

Systemic and local immune response of cows to intramammary infection with *Staphylococcus aureus*

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SUMMARY

The association between *Staphylococcus aureus* chronic mammary gland infection and the resulting immune response expressed by the production of specific IgG and IgA antibodies in blood and milk was studied in Israeli Holstein cows. Specific antibodies of the IgG class were detected in sera of 82.6 per cent of the cows chronically infected by *S aureus*, while in 17.4 per cent no such antibodies could be detected. Specific IgG antibodies to *S aureus* were neither detected in sera of cows free of mammary infection nor in those infected with different coagulase-negative staphylococci (CNS) such as *S intermedius*, *S chromogenes* or *S haemolyticus*. In milk, specific IgG antibodies to *S aureus* were detected only in cows with positive serology. The end point dilutions in the milk were 5 to 30 per cent of that of blood from the same cow. No significant difference in IgG titres was found in the same cow if the quarter was infected with *S aureus* or not. Specific antibodies to *S aureus* of the IgA class could not be detected in the sera of any of the cows included in this study. In milk, a specific IgA antibody was detected only in the samples from the *S aureus* infected quarters in which *S aureus* was isolated at the time of the experiment. In the same cow, quarters infected by *S aureus* were found to have a significantly higher IgA titre ($P < 0.0001$) than that of the non-infected ones. © 2000 Harcourt Publishers Ltd

STAPHYLOCOCCUS aureus is the major udder pathogen causing subclinical, chronic udder mastitis with high economic losses to farmers. This type of mastitis impairs alveolar function, reduces milk yield and has a deleterious effect on milk composition, one of which is an increase in milk somatic cells (Gudding et al 1984, Nickerson 1989). This increase of cells, mainly polymorphonuclear neutrophils (PMNs), as well as the secretion of viable bacteria in the milk, indicate a lack of sufficient phagocytosis and/or killing of bacteria. This failure of the immune system to confront mastitic pathogens successfully may result in chronic infection. One mechanism of the immune system to increase phagocytosis and killing are specific antibodies of the appropriate immunoglobulin (Ig) in sufficient level at the site of infection. While IgM and IgG isotypes are the main Igs attributed with this mode of action, IgA isotype is not. Macrophage contains receptors for IgG₁ (Howard et al 1980, Norcross 1991) while ruminant neutrophils have surface receptors for IgG₂ (McGuire et al 1979). Immunoglobulin A is the main Ig of the seromucous tissues, defending the exposed external surfaces by blocking the adherence of microorganisms and their toxins to epithelial cells. In colostrum and in milk, secretory IgA (sIgA) is of relevance since it provides antibodies in the intestine of the newborn animal. Previous works showed that *S aureus* opsonised with sIgA purified from human breast milk (Avery and Gordon 1991) were not phagocytised by PMN to any greater extent than unopsonised bacteria. It was also showed that *Actinomyces pyogenes*

opsonised with milk whey containing specific Ig antibodies was positively correlated with titres of IgG₂ and IgM but not with IgG₁ or IgA antibodies (Watson 1989).

The enzyme-linked immunosorbent assay (ELISA) is the primary sensitive method for detecting antibodies to *S aureus* in milk (Matsushita et al 1990, Grove and Jones 1992, Hicks et al 1994). In previous studies, ELISA kit ProStaph I (ProScience Corp, Sterling, VA, USA) was used and a positive sample was determined as the optical density (OD) equal to or greater than that of a positive control. However, this method did not allow calculation of bovine skimmed milk titres containing IgA antibody.

The objective of this study was to study the association between *S aureus* chronic mammary gland infection and the production of specific IgG and IgA antibody titres in blood and milk to *S aureus*, using an ELISA.

MATERIALS AND METHODS

Animals

Second to third lactation Israeli Holstein cows in their mid-lactation stages were included in this study. Group 1 included 26 cows chronically infected with *S aureus* at least in one quarter and the other remaining quarters with different species of CNS. Group 2 was composed of 17 cows free of intramammary infection in all quarters. Group 3 included 30 cows chronically infected with different species of CNS. Udder condition at the time of the study was confirmed after three consecutive bacteriological examinations of weekly quarter milk samples. Quarters were defined as infected with *S aureus* or CNS if at least two out of the three

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bacteriological examinations were the same and no other bacteria were found. Cows were milked three times daily. Food was offered in mangers located in the herd.

Bacteriological examinations

Duplicate quarter foremilk samples were taken aseptically according to the International Dairy Federation (1985) procedures and submitted to the laboratory within 1 hour. Bacteriological analysis was performed according to accepted standards (Barnes-Pallesen et al 1987). A 0.01-ml milk sample was spread over blood-agar plates (Bacto-Agar; Difco Laboratory, Detroit, MI, USA) containing 5 per cent of sheep red blood cells. All plates were incubated at 37°C and examined for growth at 18 and 42 hours. Colonies suspected as staphylococci were tested for coagulase (tube test) (Anilab, Rehovot, Israel) and the strains were identified by ID 32 Staph (Bio Merieux S.A., Marcy-l'Etoile, France). Colonies were considered as *S aureus* if they showed coagulase activity (positive) and so were identified by the ID 32 API > 98 per cent and τ > 65 per cent. *S aureus* isolates were further phage-typed (Blair and Williams 1961), with phages issued by the International Reference Laboratory, Collindale, UK, using a modified procedure (Samra and Gadba 1993).

Immunological assay

ELISA. Ninety-six well immunoplates (Nunc, Kamstrup, Denmark) were coated overnight at 4°C with 200 μ l well⁻¹ of 5×10^8 *S aureus* killed by agitation in carbonate-bicarbonate buffer (pH 9.6). When an assay was performed, 200 μ l of 3 per cent casein was added and incubated for 40 minutes at room temperature. The plates were washed three times (5 minutes each time) with PBS (pH 7.6) containing 0.5 per cent Tween 20, dried and 100 μ l of the tested blood (1:2000) or milk (1:50) serum was added in duplicate and incubated for 1 hour at 37°C. The plates were washed and 1:1500 dilution of affinity purified goat anti-bovine IgG(γ) (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), or 1:3000 sheep anti-bovine IgG₁-heavy chain specific or sheep anti-bovine IgG₂-heavy chain specific together (Lazae Biologicals, LA, USA), or 1:1500 sheep anti-bovine IgA-alpha chain-specific (Bethyl, Montgomery, TX, USA) peroxidase conjugate, was added to each well and incubated for 1 hour at 37°C. The bound antibodies were detected by adding 100 μ l of 2,2'-azino-di (3-ethyl-benzthiazolin sulfonate) (ABTS) peroxidase substrate (Kirkegaard and Perry Laboratories). The respective plates were read in a microplate autoreader (Dynatech MR5000, Guernsey, Channel Islands, UK) at 410 nm. Samples were added to each plate, derived from positive and negative serum standards prepared respectively from hyperimmune and intramammary challenged or unimmunized, non-infected heifers. In order to modify the OD to titre, a linear regression was calculated as described (Leitner et al 1990, 1994). The sensitivity (> 90 per cent) and the specificity (> 90 per cent) of the ELISA were estimated previously to this study (data not shown). It was calculated by testing blood and milk samples from 45 cows known to be chronically infected with *S aureus*, 50 samples from cows known to be free of infection or infected chronically with other bacteria such as CNS and 10 heifers vaccinated with killed *S aureus* vaccine. Because

of the different working dilutions of the sera derived from blood (1:2000) or milk (1:50), the titre scales were different, thus the results were calculated to end point dilution.

Study layout

Three weekly consecutive bacteriological examinations in quarter milk samples were carried out in order to confirm the udders bacteriological condition. Samples of blood and quarters milk were collected from each cow. Serum and skimmed milk were separated by centrifugation (1000 g, 10 minutes, 4°C) and stored at -20°C. In addition, somatic cell count (SCC) was determined by a Coulter Counter (CC), (Z1 model, Coulter Electronics Limited, Luton, UK). Antibody titres were determined by the specific ELISA developed.

Statistical analysis

Data was analysed using the SAS general linear model (GLM) procedure (SAS/STAT® 1990). Dependent variables were blood IgG, IgG₁ and IgG₂, and milk IgG, IgG₁, IgG₂ and IgA antibodies. The effects on the dependent variables were cow, quarter and *S aureus* infection.

RESULTS

Of the 26 cows known as chronically infected by *S aureus* prior to the time of the experiment (group 1), 23 were confirmed to be bacteriologically positive while in three cows no *S aureus* was isolated. In the sera 19 out of the 23 cows (82.6 per cent), specific IgG antibodies were detected while in the other four cows no such antibodies could be found (Table 1). Specific IgG antibodies also could be detected in the remaining three cows in which *S aureus* was not isolated at the time of the experiment. No specific IgG antibodies were detected in sera of the cows free of mammary infection (group 2) or in the cows that were infected with different CNS such as: *S intermedius*, *S chromogenes* or *S haemolyticus*, but not with *S aureus* (group 3) (Table 1). IgG₁ and IgG₂ antibodies were in parallel to the total IgG, while that of the IgG₁ was somewhat lower than IgG₂ (Table 2). Specific antibodies of the IgA class could not be found in the sera of any cow participating in this study.

In milk, specific IgG antibodies to *S aureus* were detected only in cows where their sera were positive. Comparing the IgG titre in the milk between quarters infected and non-infected by *S aureus* of the same cow, quarters infected had a higher titre than that of the non-infected ones, but the differences were not significant (Table 2). The end point dilutions in the milk of the IgG were 5 to 30 per cent of that of the blood from the same cow. IgG₁ antibodies were in parallel to the total IgG while that of the IgG₂ were parallel in the quarters infected with *S aureus* but significantly lower ($P < 0.01$) in the quarters infected with the different CNS (Table 2). Specific IgA antibodies in the milk were detected only from cows in which *S aureus* was isolated at the time of the experiment (23/26 in group 1). Furthermore, it was found almost entirely in the *S aureus* infected quarters and only in three quarters where no *S aureus* were isolated, IgA specific antibodies were found with very low titre. Moreover, specific IgA antibodies were detected in two out of the four cows

TABLE 1: Distribution of cows according to mammary gland *S aureus* infection and antibody detection in blood sera and skimmed milk

Group	Cows	<i>S aureus</i>		Antibody		
		Prior*	Present†	Blood IgG	Milk IgG	Milk IgA
1	19	+	+	+	+	+
	4	+	+	-	-	+(2); -(2)
	3	+	-	+	+	-
2	17	-	-	-	-	-
3	30	-	-	-	-	-

**S aureus* was isolated from the cow udder prior to the time of the experiment.

†Quarter bacteriological classification was confirmed after three consecutive examinations of quarter milk samples, a week between each sampling, of cows known to be chronically infected by *S aureus*.

TABLE 2: Range of antibody end point dilutions of IgG, IgG₁, IgG₂ and IgA in sera of 26 cows chronically infected with *S aureus* and in their milk according to the infection agent

Cow	Blood			Quarter*	Milk			
	IgG	IgG ₁	IgG ₂		IgG	IgG ₁	IgG ₂	IgA
26	0-1:32,000	0-1:16,000	0-1:32,000	<i>S aureus</i> (41) CNS (51)	0-1:1600 0-1:800 NS	0-1:1600 0-1:800 NS	0-1:1600 0-1:400 <0.01	1:100-1:800 0-1:20 <0.0001
P								

*Quarter bacteriological classification; infected with *S aureus* or with CNS.

infected with *S aureus* in which specific IgG antibodies were not detected, either in sera or milk. In the remaining three cows in group 1 in whose milk *S aureus* was not isolated, no specific IgA antibodies were detected in any of the quarters, although specific IgG antibodies were detected (Table 1). When comparing the IgA titre in the milk between quarters infected and non-infected by *S aureus* of the same cow, quarters infected were found to have a significant higher titre ($P < 0.0001$) than that of the non-infected ones (Table 2).

DISCUSSION

Macrophage contains receptors for IgG₁ (Howard et al 1980, Norcross 1991) while ruminant neutrophils have surface receptors for IgG₂ (McGuire et al 1979).

IgG is the principal component of the mammary gland immune response, responsible for promoting phagocytosis of neutrophils (Miller et al 1988, Watson 1989, Avery and Gordon 1991). However, as found in this work, the level of the specific total IgG antibodies in the milk was low compared with that of the blood and there was no significant difference between whether the gland was infected or not by *S aureus*. IgG₁ isotype was in parallel to the total IgG in blood and in milk, while that of the IgG₂ isotype was in parallel to the total IgG in blood and milk of the infected quarters with *S aureus* but not in the ones infected with the different CNS. Antibody isotypes cannot be compared quantitatively because each isotype was tested on a different second antibody. These results strongly suggest that in subclinical chronic mastitis caused by *S aureus*, selective transport of IgG and IgG₁ into the infected gland does not occur, however, that of the IgG₂ does occur. This is in agreement with the results found after an experimental intramammary challenge with oyster glycogen (Guidry et al 1980a) or live *S aureus* (Guidry et al 1980b). In their study, the total Ig,

mainly IgG₁ and also IgG₂, IgM and IgA, increased in the milk after challenge in both infected and non-infected quarters, while bovine serum albumin and SCC increased only in the infected quarter. This indicates that the increase of Ig was due to an increased transport to both the infected as well as to the non-infected gland with *S aureus*. These results suggest that the systemic immune system is involved in the production of IgG and not the local mammary gland as reported previously (Butler 1983), and in order to have IgG isotype antibodies in milk, *S aureus* must stimulate the cows systemic immune system. IgG₁, an effective opsonin of *Mo* are selectively transported through the epithelial cells (Butler 1983, Colditz and Watson 1985), while IgG₂ gain access by a neutrophil bound to the Fc receptor (Colditz and Watson 1985). Thus, the increase in the specific IgG₂ isotype in the *S aureus* infected quarters is probably due to elevation of the neutrophil.

IgA isotype is not among the immunoglobulin classes that increase phagocytosis, as do IgG, in milk, shown by previous studies (Avery and Gordon 1991). However, as found in this study, IgA antibody was the major Ig isotype in most of the *S aureus* infected quarters with subclinical chronic mastitis but not in the non-infected ones. These results suggest that the production of this isotype is local and because IgA is not a good opsonin for phagocytosis, it may have other functions in controlling the severity of the infection such as immune exclusion, preventing the adherence of the bacteria to the epithelial cells. There is a need for better understanding of the local and systemic immune response to udder pathogens in order to minimise the damage they cause.

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