Journal of Dairy Research (2003) **70** 379–386. © *Proprietors of Journal of Dairy Research 2003*
DOI: 10.1017/S0022029903006484 *Printed in the United Kingdom*

Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis

Ulrika Grönlund[1], Cecilia Hultén[2], Peter D. Eckersall[3], Caroline Hogarth[3] and Karin Persson Waller[4*]

[1] Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Uppsala, Sweden
[2] Department of Clinical Chemistry, Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Uppsala, Sweden
[3] Department of Veterinary Clinical Studies, University of Glasgow Veterinary School, Glasgow, United Kingdom
[4] Department of Ruminant and Porcine Diseases, National Veterinary Institute, and Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Uppsala, Sweden

Received 1 October 2002 and accepted for publication 19 December 2002

Local and systemic changes in the acute phase proteins, haptoglobin and serum amyloid A (SAA), were studied in six dairy cows during the acute and chronic phases of experimentally induced *Staphylococcus aureus* mastitis. Haptoglobin and SAA were measured in serum, and in milk from infected and healthy control udder quarters within each cow. Concentrations of haptoglobin and SAA increased rapidly in both serum and milk during the acute phase of mastitis and followed a similar pattern. Significantly raised milk concentrations of SAA were also found during chronic subclinical mastitis. Serum concentrations of SAA also tended to be higher during the chronic phase than pre-infection. Increases in milk haptoglobin and SAA were specific for the infected udder quarters. In conclusion, measurement of SAA in milk samples could be a useful tool in diagnosing mastitis.

Keywords: Dairy cow, acute phase proteins, diagnosis.

Mastitis is one of the main disease problems in dairy cows worldwide. One of the major udder pathogens is *Staphylococcus aureus* (*Staph. aureus*), which is contagious and spreads easily in a herd (e.g., Fox & Gay, 1993). It causes inflammation of the mammary gland, ranging from peracute to chronic but the peracute form is rare. A variety of virulence factors increases the ability of *Staph. aureus* to survive in the mammary gland (Jonsson & Wadström, 1993). Consequently, the bacteriological cure rate of clinical *Staph. aureus* mastitis is often low (e.g., Pyörälä & Pyörälä, 1998; Sol et al. 2000). If the acute *Staph. aureus* mastitis is not successfully cured, the disease may pass into a chronic, subclinical mastitis characterized by elevated milk somatic cell count (SCC). Chronically infected cows shed bacteria intermittently, and are an important source of infection (Sears et al. 1990). It is therefore desirable to eliminate this disease from a herd despite the great economic cost and practical effort that may be required. To achieve this it is necessary to have valid diagnostic tools to identify infected animals. However, reliable diagnostic tests suitable for automated detection of subclinical mastitis are not available and are urgently needed.

All forms of tissue injury due to infection, trauma, or inflammation cause a series of events called the acute phase response, and a prominent feature of this response is the increased production of acute phase proteins (APP) by the liver. Several authors describe the bovine acute phase response in serum during acute clinical mastitis (Spooner & Miller, 1971; Conner et al. 1986; Skinner et al. 1991; Hirvonen et al. 1996, 1999; Eckersall et al. 2001). The studies show that infected cows have increased serum concentrations of serum amyloid A (SAA), haptoglobin, fibrinogen, α1-acid glycoprotein, ceruloplasmin, and α1-proteinase inhibitor, of which SAA and haptoglobin are considered to be the most prominent markers of inflammation.

The acute phase response measured in serum is nonspecific, as it can be activated by a number of stimuli. Therefore, measurement of serum concentrations of APP is

*For correspondence; e-mail: Karin.Persson.Waller@sva.se

https://doi.org/10.1017/S0022029903006484 Published online by Cambridge University Press

not a specific test for diagnosis and prognosis of mastitis under field conditions, and investigations of the value of APP analyses in mastitis diagnostics have focused on the local inflammatory process in the mammary gland and APP measurements in milk. Recently, haptoglobin and SAA were detected in milk from natural cases of acute clinical mastitis (Eckersall et al. 2001) and a mammary-associated serum amyloid A3 was identified in bovine colostrum (McDonald et al. 2001). However, there have been no reports of these, or other, APP in milk during chronic subclinical mastitis in comparison with healthy udder quarters within the same animal. Moreover, there are no detailed studies on the dynamics of systemic and local levels of haptoglobin and SAA as mastitis passes from the acute to the chronic phase.

The aim of the present study was to describe longitudinally the local and systemic events of the acute phase response that occur during acute and chronic phases of mastitis, with emphasis on how chronic mastitis affects the local and systemic acute phase responses. A model of experimentally induced *Staph. aureus* mastitis was used, in which an acute inflammation was transformed to a chronic phase using a treatment regime with a poor cure rate (Jarp et al. 1989). Changes of SAA and haptoglobin concentrations were measured in serum samples and milk samples both from infected udder quarters and from healthy udder quarters within each cow. A within-cow control was selected to determine whether changes associated with local and systemic events during mastitis in one udder quarter can be observed also in healthy quarters.

Materials and Methods

Experimental procedure

The Ethical Committee for Animal Experiments, Uppsala, Sweden, approved the experimental protocol in advance of this study. The experiment lasted 5 weeks.

Animals

Six clinically healthy dairy cows (four of the Swedish Red and White breed and two of the Swedish Holstein breed) were used. They had no history of udder diseases, and were in their second or third lactations. They were in mid-lactation with a daily production of 20–25 kg milk at the start of the experiment. Quarter milk samples were bacteriologically negative with cow-composite SCC <150 000 cells/ml before the experiment started. Cows were tethered in tie stalls, milked twice a day, at 7.30 and 16.00, and fed according to Swedish standards (Spörndly, 1995).

Infection

Each cow was inoculated once, 2 h after morning milking on day 0, with 100 000 colony-forming units (cfu) of a penicillin-sensitive (MIC50 0·06 μg/ml) *Staph. aureus* strain (SA 14391), producing both α- and β-toxins, suspended in 5 ml of phosphate-buffered saline (PBS), through the teat canal in one randomly selected udder quarter per cow. The *Staph. aureus* strain was isolated from a case of acute clinical bovine mastitis, and was provided by the Department of Mastitis and Diagnostic Products, National Veterinary Institute, Uppsala, Sweden, and prepared according to Persson et al. (1995). One randomly selected quarter per cow acted as a non-infected, healthy control. To minimize any adverse effects on animal welfare, cows were treated intramuscularly with benzyl penicillin procaine at 20 mg/kg body weight (Penovet; Boehringer Ingelheim Vetmedica) once a day for 3 d, starting 24 h after infection.

Clinical recordings

Cows were clinically examined once a day for 2 d before infection, twice a day during the first 5 d post-infection (p.i.) and thereafter once a day for the following 3 d and then twice a week throughout the study, i.e., day 2 and day 1 before infection, and at 6, 24, 32, 48, 54, 72, 80, 96, 104, 120, 128, 144, 168, 192 h p.i., and on days 12, 15, 19, 22, 26, 29, 33 and 36 p.i. All examinations were done just after the milking procedure was finished to facilitate the udder investigation and maximize detection of increases in SCC (Östensson et al. 1988). The following parameters were recorded: appetite, rectal temperature, udder consistency and milk appearance. At each occasion one of two persons scored the clinical symptoms throughout the study.

Milk samples

Stripping milk samples were aseptically collected in sterile polystyrene tubes (Nunc™ Brand Products, Nalge Nunc International, Naperville, USA) from the infected and the control quarters after morning and afternoon milking in connection with the clinical examination. For bacteriological analyses of milk samples, sampling after milking is the recommended procedure by the Department of Mastitis and Diagnostic Products, National Veterinary Institute owing to a lower risk for false positive test results. Sears et al. (1991) also report that the test specificity is better in post-milking than in pre-milking samples. Milk samples were mixed thoroughly, frozen in aliquots and stored at −20 °C until determination of APP. Fresh milk from the following time points: day 2 and day 1 before infection, and 24, 48, 72, 96, 120, 168, 192 h p.i., and on days 12, 15, 19, 22, 26, 29, 33 and 36 p.i. were analysed for bacteriological growth and SCC. Bacteriological examinations were according to standard procedures at the Department of Mastitis and Diagnostic Products in line with recommendations from the International Dairy Federation (1981). From each milk sample, 10 μl was cultivated on a blood agar plate for 24 and 48 h at 37 °C and the numbers of *Staph. aureus* cfu (αβ+) were counted. For determination

https://doi.org/10.1017/S0022029903006484 Published online by Cambridge University Press

of SCC, stripping milk samples (2 ml) were preserved with 400 µg bronopol (2-bromo-2-nitro-1,3-propanediol; Sigma-Aldrich Sweden AB, Stockholm, Sweden) per ml and analysed with a Fossomatic cell counter (Foss Electric Ltd, Hillerød, Denmark). Any clots present in the samples were carefully avoided when loading the counter.

Blood samples

Blood samples were taken from the jugular vein in Venoject® tubes (Terumo Europe N.V., Leuven, Belgium) without additives at the same time points as the stripping milk. These samples were centrifuged and the serum was stored at −20 °C until analysed for APP.

Analyses of haptoglobin and serum amyloid A (SAA) in serum and milk

The concentration of haptoglobin in serum was determined using a commercial kit based on haemoglobin-binding capacity (Phase™ Haptoglobin, Tridelta Development Ltd., Greystones, Wicklow, Ireland) on an automated biochemical analyser (Coba's Mira, Hoffman-La Roche, Basel, Switzerland). The working range of the assay was 50–6000 mg/l and the intra- and inter-assay CV was <4%. Concentrations of haptoglobin in milk were determined by immunoassay (International Patent Application Number: PCT/GB00/03925). Initially, the method by Eckersall et al. (2001) was used to measure the concentration of haptoglobin in milk, but the high limit of detection of this immunoassay meant that most samples gave a negative response. An ELISA was therefore developed and used to quantify the low haptoglobin concentrations in the milk samples taken during the experiment. Materials for the ELISA were obtained from Sigma Chemicals Ltd, Poole, UK unless otherwise stated.

Each well of microtitre plates (NUNC Brand Products, Nottingham, UK) was coated with 100 µl haemoglobin prepared according to Makimura & Suzuki (1982), diluted in bicarbonate buffer (10 mM, pH 9·6) to a final concentration of 2·5 g/l, and left to incubate at 4 °C overnight. Plates were decanted and washed three times with 50 mM-tris buffered saline pH 7·4 (TBS). Unoccupied binding sites were blocked by addition of 200 µl of 5% (w/v) dried milk diluted in TBS to each well, and incubating the plate for 1 h at 37 °C. Plates were washed three times with TBS containing 0·005% Tween 20 (TBST). Standards, controls and test samples were all diluted 1 : 20 with TBST. This dilution was used in order to balance the matrix effect of milk as a sample fluid with the sensitivity of the assay. The diluted standard, control or test samples (100 µl) were added to duplicate wells and the plate was incubated for 1 h at 37 °C. The plate was washed three times with TBST and then incubated for 1 h at 37 °C after addition to each well of 100 µl of horseradish peroxidase labelled anti-haptoglobin antibody conjugate (a gift of Tridelta Development Ltd, Ireland) diluted 1 : 400 in TBST. After incubation, the wells were washed as before, and 200 µl of tetra methyl benzidine (TMB) substrate solution (Kirkegaard & Perry Laboratories, Maryland, USA) was added to each well. The plate was incubated at room temperature for 20 min, the reaction was terminated by addition of 50 µl 2 M-H_2SO_4 to each well and the absorbance was read at 450 nm. Haptoglobin concentration in milk samples was determined by comparison of the optical density in the sample wells with those of a standard curve constructed by serially diluting a milk sample with a known amount of haptoglobin (340 mg/l), determined by immunodiffusion, to give standards after dilution at 17, 8·5, 4·25, 2·1, 1·05 and 0·5 mg/l, respectively; concentration was calculated using Revelation™ immunoassay software (Dynex Technologies Inc, Virginia, USA). The intra-assay CV was determined by calculation of the CV of duplicate pairs for each immunoassay plate according to Fraser (1986) and had a median value of 7·3% and a range of 3·4–10·1%.

The inter-assay CV was 9·8% (n=8) and 26% (n=8) at haptoglobin concentrations of 219 mg/l and 25·5 mg/l, respectively.

Concentrations of SAA in serum and milk were determined using a commercial ELISA test (Phase™ Serum Amyloid A Assay, Tridelta Development Ltd). The working range of the assay was 1·4–1200 mg/l for serum diluted 1 : 500 or 1 : 800, and 0·6–750 mg/l for milk diluted 1 : 200 or 1 : 500. The sample diluent was provided in the test kit. Intra- and inter-assay CV was <10% within these ranges.

Statistical methods

The study was divided into three periods: pre-infection (day 2 and day 1), acute phase (from day 0 up to and including day 7) and chronic phase (from day 22 p.i. and onwards). Results are presented as median and range, and/or mean and SD of the variables. The general linear model procedure with a repeated measurement approach in the SAS system (SAS Institute, Cary, NC, USA) was used to evaluate differences between the three different phases (pre-infection, acute and chronic), and between infected and control quarters within the different phases. Repeated measurement analysis was based on individual values and infected and control measurements on the same cow were analysed as dependent measures. The given detection limit of the test was used when the measured values were below the detection limit. No data transformation was necessary as the diagnostic residual analysis indicated a reasonable fit. Pre-infection haptoglobin and SAA levels were considered as the baseline value, and a cut-off value, indicating supra-normal levels in this study, was calculated from the baseline as the upper confidence limit using α=0·05.

Results

Systemic signs

All cows were clinically healthy at the start of the experiment with rectal temperatures <39 °C. Two of the cows

https://doi.org/10.1017/S0022029903006484 Published online by Cambridge University Press

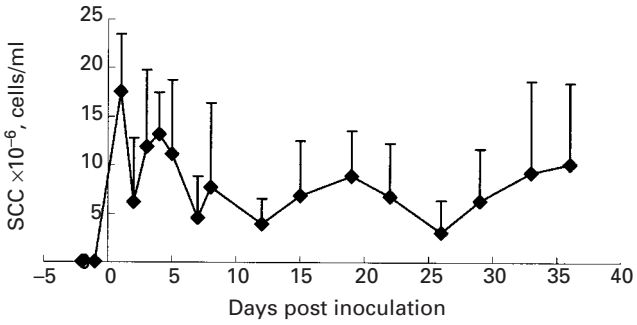

Fig. 1. Mean (±SD) somatic cell count (SCC) in milk samples from udder quarters inoculated with *Staph. aureus* at day 0 (n=6 cows).

had mild to moderate decreases in appetite at 24 h post infection (p.i.) and one of these cows remained mildly affected until day 4 p.i. At 24 h p.i. all cows had a rectal temperature >39·0 °C (39·0–41·7 °C). Maximum temperatures were observed at 24 h for 5 out of 6 cows, and at 56 h for the remaining cow. Temperatures returned to normal quickly except in one cow, which had a temperature >39 °C for 6 d. The mean±SD duration for rectal temperature >39·0 °C was 2±2 d.

Local clinical signs, milk somatic cell count and bacteriology

Before infection, the udder consistency and milk appearance were normal and no bacterial growth was found in the milk from selected udder quarters. SCC in stripping milk from the selected udder quarters ranged from 25×10^3 to 292×10^3 cells/ml (median=58×10^3 cells/ml).

Infected quarters. Acute clinical mastitis developed in all infected udder quarters. At 24 h p.i. all quarters were swollen, and they stayed swollen for on average 18±9 d. At 32 h p.i., changes in milk appearance were observed in all quarters. The changes lasted for 6±7 d, on average. From day 22 p.i. and onwards, five of six cows had chronic, subclinical mastitis. The sixth cow recovered such that SCC returned to that before infection, with no growth of *Staph. aureus* at any sampling from 4 d p.i. This cow was excluded from the investigation of the chronic phase. Another cow developed clinical mastitis on day 33 p.i. and was excluded from the study thereafter.

The dynamics of SCC in infected quarters is shown in Fig. 1. At 24 h p.i. SCC was markedly higher than pre-infection levels and stayed elevated throughout the study. During the acute phase, SCC ranged from 56×10^3 to 22×10^6 cells/ml (median=11×10^6 cells/ml) and the levels were higher than before infection (P<0·0001). During the chronic phase, SCC ranged from 607×10^3 to 26×10^6 cells/ml (median=8×10^6 cells/ml), which was higher than the pre-infection counts (P=0·0003).

At 24 and 48 h p.i., *Staph. aureus* was detected in milk from all infected udder quarters but the numbers of cfu varied. At 24 h p.i., >10 000 cfu/ml was found in 5 out of 6 quarters, while 3200 cfu/ml was found in one quarter. At 48 h p.i., ⩽100 cfu/ml was found in two quarters while the other quarters contained 100, 300, 1500 or 6000 cfu/ml. Numbers of bacteria declined further, and at day 5, 6 and 7 p.i. no growth was found in five out of six infected udder quarters. During the first 7 d (acute phase), only 52% (22/42) of the milk samples cultured positive for *Staph. aureus*. At day 8 p.i., two of the udder quarters were *Staph. aureus* positive, and from day 12 and onwards, one udder quarter (cow 240) was consistently negative while all other quarters were positive on at least four occasions each. From day 22 onwards (chronic phase), *Staph. aureus* was detected in 70% (16/23) of the milk samples collected from the five infected udder quarters.

Control quarters. Control udder quarters remained normal throughout the study, i.e., with no changes in milk appearance and udder consistency. During the study, their SCC ranged from 22×10^3 to 445×10^3 cells/ml with a median of 80×10^3 cells/ml. A few (<5) of the samples, originating from different cows, contained slightly elevated SCC (>200×10^3 cells/ml), which may have been due to a short-lasting low-grade inflammation in that quarter. Moreover, stripping milk is considered to have slightly higher SCC than fore milk (Östensson et al. 1988). No statistically significant differences were observed between pre-infection, acute and chronic phases. No specific bacterial growth was recorded in milk from control udder quarters.

Concentrations of haptoglobin and serum amyloid A in serum

Calculated cut-off values for indicating supra-normal levels were 57 mg/l and 4·1 mg/l, for haptoglobin and SAA, respectively. Haptoglobin and SAA concentrations (Fig. 2) peaked during the acute phase and, from 24 h p.i., all cows had supra-normal haptoglobin and SAA values. Highest haptoglobin and SAA concentrations were found at 72–80 h p.i. and 54–104 h p.i., respectively, and ranged from 960 to 2190 mg/l for haptoglobin, and from 487 to >1200 mg/l for SAA. During the chronic phase, all samples had supra-normal haptoglobin concentrations, while 77% of the samples had supra-normal SAA concentrations. Maximal chronic phase concentrations for haptoglobin and SAA ranged from 70 to 320 mg/l and 6·4 to 41·2 mg/l, respectively, and were randomly distributed during the chronic phase.

Descriptive statistics for haptoglobin and SAA during the different phases are shown in Table 1. Acute phase levels of both haptoglobin and SAA were higher than pre-infection (P=0·0014 and P=0·007, respectively), and higher than during the chronic phase (P=0·0003 and

https://doi.org/10.1017/S0022029903006484 Published online by Cambridge University Press

Table 1. Serum concentrations of haptoglobin and serum amyloid A before and after (acute and chronic phase) intramammary inoculation with *Staph. aureus* ($n=6$ cows)

	Haptoglobin (mg/l)				Serum amyloid A (mg/l)			
Phase	Samples detectable†	Range	Median	Mean ± SD‡	Samples detectable†	Range	Median	Mean ± SD‡
Pre-infection	9/12	<50–70	50	52·5 ± 6·2[a]	6/12	<1·4–6·4	1·7	2·82 ± 1·8[a]
Acute	78/78	50–2190	965	945·5 ± 651·1[b]	76/78	<1·4–>1200	262	376·9 ± 352·0[b]
Chronic	22/22	60–320	70	83·2 ± 54·7[a]	21/22	<1·4–41·2	7·2	11·3 ± 11·0[a]§

† Number of samples with a concentration greater than the lowest detection limit (DL) of the assay used/total number of samples; DL for haptoglobin = 50 mg/l, DL for serum amyloid A = 1·4 mg/l

‡ Values within a column without a common superscript differ significantly at $P<0·05$

§ The pre-infection and chronic phase values tended to be different ($P<0·1$)

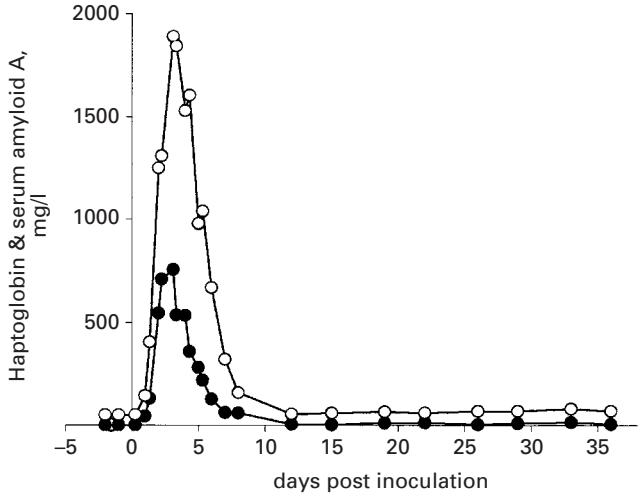

Fig. 2. Median serum concentrations of haptoglobin (–○–) and serum amyloid A (–●–). At day 0, one udder quarter per cow was inoculated with *Staph. aureus* ($n=6$ cows).

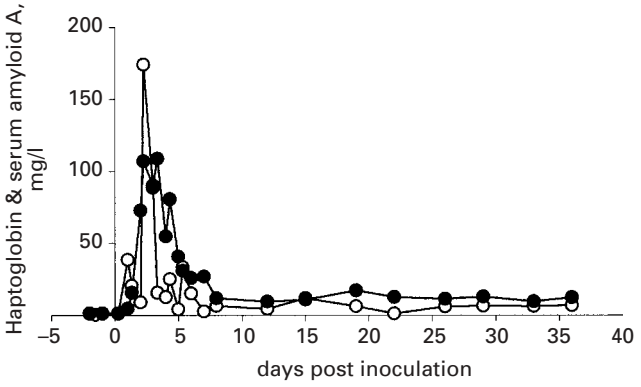

Fig. 3. Median concentrations of haptoglobin (–○–) and serum amyloid A (–●–) in milk samples from udder quarters inoculated with *Staph. aureus* at day 0 ($n=6$ cows).

$P=0·0016$, respectively). Chronic phase levels of haptoglobin were not significantly different from pre-infection, while the corresponding SAA levels tended to be higher than pre-infection ($P=0·06$).

Concentrations of haptoglobin and serum amyloid A in milk

Calculated cut-off values, indicating supra-normal levels, were 4·3 mg/l and 1·4 mg/l for haptoglobin and SAA, respectively.

Infected quarters. In milk from infected quarters (Fig. 3), haptoglobin and SAA concentrations peaked during the acute phase. At 24 h p.i., all samples contained supra-normal haptoglobin concentrations, and five out of six infected quarters had supra-normal milk SAA levels (one sample from 24 h p.i. was missing). Maximal values of haptoglobin and SAA were found 54–80 h p.i. and 48–120 h p.i., respectively. They ranged from 52 to 323 mg/l for haptoglobin and from 34 to 286 mg/l for SAA. During the chronic phase, 62% (13/21) of the samples from infected quarters had supra-normal haptoglobin concentrations, and 91% (21/23) of these samples had supra-normal SAA concentrations. Maximal concentrations of haptoglobin and SAA during this phase were 17 to 26 mg/l and 10·8 to 43 mg/l, respectively, and these values were randomly detected during the chronic phase.

Descriptive statistics for haptoglobin and SAA during the various phases are shown in Table 2. During the acute phase, haptoglobin concentrations in infected quarters tended to be higher than pre-infection ($P=0·07$) and lower than during the chronic phase ($P=0·09$), and was higher than in control samples ($P=0·0013$). During the chronic phase, haptoglobin concentrations in infected quarters were not significantly different from either pre-infection or control quarters. Acute phase SAA concentrations in infected quarters were higher than pre-infection ($P=0·006$) and chronic phase ($P=0·006$), and also higher than in control quarters ($P<0·0001$). In the chronic phase, SAA concentrations in the infected quarters were higher than pre-infection ($P=0·01$), and higher than in control quarters ($P=0·004$).

https://doi.org/10.1017/S0022029903006484 Published online by Cambridge University Press

Table 2. Haptoglobin and serum amyloid A concentrations in milk samples from infected and control udder quarters before and after (acute and chronic phase) intramammary inoculation with *Staph. aureus* ($n=6$ cows)

	Haptoglobin (mg/l)				Serum amyloid A (mg/l)			
Phase	Samples detectable†	Range	Median	Mean ± SD‡	Samples detectable†	Range	Median	Mean ± SD‡
Pre-infection	1/24	All <1·2 except one sample (22)	<1·2	2·1 ± 4·3[a]	1/24	All <1·4 except one sample (2·3)	<1·4	1·1 ± 0·5[a]
Acute								
-infected	61/74	<1·2–323	19·9	40·3 ± 62·1[a]§‖	70/76	<1·4–286·3	33·0	61·8 ± 66·3[b]‖
-control	4/75	<1·2–11	<1·2	1·4 ± 1·1[a]	10/77	<0·6–19·8	<0·6	1·0 ± 2·2[a]
Chronic								
-infected	18/21	<1·2–28	7·7	9·7 ± 9·0[a]	21/23	<1·4–43	13·1	15·2 ± 10·8[c]‖
-control	2/24	All <1·2 except two samples (2, 63)	<1·2	3·5 ± 11·9[a]	0/25	All <1·4	<1·4	0·7 ± 0·3[a]

† Number of samples with a concentration greater than the lowest detection limit (DL) of the assay used/total number of samples; DL for haptoglobin = 1·2 mg/l, DL for serum amyloid A = 0·6 mg/l or 1·4 mg/l. Differences in the number of samples are due to missing samples
‡ Values within a column without a common superscript differ significantly within quarter at $P<0·05$
§ The acute phase value tended to differ from pre-infection and chronic phase values ($P<0·1$)
‖ Infected and control udder quarters in that phase differ significantly at $P<0·05$

Control quarters. Out of 118 milk samples analysed from control udder quarters during the whole experiment (including the period between acute and chronic phases), supranormal concentrations of haptoglobin were only detected in six samples from four cows. Concentrations in these samples ranged from 8·3 to 63 mg/l (median = 18 mg/l). Elevated SAA levels were found in 7 samples (from four cows) out of 123 samples and ranged from 1·8 to 20 mg/l (median = 2·4 mg/l). No statistical differences were found in the haptoglobin or SAA concentrations in milk from control quarters for pre-infection, acute and chronic phases (Table 2).

Discussion

Milk haptoglobin and SAA were prominent and sensitive markers of acute clinical *Staph. aureus* mastitis, and supranormal milk SAA levels were also found during chronic subclinical mastitis. Moreover, the increase of these APP in milk was specific for the infected udder quarters because levels in the control quarters were unaffected or, on a few occasions, only slightly raised. Such an increase could, for example, be induced by a low-grade, short-lasting local inflammatory reaction due to mechanically induced tissue damage. The *Staph. aureus* mastitis model used in this study was successfully established. All infected quarters developed acute clinical mastitis, and most of them later progressed to chronic subclinical mastitis. The model was therefore suitable for longitudinal studies of acute and chronic inflammatory responses in serum and the mammary gland.

Longitudinal dynamics of the acute phase response were similar in milk and serum, although the relative increase was greater in serum. Pre-infection levels in milk were below the detection limit for both haptoglobin and SAA in all but one sample. For haptoglobin, this agrees with previous findings (Eckersall et al. 2001). However, the SAA assay used in that study had a lower detection limit (0·2 mg/l) than ours (0·6 mg/l), and a greater number (8/32) of SAA positive milk samples was detected among healthy cows. Several authors have stated that haptoglobin is not found in serum from healthy cattle (Spooner & Miller, 1971; Hirvonen et al. 1996; Eckersall et al. 2001) but, in the present experiment, haptoglobin was detected in 75% of the pre-infection serum samples owing to a more sensitive assay.

During acute mastitis, haptoglobin and SAA levels increased rapidly in milk from infected quarters and pronounced peaks occurred about the same time as in serum. The start of the increase coincided with the onset of clinical signs of acute mastitis and the increase in SCC. Median concentrations of APP in milk during the acute phase were higher than those described by Eckersall et al. (2001), probably owing to differences in severity of the disease. It is not clear whether APP present in milk are locally produced or whether their presence is caused by an influx from blood due to increased permeability of the blood-mammary barrier during mastitis. However, the recent discovery of a mammary-associated SAA3 expressed and secreted in colostrum (McDonald et al. 2001) supports the suggestion that they are produced locally.

Although for veterinarians and skilled stockmen, acute clinical mastitis is not difficult to diagnose, a more refined technique is needed to detect chronic subclinical mastitis. One way to monitor udder health closely, and to investigate subclinical mastitis under field conditions, would be to analyse SCC in quarter-milk samples, but that procedure

https://doi.org/10.1017/S0022029903006484 Published online by Cambridge University Press

would be expensive. Many farmers now measure SCC regularly, but this is done in composite milk, and a moderate SCC increase in one quarter may go undetected. The present results identify potential for milk SAA analyses in diagnosis of chronic subclinical *Staph. aureus* mastitis. During the chronic phase, when the cows had subclinical mastitis, haptoglobin and SAA were detected in >85% of the milk samples from infected quarters, but only the SAA levels were significantly higher than pre-infection and healthy control quarters. SCC during the chronic phase was $>600\times10^3$ cells per ml (median $=8\times10^6$ cells/ml), and further studies are needed to evaluate the milk APP response during both milder and more long-lasting cases of mastitis. Moreover, the APP response during mastitis associated with other udder pathogens should also be investigated, as well as the effect of different milk fractions and various physiological factors, such as stage of lactation.

Dramatic increases in serum concentrations of both haptoglobin and SAA were seen during the acute phase, in agreement with other studies (Conner et al. 1986; Hirvonen et al. 1996; Hirvonen et al. 1999). However, increased serum concentrations of SAA and haptoglobin are non-specific and can be due to other acute inflammatory conditions (Conner et al. 1988; Heegaard et al. 2000). Serum APP levels were much lower during the chronic phase, but supranormal haptoglobin values were found at all sampling points, and supranormal SAA levels were detected in most samples also during the chronic phase. Thus, even though the number of cows in this study was small, the results indicate that chronic subclinical mastitis gave sufficient stimulus to evoke a systemic acute phase response. Chronic inflammatory conditions have not been so extensively studied, but according to Gabay & Kushner (1999), the acute phase response is persistent in chronic diseases, and increased serum concentrations of haptoglobin and SAA have been observed during chronic conditions (e.g., Alsemgeest et al. 1994; Horadagoda et al. 1999). The acute phase response is characterized by behavioural, clinical, haematological, metabolic, neurological and immunological changes (Gruys et al. 1994) that are well studied during acute conditions. However, a biological effect of such changes could also occur during chronic diseases. For example, both subclinical and clinical mastitis can adversely affect reproductive performance (Barker et al. 1998; Schrick et al. 2001).

In conclusion, this study showed that haptoglobin and SAA in milk were sensitive markers of acute mastitis, and that milk SAA also could be used to detect subclinical chronic mastitis. At present, increased SCC is often the only characteristic of chronic subclinical mastitis. The increased use of automatic milking systems has created a need for new monitoring systems for early and accurate diagnosis of mastitis. Field studies are needed to see whether measurement of these APP in milk might meet the requirement. Moreover, for use in on-line systems, new rapid methods of analysis must be developed. Studies are also needed to determine whether APP present in mastitic milk are of systemic or local origin.

We thank Patrik Öhagen, Department of Ruminant Medicine and Veterinary Epidemiology, Swedish University of Agricultural Sciences, for statistical assistance. Financial assistance from the Swedish Council for Forestry and Agricultural Research is gratefully acknowledged.

References

Alsemgeest SP, Kalsbeek HC, Wensing T, Koeman JP, Van Ederen AM & Gruys E 1994 Concentrations of serum amyloid-A (SAA) and haptoglobin (HP) as parameters of inflammatory diseases in cattle. *Veterinary Quarterly* **16** 21–23

Barker AR, Schrick FN, Lewis MJ, Dowlen HH & Oliver SP 1998 Influence of clinical mastitis during early lactation on reproductive performance of Jersey cows. *Journal of Dairy Science* **81** 1285–1290

Conner JG, Eckersall PD, Doherty M & Douglas TA 1986 Acute phase response and mastitis in the cow. *Research in Veterinary Science* **41** 126–128

Conner JG, Eckersall PD, Wiseman A, Aitchison TC & Douglas TA 1988 Bovine acute phase response following turpentine injection. *Research in Veterinary Science* **44** 82–88

Eckersall PD, Young FJ, McComb C, Hogarth CJ, Safi S, Weber A, McDonald T, Nolan AM & Fitzpatrick JL 2001 Acute phase proteins in serum and milk from dairy cows with clinical mastitis. *Veterinary Record* **148** 35–41

Fraser CG 1986 *Interpretation of Clinical Chemistry Data*, pp. 77–81. Oxford: Blackwell Scientific Publications

Fox LK & Gay JM 1993 Contagious mastitis. *Veterinary Clinics of North America: Food Animal Practice* **9** 475–487

Gabay C & Kushner I 1999 Acute-phase proteins and other systemic responses to inflammation. *New England Journal Medicine* **340** 448–454

Gruys E, Obwolo MJ & Toussaint MJ 1994 Diagnostic significance of the major acute phase proteins in veterinary clinical chemistry: a review. *Veterinary Bulletin* **64** 1009–1018

Heegaard PM, Godson DL, Toussaint MJ, Toornehooj K, Larsen LE, Viuff B & Roonsholt L 2000 The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus. *Veterinary Immunology and Immunopathology* **77** 151–159

Hirvonen J, Pyörälä S & Jousimies-Somer H 1996 Acute phase response in heifers with experimentally induced mastitis. *Journal of Dairy Research* **63** 351–360

Hirvonen J, Eklund K, Teppo AM, Huszenicza G, Kulcsar M, Saloniemi H & Pyörälä S 1999 Acute phase response in dairy cows with experimentally induced Escherichia coli mastitis. *Acta Veterinaria Scandinavica* **40** 35–46

Horadagoda NU, Knox KM, Gibbs HA, Reid SW, Horadagoda A, Edwards SE & Eckersall PD 1999 Acute phase proteins in cattle: discrimination between acute and chronic inflammation. *Veterinary Record* **144** 437–441

International Dairy Federation 1981 Laboratory methods for use in mastitis work. Document No. 132, International Dairy Federation, Brussels, Belgium

Jarp J, Bugge HP & Larsen S 1989 Clinical trial of three therapeutic regimens for bovine mastitis. *Veterinary Record* **124** 630–634

Jonsson P & Wadström T 1993 Staphylococcus. In *Pathogenesis of Bacterial Infections in Animals*, pp. 21–35 (Eds Gyles CL & Thoen CO). Ames, Iowa: Iowa State University Press

Makimura S & Suzuki N 1982 Quantitative determination of bovine serum haptoglobin and its elevation in some inflammatory diseases. *Japanese Journal of Veterinary Science* **44** 15–21

https://doi.org/10.1017/S0022029903006484 Published online by Cambridge University Press

McDonald TL, Larson MA, Mack DR & Weber A 2001 Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrum. *Veterinary Immunology and Immunopathology* **83** 203–211

Persson K, Amolina B & Jonsson P 1995 Inflammation in the bovine teat cistern induced by *Staphylococcus aureus*. *Journal of Veterinary Medicine B* **42** 435–442

Pyörälä SH & Pyörälä EO 1998 Efficacy of parenteral administration of three antimicrobial agents in treatment of clinical mastitis in lactating cows: 487 cases (1989–1995). *Journal of the American Veterinary Medical Association* **212** 407–412

Schrick FN, Hockett ME, Saxton AM, Lewis MJ, Dowlen HH & Oliver SP 2001 Influence of subclinical mastitis during early lactation on reproductive parameters. *Journal of Dairy Science* **84** 1407–1412

Sears PM, Smith BS, English PB, Herer PS & Gonzalez RN 1990 Shedding pattern of *Staphylococcus aureus* from bovine intramammary infections. *Journal of Dairy Science* **73** 2785–2789

Sears PM, Wilson DJ, Gonzalez RN & Hancock DD 1991 Microbiological results from milk samples obtained premilking and postmilking for the diagnosis of bovine intramammary infections. *Journal of Dairy Science* **74** 4183–4188

Skinner JG, Brown RA & Roberts L 1991 Bovine haptoglobin response in clinically defined field conditions. *Veterinary Record* **128** 147–149

Sol J, Sampimon OC, Barkema HW & Schukken YH 2000 Factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus*. *Journal of Dairy Science* **83** 278–284

Spooner RL & Miller JK 1971 The measurement of haemoglobin reactive protein in ruminants as an aid to the diagnosis of acute inflammation. *Veterinary Record* **88** 2–4

Spörndly R 1995 [Feeds tables for ruminants 1995], pp. 1–97 (Ed. R Spörndly). Uppsala: Rapport 235 Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences

Östensson K, Hageltorn M & Åström G 1988 Differential cell counting in fraction-collected milk from dairy cows. *Acta Veterinaria Scandinavica* **29** 493–500

https://doi.org/10.1017/S0022029903006484 Published online by Cambridge University Press