Pathogenesis of \textit{Streptococcus zooepidemicus} infection after intratracheal inoculation in llamas

Christopher K. Cebra, VMD, MS; Jerry R. Heidel, DVM, PhD; Margaret L. Cebra, VMD, MS; Susan J. Tornquist, DVM, PhD; Bradford B. Smith, DVM, PhD

Objectives—To test whether generalized \textit{Streptococcus zooepidemicus} infection could be induced by intratracheal inoculation in llamas and to characterize this infection.

Animals—6 test and 3 control llamas.

Procedure—Test llamas received 1 of 3 dosages of \textit{S.

Result—Infection induced fever, anorexia, and signs of depression. Five of 6 infected llamas developed specific signs of inflammation in the thorax or abdomen, bacteremia, neutrophilic leukocytosis with toxic changes and high band neutrophil cell counts, hyperfibrinogenemia, and high peritoneal fluid WBC counts and protein concentrations. On development of bacteremia, llamas had significant decreases in serum iron (from 118 ± 25 to 6 ± 4 µg/ml) and increases in serum glucose (from 131 ± 5 to 253 ± 48 mg/dl) concentrations.

Conclusions and Clinical Relevance—\textit{Streptococcus zooepidemicus} spreads rapidly to other body compartments after intratracheal inoculation, whereas control llamas received sterile culture medium. Physical examination variables and results of clinical and pathologic analyses of blood, peritoneal fluid, and tracheal wash fluid were compared in test llamas between, before, and during the development of bacteremia and with control llamas. Bacteriologic culture was performed on all collected body fluids and tissue specimens that were collected at necropsy. Tissue specimens that were collected at necropsy were examined histologically.

Received Aug 27, 1999
Accepted Jan 21, 2000.
From the Departments of Large Animal Clinical Sciences (C. Cebra, M.Cebra) and Veterinary Biomedical Sciences (Heidel, Tornquist, Smith), College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331-4802.
40 and 80 kg, were used. These llamas were determined by physical examination and clinicopathologic testing (CBC, serum biochemical analysis, bacteriologic culture of blood samples, transtracheal wash evaluation, and peritoneal fluid analysis) to be free of disease and were randomly divided into 3 groups. One llama in each group was chosen randomly as the control llama, whereas the other 2 were test subjects.

**Inoculum**—*Streptococcus zooepidemicus* was grown in 3 separate overnight cultures, 1 for each test group, using conventional laboratory techniques. The organism originally had been isolated from the uterus of a horse. Organisms were quantified prior to inoculation by serial dilution and colony counting. For group 1, 10 ml of the culture broth was used, yielding an inoculum of approximately 2 x 10⁹ bacteria. For group 2, 1 ml of culture broth was diluted in 9 ml of sterile saline (0.9% NaCl) solution, yielding an inoculum of approximately 2 x 10⁸ bacteria. For group 3, 1 µl of culture broth was diluted in 10 ml of saline solution, yielding an inoculum of approximately 4 x 10⁷ bacteria. The control inoculum for each group was made by mixing sterile broth and sterile saline solution in the same ratios as used for the test inocula. Each inoculum was administered by intratracheal injection.

**Experimental protocol**—The protocol was approved by the Oregon State University Institutional Animal Care and Use Committee. Llamas were housed in groups or pairs in an indoor barrier isolation facility, with control llamas isolated from infected llamas. Rectal temperature and heart and respiratory rates were obtained daily for the 2 days before inoculation, twice a day for the first 7 days after inoculation, and once a day thereafter. Appetite, attitude, abnormal pulmonary sounds, and gastric motility were assessed subjectively at the same times.

Jugular blood was collected for CBC and serum biochemical analysis on days –2, 0, 1, 4, 7, and 13 (with inoculation occurring just after sample collection on day 0). Blood for aerobic and anaerobic bacteriologic culture and transtracheal wash fluid were collected on all of these days except day 0, and peritoneal fluid was collected on days 7, 10, 13, and after euthanasia. Necropsy was performed and selected tissue specimens were collected for histologic examination. Sections of lung, liver, spleen, and mesenteric lymph node were collected for aerobic and anaerobic bacteriologic culture. Pleural fluid was collected during necropsy from the test llamas in the first group.

Test llamas in the first 2 groups were euthanatized by an IV injection of pentobarbital as soon as 1 llama of the group appeared weak, or had signs of dyspnea or discomfort. One llama in the third group was euthanatized on the basis of these criteria, whereas the other llama was euthanatized 13 days after inoculation without having signs of weakness or distress. The first 2 control llamas were euthanatized 4 days after inoculation, and the last llama 13 days after inoculation.

**Sample collection**—Llamas were sedated with butorphanol tartrate (0.08 mg/kg of body weight, IM). Jugular, transtracheal, and abdominal sites were clipped and steriley prepared before each sample collection. Transtracheal wash samples were collected by introducing a 10-gauge needle between adjacent tracheal rings, passing a length of sterile polyethylene tubing through the needle into the tracheal lumen, introducing 20 ml of sterile saline solution through the tubing, and retrieving the sample. Peritoneal fluid samples were collected by abdominocentesis; a sterile teat cannula was introduced through the ventral surface of the body wall into the peritoneal cavity. Ultrasonographic examination of the abdomen was performed prior to collection of the first sample to identify a site of fluid accumulation on the lateral or medial side of the third gastric compartment to be used for sample collection. Transtracheal wash fluid and peritoneal fluid were collected in clot tubes for bacteriologic culture, and blood was collected, for biochemical analysis. In addition, 20 ml of blood were collected using sterile techniques, and divided between 2 vials, each containing 100 ml of Columbia broth for bacteriologic culture. All 3 fluids were collected in tubes coated with potassium EDTA for cytologic analysis.

**Statistical analysis**—To test for effects of collecting samples, data from control llamas were tested for significant changes over time, using a 1-way ANOVA. To test for effects of infection, preinfection data from test llamas were compared with data from the first day of detectable bacteremia, using paired t-tests, and data from test llamas on the first day of detectable bacteremia were compared with data from control llamas (on the fourth day after sham infection, chosen because all samples were collected that day), using unpaired t-tests. Choosing the first detectable day of bacteremia rather than a specific day after inoculation was done to compensate for the different rates of development of disease with the different bacterial inocula. Data from the test llama that did not develop bacteremia were excluded from all calculations. For all calculations, significance was determined when values were P < 0.05.

**Results**

**Clinical signs**—Control llamas did not have any signs of disease throughout the trial. Four of the 6 test llamas developed progressive disease characterized by all or some of the following clinical signs: fever (39.2 to 40.7 C), anorexia, depression, weakness, recumbency, tachycardia, tachypnea, dyspnea, cough, nasal discharge, abnormal respiratory sounds, including crackles and pleural friction rubs, hunched posture, gastric atony, tenesmus, colic, and diarrhea. Respiratory signs were subtle in most llamas and detectable only briefly. Weakness, recumbency, dyspnea, pleural friction rubs, hunched posture, and colic were grounds for immediate euthanasia. Fever and signs of depression began 8 hours after inoculation in 2 group-1 llamas and progressed until euthanasia was performed 48 hours after infection. Two llamas in group 2 developed fever 24 hours after infection that persisted until euthanasia on day 5. Whereas 1 of these llamas also developed progressive disease, the other llama had a lower fever (< 39.4 C) throughout and appeared to be recovering despite a single episode of high fever (40.7 C) and diarrhea prior to euthanasia. Rectal temperatures of llamas with bacteremia were significantly higher than preinfection temperatures (P = 0.001) or rectal temperatures of control llamas (P = 0.002). Clinical signs of disease were first observed 24 hours after infection in 1 llama in group 3 and progressed slowly until euthanasia on day 7. The other test llama in group 3 had mild abnormal pulmonary sounds on day 2 and fever on day 7 (coincident with the only positive culture result from that llama) but, otherwise, appeared completely unaffected. Because of this llama’s apparent rapid clearance of the infection, data from the llama were not included in calculations for statistical comparisons.

**Clinical pathologic findings**—There were no significant changes over time in the clinical pathologic data from the control llamas. Nonsignificant hematologic changes that were observed in test llamas at the
Table 1—Mean ± SD for selected clinicopathologic data from llamas before and after intratracheal inoculation with Streptococcus zooepidemicus and from control llamas

<table>
<thead>
<tr>
<th>Variables</th>
<th>Test llamas (n = 5)</th>
<th>Control llamas (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC (&lt;10⁵ cells/µl)</td>
<td>18.6 ± 3.5</td>
<td>24.7 ± 11.7</td>
</tr>
<tr>
<td>Band neutrophils (&lt;10⁵ cells/µl)</td>
<td>0 ± 0</td>
<td>6.6 ± 6.7</td>
</tr>
<tr>
<td>Lymphocytes (&gt;10⁵ cells/µl)</td>
<td>5.3 ± 1.0</td>
<td>2.8 ± 1.3</td>
</tr>
<tr>
<td>Total plasma protein (g/dl)</td>
<td>5.8 ± 0.2</td>
<td>6.2 ± 0.3*</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>200 ± 140</td>
<td>560 ± 320*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>131 ± 5</td>
<td>253 ± 481*</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.2 ± 0.2</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>151 ± 5</td>
<td>147 ± 5</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>5.0 ± 0.7</td>
<td>4.0 ± 0.8*</td>
</tr>
<tr>
<td>Calcium (g/dl)</td>
<td>9.5 ± 0.2</td>
<td>8.7 ± 0.31</td>
</tr>
<tr>
<td>Total CO₂ (mmol/L)</td>
<td>26.6 ± 3.0</td>
<td>23.5 ± 1.7</td>
</tr>
<tr>
<td>Iron (µg/dl)</td>
<td>114 ± 26</td>
<td>6 ± 515</td>
</tr>
<tr>
<td>Peritoneal fluid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/dl)</td>
<td>3.1 ± 0.7</td>
<td>5.4 ± 0.8*</td>
</tr>
<tr>
<td>WBC (&gt;10⁵ cells/µl)</td>
<td>3,125 ± 2,229</td>
<td>39,631 ± 60,454</td>
</tr>
</tbody>
</table>

*Significantly (P < 0.05) different from before infection value. †Significantly (P < 0.01) different from before infection value. ‡Significantly (P < 0.05) different from control value. §Significantly (P < 0.01) different from control value. n/N = 4 for the test llamas.

The time of bacteremia included increasing leukocyte and band neutrophil counts and decreasing lymphocyte counts (Table 1). Changes in the total leukocyte counts appeared to be temporary; all 5 llamas developed leukocytosis on the day after infection, but total counts returned to reference range values by day 4 or 7. Band neutrophils also became an increasingly greater contributor to the total leukocyte counts on days 4 and 7.

Plasma fibrinogen concentrations increased to >700 mg/dl on day 4 in all 3 llamas that were not euthanatized before that time. Total plasma protein concentration increased similarly. In contrast, serum albumin concentrations decreased after infection. The increases in plasma fibrinogen (P = 0.049) and total protein concentrations (P = 0.045) from test llamas before infection to the time of detectable bacteremia were significant, but the decrease in serum albumin concentration was not (P = 0.14; Table 1).

All test llamas had serum glucose concentrations >190 mg/dl in all samples obtained after infection. Glucose concentration at the time of bacteremia was significantly higher than before infection (P = 0.006; Table 1). Serum iron concentrations were ≤20 µg/dl in all samples obtained after infection from the llamas in groups 1 and 2. Serum iron concentrations decreased more gradually in the group-3 llamas but attained a low concentration by day 4. Serum iron concentration at the time of bacteremia was significantly lower than before infection (P = 0.001). Serum iron concentrations were lower (P = 0.001) and glucose concentrations higher (P = 0.013) for llamas with bacteremia than for control llamas. Three llamas in groups 2 and 3 had lower serum creatine kinase and aspartate aminotransferase (AST) activities on days 1 and 4 and day 4 after infection, respectively, but only the difference in AST between preinfection and bacteremic samples was significant (P = 0.048). Llamas with bacteremia also had significantly lower serum potassium (P = 0.017) and calcium (P = 0.006) concentrations than their preinfection values, and serum total carbon dioxide concentrations were lower than control llamas (P = 0.035).

Transtracheal wash usually yielded little fluid and did not reveal evidence of pulmonary inflammation. Peritoneal fluid analysis yielded more evidence of inflammation. All 5 test llamas that developed bacteremia had increases in peritoneal fluid protein concentration from the first sample to the last. Final protein concentration was >5 g/dl in 3 test llamas (and no control llamas), all of which had final values more than double their initial values. Llamas with bacteremia had a significant increase in peritoneal fluid protein concentration (P = 0.027). Peritoneal fluid cell count also increased but not significantly (Table 1). Test llamas with detectable bacteremia also had significantly higher peritoneal fluid protein concentrations than control llamas (P = 0.008). The 2 llamas in the first group had counts >5000 cells/µl, and the other 3 llamas had counts >10,000 cells/µl. However, 3 llamas (2 test llamas and 1 control) had initial peritoneal fluid cell counts between 3,300 and 5,900 cells/µl. Cell counts were higher in llamas with clinical evidence of abdominal pain and gross evidence of peritonitis at necropsy. Intracellular and extracellular bacterial cocci were visible in all 4 peritoneal fluid samples with positive bacteriologic culture results.

**Bacterial Isolation—Streptococcus zooepidemicus** was not isolated from any of the control llamas or from any of the preinfection samples from the test llamas. Transtracheal wash samples had positive bacteriologic culture results for S. zooepidemicus at all time points for 5 of the 6 llamas and at 1 time point for the sixth. Four llamas had evidence of diffuse infection on or near the day of euthanasia, as the organism was isolated from all tissue and fluid samples that were tested. Llamas with positive tissue specimen bacteriologic culture results included 2 in the group with the largest inoculum and 1 each from the other 2 groups. The other 2 llamas appeared to have cleared the infection, except from the inoculation site. *Escherichia coli* also was isolated from
several of the body fluids and tissue specimens collected at necropsy from 2 llamas given the largest inoculum. *Streptococcus zooepidemicus* bacteremia was first detected on day 1 in the first group, day 4 in the second group, and day 7 in the third group for llamas with positive tissue specimen bacteriologic culture results; those llamas were euthanatized within 24 hours of detection of bacteremia. Of the 2 llamas with negative tissue specimen bacteriologic culture results, 1 had transient bacteremia (low numbers of *S. zooepidemicus* on day 1 only), and the other never had evidence of disseminated infection.

**Necropsy lesions**—Important gross or histologic abnormalities were not identified in any of the control llamas. The 2 test llamas from which *S. zooepidemicus* could not be isolated after death also had no lesions. Other test llamas had peritonitis, pleuritis, or both, with fibrinous exudates and multiple adhesions between serosal surfaces of viscera and body wall surfaces. Histologic lesions consisted predominately of fibrin and neutrophil accumulations on the serosal surfaces of thoracic and abdominal visceras. One llama had pulmonary parenchymal lesions characterized by thickening of interlobular septa caused by fibrin, neutrophil infiltration, and edema. Gastric ulcers were not found in any of the llamas.

**Discussion**

The ease with which streptococcal bacteremia was induced in these llamas after experimental inoculation suggests that naturally occurring infections in camelids may only require small inocula. The largest inoculum in this trial was a fifth that used to induce mild chronic disease in foals,13 yet caused severe peracute disease in llamas. The lower doses, with as few as 40,000 colony-forming units of bacteria (1 µl of media), also were capable of causing bacteremia and severe disease, although more slowly and less reliably. Given this evidence of susceptibility, limiting exposure of camelids to sources of *Streptococcus zooepidemicus* would appear prudent.

Why these llamas were so sensitive to *Streptococcus zooepidemicus* infection remains unknown. It is a common perception that llamas and alpacas succumb easily to bacterial infection, but no scientific explanation has been provided for this belief, as experimental data about nonmycobacterial microbial infections are lacking. This trial revealed that llamas have similar clinicopathologic responses to gran-positive bacteremia as other species but appear to have difficulty clearing bacteria once they gain access to the blood. Their inability to clear the infection may have been the result of exposure to novel pathogens and lack of an amnestic immune response. Camelids in North and South America are managed differently and, hence, are exposed to different pathogens. Specifically, camelids in North America are unlikely to have a strong initial immune response to *S. zooepidemicus* infection, because it does not appear to be a common oral or respiratory tract commensal here.14,15 This is in contrast to observations about camelids in South America and may be the result of less exposure to horses, pigs, or other common hosts of the organism. There is further information implicating the role of horses in natural infection: transmission of *S. zooepidemicus* from horses to humans14,15 and sheep15 has been suspected, and we have observed streptococcal peritonitis and pleuritis in a llama shortly after it was housed with horses. The ability of the organism to adapt from an equine to a camelid host was demonstrated in this trial; its importance in natural infections remains to be established.

The lesions and clinical signs closely resembled those seen with naturally occurring infections.5,7 Despite the organism being introduced into the trachea and being isolated from the blood and the parenchyma of various thoracic and abdominal tissue specimens after death, gross and microscopic inflammatory changes were found almost exclusively upon serosal surfaces. *Streptococcus zooepidemicus* causes similar serositis in other susceptible species14,16,17 but usually also causes parenchymal disease. Serositis in camels is easier to explain than the lack of pneumonia. Pulmonary infections may quickly spread to the pleura (as well as other more distant sites) of other domestic large animals, because their visceral pleura receives its arterial blood from the bronchial artery.16 Once in the pleura or peritoneal space, bacteria tend to replicate rapidly, because the immune response is weaker than in more vascular organs.18 With a less extensive omentum than cattle,19 camels tend to develop fibrinous exudates in response to abdominal infections. The organism is able to survive within the exudate as a facultative anaerobe and may even have greater cytotoxic activity in the low oxygen tension environment.20 It is likely that all these factors are important in the pathogenesis of this infection.

Because of the lack of parenchymal disease in these llamas, most clinical signs and clinical pathologic abnormalities were nonspecific and referable to the inflammatory response (CBC changes, fever, anorexia, depression, tachycardia, tachypnea, hunched posture, colic, tenesmus), not dysfunction of specific organs. Clinical signs of infection also varied among llamas, with a broad spectrum of possible appearances. Several other conditions, including heat stress, gastric ulceration, intestinal impaction, and other infectious diseases, can cause similar clinical signs and are more widely known than *S. zooepidemicus* infection.21 Without bacteriologic culture of blood, tissue, or body cavity fluids, streptococcal infection easily could be misdiagnosed as 1 of these other diseases. It is also possible that the stress and debilitation caused by streptococcal infection contributes to the development of these other diseases (as witnessed by the development of hyperthermia, evidence of stress, and gram-negative sepsis in several of the test llamas). In any case, veterinarians should consider streptococcal disease as a differential diagnosis in any camelid with fever, signs of depression, or other clinical signs referable to sepsis or infection of the pleural or peritoneal cavities.

This model provided information about llamas' acute response to bacterial infection. Knowledge of this response may aid in differentiating this disease from other causes of fever, signs of depression, and colic. Hematologic changes included neutrophilic leukocytosis, high band cell counts, toxic changes in neu-
trophils, and hyperfibrinogenemia. Neutrophilic leukocytosis also was present in camels before infection, probably as the result of the stress response,2 whereas the other hematologic changes were specific to infected camels. Peritoneal fluid nucleated cell counts and protein concentrations rose consistently in llamas that developed clinical disease. Some healthy camels also had counts > 5000 cells/µl, but protein concentrations > 5 gm/dl, cell counts > 10,000 cells/µl, and presence of bacteria all were specific to peritoneal fluid from infected llamas. Iron sequestration was the most consistent and specific serum biochemical change. Iron sequestration as the result of acute infection has not been described previously in llamas, but this and the other hematologic abnormalities were similar to those seen in other species with gram-positive bacterial infection.11 Hematologic and peritoneal fluid abnormalities also were similar to those seen in the few reports of this disease, except that llamas given the larger inocula developed severe disease before they developed hyperfibrinogenemia and high peritoneal fluid cell counts. Because similar inflammatory changes are likely to develop in camels exposed to other bacterial pathogens, this model may be useful for further research on camels’ inflammatory and immune responses.

We believe this model provides a plausible explanation for some animals with natural infection with S. zooepidemicus. The subtle and transient nature of respiratory signs may explain why this route of infection has not been considered in the past; it is remarkable that gastrointestinal signs were more prominent than respiratory signs, despite the route of infection. It was important to establish the respiratory route of infection for 2 reasons. First, it indicates that prophylactic measures should be directed toward decreasing damage and increasing immunity in the airway. Dust control, elimination of other respiratory pathogens, and vaccines that increase immunity in the airway. Dust control, elimination of other respiratory pathogens, and vaccines that increase immunity in the airway. Dust control, elimination of other respiratory pathogens, and vaccines that increase immunity in the airway.

References