Bovine respiratory syncytial virus-specific IgE is associated with interleukin-2 and -4, and interferon-γ expression in pulmonary lymph of experimentally infected calves

Laurel J. Gershwin, DVM, PhD; Robert A. Gunther PhD; Mark L. Anderson, DVM, PhD; Amelia R. Woolums, DVM, PhD; Kerrie McArthur-Vaughan, BS; K. Erin Randel, BS; Gabrielle A. Boyle, BS; Kathleen E. Friebertshauser, BS; Pat S. McInturff, BS

Objective—To study the local immune response of calves to bovine respiratory syncytial virus (BRSV) infection with emphasis on IgE production and cytokine gene expression in pulmonary lymph.

Animals—Twelve 6- to 8-week-old Holstein bull calves. Six similar control calves were mock infected to obtain control data.

Procedure—Lymphatic cannulation surgery was performed on 12 calves to create a long-term thoracic lymph fistula draining to the exterior. Cannulated calves were exposed to virulent BRSV by aerosol. Lymph fluid collected daily was assayed for BRSV and isotype-specific IgE antibody, total IgG, IgA, IgM, and protein concentrations. Interleukin-4 (IL-4), interleukin-2 (IL-2), and interferon-γ were semi-quantitated by reverse transcription-polymerase chain reaction (RT-PCR). Cell counts and fluorescence-activated cell scanner (FACSCAN) analysis of T-cell subsets were performed on lymph cells.

Results—Calves had clinical signs of respiratory tract disease during days 5 to 10 after infection and shed virus. Bovine respiratory syncytial virus-specific IgE in infected calves was significantly increased over baseline on day 9 after infection. Mean virus-specific IgE concentrations strongly correlated with increases in severity of clinical disease (r = 0.903). Expression of IL-2, IL-4, and interferon-γ was variably present in infected and control calves, with IL-4 expression most consistent during early infection.

Conclusions and Clinical Relevance—Infection with BRSV was associated with production of BRSV-specific IgE, and IL-4 message was commonly found in lymph cells of infected calves. This finding supports the concept that BRSV-induced pathophysiology involves a T helper cell type-2 response. Effective therapeutic and prophylactic strategies could, therefore, be developed using immunomodulation to shift the immune response more toward a T helper cell type-1 response. (*Am J Vet Res* 2000;61:291–298)

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From the Departments of Pathology, Microbiology, and Immunology (Gershwin, Woolums, McArthur-Vaughan, Randel, Boyle, Friebertshauser) and Medicine and Epidemiology (McInturff), School of Veterinary Medicine; the Department of Surgery, School of Medicine (Gunther), University of California; and the California Veterinary Diagnostic Laboratory (Anderson), Davis, CA 95616. Dr. Woolums' present address is the Department of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA 30602.

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Bovine respiratory syncytial virus (BRSV) was determined to be a cause of acute respiratory disease in cattle more than 20 years ago.[1-3] Yet pathogenic mechanisms underlying the variability of the respiratory disease caused by this paramyxovirus are still unresolved. Outbreaks of BRSV are common in 2- to 4-month-old calves, but the disease also is found in feedlot steers and adult dairy cows. In the field, BRSV causes a mild, almost subclinical form of respiratory disease characterized by an increase in body temperature and coughing, without pneumonia. Bovine respiratory syncytial virus infection can also cause severe interstitial pneumonia with dyspnea, wheezes, and sometimes death.[4] Although secondary bacterial infection is commonly associated with virus infections of bovine lungs, this severe form of the disease can develop without such complications.[5] Similarly, we have found in experimentally induced BRSV infections that there is variability in the severity of clinical signs of different calves exposed with the same virus exposure method and inoculum. Clearly some BRSV isolates are more virulent than others and, thus, cause greater disease. There is nonetheless variability in disease expression when a single isolate is used to experimentally infect calves. Thus, host factors have been implicated as having a role in the pathogenesis of respiratory disease caused by BRSV.

Immunologic responses have been implicated in the pathogenesis of BRSV infection and human **respiratory syncytial virus (RSV)**, a disease caused by a related virus. Children infected with RSV have a similar dichotomy of signs. Wellivier et al[6] measured high concentrations of histamine and RSV-specific IgE in the nasopharyngeal secretions of children who had the most severe form of the disease, with wheezing and dyspnea. Whereas children with upper respiratory disease (signs limited to rhinitis and fever) did not have an IgE response to the virus.[6] Stewart and Gershwin found a correlation between disease severity and the production of BRSV-specific IgE in experimentally infected calves, providing evidence that a type-1 hypersensitivity response can have a role in BRSV pathogenesis.[7,8]

Having developed and characterized an infection model for induction of experimental BRSV and a lymph cannulation procedure that allows access to lymph that is draining the lung, we sought to combine these procedures to evaluate changes in the immune response during BRSV infection.[9,10] Our study was intended to provide an insight into the mechanism of pathogenesis of the severe form of the disease. To further examine the

potential role for IgE, and secondarily an associated **T helper cell-2 (Th2)** response, we chose to administer 2 immunomodulators to subgroups of infected and control calves. ***Bordetella pertussis* (Bp)** is recognized as an immunomodulator facilitating a Th2-induced IgE response to inhaled antigen when given simultaneously with injected antigen,[11-14] whereas **complete Freund's adjuvant (CFA)** is known to facilitate a **T helper cell 1 (Th1)** response, stimulated by the mycobacteria extract that it contains.[15] Thus, we sought to determine whether the type of immune response elicited by infection could be modified and, if so, whether the modification could change the cytokine pattern, IgE production, and the clinical course of disease.

Materials and Methods

Calves—Twelve 6- to 8-week-old conventionally reared, colostrum-fed Holstein bull calves were obtained from a local dairy during a period of 3 years. Body weights ranged from 110 to 160 kg. Calves were studied individually and housed in a screened-in barn where alfalfa hay, water, and a salt block were provided. Control data were obtained from an additional 6 mock-infected calves, 5 of which provided data for a description of the thoracic lymphatic cannulation technique.[10]

Surgical cannulation—Thoracic lymph fistulas were prepared in calves using an adaptation of the method for sheep lymph cannulation by Staub et al.[16] The calf-adapted method[10] involves placement of a silastic catheter in the efferent lymphatic of the caudal mediastinal lymph node during a right dorsolateral thoracotomy. After recovery from surgery calves were observed 3 to 4 times a day to evaluate appetite, attitude, and patency of the cannula. When samples were not being collected, the cannula was allowed to drip freely while being held away from the thorax by tape fixation to a plastic tube holder that had been sutured into the skin on the lateral area of the thorax.

BRSV infection—Calves were infected on day 0 (5 days after the lymphatic cannulation surgery) with a virulent field isolate of BRSV (CA-1) by aerosol and face mask.[9] The titer of virus used for each calf varied slightly, because calves were studied successively and, therefore, did not receive the same inoculum. The inoculum varied from 3.2×10^3 to 5.6×10^4 viruses. Control calves were mock infected with aerosol of tissue culture medium. Four of the 12 infected calves and 2 of the 6 control calves received 1×10^6 killed Bp cells intramuscularly as a Th2 immunomodulator on day 0. Three of the 12 infected calves and 1 of the 6 control calves received CFA intramuscularly as a Th1 immunomodulator on day 0. Clinical scores were assigned on the basis of a numeric system developed by Collie and modified by us.[17,18] Briefly, each calf was observed daily by an investigator/veterinarian for signs of depression, appetite, the presence, quantity, and nature of nasal and ocular exudate, cough, adenitis, and rectal temperature. Heart rate, respiratory rate and lung sounds were evaluated by ascultation. A score was assigned for each day of the experiment.

Lymph collection and processing—Pulmonary lymph samples were collected daily from the day of surgery (day –5) until day 10 or until the lymph cannula ceased flowing. Lymph was collected into a heparinized 50-ml plastic centrifuge tube and processed by centrifugation to separate the cell pellet from the fluid lymph. On days –4, –1, 1, 4, 7, and 10, aliquots of cells were used for **fluorescence-activated cell scanner (FACSCAN)** analysis. The remainder of the cells was used for RNA preparation after a smear was obtained for cytologic evaluation.

Blood sample collection and processing—Blood was collected from the jugular vein into evacuated blood collection tubes without coagulant to obtain serum samples on days –7 (2 days before cannulation), 0 (day of infection), day 7, and day 10. Tubes containing the anticoagulant EDTA were used for CBC determination. Heparinized tubes were used for FACSCAN analysis on days –7, –1,4, 7, and 10.

Antibody assays—Total IgG, IgA, and IgM concentrations in pulmonary lymph samples were measured by single radial immunodiffusion. Total IgE concentration in lymph was measured by a double antibody sandwich ELISA, using monoclonal bovine IgE antiserum.[19] Immunoglobulin E units were read from a standard curve.[20] Bovine respiratory syncytial virus-specific IgG, IgA, IgM, and IgE were performed by ELISA on lymph and serum samples (days 0, 7, 10 only), using sucrose density gradient purified BRSV as antigen.[7,21] Positive control sera for IgG, IgA, and IgM consisted of pooled serum from hyperimmunized and infected calves. The positive control serum was collected from a calf immunized with BRSV in aluminum hydroxide adjuvant and evaluated for the presence of antigen-specific IgE by Prausnitz-Kustner (P-K) testing.

Lymphocyte labeling and FACSCAN analysis—Lymphocytes obtained from pulmonary lymph for FACSCAN analysis, as well as lymphocytes obtained from blood on days –7, –1, 4, 7, and 10, were prepared for analysis.[10] Briefly, the aliquot of cells obtained from lymph or after separation of blood on a ficoll-hypaque gradient, was counted, centrifuged, and resuspended in **phosphate-buffered saline (0.9% NaCl) solution (PBSS)**. In separate tubes, 10^7 cells were resuspended in 100 µl of the following primary bovine antiserum: CD4 (Ilrad-A26) antiserum, CD8 (Ilrad-A12) antiserum, CD2 (Ilrad-A17) antiserum, and Ig antiserum, to quantify T helper cells, T cytotoxic/suppressor cells, total T cells, and total B lymphocytes, respectively. Incubations of 30 minutes on ice were followed by incubation with 100 ml of fluorescent goat mouse-Ig antiserum. After another incubation of 30 minutes, cells were washed twice and resuspended in 450 ml of PBSS and 150 ml of neutral-buffered 10% formalin and assayed within 24 hours.

Detection of cytokine gene expression—RNA was obtained from lymphocytes in pulmonary lymph samples. Daily collection of lymph into a plastic 50-ml centrifuge tube containing 0.5 ml of preservative-free heparin was followed by centrifugation to separate the cell pellet from the lymph fluid. The cell pellet was resuspended into guanidinium isothiocyanate buffer, vortexed lightly, and frozen at –80 C until further processed. **Reverse transcription-polymerase chain reaction (RT-PCR)** was performed, using a commercially available kit.[a] Reverse transcription of 1 mg of RNA was performed, using murine leukemia virus reverse transcriptase in the following reaction: 1 cycle of 10' at 25 C, 30' at 42 C, and 5' at 98 C. The generation of **complementary DNA (cDNA)** was accomplished using 2.5 m*M* oligo (dT) primers in a total reaction volume of 20 ml. Polymerase chain reactions for IL-2, IL-4, and **interferon-γ (IFN-γ)** products were performed, using the following conditions: 1 cycle of 2' at 95 C, 35 cycles of 1' at 95 C, 1' at 55 C, 2' at 72 C, and 1 cycle of 7' at 72 C. Polymerase chain reaction for the actin housekeeping product was performed, using the following conditions: 1 cycle of 2' at 95 C, 24 cycles of 1' at 95 C, 1' at 65 C, 45" at 72 C, and 1 cycle of 7' at 72 C. The total volumes for PCR were 100 µl (**Table 1**).

The resulting PCR products were analyzed by electrophoresis on a 1.8% agarose gel against a 100 base pair ladder. Bands were viewed using ethidium bromide under ultraviolet illumination. Actin was included as the housekeeping gene, to assess the quality of cDNA, and as a control for PCR. Other controls included an external positive control that consisted of a

Table 1—Reverse transcription-polymerase chain reaction primers used for the expression of cytokine gene expression from RNA of lymphocytes in pulmonary lymph of bovine respiratory syncytial virus (BRSV)-infected calves and control calves

Primers		Sequence	Reference No.
IFN-γ 300 bp	Sense	5′ TTCAGAGCCAAATTGTCTTCC 3′	29
	Antisense	5′ CATTACGTTGATGCTCTC CG 3′	
IL-2 303 bp	Sense	5′ ACA TTTGACTTTTACGCGC 3′	30
	Antisense	5′ GAGAGGCACTTAGTGATC 3′	
IL-4 303 bp	Sense	5′ GTCCATGGACACAAGTGTGATA 3′	31
	Antisense	5′ TTCCAAGAGGTCTTTCAGCGT 3′	
Actin 390 bp	Sense	5′ CCTTTTACAACGAGCTGCGTGTG 3′	32
	Antisense	5′ ACGTAGCAGAGCTTCTCCTTGATG 3′	

IFN-γ = Interferon-γ. IL-2 = Interleukin-2. IL-4 = Interleukin-4. bp = Base pairs.

known quantity of cDNA. The cDNA was made, using the same PCR conditions as described, and was used for the standardization of PCR products from gel to gel. Blood mononuclear cells incubated with **concanavalin A (Con A)** for 18 hours were also subjected to the same PCR conditions to provide a positive RT-PCR (internal) control for the detection of amplified IL-2, IL-4, IFN-γ, and actin messenger RNA. The semiquantitation of band densities was performed, using an image analysis system.[b] Band densities were measured as a percentage of the internal Con A control for each cytokine. The finding of any band quantified as 10% or more of the standard value of the internal control on the same gel was considered as a positive result for cytokine message in that particular sample on a particular day of infection.

Virus shedding—Each day beginning on day 1 a sterile swab was inserted deeply into the nasal cavity to obtain a caudal nasopharyngeal swab specimen for virus isolation. The swab was immediately placed into a 15 ml conical plastic centrifuge tube containing tissue culture medium on ice. After transport to the laboratory the medium was filtered through a syringe-top 0.2 µm-diameter millipore filter[c] onto replicate wells on a slide on which bovine turbinate cells were growing. Positive and negative control wells consisted of cells inoculated with stock virus or uninoculated, respectively. After incubation for 4 to 5 days (when cytopathic effect was evident) the slides were fixed for 1 minute in acetone and stained with fluorescein isothiocyanate conjugated rabbit anti-human RSV[d] and observed for intracytoplasmic staining with epiillumination microscopy.

Pathologic findings in lung tissues—Three of the control calves were euthanatized with pentobarbital sodium and necropsied on day 10 to observe and ascertain that the lung cannulation procedure did not create any lesions that may influence the variables examined.[10] Three of the infected calves were also necropsied on day 10 to confirm and describe the lesions that resulted from BRSV infection. A fourth infected calf was euthanatized in a moribund state and necropsied on day 7. Briefly, gross pathologic evidence showing consolidation and interstitial emphysema was expected. On histologic examination, interstitial cellular infiltration, bronchiolitis, alveolitis, and synctia were expected as lesions characteristic of BRSV infection. Other calves were not necropsied, because the course of experimental disease and virus shedding was predicable for those calves with patent cannulas on day 10, and it was desirable to continue to follow immune variables for additional days (data not presented). The lungs were removed, and the left lung was perfused with 10% neutral-buffered formalin. The right lung was used to provide samples for aerobic bacterial culturing; ELISA and immunohistochemical staining for BRSV; and immunofluorescence detection of other infectious bovine rhinotracheitis, parainfluenza 3, and bovine viral diarrhea viruses. The pathologic examination protocol has been reported in detail elsewhere.[18]

Statistical analyses—Multivariate ANOVA with 1 grouping variable (infection status) and 1 repeated measure (day) was used to model clinical score, leukocyte numbers in pulmonary lymph as fraction of baseline, lymph flow rate, lymph protein concentration, lymph total IgA, IgE, IgG, and IgM concentrations, lymph BRSV-specific IgA, IgE, IgG, and IgM concentrations, numbers of T cells in lymph and serum samples ($CD2^+$, $CD4^+$, $CD8^+$, and the ratio of $CD4^+$:$CD8^+$), and numbers of B-cells in blood and lymph. The assumption of multivariate normality was reasonable (tests for violations of univariate normality were nonsignificant for all data except leukocyte numbers, to which a log-transformation was applied to achieve normality).[22,23]

Significance of multivariate ANOVA main effects and interactions among grouping variables and day was set at the $P < 0.05$. Significant interactions were further analyzed for significant differences between infected and control calves at the 0.05 joint confidence level, using Bonferroni multiple comparisons procedures.[22,23] Appropriateness of including data from calves that were treated with immunomodulators with data from calves that were not treated with immunomodulators was determined by considering the significance of immunomodulator main effect and interaction terms when included in the model. Data of calves were grouped together if the main effect and interaction terms were nonsignificant. These data are presented mean ± SEM.

Concentrations of BRSV-specific IgM and IgG in serum samples were analyzed using paired t-tests to compare samples on, before, and after infection and days. The significance of a linear correlation between daily mean BRSV-specific IgG, BRSV-specific IgE, and clinical score was tested, using a Wald statistic.

Results

Lymph cell count—Daily pulmonary lymph total WBC counts were evaluated as a percentage of baseline values (ie, values on day 0 before infection). Infected and control calves had an overall decline in cell counts, but the infected calves had predominant fluctuations above and below baseline values, whereas the control calves had a progressive decline below baseline values. Cell count (measured as a percentage above or below day 0 values) for infected calves on day 7 was 5.41 ± 8.95%, compared with –21.46 ± 24.40% for control calves. For day 10, these values were –17.7 ± 13.84% for infected calves and –44.78 ± 15.56% for control calves. Standard error values indicated high variability, and the differences between infected and control calves were not significant on any day.

Lymph flow rates—Pulmonary lymph flow rate for infected calves varied from a low value of 11.0 ± 1.80 ml/h on day 1 to a high value of 26.3 ± 4.67 ml/h on day 8. Flow rates of control calves varied from a low value of 13.1 ± 4.78 ml/h on day 2 to a high value of 19.3 ± 8.1 ml/h on day 8. On no day was the difference between the flow rate in infected and control calves significant.

Lymph protein concentration—Pulmonary lymph protein concentrations varied from baseline values of 4.24 ± 0.24 mg/ml for infected calves and 4.07 ± 0.31 mg/ml for control calves to 3.64 ± 0.34 mg/ml and 4.42 ± 0.51 mg/ml, respectively on day 10. There was no significant difference in protein concentration between groups at any time during the study.

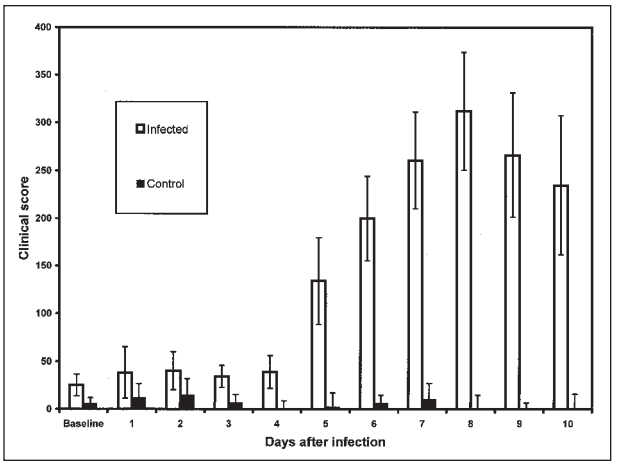

Figure 1—Mean (± SEM) clinical sign scores vs days after infection for bovine respiratory syncytial virus (BRSV)-infected (n = 12) and mock-infected control (6) calves. Baseline values represent scores before infection.

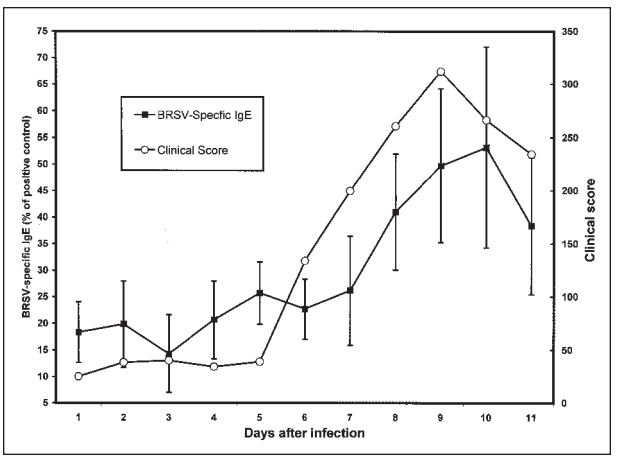

Figure 2—Mean (± SEM) BRSV-specific IgE concentrations expressed as a percentage of positive control value and clinical scores vs days after infection for BRSV-infected calves (n = 12).

Clinical signs—The clinical course of disease in experimentally infected calves in our study has been reported.[24] On the basis of daily clinical scores, there was a gradual progression of disease severity on days 5 through 8 in infected calves with continued signs of disease through day 10 when the experiment was terminated (**Fig 1**). Control calves remained clinically normal throughout the observation period. Differences in clinical scores of infected and control calves were significant for days 5 through 10.

Total and BRSV-specific Ig concentrations—During the 10-day period, there was no significant difference change in total IgG concentrations in pulmonary lymph of control and infected calves, compared with baseline values. Baseline values for lymph total IgG concentrations in control calves were, in general, less than those for the infected group.

To normalize the effect of preexisting BRSV-specific antibodies obtained from passive transfer via colostrum ingestion, each calf served as its own control and data on BRSV-specific IgG concentrations were evaluated for an increase compared with baseline values during the 10 day period. There was no significant increase in lymph or serum BRSV-specific IgG concentrations in infected calves or control calves.

Pulmonary lymph total IgM concentration in infected calves varied from a baseline value of 33.69 ± 4.45 mg/ml to a high value of 95.15 ± 33.27 mg/ml on day 9. Mean concentrations for control calves varied from a baseline value of 27.76 ± 3.13 mg/ml to a high value of 43.50 ± 8.35 mg/ml on day 8. On days 8, 9, and 10 there were significantly greater lymph total IgM concentrations in infected calves than in control calves. Lymph IgM concentrations from infected calves treated with CFA varied from a baseline value of 35 ± 7.77 mg/ml to high values of 115.5 ± 89.50 mg/ml on day 5, 119 ± 66.00 mg/ml on day 6, and 129.5 ± 95.50 mg/ml on day 8.

In infected calves, lymph BRSV-specific IgM concentrations (expressed as percent of a positive control values × 100) increased during the 10-day period. There was a greater increase in lymph BRSV-specific IgM concentrations in infected calves treated with CFA, compared with infected calves that were not treated (49.3 ± 6.72 mg/ml on day 0 to 90.2 mg/ml on day 7 compared with 45.1 ± 7.41 mg/ml on day 0 to 51.1 ± 16.40 mg/ml on day 7, respectively). Infected calves treated with Bp did not have an increase in lymph BRSV-specific IgM concentrations over baseline values. Control calves did not have an increase in lymph BRSV-specific IgM concentrations over time.

Pulmonary lymph total IgA concentrations were generally higher in infected calves than in control calves. When data were expressed as a percent change from the day 0 value to account for differences in baseline values, there was no significant difference between control and infected calves. There was also no significant change over time in lymph total IgA concentrations within either group.

Lymph BRSV-specific IgA concentration in infected calves had no significant change (expressed as percent of a positive control values × 100) over time. However, there was a gradual increase in lymph BRSV-specific IgA concentration from a baseline value of 49.2 ± 6.67 mg/ml to a high value of 68.1 ± 10.35 mg/ml on day 9. Control calves did not have an increase in lymph BRSV-specific IgA concentrations over time.

Pulmonary lymph total IgE concentrations did not change significantly over time in either group. Lymph total IgE concentrations ranged from 1.19 ± 0.55 to 2.50 ± 0.69 U in infected calves and from 1.73 ± 0.24 to 3.39 ± 0.96 U in control calves. Modulation by treatment with Bp cells or CFA had no effect on lymph total IgE concentrations.

Lymph BRSV-Specific IgE concentrations (expressed as percent of a positive control values × 100) increased significantly from a baseline value of 18.35 ± 5.71 to a high value of 53.15 ± 18.90 U on day 9 in infected calves (**Fig 2**). Lymph BRSV-Specific IgE concentrations did not change significantly from baseline values over time in control calves.

Infected calves treated with CFA generally had lower pulmonary lymph BRSV-specific IgE concentrations,

compared with those not treated with CFA. Two of the 3 infected calves that were treated with CFA failed to have an increase in lymph BRSV-specific IgE concentrations over baseline values, whereas the third calf had a transient response of 60 % of positive control in lymph on day 8. Two of the 4 infected calves treated with Bp cells had a virus-specific response of > 50 % of positive control in lymph on days 7 to 9. In addition, there was a positive correlation between mean lymph BRSV-specific IgE concentration and mean clinical score (r = 0.905; Fig 2).

Evaluation of serum BRSV-specific IgE concentrations on days 0, 7, and 10 revealed a change similar to that of pulmonary lymph concentrations. However, serum BRSV-specific IgE concentrations were generally 2 to 3 times greater than in the corresponding lymph concentrations, as reflected by mean group values.

Lymphocyte subpopulations in lymph and blood—There were no significant changes between percentages of CD2, CD4, CD8, and surface Ig-bearing cells in pulmonary lymph or blood of infected or control calves on days 4, 7, and 10, compared with baseline values. In lymph, percentage of $CD2^+$ cells varied from 62 ± 10% to 80 ± 4% in infected calves and from 57 ± 5% to 67 ± 6% in control calves, $CD4^+$ cells varied from 33 ± 7% to 48 ± 5% in infected calves and from 42 ± 5% to 50 ± 3% in control calves, and $CD8^+$ cells varied from 26 ± 6% to 33 ± 8% in infected calves and from 17 ± 2% to 23 ± 2% in control calves. Differences between groups and over time were not significant.

In infected calves, the pulmonary lymph-to-blood ratio of $CD4^+$ cells on days 4 and 7 increased, and returned to baseline values on day 10. The ratio of $CD4^+$:$CD8^+$ cells in lymph on day 0 was 2.02 ± 0.34 for infected calves and 2.82 ± 0.63 for control calves. There was no significant change over time, although the ratio of $CD4^+$:$CD8^+$ cells in lymph decreased to 1.87 ± 0.81 in infected calves and 1.91 ± 0.26 in control calves on day 10. Baseline values for B cells in lymph as determined by FACSCAN for surface Ig bearing cells, were higher in control calves than in infected calves (13 ± 5% vs 5 ± 2%). This difference was not statistically significant ($P > 0.05$). Evaluation of the daily data as a percent of baseline values indicated that on day 4 the infected calves had an increased number of B cells in lymph, which was not apparent in control calves. Mean percentage of B cells in lymph of infected calves increased to 2.86 times baseline values, whereas the mean percentage of B cells in lymph of control calves decreased slightly to 0.64 times baseline values. On day 7 both groups had a slight decrease from baseline values (0.89 times baseline value in infected calves, compared with 0.84 in control calves).

In infected and control calves, comparison between percentages of $CD2^+$ lymphocytes in blood versus pulmonary lymph revealed that the number of $CD2^+$ lymphocytes in lymph were approximately 1.5 to 2 times greater than in blood (**Table 2**). This was also true for $CD4^+$ and $CD8^+$ lymphocytes in infected and control calves.

Cytokine gene expression in lymphocytes from lymph—Two of 5 control calves had lymphocyte IL-4 gene expression during days –1 through 4, and none had IL-4 expression after day 4. In contrast, 4 of 8 infected calves had lymphocyte IL-4 gene expression during days –1 through 7, and 2 of 8 infected calves had lymphocyte IL-4 gene expression during days 8 through 10.

Of the control calves, 5 of 5 had lymphocyte IL-2 gene expression during days –1 through 7 and 3 of 4 had lymphocyte IL-2 expression during days 8 through 10. Of the infected calves, 8 of 8 had lymphocyte IL-2 gene expression during days –1 through 10.

Gene expression for IFN-γ was detected in lym-

Table 2—Percentage of T lymphocyte subsets in blood and pulmonary lymph samples from BRSV-infected and control calves

	Infected calves (n = 12)				Control calves (n = 6)			
	d*				d			
Variables	**0**	**4**	**7**	**10**	**0**	**4**	**7**	**10**
Blood								
CD2	42.6	41.7	28.2	45.6	40.6	46.9	47.3	36.9
CD4	28.1	21.0	23.7	20.4	21.3	21.1	25.2	19.3
CD8	16.9	10.1	12.8	19.2	15.4	17.0	9.0	10.5
CD4:CD8	2.17	2.78	2.38	2.02	1.83	1.75	2.39	2.23
Pulmonary lymph								
CD2	68.7	75.5	76.0	63.6	56.8	60.8	65.1	67.0
CD4	44.3	47.6	48.0	37.2	42.6	46.9	50.0	41.3
CD8	31.2	24.1	27.0	32.1	17.7	18.9	18.8	21.8
CD4:CD8	2.02	2.54	2.07	1.87	2.82	2.56	2.91	1.91

*Day 0 indicates values before infection; days 4, 7, and 10 represent values after infection.

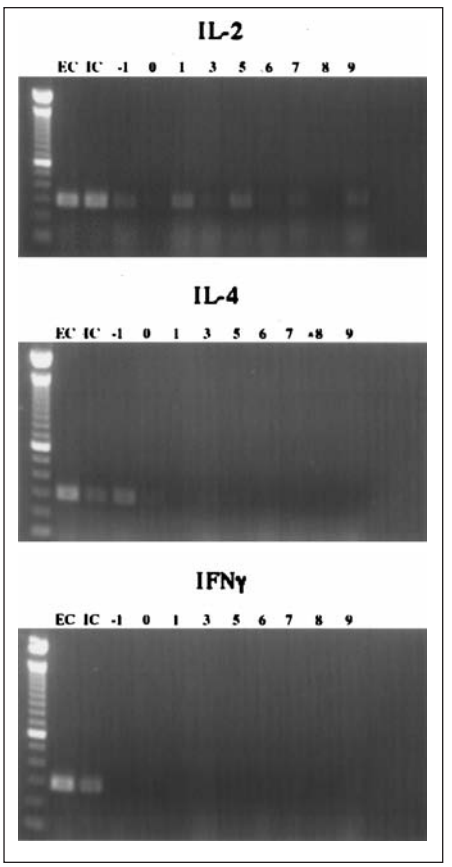

Figure 3—Gel electrophoresis results of reverse transcription-polymerase chain reaction (RT-PCR) products from for daily lymph samples of a representative mock-infected control calf. EC = external control, IC = internal control, numbers at top of lanes indicate day after mock infection. Actin bands (not shown) were observed. The DNA ladder (left) is 100 base pairs (bp). Interleukin-2 (IL-2), IL-4, and interferon-γ (IFN-γ) bands are 300 bp.

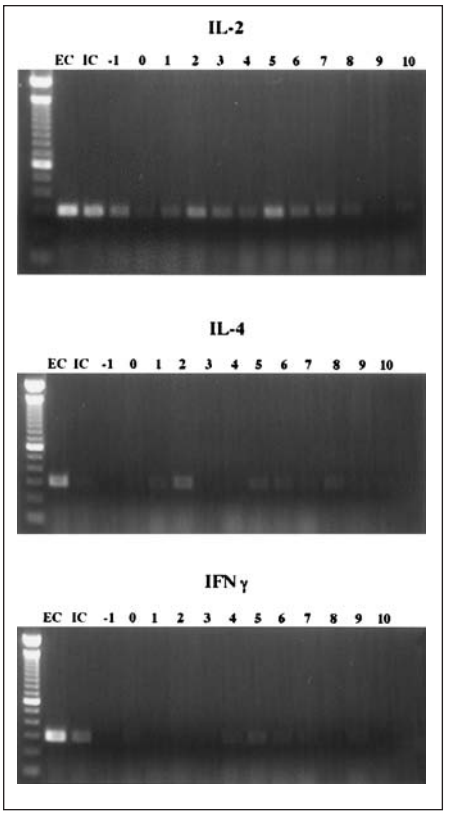

Figure 4—RT-PCR products are shown for daily lymph samples of a representative infected calf. Actin bands (not shown) were present. DNA ladder (left) is 100 bp. IL-2, IL-4, and IFN-γ bands are 300 bp. *See* Figure 3 for key.

phocytes from 4 of 5 control calves during days –1 through 4, from 5 of 5 control calves during days 5 through 7, and from 4 of 5 control calves during days 8 through 10. Of the infected calves, 8 of 9 had lymphocyte IFN-γ gene expression during days –1 through 4, 9 of 9 had lymphocyte IFN-γ gene expression during days 5 through 7, and 5 of 6 had lymphocyte IFN-γ gene expression during days 8 through 10.

Band densities varied from 0 to 110% for IL-2, from 0 to 74% for IL-4, and from 0 to 160% for IFN-γ. Treatment of control calves with Bp cells or CFA did not appear to influence their cytokine expression when compared with untreated control calves.

Three of 4 infected calves that were treated with Bp cells had lymphocyte IL-4 gene expression at some time during days –1 through 10, whereas neither of 2 infected calves that were treated with CFA had lymphocyte IL-4 gene expression on any day. Lymph was not available throughout the entire collection period from the third infected calf that was treated with CFA because of cannula obstruction. In comparison, all tested infected calves that were not treated had lymphocyte IL-4 gene expression (**Figs 3 and 4**).

Virus shedding—All infected calves shed virus at some time during the infection. Virus cultures results were positive for BRSV for most infected calves from days 5 through 9, with at least 3 infected calves shedding virus as early as day 4. On days 3, 4, 7, and 10 for 1 calf and on days 6 and 8 for another, the slides were inconclusive because of problems with contamination or cell viability.

Pathologic findings in lung tissues—Four calves from the infected group were necropsied, and lungs were examined for gross and histopathologic characteristics of BRSV infection and for bacteriologic culturing. Results of these findings are included in another report that describes the infection model that was used herein.[24] One calf had been euthanized on day 7 of infection because of its moribund state. That calf had extensive emphysema with a large bulla in the right caudal lobe. A common histologic feature in infected calves was proliferative bronchiolitis and alveolitis, with bronchiolar and alveolar syncytia. There was no evidence of infection associated with the surgically placed cannulas of calves included in our study. Bacterial growth was found in only 1 of the infected calves; this calf had small numbers of *Pasteurella multocida*. Tissues taken at necropsy were examined by immunofluorescence for infectious bovine rhinotracheitis, Parainfluenza 3 virus, and bovine viral diarrhea viruses and were found to have negative results for these agents. Control calves included in our study that were necropsied did not have any signs of disease, and the cannulas were neither associated with inflammation nor infection.[10]

Discussion

It has been established from the results of human infant studies, as well as results of studies using a mouse model, that RSV infection is associated predominantly with Th2 responses.[25-27] A consequence of the predominant Th2 response is the development of virus-specific IgE in infected human infants and increased severity of disease.[6] Results of our study demonstrated a similar IgE response to BRSV infection in calves.[7,8]

Creation of a lymphatic fistula draining the caudal mediastinal lymph node provides an opportunity to measure cellular and humoral variables continuously in lymph from the lung. In our study we used this method to measure the cytokine responses, T cell subset fluctuations, and antibody production before and during experimental BRSV infection. When variables were statistically compared with mock-infected control calves, the most important observation identified was a strong correlation between BRSV-specific IgE and the development of clinical disease. The temporal pattern of cytokine production, although supportive of IgE production, was not an exclusively polarized Th2 response. Interleukin-4 cytokine gene expression was accompanied by IgE production in lymph draining the lung of calves infected with BRSV, but prior to infection there was evidence of IL-4 gene expression in several calves. Although the use of Bp cell treatment to upregulate a Th2 type response was not 100% effective, it is notable that 3 out of 4 infected calves treated with Bp cells had evidence of IL-4 gene expression during infection, whereas IL-4 was not detected in control calves that were treated with Bp cells. Moreover, treatment with CFA was not associated with IL-4 gene expression in infected calves. This indicates that a Th1 response inducer may be useful in altering the immune response to BRSV infection.

Results of another of our studies indicates that there is a lack of IL-4 expression in control calves.[e] However, analysis of additional samples in our study reported here revealed IL-4 expression in 2 of 5 control calves on days –1 through 4. The influence of vari-

ability among out-bred calves may be a factor responsible for IL-4 expression in 2 control calves. This response was most likely initiated by immune stimulation resulting from the surgical cannulation procedure that took place on day –5 or the mock-infection that consisted of an aerosol of spent tissue culture medium on day 0. In addition, these calves had positive results for expression of IL-2 and γ-interferon genes during the period of observation. These data differ from those of McInnes et al,[28] who reported on cytokine expression in lymph node cells and blood mononuclear cells from calves euthanatized 7 days after experimental BRSV infection. There are important differences between that study and our study. First, our studies followed cytokine expression daily from preinfection through postinfection day 10 and used pulmonary lymph-derived RNA, whereas the McInnes study examined cells from lymph nodes and lung at a single time point after infection (during necropsy examination). Second, that study used gnotobiotic calves that were 10 days old when infected, whereas calves in our study were 6 to 8 weeks old at the time of infection and were conventionally reared. Interleukin-2 gene expression was detected in control and BRSV-infected calves, whereas McInnes et al detected IL-2 only in infected calves. This difference most likely reflects the gnotobiotic background of the calves in the McInnes study, which would be expected to result in minimum antigenic stimulation. In addition, no mock infection was described for control calves in that study. In contrast, our conventionally reared calves had been exposed to a variety of environmental antigens as well as treated with a spent tissue culture fluid aerosol. In both studies, γ-interferon gene expression was detected in control and infected calves. Despite these differences, results of both studies indicate that although there is a strong IL-4 response to BRSV infection, it is not a highly polarized Th2 response.

Although significant differences in $CD4^+$ and $CD8^+$ T-cell numbers were not apparent when mock-infected control calves were compared with BRSV infected calves, there was a decrease in $CD4^+$ cells on day 10. By day 10 there were fewer data points than at earlier times, because of the cessation of lymph flow in several calves. In a study on 15 hospitalized infants with RSV infection and 17 healthy infants, infection with RSV was associated with a significant increase in B cells and a decrease in $CD8^+$ T cells.[25] Although there were no significant differences in B cell numbers when infected calves were compared with controls, on day 4 of infection there was an increase in B cell numbers in infected calves. The B cell percentage decreased to 0.64 times baseline in control calves, whereas that of infected calves increased. Because samples were obtained from naturally infected infants in the study on human infants, the relationship between the day of infection and the day of sampling is unknown, hence the bovine and human studies, although not directly comparable, indicate similar changes in B cell fluctuation if one assumes that samples were obtained from infants early in infection.

In summary, in our study we used a novel means of evaluating immune variables in cells and fluid from lung-derived lymph, combined with a well established infection model that results in moderate-to-severe clinical disease. We have demonstrated a temporal reflection of Th1 and Th2 cytokine profiles in pulmonary lymph before and during infection with BRSV. These results indicate that conventionally reared outbred calves do not have highly polarized Th1 and Th2 responses of syngeneic mice infected with RSV. Larger numbers of calves must be studied to fully appreciate the role of immunomodulators in the response to BRSV infection.

[a]GenAmp RNA PCR Core kit, Perkin Elmer, Norwalk, Conn.
[b]Alpha Image 2000 image analysis system, Alpha Innotech Corp, San Leandro, Calif.
[c]lab-Tek slides, Baxter Scientific Products, Brisbane, Calif.
[d]Fluorescein-conjugated anti-BRSV, American Bio Research Labs, Seymour, Tenn.
[e]Gershwin L, Gunther R, Randel E, et al. Cytokine production by lymphocytes in pulmonary efferent lymph in bovine respiratory syncytial virus infected calves compared to uninfected calves. *J Interferon Cytokine Res* 1996;16:6.

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