Specificity of Bovine Serum Antibody to Capsular Carbohydrate Antigens from *Pasteurella haemolytica*†

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A more complete understanding of the bovine immune response to antigens of *Pasteurella haemolytica* biotype A, serotype 1, will improve control of bovine respiratory disease (BRD). Sera were obtained from blood samples of calves as they transited the market system of eastern Tennessee and were transported to a feedlot in Texas. The clinical histories and performance data were recorded and compared with serologic findings. The calves underwent a natural challenge of BRD. Serologic and bacteriologic evaluation indicated that *P. haemolytica* A1 was a significant component of the challenge. Serum antibody titers against *P. haemolytica* A1 capsular antigens (in enzyme-linked immunosorbent assay and hemolysin-in-gel test) increased by day 15 and continued at high levels through day 56. The animals that remained free of BRD had higher initial serum antibody concentrations than those that succumbed to BRD. The specificity of the immunoglobulin G subclass 1 (IgG1) ant capsular antibody to *P. haemolytica* A1 increased from day 8 to day 29 as evidenced by a decrease in *P. haemolytica* A2 absorption inhibition from 60% (day 8) to 15% (day 29). However, IgA, IgG2, and IgM were more serotype specific on both days 8 and 29. There were no significant changes in anti-*P. haemolytica* A2 antibody titers. Both in vitro complement-dependent bacteriolysis and C3 deposition on the surface of the bacteria increased significantly (*P < 0.01*) in a serotype-specific fashion from day 8 to day 29. These calves showed a humoral immune response to capsular polysaccharide antigens of *P. haemolytica* A1. Such a response may be an important component of immunity to BRD.

Pneumonic pasteurellosis continues to be a significant component of the bovine respiratory disease (BRD) complex (13, 28, 38). The complicated pathogenesis and multiple immune mechanisms associated with the BRD complex have been studied (for recent reviews, see references 7 and 13). A combination of environmental stressors and multiple infectious agents often results in the development of shipping fever (7, 13, 23).

One common sequela of severe shipping fever is a fibrinous pleuroneumonia (7). *Pasteurella haemolytica* biotype A, serotype 1, has been isolated most frequently from pneumatic lesions (7). Although other bacteria (*Pasteurella multocida, Haemophilus somnus, Mycoplasma spp.,* and *Chlamydia psittaci*) also have been isolated from the respiratory tracts of feedlot cattle (9), *P. haemolytica* has been recognized as the most significant bacterial species associated with the fibrinous pleuroneumonia of shipping fever (7, 9). The serotypes of *P. haemolytica* have been determined by indirect hemagglutination procedures (1, 2, 17, 37, 41). Serotypes 1 and 2 of *P. haemolytica* (biotype A) have been the most common serotypes isolated from cattle (15, 16, 37). Serotype 2 generally has not been considered to be pathogenic for cattle and has been isolated from nasal swabs of healthy cattle (15). Upon stress (including shipment) or viral infection of the respiratory tract, serotype 1 has been observed as the most common isolate (14). The control of *P. haemolytica* serotype 1 infections may significantly reduce the losses caused by the BRD complex (7).

Cross-reactivity between serovars has been recognized by indirect hemagglutination (1, 2), rapid plate agglutination (16), and enzyme-linked immunosorbent techniques (4). Tsai et al. (41), using crossed electrophoresis, demonstrated reciprocal cross-reactions between serovars A1 and A2. The antibodies used in crossed immunoelectrophoresis were obtained by immunization of rabbits and subsequent purification (41). In rabbits, the serovar- and biovar-specific antigens of *P. haemolytica*, presumably of the lipopolysaccharide complex (41), were the most antigenic. The specificity of the bovine humoral response to *P. haemolytica* serovars has not been extensively investigated.

Numerous serologic assays to quantitate antibodies to different *P. haemolytica* antigens have been constructed (for a review, see reference 7). One antigen preparation employed was the carbohydrate-protein subunit (CPS; 26). High levels of antibody to this CPS antigen have been correlated positively to resistance to pneumonic pasteurellosis (5, 6, 8). The highest levels of protective antibodies to bacterial surface proteins of CPS were consistently observed in cattle vaccinated with live organisms or bacterins in Freund-type adjuvants (3, 6, 7).

The purpose of this study was to evaluate the specificity and related antimicrobial function of bovine serum antibodies to *P. haemolytica* serovars A1 and A2. The demonstration of such changes in specificity or function or both of the serum antibodies may be important in immunity to pneumonic pasteurellosis. These immunoglobulins were obtained from serum specimens from calves that were naturally

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exposed in traditional market, transit, and feedlot conditions.

MATERIALS AND METHODS

Experimental design. Weaned calves were purchased and assembled at an order-buyer sale barn in eastern Tennessee. This market-transit system has been described previously (27, 36). At the time of initial assembly, the calves were vaccinated for infectious bovine rhinotracheitis and parainfluenza virus 3 infection with a modified live, intramuscular immunogen. After co mingling for 48 h, the 116 calves were transported by truck to a feedlot in Bushland, Texas (1,885 km). The cattle were weighed and given access to water upon arrival. At this time, the calves were immunized with a four-way cross-sectional bacterin-toxoid. Oral anthelmintic, pour-on insecticide, and intramuscular vitamins A and D also were administered. Blood for serum specimens was collected 24 h after arrival at the feedlot (day 1) and subsequently on days 8, 15, 29, and 56. Calves were observed at least twice daily for clinical illness. Depressed calves or calves with nasal and/or ocular discharge were separated for closer examination. If rectal temperature was greater than 40.0°C, the calf was treated intramuscularly with erythromycin (22 mg/kg of body weight; once per day for 3 days). If the treatment did not reduce the rectal temperature within 48 h, oxytetracycline (11 mg/kg; intramuscular injection, once per day for 3 days) was administered. All calves that died were subjected to complete necropsy, and samples were taken for viral and bacterial isolation. Nasal swabs for Pasteurella isolation were obtained from all calves on day 1 in the feedlot.

The serum specimens collected were stored at −20°C until assayed by enzyme-linked immunosorbent assay (ELISA) or hemolysin-in-gel test (HIGT) for antibodies to capsular polysaccharide antigen from P. haemolytica serotypes A1 and A2 (see below). Serum samples from selected calves also were examined for antibodies to P. multocida A3 and core glycolipids from Escherichia coli J5. In addition, most of these selected serum specimens were absorbed with each of the bacterial antigens to determine relative specificity of the humoral response to the protective antigens. These serum specimens were examined further for their ability to activate the complement system.

ELISA. The semiquantitation of serum antibody was performed by an ELISA as described previously, with minor modifications (5). Volumes of 100 µl were used for all solutions. The reactions were performed at room temperature (24 to 26°C). After coating the wells of 96-well microdilution plates (Immuno 1; Dynatech Laboratories, Inc., Alexandria, Va.) with the appropriate antigen (P. haemolytica A1 or A2 capsular polysaccharide) in carbonate buffer (pH 9.6), the wells were washed with 0.5% bovine serum albumin in phosphate-buffered saline (PBS; pH 7.4). Dilutions of unknown or control serum specimens (from 1:100 to 1:20,000) were constructed in a semilogarithmic pattern. Dilutions were made in PBS. Subsequent washes were performed by using PBS with 0.5% bovine serum albumin. The serum specimens were incubated in the wells for 30 min, the wells were washed three times, and rabbit anti-bovine immunoglobulin G (IgG) (heavy- and light-chain specific; Organon Teknika-Cappel, West Chester, Pa.) horseradish peroxidase conjugates were added at a 1:500 dilution. Some ELISAs were done by using anti-bovine IgA, IgG subclass 1 (IgG1), IgG2, or IgM (heavy-chain specific) conjugates (Bethyl Laboratories, Montgomery, Tex.). The appropriate conjugates were incubated in the wells for 30 min, the wells were washed again three times, and the colorimetric substrate o-phenylenediamine with hydrogen peroxide was added. The enzymatic reaction was stopped at 30 min with 2 M sulfuric acid. A405 was recorded by using an automated spectrophotometer (model EL 309; Bio-Tek Instruments, Inc., Winooski, Vt.). Pooled hyperimmune serum was used as a positive control (29), and fetal bovine serum (FBS) was employed as a negative control (5). An absorption value greater than 3 standard deviations above the mean of the negative control was considered positive. Data are reported as log2 ELISA titers.

Bacterial antigen preparation. P. haemolytica A1 was originally isolated from the lungs of a holstein heifer calf that died of acute bronchopneumonia. Isolation, culture, and preservation of this isolate have been previously described (30). P. haemolytica A2 and P. multocida type A3 were bovine isolates obtained from W. E. Bailie (Department of Laboratory Medicine, Kansas State University, Manhattan, Kans.). The E. coli J5 lipopolysaccharide was purchased from List Biological Laboratories, Campbell, Calif. In all cases, the bacteria were cultured and the carbohydrate-protein subunit was extracted from late-logarithmic-phase organisms as described previously (19), except that RPMI 1640 with 5% FBS was used for the growth medium. Protein was precipitated from these extracted preparations by the addition of 15% polyethylene glycol (average molecular mass, 8,000 daltons; Sigma Chemical Company, St. Louis, Mo.). The carbohydrate remaining in the extraction was quantitated by H2SO4-cystine shift and standardized to 10 µg per ml with carbonate buffers (pH 9.6; 29). This procedure resulted in a reduction of protein concentration of 98%, as determined by a modified colorimetric micromethod of Lowry and confirmed by quantitative polyacrylamide gel electrophoresis (see below). The presence of antigenic material was confirmed by the formation of a specific immunoprecipitate with bovine immune serum. These preparations were employed as the polysaccharide-enriched antigens in the ELISA, designated C-A1 and C-A2. The J5 lipopolysaccharide was dissolved in carbonate buffer at 10 µg/ml as another ELISA antigen preparation.

Absorption procedures. For completion of the serologic studies, it was desirable to absorb antibodies from diluted serum with the bacteria. Serum samples from six calves free of BRD and serum samples from six calves that recovered from BRD were randomly selected. This was accomplished by using approximately 1.5 × 107 log-phase, washed bacteria. The diluted serum (1.0 ml) (1:100 in PBS with 0.01% sodium azide) was added to the cell pellet in 1.5-ml Eppendorf tubes and incubated with gentle agitation for 60 min at 20°C. Following centrifugation at 9,000 × g (relative centrifugal force) for 15 min, the serum dilution was transferred to a fresh pellet and the process was repeated. Repetition of this procedure four times removed >99% of specific antibody, as determined by the ELISAs described here.

Polyacrylamide gel electrophoresis. The methods for vertical slab gels which were used for discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 2% stacking gels and 10% resolving gels were previously described (26). Protein detection was accomplished by silver staining (32).

Complement-dependent growth-inhibiting (bactericidal) antibody activity. Reaction mixtures were set up by using techniques similar to those described by Hassan-King et al. (22). Briefly, in sterile polypropylene tubes (10 by 120 mm), 100 µl of diluted serum (1:100 in Hanks balanced salt
solution) was combined with 100 μl of 5% (vol/vol) fresh guinea pig serum and 5% (vol/vol) FBS. Washed, log-phase *P. haemolytica* serotype A1 cells (100 μl; optical density at 650 nm, 0.25) were added to this mixture. The reaction volume was increased to 1.0 ml with RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.). The reaction suspension was incubated for 30 min with slow rotation in a 37°C incubator. Then, 1 μCl of [3H]thymidine (New England Nuclear Corp., Boston, Mass.) in 100 μl of RPMI 1640 was added to the culture. Following further incubation for 2 h, the bacteria were washed twice with cold PBS and extracted with trichloroacetic acid, as described previously (22). The resolubilized lysate was combined with 2.0 ml of scintillation cocktail (Biosafe II; Research Products International Corp., Mount Prospect, Ill.), and trichromine incorporation was quantitated by liquid scintillation (model LS 6800; Beckman Instruments, Arlington Heights, Ill.). Bactericidal activity was expressed as [(cpm in control – cpm in sample)/(cpm in control)] × 100, where cpm indicates counts per minute. Controls included heat-inactivated (56°C for 30 min) FBS as a test sample (the negative control was the above with complement), a heat-inactivated combination of FBS plus guinea pig serum complement source with immune serum (to assure complement dependence), hyperimmune serum with active complement (positive control), and inactivated FBS with inactivated complement (as a complete negative control).

**HIGT.** HIGT was performed as reported previously (31), a modification of the methods of Nielson et al. (34). Briefly, sensitizing antigen was prepared by warm-saline extraction and adsorbed onto fresh sheep erythrocytes. A 1% suspension of these sensitized cells was mixed with 2% agarose to which 10% fresh guinea pig serum had been added. The resulting agar suspension was poured onto plates and allowed to solidify. Serum (diluted 1:2) was added to wells cut in the agar. The plates were incubated first at 4°C for 18 h and then at 37°C for 2 h. The radius of each hemolytic zone of each plate was measured in millimeters and recorded as the HIGT value. Negative and positive control serum specimens were assayed on each plate. Also, the HIGT was prepared with nonsensitized sheep erythrocytes, and each serum sample was assayed to determine that hemolysis was not attributable to nonspecific or anti-erythrocyte activity.

**Quantitation of bound C3 fragments.** C3 fragments bound to bacteria after serum and complement treatment were quantitated by using a modified enzyme immunoassay (ELISA), as described previously (21). The IgG fraction of goat antiserum to guinea pig C3 was prepared by caprylic acid precipitation (40) and conjugated with horseradish peroxidase by the periodate method (42).

For the ELISA, bacteria were treated with serum and complement as above, incubated as above, and washed three times with PBS containing 1% sodium dodecyl sulfate to remove noncovalently associated C3. Then, 50 μl of this bacterial suspension (without [3H]thymidine) containing ~10⁸ bacteria was added in triplicate to U-bottomed 96-well ELISA plates (Linbro; Flow Laboratories, Inc., McLean, Va.). Bacteria were attached by desiccation at 37°C overnight. Nonspecific binding sites were blocked with 200 μl of 0.5% bovine serum albumin for 30 min at 37°C, and after two washes with 0.05% Tween 80 (Union Carbide Corp., Greenwich, Conn.) in PBS, 100 μl of peroxidase-conjugated goat antiserum to C3 (1:500 in 1% bovine serum albumin and 1% Tween) was added. After incubation for 30 min at room temperature (~23°C) and two further washes, 200 μl of substrate (0.37 M o-phenylenediamine and 0.025% H₂O₂ in 0.1 M citrate buffer) was added. The reaction was stopped after 30 min by the addition of 50 μl of 2.0 M H₂SO₄. A portion (200 μl) of the reaction mixture was transferred to a new plate, and the optical density at 490 nm was measured as above. Nonspecific binding was determined in parallel by incubation of bacteria in heat-inactivated serum, and this value was subtracted from total binding to give specific binding.

**Statistical data analysis.** Serologic data were compiled by health status and date. Results were subjected to analysis of variance for an unbalanced design. The Student-Neuman-Keuls procedure was used to statistically separate differences by health status and date. Least-squares estimates of marginal means were calculated for comparison. This is a calculation of expected means had the trials contained equal numbers of sick and healthy animals. The significance level for all calculations was *P* < 0.05 unless described otherwise. The procedures have been described by Ott (35).

**RESULTS**

The calves in this study underwent a severe natural challenge of BRD (59.5% morbidity and 11.2% mortality). A total of 56 calves were diagnosed with BRD and recovered after treatment. Three calves had symptoms of diarrhea. A total of 57 calves were free of BRD. Nasal cultures for *P. haemolytica* were positive for 35.3% (41 of 116) of all calves on day 1 in the feedlot and 12 of 13 calves that died (one from acute bloat). One animal with BRD died after being treated for 15 days, and *Actinomyces pyogenes* was isolated from lung tissue. Bacterial isolation from lung tissue demonstrated *Pasteurella* spp. from all of the 12 remaining calves. *P. haemolytica* cultured from 10 of the 12 calves with fatal pneumonic pasteurellosis was serotype A1. One calf had a mixed *P. haemolytica* A1 and *P. multocida A3* infection. Pure culture of *P. multocida A3* was isolated from the lungs of the other two calves.

The results of the ELISAs against the polysaccharide capsular antigens of *P. haemolytica* A1 and A2 (C-A1 and C-A2, respectively) support the severity of the challenge in the calves. These data are depicted in Fig. 1 through 3. There was a slight increase of antibody to *P. haemolytica* C-A1 among calves both with and without BRD (see Fig. 1 and 3). In general, the calves free of BRD had slightly higher titers than calves with BRD (B < 0.05; Fig. 1). However, only the differences on days 15, 29, and 56 were significantly higher.
(P < 0.05; Fig. 1). Total serum antibody to \textit{P. haemolytica} C-A2 changed little or decreased slightly (Fig. 2). The only statistically significant comparison observed was that calves free of BRD had higher titers to C-A2 than calves that had recovered from BRD at day 56 (Fig. 2). The HIGT results also indicated a humoral response to \textit{P. haemolytica} A1 (Fig. 3).

After observing these data, we were interested in whether these same trends would be present by isotype. Serum specimens from 12 calves (6 free of BRD and 6 recovered from BRD) were selected randomly for further analysis. The results of these ELISAs are represented in Tables 1 and 2. Again, the serology supports challenge with \textit{P. haemolytica} A1. The antibodies of all isotypes increased significantly (P < 0.05). Both IgA and IgG2 titers were higher in calves with BRD on days 29 and 56. There was very little change in the antibody levels to the \textit{P. haemolytica} C-A2. There were no changes in these trends when the titers were averaged for all calves (data not shown).

To further examine the nature of the humoral response to the \textit{P. haemolytica} antigens, the same 12 sets of serum samples were subjected to absorption with \textit{P. haemolytica} A1 and A2, \textit{P. multocida} A3, and lipopolysaccharide (with characteristic exposed core glycolipids of \textit{E coli} J5). These treatments were performed on the serum specimens from days 8 and 29 (designated S8 and S29, respectively). The results are presented in Fig. 4 and Table 3. Most of the IgG1 reactivity towards C-A1 in S8 could be absorbed with one treatment of either \textit{P. haemolytica} A1 or A2 cells. However, \textit{P. haemolytica} A2 could no longer absorb this antibody activity in S29. Absorption of S8 with \textit{P. haemolytica} A1 inhibited the IgG1 reactivity by 79%, whereas \textit{P. haemolytica} A2 absorbed 60% of the IgG1 activity. Absorption of S29 with \textit{P. haemolytica} A2 could only inhibit the logarithmic titer by 16%, whereas \textit{P. haemolytica} A1 inhibited the same reaction by 79% again. The same relationship was not observed with respect to IgG2; only \textit{P. haemolytica} A1 could significantly absorb this IgG2 reactivity.

In both IgA and IgM ELISAs for C-A1 antigen, increased inhibition by the serotype-specific absorbant (P < 0.05) between S8 and S29 was observed. There was no significant change in the level of inhibition of anti-C-A2 antibody reactivity, but the absorption was observed as generally serotype specific. The inhibition of serum IgA and IgG1 ELISA antibody activity in S8 and S29 decreased significantly (P <0.05) after absorption with strain J5 core glycolipids. However, the inhibition of IgG2 and IgM activity caused by J5 core glycolipid absorption did not significantly change.

The ability of the antibodies (in the same 12 serum specimens) to induce complement-mediated lysis of log-phase \textit{P. haemolytica} A1 organisms was also investigated. The results are presented in Table 4. The percents inhibition of bacterial growth (i.e., complement-dependent bacteriolyis) and C3 deposition on the surfaces of bacteria were significantly greater in S29 than in S8 (P < 0.01). In these assays, only absorption with \textit{P. haemolytica} A1 reduced bactericidal activity and C3 deposition. The marked increase in this bactericidal effect (38.2 to 95.1%) was serotype specific. Bactericidal assays using \textit{P. haemolytica} A2 as a target were also performed, with no significant difference in activity observed between S9 and S29 (54.7 to 59.3%). This

**TABLE 1.** Logarithmic serum ELISA titers by isotype and by date in feedlot to \textit{P. haemolytica} C-A1

<table>
<thead>
<tr>
<th>Day in feedlot</th>
<th>Healthy IgA</th>
<th>BRD IgA</th>
<th>Healthy IgG1</th>
<th>BRD IgG1</th>
<th>Healthy IgG2</th>
<th>BRD IgG2</th>
<th>Healthy IgM</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>2.60c</td>
<td>2.70a</td>
<td>3.30c</td>
<td>3.11c</td>
<td>1.52c</td>
<td>1.00c</td>
<td>1.17c</td>
<td>1.16c</td>
</tr>
<tr>
<td>8</td>
<td>2.13c</td>
<td>2.80d</td>
<td>3.57c</td>
<td>2.14c</td>
<td>3.10d</td>
<td>1.92b</td>
<td>3.41c</td>
<td>3.30c</td>
</tr>
<tr>
<td>15</td>
<td>1.17c</td>
<td>2.70b</td>
<td>3.41c</td>
<td>3.11c</td>
<td>3.67c</td>
<td>2.81b</td>
<td>3.16d</td>
<td>2.43b</td>
</tr>
<tr>
<td>29</td>
<td>1.40c</td>
<td>3.00f</td>
<td>3.61c</td>
<td>3.86d</td>
<td>3.10d</td>
<td>4.20c</td>
<td>2.97d</td>
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</tr>
<tr>
<td>56</td>
<td>1.50c</td>
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<td>3.81d</td>
<td>3.79d</td>
<td>3.43d</td>
<td>4.16c</td>
<td>3.08d</td>
<td>1.17c</td>
</tr>
</tbody>
</table>

* A different letter indicates a significantly different comparison by the Student-Newman-Keuls procedure (P < 0.05).
activity was also specific, as evidenced in S29 by significant serum bactericidal activity reduction from 59.3 to 32.4% by *P. haemolytica* A2 absorption (*P* < 0.05) but no significant reductions in activity by other absorbents.

**DISCUSSION**

Confer et al. (8) demonstrated that serum antibodies to capsular polysaccharides were correlated with a reduction in lesion severity after transthoracic challenge with *P. haemolytica*. Furthermore, *P. haemolytica* A1 epitopes not sensitive to periodate oxidation were reported as probably of some importance in resistance to pneumonic pasteurellosis. These authors also described quantitative and qualitative differences among humoral responses to capsular polysaccharides between different experimental immunogens. Furthermore, a higher percentage (65.4%) of reduction of the antibody activity in an anti-CPS ELISA was observed after capsular carbohydrate absorption of serum specimens from saline extraction-vaccinated calves than after absorption of serum specimens from calves immunized with a bacterin in aluminum hydroxide (47.1%), a bacterin in Freund incomplete adjuvant (40.5%), or live vaccine (25.0%) (8). Much of the antibody response to saline extraction vaccines was found to be directed against carbohydrate determinants and probably irrelevant for protection. In another study, high or rapidly increasing levels of anti-CPS IgG1 were associated with freedom from BRD (31) in feedlot calves. Because of these data, we were interested in further study of the specificity of the bovine immune response to capsular polysaccharide antigens, especially since the change in specificity occurs with natural exposure.

In this serologic study, the antigen preparation was unique. Beginning with the warm-saline extraction of *Pasteurella* spp. organisms in the early exponential phase of growth (19), the protein was precipitated by the addition of 15% polyethylene glycol. After this nearly complete precipitation of protein (98%), many carbohydrate-associated determinants were still present. Confer et al. (8) reported difficulties in separating protein and carbohydrate components of this extract. It is likely that many protective carbohydrate-associated epitopes are associated closely with these precipitated peptides of the saline-extracted antigens (8, 33). Nevertheless, there was antigenic polysaccharide material in the ELISA antigens used in these studies. Also, the humoral responses of these calves to these antigens indicate that the antigens were recognized by the bovine immune system.

The titers of all calves against serotype A1 antigens increased throughout the first 29 days in the feedlot. These increases were significant (*P* < 0.05) and were also observed to be slightly greater in calves free of BRD than in convalescent calves. These increases were reflected in all of the

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**TABLE 2. Logarithmic serum ELISA titers by isotype and by date in feedlot to *P. haemolytica* C-A2**

<table>
<thead>
<tr>
<th>Day in feedlot</th>
<th>IgA</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgM</th>
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</thead>
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<tr>
<td></td>
<td>Healthy</td>
<td>BRD</td>
<td>Healthy</td>
<td>BRD</td>
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<tr>
<td>1</td>
<td>0.67b</td>
<td>2.00c</td>
<td>3.10c</td>
<td>2.10c</td>
</tr>
<tr>
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<td>1.63c</td>
<td>2.60d</td>
<td>2.13b</td>
<td>2.30c</td>
</tr>
<tr>
<td>15</td>
<td>0.87b</td>
<td>2.20d</td>
<td>2.97c</td>
<td>3.20c</td>
</tr>
<tr>
<td>29</td>
<td>2.04c</td>
<td>2.56d</td>
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<td>1.40c</td>
<td>2.00c</td>
<td>3.00c</td>
<td>3.31c</td>
</tr>
</tbody>
</table>

*a A different letter indicates a significantly different comparison by the Student-Newman-Keuls procedure (*P* < 0.05).

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**FIG. 4. Log of ELISA titers (IgG1 and IgG2) for *P. haemolytica* C-A1 antigen.** The serum specimens were absorbed with *P. haemolytica* A1 or A2 or *P. multocida* A3 or were unabsorbed. Serum specimens from days 8 and 29 were selected for these assays. **a** Statistically significant difference in titer between healthy calves and calves with BRD (*P* < 0.05).
TABLE 3. Reduction of logarithmic serum ELISA titers by isotype for Pasteurella species antigen after immunoabsorption

<table>
<thead>
<tr>
<th>Absorbent*</th>
<th>Antigen</th>
<th>Isotype</th>
<th>% Reduction</th>
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<tbody>
<tr>
<td>NA</td>
<td>C-A1</td>
<td>lgA</td>
<td>0</td>
</tr>
<tr>
<td>PmA1</td>
<td>C-A1</td>
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<td>C-A1</td>
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<td>76</td>
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<td>lgM</td>
<td>19</td>
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<td>lgG1</td>
<td>0</td>
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<td>11</td>
</tr>
<tr>
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<td>C-A2</td>
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<td>JSe</td>
<td>C-A1</td>
<td>lgG1</td>
<td>72</td>
</tr>
<tr>
<td>JSe</td>
<td>C-A1</td>
<td>lgIG2</td>
<td>9</td>
</tr>
<tr>
<td>JSe</td>
<td>C-A1</td>
<td>lgM</td>
<td>23</td>
</tr>
</tbody>
</table>

*NA, Not absorbed.

Indicates significant increase in inhibition of antibody activity in the ELISA (P < 0.05).

Indicates significant decrease in inhibition of antibody activity in the ELISA (P < 0.05).

Phase organisms are encapsulated to the greatest extent (19). By four sequential absorptions, 98% of the ELISA antibody activity to the polysaccharide antigen could be removed from a hyperimmune positive control serum. This ability to absorb reactivity by intact bacterial cells probably indicates that these antibodies are important surface epitopes.

The homologous absorption by either P. haemolytica A1 or A2 was expected and demonstrated in all isotypes. However, some of the antibody activity in these serum specimens was not serotype specific, because heterologous absorption removed variable degrees of reactivity. This was most apparent in the absorption of IgG1 from serum samples obtained on day 8. The IgG1 reactivity toward C-A1 was greatly reduced by absorption with both P. haemolytica A1 and A2 and to a lesser degree by absorption with P. multocida A3 and E. coli J5 core glycolipids. However, by day 29, the ELISA-determined antibody reactivity within the IgG1 isotype was much more serotype specific. Interestingly, the day 8 IgG2 activity against C-A1 was also reduced by heterologous absorption with E. coli J5 core glycolipids and to a lesser extent by absorption with P. haemolytica A2 and P. multocida A3. The antibody to C-A2 was reduced by homologous absorption and somewhat by heterologous absorption, but the titers and patterns did not change significantly in any of the isotypes.

The increasing concentration and specificity of the anti-C-A1 antibody were associated with an increase in complement-dependent bactericidal activity. This increase in bactericidal antibody was, in turn, associated with an increase in C3 deposition on the surfaces of log-phase P. haemolytica A1 cells. Absorption assays indicated that increased complement-mediated bacteriolysis was serotype specific. This complement-dependent bactericidal property of the serum specimens was observed in S8, samples, but the specificity increased in S29.

Activation of the complement system leads to deposition of C3b on the surfaces of bacteria by covalent esters or amide bonds (18, 24, 25). This is significant, because further complement activation of the terminal lytic cascade may actually lyse the bacteria and/or the C3b fragments greatly enhance opsonization of organisms coated with immunoglobulin. These phenomena facilitate more effective clearance (22). These effector mechanisms may be very important in immunity to pneumonic pasteurellosis. All of the bovine isotypes can mediate some activation of the bovine complement system (30), but IgG1 and IgM were most effective in complement activation. Also, depletion of murine complement with cobra venom factor 3 reduced hepatic clearance of P. haemolytica A1 in passively immunized mice (30). Antibodies able to react as strongly positive in a complement-dependent HIGT (probably IgG1) were also associated with freedom from BRD in vaccinated calves (31). Czyzynski et al. reported that in vivo phagocytosis and killing of P. haemolytica by bovine neutrophils was enhanced by serum opsonization (10). Perhaps complement-associated enhanced opsonization and membrane damage could increase this cell-mediated killing of P. haemolytica. Further studies of these phenomena are needed.

The pattern of C3b deposition and degradation determines the nature of ligand-receptor interactions between an opsonized bacterium and phagocytic effector cells. In human studies, serum specimens with relatively low affinity and specificity mediated C3b deposition, but C3b was not converted to iC3b (11, 20). Such conversion is important, because C3b and iC3b react with different receptors. The C3b interacts with the C3b receptor, CR1 (11), whereas the

TABLE 4. Complement-dependent bactericidal activity and percentage of complement deposition of serum antibodies from feedlot calves

<table>
<thead>
<tr>
<th>Absorbent*</th>
<th>% Inhibition of bacterial growth</th>
<th>% Total C3 deposition on bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S8</td>
<td>S29</td>
</tr>
<tr>
<td>NA</td>
<td>38.2</td>
<td>95.1b</td>
</tr>
<tr>
<td>P. haemolytica A1</td>
<td>30.0</td>
<td>36.8d</td>
</tr>
<tr>
<td>P. haemolytica A2</td>
<td>42.2</td>
<td>85.1c</td>
</tr>
<tr>
<td>P. multocida A3</td>
<td>41.6</td>
<td>81.4a</td>
</tr>
</tbody>
</table>

*NA, Not absorbed.

Indicates significant increase in bactericidal activity or C3 deposition from day 8 to 29 for a treatment (absorbent).

Indicates significant decrease in bactericidal activity or C3 deposition compared with that of nonabsorbed serum.

Indicates significant reduction in bactericidal activity or C3 deposition of the absorbed serum compared with that of nonabsorbed serum.

Indicates significant decrease in bactericidal activity or C3 deposition compared with that of nonabsorbed serum.
iC3b reacts with CR3 receptors (19). The interaction with iC3b and CR3 (mediated by antibodies of high affinity and great specificity) was related to enhanced phagocytic clearance and stimulation of intracellular microbicidal mechanisms (20). Therefore, further investigation of these phenomena in the bovine is warranted.

We have demonstrated that in the course of natural exposure to Pasteurella spp., feedlot calves undergo a humoral immune response to carbohydrate antigens of the capsular material. The specificity (and probably affinity) of these antibodies increases during the course of this response. Further, these responses are associated with a serotype-specific increase in bactericidal activity and C3 deposition. Other antigens are also important in immunity to pneumonic pasteurellosis (7). The role of antibodies against polysaccharide antigens is but one component of an orchestrated response. Therefore, continued investigation of the antibody response to several antigenic components in vaccinated or convalescent calves and interaction with appropriate effector mechanisms is necessary.

LITERATURE CITED


