was no difference between MC- and VC-group calves. The ratios of IL-4-to-IFN-γ expression, which were calculated as a measure of Th2-to-Th1 bias, were significantly lower in MC-group calves than in control- (P = 0.036) and VC-group calves (P = 0.016; Fig 3), consistent with an increase in Th1-type responses (ie, increased expression of IFN-γ mRNA), compared with the other 2 groups.

Cytokine mRNA expression for IL-4 and IFN-γ from BAL fluid cells generally paralleled the findings in the lung in which expression of IL-4 mRNA was significantly (P = 0.037) higher in VC-group calves than MC- and control-group calves and expression of IFN-γ mRNA was significantly (P = 0.057) higher in MC-group calves than control-group calves (Fig 4). When the ratios of IL-4-to-IFN-γ were evaluated, the values were significantly (P = 0.057) lower in MC-group calves than in control- or VC-group calves, indicating an increase in IFN-γ mRNA expression in MC-group calves. There was no significant difference in the ratio of IL-4-to-IFN-γ between control- and VC-group calves.

Because of destruction of 2 centrifuge tubes during RNA purification, pharyngeal tonsil RNA samples were available for only 2 VC-group calves. Messenger RNA expression for IL-4 in the pharyngeal tonsil was significantly (P = 0.036) higher in MC-group calves than that in control-group calves (Fig 5). Expression of IL-4 mRNA in VC-group calves was lower than that in MC-group calves, although this difference was not significant. Similarly, expression of IFN-γ mRNA in MC-group calves was significantly (P = 0.036) higher than that in control-group calves and was also higher than that in VC-group calves, but this difference was not significant. The ratio of IL-4-to-IFN-γ was significantly (P = 0.036) higher in MC-group calves than in control-group calves. There was no significant difference in the ratio of IL-4-to-IFN-γ between MC- and VC-group calves.

No significant differences in IL-4 mRNA expression in the tracheobronchial lymph node were observed among groups (Fig 6). Expression of IFN-γ mRNA was significantly (P = 0.016) higher in MC-group calves than that in VC-group calves; there was no difference in IFN-γ mRNA expression between control-group calves and MC- or VC-group calves. Although there was no significant difference in the ratio of IL-4-to-IFN-γ among groups, with the exception of the outlier (calf 672), MC-group calves had lower IL-4-to-IFN-γ ratios in the tracheobronchial lymph node. If the outlier was removed, then the ratio of IL-4-to-IFN-γ was significantly (P = 0.029) lower in MC-group calves than in VC-group calves, indicating a Th1 bias in mRNA expression in MC-group calves, compared with VC-group calves.

**Discussion**

The T-helper cell cytokine response to IM vaccination has been found to influence the response of cattle to subsequent BRSV challenge. To the authors’ knowledge, this is the first study comparing cytokine gene expression profiles from various sites of calves given intranasal BRSV vaccine prior to challenge. A significant decrease in clinical scores in vaccinated calves, compared with mock-vaccinated calves, indicated that modified-live BRSV vaccine administered intranasally effectively protected calves from disease caused by BRSV infection. Results of the study reported here provide new insight into mechanisms of protection afforded by mucosal BRSV vaccination of cattle.
Results of other studies\textsuperscript{20,28} suggest that TNF-\(\alpha\) may be involved in both the pathogenesis of disease caused by RSV and protection against RSV infection. Expression of TNF-\(\alpha\) has been associated with antiviral effects, both in human peripheral blood mononuclear cells (PBMC) exposed to RSV in vitro\textsuperscript{29,30} and in mice challenged with RSV.\textsuperscript{31} Results of the study reported here indicated that TNF-\(\alpha\) mRNA expression increased in the lungs of BRSV-infected calves and are consistent with those of Røntved et al\textsuperscript{21} who quantified TNF-\(\alpha\) with results of ELISA in calves experimentally infected with BRSV. Decreased expression of pulmonary TNF-\(\alpha\) mRNA in vaccinated calves, compared with mock-vaccinated calves, was consistent with decreased pulmonary inflammation in the vaccinated calves. Similar results also appeared in BAL fluid cells in which decreased expression of TNF-\(\alpha\) mRNA was detected in VC-group calves, compared with MC-group calves. The results of our study indicate that expression of TNF-\(\alpha\) mRNA in BAL fluid cells can accurately predict expression of TNF-\(\alpha\) mRNA in the lung.

Evidence that differential use of subsets of T-helper cells can occur after vaccination is documented in mouse models of RSV.\textsuperscript{32,33} In general, immunization that leads to a dominant IL-4 response from T cells is associated with disease severity, whereas immunization that leads to a dominant IFN-\(\gamma\) response is protective.\textsuperscript{8} Graham et al\textsuperscript{32} indicated that the formulation and route of delivery of a vaccine could influence the pattern of cytokine expression in mouse lungs after RSV challenge. Challenge of mice primed with live RSV by parenteral or mucosal routes induced type 1 cytokine response. Studies in human infants infected with RSV have also indicated a role for T-helper cell cytokines in disease outcome. Roman et al\textsuperscript{34} reported decreased production of IFN-\(\gamma\) and IL-4 by cultured PBMC from infants with acute RSV bronchiolitis, compared with healthy infants. Because production of IFN-\(\gamma\) was more depressed than production of IL-4, the authors of that study concluded that this represented an imbalance in cytokine production favoring a Th2 profile.

Results of several studies\textsuperscript{10,12,20} indicate that the relative balance of Th1 and Th2 cytokines influences the outcome of BRSV infection in cattle. Production of BRSV-specific IgE is associated with expression of IL-4 mRNA and with clinical signs of increased severity in calves challenged with BRSV.\textsuperscript{12} In calves vaccinated IM with modified-live BRSV vaccine, IFN-\(\gamma\) production by PBMC increased coincident with protection against clinical signs of disease following BRSV challenge.\textsuperscript{13} Results of 1 study\textsuperscript{20} with a model of vaccine-enhanced disease indicated that calves vaccinated IM with formalin-inactivated BRSV vaccine developed more severe clinical signs after BRSV challenge than mock-vaccinated control calves, and IFN-\(\gamma\) production by PBMC was decreased in calves that received the formalin-inactivated vaccine, compared with mock-vaccinated control calves.

Given that increased IFN-\(\gamma\) production has been associated with less severe disease caused by BRSV infection, results of the study reported here and a previous study\textsuperscript{23} indicating decreased concentrations of IFN-\(\gamma\) mRNA and protein\textsuperscript{22} in the lungs and BAL fluid cells of VC-group calves were unexpected. These results may suggest that IFN-\(\gamma\) production in the lung is not related to protection induced by intranasal vaccination. Alternatively, because the kinetics of the immune response were not evaluated during these studies, it may be that expression of IFN-\(\gamma\) was high in VC-group calves earlier than day 7 after BRSV challenge when cytokine mRNA and protein concentrations were measured in these studies. Further research to characterize cytokine expression earlier in the course of infection is required to clarify the role of IFN-\(\gamma\) production in the lung in vaccine-induced protection.

There was no significant difference in the concentrations of mRNA for IL-4 or IFN-\(\gamma\) in the pharyngeal tonsil between MC- and VC-group calves. The small sample size in the VC group may have contributed to this finding. From the available data, it is not possible to associate changes in cytokine expression in the pharyngeal tonsil on day 7 after challenge with vaccine-induced protection. However, because concentrations of mRNA for both IL-4 and IFN-\(\gamma\) were significantly increased in the pharyngeal tonsil in MC- and VC-group calves, compared with mock-challenged control calves, the data nonetheless confirm that the pharyngeal tonsil is a site of immune activity during BRSV infection and may be amenable to targeted stimulation via novel vaccine strategies.

Cytokine mRNA concentrations in the tracheobronchial lymph node differed from those observed in the pharyngeal tonsil. Although no significant differences were found among IL-4 mRNA concentrations, the relative concentrations of IFN-\(\gamma\) mRNA were significantly higher in MC-group calves than in VC-group calves. When the calf with the highest IL-4-to-IFN-\(\gamma\) ratio was included, no significant differences were observed in IL-4-to-IFN-\(\gamma\) ratio values between MC- and VC-group calves; however, after removal of this calf from the analysis, the IL-4-to-IFN-\(\gamma\) was significantly higher in VC-group calves. Results of our study indicated that the mRNA concentration for IFN-\(\gamma\) was low in the tracheobronchial lymph node. This was in contrast to previously reported findings\textsuperscript{22} that IFN-\(\gamma\) production (as measured by ELISA) was higher in mononuclear cells isolated from the tracheobronchial lymph node of VC-group calves. Although we cannot rule out a disjunction between mRNA transcription and protein production, it is more likely that, given the tight regulation accompanying IFN-\(\gamma\) production, these findings are related to the timing of sampling. Concentrations of mRNA for IFN-\(\gamma\) in the tracheobronchial lymph node may have been higher in VC-group calves earlier than day 7 after BRSV challenge, peaking before protein concentrations.

Differences in cytokine expression in the pharyngeal tonsil, compared with those observed in the tracheobronchial lymph node, may be related to localization of the immune response. Variation between peripheral and local immune responses is well supported in diseases caused by infectious organisms that have a predilection for specific sites within the host. Results of the study reported here do not permit a more
in-depth assessment of this possibility. Nonetheless, these findings are consistent with differences in kinetics of cytokine responses in the nasopharyngeal region as compared with the bronchiolar region.

In our study, a reverse transcriptase-competitive PCR assay was used to generate, evaluate, and compare relative mRNA expression within and among treatment groups, and from these comparisons directional changes in the immune response were postulated. We chose to use relative values rather than define responses in absolute terms because of the inherent variability in immune responses of outbred species (ie, calves). The lack of evidence directly associating the absolute value of mRNA concentrations with the intensity of an immune response, and because of the constraints involved in the use of techniques aimed at specifically quantifying copy numbers of mRNA transcripts. Furthermore, data obtained from transcription studies must be quantified with the caveat that transcription may not necessarily equate to the production of a functional protein. Nonetheless, the approach used herein provided useful and reliable information that is relevant to understanding the nature of T-helper cell responses in calves receiving intranasal BRSV vaccination.

Results of the study reported here clearly support our hypothesis that BRSV infection leads to increased expression of mRNA for TNF-α in the lung and BAL fluid cells, and that mRNA expression for TNF-α at these sites are downregulated in vaccinated calves. However, polarization to a Th2 response (evidenced by increased expression of mRNA for IL-4) in mock-vaccinated calves following BRSV challenge, compared with vaccinated calves, was not clearly confirmed. Further investigation of the kinetics of the host response may help to resolve this issue; this seems likely given that higher concentrations of IFN-γ protein have been observed at various sites of VC-group calves on day 7 after BRSV challenge. Alternatively, a rigid interpretation of the Th1 and Th2 paradigm may be insufficient to fully explain immunologic events that occur in response to BRSV challenge in calves vaccinated intranasally. Results of other studies indicate a unique association between IL-4 and IFN-γ expression in cattle infected with nematodes27,35 or bovine viral diarrhea virus28 in which increased expression of both cytokine mRNAs were observed. Thus, additional research is required to better characterize which components of the immune response are responsible for the protection induced by intranasal vaccination. In addition to T-helper cells, it is likely that other types of cells, such as CD8+ cytotoxic T lymphocytes, γδ T cells, natural killer cells, B cells, and epithelial cells also contribute to the host response, thereby influencing the modulation of subsequent immune events.37-39 Moreover, although the focus of this study was the cell-mediated immune response to intranasal BRSV vaccination, mucosal antibody production may have also influenced the response to BRSV challenge in VC-group calves.

References


