Effect of infection with bovine respiratory syncytial virus on pulmonary clearance of an inhaled antigen in calves

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Objective—To evaluate the effect of infection with bovine respiratory syncytial virus (BRSV) on clearance of inhaled antigens from the lungs of calves.

Animals—Eleven 6- to 8-week-old Holstein bull calves.

Procedures—Aerosolized 99mTc-technetium (99mTc)-labeled diethylene triamine pentacetate (DTPA; 3 calves), commonly used to measure integrity of the pulmonary epithelium, and 99mTc-labeled ovalbumin (OA; 8 calves), commonly used as a prototype allergen, were used to evaluate pulmonary clearance before, during, and after experimentally induced infection with BRSV or sham inoculation with BRSV. Uptake in plasma (6 calves) and lung-effluent lymph (1 calf) was examined.

Results—Clearance of 99mTc-DTPA was significantly increased during BRSV infection; clearance of 99mTc-OA was decreased on day 7 after inoculation. Clearance time was correlated with severity of clinical disease, and amounts of 99mTc-OA in plasma and lymph were inversely correlated with clearance time. Minimum amounts of 99mTc-OA were detected at time points when pulmonary clearance of 99mTc-OA was most delayed.

Conclusions and Clinical Relevance—BRSV caused infection of the respiratory tract with peak signs of clinical disease at 7 or 8 days after inoculation. Concurrently, there was a diminished ability to move inhaled protein antigen out of the lungs. Prolonged exposure to inhaled antigens during BRSV infection may enhance antigen presentation with consequent allergic sensitization and development of chronic inflammatory lung disease.

Impact for Human Medicine—Infection of humans with respiratory syncytial virus early after birth is associated with subsequent development of allergic asthma. Results for BRSV infection in these calves suggested a supportive mechanism for this scenario. (Am J Vet Res 2008;69:416–422)

Bovine respiratory syncytial virus is an important pathogen of the respiratory tract in cattle, with an infection and disease pattern that remarkably parallels that of the closely related RSV in humans. There is epidemiologic evidence that infection with RSV influences the development of allergic sensitization and, potentially, asthma in humans. Although cattle do not develop the clinical syndrome referred to as asthma, chronic lung disease is a common sequel to calfhood respiratory tract disease. Moreover, exposure of cattle to environmental antigenic inhalants is an unavoidable consequence of housing conditions. In other studies, conducted by our laboratory group, there was an increase in disease severity in calves infected with BRSV and exposed by aerosol to the thermophilic actinomycete *Saccharopolyspora rectivirgula* (formerly known as *Micropolyspora faeni*). This organism is often found in moldy hay dust and has been associated with Farmer's lung in humans and cattle.

Similarly to RSV in humans, BRSV causes an acute and often severe infection of the lungs in young cattle and less severe infection of the nasal cavities and trachea in adult cattle. Pathogenic processes that are made more severe by the immune response of the host have been ascribed to a preponderance of a Th2-mediated mechanism. Analysis of results of an aforementioned study conducted by our laboratory group suggests that BRSV infection alters the persistence of antigen in the

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>RSV</th>
<th>Respiratory syncytial virus</th>
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<tr>
<td>BRSV</td>
<td>Bovine respiratory syncytial virus</td>
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<tr>
<td>Th</td>
<td>T-helper</td>
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<tr>
<td>99mTc-DTPA</td>
<td>99mTc-Technetium-labeled diethylene triamine pentacetate</td>
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<tr>
<td>OA</td>
<td>Ovalbumin</td>
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<td>99mTc-OA</td>
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lungs, thereby providing increased access to antigen by antigen-presenting cells in the lungs. To examine this hypothesis, we used scintigraphic evaluation of clearance of a radioactively labeled aerosol from the lungs of calves before and after experimentally induced infection with BRSV. In the study, 99mTc-DTPA, a small molecule commonly used to evaluate the integrity of the pulmonary epithelium, was administered to calves by nebulization, and clearance was evaluated by use of a γ camera. Next, clearance of a larger molecule (ie, OA [45 kDa]), also labeled with 99mTc, was evaluated. In another study conducted by our laboratory group, we used OA, a common prototype allergen, to evaluate immune responses to an aerosolized antigen in calves.

Evaluation of pulmonary clearance of 99mTc-labeled molecules is a useful clinical tool as well as a method by which hypotheses on alterations in pathophysiologic processes of the lungs can be addressed. Studies have been performed to evaluate clearance of 99mTc-DTPA in sheep. In 1 study, sheep with chronic lymphatic fistulae in the lungs were used to evaluate clearance of 99mTc-DTPA from air spaces into pulmonary lymph. In another study, appearance of 99mTc-albumin in pulmonary lymph was examined in sheep whose lungs were injured by IV infusion of oleic acid. Clearance of 99mTc-DTPA has also been evaluated in anesthetized dogs. Mucociliary clearance of a 99mTc-labeled sulfur colloid complex was evaluated in a study in calves, but investigators in that study measured clearance that focused on the larynx. To our knowledge, the effect of viral infection of the respiratory tract on clearance of 99mTc-DTPA or protein antigen from the lungs of calves has not been addressed.

In the study reported here, calves were exposed to aerosolized 99mTc-DTPA or 99mTc-OA to establish the amount of time required to clear the material from the lungs. Calves were then inoculated with BRSV to induce experimental infection, and clearance was examined on several days after inoculation to evaluate the effect of infection on clearance of the aerosolized protein from the lungs. In a subset of calves, the temporal appearance of 99mTc-OA in plasma (8 calves) and pulmonary lymph (1 calf) was also examined to evaluate the effect of viral infection of the respiratory tract on movement of 99mTc-OA from the lungs into blood and lymph.

Materials and Methods

Animals—Eleven conventionally raised 6- to 8-week-old Holstein bull calves were used in the study; each calf served as its own control animal. Calves were assessed at the beginning of the study by use of an indirect immunofluorescence assay performed by personnel at the Center for Animal Health and Food Safety at the University of California, Davis; all calves were seronegative for BRSV. Calves were housed at the Center for Laboratory Animal Medicine at the University of California, Davis. Calves were fed alfalfa hay, and salt and water were available ad libitum. The study was conducted in accordance with a protocol approved by an institutional animal care committee.

Schedule of procedures—Clearance of 99mTc-DTPA was examined in 2 calves (Nos. 1 and 2) before inoculation with BRSV (day of inoculation was designated as day 0) and on 5 days during the course of the disease. Clearance was evaluated in calves 1 and 2 on days –3, 2, 4, 7, 9, and 16. Clearance of 99mTc-OA was evaluated in 6 calves (Nos. 3 through 8) before inoculation with BRSV (day –3) and at various time points during the course of the disease (calves 3 and 4, day 7; calves 5 and 6, days 7, 11, and 16; and calves 7 and 8, days 4, 7, 9, and 11). Additionally, a lymphatic cannula was surgically implanted in calf 8 on day –4 for subsequent collection of efferent lung lymph. To examine the effect of repeated administration of OA aerosol on subsequent OA clearance from the lungs, 2 additional calves (Nos. 9 and 10) were exposed via aerosol to 99mTc-OA; clearance was measured before inoculation (days –19, –12, –9, –7, and –5) and during infection (days 5, 7, 9, and 14). Clearance of 99mTc-OA in a control calf (No. 11) was measured before (days –19, –12, –9, –7, and –5) and after (days 5, 7, 9, and 14) mock inoculation. Baseline clearance was designated as values obtained on day –3 for calves 1 through 8 and day –5 for calves 9 through 11. Finally, 99mTc-OA concentrations were measured in plasma obtained from calves 7 through 11 and in lymph collected from calf 8.

Clearance of 99mTc-DTPA and 99mTc-OA—Food was withheld from all calves beginning at noon the day before imaging procedures. The 99mTc-DTPA was purchased commercially, and OA was labeled with 99mTc by use of a technique described elsewhere. Appropriate precautions for handling of Tc (as dictated by the campus environmental health and safety officer) were adhered to, including use of a dosimeter for all personnel that handled an animal or were present in the room during 99mTc aerosol exposures. Each of the 2 calves received a 100 mCi dose of 99mTc-DTPA. The other calves received 0.4 mg of OA labeled with 60 mCi of Tc in a volume of 3.5 mL.

All aerosols were administered through a bronchoscope. Each unsedated calf was positioned in a stanchion in front of a γ camera while the 99mTc-OA or 99mTc-DTPA was nebulized. The nebulizer unit contained a aerosol delivery system and was connected to an oxygen source. It delivered an aerosol that had an aerodynamic diameter of approximately 1 μm, which was administered through an endotracheal tube. The cuff on the endotracheal tube was inflated to prevent escape of Tc into the room, thus making it a closed circuit. Air exhaled by each calf was passed through a breathing circuit filter before being returned to the room. The nebulizer and filter were contained in a lead-lined box. Each calf remained attached to the breathing circuit for 5 minutes after nebulization to allow the filter unit to collect any 99mTc-OA or 99mTc-DTPA that had not been taken up in the lungs. Lateral images were obtained of the right lung field with a large field-of-view γ camera fitted with a parallel-hole low-energy collimator linked to a dedicated computer system. Data were stored on magnetic tape for subsequent analysis with software.

A 99mTc sample was taped to the camera face and shielded with lead; images were then continuously acquired for the first 15 minutes after the beginning of aerosol administration. One-minute images were acquired for 30 minutes after nebulization and at 30-minute intervals until counts were noticeably less than half
of the initial counts (after the observed half-life). Data were expressed as the number of minutes it required for half of the nebulized $^{99m}$Tc-OA or $^{99m}$Tc-DTPA to leave the lungs (ie, half-life).

Measurement of $^{99m}$Tc-OA concentrations in plasma and lymph after aerosol exposure—For calves 7 through 11, plasma samples were obtained at 1-hour intervals until 4 hours after aerosol exposure. For calves 9 and 10, additional samples of plasma were obtained at 3- to 10-minute intervals for the first hour. Lymph samples were collected from calf 8 at 30-minute intervals until 7 hours after aerosol exposure. Plasma and lymph samples were evaluated for $^{99m}$Tc-OA concentrations. Blood samples were collected into heparinized tubes, which were then centrifuged. Plasma was harvested, and an aliquot (0.5 mL) was placed into a counter tube with 0.5 mL of PBS solution. To evaluate $^{99m}$Tc-OA without interference from unbound $^{99m}$Tc, differential centrifugation was performed. A direct count was made from the aforementioned sample (bound $^{99m}$Tc + unbound $^{99m}$Tc). An ultrafiltration device with a molecular exclusion limit of 10 kd was used to separate bound $^{99m}$Tc from unbound (filtrate) $^{99m}$Tc. Then, 1.0 mL of the concentrate was counted to determine the amount of bound $^{99m}$Tc. The identical procedure was performed on lymph samples. Results for plasma and lymph were reported as adjusted data (percentage of the $^{99m}$Tc-OA counts in the lungs measured 15 minutes after beginning of aerosol exposure).

Experimental infection with BRSV—Calves were inoculated with a virulent field isolate of BRSV (CA-1) grown on bovine turbinate cells and prepared as described elsewhere. A representative sample was assayed to determine the titer of the virus preparation; titer of the virus used was $4 \times 10^5$ TCID$_{50}$/mL to $5 \times 10^7$ TCID$_{50}$/mL. Calves received 5 mL of virus suspension by aerosol via a face mask, as described. The mock-infected calf received aerosol of spent tissue culture medium without virus.

Evaluation of clinical signs—A score for clinical signs was determined each day for each calf. A physical examination was performed by an investigator (LJG), and scoring of clinical signs was modified from a method reported elsewhere. Clinical scores were determined by assignment of points based on variables such as rectal temperature, coughing, nasal exudate, results of lung auscultation, dyspnea, wheezing, anorexia, and lethargy. Scores were modified to decrease the emphasis of rectal temperature (decreased from multiplica-

Blood gas analysis—On days 0 and 7, a sample of arterial blood was collected from the auricular artery of each BRSV-inoculated calf. Samples were analyzed by use of a blood gas analyzer within minutes after collection.

Shedding of BRSV—Virus shedding was monitored by evaluation of nasopharyngeal swab specimens. Samples were inoculated onto bovine turbinate cells grown on slides. Slides were then fixed by incubation with acetone for 1 minute, and viral antigen was detected by staining of slides with fluorescein isothiocyanate-conjugated rabbit anti-RSV. Positive (infected) and negative (uninfected) control samples were evaluated on the same slides.

Figure 1—Gamma camera image of $^{99m}$Tc-DTPA (A and B) and $^{99m}$Tc-OA (C and D) in the lungs of representative calves 15 minutes after aerosol exposure on day 3 (A and C) and on day 7 (B and D). The images were obtained as a lateral view on standing calves. The $^{99m}$Tc-DTPA and $^{99m}$Tc–OA are evident in the lungs as an increase in opacity. The lung volume containing $^{99m}$Tc-DTPA has diminished on day 7 after inoculation with BRSV, compared with results for day 3; this indicates that more of the $^{99m}$Tc–DTPA has been cleared from the lung. In contrast, the opacity in the lung on day 7 indicates that clearence of $^{99m}$Tc–OA has not been altered, compared with clearance on day 3. Day 0 = Day of BRSV inoculation.
**Results**

**Clearance of 99mTc-DTPA from the lungs**—Clearance of 99mTc-DTPA was examined only in calves 1 and 2. Enhanced clearance of 99mTc-DTPA from the lungs was evident at 15 minutes after aerosol exposure on day 7 (Figure 1). The number of subjects exposed to 99mTc-DTPA was insufficient for statistical analysis; however, mean values for clearance half-time on days –3 and 7 were calculated (Figure 2). There was increased clearance of 99mTc-DTPA in both calves on day 7, compared with baseline clearance measured before inoculation.

**Clearance of 99mTc-OA from the lungs**—Clearance of OA, a larger molecule (45 kd) than DTPA, was examined in 8 calves. This larger molecule induced a clearance pattern that differed from the pattern for DTPA, as indicated by evaluation of images obtained on days –3 and 7 (Figure 1). In calves 3 to 8, the time required to clear 99mTc-OA from the lungs was greater on day 7 after inoculation. Mean clearance half-time for 99mTc-OA at baseline was 675.95 minutes, which was significantly (P = 0.043) less than the value of 1,044.37 minutes on day 7.

To evaluate the possibility that repeated exposure to 99mTc-OA aerosol would influence clearance independent of BRSV infection, 2 additional inoculated calves (Nos. 9 and 10) and the mock-infected calf (No. 11) were tested for clearance of 99mTc-OA aerosol on multiple days before and after inoculation. Those calves failed to have a significant increase in clearance time on any day during the preinoculation testing, yet calves 9 and 10 both had an increase in clearance time after inoculation, similar to results for calves that received only the single baseline exposure before inoculation. The mock-infected calf did not have an increase in clearance time after mock inoculation (Figure 3). Mean 99mTc-OA clearance time for all 8 calves exposed to 99mTc-OA aerosol before...
and on day 7 after inoculation revealed a significant (P = 0.011) enhancement in persistence of OA in the lungs (Figure 2).

Detection of 99mTc-OA in lymph and plasma—Plasma and lymph data were adjusted for each calf and reported as a percentage of the counts per minute in the lungs of each respective calf 15 minutes after the 99mTc-OA aerosol. For those calves in which the plasma concentration of 99mTc-OA was measured, the peak of 99mTc-OA was detected between 10 and 15 minutes after beginning of aerosol administration, with a gradual decrease during the next 4 hours (Figure 3). Generally on day 7, the amount of 99mTc-OA in plasma had decreased substantially below the amount at baseline, and on days 9, 11, and 14, the amount of 99mTc-OA in plasma was still less than the amount at baseline. The value for 99mTc-OA measured in plasma was greatest for most calves at baseline through 5 hours after aerosol administration. Infection diminished the amount of detectable 99mTc-OA in plasma. The number of calves was insufficient for statistical evaluation.

Samples of lymph were obtained from the cannulated calf (No. 8) at 30-minute intervals for 7 hours on each day that clearance was evaluated. On day –3 before inoculation, the peak concentration of 99mTc-OA was detected between 10 and 15 minutes after beginning of aerosol administration, with a gradual decrease during the next 4 hours (Figure 3). Generally on day 7, the amount of 99mTc-OA in plasma had decreased substantially below the amount at baseline, and on days 9, 11, and 14, the amount of 99mTc-OA in plasma was still less than the amount at baseline. The value for 99mTc-OA measured in plasma was greatest for most calves at baseline through 5 hours after aerosol administration. Infection diminished the amount of detectable 99mTc-OA in plasma. The number of calves was insufficient for statistical evaluation.

In call 8, plasma concentrations of 99mTc-OA were measured, but at 1-hour intervals (Figure 3). The pattern for concentrations in plasma differed from that of concentrations in lymph. Beginning at 1 hour after 99mTc-OA aerosol exposure on day –3, values for 99mTc-OA were greater than values for all days after inoculation until 6 hours after aerosol exposure; on day 4, there was a peak in detectable 99mTc-OA at 6 hours after aerosol exposure. Similar to results for the other calves on days 7, 9, and 11, the adjusted 99mTc-OA concentration detected in plasma failed to reach 15% at any time point.

Clinical signs and blood gas concentrations—All BRSV-inoculated calves developed clinical signs of disease. Significant differences were apparent in mean clinical scores from days 6 through 9. Differences were most evident when comparing scores for days 8 and 9 with scores for days 0 through 4. A correlation of mean clinical score versus 99mTc-OA clearance half-time revealed a significant positive correlation (R² = 0.614). The temporal relationship between severity of clinical disease and clearance half-time revealed an increase in both beginning at day 5 and peaking on days 7 and 8 (Figure 5). Analysis of arterial blood gas data revealed a significant difference between baseline values and values on day 7. Mean PaO₂ was 87.9 mm Hg at baseline, and it decreased significantly (P = 0.032) to 68.4 mm Hg on day 7. Mean PaCO₂ increased significantly (P = 0.029) from 33.18 mm Hg at baseline to 39.7 mm Hg on day 7.

Shedding of BRSV—All calves shed virus at some time point during the 10 days after inoculation. Generally, virus was detectable on days 5 and 6, but some calves shed virus as early as day 3, and several were still shedding virus on day 8 (data not shown).

Antibody titers against BRSV—Calves were chosen on the basis of a very low (seronegative or < 4 on the indirect immunofluorescence assay) maternal-derived titer against BRSV. By day 21 (or sooner), all calves seroconverted to BRSV, with most calves having a titer > 360 (data not shown).
plasma and lymph, the concentration of \(^{99m}\text{Tc-OA}\) on days 7, 9, and 11 were greatly reduced (by approx a third) from those at baseline. Although the data for the lymph was from only 1 calf, the pattern in plasma was representative of data from 8 calves in which plasma concentrations of \(^{99m}\text{Tc-OA}\) were measured.

Analysis of data obtained from calves exposed to \(^{99m}\text{Tc-OA}\) multiple times before and after inoculation with BRSV supported the conclusion that the effects of viral infection, rather than a developing immune response, caused a delay in clearance of OA from the lungs. It is unlikely that the small amount of OA administered repeatedly by aerosol exposure would have caused antibody production within the time frame of this study. However, the persistence of OA in the lungs may serve to prime an immune response for an increase in responsiveness to inhaled antigen in the days and weeks following acute BRSV infection. This hypothesis is supported by other experiments\(^{22}\) in which mice and guinea pigs were experimentally infected with human RSV and exposed to OA-containing aerosol.

Examination of plasma concentrations of \(^{99m}\text{Tc-OA}\) in conjunction with the clearance data for \(^{99m}\text{Tc-OA}\) suggested that as the clearance time decreased, so did the amount of \(^{99m}\text{Tc-OA}\) detectable in the plasma (Figure 3). This suggests that during the period of most severe pathologic changes in the lungs, the movement of solutes from the airspace into the vascular system may be compromised. In addition, results for the single mock-infected calf further indicated that the BRSV infection, rather than repeated \(^{99m}\text{Tc-OA}\) aerosol exposure, was responsible for the increase in clearance time from the lungs.

During acute RSV infection in human infants, experimental infection in mice, and BRSV infection in calves, chemokines, cytokines (Th2), and inflammatory cells increase in the lungs, which creates an environment that facilitates antigen presentation and development of immune responses to inhaled allergens.\(^{7,22-25}\) Experimentally induced RSV infection in primed mice can stimulate a Th2 response and increased amounts of IgE in mice exposed by aerosol to OA.\(^{24}\) Our studies\(^{13,24}\) in cattle and in mouse RSV experiments indicate that vaccination against BRSV or RSV may fail to protect and instead can induce a Th2 environment.\(^{26}\) Additional studies will be

Discussion

In other studies\(^{19,20}\) conducted by our laboratory group in which we used the same method to induce BRSV infection, we determined that clinical signs began on day 4 and gradually increase to day 7 or 8, after which most calves continue to get better until day 10 when the disease is mostly resolved. Calves in the study reported here had this expected course of disease. Clinical signs and blood gas concentrations during the peak days of illness indicated moderate to severe disease in all infected calves. Other indicators of successful infection included seroconversion and shedding of BRSV.

The hypothesis that BRSV infection alters the clearance of inhaled antigen was substantiated. Size of the nebulized molecules had a noticeable effect on whether clearance time from the lungs was increased or decreased. Altered permeability of bronchial epithelium, perhaps by viral effects on tight junctions, increased the movement of the small molecule DPTA from the lungs, whereas the larger molecule OA was delayed in passage from the lungs at similar time points. On the basis of other studies\(^{19,20}\) conducted by our laboratory group in which we used an identical experimental protocol and virus isolate, we know that there is some loss of virus-infected epithelium into small airways as well as the development of interstitial pneumonia. Indeed, the decrease in clearance of \(^{99m}\text{Tc-OA}\) was most pronounced when the clinical signs were greatest. It is possible that aerosolized OA molecules were trapped in the lungs by an intense inflammatory response.

The greatest concentration of \(^{99m}\text{Tc-OA}\) was detected in plasma prior to inoculation at baseline, and the amount detected in plasma diminished each day as the clearance time increased. In contrast in lymph, there was a large increase in \(^{99m}\text{Tc-OA}\) concentration on day 4 after inoculation with a subsequent return to concentrations less than those at baseline, which essentially paralleled concentrations detected in plasma by day 7. In contrast, plasma concentrations of \(^{99m}\text{Tc-OA}\) on day 4 were less than concentrations at baseline until 6 hours after aerosol exposure (Figure 4). For both
required to define the immunologic ramifications of these results.

References

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