Host response in bovine mastitis experimentally induced with

Staphylococcus chromogenes

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1. Introduction

An experimental mastitis model was developed to study host response to intramammary infection in cows caused by Staphylococcus chromogenes. CNS intramammary infections have become very common in modern dairy herds, and they can remain persistent in the mammary gland. More information would be needed about the pathophysiology of CNS mastitis, and an experimental mastitis model is a means for this research. Six primiparous Holstein–Friesian cows were challenged with S. chromogenes 4 weeks after calving. One udder quarter of each cow was inoculated with 2.1 × 10^6 cfu of S. chromogenes. All cows became infected and clinical signs were mild. Milk production of the challenged quarter decreased on average by 16.3% during 7 days post-challenge. Cows eliminated bacteria in a few days, except for one cow which developed persistent mastitis. Milk indicators of inflammation, SCC and N-acetyl-β-D-glucosaminidase (NAGase) returned to normal within a week. Milk NAGase activity increased moderately, which reflects minor tissue damage in the udder. Concentrations of serum amyloid A (SAA) and milk amyloid A (MAA) were both elevated at 12 h PC. MAA was affected by the milking times, and was at its highest before the morning milking. In our experimental model, systemic acute phase protein response with SAA occurred as an on–off type reaction. In conclusion, this experimental model could be used to study host response in CNS mastitis caused by the main CNS species and also for comparison of the host response in a mild intramammary infection and in more severe mastitis models.
milk (Oliver et al., 2003). A number of CNS species have been isolated in bovine mastitis, the most common being S. chromogenes, S. simulans and S. epidermidis (Taponen et al., 2006; Matthews et al., 1991; Aarestrup et al., 1995). Recent studies have shown some differences in the pathogenesis of mastitis caused by different CNS species (Almeida and Oliver, 2001; Zhang and Maddox, 2000).

Infection dynamics of bovine CNS mastitis have not been studied using experimental infection models. Reports on experimentally induced CNS intramammary infection in sheep are available. In sheep, experimental CNS mastitis was shown to cause mild clinical or subclinical mastitis. Infection persisted in some animals over the whole study period, which ranged from 6 days to 10 weeks after inoculation (Winter and Colditz, 2002; Winter et al., 2003; Burriel, 1997).

The aim of this pilot study was to investigate the host response in bovine intramammary infection caused by S. chromogenes, using an experimental model. Concentrations of serum amyloid A in milk and blood, as well somatic cell count (SCC), and N-acetyl-β-D-glucosaminidase (NAGase) activity in the milk were determined. Bacterial elimination rates and clinical signs were also investigated.

2. Materials and methods

2.1. Study animals

Six primiparous Holstein–Friesian cows were used as experimental animals. They were 30 months old (from 27 to 31 months) at parturition. One udder quarter of each cow was experimentally infected by S. chromogenes 4 weeks after calving. One cow was excluded from the study due to S. aureus mastitis in another quarter. The cows were kept in a tie-stall barn and fed with silage and concentrates according to Finnish feeding recommendations. At the beginning of the study all the cows were clinically healthy, except for one cow that had mild laminitis. Udder quarters of all cows had a low somatic cell count in their milk (<100,000 cells/ml) and they were free from bacterial growth in two subsequent samplings before the experimental infection. The cows did not receive any medical treatment during the study. The Ethics Committee of Helsinki University approved the study protocol.

2.2. Inoculation procedure

The S. chromogenes strain was isolated from a case of clinical mastitis in a dairy cow. The strain was identified with the API Staph ID 32 test. The strain was stored at −80 °C (Protect Bacterial Preservers®) and cultured on TSH-blood agar (bioMérieux, France) at 37 °C for 18 h. Two colonies were transferred to Müller–Hinton broth and cultured at 37 °C for 18 h. The density of the bacterial suspension was determined with a spectrophotometer (Stasar, Gilford Instrument Laboratories Inc., Ohio) at 550 nm and using application of McFarland standard (bioMérieux, France). The bacterial culture was pelleted by centrifugation and washed with phosphate buffered saline (PBS) several times. The suspension was diluted in saline to 300,000 cfu (colony forming units)/ml. The inoculate contained 2.1 × 10⁸ cfu in 7 ml of saline. The suspension was cultured on a blood agar plate in a dilution series and colonies were assessed to determine the final inoculum dose.

The infection dose used was based on a preliminary study in two cows with different doses of the same S. chromogenes strain. The aim of the study was to induce clinical mastitis. The first preliminary testing with a dose of 50,000 cfu did not provoke any clinical signs and bacteria were eliminated from the quarters within 6 h post-inoculation. In the final experiment, one udder quarter of each cow was used as the experimental quarter and another quarter as a control quarter. The quarters were infused through the teat canal within 30 min of the morning milking, using a blunt cannula. Prior to infusion, the teat end was disinfected with chlorhexidin. After the infusion, the teat was gently closed with the fingers and the inoculation dose massaged upwards.

2.3. Milk and blood samples

Milk samples were taken from the experimental and control quarters for bacteriological culturing, SCC, and determination of NAGase activity and milk amyloid A (MAA). Aseptic milk samples were collected 2 h before the challenge and then at 8, 12, 22, 30, 46, 54, 72, 78, and 96 h, and 7th and 14th day after the challenge. A volume of 100 µl milk was cultured on blood–esculin agar (TSH-agar) and several dilutions of the milk samples cultured for bacterial counting; the detection limit for bacterial growth was 10 cfu/ml. Colonies were identified as CNS using standard procedures (Hogan et al., 1999) and in unclear cases additionally with the API Staph ID 32 test.

SCC was determined by a fluoro-optical method using the Fossomatic-instrument in Valio Ltd. Laboratories, Finland. Milk samples were stored frozen at −80 °C for later determinations of milk NAGase activity and MAA. Milk NAGase activity was measured by fluorogenic method (Kitchen et al., 1978) using an in-house microplate modification developed by Mattila and Sandholm (1985). Using this method, NAGase activity of normal milk (SCC below 100,000 cells/ml) is 0.049–0.062 pmol 4-MU/min/µl of milk at 25 °C. Inter-assay and intra-assay CV for the NAGase activity were <4.8% for the high control and <6.6% for the low control.

Blood samples were collected 2 h before the challenge and at 12, 22, 30, 34, 46, 54, 72 and 96 h PC. Serum was separated and serum samples stored were frozen at −80 °C for later determination of SAA. Concentrations of SAA and MAA were determined using a commercial kit (Tridelta Development, Wicklow, Ireland). The detection limit of the kit was 0.005 mg/ml. Serum and milk samples were initially diluted 1:500 and 1:50 respectively. Dilutions 1:1000 and 1:100 were used if results were over the range of the standard curve (75 mg/l and 7.5 mg/l respectively). The inter-assay and intra-assay CV for the SAA and MAA analyses were <10% and <5%.

2.4. Clinical observations

The cows were examined clinically at every sampling. Clinical status consisted of general attitude of the cow, appetite, body temperature, rumen function, consistency
of the udder and milk appearance. Signs were divided into three groups: systemic signs, local signs and milk appearance. The scoring system was adapted from Anderson et al. (1986) with slight modifications (scoring from 1 to 3, half numbers also used). Signs were scored according to their severity (1 = no signs or changes and 3 = severe signs or changes).

2.5. Statistical methods

Descriptive statistics were performed using SPSS 13.0 software. Results are presented as mean (±S.E.M.) and median (range) of the variables. A Wilcoxon Signed Ranks test was used to test the statistical significance of the differences between the challenged and control quarters.

3. Results

3.1. Clinical signs

All cows became infected with S. chromogenes and developed mastitis. Clinical signs were mild. The body temperatures of all the cows were normal during the entire study period. One cow had poor appetite at 30 and 54 h PC (post-challenge) and her systemic signs were scored from 1.5 to 2. The other cows did not show any systemic signs (score 1). All cows had mild local signs in the challenged quarter at 12–78 h PC (scores 1.5–2); these included swelling, increased firmness and heat of the quarter. Two cows had very mild local signs (maximum score 1.5), and only some heat and slight firmness was recorded. Only one cow exhibited mild milk changes (clots, colour changes) 22 h PC.

No significant differences in the daily total milk yields during the experiment were recorded. The milk yields of the challenged quarters were slightly decreased at 30 and 46 h PC (consecutive milking times) in all cows compared with the control quarters. The milk yield ratio, calculated as the challenged quarter milk yield per the control quarter milk yield, was 1.02 before challenge. The decrease in the milk yield ratio was on average 0.17 (range 0.08–0.33).

3.2. Bacterial counts

Bacterial growth peaked in the quarters at the first sampling 8 h PC (220–19,500 cfu/ml). Bacteria were eliminated fast and at 46 h PC no bacteria were isolated from the milk samples (Fig. 1). One cow (number 11) developed persistent mastitis. After the first peak only few colonies of bacteria were isolated from this quarter, but at 7th day PC the number of bacteria increased to 1300 cfu/ml.

3.3. Indicators of inflammation in the milk

The SCC increased to >1.2 × 10^6 cells/ml in the milk of all infected quarters. The mean SCC value exceeded 0.15 × 10^6 cells/ml at 8 h PC. SCC peaked at 30 h PC (from 0.93 × 10^6 to 7.29 × 10^6 cells/ml; median 2.39 × 10^6 cells/ml) and then started to decrease to under 0.15 × 10^6 cells/ml at day 7 (Fig. 1). SCC in the infected quarter of the cow with persistent infection (11) did not rise at the time when the bacterial count started to increase again.

NAGase activity of the milk first decreased and then started to increase (Fig. 2), peaking at 22–46 h PC (0.15–0.3 pmol 4-MU/min/μl). In the cow with persistent infection, changes in the NAGase activity in the milk were similar to those in SCC. By the 7th day PC the NAGase activity had returned to the basic level in all cows (0.05–0.11 pmol 4-MU/min/μl).

MAA concentrations in the milk before challenge were under the detection limit. MAA was slightly increased at 22 h after the challenge and then increased during the next 3 days (Fig. 3). MAA peaked at 54 h PC (13.94 mg/ml). MAA fluctuated between milking times and was the highest before the morning milking. Two cows (7 and 10), had a low concentration of MAA compared with the others (maximum value 2.83 mg/l); these cows also eliminated the infection fast.

Before the challenge the serum concentration SAA was 0.3–10.1 mg/l (median 2.41 mg/l). The concentration of SAA increased in serum after the challenge, peaking at 46 h PC (Fig. 4).

Fig. 1. Mean (±S.E.M.) SCC ( ×10^6 cells/ml; ●) and mean (±S.E.M.) bacterial growth (log cfu/ml; ○) in the milk after experimental intramammary induction with S. chromogenes.
This study describes an experimental model for bovine CNS mastitis for the first time. The disease was mild, only one cow showing systemic signs. The local signs seen in the infected udder quarters were mild in all cows. All cows except one eliminated infection from the challenged quarters within the follow-up period of 14 days. The challenge dose used in the present study was high as compared with doses used to induce *S. aureus* or *E. coli* mastitis (Schukken et al., 1999; Hyvönen et al., 2006). This may cause a rapid immune response that enhances the elimination of bacteria. However, in the preliminary challenge test we failed to infect cows with lower doses of this CNS. It seems that a high dose is required to induce CNS mastitis and it may be difficult to induce clinical CNS mastitis. The number of cows included in this study was low and only one species of CNS was used, so our results should be considered as preliminary.

*S. chromogenes* was selected for the experiment because it is one of the most common species of CNS isolated from bovine mastitis. Furthermore, some in vitro studies indicated *S. chromogenes* to be more virulent than other CNS species (Zhang and Maddox, 2000). In this study *S. chromogenes* caused hardly any systemic signs, which is typical of CNS mastitis. Local signs were also mild, but some damage to the udder quarter was present as milk yield in the challenged quarters decreased on average by 16.3%. All cows spontaneously eliminated the infection, except one which developed persistent mastitis. In all three studies on experimental bovine mastitis with CNS (*S. epidermidis*), only 40% of the sheep eliminated infection (Winter and Colditz, 2002; Winter et al., 2003; Burriel, 1997). The difference may be attributable to the different animal species or differences in the virulence of the bacterial species. Winter and Colditz (2002) reported increasing content of cytokines IL-1β, IL-6 and IL-8 in milk following a *S. epidermidis* challenge, which was induced with an equal inoculum dose as in our study on dairy cows.

Somatic cells invade the milk after alarm of the immune system. SCC of the infected quarter can be relatively low in CNS mastitis compared with mastitis caused by major pathogens (Djabri et al., 2002). In the Finnish mastitis survey, SCC was >300,000 cells/ml in 18% of the quarters infected by CNS only (Pitkälä et al., 2004). In our experiment, the SCC curve was of a similar shape although much lower than in experimental studies with major pathogens (Bannerman et al., 2005). Milk SCC did not rise in the quarter with persisting infection at the time when the bacterial growth started to increase, which may indicate that the infection did not trigger any immune response.

NAGase is a lysosomal enzyme and reflects udder tissue damage due to inflammation. In our experimental model, milk NAGase activity increased only moderately, which indicated the mild nature of CNS intramammary infection. In more severe infections, such as mastitis due to *E. coli*, NAGase activity in the milk can be 9–10 times as high as in the normal milk, suggesting considerable tissue damage (Hyvönen et al., 2006).

Acute phase proteins (APP) are involved in early state response to infection. Stimulated by pro-inflammatory cytokines, SAA is excreted from the liver, but is also produced locally in the mammary epithelial cells (Weber et al., 2006). SAA has been suggested to have many immunological roles: it activates leucocytes by chemotaxis, increases phagocytosis and is able to enhance leucocyte adhesion to the endothelial cells (Suffredini et al., 1999). In this study, concentrations of SAA and MAA were both elevated at 12 h PC. MAA was affected by milking times, and was at its highest before the morning milking, reflecting the longer milking interval. This could probably be seen in this mild infection model due to the relatively low rise of MAA, but was not noticed in a more severe experimental mastitis model with *E. coli* (Jacobson et al., 2005; Hyvönen et al., 2006). The time of sampling in relation to milking should perhaps be taken into account when interpreting low MAA concentrations. Grönlund et al. (2005) for example reported great variation in MAA concentrations in spontaneous subclinical mastitis; sampling time could have some confounding effect in their study.

In our study MAA concentration continued to increase in the milk, even though bacteria had already been eliminated from the quarters. Milk SCC started to decrease before MAA, which continued to fluctuate for much longer. Compared with MAA and SAA concentrations in experimental *E. coli* mastitis determined in the same laboratory with the same assay (Hyvönen et al., 2006), MAA was 100 times lower and SAA 3 times lower in *S. chromogenes* mastitis. In two cows SAA remained at the basic level over...
the whole experimental period; one of them was the cow which developed persistent mastitis. This could be linked to the very mild local signs in the udder seen in these two cows. It is possible that local proinflammatory cytokine response in the affected quarters was not high enough to provoke systemic production of SAA. Different patterns could be recognized in systemic and local SAA response. Two cows with the highest and clearest systemic response hardly showed local MAA reaction and eliminated bacteria quickly (cow nos. 7 and 10). On the contrary, two cows (nos. 9 and 11) with no systemic reaction exhibited moderate local MAA response and the other developed persistent infection. It might indicate that quality of the inflammatory response could play an important role for the outcome of infection. The possible role of an on–off type of systemic inflammatory response is interesting and merits further study.

In conclusion, with this infection model, in vivo virulence of different CNS species and host response of the mammary gland to CNS infection could be investigated. One interesting aspect to study would be the development of persistent intramammary infection and possible host or pathogen factors involved in that. CNS mastitis model is mild and it can also be used for comparison with more severe mastitis models.

Conflict of interest

None.

References


