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Short communication

The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus

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Abstract

The ability of a pure virus infection to induce an acute phase protein response is of interest as viral infections are normally considered to be less efficient in inducing an acute phase protein response than bacterial infections. This was studied in a bovine model for infection with bovine respiratory syncytial virus (BRSV), analysing the induction of the two most dominant bovine acute phase proteins haptoglobin and serum amyloid A (SAA). Strong and reproducible acute phase responses were detected for both proteins, peaking at around 7–8 days after inoculation of BRSV, while no response was seen in mock-inoculated control animals. The serum concentrations reached for SAA and haptoglobin during the BRSV-induced acute phase response were generally the same or higher than previously reported for bacterial infections in calves. The magnitude and the duration of the haptoglobin response was found to correlate well with the severity of clinical signs (fever) and with the extent of lung consolidation while SAA responded most rapidly to infection.

Keywords: Bovine respiratory syncytial virus infection; Bovine acute phase response; Bovine haptoglobin; Bovine serum amyloid A

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1. Introduction

The acute phase serum protein response is a well-known general indicator of inflammation, trauma and other pathological conditions (Kushner and Rzewnicki, 1994; Baumann and Gauldie, 1994) and its relevance for the monitoring of the health status of domestic animals is being increasingly realised (Eckersall et al., 1999; Gruys et al., 1994). The serum concentration of individual acute phase serum proteins increases from 2 to 3 times to several hundred times (depending on the protein in question) during the acute phase of an inflammation (Kushner, 1982). Haptoglobin and SAA are major acute phase proteins in most species studied (Mackiewicz et al., 1993).

To evaluate the usefulness of these markers of inflammation for diagnosis of disease, and as markers of severity of disease in domestic animals, it is important to define precisely the disease conditions leading to acute phase responses of different proteins in the species in question. The bovine acute phase protein reaction has been reviewed most recently by Eckersall and Conner (1988) and Gruys et al. (1994). Haptoglobin concentrations in healthy cattle are often undetectable (Makimura and Suzuki, 1982; Conner et al., 1986; Eckersall and Conner, 1988) but during an acute phase response bovine haptoglobin can increase 50–100 times (Conner et al., 1988, 1989; Godson et al., 1996; Gruys et al., 1993), making it the most prominent acute phase protein in cattle (Alsemgeest et al., 1994). On the other hand, SAA is a remarkably moderate acute phase protein in cattle increasing around 2–5 times during an acute phase response (Boosman et al., 1989; Gruys et al., 1993; Alsemgeest et al., 1994; Werling et al., 1996). Nevertheless, SAA seems to react faster than haptoglobin in response to an acute phase protein inducing event (Gruys et al., 1993; Horadagoda et al., 1994) and the difference between serum concentrations induced by acute and chronic inflammation, respectively, was found to be bigger for SAA than for haptoglobin in naturally infected cattle (Alsemgeest et al., 1994; Horadagoda et al., 1999).

The generally held view is that viral infections lead to weak acute phase reactions if any (van Leeuwen and van Rijswijk, 1994), a fact that has been exploited in human clinical medicine to discriminate between viral and bacterial infections, e.g. in respiratory infections (van Leeuwen and van Rijswijk, 1994). Spooner and Miller (1971) reported the absence of elevated haptoglobin in clinical cases of rinderpest virus infection, whereas Höfner et al. (1994) demonstrated a haptoglobin response in foot- and mouth-disease virus infected cattle.

It was the aim of the present study to determine the acute phase protein (SAA and haptoglobin) inducing ability of the common bovine respiratory pathogen BRSV (bovine respiratory syncytial virus), using a well-characterised experimental infection model (Tjørnehøj, 2000). Furthermore, the usefulness of haptoglobin and SAA as clinical parameters reflecting the establishment, development and severity of a BRSV infection was examined.

2. Materials and methods

Animals. Conventional, colostrum-fed 7–14 days old male Jersey calves were purchased from two self-supplementary herds and reared in isolation units following

normal management procedures for dairy calves. Before challenge, all calves were tested as previously described (Rønsholt et al., 1996) for persistent infection with bovine virus diarrhea virus (BVDV), and all animals were negative.

Inoculations. The BRSV inoculum consisted of a third or fourth passage of BRSV isolate 2022 in fetal bovine lung cell culture (FBL) or of uninfected FBL cultures (mock infection (controls)). The original BRSV material was lung lavage fluid from a 1-month-old field calf with severe BRSV-related pneumonia (Viuff et al., 1996), and was passaged once in a 1-month-old colostrum-deprived calf before inoculation into FBL cells. The inocula were tested for and found free from other viruses, bacteria and *Mycoplasma* spp. as described in Tjørnehøj (2000). The calves were inoculated once by combined aerosol exposure (Waechtomat inhalator VM 82, most droplets less than 3 μm) and intratracheal injection. The inoculum (5 ml in phosphate buffered saline (PBS)) was administered over 10 min through a mask covering nostrils and mouth. The intratracheal inoculum (20 ml in PBS) was injected into the trachea during provoked heavy inhalation. The dose was $10^{4.6}$–$10^{5.2}$ $TCID_{50}$ of BRSV in each type of inoculation.

Control calves and BRSV-infected calves were kept in separate isolation rooms during challenge. Clinical signs were monitored daily. Unstabilized blood samples (approximately 8 ml) were obtained by jugular venipuncture using Veno-ject tubes. The blood was allowed to clot at 4°C, and serum was extracted the following day and stored at −20°C.

Calves were regularly blood sampled for up to 15 days after infection (see Fig. 1) and then euthanised. In one experiment (Fig. 1B) where the calves were blood sampled daily, all animals received 2.5 mg enrofloxacin (Baytril®, Bayer) per kilogram body weight from the day before BRSV-infection until euthanasia.

Post-infection analyses. Maximal temperature and days with fever (i.e. rectal temperature above 39.2°C) were recorded.

Post-mortem analyses. Calves were anaesthetized with pentobarbital (50 mg/kg) and euthanized by bleeding out through *V. axillaris* or *V. iliaca* externa. The lungs were immediately removed, and macroscopic lesions scored between 0 and 5 as described by Tjørnehøj (2000).

Tissue from the right lung of all calves was tested for the presence of BRSV, BVDV, parainfluenza 3 virus, and bovine corona virus as described by Uttenthal et al. (1996). Lung-, spleen- and liver-samples from all calves were investigated for aerobic and anaerobic bacteria by conventional techniques, and bronchial swabs were examined for *Mycoplasma* spp. by culture.

Serum haptoglobin was determined by a monoclonal antibody-based capture ELISA as described previously (Godson et al., 1996). Briefly, to coat plates with antibody, ascites fluid containing monoclonal antibody (1D1) to bovine haptoglobin, diluted to 1/1000 in 0.05 M carbonate buffer (pH 9.3), was added to Immulon II microtitre plates (Dynatech Laboratories, Chantilly, VA) and incubated at 4°C overnight. Plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked for 1 h with PBST plus 0.5% gelatin. Sera were diluted in PBST (1/100, 1/300, and 1/900) and incubated in the plate overnight. All incubations were carried out at room temperature and all incubation volumes were 100 μl. After washing, the biotinylated IgG of the monoclonal antibody 1D1 (0.5 μg/ml) (prepared as described in Godson et al. (1996)) was added to the plates

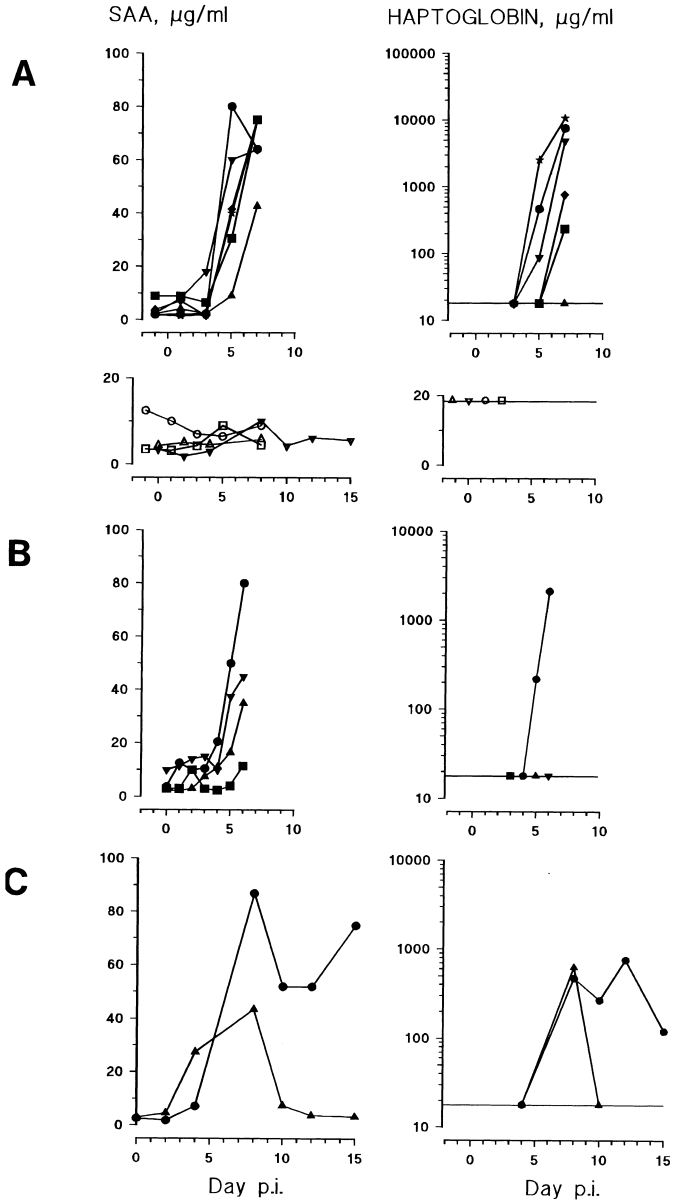

Fig. 1. (A) SAA (left-hand side) and haptoglobin (right-hand side). Infected animals: (★) 1; (●) 2; (▼) 3; (◆) 4; (▲) 5; (■) 6. Control animals: (○) 11; (□) 8; (▽) 9; (△) 10. Horizontal line depicts the detection limit of the assay (haptoglobin). None of the control animals had haptoglobin levels above the detection limit of the assay. (B) SAA (left-hand side) and haptoglobin (right-hand side). Infected animal: (●) 11; (■) 12; (▲) 13; (▼) 14. Horizontal line depicts the detection limit of the assay (haptoglobin). (C) SAA (left-hand side) and haptoglobin (right-hand side). Infected animals: (●) 15; (▲) 16. Horizontal line depicts the detection limit of the assay (haptoglobin).

for 2 h. Detection of bound antibody was accomplished by addition of a 1/2000 dilution of streptavidin-alkaline phosphatase (Gibco/BRL) for 2 h, followed by washing and the addition of the color substrate *p*-nitrophenylphosphate di(TRIS) salt (Sigma) in 0.5 mM $MgCl_2$ (pH 9.8) with 1% diethanolamine. Absorbance was 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 to a standard curve generated from dilutions of antibody affinity-purified bovine haptoglobin (Godson et al., 1996). The detection limit of the assay was defined as two times the standard deviation of the background OD.

Serum amyloid A was determined by a commercially obtained ELISA kit (Phase SAA kit, Tridelta) according to the manufacturer's instructions. This is a sandwich type assay in which sera were diluted 1/500 with the kit diluent and applied together with biotinylated anti-SAA antibody and incubated for 1 h at 37°C. After washing in kit wash buffer, streptavidin-peroxidase was added and incubated at room temperature for 30 min before wash and colour development with the tetramethylbenzidine substrate of the kit. Colour was stopped with the stop reagent and read at 490 nm with 650 nm as background correction. All plates included the kit standard in five dilutions, ranging from 2.3 to 37.5 ng/ml and a blind sample. All samples were run in duplicate.

3. Results and discussion

The acute phase response of the two most prominent (Gruys et al., 1994; Eckersall et al., 1999) bovine acute phase proteins, SAA and haptoglobin, was studied in young calves after experimental infection with BRSV. Although most animals including controls had *Mycoplasma* spp. at necropsy, the infection method (Tjørnehøj, 2000) reproduced disease through monoinfection with BRSV as indicated by the lack of pathological lesions and clinical signs in the control animals (see Table 1). In three calves out of 16 included in this study, either *P. multocida* or *P. avium* (both common respiratory (co-)pathogens in cattle (Bisgaard et al., 1991)) were found upon necroscopy.

The serum concentration of both SAA and haptoglobin remained low in all mock-infected control animals at all times with SAA concentrations below 17 μg/ml and haptoglobin concentrations below the detection limit of the ELISA (18 μg/ml, Fig. 1A). The SAA concentration became elevated in 11 out of 12 experimentally infected animals within 6 days post infection (p.i.) (Fig. 1A–C). In most cases this elevation was detectable at day 5 p.i, and peaked around day 5–8 p.i. Peak concentrations were in the 60–80 μg/ml range which is 5–7 times the normal SAA concentration as defined by the four control animals. In one of the two animals followed for 15 days p.i., a secondary response was noted on day 15 p.i. (animal No. 15, Fig. 1C). Generally, changes in haptoglobin concentrations followed the changes in the SAA concentrations seen in individual animals, although low SAA responders did not show any haptoglobin response at all (Fig. 1A: animal No. 5, Fig. 1B: animal Nos. 12–14). The maximum response of haptoglobin was seen on day 6–7 p.i. and reached 8–10 mg/ml, which is approximately 500 times the detection limit of the assay (18 μg/ml). The secondary response of animal No. 15 was also seen with haptoglobin, although the second peak was at day 12 p.i.

Table 1
Clinical signs, pathological lesions and agents isolated at necroscopy in individual BRSV-infected and control calves

Calf no.[a]	Killed at day p.i.	Pathology (lesion) score	Fever days (p.i. days)	Maximum temperature (°C)	BRSV at necropsy	Other agents at necropsy	SAA rank	Haptoglobin rank
1	7	4	4 (4–7)	40.2	Yes	M[b]	3	1
2	7	5	3 (5–7)	40.6	Yes	M, Pm[c]	1	2
3	7	3	3 (5–7)	40.2	Yes	Pm	2	3
4	7	2	2 (6–7)	39.6	Yes	M	4	4
5	7	3	1 (6)	39.6	Yes	M	6	nr[d]
6	7	3	–	–	Yes	–	5	5
7 (control)	8	0	–	–	No	M	–	–
8 (control)	8	1	–	–	No	M	–	–
9 (control)	15	1	–	–	No	M	–	–
10 (control)	8	1	–	–	No	M	–	–
11	6	4	2 (5–6)	40.0	Yes	–	1	1
12	6	1	1 (6)	39.5	Yes	–	nr	nr
13	6	2	–	–	Yes	M	3	nr
14	6	0	–	–	Yes	–	2	nr
15	15	4	9 (5–13)	40.9	No	M, Pa[e]	1	1
16	15	1	3 (5–7)	39.5	No	M	2	2

[a] Animals are listed according to number of fever days. If number of fever days are identical, the animal with highest lesion score is listed first. The acute phase rank is derived from the area under the acute phase response curve of the protein in question until the day of sacrifice, with 1 being the biggest area.
[b] *Mycoplasma dispar, Mycoplasma bovirhinis* or *Ureaplasma diversum.*
[c] *P. multocida.*
[d] No response could be detected.
[e] *P. avium.*

The two fastest SAA responders (animals Nos. 2 and 3) in Fig. 1A also belonged to the group of the three fastest haptoglobin responders; these animals were found to be infected with *P. multocida* at necropsy. Bacterial infection (with *P. avium*) was also found in animal No. 15, probably being the cause of the secondary responses of haptoglobin and SAA in this animal (Fig. 1C). The other two animals from which bacteria (*P. multocida*) were isolated were followed for 8 days only and thus a putative secondary acute phase response was not detected.

The response to BRSV-infection of both SAA and haptoglobin, developing during days 4–8 p.i. coincided with development of fever in most animals (Table 1). One exception was animal No. 5 (Fig. 1A) which was the lowest acute phase responder with both SAA and haptoglobin but did show a significant pathology score of three and had 1 fever day in contrast to animal No. 6 which had no fever days (but a pathology score of three) and which had a higher acute phase response with both proteins.

Generally, the acute phase responses could be ranked after the area under the response curve and correlated with the number of days with fever (see Table 1). Although in some cases with mild clinical signs (animals 12–14, Fig. 1B) haptoglobin did not react while

SAA showed a low response, the magnitudes of the SAA responses of these animals were not correlated to either fever or pathology score (Table 1), and generally the haptoglobin concentrations correlated better with disease severity. As a rough guideline, severe BRSV infection correlated with haptoglobin concentrations above 1 mg/ml at day 7 p.i. while less severe infection correlated with concentrations above the detection limit but below 1 mg/ml at day 7 p.i. (compare Table 1 and Fig. 1A–C). For SAA, concentrations around 30–40 μg/ml were associated with mild disease and severe disease resulted in concentrations at or above 60 μg/ml.

Although no reports have been published previously on the induction of SAA after viral infection in cattle, the SAA and haptoglobin concentrations reported here are within previously reported ranges for normal and acute phase concentrations in cattle after various experimental bacterial infections and aseptic inflammations (Werling et al., 1996; Boosman et al., 1989; Alsemgeest et al., 1994; Horadagoda et al., 1999; Conner et al., 1986; Eckersall and Conner, 1988; Skinner et al., 1991; Gruys et al., 1993; McNair et al., 1997; Nakagawa et al., 1997).

The haptoglobin concentration was demonstrated before to correlate with severity of clinical signs in mastitis cases (Makimura and Suzuki, 1982; Spooner and Miller, 1971; Conner et al., 1986) and in an experimental study of *Haemophilus somnus* infection (McNair et al., 1997). Skinner et al. (1991) found that haptoglobin concentrations of more than 200 μg/ml indicated mild infection, values above 400 μg/ml diagnosed severe infection, while extended pathological conditions were typically associated with haptoglobin concentrations in the 1–2 mg/ml range (Skinner et al., 1991; Saini et al., 1998). In Godson et al. (1996), maximum haptoglobin concentrations correlated with maximum rectal temperature, sick score, weight change and decreased zinc concentration as well as with death after experimental herpesvirus type-1/*P. haemolytica* co-infection. Alsemgeest et al. (1994) found that bovine SAA was a more sensitive indicator of acute disease than haptoglobin. Also, while a bovine SAA response was found within 360 min after intravenous LPS, no increase in haptoglobin was found in the same time frame (Werling et al., 1996). Similarly, Horadagoda et al. (1993, 1994) found SAA to react more rapidly than haptoglobin to experimental *P. haemolytica* infection. The difference in the inducibility of SAA and haptoglobin by acute and chronic disorders, respectively, was recently confirmed in cattle with various acute or chronic diseases (Horadagoda et al., 1999) and SAA was also reacting more rapidly and sensitively than haptoglobin in the present study (see above). In man, SAA is the acute phase protein that is most sensitive to viral infections (see e.g. van Leeuwen and van Rijswijk, 1994).

In conclusion, this model of BRSV infection (Tjørnehøj, 2000) leads to strong and reproducible SAA and haptoglobin acute phase responses, the magnitude of which correlated well with the severity of disease, even if infection with BRSV is not believed to have a viraemic phase and leads to pathological changes that do not involve actual tissue damage (Collins et al., 1996). In other studies, viral infection, either experimental (bovine herpes virus followed for 4 days after infection (Godson et al., 1996)) or natural (rinderpest virus (Spooner and Miller, 1971)) did not give rise to elevated haptoglobin. However, an acute phase haptoglobin response was observed in foot-and-mouth disease virus infections (Höfner et al., 1994), simultaneously with or a couple of days later than viraemia and clinical disease. Also, bovine herpes virus infections did lead to a

haptoglobin response when animals were followed for more than 4 days p.i. (Godson et al., unpublished results).

Haptoglobin reached maximum concentrations up to above 10 mg/ml while SAA increased up to around 80 μg/ml, both proteins peaking around day 8 p.i., and setting off at day 5 p.i. Haptoglobin reacted with the biggest relative increase but SAA was most sensitive to BRSV-infection. None of the control animals showed any sign at any time of haptoglobin above the detection limit of the assay or SAA above normal levels even if *Mycoplasma* spp. were isolated from all control animals. It must be concluded therefore that bovine haptoglobin and SAA can react to viral infection. Considering the timing of the response and the correlation between the magnitude of the response and the severity of the infection, it can be established that viruses causing only local pathology can indeed induce acute phase responses, most probably indirectly via virus-mediated tissue perturbations.

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