The Immune Response during Acute and Chronic Phase of Bovine Mastitis

with emphasis on *Staphylococcus aureus* infection

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Till Miriam

“Despite our monumental achievements in philosophy, technology and the arts, to bacteria humans are no more than an organic mass to be utilised for growth and reproduction.”

Sokurenko et al.

Abstract

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The aims of this thesis were to describe the innate and adapted immune response during acute and chronic phase of bovine *Staphylococcus aureus* (*S. aureus*) mastitis, and to investigate why the infection often becomes persistent. The potential of the milk acute phase proteins (APP) haptoglobin and serum amyloid A (SAA) as indicators of chronic sub-clinical mastitis was also evaluated, as well as the preventive and therapeutic effects of the immunomodulator ß1,3-glucan against intramammary *S. aureus* infection.

After intramammary inoculation of *S. aureus*, acute clinical mastitis developed and was transformed to chronic sub-clinical mastitis with controlled use of penicillin. Blood and milk samples from infected and healthy quarters were collected during five weeks, and analysed for APP and lymphocyte sub-populations. The most prominent features were increased APP concentrations in serum, and in milk from infected quarters, but not in milk from control quarters, during both acute and chronic phase of mastitis, and an increased proportion of B-lymphocytes and cellular expression of B-cell antigen in blood, infected and healthy quarters. The results indicate that both clinical and sub-clinical mastitis exert effects on local, as well as systemic, innate and adapted immune responses. The B-cell response could be one explanation why the immune system failed to eliminate the infection.

When studying naturally occurring cases of chronic sub-clinical mastitis, a large variation in expression of APP in milk, and a discrepancy between the levels of APP and adenosine triphosphate (ATP), an indirect measurement of the milk somatic cell count, was observed. In most cases, healthy cows had undetectable levels of milk APP. The results indicate that milk haptoglobin and SAA can be used as indicators of udder health.

Intramammary infusions of ß1,3-glucan failed to prevent experimental *S. aureus* infection at drying-off, and to eliminate *S. aureus* infection in cows with chronic sub-clinical mastitis. However, an immunostimulating effect was observed as the expression of MHC class II was increased on lymphocytes from *S. aureus*-infected quarters. Prevention and elimination of intramammary *S. aureus* infections using immunomodulators, like ß1,3-glucan, need further studies.

Keywords: bovine, mastitis, *Staphylococcus aureus*, immunity, acute phase response, chronic, sub-clinical, acute phase proteins, haptoglobin, serum amyloid A, lymphocyte, immunomodulation, ß1,3-glucan.

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“Att inse att man är okunnig är ett bra steg mot kunskap”

Benjamin Disraeli

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Papers I-IV

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Grönlund, U., Hultén, C., Eckersall, P.D., Hogarth, C.J. & Persson Waller, K. 2003. Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis.
Journal of Dairy Research 70, 379-386.

II. Grönlund, U, Johannisson, A. & Persson Waller, K. 2004. Changes in lymphocyte sub-populations during acute and chronic phases of *Staphylococcus aureus* induced bovine mastitis.
Manuscript submitted for publication

III. Grönlund, U, Hallén Sandgren, C. & Persson Waller, K. 2004. Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis.
Manuscript submitted for publication

IV. Persson Waller, K. Grönlund, U. &. Johannisson, A. 2003. Infusion of ß1,3-glucan for prevention and treatment of *Staphylococcus aureus* mastitis.
Journal of Veterinary Medicine, series *B 50*, 1-7.

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Abbreviations

APC	antigen-presenting cell
APP	acute phase proteins
APR	acute phase response
ATP	adenosine triphosphate
CD	cluster of differentiation
CFU	colony-forming units
CMT	California Mastitis Test
CPR	chronic phase response
CSCC	composite somatic cell count
EC	electrical conductivity
ELISA	enzyme-linked immunosorbent assay
Ig	immunoglobulin
IL	interleukin
MHC	major histocompatibility complex
NK	natural killer
NAGase	N-acetyl-ß-D-glucosaminidase
PBS	phosphate-buffered saline
pi	post-infection
SCC	milk somatic cell counts
SAA	serum amyloid A
UDS	udder disease score
WC	Workshop Cluster

Introduction

This thesis starts with a general introduction to the immune response, with focus on bovine innate and acquired immunity. For the innate immune system, the phenomena acute and chronic phase response is emphasized. Thereafter, different aspects of bovine mastitis, especially mastitis caused by *Staphylococcus aureus* (*S. aureus*) are reviewed. The development of chronic, sub-clinical mastitis caused by *S. aureus* is a fascinating phenomenon. Despite a prominent initial host response trying to eliminate the invaders, the outcome is often a balanced relationship between the host and the microbes. Attempts to eliminate and prevent intramammary infection through immunomodulation will also be describe.

General aspects on acute and chronic phase response

As a part of the innate host defence system, the acute phase response (APR) is responsible for a quick adaptation of the body to a defence situation, and prevents tissue damage, isolates and destroys the invading pathogens, and promotes tissue repair (Whicher & Westacott, 1992). The APR is a stereotyped response, which is the same regardless of the underlying cause (inflammation, trauma, infection, tumour) and comprises a cascade of events that include behavioural, haematological, metabolic, biochemical and immunological changes that is well-orchestrated by a complex array of hormones and cytokines. Macrophages are the cell type that is most commonly associated with initiation of the APR. They recognise conserved microbial structures, carbohydrates and lipids, which are shared by Gram-negative and Gram-positive bacteria, and fungi (Suffredini *et al.*, 1999; Kehrli, Jr. & Harp, 2001). Upon this recognition, macrophages become stimulated and start to produce and release cytokines that trigger the APR, and also initiate adapted immunity.

In spite of its designation as an acute phenomenon, the APR is maintained as long as the inflammatory process is active, and therefore, it may be persistent also in chronic disease (Gabay & Kushner, 1999). Bengmark (2001) describes a similar process as in APR in human patients with chronic sub-clinical diseases as chronic phase response (CPR). In CPR, the extent of the above-mentioned changes differs from APR.

Bovine acute phase proteins

During the APR, there is a dramatic effect on the function and metabolism of the liver, and a prominent feature is the shift in protein synthesis. In cattle, the pro-inflammatory cytokines*, i.e.* interleukin (IL)-6, IL-1, and tumour necrosis factor-α (TNF-α), released from activated macrophages stimulate the hepatocytes to increase their production of plasma α- and β-proteins (Godson *et al.*, 1995; Nakagawa-Tosa *et al.*, 1995; Alsemgeest *et al.*, 1996; Yoshioka *et al.*, 2002). These proteins will rapidly increase in serum and are therefore called positive acute phase proteins (APP), and will further on be mentioned only as APP. They are synthesised at the expense of other plasma proteins, like albumin, which also

are called negative APP as they decrease in serum during the APR (Conner *et al.*, 1988).

The production of APP is, like the APR in general, a non-specific response, which occurs regardless of type of stimulus, and different combinations of the pro-inflammatory cytokines induce different APP (Mackiewicz *et al.*, 1991; Baumann & Gauldie, 1994). In addition, in humans and rodents, APP can be regulated by other cytokines than the pro-inflammatory, and also by growth factors and hormones, where glucocorticosteroids have gained great interest (Shim, 1976; Waage, Slupphaug & Shalaby, 1990; Baumann & Gauldie, 1994; Murata *et al.*, 2004). Extrahepatic expression of haptoglobin is found in mice Kalmovarin *et al.* (1991) and Hiss *et al.* (2003) reported presence of haptoglobin mRNA also in the bovine mammary gland. Extrahepatic synthesis of different SAA isoforms has been demonstrated in several species (Ramadori, Sipe & Colten, 1985; Meek & Benditt, 1986; Benditt & Meek, 1989; Rygg, Husby & Marhaug, 1993), and in cattle, a specific mammary–associated isoform was found in colostrum and milk (McDonald *et al.*, 2001).

The APP constitutes of a heterogeneous group of proteins, which are species specific. In the bovine, they consist of haptoglobin, serum amyloid A (SAA), α_1-acid glycoprotein, α_1-antitrypsin, fibrinogen and ceruloplasmin (Eckersall & Conner, 1988). However, only haptoglobin and SAA are considered as major APP, which means that their concentrations increase over 100 times after stimulation, whereas healthy animals have very low levels (Conner *et al.*, 1986; Eckersall & Conner, 1988; Conner *et al.*, 1988; Gruys *et al.*, 1993; Alsemgeest *et al.*, 1994). SAA is considered the most sensitive of the two as it is detected earlier in blood than haptoglobin (Horadagoda *et al.*, 1994). With exception for ceruloplasmin, which is a minor APP, the other APP are moderate APP as they only increase two to three fold during the APR (Conner *et al.*, 1986) (Eckersall & Conner, 1988; Conner *et al.*, 1988; Skinner, Brown & Roberts, 1991; Hirvonen, Pyörälä & Jousimies-Somer, 1996).

APP are considered as mediators, inhibitors and scavengers in the inflammatory process, but there functions, especially in bovine, are not fully understood (Whicher & Westacott, 1992). Most of the research on APP functions has been done in *in vitro* systems with proteins from humans or mice. The main functions of both haptoglobin and SAA are believed to be removal of cell-derived products released from damaged tissues and macrophages (Whicher & Westacott, 1992). In addition, haptoglobin binds haemoglobin strongly, and the complex is rapidly removed from circulation by the reticuloendothelial system to conserve haemoglobin iron (Hwang & Greer, 1980). Thereby the toxic and pro-inflammatory effects of free haemoglobin are eliminated (Wagener *et al.*, 2001). Haptoglobin can also exert immunomodulatory effects, like inhibition of chemotaxis and phagocytosis of leukocytes (Rossbacher, Wagner & Pasternack, 1999). In blood, SAA is bound to lipids and form a lipoprotein complex and is involved in cholesterol metabolism by removal of cholesterol from the inflammatory site. Beside its role as a scavenger, SAA is considered to have both immunosuppressive, by inhibiting neutrophil migration and function, and

immunostimulating effects, by inducing T-lymphocyte migration (Whicher & Westacott, 1992; Xu *et al.*, 1995; Gatt *et al.*, 1998; Suffredini *et al.*, 1999).

Over the last three decades, measurement of serum APP in cattle has been evaluated as a diagnostic tool for veterinarians and the main focus has been on haptoglobin and SAA. However, the analyses are still used only in research and have not reached the clinical veterinary practice, due to time-consuming, troublesome and costly methods of analysis. In cattle, elevated levels of haptoglobin and SAA have been found in cows and calves with a variety of diseases, during both natural and experimental conditions as reviewed by Murata *et al.* (2004). Thus, these APP are considered to be valuable inflammatory markers, and an aid when distinguishing between acute and chronic inflammation (Gruys *et al.*, 1993; Alsemgeest *et al.*, 1994; Horadagoda *et al.*, 1999). However, chronic diseases have gained little attention in APP research. Elevated levels of haptoglobin and SAA have also been detected in cattle during stressful situations (Murata & Miyamoto, 1993; Alsemgeest *et al.*, 1995).

General aspects on the bovine immune system

For survival, the host has to effectively exclude invading pathogens and is therefore dependent on successful defence mechanisms that can be divided into innate and adapted immunity. The innate immunity consists of a number of mechanisms, which in a non-specific manner recognise micro-organisms. In contrast, the adapted immunity is specific, and recognises specific determinants of pathogens that leads to selective elimination. In addition, the effects of adapted immunity are augmented by repeated exposure to the same antigen.

Innate immunity

The first line of defence against invading microorganisms is the physical barriers, like skin, self-cleaning processes (*e.g.* sneezing and mucus flow), and the normal bacterial flora (Tizard, 2000). The second line of defence is the inflammatory response, which results in increased vascular permeability and increased blood flow in affected tissues followed by an accumulation of leukocytes and certain soluble factors. Neutrophils, monocytes and natural killer (NK) cells migrate to affected areas guided by chemotactic factors, like complement factor C5a and IL-8 as reviewed by Paape *et al.* (2002). Neutrophils and macrophages are the functional phagocytes of the body (Paape *et al.*, 2002), whereas NK-cells recognise cells that fail to express major histocompatibility complex (MHC) class I molecules, and lyse these cells through various killing mechanisms (Paape *et al.*, 2000). The antimicrobial factors involved in innate immunity are for example complement, antibodies, lysozyme, and transferrin, of which some are considered to be both non-specific and specific (Tizard, 2000).

Monocytes constitute 2 to 7% of the bovine blood leukocytes (Jain, 1986). When monocytes leave the circulation and enter tissues, they mature and become macrophages. Macrophages recognise bacterial compounds, as mentioned earlier, engulf the invading micro-organisms and then kill the bacteria (Suffredini *et al.*, 1999). Macrophages also play a vital role as antigen-presenting cells (APC),

where they process the engulfed bacteria and present the antigen in association with the MHC class II molecule on the cell surface to specific T-lymphocytes (Tizard, 2000). This recognition activates both T-cells and APC, and their production of cytokines is triggered. The release of different combinations of cytokines at different time points during the immune response is a very complex phenomena, and depends on many factors, such as type of antigen and type of APC (Brown, Rice-Ficht & Estes, 1998). Other cell types, like B-lymphocytes and dendritic cells, can also function as APC. In addition to the above, macrophages are scavengers by ingesting damaged cells, connective tissue matrix and apoptopic neutrophils, and thereby they encapsulate damaging chemicals and minimise the tissue damage (Sipe, 1985).

In bovine blood, 15 to 45% of the leukocytes are neutrophils (Jain, 1986). They are the first cells to migrate from blood into an inflamed area after initiation of the inflammation. The main functions of neutrophils are phagocytosis and intracellular killing of engulfed bacteria by two distinct mechanisms, the respiratory burst and digestion by lysosomal enzymes (Woessner, 1992). The most important antibacterial mechanism is the respiratory burst that is also called the myeloperoxidase-hydrogen peroxide-halide system (Klebanoff, 1970). When a foreign particle is bound to the neutrophil surface, it triggers a synthesis of hydrogen peroxide that together with halide ions under influence of myeloperoxidase form highly reactive hypohalides. These hypohalides kill bacteria by oxidising their proteins and the lysosomal enzymes enhance the effect by destroying the bacterial cell wall (Tizard, 2000). The lysosomal enzymes, such as the myeloperoxidase, are released from cytoplasmic granules into the phagosome, where the bacteria are trapped. After ingestion and release of their granular contents, the neutrophils undergo apoptosis and die (Paape *et al.*, 2002).

Adapted immunity

The third line of defence consists of the specific immune defence, *i.e.* T- and B-lymphocytes. When the naive lymphocyte for the first time encounters and recognises its specific antigen, the lymphocyte will be primed. Lymphocyte trafficking is different from that of monocytes and neutrophils. The latter cell types leave the blood and migrate to affected tissues and stay there (Kehrli, Jr. & Harp, 2001). In contrast, lymphocytes recirculate, *i.e.* they continually patrol the body for foreign antigens, in different ways depending on if they are primed or naive cells. Primed lymphocytes home back to its predilection organ, which is the tissue or the draining lymph node of that tissue where they first encountered the antigen (Mackay, 1992). They circulate from blood to the predilection organ and back to blood via lymphatic vessels and lymph nodes (Springer, 1994), but naive lymphocytes only traffic the route from blood to peripheral lymph nodes and back to blood via efferent lymph. Lymphocyte trafficking is directed by homing receptors, *i.e.* cell-surface molecules that selectively interact with molecules on endothelial cells, and these receptors may be up-regulated during inflammation (Dailey, 1998).

Depending on their T-cell receptors, T-lymphocytes can be subdivided into αβ and γδ T-lymphocytes, where both T-helper cells (CD4+) and T-

cytotoxic/suppressor cells (CD8+) express the αβ T-cell receptor. In bovine blood, the CD4+ cell is the dominating phenotype regardless of lactation stage (Yang, Mather & Rabinovsky, 1988; Shafer-Weaver, Pighetti & Sordillo, 1996). After recognition of specific antigens bound to MHC class II molecules on APC, CD4+ cells become activated and start to produce and release cytokines. In general, the prominent role of CD4+ cells is to stimulate the immune response towards specific effector mechanisms by release of different combinations of cytokines (Roitt, Brostoff & Male, 1998) T-cytotoxic cells recognise specific antigens in conjunction with MHC class I molecules on the cell surface of infected cells. Roitt, Brostoff & Male (1998) describe that T-cytotoxic cells mainly kill infected cells through induction of apoptosis or lysis. According to (Tizard, 2000) it is very difficult to distinguish T-suppressor cells from T-cytotoxic cells. They are characterised by the cytokines they secrete, and it is through these cytokines that they are considered to suppress the immune response. However, the suppressing effects of the immune system are not only attributed to CD8+ lymphocytes, but also to several other cell types (Tizard, 2000). In cattle, a suppressing function is described for CD8+ blood lymphocytes, and (Shafer-Weaver & Sordillo, 1997) reported that these cells produce cytokines associated with the suppressor phenotype, and have only limited cytotoxic activity.

In bovine blood, 10 to 15% of the leukocytes are γδT-lymphocytes, which is a high proportion compared to in humans and mice (Wyatt *et al.*, 1994; Wilson *et al.*, 1996; Pollock & Welsh, 2002). In addition, cattle have sub-populations of γδT-cells that differ in cellular phenotype and may have different tissue distributions, and the majority of γδT-cells in peripheral blood express the Workshop Cluster 1 (WC1) molecule on their surface (Pollock & Welsh, 2002). The roles of these lymphocytes are not fully understood, but in cattle, as in many other species, they migrate preferentially to epithelial surfaces and do not recirculate extensively (Mackay & Hein, 1989). In ruminants, as many as 90% of the intraepithelial T-lymphocytes carry the γδ receptor (Tizard, 2000). Studies on bovine γδT-lymphocytes suggest that they can act as APC and produce an array of cytokines, and that they are cytotoxic to infected cells, but not necessarily MHC-restricted (Pollock & Welsh, 2002). These results taken together imply a role for γδT-lymphocytes in the defence against intracellular infections.

B-lymphocytes have two important roles as they can both respond to specific antigens and act as APC (Tizard, 2000). B-cells recognise specific antigens through their surface receptors, which are antibodies, and become activated when the antigen has bound to the receptor. Upon activation, which in general requires help from T-helper cells, the B-cells multiply and differentiate into plasma cells. The primary role of plasma cells is to produce large amounts of antigen specific antibodies, which are a soluble form of the receptor molecule. In cattle, antibodies, also referred to as immunoglobulins (Ig), are divided into four classes, IgM, IgG, IgA and IgE (Tizard, 2000). IgG can be further subdivided into IgG_1, IgG_{2a} and IgG_{2b}, and in contrast to other mammalian species, IgG is the predominant Ig in bovine colostrum and milk rather than IgA (Kehrli, Jr. & Harp, 2001). The main function of Ig is to facilitate phagocytosis by acting as opsonins. Moreover, they can activate complement and mediate inflammatory reactions, prevent bacterial

colonisation, neutralize toxins, and trigger NK-cells to kill antibody-coated (Tizard, 2000).

Bovine mastitis

In dairy cows worldwide, mastitis is one of the main diseases with considerable economic consequences for the farmers due to discarded milk, lower production, increased culling rate and penalty for high milk somatic cell count (SCC) (Smith & Hogan, 2001). The cow can become affected at any time of lactation, and even get an intramammary infection before her first lactation starts. However, there are periods in the cow's life when she is more susceptible to udder infections and mastitis, *i.e.* at drying off and around calving (Oliver & Mitchell, 1983). The reasons for increased susceptibility are not fully understood, but are probably associated with changes in hormonal levels, management and feeding (Oliver & Sordillo, 1988; Mallard *et al.*, 1998; Dosogne, Massart-Leen & Burvenich, 2000).

In Sweden, the estimated prevalence of infectious mastitis among dairy cows is 33%, and the estimated yearly incidence is 68% (Swedish Dairy Association, 2003). Most of these are sub-clinical cases of mastitis, but the degree of the inflammatory response in the mammary gland can vary from acute clinical mastitis to chronic sub-clinical mastitis. In acute clinical mastitis the classical symptoms of inflammation are obvious and no diagnostic tests are needed for disease confirmation, whereas in chronic sub-clinical mastitis the only symptom is an increased SCC in the affected udder quarter.

Mastitis is often associated with bacterial infections, and the most common udder pathogen in Sweden is *S. aureus*. Accurate and rapid diagnosis of infected cows is needed for a successful control program to ensure a good udder health in a herd. In such a program, identification of herd-specific pre-disposing factors is an important step towards prevention of udder infections. Another part of the program is selective use of antibiotics. However, it would also be beneficial if it was possible to stimulate the immune system of the cow by using vaccines, or non-specific immunomodulators, to ensure a higher proportion of self-cure, reducing the use of antibiotics.

Udder pathogens

Different microbes evoke different inflammatory responses due to different virulence factors. Gram-positive bacteria, mainly staphylococci and streptococci, cause about 64% of the clinical cases of mastitis in Sweden, and among these, the dominating microbe is *S. aureus* (Ekman *et al.*, 2004). In cases of sub-clinical mastitis, *S. aureus* and coagulase negative staphylococci are the most common findings (Swedish Dairy Association, 2003).

S. aureus

Contagious udder pathogens, like *S. aureus, Streptococcus (Str.) agalactiae, Str. dysgalactiae,* and *Corynebacterium bovis,* are characterised by an ability to colonise the teat canal and adhere to epithelial cells (Davidson, 1961; Olmsted &

Norcross, 1992). For these pathogens, the udder is the primary reservoir and the disease is transmitted from infected to non-infected cows during the milking process. Therefore, to minimise the new infection rate, it is important to practise good milking hygiene, to have well-functioning milking equipment and to have a milking order, where healthy cows are milked before infected cows (Fox & Gay, 1993). However, *S. aureus* is not an obligate udder pathogen, as it can survive in skin lesions and on other body locations, such as vagina and tonsils, for several months (McDonald, 1984). *S. aureus* is therefore more difficult to control compared with *Str. agalactiae*, which is an obligate udder pathogen that has been eradicated from most Swedish and American herds (Fox & Gay, 1993; Swedish Dairy Association, 2003).

Intramammary infections with *S. aureus* often cause an acute episode of mild to moderate clinical mastitis. In many cases the infection is not successfully cured, which leads to the development of chronic sub-clinical mastitis (Anderson, 1983). The pathogenesis of chronic *S. aureus* mastitis is not completely understood, but *S. aureus* have a variety of virulence factors, which increase their ability to avoid the immune system and therefore survive in the mammary gland (Anderson, 1976; Jonsson & Wadström, 1993). These virulence factors can be divided into three categories according to their function (Dego, van Dijk & Nederbragt, 2002), *i.e.* factors that mediate adhesion of bacteria to host cells, promote tissue damage and spread of the bacteria, or protect the bacteria from the host immune system. Virulence factors are for example toxins, enzymes, surface proteins (like protein A), capsule and slime, and they make it possible for the bacteria to, for example, form and colonise micro-abscesses, where *S. aureus* are protected from neutrophil activity, and to suppress mitogenesis of lymphocytes (Anderson, 1976; Gudding, McDonald & Cheville, 1984; Craven & Anderson, 1984; Nonnecke & Harp, 1985; Park *et al.*, 1992; Dego, van Dijk & Nederbragt, 2002). In addition, *S. aureus* can survive inside neutrophils, and also invade and live inside other cells, *e.g.* mammary epithelial cells and macrophages (Craven & Anderson, 1984; Almeida *et al.*, 1996; Hébert *et al.*, 2000; Hensen *et al.*, 2000; Hensen *et al.*, 2000). Consequently, *S. aureus* can damage, inhibit and hide from the immune system, and they can also avoid the effects of antibiotics (Craven & Anderson, 1984). This results in a low bacteriological cure rate for *S. aureus* mastitis (Pyörälä, 1988; Sol *et al.*, 2000).

Acute and chronic phase response

Several authors have elucidated the APR during clinical mastitis by measuring APP in serum during experimentally induced and naturally occurring cases of bovine mastitis (Spooner & Miller, 1971; Conner *et al.*, 1986; Tamura *et al.*, 1989; Skinner, Brown & Roberts, 1991; Hirvonen, Pyörälä & Jousimies-Somer, 1996; Hirvonen *et al.*, 1999; Eckersall *et al.*, 2001; Ohtsuka *et al.*, 2001; Pedersen *et al.*, 2003). In experimental studies, *E. coli, Str. uberis,* and a mix of *Arcanobacterium pyogenes*, *Fusobacterium necrophorum* and *Peptostreptococcus indolicus* (*i.e.* summer mastitis) have been used. The serum concentrations of the following APP have been analysed during mastitis: haptoglobin, SAA, α_1-acid glycoprotein, α_1-antitrypsin, fibrinogen and ceruloplasmin.

Milk APP have met great interest in research on mastitis diagnostics. Besides albumin, a negative APP mentioned above, α_1-antitrypsin was the first APP measured in milk (Sandholm, Honkanen-Buzalski & Kangasniemi, 1984). Alfa1-antitrypsin has been measured in both clinical and sub-clinical mastitis, and is closely related to SCC (Honkanen-Buzalski, Katila & Sandholm, 1981; Sandholm, Honkanen-Buzalski & Kangasniemi, 1984; Mattila *et al.*, 1986). The analysis of α_1-antitrypsin has been automated, making herd screenings possible (Sandholm, Honkanen-Buzalski & Kangasniemi, 1984). However, in recent years analysis of haptoglobin and SAA in milk has gained more interest, because of their advantage as major APP. (Eckersall *et al.*, 2001) was the first to describe an increase in haptoglobin and SAA in milk from naturally occurring cases of clinical mastitis, and reported a high specificity (100%) and a relatively high sensitivity (86 and 93%, respectively) for the two APP. However, analyses of haptoglobin and SAA content in milk are at present, time-consuming and expensive, and in milk they can be measured by different techniques, but at the moment there are no reports on automated methods.

The increases in haptoglobin and SAA observed in milk are likely due to leakage from the blood as the permeability of the blood-milk barrier increases during mastitis (Eckersall, 2000). However, McDonald *et al.* (2001) found a mammary gland associated isoform of SAA, and Hiss *et al.* (2003) demonstrated haptoglobin mRNA in the mammary gland, which indicates a local production of APP in the udder.

Mammary gland immunity

The immunity of the mammary gland can also be divided into innate and adapted immunity. Macrophages are the dominating cell type in milk and tissue of healthy mammary glands, but there are also neutrophils, lymphocytes and epithelial cells (Lee, Wooding & Kemp, 1980; Sordillo & Nickerson, 1988; Östensson, Hageltorn & Åstrom, 1988). The SCC of milk from a healthy mammary gland is often $<10^5$ cells per ml (Sordillo, Shafer-Weaver & DeRosa, 1997), but within a few hours of bacterial intramammary infection the SCC increase to $>10^6$ cells/ml (Paape, Wergin & Guidry, 1981).

Innate immunity

The teat canal is the entrance for intramammary infections, and is equipped with a keratin layer, which has antibacterial properties and acts as a physical barrier (Craven & Williams, 1985). Together with the teat skin and the milk flow, the teat canal is the first line of the defence of the mammary gland. However, some bacteria are able to survive and colonise in the teat canal and can thus gain access to the teat cistern. There, they encounter the second line of defence comprised of leukocytes, and innate and specific soluble factors like complement, lactoferrin, lysozyme, immunoglobulins and the lactoperoxidase system (Sandholm *et al.*, 1995).

Macrophages are active phagocytic cells in the mammary gland, which eat bacteria, tissue debris and milk components (Sordillo & Nickerson, 1988). However, macrophages are believed to be of greatest importance for the innate

mammary immunity as APC (Politis *et al.*, 1992; Fitzpatrick *et al.*, 1992; Sordillo & Streicher, 2002). The macrophages in milk and mammary tissue recognise bacterial products and after internal processing, they become activated. As described earlier, activated macrophages produce and release pro-inflammatory cytokines that trigger the APR, and the adapted immunity. They also release chemokines, like IL-8, together with arachidonic acid metabolites, like leukotrienes, prostaglandins and platelet-activating factor. These factors greatly augment the local inflammatory process and together with complement components, mainly C5a, they guide the leukocytes to the affected mammary gland (Persson, Larsson & Hallen, 1993; Kehrli, Jr. & Harp, 2001; Sordillo & Streicher, 2002). During mastitis, the proportion of neutrophils increase dramatically and often constitute >90% of the cells (Paape *et al.*, 1991; Sandholm *et al.*, 1995). However, several studies indicate that milk neutrophils are less responsive to stimulating agents than blood neutrophils due to engulfment of milk fat and casein (Paape *et al.*, 2002).

Adapted immunity

In both udder tissues and milk of healthy mammary glands, the CD8+ T-lymphocyte is the predominant lymphocyte phenotype resulting in a CD4:CD8 ratio <1, in contrast to in blood where the ratio is >1 (Park *et al.*, 1992; Taylor *et al.*, 1994). The CD4:CD8 ratio in mammary secretions shifts to >1 at drying-off, stays >1 during the dry period and shifts back to <1 just before parturition (Asai *et al.*, 1998). From the above follows that lymphocyte trafficking of the healthy mammary gland is selective. However, the functional significance of the CD8 dominance is not fully understood. One hypothesis is that mammary CD8+ T-cells is an activated cell type, different from blood CD8+ T-cells, with a potential role in maintaining the integrity of epithelial linings by removal of damaged or infected cells (Taylor *et al.*, 1994; Asai *et al.*, 1998). In addition, Park *et al.* (1993) demonstrated that activated CD8+ lymphocytes from infected mammary glands had a suppressing effect on the proliferative response of CD4+ cells. However, during mastitis, the lymphocyte trafficking is affected, as CD4+ T-cells become the dominating sub-population during both acute and chronic *S. aureus* mastitis (Taylor *et al.*, 1997; Soltys & Quinn, 1999; Rivas *et al.*, 2000; Riollet, Rainard & Poutrel, 2001). In addition, the proportion of B-cells increases during *S. aureus* mastitis (Nickerson & Heald, 1982; Riollet, Rainard & Poutrel, 2001).

Bovine milk contains a lower number of WC1+ γδ T-lymphocytes than peripheral blood (Taylor *et al.*, 1994), and these cells are not recruited to milk during chronic *S. aureus* mastitis (Riollet, Rainard & Poutrel, 2001). This would be in accordance with their preferential migration to epithelial surfaces and an immunological role in mammary tissue but not in milk.

Mastitis diagnostics

As cows infected with contagious bacteria are the main source of infection, these cows have to be identified to stop the spread of infections in a herd. Clinical mastitis is easy to detect for veterinarians and trained dairy personnel, but detection of sub-clinical cases of mastitis can be a challenge. Diagnostic methods

are focused on detection of inflammatory products, mainly SCC, and bacterial growth. At present, most of these tests are analysed at laboratories. However, with increasing herd size and more automated milking systems there is a need for on-line and large-scale analyses for mastitis detection.

SCC and bacterial examinations

According to recommendations by the International Dairy Federation (International Dairy Federation, 1971), milk SCC and bacteriological examination are the parameters that define if an udder quarter should be regarded as inflamed/infected or not. The threshold for SCC has changed throughout the years and is still under debate. (Hamann, 2002) suggested that a SCC of 100 000 cells/ml is the physiological limit in an udder quarter, as the levels of important milk components in milk differ significantly from the physiological norm with higher SCC. However, already at 20 000 to 30 000 cells/ml the curves of milk components start to diverge from the physiological. The standard method for SCC measurement is to use an electro-optical cell counter at a laboratory. However, a cheaper SCC-related cow-side test is the California Mastitis Test (CMT).

Chronically infected quarters are often under-diagnosed if a bacteriological criterion is used, especially if the diagnosis is based on one sample. To increase efficiency, *i.e.* sensitivity, in finding udder infections, at least two consecutive samplings should be done, and to be more cost-efficient the bacterial examinations should be preceded by CMT (Pyörälä, 1988; Sears *et al.*, 1990; Hallén Sandgren, 2001). A way to combine SCC measurement with bacterial examinations, is to use the Mastistrip™ cassette (Nilsson, Holmberg & Funke, 1989). Here, milk SCC is estimated by measuring the concentration of adenosine triphosphate (ATP) (Olsson *et al.*, 1986).

Changes in milk composition as indicators of mastitis

Mammary tissue damage caused by mediators, like TNF-α, released from macrophages, and potent oxidants released during neutrophil phagocytosis will lead to impaired functions of mammary cells, and development of fibrosis with reduced milk production as a consequence (Kehrli, Jr. & Harp, 2001). Through the impaired functions of the mammary gland, the composition of milk changes during mastitis. The milk secretory cells produce less milk protein, α-lactalbumin and β-lactoglobulin, and also less lactose (Sandholm *et al.*, 1995). In healthy udders the lactose content in milk is very stable, but the concentration decreases during mastitis (Berglund *et al.*, 2003). A large-scale method for detection of lactose is available, and lactose may be a reliable indicator of mastitis (Hamann, 2002; Berglund *et al.*, 2003).

Due to increased permeability of the blood-milk barrier during mastitis, an enrichment of sodium and chloride ions occurs, as well as a decrease in the concentrations of calcium, phosphorus, magnesium, potassium and vitamins. Changes in the concentrations of sodium and chloride ions can measured using electrical conductivity (EC) (Kitchen, 1981). The analysis is automated, and can be installed on-line in the milking equipment to monitor udder health, but hand-held meters of EC are also available (Pyörälä, 2003). However, several reports

indicate that EC is no good indicator of sub-clinical mastitis, and some studies demonstrate that EC does not detect clinical mastitis well either (Pyörälä, 2003). Today, EC is the most commonly used diagnostic tools in automatic milking systems, and is often used together with changes in the flow, yield, temperature, colour and/or homogeneity of the milk, which can increase the predictive value of EC (Knappstein, Reichmuth, & Suhren, 2002).

In addition, the enzymatic and biochemical activity increases in mastitic milk (Sandholm *et al.*, 1995), and changes in various enzymes and other proteins can also be used in mastitis diagnostics. APP as indicators of mastitis have already been described in an earlier section. Due to increased permeability of blood-milk barrier during mastitis, albumin increase in milk (Giesecke & Viljoen, 1974; Honkanen-Buzalski & Sandholm, 1981). However, Emanuelson *et al.* (1987) stated that albumin has a low predictive value for mastitis detection. Enzymes derived from tissue damage and neutrophil phagocytosis increase during mastitis. Among those, N-acetyl-ß-D-glucosaminidase (NAGase) is the most commonly analysed (Kitchen, Middleton & Salmon, 1978). Although it has a good predictive value in mastitis detection and the assay has potential for herd monitoring, there is no commercially available analysis at present (Mattila, Pyorala & Sandholm, 1986; Emanuelson *et al.*, 1987; Pyörälä & Pyörälä, 1997; Pyörälä, 2003).

Immunomodulation

Large efforts have been made in mastitis research to modulate the defence mechanisms of the udder in order to decrease the susceptibility to intramammary infections and to stimulate the immune system to eliminate existing infections. Substances used for modulation are called immunomodulators and exert their effects on innate and/or adapted immunity.

Much of the focus has been on enhancing neutrophil numbers or functions. Early studies showed that intramammary devices can increase SCC, but the results varied (Kehrli, Jr. & Harp, 2001). In addition, cytokines, like granulocyte colony-stimulating factor, interferon (IFN) -γ and IL-2, have been beneficial in the cure of acute clinical mastitis, but has no effect on chronic sub-clinical mastitis as reviewed by Kehrli, Jr. & Harp (2001). Inactivated viruses, such as *Parapox ovis*, increase the IFN-γ production, and treatment with this virus has been reported to reduce the number of *S. aureus* infections (Zecconi *et al.*, 1999). In addition, biological compounds, such as ß1,3-glucan, a yeast component, and ginseng, exert their effects on both innate and adapted immunity. Intramammary infusion of dry cows with ß1,3-glucan resulted in an increased number of neutrophils and macrophages, an increased proportion of CD14+ and MHC class II+ leukocytes, and CD4+ lymphocytes, as well as in increased concentrations of IgG_1 and IgG_2 in mammary secretions (Inchaisri, Waller & Johannisson, 2000). Ginseng treatment of cows with sub-clinical *S. aureus* mastitis gave increased numbers of monocytes and blood lymphocytes together with increased phagocytic capacity of blood neutrophils (Hu *et al.*, 2001).

Vaccination is the most common way to modulate the adapted immune system, in order to recruit and activate T- and B-cells towards a specific antigen and thereby promote antibody production. Main research in this area has focused on

development of vaccines against *S. aureus, E. coli* and *Str. uberis* infections (Kehrli, Jr. & Harp, 2001). Unfortunately, results from field trials are contradictory and few vaccines give financial return (Yancey, Jr., 1999).

Aims

The overall aim of the present study was to characterise innate and adapted immune response during acute and chronic phase of bovine mastitis with emphasis on systemic and local changes during *S. aureus* infection. The specific aims were to:

- longitudinally describe changes in concentrations of haptoglobin and SAA in milk and blood as an acute clinical *S. aureus* mastitis is transformed into chronic sub-clinical mastitis during controlled conditions.

- longitudinally describe changes in proportions of certain lymphocyte sub-populations and cellular expressions of these lymphocyte surface antigens during the same conditions as stated above, and thereby be able to clarify some aspects of the development of chronic *S. aureus* mastitis.

- evaluate the reactions in a non-infected mammary gland within a cow with experimentally induced *S. aureus* mastitis with respect to changes in haptoglobin, SAA and lymphocyte sub-populations in milk.

- study the concentrations of haptoglobin and SAA in milk from cows with naturally occurring chronic sub-clinical mastitis, to further evaluate the usefulness of these APP in mastitis diagnostics.

- investigate if the immunomodulator ß1,3-glucan can prevent udder infection with *S. aureus* at drying-off, and stimulate elimination of the infection in cows with chronic sub-clinical *S. aureus* mastitis.

- evaluate the influence of ß1,3-glucan on the expression of MHC class II on bovine milk leukocytes.

Material and methods

Material and methods used in the present study are described in detail in Papers I-IV. Here, only general comments of a conceptual nature are provided.

Animals

In Papers I and II, six dairy cows were used. They were non-pregnant, and in mid-lactation with no history of udder disease and a composite SCC (CSCC) <150 000 cells/ml at the start of the experiment. Four of these cows were subsequently used in Paper IV (experiment 2). In addition, four late lactation dairy cows with no history of udder diseases and a low CSCC were included in Paper IV (experiment 1). In Paper III, cows were selected according to their udder disease score (UDS). The UDS is based on the monthly-recorded CSCC, and indicates the duration and degree of inflammation in the udder. The UDS has a 10-grade scale, where 0 is a healthy udder. In Paper III, 41 cows with UDS>5 and eleven cows with UDS=0 were selected.

In all studies, cows from both of the two most common Swedish dairy breeds, Swedish Red and White, and Swedish Holstein, were used.

The mastitis model (Papers I and II)

An important part of the experimental mastitis study was to achieve an acute clinical mastitis that was transformed to chronic sub-clinical mastitis, using an antibiotic treatment regime with a poor cure rate. This was achieved by intramammary infusion of 100 000 CFU of a penicillin-sensitive *S. aureus* strain in one udder quarter per cow 2 h after morning milking on day 0. Another randomly selected quarter per cow acted as a non-infected, healthy control. Blood samples and milk samples from infected and control udder quarters were collected at selected time points for five weeks. Milk samples were analysed for bacteriology and total SCC. In addition, serum and milk samples were analysed for the concentrations of haptoglobin and SAA, and blood and milk leukocytes were analysed for the expression of lymphocyte antigens.

Experimental designs (Papers III and IV)

In Paper III, milk samples were collected, using both test tubes and Mastistrip^{TM}, from all udder quarters of each cow. For practical reasons, the cows were only sampled once. Composite cow milk samples were produced by pooling milk from each udder quarter.

In experiment 2 of Paper IV, one randomly selected quarter per cow was infused with ß1,3-glucan immediately after the last milking at drying-off and a second time 14 days later. Forty-eight hours after the first infusion, the glucan-infused udder quarters and the contralateral quarters of each cow were infused with *S. aureu*s. The same strain was used as in Papers I and II, but the dose was only 200 CFU. A third udder quarter acted as untreated, healthy control. Milk samples were taken twice before drying-off and once weekly during six weeks after drying-off.

The samples were analysed for bacteriology, total and differential SCC and expression of MHC class II on milk leukocytes.

In experiment 2 of Paper IV, *S. aureus* infected udder quarters were treated twice, with an interval of 72 h, with intramammary infusions of ß1,3-glucan. One randomly selected quarter per cow acted as non-infected, healthy control. Milk samples were taken from infected and control quarters before and after infusions. The samples were analysed for bacteriology, total SCC and expression of MHC class II on milk leukocytes.

Analyses

Cell counts in milk and blood

In Papers I, II and IV, the milk SCC was analysed by automatic cell counter, whereas indirect calculation of SCC using ATP was used in Paper III. Milk cells were differentiated into macrophages, lymphocytes and neutrophils by using light microscopy after preparation and staining of cytospots (Paper IV). In blood, total and differential cell counts (Paper II) were analysed by an automated blood analyser.

Flow cytometry was used to further differentiate blood and milk leukocytes. Blood and milk lymphocytes were labelled with antibodies to WC1, CD4, CD8, B-cells and IL-2R antigens. In Paper IV, milk leukocytes were stained with antibodies to MHC class II molecules.

Bacterial growth in milk samples

Milk for bacteriological analysis was collected in test tubes (Papers I, II and IV) or using Mastistrip^{TM} (Paper III) and analysed according to accredited methods.

Haptoglobin and SAA in milk and serum

In Papers I and III, the analyses of milk haptoglobin and SAA differed slightly between studies. In Paper I, an ELISA for measuring haptoglobin in milk was developed to decrease the detection limit compared to the immunoassay that had been used before. This assay was later made commercially available as a test kit, which was used for analyses in Paper III. The serum concentration of haptoglobin was determined using a kit based on the haemoglobin-binding capacity of haptoglobin (Paper I). In both Papers I and III, the same ELISA kit was used to measure the SAA levels in both milk and serum, but extra data points were added to the standard curve. The number of data points and the dilutions used differed slightly between the two studies, explaining the differences in detection limits.

Statistics

In Papers I and II, the study period was divided into three periods: pre-infection (days -2 and -1), acute phase (from day 0 up to and including day 7 pi) and chronic phase (from day 22 pi and onwards). The general linear model procedure with a repeated measurement approach was used to evaluate differences between the three phases, and between infected and control quarters in each phase.

In Paper III, the Mann-Whitney test was used to calculate differences in haptoglobin and SAA levels, and the Spearman's rank correlation test was used to test for associations between haptoglobin, SAA and ATP in an udder quarter.

In Paper IV, the effects of treatment and time were evaluated using repeated measures ANOVA. Differences between time points within and between groups were calculated using t-tests.

A p-value <0.05 was considered significant in all studies, and all results presented below are significant, if nothing else is stated.

Results

Experimentally induced *S. aureus* mastitis (Papers I and II)

Acute clinical mastitis developed in all infected udder quarters, and after three weeks pi, five of six cows had chronic sub-clinical mastitis.

Systemic and local signs

The cows were mildly affected for some days with increased body temperature and slightly reduced appetite. All infected udder quarters developed symptoms of mastitis *i.e.* swelling, changes in milk appearance and udder consistency together with increased SCC. *S. aureus* was detected in milk from all infected udder quarters throughout the study. The control udder quarters remained normal during the study period.

Concentrations of haptoglobin and SAA in serum (Paper I)

In acute phase, haptoglobin and SAA levels were higher than pre-infection and chronic phase levels. However, in chronic phase, only the SAA concentration tended to be higher than pre-infection.

Concentrations of haptoglobin and SAA in milk (Paper I)

In acute phase, the haptoglobin concentration tended to be higher in infected quarters than pre-infection, and was higher than in control samples. In infected quarters, both acute and chronic phase SAA levels were higher than pre-infection, and also higher than in control quarters. No differences were observed in the haptoglobin or SAA concentrations in milk from control quarters between pre-infection, acute and chronic phases.

Total and differential cell counts in blood (Paper II)

In acute phase, the total leukocyte count was lower compared to pre-infection and chronic phase, mainly due to a decreased number of lymphocytes. The proportion of CD4+ lymphocytes was lowered in chronic phase, while the proportion of CD8+ lymphocytes increased after infection. In chronic phase, the proportion of B-lymphocytes tended to be higher than pre-infection. In addition, the cellular expression of CD4, CD8, WC1, B-cell antigen and IL-2R was higher during chronic phase than pre-infection and acute phase.

Differential cell counts in milk (Paper II)

In infected udder quarters, the proportion of CD4+ lymphocytes declined with time, but no change was observed in control quarters. However, in chronic phase the cellular expression increased in control quarters and was higher than in infected quarters. In infected quarters, the proportion of CD8+ lymphocytes decreased after infection, but there was no change in cellular expression. However, in control quarters, the CD8 expression per cell increased in chronic phase. Changes in proportions of CD4+ and CD8+ cells in infected quarters

resulted in an increased CD4:CD8 ratio in acute phase. No differences were observed in the proportion of WC1+ lymphocytes, or in their fluorescence intensity, between phases, or between infected and control quarters.

The proportion of B-cells increased with time in infected quarters and increased during chronic phase also in control quarters. The expression per cell also increased in chronic phase in both infected and control quarters. Values for expression and proportions were consistently higher in infected than in control quarters.

In both infected and control quarters, the proportion of IL-2R+ lymphocytes increased during acute phase, while the IL-2R expression per cell increased during chronic phase. There were no differences between infected and control quarters neither in proportion nor in expression of IL-2R.

Naturally occurring cases of sub-clinical mastitis (Paper III)

The control cows had ATP levels below $2x10^{-10}$ mol/ml, were bacteriologically negative, and had very few samples with detectable levels of haptoglobin (Hp) and SAA.

One hundred and forty three of the 164 udder quarter samples from cows with chronic sub-clinical mastitis had an ATP content $>2\ x10^{-10}$ mol/ml, and 98 of the 164 samples had detectable levels of one or both APP. Udder pathogens were found in nearly half of the cows. Penicillinase negative *S. aureus* were the most frequently found bacteria.

The samples from cows with chronic sub-clinical mastitis were grouped according to their ATP, haptoglobin and SAA statuses. ATP+ samples had an ATP content $>2\ x10^{-10}$ mol/ml, while Hp+ and SAA+ samples had detectable levels of haptoglobin and SAA, respectively. Out of 164 samples, 66 belonged to the ATP+Hp-SAA- group while 44 samples were ATP+Hp-SAA-. Significant correlations were observed between all three inflammatory markers.

One or both of the APP were detected in 34 of 41 (83%) composite milk samples, and haptoglobin was the most frequently found of the two. In order to find detectable levels of APP in the composite samples, more than one quarter per cow had to have a detectable content of APP.

Immunomodulation with ß1,3-glucan (Paper IV)

An infusion of ß1,3-glucan before *S. aureus* inoculation at drying off gave a slight numerical reduction of clinical symptoms and bacterial numbers, but ß1,3-glucan infusions had no therapeutic effect on udder quarters with chronic sub-clinical *S. aureus* mastitis. However, the proportion of MHC class II positive milk lymphocytes tended to increase after ß1,3-glucan infusion in those quarters.

General discussion

This thesis is mainly focused on mastitis caused by *S. aureus* infections. One reason for this is that this bacteria is the most common udder pathogen in Sweden. However, the main explanation is the fascinating development of persistent cases of mastitis associated with *S. aureus* infections. To be able to study this development, a mastitis model was created.

Methodological considerations

The mastitis model

The model, *i.e.* transforming experimentally induced acute clinical *S. aureus* mastitis into chronic sub-clinical mastitis by controlled use of antibiotics, was successful! All cows got clinical mastitis and five of them developed chronic sub-clinical mastitis. The model made it possible to longitudinally study acute and chronic inflammatory responses in serum, and in infected as well as healthy udder quarters. The results were divided into three periods because the focus of interest was the different stages of the pathogenesis of *S. aureus* mastitis. The time limits for acute, <8 days pi, and chronic, >21 days pi, were based on theory and experience, and these phases corresponded well to clinical signs. As mentioned, this model also made it possible to study the influence on the non-infected mammary gland in a cow with mastitis. It was important that these udder quarters stayed healthy throughout the experiment, and also regarding this, the model was successful.

Experimental designs

The main drawbacks of the experimental designs used in Papers I, II and IV were the small number of cows, the relatively short study periods, and the use of only one dose and one strain of *S. aureus*. This was mainly due to economical and practical reasons. The results gained from these studies are therefore representative only for this bacterial species and strain. In addition, because of the impact from hormones on immune response, and vice versa (Morale *et al.*, 2003), conclusions from these studies should only be applied to non-pregnant cows. Similar experiments with pregnant cows and also with other bacterial species and strains are needed.

In addition, the blood and milk sampling frequency and intervals were less than optimal, but were selected mainly due to practical reasons. For example, the sample handling and analyses of differential cell counts using flow cytometric analysis is time consuming and the number of sampling occasions were therefore limited. Interpretation of the milk lymphocyte results in Paper II would have been easier if it had been possible to calculate the total and differential cell counts in the residual milk. Initially, the intention was to evaluate the differential cell counts using milk cytospots for manual counting of cell types in the microscope. Unfortunately, the differentiation between cells using this technique was very difficult. Therefore, the results were too uncertain and had to be redrawn from further analyses.

Acute and chronic phase response

During the acute phase of *S. aureus* infection both haptoglobin and SAA concentrations increased markedly and simultaneously in milk and serum, and they also declined in a similar manner. However, during this period, higher SAA levels were found in serum than in milk, while a shift occurred in chronic phase with higher concentrations in milk. In contrast, serum haptoglobin concentrations were higher in serum than in milk throughout the study. A similar pattern was described by Winter *et al.* (2003), who found that SAA contents were back to normal in serum a week after intramammary infection, but were still slightly elevated in milk from infected quarters. Maybe this is caused by initiation of local synthesis of SAA after a certain time of infectious stimulus?

Haptoglobin and SAA concentrations did not increase as rapidly after the intramammary infection as shown by Pedersen *et al.* (2003). At the first sampling occasion, *i.e.* 6 h after experimental *S. aureus* infection, the concentrations of neither haptoglobin nor SAA had increased. The APP were not detected in serum and milk until 24 h pi, which is late compared to what was reported by (Pedersen *et al.*, 2003). In that study, they observed a rise in milk and serum SAA at 6 and 11 h after inoculation, respectively. However, increased milk haptoglobin was not detected until 10 h after challenge, and no rise in serum haptoglobin was demonstrated. It is likely that additional samplings between 6 and 24 h pi in the Paper I study would have given a better agreement between the studies.

The APR and CPR, together with neutrophil recruitment, are parts of the innate immune response. In Paper I, a parallel increase in SCC and SAA was observed during acute clinical mastitis. This is in accordance with the development of clinical *S. uberis* mastitis in dairy cows (Pedersen *et al.*, 2003), and sub-clinical *S. epidermidis* mastitis in ewes (Winter *et al.*, 2003). However, during CPR, as measured in Paper III, there is a discrepancy between ATP, as indicator of SCC, and APP contents. As many as 25% of the udder quarters from cows with sub-clinical mastitis were considered diseased using ATP, but not using APP (ATP+Hp-SAA-). One explanation for the difference between acute and chronic phase could be that the APP production is down-regulated during CPR compared to in APR, due to down-regulation of pro-inflammatory cytokines. The recruitment of leukocytes to the udder during chronic phase might be due to chemotactic stimulation via other inflammatory mediators that do not have an impact on APP production. The ATP content in the ATP+Hp+SAA+ group was about three times higher than in the ATP+Hp-SAA-group, and most of the bacteriologically positive quarters belonged to the ATP+Hp+SAA+ group. It may be speculated that the inflammation in the ATP+Hp-SAA- group is a self-going process without involvement of bacteria, and that this inflammation does not trigger production of APP. (Hallén Sandgren, 1991) speculated that neutrophils that arrive to an inflamed area become activated and release their granular compounds, but are non-responsive to down-regulating signals, initiating a vicious circle. Another hypothesis explaining the large proportion of udder quarters belonging to the ATP+Hp-SAA- group, is that all four quarters within the udder

are affected by a general recruitment and influx of leukocytes. This is supported by the fact that ATP+Hp-SAA- quarters were always found in udders where at least one more quarter was ATP+ and Hp+ and/or SAA+. Different mechanisms of passage into the udder could be another explanation to the discrepancy between ATP and APP. As the migration of leukocytes is an active process, and APP passively leak from blood into milk, the latter might be more effective when the inflammations wanes off and the immune response is down-regulated during persistent disease (Rhen *et al.*, 2003). The leakage of APP is due to increased permeability in the inflamed mammary gland (Eckersall *et al.*, 2001). However, local production of SAA, and probably also of haptoglobin, as a source of milk APP must also be considered (McDonald *et al.*, 2001; Hiss *et al.*, 2003). To further understand the relationship between SCC and APP, it would be beneficial if the local production of APP could be measured. However, at present, it is not possible to determine the origin of SAA and haptoglobin found in milk.

As mentioned earlier, the combination of cytokines produced influences the synthesis of APP (Mackiewicz *et al.*, 1991; Baumann & Gauldie, 1994; Alsemgeest *et al.*, 1996). Baumann & Gauldie (1994) describe regulation of haptoglobin in humans as IL-6 specific, whereas human SAA is related to IL-1 and TNF-α. If this is the case also in cattle needs further investigation. The type and strain of bacteria, amount of bacteria, as well as the duration of disease, can probably have an impact on the cytokine production due to the presence of different virulence factors. As an example, the onset of SAA production and the peak concentration occurred earlier after experimental infection with *S. uberis* and *S. epidermidis* compared to after *S. aureus* infection (Paper I). In addition, in Paper I SAA was more often detected in milk from cows with chronic sub-clinical mastitis, while in Paper III haptoglobin was more frequently found. However, the number of cases of mastitis in Paper III was too small for statistical comparisons between APP levels in different types of bacterial infections. The role of APP in the pathogenesis of mastitis must also be considered. Maybe different APP are needed in different phases of the inflammatory process? However, to my knowledge, there are no reports on functions of haptoglobin and SAA in cattle.

Eckersall *et al.* (2001) and the present study show that the levels of APP were low or undetectable in serum and milk from healthy animals. It was also clear that the increase in milk APP is specific for infected glands, which is in line with earlier studies on naturally occurring mastitis (Eckersall *et al.*, 2001). The results in Paper III, show that haptoglobin and SAA levels above DL, and ATP activity $>2 \times 10^{-10}$ mol/ml could be considered as an indication of an unhealthy udder quarter. Using these criteria, only 12% of the udder quarters from cows with chronic sub-clinical mastitis were healthy. Also in CMT negative quarters from cows with chronic mastitis, the APP content was higher than in healthy cows. Thus, it seems like the CPR affects all four quarters of cows with chronic sub-clinical mastitis.

Lymphocyte trafficking

Via its many virulence factors, *S. aureus* has many possibilities to evade the immune defence, resulting in persistent infections. One important factor, which also occurs in other pathogens, is its ability to hide inside cells (Rhen *et al.*, 2003). Persistent infections are often associated with an impairment of the immune response, due to factors related both to the bacterium and the host. In Paper II, the host's adapted immune response was monitored when acute clinical mastitis turned into chronic sub-clinical disease.

In general, changes in proportions of lymphocyte sub-populations were mostly observed in infected quarters during acute phase. In contrast, increased cellular expression of surface antigens was the dominating finding during chronic phase. Interestingly, an increased proportion of B-cells was found in both infected and control udder quarters, as well as in blood.

The mastitis model made it possible to longitudinally monitor the dynamics of lymphocyte sub-populations after *S. aureus* infection. Unfortunately, only data regarding relative proportions of these cell types were available in milk, as it was not possible to study the differential cell count. However, the milk lymphocyte population is distinct from neutrophils in the scatter plot of the flow cytometry analyses. Therefore, the changes in proportions of milk lymphocytes were not affected by the massive influx of neutrophils that occurred after intramammary infection. In blood, the total number of lymphocytes was measured and regained pre-infection levels from day 5 and onwards. Therefore, changes in proportions of cells occurring during acute and chronic phase also reflected changes in numbers of the measured sub-populations.

In milk, lymphocyte trafficking changed rapidly after *S. aureus* infection with a decrease in the proportions of CD4+ and CD8+cells at 24 h pi. According to Roitt, Brostoff & Male (1998) primed antigen-specific lymphocytes are retained in the lymph node where the antigen originally entered, when animals encounter the antigen a second time. Therefore, a temporary 24 h shut down in lymphocyte trafficking is observed. *S. aureus* is not only a common udder pathogen, but can also colonise the skin of the udder (McDonald, 1984). Therefore, a cow can be sensitised to these bacteria without a history of *S. aureus* mastitis, making it possible for primed lymphocytes to recirculate to the udder and regional lymph nodes. Thus, a hypothetic explanation to the early changes in acute phase of specific immunity in *S. aureus* mastitis could be as follows: APC in udder tissue recognises common bacterial structures on inoculated *S. aureus*, and engulf, process and present *S. aureus* antigen to CD4+ cells in regional lymph nodes, where they will be retained. The antigen specific lymphocytes become activated together with the APC, and produce and release cytokines. One of these cytokines is IL-2, which in turn up-regulate the expression of its own receptor on lymphocyte cell surfaces. On day 3 pi, this was measurable in milk from both infected and control quarters as an increased proportion of IL-2R+ lymphocytes. If the APC is a B-cell, they will start to proliferate in the lymph nodes, and then home back to the mammary gland, and mainly to the infected quarter. This was

observedas an increased proportion of B-cells on day 3 p.i. The sum of the proportions of CD4+, CD8+, WC1+ and B-lymphocytes was equal to 1 before infection and in control quarters throughout the study, but the sum was below 1 in infected quarters (data not shown). It may be speculated that this was due to an influx of CD4-/CD8- cells, for example NK-cells or WC1- γδ T-lymphocytes, to infected quarters.

Another finding that is difficult to explain is the increased expression of lymphocyte receptors, which is the most prominent feature during chronic phase. The cellular expression of IL-2R and B was up-regulated both systemically and locally. In addition, an enhanced expression of CD4 and CD8 was also observed in blood and control quarters, but not in infected quarters. Could *S. aureus* exert suppression on lymphocyte receptor expression? Maybe this could be an explanation to the hyporesponsiveness that other authors have described. For instance, Hisatsune *et al.* (1990) and Park *et al*. (1993) reported that enhanced persistency of *S. aureus* intramammary infections is due to activated CD8+ cells that induce hyporesponsiveness to antigens in milk lymphocytes. In addition, milk lymphocytes from chronically infected glands were shown also to be hyporesponsive to mitogens (Nonnecke & Harp, 1985; Harp & Nonnecke, 1986; Doymaz *et al.*, 1988). The biological relevance of increased cellular expression of surface antigens has to be further investigated.

Both acute clinical, and chronic sub-clinical, *S. aureus* mastitis had an impact on the systemic immune response and on the immune response in the opposite healthy quarters. The latter is also described by Nonnecke & Harp (1985), but to interpret these results, further studies, *e.g.* evaluating the effects of bacterial challenges in healthy glands in cows with mastitis, are needed.

Why does *S. aureus* mastitis often become chronic?

For some reasons, probably due to influence from the antigens, B-cells dominate the specific immune response to intramammary infections with *S. aureus*, seen as a drastic increase in the proportion of B-cells in infected quarters due to an influx of systemic B-cells to infected quarters and not due to local proliferation (Nickerson & Heald, 1982). Activation of the humoral response during *S. aureus* mastitis is well-established according to Lascelles *et al.* (1986), and is also demonstrated by Riollet, Rainard & Poutrel (2001) in milk from cows with chronic sub-clinical *S. aureus* mastitis. An induction of the humoral immune response may not be effective against intracellular bacteria. This, together with the immune suppression induced by *S. aureus* toxins could explain the persistency of this disease. However, the massive influx of neutrophils with their release of tissue damaging substances can also exert a negative effect on the immune system in the infected gland. This is supported by Clark & Klebanoff (1977), who reported that enzymes from neutrophils were cytotoxic to human blood leukocytes. In addition, the solid architecture of cells in healthy animals is impaired during the inflammatory process, reducing their capacity to inhibit invasion of pathogens, and some bacterial species can even benefit from the local inflammation (Rhen *et al.*, 2003).

Milk APP in mastitis diagnostics

When evaluating tools to diagnose mastitis, the true status of the udder quarter is hard to define. In general, SCC is used as the golden standard, partly because of the inconsistency of bacterial analyses, as described in the introduction. SCC can be measured indirectly using CMT or ATP, but other inflammatory markers, like NAGase, are also closely related to SCC. The disadvantage of these methods is the rather high day-to-day variation in SCC, which is probably even higher in milk from infected udder quarters (Berglund *et al.*, 2003). In addition, SCC, ATP and NAGase have to be analysed at a laboratory, and the cow-side test CMT is not suitable for screening of large numbers of cows. At present, neither of these methods is available for on-line measurements. Today, EC is the only method that is used in on-line systems, like automatic milking systems, and EC has to be combined with other milk data to be a reliable indicator of clinical and sub-clinical mastitis, as described in the introduction.

However, is measurement of inflammation, *i.e.* disease detection, the proper approach when screening udder health? Maybe it is better to look for tools that can be used as indicators of both healthy and diseased udders? From this point of view, I believe that measurements of haptoglobin and SAA fit well as tools for this dual purpose. Despite a rapid and prominent increase of haptoglobin and SAA in milk during acute clinical *S. aureus* mastitis, and the finding of increased levels also during chronic sub-clinical mastitis, the control quarters were mostly unaffected (Paper I). In addition, the variation in APP in milk from healthy cows in Paper III was very low and therefore analyses of haptoglobin and SAA in milk have a potential in udder health diagnostics. These two parameters are indicators of healthy quarters, of acute clinical mastitis as well as of chronic sub-clinical mastitis. The results also indicate that analysis must be done on udder quarter level and not in composite samples, which is possible to do in on-line systems. However, today, the APP analyses are time consuming, costly and have to be done at a laboratory. Thus, new analysing techniques have to be developed before APP analyses can be cost-effective for the dairy farmers. In addition, a better understanding of the relationship between milk SCC and APP, and the true inflammatory status of the udder quarter is needed.

Immunomodulation in connection with *S. aureus* mastitis

In studies on shipping fever in heifers, Pedroso (1994) reported a preventive effect of subcutaneous injection of glucan. In addition, (Buddle, Pulford & Ralston, 1988) reported a decreased bacterial count in milk from udder quarters intramammarily challenged with *S. haemolyticus*, when the ewes had been subcutaneously treated with glucan. However, in Paper IV, intramammary infusion of ß1,3-glucan had no therapeutic effect on cows with chronic sub-clinical mastitis, and only a small preventive effect on *S. aureus* infection at drying off.

Evaluation of the effects of an immunomodulating substance, like ß1,3-glucan, is not an easy task. Dose, administration route and stage of lactation could have

influenced the results. The dose used was based on earlier studies by Inchaisri, Waller & Johannisson (2000), but in that study only a few cows were used, which were free from udder infections. It is possible that another dose regime, and/or administration routine, like subcutaneous injections, would have been more effective against *S. aureus* infections. However, an immunostimulationg effect was observed as the proportion of MHC class II positive milk lymphocytes tended to increase after glucan administration in cows with chronic sub-clinical mastitis, but the biological benefit of this change is difficult to interpret. As *S. aureus* acts as an intracellular bacteria, it would probably be more beneficial to stimulate CD8+ cytotoxic lymphocytes, or maybe NK-cells or γδT-cells, to increase killing of infected cells.

Before a substance can be presented as useful or not as an immunomodulator intense *in vivo* experiments and field studies are needed. A vaccine can be promising in experimental studies on a relatively small number of animals, but its preventive effects are often modest when tested in field studies on naturally occurring cases of infections when the variation in the animal population is large. Substantial research funding is a must when immunomodulators are evaluated.

Concluding remarks

The CPR has not been extensively studied in veterinary medicine, and to my knowledge, Papers I and III are the first to describe CPR in milk and serum during sub-clinical mastitis. However, increased serum concentrations of haptoglobin and SAA during different chronic conditions in cattle have been observed earlier (Alsemgeest *et al.*, 1994; Horadagoda *et al.*, 1999). In the present study it was evident that the inflammation was sufficiently intense to induce a measurable haptoglobin and SAA production in serum even during such a mild and local process as chronic sub-clinical mastitis. In addition, alterations in adapted immunity were also demonstrated in peripheral blood during chronic sub-clinical *S. aureus* mastitis, giving further proof of a systemic impact from the local inflammatory process.

The above mentioned results may be an example of the involvement of the neuroendocrine-immune system. According to (Morale *et al.,* 2003), the communication between the neuroendocrine and immune systems plays a pivotal role in health and disease. Mainly the endocrine-immune system has been elucidated during bovine mastitis (Shuster & Harmon, 1992; Hockett *et al.*, 2000; Diez-Fraile, Meyer & Burvenich, 2003) with reports on increased levels of cortisol, prostaglandins and stress hormones. Morale *et al.* (2003), also describe how the immune system affects the nervous system via cytokines that induce fatigue, depressed activity, and excessive sleep, and further research is needed in this area in relation to mastitis. With the relationship between neuroendocrine and immune systems in mind, questions arise regarding if sub-clinical mastitis also can have an effect on other organ systems. In support for this, both sub-clinical and clinical mastitis have been reported to have a negative impact on reproductive performances (Barker *et al.*, 1998; Oliver *et al.*, 2000; Schrick *et al.*, 2001). In humans, it has been discussed that the body suffers from general hypermetabolism

with higher resting energy expenditure during CPR (Bengmark, 2001). From this it can be speculated that it is energy consuming to, for instance, produce APP and up-regulate lymphocyte receptors. An increased susceptibility to disease is also associated with nutrient deficiency in cattle (reviewed *e.g.* by Østergaard & Sørensen (1998) and Rukkwamsuk, Kruip & Wensing (1999). Since a lactating cow is already having a very high workload, a chronic infection may be the factor that tips the scale, starting a vicious circle, where the persistent disease further increases the cow's nutrient requirements making her deficient and more disease susceptible. Rhen *et al.* (2003) describe chronic disease in the following way: "For many pathogens, acute infection is merely a prelude to a more long-lasting association to the host, and different to normal microbial flora of the host, persistent pathogens are a burden to the carrier, despite a sub-clinical appearance of disease".

Worldwide, a lot of effort has been focused on how to minimise the effects of chronic sub-clinical mastitis, for example by preventing intramammary infection using vaccines, or to eliminate ongoing infections by treatment with antibiotics, or to stimulate the immune system of an animal with non-specific immunomodulators. Despite all these efforts the bacteria are ahead of us. To win the battle against *S. aureus* and decrease the number of cows suffering from chronic sub-clinical mastitis it is essential that all infected cows are rapidly detected and isolated from other cows, and that proper milking management and other preventive measures are used to minimise the spread of bacteria.

Conclusions

- Serum concentrations of haptoglobin and SAA increased rapidly and prominently during acute clinical *S. aureus* mastitis, and SAA stayed slightly elevated also when the mastitis had been transformed into a chronic sub-clinical phase.

- Concentrations of haptoglobin and SAA in milk from infected udder quarters increased rapidly and prominently after *S. aureus* inoculation, and SAA levels stayed elevated also when chronic sub-clinical mastitis had developed, but healthy control quarters was not affected throughout the study.

- During acute clinical *S. aureus* mastitis, changes in proportions of lymphocyte sub-populations occurred mainly in infected quarters, while increased cellular expression of the measured lymphocyte surface antigens was the main finding in chronic phase, and was observed in blood, and in both infected and healthy control quarters.

- The main finding during acute and chronic phase of *S. aureus* mastitis was a drastic increase in the proportion of B-cells and expression of B-cell antigen, which occurred in blood, infected and control quarters. A preferential induction of the humoral immune response may not be sufficient to eliminate intracellular bacteria, like *S. aureus*, which could be one explanation to the persistency of *S. aureus* mastitis.

- Based on the study on healthy cows and cows with naturally occurring cases of chronic subclinical mastitis, milk haptoglobin and SAA were considered to be good indicators of healthy udder quarters, and also to have a potential as indicators of chronic sub-clinical mastitis. The large variation in ATP, haptoglobin and SAA within quarter samples from cows with chronic subclinical mastitis suggests that different mechanisms regulate migration of leukocytes into the udder compared to the influx and/or secretion of haptoglobin and SAA into milk.

- Intramammary infusion of ß1,3-glucan had a slight, but not statistically significant, positive effect on the clinical and anti-bacterial response to *S. aureus* infection at drying off, but had no therapeutic effect in udder quarters of cows with chronic sub-clinical *S. aureus* mastitis.

- The proportion of MHC class II+ milk lymphocytes tended to increase after intramammary infusion of ß1,3-glucan in udder quarters with chronic subclinical *S. aureus* mastitis, indicating a stimulation of the antigen presenting ability.

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Populärvetenskaplig sammanfattning

Bland dagens mjölkkor är juverinflammationer, så kallade mastiter, den vanligaste förekommande sjukdomen. För mjölkbonden är mastit en kostsam affär då till exempel mastitmjölk ibland måste kastas och ger sämre betalt om den skickas till mejeriet. Dessutom mjölkar kor med mastit sämre och måste ofta slaktas i förväg. Orsaken till mastit är oftast bakterier som har fått fäste i spenen och sedan kan vandra in i juvervävnaden. Juverinflammationer som har synliga symptom som till exempel flockor i mjölken och svullet och ömt juver, kallas klinisk mastit. Juverinflammationen kan också vara subklinisk, vilket betyder utan symptom, och det enda sätt att finna en sådan juverdel är genom att mäta antalet celler i mjölken. Efter att bakterierna har invaderat juvret, stiger celltalet i mjölken markant från ca 100 000 celler per ml till över 1 miljon celler per ml. Det är tyvärr vanligt att en klinisk mastit övergår i subklinisk kronisk (långvarig) form på grund av att kon har misslyckats att göra sig av med bakterierna. Kronisk subklinisk mastit är den vanligaste formen i Sverige och den bakterie som oftast orsakar detta är *Staphylococcus aureus* (*S. aureus*).

Det immunsvar som sker när bakterier har invaderat juvret kan indelas i en medfödd och en förvärvad del. De inflammatoriska celler, framförallt vita blodkroppar, som tillhör det medfödda immunförsvaret reagerar snabbast och startar hela inflammationsprocessen. Denna avhandling behandlar hur delar av det medfödda och förvärvade immunförsvaret reagerar vid en juverinfektion och hur det förändras, när mastiten går från att varit klinisk till att bli kronisk subklinisk. Dessutom ville vi finna orsaker till varför just *S. aureus* mastiter blir kroniska och undersöka om vi kunde stimulera juvrets immunförsvar så att det kunde eliminera *S. aureus* bakterierna utan antibiotika.

Det så kallade akutfassvaret, som är en del av det medfödda immunförsvaret, stimuleras kraftigt vid en infektion vilket leder till ett antal förändringar i kroppen som har till uppgift att lindra och reparera skadan av infektionen. En av förändringarna är att levern ställer om sin proteinproduktion vilket visar sig som en ökning i blodet av så kallade akutfasproteiner (APP), som inte finns i blodet hos friska individer. APP är artspecifika och hos kor är haptoglobin och serum amyloid A (SAA) de APP som ökar snabbast och mest efter en infektion. I två av studierna, mätte vi haptoglobin och SAA i blod och/eller mjölk. I den första, som var en experimentell studie där vi sprutade in *S. aureus* i en juverdel, såg vi att dessa två APP ökade kraftigt efter infektion, då korna hade klinisk mastit. Efter ungefär en vecka sjönk värdena betydligt, och efter tre veckor, när korna hade utvecklat kronisk subklinisk mastit, var SAA värdena fortfarande höga i både blod och mjölk. Det speciella för APP koncentrationen i mjölk var att den endast ökade i den juverdel som hade blivit infekterad och inte i den friska kontrolljuverdelen. Baserat på dessa fynd ville vi gå vidare och undersöka om APP i mjölk kan användas för att hitta kor med kronisk subklinisk mastit. Då *S. aureus* är en smittsam bakterie är det viktigt att snabbt och säkert hitta alla kor som är infekterade. I denna studie samlades mjölkprover in från kor som haft förhöjda celltal i mjölken under lång tid och som därför ansågs ha kronisk subklinisk mastit. Haptoglobin- och SAA-värdena i mjölk varierade mycket mellan

juverdelarna från dessa kor. Även celltalet, mätt i form av adenosin trifosfat (ATP), varierade. Förhöjda nivåer av haptoglobin detekterades oftare än SAA, och i flera juverdelar var ATP-nivåerna höga, men inte APP-värdena. Det tycks som om det under kronisk mastit är olika substanser som stimulerar cellinvandring till juvret jämfört med APP-produktion. Däremot var det väldigt liten variation i APP och ATP bland resultaten från prover som togs från friska kor, det vill säga kor som under längre tid haft väldigt låga cellkoncentrationer i mjölken. Haptoglobin- och SAA-resultaten från dessa friska kor gav riktvärden för hälsa. I mjölk från friska juverdelar ska dessa två APP vara under detektionsnivå och i och med detta skulle dessa två proteiner kunna användas för att upptäcka kronisk subklinisk mastit.

Vi undersökte det förvärvade immunsvaret i samma experimentella studie som nämndes ovan där *S. aureus* sprutades in i en juverdel. Blod- och mjölkprover analyserades för olika undergrupper av en sorts vita blodkroppar som kallas lymfocyter. Vi mätte både hur stor andelen var olika slags lymfocyter samt hur stort det specifika uttrycket av ytreceptorer var per lymfocyt för varje lymfocyttyp. När korna hade klinisk mastit förändrades framförallt andelen av de olika lymfocyterna i mjölk från den infekterade juverdelen. Men när korna hade utvecklat kronisk subklinisk mastit var det uttrycket per lymfocyt som hade ökat och då framförallt på lymfocyter i blod och i den friska juverdelen. I blod liksom i mjölk från både friska och sjuka juverdelar ökade andelen av en lymfocytundergrupp som kallas B-celler markant. B-cellernas främsta uppgift är att producera antikroppar men eftersom *S. aureus* har möjlighet att leva inuti vita blodkroppar så kan de inte bli igenkända med antikroppar. Detta fynd kan vara en förklaring till varför *S. aureus*-mastiter ofta blir kroniska.

Immunförsvaret har svårt att göra sig av med *S. aureus*-bakterier och eftersom de kan gömma sig inuti celler är det också svårt att döda dem med antibiotika. Detta ledde till att vi ville test om en biologisk substans, ß1,3-glukan, från väggen av en jästsvamp, kunde förhindra eller hjälpa immunförsvaret att eliminera en *S. aureus*-infektion. β1,3-glukan har i tidigare försök förhindrat eller hämmat vissa infektioner i samband med stimulering av immunförsvaret. I vår studie sprutade vi in detta ämne i kons juver innan vi sprutade in bakterierna för att se om β1,3-glukan kunde förhindra infektion i samband med att korna skulle sinläggas. Vi behandlade även kor med kronisk subklinisk *S. aureus*-mastit på liknande sätt för att se om ß1,3-glukan kunde stimulera immunförsvaret och göra juverdelen frisk. Resultaten visade att β1,3-glukan hade en viss immunstimulerande effekt på mjölklymfocyter men vi såg ingen behandlingseffekt ochs endast en liten förebyggande effekt.