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Serum haptoglobin as an indicator of the acute phase response in bovine respiratory disease

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Abstract

The early stages of the host response to infectious agents include a number of physiologic changes, collectively known as the acute phase response. The acute phase response is comprised of reactions localized at the site of infection, as well as the initiation of systemic responses, which include a rapid increase in the serum concentration of some proteins, known as acute phase proteins (APP). Using polyacrylamide gel electrophoresis, we detected two APP of approximately 22 and 37 kDa molecular weight in sera obtained from cattle with bovine respiratory disease (BRD). Based on their presence in the sera of sick, but not normal animals, the molecular weights, N-terminal amino acid sequence analysis, and the ability to bind hemoglobin, we identified these proteins as the α and β subunits of haptoglobin. The haptoglobin molecule and the α subunit were isolated from serum, purified, and used to produce monoclonal and polyclonal antibodies. With these reagents, an enzyme linked immunosorbent assay was developed to measure the concentration of haptoglobin in bovine serum. Using an experimental model of BRD induced by a sequential challenge of calves with bovine herpesvirus type-1 and *Pasteurella haemolytica*, we

Abbreviations: SDS-PAGE = Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; APP = Acute phase protein; BRD = Bovine respiratory disease; BHV-1 = Bovine herpesvirus-1; ELISA = Enzyme linked immunosorbent assay; HPLC = High pressure liquid chromatography; PBST = Phosphate buffered saline with 0.5% Tween 20

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observed a temporal relationship between the increase in haptoglobin concentration in serum and the onset of bacterial infection. The haptoglobin concentration ranged from undetectable in the serum of most calves prior to challenge, to greater than 1 mg ml^{-1} in over one-third of the calves at the height of disease. Furthermore, the concentration of haptoglobin was associated significantly with other measures of the severity of disease. Together, these results indicate that quantification of acute phase proteins in animals with BRD could be a valuable diagnostic and prognostic aid.

Keywords: Acute phase response; Acute phase protein; Bovine respiratory disease; Haptoglobin

1. Introduction

Infectious disease is, by definition, the consequence of infection by microorganisms, such as bacteria, viruses, or parasites. However, the manifestations of infectious disease are the result of both the action of the infecting organism and the animal's response to that infection. Hence, the study of disease requires not only attention to the characteristics of the microorganism, but also to the means by which the host responds to its presence. Every animal responds to the presence of a foreign organism by invoking a number of physiological changes, including rapid inflammatory changes collectively known as the acute phase response. The acute phase response is characterized by localized changes such as the aggregation of platelets and clot formation, dilatation and leakage of blood vessels, accumulation of leukocytes, and activation of stromal cells to release biological response modifiers. The release of mediators by resident and infiltrating cells then results in the initiation of systemic responses, including fever, leukocytosis, activation of complement and clotting systems, alterations in the plasma concentration of trace minerals, and changes in liver metabolism, including the production of a set of proteins called acute phase proteins (APP) (Heinrich et al., 1990). Therefore, we initiated a study to evaluate the acute phase response of cattle because it may provide insight into the pathogenic mechanisms invoked in different diseases.

The serum profile of APP varies between species and is dependent on the type of inflammatory stimulus (Eckersall and Conner, 1988; Conner et al., 1988). In cattle, haptoglobin, ceruloplasmin, α-1 proteinase inhibitor, fibrinogen and α-1 acid glycoprotein (seromucoid) have all been shown to increase during the acute phase response (Conner et al., 1986; Conner et al., 1988; Conner et al., 1989; Itoh et al., 1990; Skinner et al., 1991; Morimatsu et al., 1992; Motoi et al., 1992). Infection with *Pasteurella haemolytica* raised the levels of haptoglobin, α-1 proteinase inhibitor, and seromucoid, but not that of ceruloplasmin (Conner et al., 1989), whereas *Salmonella dublin* infections in calves and suppurative inflammation of the mammary gland in cows increased the levels of ceruloplasmin in serum (Piercy, 1979; Conner et al., 1986). Therefore, description of the acute phase response for individual diseases may define a profile of changes characteristic of that disease, which will assist in identifying the pathogenic mechanisms and aid diagnosis. Alternatively, measurement of the acute phase response may reflect the severity of the disease process and serve as a prognostic indicator.

Bovine respiratory disease (BRD) continues to be one of the most important disease problems of cattle. Assessment of the acute phase response during BRD could be a

valuable aid in the diagnosis and prognosis of this condition. In this study we investigated serum protein profiles from animals with BRD to identify bovine APP which may be used as indicators of disease. We isolated two APP, identified them as the α and β subunits of haptoglobin, and developed an ELISA to measure haptoglobin concentration in serum. This assay was used to describe the kinetics of haptoglobin production in an experimental model of BRD.

2. Materials and methods

2.1. Polyacrylamide gel electrophoresis

Serum samples were obtained by centrifugation of clotted blood from feedlot calves showing typical signs of the early stages of BRD, from normal calves, and from calves that had been experimentally infected with BHV-1 and *P. haemolytica*. All sera were stored at −20°C. Serum samples were diluted 1:10 in Laemmli reducing buffer containing 2-mercaptoethanol(2ME)/bromophenol blue, and boiled for 5 min before resolving on a 15.0% acrylamide gel with a 3% stacking gel using the mini gel system (Bio-Rad Laboratories Ltd., Mississauga, Ont., Canada) and standard procedures (Laemmli, 1970). Protein bands were visualized by staining with Coomassie blue or by Western blot analysis. Proteins were electroblotted to Immobilon-PVD membrane (Millipore, Bedford, MA), and allowed to react with polyclonal or monoclonal antibodies for 1 h. Visualization of this reaction was carried out by incubating the blots in a solution of horseradish peroxidase conjugated goat anti-rabbit (or mouse) immunoglobulins (Gibco/BRL, Burlington, Ont., Canada) followed by the addition of 4-chloro-1-naphthol substrate (Bio-Rad).

2.2. Amino acid sequence analysis

Serum samples containing APP were subjected to procedures for the preparation of samples for sequencing according to the method described by Moss et al. (1988). After electrophoresis, the area of the gel containing proteins of molecular weights of approximately 10 000–50 000 Da was cut out, washed in glycine–Tris–methanol (electrophoretic blot transfer) buffer, and transferred to Immobilon-PVD membrane (Millipore, Bedford, MA). The blot was then stained in Coomassie blue to identify the relevant protein bands, which were cut from the blots, sealed in vials, and submitted for amino acid sequence analysis (National Research Council of Canada Protein Laboratory, Ottawa, Ont.).

2.3. Purification of the 22 kDa acute phase protein

Acute phase serum was obtained from a calf undergoing acute disease caused by *Haemophilus somnus*. Serum was precipitated with ammonium sulfate (40% vol/vol). The supernatant was dialyzed against double-distilled water, lyophilized, resuspended in 1 M guanidine HCl, dialyzed again against double-distilled water, concentrated by

ultrafiltration, and further clarified by centrifugation. The resulting supernatant containing both the 37 and the 22 kDa proteins was purified by affinity chromatography on an agarose–hemoglobin matrix (Sigma Chemical Co., St. Louis, MO, USA). Bound material was eluted with 3.5 M guanidine (pH 5.0). The guanidine was then removed by dialysis.

The 22 kDa acute phase protein was further purified by reversed phase high pressure liquid chromatography (HPLC) on a Vydac C-4 reversed phase column equilibrated with 0.05 M KH_2PO_4/0.07% 2ME buffer. The protein was eluted with a linear gradient (0–100%) of acetonitrile:water (9:1) containing 0.07% 2ME. The presence of the 22 kDa acute phase protein in the eluent was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using rabbit anti-22 kDa antibody.

2.4. Production of antibodies to acute phase proteins

Antibodies specific for acute phase proteins were first produced by immunizing rabbits with selected protein bands obtained by SDS-PAGE of serum from sick cattle. The areas of gel containing the acute phase proteins were excised, washed in double-distilled water, diced and emulsified in Freund's complete adjuvant (FCA), and used to immunize rabbits.

Rabbit antibodies specific to the purified bovine 22 kDa acute phase protein were produced by first immunizing with 25 μg of purified 22 kDa protein formulated in FCA containing 3% alum. The vaccine was administered by intramuscular injection at multiple sites. For subsequent immunizations, the 22 kDa protein was emulsified in incomplete Freund's adjuvant (IFA). The presence of antibody in serum of immunized rabbits was determined by reactivity in Western blots of serum from sick calves.

Monoclonal antibodies (mAbs) against the 22 kDa acute phase protein were produced following standard procedures (Deregt et al., 1987). The selection of hybridomas producing antibodies specific for the 22 kDa acute phase protein was performed by screening supernatants using an ELISA method with purified 22 kDa protein coating the plates. Selected hybridomas were subcloned by limiting dilution. The hybridoma clone designated 1D1 was selected for production of high levels of antibody and was transferred to BALB/c, IFA-injected mice for growth as monoclonal plasmacytomas. Monoclonal antibody was purified from ascites fluid using a Protein G Sepharose 4 Fast Flow column (Pharmacia LKB Biotechnology, Uppsula, Sweden). The purified immunoglobulin was biotinylated using biotin amidocaproate *N*-hydroxysuccinate ester (Sigma).

1D1 IgG was also coupled to Affi-Gel 10 (Bio-Rad) to produce an affinity column. Several aliquots of acute phase serum were passed through the column, using 0.1 M HEPES (pH 8.0) to equilibrate. Bound haptoglobin was eluted with 100 mM ethanolamine (pH 11).

To confirm the identity of the protein bound by 1D1 and the rabbit antibodies, serum from a sick calf was first incubated with hemoglobin coupled to agarose beads (Sigma) for 1 h at room temperature. The supernatant was removed from the matrix and then incubated for 1 h on ice with Protein G-Sepharose CL-4 beads (Pharmacia), previously

reacted with either 1D1 monoclonal antibody or rabbit anti-bovine haptoglobin serum. The agarose beads were then washed four times, boiled in sample buffer, and run on a 12% SDS-PAGE gel.

2.5. ELISA method

The final ELISA method used to test experimental samples was as follows. Ascites 1D1 diluted to 10 $\mu g\,ml^{-1}$ Ig in 0.05 M carbonate buffer (pH 9.3) was added to Immulon II microtiter plates (Dynatech Laboratories, Chantilly, VA) and incubated at 4°C overnight. Plates were washed with 0.01 M phosphate buffered saline containing 0.05% Tween 20 (PBST) and blocked for 1 h with PBST plus 0.5% gelatin. Serum samples were diluted in PBST (1/100, 1/200, and 1/400) and incubated in the plate for 2 h at 37°C. After washing, biotinylated 1D1 IgG (0.5 $\mu g\,ml^{-1}$) was added to the plates for 90 min at 37°C. Detection of bound antibody was accomplished by addition of a 1/1000 dilution of strepavidin–horseradish peroxidase complex (Amersham Canada Ltd., Oakville, Ont.) for 1 h at 37°C, followed by washing and the addition of the color substrate 2,2′-azino-di[3-ethyl-benzthiazolinsulfonate(6)] (Sigma) (150 $\mu g\,ml^{-1}$) in citrate buffer (pH 4.0) with 0.03% H_2O_2. Alternatively, a strepavidin–alkaline phosphatase conjugate (Gibco/BRL) was added at a 1/2000 dilution, followed by addition of the substrate *p*-nitrophenylphosphate di(TRIS) salt (Sigma) in 0.5 mM $MgCl_2$ (pH 9.8) with 1% diethanolamine. Both color reactions were measured on a Bio-Rad 3550 microplate reader at a wavelength of 405 nm (reference wavelength 490 nm). The concentration of haptoglobin in serum samples was determined by comparison of absorbances with a standard curve generated from dilutions of antibody affinity-purified haptoglobin.

2.6. Experimental infection of calves

Bovine respiratory disease was experimentally reproduced (following the guidelines of the Canadian Council of Animal Care), by challenging seronegative animals with bovine herpesvirus (BHV)-1 and *P. haemolytica* as previously described (Jericho et al., 1982). Briefly, on Day 0, Hereford calves, 7–9 months old, were individually exposed to an aerosol of BHV-1 (10^7 plaque forming units ml^{-1}) for 4 min using a nebulizer. On Day 4, BHV-1 challenged calves were exposed to aerosolized *P. haemolytica* A-1 strain B122 (2×10^8 colony forming units ml^{-1}) for 4 min using a nebulizer. This model mimics the clinical signs and pathological changes associated with the naturally occurring fibrinous pneumonia observed in feedlots. The clinical condition of individual calves was monitored and scored daily by a veterinarian according to previously reported criteria for BRD (Babiuk et al., 1987). Blood samples for determination of plasma zinc levels and serum acute phase proteins were collected daily. Zinc levels in plasma were determined by atomic absorption spectroscopy (Butrimovitz and Purdy, 1977) performed by the Animal and Poultry Science Department of the University of Saskatchewan.

2.7. Statistical analysis

Results from the BRD experiment were analyzed using the BMDP software package. Simple regression analysis was performed to compare Day 8 haptoglobin concentration (or the last recorded haptoglobin concentration for animals which died prior to Day 8) with zinc concentration on the same day, the maximum sick score after bacterial challenge, the maximum temperature after bacterial challenge, and the weight change over the course of the trial. One-way analysis of variance was also performed to compare the above measurements in animals which survived against those of animals which died.

3. Results

3.1. Identification and purification of acute phase proteins from calves with BRD

Acute phase proteins have been defined as those proteins which are present in plasma of acutely ill animals, but are absent or detected at lower levels in normal animals. Using SDS-PAGE analysis, serum obtained from sick animals contained two dominant proteins with molecular weights of approximately 37 and 22 kDa that were either absent, or present in lower concentration, in serum from normal animals (Fig. 1). Amino acid sequence analysis of the N termini of the acute phase proteins strongly suggests that the

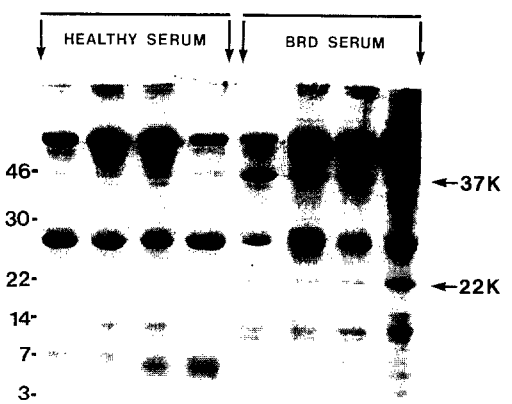

Fig. 1. Comparison of serum protein profiles of healthy and sick calves. Serum was collected from four healthy (healthy serum) calves and four calves with bovine respiratory disease (BRD serum). Protein profiles were compared after electrophoresis of serum samples on a 12.5% SDS-PAGE gel. The arrows indicate proteins which were present in the serum of sick calves but were absent, or in low concentration, in the serum of healthy calves. Molecular weight markers are indicated to the left of the figure.

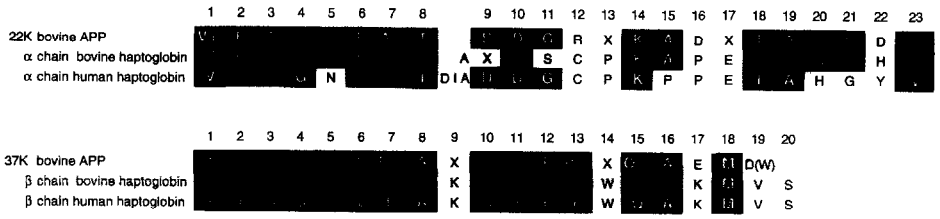

Fig. 2. Amino acid sequence analysis of bovine acute phase proteins. Serum from a sick calf was subjected to SDS-PAGE eletrophoresis and then electroblotted to Immobilon-PVD membrane. Protein bands of approximately 22 and 37 kDa were exised from the blot, the N-terminal amino acids sequenced, and a comparison made between these sequences and those reported for human and bovine haptoglobin (Kurosky et al., 1980; Morimatsu et al., 1991). The dark shaded areas denote homologous sequences, light shaded areas denote conservative amino acid changes, and unshaded areas denote mismatch between sequences. Potential alternative amino acids are indicated by parentheses, while the letter X designates an undetermined amino acid.

proteins are haptoglobin related. Using both Genbank and protein sequence databases, homology of the 37 kDa bovine acute phase protein with the β subunit of haptoglobin from several species including rat, human, and chimpanzee was identified (results not shown). The 22 kDa acute phase protein is homologous with the first eight amino acids of the α chain bovine haptoglobin sequence reported by Morimatsu et al. (1991). Alignment of the sequences of the 22 and 37 kDa proteins with bovine and human haptoglobin sequences as shown in Fig. 2 demonstrate this homology.

Having tentatively identified the acute phase protein as haptoglobin, we used the hemoglobin-binding activity of haptoglobin to purify larger amounts of the protein. Serum from an acutely ill calf was passed through a hemoglobin sepharose column, and the bound haptoglobin was recovered by guanidine treatment of the column. This haptoglobin preparation reacted with antibodies raised against the 22 and 37 kDa gel bands of serum from sick calves in a Western blot (not shown). Further purification steps resulted in isolation of the α subunit (22 kDa protein); however, the β subunit could not be isolated as an individual protein, as it appeared to become unstable after the α–β complexes were disassociated. The purified α subunit was then used to immunize rabbits and mice to produce polyclonal and monoclonal antibodies, respectively. The resulting monoclonal antibody, 1D1, reacted in Western blot with a 22 kDa protein in purified haptoglobin and acute phase serum, but not normal serum (Fig. 3).

To confirm the identity of the protein bound by 1D1 and the rabbit antibodies as haptoglobin, the interaction between hemoglobin and antibody binding was examined. Hemoglobin bound 22 and 37 kDa proteins in acute phase serum (Fig. 4, Lanes c and e), but not normal serum (not shown). Antibody could reduce, but not completely block hemoglobin binding (not shown). However, when acute phase serum was preincubated with hemoglobin, it was cleared of the 22 and 37 kDa proteins that were bound by either monoclonal antibody (Fig. 4, compare Lane d with Lane i) or polyclonal antiserum (Fig. 4, compare Lane f with Lane k). The presence of other proteins brought down by the antibody–Sepharose beads could be explained by examination of Sepharose bead plus bovine serum (Lane g) and antibody–Sepharose (Lanes h and j) controls.

An anti-α subunit antibody-affinity column, using the 1D1 monoclonal antibody, was then used to purify haptoglobin from the serum of a sick calf. This procedure resulted in

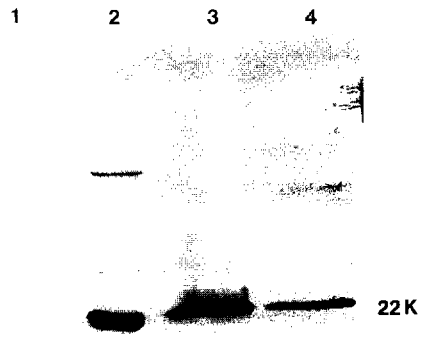

Fig. 3. Reactivity of the monoclonal antibody 1D1. To determine the specificity of the monoclonal antibody 1D1, it was reacted in a Western blot of normal bovine serum (Lane 1), purified bovine haptoglobin (Lane 2), serum from a sick calf (Lane 3), purified α subunit of bovine haptoglobin (Lane 4).

a haptoglobin preparation with less hemoglobin contamination than the one prepared with the hemoglobin affinity column. The purity of haptoglobin in this preparation was 70%, as determined by densitometric analysis of a Coomassie blue stained SDS-PAGE gel. The protein concentration was 12.6 mg ml^{-1} as determined by the Bradford method (Bio-Rad Protein Assay).

Fig. 4. Hemoglobin and antibodies bind the same proteins. To investigate the interaction between hemoglobin and antibody binding of the 22 and 37 kDa proteins, serum from a sick calf was incubated with hemoglobin bound to agarose beads. The supernatant from that reaction was then incubated with 1D1 monoclonal antibody or rabbit anti-bovine haptoglobin antibody bound to Protein G Sepharose CL-4B beads. The agarose beads were then washed and run on a 12% SDS-PAGE gel to identify bound proteins. Lane a, agarose alone + serum; Lane b, hemoglobin–agarose, no serum; Lane c, hemoglobin–agarose + serum; Lane d, 1D1–Sepharose + supernatant from reaction c; Lane e, hemoglobin–agarose + serum (as in c); Lane f, rabbit antibody–Sepharaose + supernatant from reaction e; Lane g, Protein G–Sepharaose alone + serum; Lane h, 1D1–Sepharose, no serum; Lane i, 1D1–Sepharose + serum; Lane j, rabbit antibody–Sepharose, no serum; Lane k, rabbit antibody–Sepharose + serum. Molecular weight markers are indicated to the left of the figure.

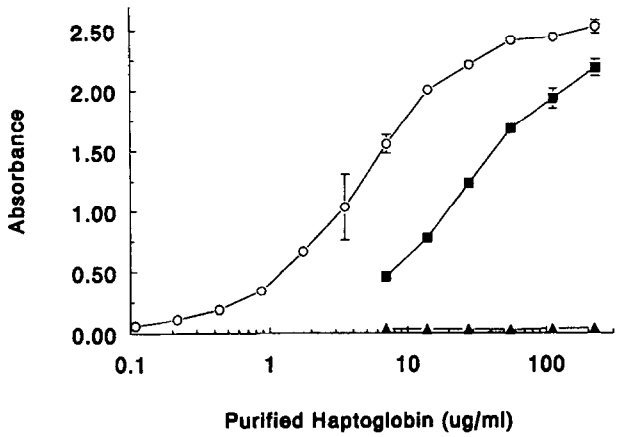

Fig. 5. Standard curve for the haptoglobin ELISA. A capture ELISA procedure was developed using monoclonal antibody 1D1. The relationship between the concentration of antibody affinity purified haptoglobin (○) and absorbance using this method is shown. Doubling dilutions (1/100–1/3200) of serum from a sick calf (■) and from a normal calf (▲) are included for comparison.

3.2. Development of the ELISA

A capture ELISA was initially developed using the 1D1 monoclonal antibody as the capture antibody, and the polyclonal antibody as the detection antibody. We found that

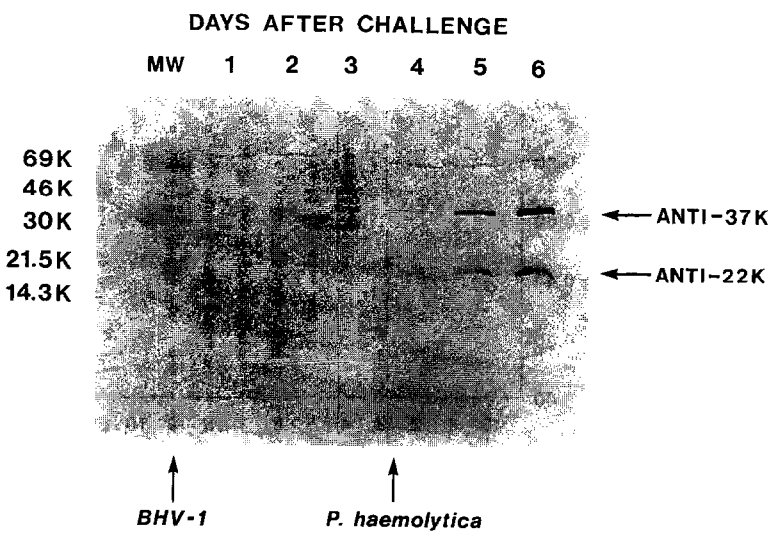

Fig. 6. Appearance of serum acute phase proteins during experimentally induced bovine repiratory disease. Samples were obtained from calves challenged with BHV-1 on Day 0 and *P. haemolytica* on Day 4. This figure shows a representative Western blot of serum collected from one animal on different days after challenge. Acute phase proteins were identified by using a mixture of rabbit antibodies raised against gel bands corresponding to the 22 and 37 kDa acute proteins.

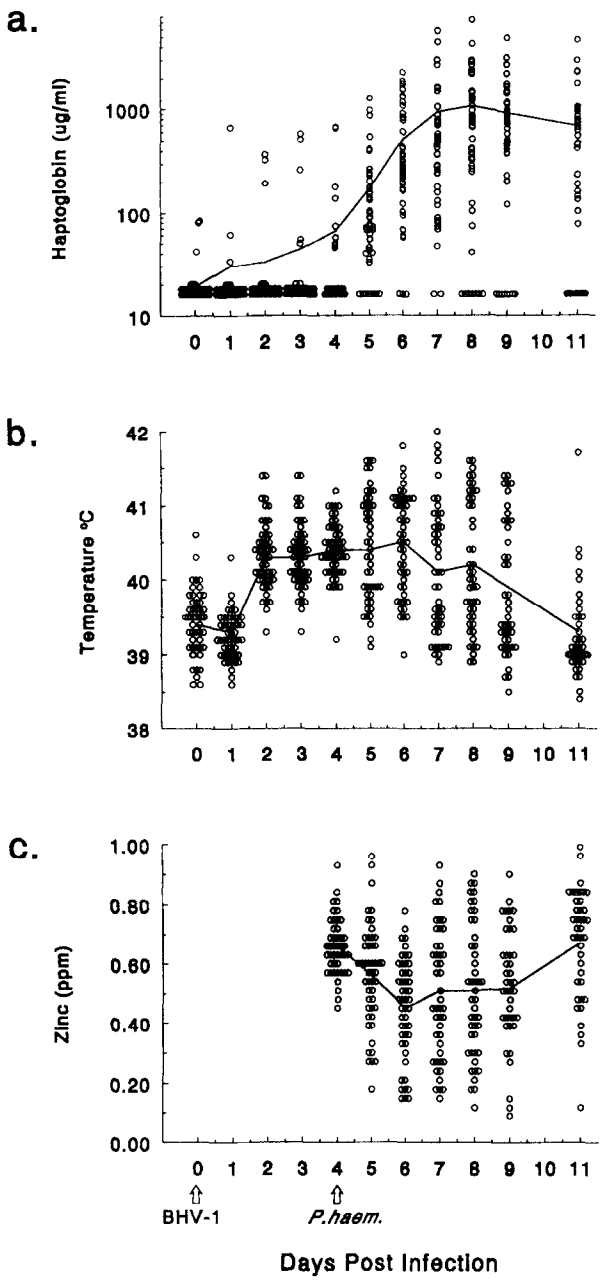

Fig. 7. Measurement of the acute phase response during bovine respiratory disease. Forty-nine calves were challenged with BHV-1 on Day 0 and *P. haemolytica* on Day 4. (a) Serum haptoglobin concentration determined by ELISA. (b) Calves were observed daily and rectal temperature recorded. (c) Plasma zinc concentration determined by atomic absorption spectroscopy. Individual results (open circles) and the mean (solid line) are shown.

using biotinylated 1D1 monoclonal antibody as the detection reagent gave similar results with less non-specific binding (data not shown). The working range of the assay was 0.5–50 $\mu g\,ml^{-1}$ of purified haptoglobin (Fig. 5).

3.3. Association between clinical disease and serum APP during experimentally induced BRD

To confirm the 22 and 37 kDa proteins as acute phase proteins, we examined the temporal relationship between the development of disease and the presence of these proteins in serum. We induced BRD with an initial challenge of BHV-1 followed 4 days later by a challenge with *P. haemolytica*. Protein bands corresponding to the 37 and 22 kDa proteins were not detected in Western blots of serum samples collected prior to challenge, or on Days 1, 2, or 3 after BHV-1 challenge. Only in a few instances were we able to detect a protein band that reacted with the anti-37 kDa protein in serum samples obtained 4 days after BHV-1 challenge, just prior to infection with *P. haemolytica*. However, both proteins (37 and 22 K) were detected in serum samples from all animals after *P. haemolytica* challenge (Fig. 6). Although Western blot analysis of serum was not intended to be quantitative, it was clear that the intensity of reactivity of the antibodies against APP increased as clinical manifestations of fibrinous pneumonia became more evident.

To more precisely correlate haptoglobin levels with disease, we followed 49 experimentally infected animals throughout the course of infection. At the beginning of this trial, serum haptoglobin was detectable (over 30 $\mu g\,ml^{-1}$) in only three animals, none of which had levels exceeding 100 $\mu g\,ml^{-1}$ (Fig. 7(a)). By Day 4, just 11 animals had detectable amounts of haptoglobin and there was a only a small rise in mean haptoglobin concentration. However, on Day 5, 24 h after bacterial challenge, all but seven of the

Table 1
Association of haptoglobin concentration with other measures of disease severity

Parameter	Clinical outcome [a]				Correlation with haptoglobin [b] r^2
	Alive		Dead		
	Mean	95% *CI*	Mean	95% *CI*	
Haptoglobin [c] ($\mu g\,ml^{-1}$)	661	(414–908)	2812	(1579–4045)	–
Sick score [d]	1.17	(0.9–1.4)	3.1	(2.8–3.4)	0.481
Temperature [e] (°C)	40.7	(40.5–41.0)	41.5	(41.4–41.6)	0.345
Weight change [f] (kg)	3.4	(0.7–6.2)	12.8	(16.3–9.2)	0.391
Zinc [g] (ppm)	0.57	(0.50–0.63)	0.19	(0.16–0.22)	0.333

[a] Forty-nine calves were challenged with BHV-1 on Day 0 and with *P. haemolytica* on Day 4. The data were grouped according to whether the animal survived the challenge. Ten animals died in the trial. Results are expressed as the mean and 95% confidence interval for each parameter. All measures of disease severity were significantly different between the two groups ($P < 0.001$).[b] All correlations were significant ($P < 0.001$).[c] Day 8 serum haptoglobin concentration determined by ELISA.[d] Maximum sick score after *P. haemolytica* challenge: 0, healthy; 4, severe disease.[e] Maximum rectal temperature after *P. haemolytica* challenge.[f] Difference in weight from the start of the trial to its completion on Day 11.[g] Day 8 plasma zinc concentration determined by atomic absorption spectroscopy.

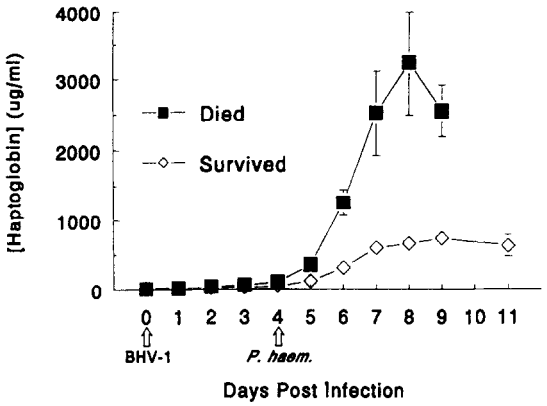

Fig. 8. Haptoglobin concentration in calves which survived and calves which died with BRD. Forty-nine calves were challenged with BHV-1 on Day 0 and *P. haemolytica* on Day 4. Serum haptoglobin concentration was determined by ELISA. The data from the calves were then grouped based on whether the animal died or survived. Ten calves died. The mean (± SE) haptoglobin concentration for each group is shown.

animals had elevated haptoglobin levels. The mean serum concentration of haptoglobin continued to increase, reaching a peak on Day 8 of the experiment (4 days after *P. haemolytica* challenge). In contrast, mean body temperature was elevated by 48 h after viral infection and remained so until it began to decline after Day 6 (Fig. 7(b)). Plasma zinc concentration, which was measured only from Day 4 on, declined after bacterial challenge until Day 6 and then slowly recovered to prechallenge levels by Day 11 (Fig. 7(c)).

When compared with other measures of disease severity, haptoglobin concentration was highly correlated with subjective clinical examination (sick score), body temperature, weight change, and plasma zinc concentration (Table 1) ($P < 0.001$ in all cases). Since we had seen a relationship between haptoglobin concentration and the course of infection, we then attempted to determine if haptoglobin concentration could be used to discriminate between clinical outcomes, in this case, death versus survival (Fig. 8). Haptoglobin levels were significantly different ($P < 0.001$) between the two outcomes (Table 1), as were the other measures of disease severity.

4. Discussion

Since the acute phase response is intimately associated with the infection process, the evaluation of the acute phase response could be a valuable aid in the management of infectious diseases (Eckersall and Conner, 1988; Saini and Webert, 1991). However, the availability of suitable reagents for evaluating this response in cattle is limited. In addition, very little is known about the type of acute phase reaction mounted in response to specific pathogens or in specific clinical conditions. Therefore, the objective of this project was to identify acute phase proteins associated with bovine respiratory disease and to develop methods to measure them. With these tools we could begin to develop a

better understanding of the relationship between the acute phase response and different disease processes.

We have shown that two proteins that were either not present, or present in very low concentration, in serum of normal animals, became increasingly prominent in serum samples from animals as clinical disease developed. The sequences of the 22 kDa and 37 kDa acute phase proteins were homologous with the first eight amino acids of the reported sequences of the α and β chains of bovine haptoglobin, respectively (Morimatsu et al., 1991). That the absolute sequence homology of the α chain falls to 70% for the sequenced 23 amino acids may be attributed to differences arising from the method of gas-phase amino acid sequencing. This method is extremely sensitive and allows long sequences to be generated. However, comparisons are known to be subject to variation because of equipment or technical differences, which become more pronounced with each cycle of sequencing. Analysis of the chromatograms from the protein sequencer precluded the possibility that contaminating proteins produced the sequence differences. Comparison of both bovine α chain sequences with the reported sequence for human α chain haptoglobin (Kurosky et al., 1980) indicated an absolute homology of over 50% over the full sequence available for the N terminal. When conservative amino acid substitutions are introduced, there is significant homology over the first eight amino acids. The homology between the bovine and human β chain sequences is more obvious, and together, these results suggest strongly that the 22 kDa and 37 kDa acute phase proteins are indeed the α and β subunits of haptoglobin.

Further evidence for the identity of the acute phase proteins is based on the known function of haptoglobin to bind hemoglobin (Morimatsu et al., 1991). Bovine hemoglobin specifically bound proteins of 22 and 37 kDa in acute phase serum (Fig. 4), but not normal serum. Incubation of acute phase serum with hemoglobin removed the 22 kDa and 37 kDa proteins which react with both the monoclonal and polyclonal antibodies (Fig. 4). The monoclonal antibody, raised against purified 22 kDa protein, reacted with the corresponding protein in Western blots of purified haptoglobin and acute phase serum, but not normal serum (Fig. 3). Therefore, we conclude that the 22 kDa and 37 kDa acute phase proteins are, respectively, the α and β subunits of haptoglobin, and that the antibodies used in this report bind to bovine haptoglobin.

Using these reagents, a sensitive and specific immunoassay to determine the concentration of haptoglobin in serum was developed. Use of the monoclonal antibody 1D1 as both capture and detection antibody resulted in less nonspecific binding compared to using rabbit antiserum as the detection antibody. That we were able to use the same antibody for both purposes is probably related to the fact that haptoglobin exists as multimeric complexes (Eckersall and Conner, 1990; Morimatsu et al., 1991) and thus presents epitopes in addition to the one bound to the capture antibody. The similarity of the slopes of concentration versus absorbance for purified haptoglobin and acute phase serum (Fig. 5) indicate that the purified haptoglobin is recognized in a similar fashion to haptoglobin in serum, and is thus a suitable standard for calculation of concentration.

Bovine serum haptoglobin concentration has been reported to be increased in a number of disease situations (Makimura and Suzuki, 1982; Conner et al., 1986; Conner et al., 1989; Skinner et al., 1991). In these studies, haptoglobin concentration was determined based on its ability to bind hemoglobin (Makimura and Suzuki, 1982), a

procedure which is rather cumbersome and precludes the assessment of large numbers of samples. Thus, to date, the correlation of elevated haptoglobin concentration with specific diseases is based on relatively few animals for each disease condition, or measurement of haptoglobin concentration at only one time point in the course of the disease. A radial immunodiffusion assay has been developed which simplifies haptoglobin analysis (Morimatsu et al., 1992), but this assay is still not amenable to analysis of large numbers of samples. Monoclonal antibodies to haptoglobin have been reported recently, but they were not used to quantify haptoglobin in specific disease conditions (Sheffield et al., 1994). By developing a sensitive and relatively simple ELISA, we were able to monitor the serum haptoglobin concentration of 49 animals sampled daily over the course of an experimental BRD infection. In previous reports, haptoglobin levels of greater than 1 mg ml^{-1} were detected in infectious conditions such as mastitis, metritis, and *P. haemolytica* infection (Makimura and Suzuki, 1982; Conner et al., 1986; Conner et al., 1989; Skinner et al., 1991). With the ELISA, we measured a mean peak level of 1.1 mg ml^{-1} of haptoglobin on Day 8 of the experiment, with two animals having concentrations of haptoglobin in excess of 4 mg ml^{-1}. Similar to previous reports, haptoglobin levels were undetectable (less than 30 $\mu g\,ml^{-1}$) in animals prior to challenge. This data provides us with valuable information regarding the induction of the acute phase response, and in particular the kinetics of haptoglobin production, in this example of virus-predisposed bacterial pneumonia.

While an increase in the concentration of APP is a well recognized phenomenon during the response to infectious agents, the proteins associated with the acute phase response vary between different animal species. In addition, APP profiles differ within a species according to the infectious stimuli. In this model of virus-induced bacterial pneumonia, few animals responded to the viral challenge with increased haptoglobin levels. Over the first 4 days after BHV-1 exposure, only 10% of animals developed haptoglobin concentrations greater than 100 $\mu g\,ml^{-1}$. However, 24 h after *P. haemolytica* challenge, 43% of the animals had haptoglobin levels in excess of 100 $\mu g\,ml^{-1}$, and by 3 days after challenge, this proportion reached 84%. Thus, haptoglobin induction appeared to be related to the onset of bacterial infection. The induction of this response was detectable by 24 h after bacterial challenge, but the proportion of animals responding and the mean haptoglobin concentration continued to rise for another 2–3 days. From the information in Fig. 7, it can be seen that the kinetics of fever and haptoglobin induction differ, providing a distinguishing feature between viral and bacterial disease.

Another aspect of the acute phase response is the change in plasma trace mineral concentration. Decreases in zinc concentration have been noted previously in association with bacterial, but not viral challenge (unpublished data). The magnitude of the change in zinc concentration is not as great as that of haptoglobin (Fig. 7). In addition, the mean zinc concentration was lowest on Day 6, which was somewhat sooner than when haptoglobin levels peaked on Day 8. Thus, different aspects of the acute phase response may be manifested at different times, and the relationship between these different components of the acute phase response may be important in establishing the profile of changes associated with different disease processes.

Since the induction of haptoglobin production was temporally associated with the development of disease, we were also interested to see if the level of haptoglobin was

associated with the severity of disease. Increased concentrations of haptoglobin were significantly associated with other measures of disease severity such as fever (after bacterial challenge), sick score, and weight loss (Table 1). While increased haptoglobin levels were detected in some animals that did not show apparent illness, all animals which were clinically sick had elevated haptoglobin levels. Thus haptoglobin measurement may detect infected animals before clinical signs of disease become apparent. Furthermore, the concentration of haptoglobin on Day 8 was significantly higher in animals that subsequently died, compared to those that recovered. Thus, measurement of haptoglobin concentration may serve as a prognostic aid in determining the severity of disease.

The lack of rapid and accurate means of diagnosis of infectious conditions in cattle translates into inadequate management of the disease, with obvious economic consequences. The ability to measure acute phase proteins and proinflammatory cytokines will be instrumental in further characterization of the acute phase response in cattle. This will be of value, not only as a practical aid to determining the diagnosis and prognosis of infectious disease, but also by contributing to the understanding of the pathogenesis of various diseases, including BRD.

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