Parameters for natural resistance in bovine milk

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Voor jou,
jullie

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Alles kan, als je het maar laat gebeuren

(Mary Poppins)

Chapter 1

General Introduction
Aims and Outline

1.1 THE BOVINE MAMMARY GLAND

The macroscopic anatomy of the mammary gland (udder) differs among different species, but the microscopic anatomy is very similar among species. Development of the bovine udder starts early in the fetal life and by the sixth month, the udder of the fetus is almost fully developed with 4 separate quarters and a median ligament, teat and gland cisterns. The development of milk ducts and the milk secreting tissue take place between puberty and parturition. The udder continues to increase in cell numbers throughout the first 5 lactations, and the milk producing capacity increases correspondingly. This is not always fully utilized, since the average productive life time of many cows today can be as short as 3 lactations. The udder is a very big organ weighing around 50 kg (up to 100 kg), including milk and blood. Therefore, the udder is very well attached to the skeleton and muscles by ligaments that are composed of elastic fibrous tissue and connective tissue. The udder consists of secreting tissue and connective tissue. The amount of secreting tissue, and thus the number of secreting cells is the limiting factor for the milk producing capacity of the udder. The milk is synthesized by the secretory cells that are arranged as a single layer on a basal membrane in a spherical structure called alveolus. The diameter of each alveolus is about 50 - 250 mm (McClellan et al., 2008). Several hundreds alveoli together form a lobule. The milk, which is continuously synthesized in the alveolar area, is stored in the alveoli, milk ducts, udder and teat cistern between milkings. Most of the milk (60 - 80%) is stored in the alveoli and small milk ducts, while the cistern only contains 20 - 40%. The teat consists of a teat cistern and a teat canal. Where the teat cistern and teat canal meet, 6 - 10 longitudinal folds form the so called Furstenberg's rosette, which is involved in the local (immune) defense against mastitis. The teat canal is surrounded by bundles of smooth muscle fibers, longitudinal as well as circular. Between milkings the smooth muscles function to keep the teat canal closed. In case of an infection, pathogens such as bacteria can enter an udder through the teat sphincter. The teat canal is lined with keratin or keratin like substances that act as a barrier for pathogenic bacteria. The keratin is sticky and bacteria that enter the sphincter are caught on the keratin. Approximately 40% of the keratin lining, with any bacteria stuck to it, is removed at each milking and, therefore, it requires constant regeneration. Consequently, it is important to ensure that there is closure of the teat canal post-milking. Besides keratin removal, a more patent teat orifice and milk leaking can increase the risk for mastitis (Fox, 2009). The udder of a cow consists of 4 quarters. Milk that is synthesized in one quarter cannot pass over to any of the other quarters. The right and left side of the udder are separated by a median ligament, while the front and the hind quarters are more diffusely separated. The udder is very well supported by blood vessels, arteries and veins and right and left udder halves generally have their own arterial blood supply. There are some small arterial connections that pass from one half to the other. The primary function of the arterial system is to provide a continuous supply of nutrients to the milk synthesizing cells. To produce 1 liter of milk, around 500 liter of blood has to pass through the udder. When the cow is producing 40 liter of milk per day, some 20,000 liter of blood is circulating through the udder. The udder also contains an elaborate lymphatic

system that serves as a filter that destroys foreign substances (including microbes), but also provides a source of lymphocytes to fight infections. Figure 1 shows the anatomy of the bovine udder.

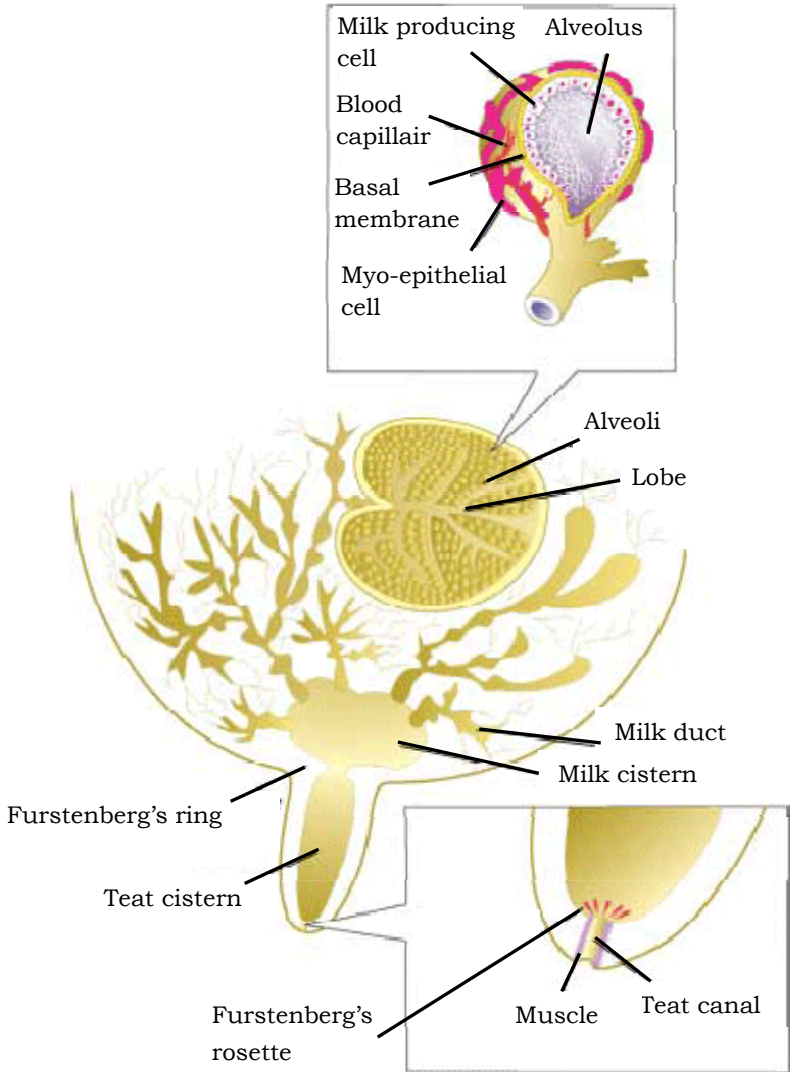

Figure 1. Schematic overview of the udder. Adapted from DeLaval (http://du.delaval.nl).

After bacteria breach the teat end, they can be taken up and destroyed by white blood cells (leukocytes). Leukocyte chemotaxis is one of the major factors involved in migration of these cells towards the site of inflammation. Several studies have shown a strong relationship between early leukocyte influx and outcome of infection (Kornalijnslijper et al., 2004).

1.2 MASTITIS

Mastitis is one of the most important health problems in dairy cattle and is responsible for reduced animal welfare, the use of antibiotics and considerable economic losses (Halasa et al., 2009; Huijps et al., 2008). Annually about 25% of all dairy cows develop clinical mastitis, but also subclinical mastitis causes many problems, such as reduced milk production, a higher somatic cell count (SCC) and use of more antibiotics. Clinical mastitis (CM) includes externally observable phenomena to the udder, such as reddening and swelling of the infected quarter(s) and the presence of whitish clots or flakes in the milk. In extreme cases CM can result in death of the cow. Subclinical mastitis is observed by elevated SCC levels and the presence of pathogens in the milk. It is well known that bacterial infections, management and cow factors are interdependent and influence mastitis incidence. Major bacterial mastitis pathogens are *Escherichia coli, Streptococcus uberis, Streptococcus dysgalactiae,* and *Staphylococcus aureus. Escherichia coli* causes transient peracute or acute mastitis (reviewed by Burvenich et al., 2003; Suojala et al., 2010). *Escherichia coli* are associated with environmental mastitis. *Staphylococcus aureus* infection is associated with contagious mastitis. It can cause peracute, acute, chronic, and subclinical mastitis with the chronic subclinical form being predominant. Management factors like feed, hygiene, housing, purchase policy and milking technique can all influence mastitis incidence (Nyman et al., 2009). Cow factors also play a major role in mastitis susceptibility. Many studies indicate that the severity of *E. coli* mastitis is mainly determined by cow factors rather than by *E. coli* pathogenicity (reviewed by Burvenich et al., 2003). Of these cow factors the host (innate) defense status is of major importance in determining the course of an infection (Stelwagen et al., 2009). Besides physical barriers, like the keratin plug, this innate defense involves humoral and cellular components.

1.3 INNATE IMMUNITY AND MASTITIS

1.3.1 Overview

The immune system of the mammary gland must serve a dual function in that it provides passive immunity for the neonate and protection of the organ itself against pathogenic insults (Stelwagen et al., 2009). Physical resistance against infection is based on the structure and function of the teat, besides an intact epidermal (skin) barrier. Once a pathogen has passed the physical barriers it will initially face the resident defenses in the mammary gland. These include resident phagocytic cells like macrophages and neutrophils, natural antibodies (NAb), complement, acute phase proteins and antibacterial peptides, etc. Depending on numbers and characteristics of the pathogen these resident defense mechanisms may already effectively eliminate the invading pathogen. In that case the inflammatory response will remain (very) limited. If the pathogen is not removed by these resident immune mechanisms, the next step in the host defense involves detection of the pathogen by the pattern recognition receptors (PRRs) on immune cells. This in turn results in induction of extra defense mechanisms, like a more activated phenotype of

phagocytic cells, the production of signaling molecules, like tumor necrosis factor α (TNF-α), and recruitment of immune molecules and cells (mostly neutrophils) from the blood to the site of infection (inflammation). Neutrophils contribute to host defenses through their ability to phagocytose and kill bacteria. This is the most abundant cell type of the innate immune system, and it is among the earliest leukocyte types recruited to the site of infection. Nutrition and nutritional strategies can have a clear influence on mastitis incidence (Compton et al., 2007; Svensson et al., 2006). Associations between blood serum concentration of different metabolites and udder health have been found (Nyman et al., 2008). Low dietary selenium or vitamine E is associated with a high incidence of mastitis and retained fetal membranes (reviewed by Goff, 2006), and can impair the function of neutrophils (Weiss et al., 2009) that is essential for resistance to mastitis. Additionally, nutrition strategy is of importance to reduce the negative energy balance and other metabolic diseases in dairy cows. Increasing evidence is showing a connection between metabolic diseases and impaired immune function, leading to infectious diseases (reviewed by Goff, 2006; Heinrichs et al., 2009). A proper (innate) immune system can thus form a great contribution to an efficient control of mastitis and other diseases. In the following part the role in the innate immune system of the mammary gland will be discussed in more detail.

1.3.2 Receptors: sensing the pathogen

In recent years progress has been made in understanding how the innate immune system senses "danger signals" from microbes. The cells of the innate immune system carry so called "Pathogen Recognition Receptors" (PRR) that recognize conserved bacterial structures called Pathogen Associated Molecular Patterns (PAMP). An important class of PRR is the Toll-like receptors (TLR). In relation to mastitis different TLR were found to bind different PAMP, e.g. TLR2 binds peptidoglycan and lipoteichoic acid (LTA) from Gram-positive bacteria like *S. aureus* (Van Amersfoort et al., 2003). Toll like receptor 4 binds lipopolysaccharide (LPS) from Gram-negative bacteria like *E. coli*. Lipopolysaccharide binding protein (LBP) and soluble CD14 (sCD14) strongly promote the binding of LPS to TLR4. In experimental infection of the mammary gland with pathogens like *E. coli*, *S. aureus*, *Klebsiella pneumonia*, *Serratia marcescens* and *S. uberis*, these bacteria markedly increased the concentrations of LPS binding protein (LBP) and soluble CD14 (sCD14) (Bannerman et al., 2004a, b, c). Single nucleotide polymorphisms (SNP) in the gene for bovine TLR4 have been found that affect the susceptibility to mastitis and paratuberculosis (Bhide et al., 2009; Wang et al., 2007). Susceptibility to paratuberculosis is also associated with polymorphisms in another intracellular PRR (CARD15/NOD2) (Pinedo et al., 2009). Binding of PRR to PAMP results in a cascade of signals initiating inflammation. This is a normal and essential function crucial to the recruitment of bovine neutrophils and the initiation of further effective immune defense. In contrast to other species, bovine neutrophils are not attracted directly by bacteria or bacterial products (Gray et al., 1982). Once the pathogen has been sensed by the immune system, signaling molecules will be released that induce the production and recruitment of effector immune molecules and cells. The most important ones are discussed below.

1.3.3 Soluble signaling molecules and inflammation

After the invading pathogen has been detected (sensed) by the immune system of the host, signaling occurs. In relation to mastitis, upon contact of their PRR with microbes, resident macrophages and mammary epithelial cells were found to release pro-inflammatory cytokines, like interleukin (IL)-1β, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and also the complement factor C5a and chemokines (like IL-8) that play important roles in the induction of an innate immune response (reviewed by Bannerman, 2009). These pro-inflammatory molecules induce retraction of the endothelial cells that line the vessel walls, resulting in an increased vascular permeability. This enables the transfer of larger molecules, (like (natural) antibodies) from the blood to the infected site. Also immune cells, in particular neutrophils, will migrate in large numbers from the blood, between the endothelial cells, towards the site of infection. This process is called "inflammation". The intensity of the inflammation is dependent on the kind of pathogen and its quantity. In other words, if the resident and initially induced immune mechanisms have been sufficient to control the pathogen, the inflammatory response will be small or remain absent. If on the other hand the pathogen is expanding, an inflammatory response will occur. This will greatly enhance the ability of the host to destroy the pathogen, but may result in damage to the host tissues (collateral damage).

1.3.4 Effector cells

The cellular innate immune defense comprises neutrophils, macrophages, dendritic cells and natural killer (NK) cells (Rainard and Riollet, 2006). Natural killer cells are particularly important for the destruction of infected cells. They can express bactericidal activity against *S. aureus* upon stimulation with IL-2 (Sordillo et al., 2005) and can also be good producers of interferon γ (IFN-γ). This IFN-γ is a key cytokine for the activation of neutrophils (Sordillo and Babiuk, 1991) and macrophages that are the most effective cell types acting against bacterial pathogens. Interferon-γ induces nitric oxide synthase (iNOS) expression in local macrophages at the infected site. This iNOS expression generally results in the production of nitric oxide (NO) that is a key component in the antibacterial activity of macrophages.

Neutrophils are the most abundant white blood cell type in the blood and they play a crucial role in the clearance of bacterial infections in general and also in clearing intramammary infections. Merhzad et al. (2004, 2005 and 2009) demonstrated that the severity of experimental clinical mastitis is related to the quantity and quality of neutrophils and speed of recruitment to the site of infection. Neutrophils are, therefore, considered to be the most important recruited effector cells during mastitis. In relation to mastitis neutrophils were found to phagocytose and kill the ingested bacteria. The ingested bacteria will be killed by an oxygen-dependent or an oxygen-independent system, or both. The oxygen-dependent system results in the formation of hydrogen peroxide (H_2O_2) and hydroxyl (OH) radicals. In addition, nitric oxide (NO) can combine with hydrogen peroxide to form peroxynitrite radicals (Tizard, 2004). These compounds are very microbicidal because they are powerful oxidizing agents that oxidize most of the chemical groups found in proteins,

enzymes, carbohydrates, DNA, and lipids. In addition, lipid oxidation can break down cytoplasmic membranes of the invading pathogens.

The oxygen-independent system consists of cationic proteins that alter cytoplasmic membranes. For instance lysozyme that breaks down peptidoglycan, lactoferrin that deprives bacteria of essential iron and various digestive enzymes that exhibit antimicrobial activity by breaking down proteins, RNA, phosphate compounds, lipids, and carbohydrates. In milk, where the oxygen concentration is low, oxygen-independent killing mechanisms may be relatively more important than in other tissues (Paape et al., 2003). The phagocytosis and bacterial killing by neutrophils can be enhanced by their exposure to pro-inflammatory cytokines like IL-1β, TNF-α or IFN-γ from the inflamed tissue (Sample and Czuprynski, 1991; Sordillo et al., 2005). The viability and activity of milk neutrophils correlates with bacterial clearance (Mehrzad et al., 2004). Milk neutrophils (and macrophages) are generally less functional than blood neutrophils (Mehrzad et al., 2009). Different cow factors (e.g. lactation stage and parity) and environmental factors e.g. vitamin E (Weiss et al., 2009) or selenium deficiency, are known to influence the activity of neutrophils.

Somatic cell counts

The alveolar epithelial cells of the mammary gland are highly active secretory cells that are normally subjected to continuous high turnover and therefore must be replaced continually with new cells. These displaced cells are discharged into the milk as a normal process. In response to irritation, whether physical or microbial, leukocytes enter the milk from the blood and perialveolar interstitium. The displaced alveolar epithelial cells and leucocytes comprise the somatic cells of the milk. The measurement of the number of somatic cells in milk is known as the SCC of milk samples, and is one of the techniques used to monitor the level or occurrence of subclinical mastitis in herds or individual cows or quarters. The prevalence of subclinical infection is difficult to ascertain, as this can only be determined by the isolation of bacteria from the milk. However, SCC is often used as an indirect estimation. The ability to correctly interpret SCC depends on an understanding of the factors that may affect them, like infection status, season, age, stage of lactation, diurnal variation, day to day variation, stress, management and husbandry aspects, effects of quarter and milk fraction.

Milk cell concentrations vary widely as a function of the lactation cycle. In milk from full lactation healthy udders only few leucocytes are present. In the uninfected quarters SCC is generally < 100.000 cells/mL with a low proportion of neutrophils. As lactation progresses usually an increase in SCC and in the proportion of neutrophils (up to 40% near drying-off) occurs (Stelwagen et al., 2009). In the early dry period SCC is 2 - 5 x 10^6 cells/mL, while during most of the dry period, SSC is 1 - 3 x 10^6 cells/mL (Jensen and Eberhart, 1981; McDonald and Anderson, 1981a). At parturition, SCC is usually >1 x 10^6 cells/mL, with a decrease to 10^5 cells/mL in the 7 - 10 days after calving (Jensen and Eberhart, 1981; McDonald and Anderson, 1981b). Macrophages are the dominant cell type in mammary secretions of (healthy) dry and lactating cows while neutrophils are dominant in colostrum (Lee et al., 1980). High concentrations of lymphocytes are present in the secretion of involuted

udders, but they decrease to very low numbers during the week preceding calving (McDonald and Anderson, 1981b), or at calving (Jensen and Eberhart, 1981). The period in which the cow is most sensitive to mastitis is in the first 1 - 2 months after calving, when certain humoral and cellular defenses can be impaired (Sordillo et al., 2005). The dry period, in which the cow is least susceptible to mastitis, is characterized by a high SCC and a high lactoferrin concentration. The most important factor affecting the SCC of the milk from an individual quarter, and consequently the cow and the herd, is the infection status of the quarter.

1.3.5 Effector molecules

Lactoferrin
Lactoferrin has iron-chelating properties, thereby making this essential element unavailable to microbes that limits their infectious properties (Legrand et al., 2004). In relation to mastitis it was found that very low concentrations (20 - 200 µg/mL) are present in the milk of lactating cows compared to concentrations found in human (2 mg/mL) or sow milk (0.5 mg/mL). These concentrations in milk are most likely too low to be effective initially, but concentrations are strongly increased during acute mastitis and then can reach concentrations (5 - 6 mg/mL) that are likely to work bacteriostaticaly. The lactoferrin concentration in colostrum (2 - 4 mg/mL) and non-lactating mammary glands are high (20 - 100 mg/mL) and may play a role in protection against bacterial infection (Molenaar et al., 1996). The role in colostrum is probably limited due to the presence of citrate that counteracts the effect of lactoferrin (Smith and Schanbacher, 1997). Among different bacteria species *E. coli* is most susceptible to lactoferrin, followed by *S. aureus*, while *Streptococci* are generally resistant.

Lysozyme
Lysozyme is a protein that can cleave the peptidoglycan layers of the cell walls of Gram-negative and Gram-positive bacteria. However, bovine milk contains only 0.13 µg/mL milk compared to 10 mg/mL in human milk (Reiter, 1985). Most likely, lysozyme is not a significant defense mechanism of the bovine udder.

Xanthine oxidase
The enzyme xanthine oxidase is present in the membrane of milk fat globules. It catalyses the formation of NO from inorganic nitrite that in turn leads to the production of a bactericidal component important for general immune defense against bacteria. Bovine milk has a noticeable xanthine oxidase activity and was shown to be bacteriostatic to *E. coli* after the addition of nitrite (Hancock et al., 2002).

Complement
The complement system consists of a series of blood proteins that interact as an enzyme cascade and function as a component of the acute inflammatory response. Several different complement pathways are distinguished. Activation of the

complement system can result in direct killing of the pathogen or enhanced killing by other immune mechanisms. The “classical” complement pathway requires antibodies bound to a microbe. The other two pathways (the “mannose” and “alternative” pathways) can interact directly with microbes.

In relation to mastitis low, but significant, complement concentrations were found in glands that do not show clinical signs of inflammation. The classical pathway of complement activation is not functional in the healthy udder due to lack of the complement component C1q. Milk concentrations of complement factor C5 vary widely among cows and are 0.2 - 1.9% of the concentration in blood. The proteolyticaly derived fragment C5a is a strong inducer of neutrophil infiltration. This fragment is detected after inoculation of the mammary gland with *E. coli*, but not after inoculation with *S. aureus* (Riollet et al., 2000). The role of the complement system in the initiation of inflammation remains uncertain, but is very likely to contribute to the immune defense once inflammation has started.

Antibacterial peptides

Host anti-microbial peptides are also induced in large quantities during mastitis. These peptides can have direct killing effects (especially in the phagosome of phagocytic cells) on bacteria, fungi and viruses or induce immune modulating effects (extracellular) (reviewed by Kruse and Kristensen, 2008). The main sources of local anti-microbial peptides production are granulocytes, monocytes, macrophages, platelets and (mammary) epithelial cells.

Natural antibodies

In animals without intentional antigenic stimulation, spontaneously occurring (“background”) antibody-secreting B cells exist in various lymphoid organs (Avrameas, 1991), including the mammary gland. The natural antibodies (NAb) produced by these cells develop already early in life in the absence of external antigens and provide the individual with specificities for all antigens towards which the individual can respond (Avrameas, 1991; Pastoret et al., 1998). A high proportion of NAb binds PAMP that represent antigens that are shared by ’classes’ of microbes and serve as targets for identification of microbes by the innate immune system (Kohler et al., 2003). Important PAMP are lipopolysaccharide (LPS) present on gram negative (entero)bacteria, such as *E. coli* or *Salmonella spp*; lipoteichoic acid (LTA) present on gram-positive bacteria, such as *Staphylococcus aureus*; or peptidoglycan (PGN) present on gram-negative as well as gram-positive bacteria. The broad reactivity of individual NAb, and of the NAb population as a whole (Casali and Notkins, 1989), is important because it provides pre-existing antibody reactivity that allows animals to rapidly recognize and be protected against pathogens that have not been encountered previously. Although this antibody reactivity tends to be of low affinity (Casali and Notkins, 1989), it could provide key protection during the period between the onset of infection and the emergence of the adaptive immune response. These NAb thereby constitute a potentially important humoral component of innate immunity (Baumgarth et al., 2005; Elluru et al., 2008). It has been described by Jerne (1984) that upon arrival in an animal, any antigen is a 1000-fold more likely to encounter circulating NAb than antigen receptors expressed on B cells. Thus,

Jerne (1984) suggested that the initial step leading to any protective antibody formation is dependent on these NAb. These NAb, thereby, provide immediate, early and broad protection against pathogens. These antibodies can be produced spontaneously by B1 cells or after contact to cell wall fragments of autologous gut bacteria. These B cells do not require T cell help for the stimulation of antibody production and are considered to be part of the innate immune system more than of the adaptive immune system.

Natural antibodies are present in bovine milk and plasma (Van Knegsel et al., 2007). Furthermore, NAb titers in blood and milk increase in ageing cattle (Srinivasan et al., 1999; Van Knegsel et al., 2007). This suggests that NAb are the cumulative result of antigenic stimulation, either by continuous polyclonal stimulation by exogenous microbes, or self-antigens, or both (Avrameas, 1991; Tomer and Shoenfeld, 1988).

Natural antibodies, either alone or in cooperation with the complement system, are thought to provide immediate early and broad protection against pathogens, before adaptive antibodies are developed in the course of an infection. After binding of NAb to an invading microbe, the opsonized microbe can be taken up much more efficiently by phagocytic cells such as neutrophils and macrophages than without opsonization. These cells can subsequently kill the ingested microbes. Antigen uptake, processing and presentation via B cells or dendrites may be enhanced by NAb, and even more when NAb and complement factor C3b are cooperating as shown in figure 2. In this way NAb may act as an adjuvant for specific immunity that will help inducing an adaptive immune response. Indeed, NAb binding specific pathogens or antigens have been proposed to enhance protection against subsequent infection (reviewed by Kohler et al., 2003).

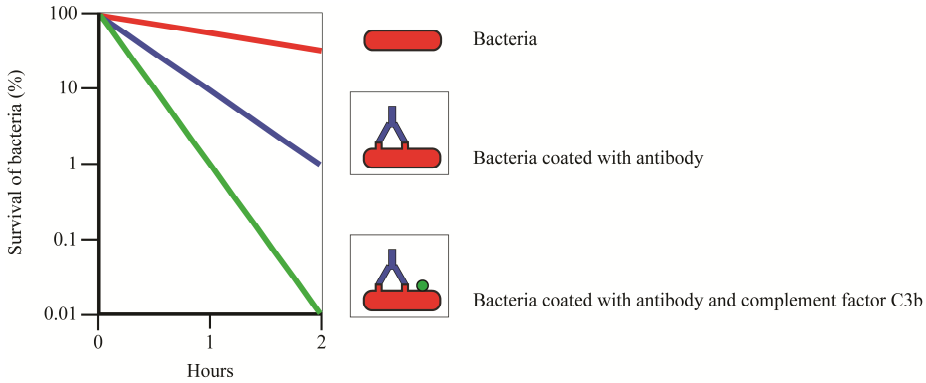

Figure 2. Effect of antibody and complement on removal of virulent bacteria. Adapted from Roitt et al. (1995).

Antibody isotypes

Besides total NAb, (sub)classes of antibodies (isotypes) are important to consider separately as the different requirements for protection from different infectious agents is reflected in the distinct properties of the antibody isotypes. The constant regions of the different isotypes determine their interactions with other molecules

and cells of the immune system. Four antibody isotypes are known in cattle: IgG1, IgG2, IgA and IgM (Figure 3). In mammals, NAb are mostly of the IgM isotype class, but IgG and IgA NAb have been described as well (Boes, 2000). IgG1 is the primary isotype found in milk of healthy cows (Korhonen et al., 2000). Each of the isotypes differ in physiochemical and biological properties (Gershwin et al., 1995; Musoke et al., 1987). Antibodies can diffuse through mammary endothelium (Östensson and Lun, 2008). Furthermore, IgA can be produced locally in the mammary tissue. The translocation of IgA across the mammary epithelial layer is facilitated by the polymeric Ig receptor (pIgR) expressed on the mucosal epithelium (Pastoret et al., 1998). IgM can be transferred by diffusion from blood to milk and probably also be produced locally by cells in the mammary tissue (Östensson and Lun, 2008). The isotype IgG1, on the other hand, can be selectively transferred by active transport from serum using the FcRn receptor, which is expressed on epithelial cells (Kacskovics et al., 2000).

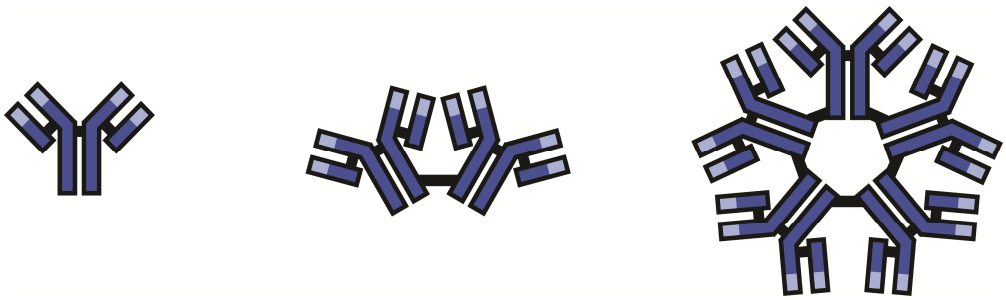

Figure 3. From left to right: monomeric IgG, dimeric IgA and pentameric IgM; Adapted from: Roitt et al. (1995).

Natural IgM are polyreactive in early protection against pathogens and its role as a regulator in the immune system to prevent autoimmunity has recently been reviewed (Ehrenstein and Notley, 2010). Natural IgM antibodies have characteristic properties, like low affinity, cross-reactivity and pentameric structure, and they have a currently well accepted role in protection against pathogens and in immune regulation (Ehrenstein and Notley, 2010). Due to their pentameric structure IgM can display considerable avidity in spite of low binding affinity. Thus, these IgM NAb are also likely to have a prominent role in the immune response against infection involved in the induction of mastitis.

In milk, antibody levels are, on average, lower than in serum: for IgM 0.086 and 3.05 mg/mL, for IgG1 0.58 and 11.2 mg/mL; for IgG2 0.005 and 9.2 mg/mL and for IgA 0.081 and 0.37 mg/mL, respectively (Pastoret et al., 1998). Because of milk 'continuously' flushing out of a lactating udder, the antibody concentrations are generally quite low (< 1 mg/mL), but this can increase considerably during mastitis, with total antibody levels reaching up to 80 mg/mL.

The isotypes IgG1, IgG2 and IgM in cows can function as opsonins that prepare bacteria for phagocytosis by neutrophils and macrophages. These antibodies bind bacterial pathogens directly or with the C3b component of complement (Howard et al., 1980). Neutrophils and macrophages can bind to antibody-bacteria complexes

and antibody-C3b-bacteria complexes via their Fc receptors and subsequently more effectively phagocytose the invading bacteria. IgA in milk appears to contribute to agglutination of invading bacteria that can prevent the spread of bacterial disease in the bovine mammary gland (Korhonen et al., 2000). IgA also plays a role in inhibiting bacterial multiplication, toxin neutralization, and preventing bacterial colonization of epithelial surfaces (Nickerson, 1985; Sheldrake et al., 1984).

In conclusion, various (if not all) of the before described components of the immune system are implicated in an infected udder and provide the available toolbox with which the pathogen-induced immunity will provide local defense against the development of mastitis, as illustrated in Figure 4. Basal levels of immune components, like for instance phagocytic cells and NAb, are present in a healthy udder, ready for immediate defense against invading pathogens. Immune components present in milk could reflect resistance to mastitis and possibly also other diseases.

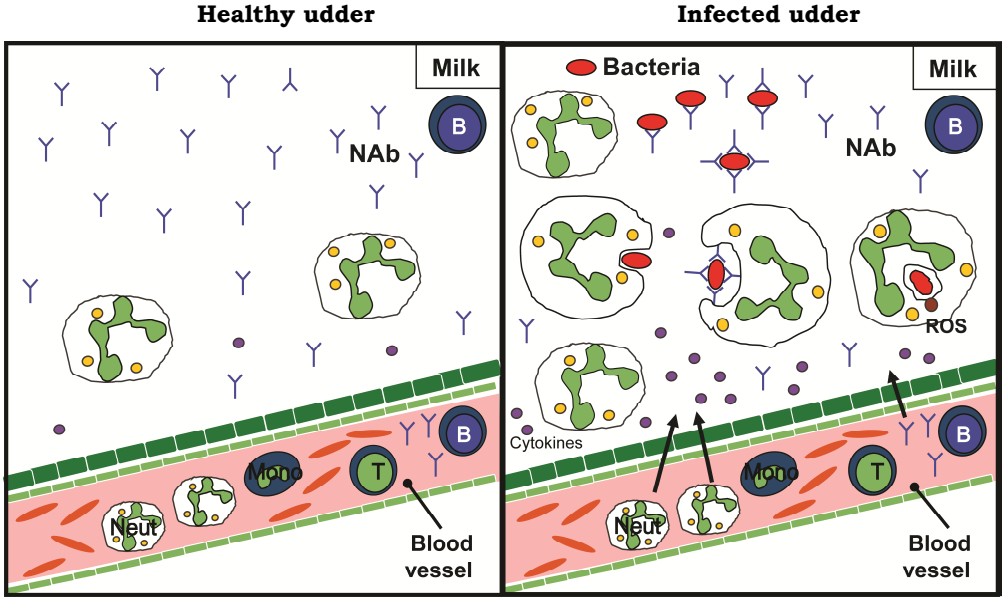

Figure 4. Schematic reflection of udder with milk, and blood vessel, including possible present immune parameters. Picture on the left represents a healthy udder; picture on the right represents infected udder with bacteria and possible opsonization by (natural) antibodies (NAb), and phagocytosis of bacteria. ROS = reactive oxygen species (oxygen radicals). In an infected udder cytokines can induce recruitment of cells to the milk, possibly resulting in inflammation.

1.4 BREEDING FOR REDUCED MASTITIS

Genetic variation of mastitis resistance is well established in dairy cattle. Many studies have focused on the polygenic variation of the trait, by estimating heritabilities and genetic correlations among phenotypic traits related to mastitis, such as SCC and clinical cases of disease. Using breeding to reduce the incidence of mastitis in dairy cattle has been the subject of considerable research effort over the past 20 years (Rupp and Boichard, 2003). The genetic basis of innate and acquired immunity traits related to mastitis resistance in dairy cattle has been mainly based on antibody response and functionality of neutrophils, of which recruitment and activity are essential in the innate defense against udder infection.

Genetic evaluations of bulls for SCC in dairy cattle have become available in several countries. As SCC is genetically correlated with mastitis, selection for reduced SCC will lower the incidence of mastitis, although SCC is an immune response that should help fighting pathogens. Considerable evidence now exists that has established unfavourable genetic correlations between resistance to mastitis and milk production traits. This unfavourable genetic correlation stresses the need to consider mastitis resistance in selection. Several countries have included mastitis into breeding programmes alongside conventional traits in order to prevent the unfavourable correlated response in genetic susceptibility as a result of selection for increased productivity (Rupp and Boichard, 2003).

Selection against mastitis in dairy cattle is currently underway in several countries. One of the key components to select against clinical mastitis (CM) is the difficulty of recording the selection trait and hence the quality of the resulting data. Compared with CM, SCC is routinely recorded in several countries and it has a higher heritability than CM (Detilleux, 2002). SCC has heritability between 4 – 15%, depending on use of test-day records, lactation means or other models and the lactation stage (Detilleux, 2002, 2009). Heritability for CM was around 4% (ranging from 0.004 – 0.11 depending on model and mastitis incidence definition used (Vallimont et al., 2009), or around 5%, but could reach 15%, depending on causing bacteria species and lactation stage (Detilleux, 2009). Heritability estimates for SCS of around 10% (Windig et al., 2010), and for CM of around 2.5 - 3% (Bloemhof et al., 2009; Windig et al., 2010) were reported. Generally, a relatively high genetic correlation between CM and SCC is found (r_g = 0.30 – 0.70) that indicates that selection against high SCC will lead to a reduction in CM (Detilleux, 2002). However, to comprehensively evaluate the implications of selection, the genetic relationships among resistance to mastitis and other key performance traits need to be investigated before a breeding strategy can be employed. A better measurement of udder health can help to improve the effectiveness of selection for lower incidence of mastitis.

1.5 AIMS AND OUTLINE OF THE THESIS

1.5.1 Aims

This project aims at identifying new immune parameters, to determine the susceptibility of the individual animal for the development of mastitis and possibly also other health problems. For future routine screening of dairy cows it is important that the parameters can be determined in samples that can be obtained easily and in a non-invasive way, such as milk. When a high correlation between blood and milk parameters can be established, milk would offer a possibility to study the age-related development of systemic immunity next to local mammary gland immunity. Determining this correlation is, therefore, another aim of this project. Heritability of immune parameters will also be determined to reveal the prospects of selective breeding.

1.5.2 Outline

Based on literature a list of immune parameters that could potentially be related to mastitis or other disease susceptibility was composed. The list contains soluble mediators, like NAb, cytokines, anti-microbial peptides and complement proteins, and cellular components like B, T and NK cells, γδ-T cells and granulocytes. The quantity as well as the functional activity of the immune components could be important and can be measured in bovine blood and mostly also in milk. To be appropriate as marker of natural resistance, immune parameters should show variation among cows and repeatability within cows over time. This repeatability and variation is examined for several parameters. In *Chapter 2* repeatability and variation for concentrations of the cytokines TNF-α and IFN-γ in blood and milk samples of 20 clinically-healthy cows were estimated. Additionally, a number of *cellular* immune parameters in the same milk and blood samples were analyzed. Upon analysis of other parameters, total NAb titers to several different antigens (LPS, LTA, PGN and KLH) and isotypes (IgG1, IgM and IgA) binding LTA (in milk) best fulfilled the criteria for repeatability and variation (*Chapter 3*). To determine the prospect for selective breeding, NAb titers were determined in nearly 2000 frozen milk samples obtained during a previous study that had been setup to determine the genetic variation of fat and protein composition of milk (Milk Genomics study). *Chapter 4* describes the heritability of individual NAb *isotypes* (IgA, IgM and IgG1) binding LTA and total NAb, binding the 4 different antigens, measured in these nearly 2000 milk samples. For about 75% of the heifers that participated in the Milk Genomics study, SCC and CM data during their first lactation were available. Data analysis could, therefore, be performed to determine the relation of the different NAb titers with risk for developing CM and high SCC later in lactation (*Chapter 5*).

The Milk Genomics study had been setup to determine heritability of fat and protein composition of milk. Although additionally collected udder health data could make it possible to identify parameters that are related to mastitis susceptibility, the Milk Genomics study was not primarily designed for this. The Milk Genomics study was also not suitable to determine the relation of immune parameters with health disorders, other than mastitis, because these data were not collected. Therefore, a field study was conducted that was specifically designed to validate if NAb titers

present in milk samples taken during the first 3 months of lactation (i.e. the period with most health problems) had a prognostic value for susceptibility to CM, high SCC or other diseases later in that lactation (*Chapter 6*). In the general discussion (*Chapter 7*) a synthesis of all the results is given and the prospects to use NAb titers in milk as predictive immune parameters for susceptibility of the individual animal for mastitis and other health problems, and for selective breeding are discussed.

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Chapter 2

Cytokines TNF-α and IFN-γ, types and reactive oxygen species production of cells: repeatability and variation in bovine milk and blood

Parts of this chapter have been published in

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ABSTRACT

The defense mechanism of dairy cows against mastitis is based partly on their naturally present disease resistance capacity - their innate immune system. Various cell types and humoral factors are part of the innate immune system. Our hypothesis is that these innate immune parameters contribute to the cow's capacity to resist mastitis and possibly other diseases; that their amounts in milk can be used as objective parameters to routinely monitor, in a non-invasive manner, the level of natural resistance of dairy cows. To be appropriate as markers of natural resistance and to study heritability, immune parameters should show variation among cows and repeatability within cows over time. For this study 10 milk and blood samples from each of 20 clinically-healthy cows were taken during a 17 day period. In these samples presence of different cell types, radical production by cells, and levels of the cytokines tumor necrosis factor α and interferon γ were determined. Of all tested immune cells only the γδ-T-cells fulfilled the criteria for variation among cows and repeatability within cows over time, but this could only be demonstrated for blood and not for milk cells. Cytokine levels showed variation among cows and repeatability within cows over time in blood as well as in milk samples. However, for most samples the tumor necrosis factor α and interferon γ levels appeared to be around or below the minimal reliable detection limit of the used tests. Therefore, none of the immune parameters tested in this study were considered suitable for further study in milk samples.

INTRODUCTION

Natural disease resistance of cows can, among many other factors, play a role in preventing and fighting mastitis and other diseases. Several immune parameters have been investigated following experimental infection of the mammary gland with different pathogens. Among others, several cytokines like tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) or their mRNA levels were measured following experimental infection with *Staphylococcus aureus* or *Escherichia coli* (Bannerman *et al.*, 2004; Lee *et al.*, 2006; Shuster et al., 1997). Kornalijnslijper et al. (2004) and Mehrzad et al. (2004, 2005 and 2009) demonstrated that the severity of experimental clinical mastitis is related to the quantity and quality of neutrophils and speed of recruitment to the site of infection. Neutrophils and macrophages can produce reactive oxygen species (ROS, oxygen radicals) that help destroy phagocytosed pathogens (Pastoret et al., 1998; Tizard, 2004). However, knowledge about the relationship between different immune parameters in clinically-healthy animals and their natural resistance to diseases, is scarce. Identification of such parameters can enable the development of tests to monitor objectively in a non-invasive way the level of natural resistance to mastitis or other diseases. Monitoring of natural resistance may support the farmer in managing the herd. Based on literature, parameters were selected that might be correlated with resistance to diseases. A suitable parameter needs to show variation among different animals, otherwise it can never be related to variations in disease susceptibility. Furthermore, a parameter has to be repeatable when measured a number of times during a relatively short time-span of a few weeks. If this is not the case the parameter will most likely not be a good indicator for the cow's natural resistance. In this chapter cytokines TNF-α and IFN-γ, and cellular composition (e.g. B, T and NK cells) and ROS production by phagocytic cells were studied. The hypothesis was that basal levels of cytokines and cellular composition, and the capacity of cells to produce ROS after *ex vivo* stimulation might be related with natural resistance. The aims of this study were to investigate if these parameters meet the following two criteria; 1) show variation among cows; 2) show repeatability within cows over time.

MATERIALS AND METHODS

Animals and samples

Ten milk and blood samples were collected from each of 20 clinically-healthy, Holstein-Friesian cows from the herd at the experimental farm of Wageningen University over a period of 17 d. Selected cows were 17 clinically-healthy cows that had a somatic cell count (SCC) lower than 150,000 cells/mL at the previous test-day milk recording, and 3 clinically-healthy heifers without previous SCC measurement. At the start of the experiment the number of days in lactation ranged from 27 to 195. Six cows were in their first lactation and the oldest cow was in the seventh lactation. One cow developed clinical mastitis during the experiment and was excluded from the analysis. None of the other cows required a veterinary treatment during the experiment, and none of the cows received vaccinations.

Cows were milked twice a day, and received no other treatments. At days 1 and 3, samples were taken in the morning only, and at days 8, 10, 15 and 17, in the morning and afternoon. Milk was sampled during routine milking. Just after milking, blood samples were taken using 10 mL vacutainers with K2EDTA (BD, Plymouth, PL67BP, United Kingdom). Milk and plasma samples were aliquoted and stored at -20°C at the day of collection.

Cytokines: TNF-α and IFN-γ

Concentrations of TNF-α and IFN-γ in milk and plasma samples were determined by a sandwich ELISA that was developed in house. High binding 96-well plates (Greiner Bio-one, Kremsmünster, Austria) were coated with 1 μg/mL monoclonal mouse anti bovine TNF-α or IFN-γ capture antibody that were both developed in house, dissolved in 1x phosphate buffered saline solution (PBS). After overnight incubation at 4°C plates were emptied and blocked overnight at 4°C with 150 μL of Universal Casein Diluent/Blocker (UCDB) (Stereospecific Detection Technologies, Baesweiler, Germany). After washing (3 times) with PBS/0.05% Tween20 plates were incubated with milk or plasma samples diluted with an equal part of UCDB for 2 h at 21°C. On each plate a calibration curve of recombinant bovine IFN-γ or TNF-α (both R&D systems, Minneapolis, M.N., U.S.A) was included. Following this incubation period, plates were washed (3 times) and incubated for 1 h at 21°C with 100 μL/well of a biotinylated polyclonal chicken IgY antibody affinity purified against recombinant bovine TNF-α or IFN-γ (both produced in house) diluted in UCDB at a concentration of 0.250 μg/mL. After washing (5 times) plates were incubated for 30 min at 21°C with 100 μL/well horseradish peroxidase conjugated streptavidin (Stereospecific Detection Technologies, Baesweiler, Germany) diluted to 0.2 μg/mL in UCDB. After washing the plates (6 times) they were incubated with 100 μL/well tetramethylbenzidine (Stereospecific Detection Technologies, Baesweiler, Germany) reagent. After 20 min, the reaction was stopped by the addition of 100 μL/well of 1% hydrochloric acid and the extinctions were read at 450 nm corrected at 695 nm with a Multiskan spectrophotometer (Molecular Devices, Sunnyvale C.A., U.S.A). The corresponding concentrations (pg/mL) were subsequently calculated with use of a regression line, based on the calibration curves on every plate.

Cell isolation

Cells were isolated from fresh milk by diluting milk with an equal volume of culture medium, RPMI-1640 with HEPES and L-glutamine (RPMI) (BioWhittaker, B-4800, Verviers, Belgium) and centrifuging for 30 min at 750 *g*, after which the supernatant was discarded. Cells were resuspended in RPMI and washed twice. Blood was centrifuged for 20 min at 750 *g*, 4°C. After removing plasma (for cytokine detection) the layer of white blood cells and some of red blood cells beneath were pipetted off and transferred into a 50 mL tube. White blood cells were isolated by lysis of red blood cells with ultra-pure water (hypotonic shock) followed by rapid addition of 2x PBS for reconstitution of an isotonic environment. This procedure was repeated, and the cells were subsequently washed and resuspended in RPMI.

Radical production and cell type identification

Cells were resuspended to 10^7 cells/mL in RPMI supplemented with 2 mM L-glutamin, 10% fetal calf serum, penicillin (50 i.u./mL) and streptomycin (50 µg/mL) (complete medium) to measure reactive oxygen species (ROS) production. Intracellular ROS levels were measured using the redox sensitive dye dihydrorhodamine 123 (DHR) at 0.25 µg/mL. Cells were stimulated with 0.05 µg/mL phorbol 12-myristate 13-acetate (PMA) to prime the cells to react, in combination with 0, 5, 20 or 50 µg/mL *S. aureus*-derived peptidoglycan (PGN) (BioChemika, Buchs, CH) or left without stimulation as negative controls. To all samples, except for 1 extra negative control without any treatment, DHR was added and incubated for 1 h at 37°C. Just before fluorescence measurement in a FACScan® flow cytometer (Beckman Coulter, Epics XL-MCL, Miami, Florida USA), propidium iodide (PI, 0.1 mg/ml; Sigma, St. Louis, MO) was added to stain dead cells so that these could be excluded from the analysis. Reactive oxygen species production of white blood cells isolated from 3 morning blood samples from 10 cows was measured. Samples were tested in triplicate. At the present status, the cells isolated from milk did not permit such an analysis.

White blood and milk cells were tested for presence of CD4 (T cells), CD8 (T cells), CD21 (B cells), NK-p46 (NK cells), and WC1 (γδ-T cells) surface markers (CD markers) to identify cell types, using fluorescent dye (phycoerythrin (PE) or fluorescein isothiocyanate (FITC)) labeled antibodies (Serotec, Düsseldorf, Germany). T-cells, monocytes and granulocytes were gated by forward-sideward scatter in the case of white blood cells, but not in the case of milk cells. Cells were incubated with the antibodies for 30 min, washed and resuspended in RPMI with 1% bovine serum albumin and 0.01% sodium azide, and measured by flow cytometry.

Statistical analysis

Variation among cows of immune parameters was analyzed using a mixed model in SAS 9.1 (SAS Institute Inc., Cary, NC, USA):

$$y_{ijk} = \mu + period_i + cow_j + error_{ijk} \qquad [1]$$

where $period_i$ is fixed effect of period of measurement (i = 1-10 in case of NAb and 1-3 in case of cellular parameters), cow is random effect of cow and e is random residual effect.

Repeatability within cows over time was initially tested by calculating Pearson correlation coefficients in Statistical Analysis Software (SAS) 9.1. This was, however, comparable to the following method where repeatability (r) within cows was calculated as:

$$r = \frac{\sigma^2_{cow}}{\sigma^2_{cow} + \sigma^2_e} \qquad [2]$$

where σ^2_{cow} was variance among cows and σ^2_e residual variance (Falconer and MacKay, 1996).

RESULTS

Cytokines: TNF-α and IFN-γ

Levels of TNF-α and IFN-γ in milk and blood plasma showed significant variation among cows ($P < 0.01$) and repeatability ranged between 0.84 and 0.99 (Table 1). However, this was mostly due to 1 cow that consistently showed much higher values compared to the other cows. When the observations of that particular cow were excluded from the analysis, repeatability for IFN-γ levels decreased to 0.66 and 0.61 for plasma respectively milk, and to 0.57 for TNF-α in plasma. Repeatability for TNF-α in milk was very low (0.05). For the majority of cows the TNF-α en IFN-γ levels turned out to be around or below the minimal reliable detection limit of the test (approximately 40 pg/mL).

Table 1. Repeatability estimates of TNF-α and IFN-γ levels in milk and plasma

Parameter	Plasma	Milk
TNF-α	0.99	0.84
IFN-γ	0.99	0.99

Reactive oxygen species production and cell types

Reactive oxygen species production and cell types were analyzed for variation among cows and repeatability within cows over time. Figure 1 shows an example of FACS data on ROS production of 1 cow following different stimuli. Cells stimulated with PGN produced more ROS compared to control cells or cells primed with PMA only, as measured by DHR fluorescence.

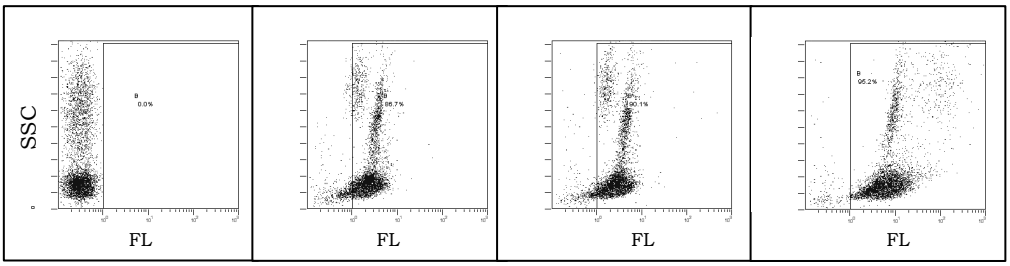

Figure 1. Reactive Oxygen Species production. Figures show, from left to right, only cells, cells plus dihydrorhodamine 123 (DHR) (0.25 µg/ml), cells plus DHR (0.25 µg/ml) plus phorbol 12-myristate 13-acetate (PMA) (0.05 µg/ml), and cells plus DHR (0.25 µg/ml) plus PMA (0.05 µg/ml) plus peptidoglycan (PGN) (50 µg/ml). The y-axis shows the sideward scatter (SSC) and the x-axis shows the level of fluorescence (FL).

Table 2 shows mean fluorescence intensity with standard deviations, variation among cows and repeatability within cows over time of the ROS measurements. The data showed high standard deviations during the 17 d sampling period.

Table 2. Mean fluorescence intensity of cells without any treatment (No), with only dihydrorhodamine 123 (DHR) added, or stimulated with DHR plus phorbol 12-myristate 13-acetate (PMA) plus 0, 5, 20 or 50 µg/ml peptidoglycan (PGN) (indicated as PGN0, PGN5, PGN20 and PGN50); standard deviation (SD), *P* value of variation among cows and repeatability within cows over time

Stimulus	No	DHR	DHR+ PMA PGN0	DHR+ PMA PGN5	DHR+ PMA PGN20	DHR+ PMA PGN50
Mean (Overall SD)	2.86 (2.98)	3.46 (0.92)	8.76 (8.22)	11.80 (7.03)	19.87 (8.64)	25.52 (11.41)
P value of variation among cows	0.18	0.69	0.94	0.54	0.68	0.23
Repeatability	0.37	0.27	0.12	0.08	0.21	0.58

No significant variation (P > 0.05) among cows was found for ROS and CD markers, except for the WC1 marker for γδ-T cells. Repeatability of ROS data ranged from 0.08 to 0.58. Repeatability of CD markers ranged from 0 (CD4 and CD8 on blood cells and NK on milk cells) to 0.84 (WC1 on blood cells) (Table 3).

Table 3. Mean percentage of cells, standard deviation (SD), *P* value of variation among cows and repeatability within cows over time for CD8, NK, CD21, CD4, WC1, and granulocytes, lymphocytes and monocytes in white blood cells

Blood cells	CD8	NK	CD21	CD4	WC1	Granulocytes	Lymphocytes	Monocytes
Mean (Overall SD)	6 (3.24)	0.59 (0.52)	33.67 (10.76)	2.12 (2.71)	3.03 (3.41)	11.25 (7.17)	53.29 (11.17)	13.66 (6.23)
P value of variation among cows	0.67	0.22	0.079	0.14	0.042	0.27	0.14	0.091
Repeatability	0	0.19	0.33	0	0.84	0.26	0.40	0.59
Milk cells[1]	CD8	NK	CD21					
Mean (Overall SD)	7.75 (10.78)	0.39 (0.48)	0.21 (0.33)					
P value of variation among cows	0.16	0.42	0.16					
Repeatability	0.46	0	0.46					

[1]For milk cells at the present status, only CD8, NK and CD21 could be properly measured.

DISCUSSION

The hypothesis was that ROS production, numbers of different cell types and cytokines might have predictive value for disease resistance, because of their microbicidal or communicative functions in relation to infection (Bannermann, 2009; Pastoret et al., 1998; Rainard and Riollet, 2006). To study the relationship between natural disease resistance and selected immune parameters, these parameters should first show variation among individual cows and repeatability within individual cows over time. Reactive oxygen species production and most cell types did, however, not comply with the criteria by not showing significant variation among cows, and only low to moderate repeatability. For cell types and to a lesser degree also for ROS production, this is probably due to the high variation observed within cows over time that lowered variation among cows and repeatability of measurements over time. Immune cells rapidly respond to infection, thereby impeding their use for studying a predictive relation with natural resistance of cows against mastitis. Only the percentage of γδ-T cells in white blood cells did comply with the criteria for variation among cows and repeatability within individual cows. However, this could not be confirmed for milk because detection of these cells in milk was technically problematic. Assessment of the percentage of γδ-T cells in individual cows would therefore require the sampling of blood that is impractical for routine screening.

Levels of TNF-α and IFN-γ demonstrated significant variation among individual cows and very high repeatability within individual cows (Table 1). However, repeatability reduced considerably when observations on a single cow with high levels of both cytokines were excluded from the analysis. In that case the repeatability for TNF-α in milk became very low (0.05). Levels of TNF-α and IFN-γ turned out to be around or below the minimal reliable detection limit of the test for the majority of cows, thereby compromising accurate distinction between these animals. Indeed, when the same tests were used to screen single milk samples of nearly 2000 clinically-healthy cows (heifers), less than 5 percent of the animals showed TNF-α and IFN-γ levels well above the minimal reliable detection limit of approximately 40 pg/mL (unpublished results). Tumor necrosis factor α and IFN-γ are key cytokines in the control of many bacterial infections. Tumor necrosis factor α induces inflammation by recruiting leukocytes, among which neutrophils, to a site of infection (Bannermann, 2009; Rainard and Riollet, 2006) to phagocytose and kill pathogens. Interferon γ is an important cytokine for the activation of neutrophils, and increased killing of pathogens (Bannermann, 2009; Sordillo and Babiuk, 1991). However, the hypothesis that differences in basal levels of cytokines among individual cows could have a relation with natural resistance and would be suitable to study their predictive value for disease resistance, could not be corroborated. This was due to the generally low levels of TNF-α and IFN-γ in the fast majority of clinically-healthy cows, despite significant variation among individual cows and generally sufficient repeatability over time.

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Let it be

(The Beatles)

Chapter 3

Natural antibodies in bovine milk and blood plasma: variability among cows, repeatability within cows, and relation between milk and plasma titers

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ABSTRACT

Innate immunity plays an important role in preventing (barrier function) or combating infection (effector function). An important humoral component of innate immunity is formed by natural antibodies (NAb). The objectives of this study were to determine presence, variation among cows and repeatability within cows over time of total NAb titers directed to the pathogen-associated molecular patterns lipopolysaccharide, lipoteichoic acid (LTA) and peptidoglycan, and the glycoprotein keyhole limpet hemocyanin in milk and plasma of individual cows. Furthermore in milk the antibody isotypes IgG1, IgG2, IgM and IgA binding LTA were analyzed. Ten milk and blood samples were obtained from each of 20 clinically-healthy dairy cows from first to seventh parity during a period of 3 weeks. Total NAb binding lipopolysaccharide, LTA, peptidoglycan, and keyhole limpet hemocyanin were detected in milk and plasma, with titers considerably higher in plasma than in milk. Total NAb titers showed significant variation among cows, and repeatability within cows over time (ranging from 0.60 to 0.93). Titers of NAb in milk and plasma were positively correlated (correlation ranging from 0.69 to 0.91). Natural antibodies in milk binding LTA were of all 4 isotypes tested, although IgG2 was on average only present at low titers. All 4 isotypes in milk binding LTA also showed variation among cows, and repeatability within cows over time (ranging from 0.84 to 0.92). We conclude that NAb can be measured in a consistent and repeatable manner in bovine milk and blood plasma.

INTRODUCTION

Innate immunity plays an important role in preventing or combating infection. Knowledge of protection from intra-mammary or other infections of cattle by (components of) the innate immune system is scarce. A potentially important humoral component of the innate immune system is formed by natural antibodies (NAb) (Baumgarth et al., 2005; Elluru et al., 2008), defined as antibodies present in healthy animals without (known or deliberate) antigenic stimulation (Star et al., 2007; Van Knegsel et al., 2007).

In mammals, NAb are thought to be derived from B1 cells (Casali and Notkins, 1989; Baumgarth et al., 2005). Natural antibodies are generally characterized by a broad specificity repertoire, with usually low binding affinity (Casali and Notkins, 1989). Titers of NAb increase with age in cattle in blood and also in milk (Srinivasan et al., 1999; Van Knegsel et al., 2007), suggesting that NAb are the cumulative result of antigenic stimulation of B1 B cells (Tomer and Shoenfeld, 1988).

A high proportion of NAb binds so-called pathogen-associated molecular patterns (PAMP). These represent antigens that are shared by 'classes' of microbes and therefore serve as targets for identification of various microbes by the immune system. Important PAMP are LPS present on gram-negative (entero)bacteriacea, such as *Escherichia coli* or *Salmonella spp.*; lipoteichoic acid (LTA) present on gram-positive bacteria, such as *Staphylococcus aureus*; and peptidoglycan (PGN) present on gram-negative and gram-positive bacteria. A relationship was suggested between NAb titers binding the antigen Keyhole Limpet Hemocyanin (KLH) or LPS, and body condition, energy balance, milk yield and plasma cholesterol concentration in the peri-partum period (Van Knegsel et al., 2007).

Natural antibodies could act as a first line of defense against invading pathogens once these pathogens passed physical barriers (Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000; Baumgarth et al., 2005). Natural antibodies in milk could therefore be potential parameters for resistance of dairy cows to mastitis and other (infectious) diseases. To be a potential parameter for disease resistance an immune parameter should be variable among individual cows, be repeatable within cows over time, and preferably show a correlation for titers measured in blood and milk. When an immune parameter does not vary among cows, while natural disease susceptibility in general does vary among cows, this immune parameter would not be a useful parameter for natural disease resistance. Repeatability of parameters is important to assess the status of natural resistance of a cow using a single or only a few measurements. Furthermore, in the case of a high correlation between blood and milk parameters, milk would offer a possibility to monitor systemic immunity in addition to local mammary immunity.

The objectives of our study were 5-fold. First, we determined the presence of NAb binding LPS, LTA, PGN, and KLH in milk and plasma of clinically-healthy, lactating dairy cows. Second, we estimated variation of NAb titers among cows in milk and plasma. Third, we measured repeatability of NAb titers within cows in milk and plasma over 3 weeks. Fourth, we investigated the correlation of the antibody titers between milk and blood plasma. Finally, we determined the isotypes of NAb in milk binding LTA. The prospects of measuring NAb titers in milk are discussed.

MATERIALS AND METHODS

Animals and samples

From the dairy herd at the experimental farm of Wageningen University, we selected seventeen clinically healthy cows that had a somatic cell count (SCC) lower than 150,000 cells/mL at the previous test-day milk recording, and three heifers without previous SCC measurement. At the start of the experiment the number of days in lactation ranged from 27 to 195. Six cows were in their first lactation and the oldest cow was in the seventh lactation. One cow developed clinical mastitis during the experiment and was excluded from the analysis. None of the other cows required a veterinary treatment during the experiment, and none of the cows received vaccinations.

A total of 10 milk and 10 blood samples were obtained from each cow during a 17-day period. Samples were taken on day 1 and 3 in the morning only, and on day 8, 10, 15 and 17 in the morning and in the afternoon each sampling day. Cows were milked twice daily, and milk was sampled during routine milking. During milking milk was collected in milk glasses. After milking the milk in the glass was mixed and collected via a tap from the glass into 50 mL tubes. Milk samples were aliquoted at the day of collection, and stored at -20°C until use. Immediately after milking, 50 mL of blood was taken from the tail vain using 10 mL K_2EDTA-vacutainers (BD, Plymouth, PL67BP, United Kingdom). Blood plasma was obtained by centrifugation of the blood for 20 min at 750 *g*, and stored at -20°C until analysis.

Total natural antibodies in plasma and milk and antibody isotypes in milk

In milk and plasma samples, NAb titers binding either *Escherichia coli*-derived LPS (L2880, serotype O55:B5), *Staphylococcus aureus*-derived LTA (L2515) from Sigma-Aldrich Inc. (St. Louis, MO), *S. aureus*-derived PGN (BioChemika, Buchs, CH), and *Megathura crenulata-derived* KLH from MP Biomedicals (Solon, Ohio) were determined. In milk also the isotypes, IgG1, IgG2, IgM and IgA of antibodies binding LTA were measured. Titers of NAb were determined using an indirect ELISA procedure, essentially as described by Van Knegsel et al. (2007). Plates were coated with 100 µL/well of either 1 µg KLH, 4 µg LPS, 5 µg LTA or 2 µg PGN per mL of carbonate buffer (10.6 g/L Na_2CO_3, pH 9.6). After washing, plates were incubated with 100 µL/well 2.5% rabbit serum in PBS with 0.05% Tween20 for at least 30 min at room temperature. Serial dilutions of plasma (1:4) or milk (1:4) in PBS, 0.05% Tween20, and 2.5% rabbit serum were added, dilutions started at 1:40 for plasma and 1:30 for milk samples for analysis of total antibodies, or dilutions started 1:4 for milk samples for isotype analysis. On each plate the same positive sample was included with 8 serial dilutions (1:2) in duplicate. Plates were incubated for 1 h at room temperature. Binding of total antibodies to LPS, LTA, PGN or KLH was detected using 100 µL/well 1:15,000 diluted Rabbit Anti-Bovine IgG (H + L) (recognizing total Ig of all isotypes) coupled to peroxidase (Sigma-Aldrich Inc.). Binding of isotype antibodies to LTA was detected using 1:16,000 diluted sheep anti-bovine IgG1 (Serotec, Düsseldorf, Germany), 1:16,000 diluted sheep anti-bovine IgM (Serotec), 1:1,600 diluted sheep anti-bovine IgG2 (Serotec), or 1:8,000 diluted sheep anti-bovine IgA (Serotec). After washing, 100 µL/well tetramethylbenzidine (71.7

μg/mL) and 0.05% H_2O_2 were added to the wells, and incubated for 10 min at room temperature. The reaction was stopped with 50 μL/well 2.5 *N* H_2SO_4. Extinctions were measured with a Multiskan (Flow, Irvine, UK) at a wave length of 450 nm. Titers were expressed as Log_2 values of the dilutions that gave an extinction closest to 50% of Emax, where Emax represents the highest mean extinction of a standard positive milk sample present in duplo on every microtiter plate (Ploegaert et al., 2007; Van Knegsel et al., 2007).

Statistical analysis

Variation among cows and repeatability within cows over time were estimated using the following mixed model:

$$Y_{ij} = \mu + Time_i + Cow_j + e_{ij} \qquad [1]$$

where Y_{ij} is the NAb titer of Cow_j at $time_i$, μ = the common mean. Time is the fixed effect of time of measurement i (i = 1, …, 10). Cow is the random effect of cow_j (j = 1,…, 19; normal, independent and identically distributed (0, σ^2_{cow})) and e_{ij} is the random residual (normal, independent and identically distributed (0, σ^2_e)). Repeated measures analysis was performed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC; version 9.1). Covariance structure used was compound symmetry ($\sigma_1 + \sigma^2 1$ (i = j)). Model assumptions regarding normality were evaluated by examining whether skewness and kurtosis were close to 0 and whether a probability plot did not show deviations from a straight line. Repeatability (r) within cows was calculated as:

$$r = \frac{\sigma^2_{cow}}{\sigma^2_{cow} + \sigma^2_e} \qquad [2]$$

where σ^2_{cow} is the variance among cows and σ^2_e is the residual variance.

Relationship between milk and blood plasma was analyzed by calculation of the Pearson correlation coefficient between the average value of the 10 observations per cow of the antibodies in milk and in blood plasma.

RESULTS

Presence of total natural antibodies in plasma and milk

Natural antibodies binding LPS, LTA, PGN or KLH, were detected in bovine milk and blood plasma at 10 time points. The NAb binding these 4 antigens were present both in plasma and milk. Figure 1 shows means and standard deviation of (Log_2) NAb titers binding LPS, LTA, PGN or KLH, in milk and in plasma from 19 cows. In milk, the lowest antibody titers were those binding LPS and averaged 1.91, whereas titers of antibodies binding LTA and PGN had the highest average at 4.53 (Table 1). In plasma, titers of antibodies binding LPS (7.91) and LTA (7.89) averaged lowest, whereas titers of antibodies binding PGN (11.41) averaged highest. Titers of NAb binding the four antigens were consistently present, but higher in plasma than in milk.

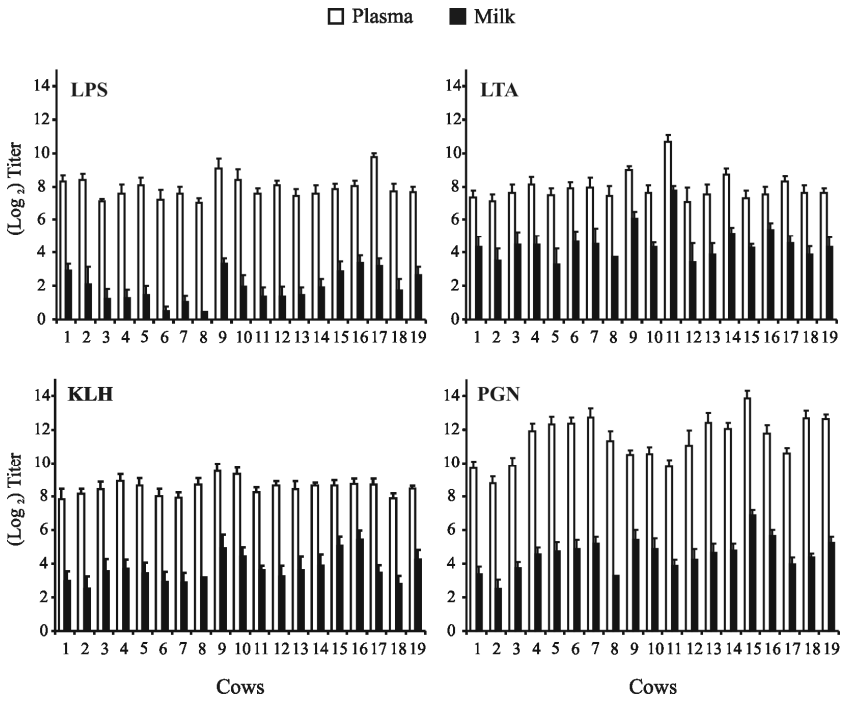

Figure 1. Mean total natural antibody (Log_2) titers binding lipopolysaccharide (LPS) (left upper), lipoteichoic acid (LTA) (right upper), peptidoglycan (PGN) (left lower) and keyhole limpet hemocyanin (KLH) (right lower) per cow in plasma (white bars) and milk (black bars) during a 17-day period. Error bars show standard deviations within cows. The numbers on the X-axis represent 19 individual cows.

Variation of total natural antibody titers among individual cows

Natural antibody titers binding LPS, LTA, PGN, and KLH were studied for variation among individual cows. Table 1 shows differences among cows of NAb titers binding LPS, LTA, PGN or KLH, in plasma and in milk. The antibody titers showed significant variation among individual cows ($P < 0.01$) for each of the antigens analyzed.

Table 1. Total natural antibody (Log_2) titers binding lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN) and keyhole limpet hemocyanin (KLH) in blood plasma and milk; Means, overall standard deviations, standard deviations (SD) within and among cows, significance of variation among cows, and estimates of repeatability

	Milk				Plasma			
Parameter	LPS[1]	LTA[1]	PGN[1]	KLH[1]	LPS[1]	LTA[1]	PGN[1]	KLH[1]
Mean	1.91	4.53	4.53	3.67	7.91	7.89	11.41	8.50
Overall SD[2]	1.02	1.11	1.03	0.85	0.73	0.90	1.34	0.55
SD within cows[3]	0.52	0.48	0.41	0.33	0.34	0.40	0.36	0.35
SD among cows[4]	0.87	1.00	0.95	0.78	0.65	0.81	1.29	0.42
Variation among cows (*P* value)	0.003	0.003	0.003	0.003	0.003	0.003	0.002	0.004
Repeatability	0.74	0.81	0.84	0.85	0.79	0.80	0.93	0.60

[1]10 samples from each of 19 cows were analyzed, and measured antibody titers were total Ig
[2]Overall SD = $\sqrt{(\sigma^2_{cow} + \sigma^2_e)}$
[3]SD within cows = $\sqrt{\sigma^2_e}$
[4]SD among cows = $\sqrt{\sigma^2_{cow}}$

Repeatability of total natural antibody titers

Natural antibody titers of cows were determined at 10 time points to assess their repeatability. As a representative example, Figure 2 shows NAb titers binding PGN in milk and in plasma of individual cows over time. Triangles show the average titer of all the cows at the different time points. Overall, Figure 2 shows little variation in mean level over time within cows. Cows that initially had higher NAb titers than other cows remained higher throughout the 3-weeks observation period. The same was true for cows with lower titers. Table 1 shows the estimated repeatability of the antibody titer measurements for the 4 antigens in plasma and in milk. In plasma, antibody titers binding the naïve (never encountered before by cows) antigen KLH showed lowest repeatability (0.60), whereas antibody titers binding PGN showed the highest repeatability (0.93). Repeatability of NAb titers in milk ranged from 0.74 (LPS) to 0.85 (KLH).

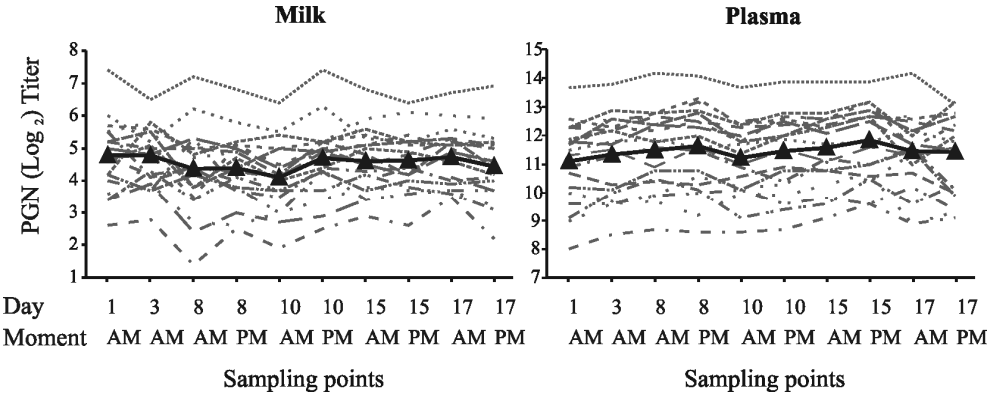

Figure 2. Total natural antibody (Log_2) titers binding peptidoglycan (PGN) from *S. aureus* in milk (left) and in plasma (right) for individual cows (n = 19) over time (10 time points); larger triangles connected with a line represent the average of all cows over time. Other lines show measurements for individual cows. The x axis shows sampling points: the days of the blood and milk sampling (numbers) and the moment of the day (morning (AM) or afternoon (PM)).

Correlation of total natural antibody titers in blood versus milk

Correlation between mean milk and blood plasma titers of NAb binding LPS, LTA, PGN, and KLH are shown in Figure 3, along with the correlation coefficients. The titers of NAb binding KLH showed the lowest correlation (0.69) between milk and blood plasma, whereas titers of NAb binding LTA showed the highest correlation (0.91).

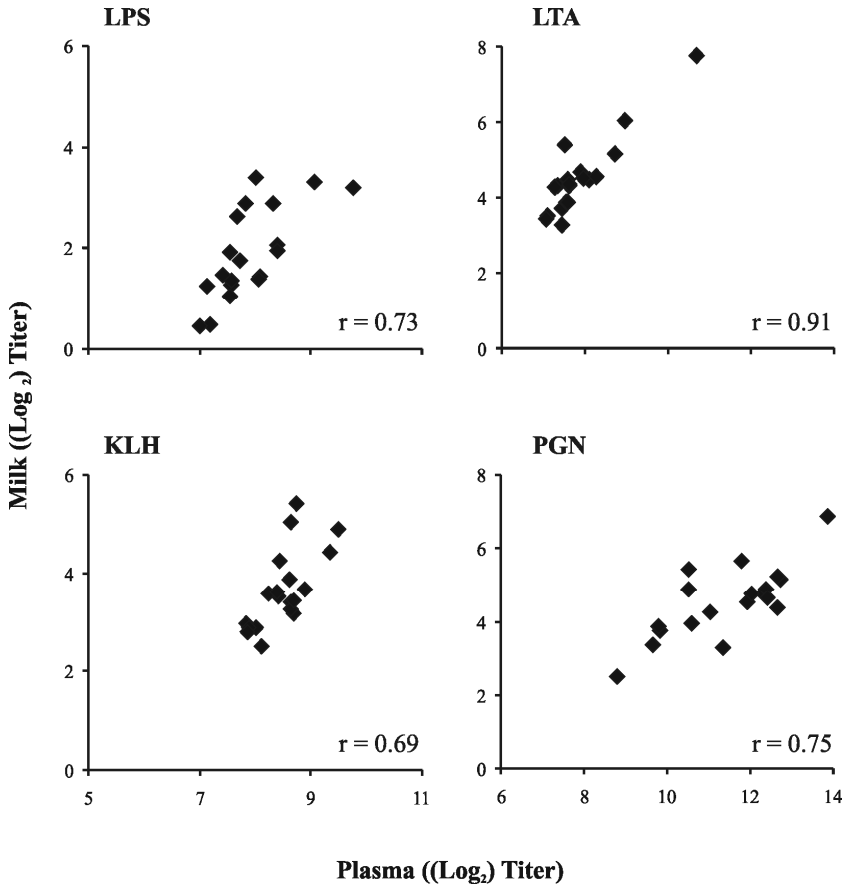

Figure 3. Correlation (r) between mean total antibody titers binding LPS (left upper; n = 19), lipoteichoic acid (LTA) (right upper; n = 19), keyhole limpet hemocyanin (KLH) (left lower; n = 19) and peptidoglycan (PGN) (right lower; n = 19) in milk and blood on 19 cows. The x axes show mean (Log_2) natural antibody titers in plasma, and y axes show the mean (Log_2) natural antibody titers in milk.

Presence of natural antibody isotypes binding lipoteichoic acid in milk

The means and standard deviation of the fractions of NAb belonging to the 4 known bovine isotype antibodies IgG1, IgG2, IgM, and IgA binding LTA, as measured in milk samples from 19 cows, are shown in Table 2. Titers of IgG2 binding LTA averaged lowest (0.05), and had the highest standard deviation. Titers of IgM binding LTA averaged highest (2.86), with IgG1 (1.76) and IgA (2.09) NAb titers being intermediate. Thus, IgG1, IgG2, IgM, and IgA isotypes of NAb binding LTA are present in milk, albeit IgG2 at low titers.

Variation among cows and repeatability of natural antibody isotypes

Table 2 shows differences among cows and repeatability within cows over time for isotype NAb titers. The (PROC MIXED) analyses revealed variation among cows ($P < 0.01$) for all 4 isotypes, and a repeatability within cows over time of at least 0.84.

Table 2. Antibody isotype (Log_2) titers for IgG1, IgG2, IgM, and IgA binding lipoteichoic acid in milk (n = 19). Means, overall standard deviations, standard deviations within and among cows, *P* value of variation among cows, and estimates of repeatability

Parameter	IgG1[1]	IgG2[1]	IgM[1]	IgA[1]
Mean	1.76	0.05	2.86	2.09
Overall SD[2]	1.27	2.15	0.80	0.92
SD within cows[3]	0.39	0.61	0.32	0.37
SD among cows[4]	1.20	2.06	0.74	0.84
Variation among cows (*P* value)	0.002	0.002	0.003	0.003
Repeatability	0.91	0.92	0.84	0.84

[1]10 samples from each of 19 cows were analyzed
[2]Overall SD = $\sqrt{(\sigma^2_{cow} + \sigma^2_e)}$
[3]SD within cows = $\sqrt{\sigma^2_e}$
[4]SD among cows = $\sqrt{\sigma^2_{cow}}$

DISCUSSION

Increased incidences of (infectious) diseases in cattle were related with suboptimal functioning of the specific immune system (Kehrli et al., 1989; Mallard et al., 1998). Little is known, however, of the role of various components of the innate immune system in preventing disease. Natural antibodies (NAb) represent an important humoral component of the innate immune system (Avrameas, 1991; Baumgarth et al., 2005; Elluru et al., 2008). They are defined as antibodies present in healthy animals without (known or deliberate) antigenic stimulation (Star et al., 2007; Van Knegsel et al., 2007). The high levels of polyreactive NAb in serum and mucosa from mammals and birds suggest important effector and regulatory functions of NAb, e.g. by enhancing various specific immune responses (Ochsenbein et al., 1999; Quan et al., 1997; Stager et al., 2003; Thornton et al., 1994). Most NAb are polyreactive to phylogenetically conserved structures, such as PAMP (Boes, 2000; Jalkanen et al., 1983; Koppenheffer, 2007). Additionally, high levels of NAb binding PAMP have been related to higher resistance to pathogens bearing these PAMP (reviewed by Kohler et al., 2003). Natural antibodies, therefore, play an important role in the first line of defense of animals against pathogens once these pathogens passed physical barriers (Baumgarth et al., 2005; Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000), and may have a relationship with resistance to mastitis and other diseases.

In plasma NAb titers can be easily detected. In milk, however, only few studies were performed. Average titers of total NAb binding LPS and KLH in bovine milk and blood were reported earlier for groups of cows with different diets, in the beginning of lactation when suffering from negative energy balance (Van Knegsel et al., 2007). We now show total NAb titers binding LPS, KLH, PGN and LTA in milk and blood plasma, as well as NAb isotypes (IgG1, IgG2, IgM, IgA) binding LTA in milk, and their variation among cows and repeatability, in individual clinically-healthy cows. Lipoteichoic acid is a commonly present PAMP on gram-positive bacteria, such as *S. aureus,* that often causes (subclinical) intra-mammary infection in cattle.
For NAb titers to be potential parameters for resistance to mastitis and other diseases, the NAb titers should be variable among individual cows, repeatable within cows over time, and preferably easy to measure in samples that can be obtained in a non-invasive way (for instance milk samples). Before embarking on the relation between NAb titers and resistance to diseases, this study focused on estimating variation and repeatability of NAb titers in cows to study their suitability as potential parameters for disease resistance. In addition, we estimated the correlation between NAb titers measured in milk and blood.

Healthy cows generally do not have an identical resistance to diseases. If all healthy cows would have equal NAb titers, then these NAb titers could not be potential predictive parameters for disease resistance of the cow. Variation in NAb titers among cows was found (Table 1). Furthermore, in milk we showed repeatability for total NAb titers ranging from 0.74 to 0.85, and in plasma 0.79 to 0.93 for the NAb binding LPS, LTA, and PGN, and 0.60 for the NAb binding KLH (Table 1). Repeatabilities of daily milk, fat, and protein yields ranging from 0.63 to 0.83, at different stages of lactation, were reported (Vasconcelos et al., 2004). Our estimates are comparable to repeatability for daily milk production traits (Vasconcelos et al., 2004). Effects of measurement errors can be reduced by taking a repeated measurement of the NAb titer. Based on our repeatability estimates, the benefit of repeating the measurement at the expense of sampling fewer animals is predicted to be low and a single measurement will suffice for determining the NAb titer.

We cannot exclude that the measured NAb titers binding LPS, LTA, and PGN might be in part the result of an active adaptive immune response. During an active adaptive immune response to an infection with specific microbes, specific antibody titers rise within 7 to 14 days and then decrease again. However, the major amount of NAb binding LPS, LTA, or PGN, in the current study of clinically healthy cows probably does not reflect a specific antibody response to a current or recent infection with specific microbes. This is because of the lack of a rise or a decrease in titers during the study, also represented in the repeatability of the measured NAb. Taken together, this study showed that, in clinically healthy cows, total NAb titers binding LPS, LTA and PGN, as well as NAb binding KLH, were relatively constant over time. Binding of plasma and milk NAb to KLH, to which animals were not primed, might reflect innate humoral immune competence. Future studies should reveal whether variation in NAb titers is predictive for differences in disease resistance among cows.
Milk is easily accessible without invasive procedures. Our results showed that, although NAb titers in plasma were considerably higher than in milk, correlation

between NAb titers in blood and milk was high, varying between 0.69 and 0.91. Therefore, NAb titers in milk give a reasonable estimation of systemic NAb titers.

Natural antibodies are usually of the IgM isotype, but IgG and IgA NAb have been described as well (Boes, 2000) that might reflect different effector functions of NAb (Korhonen et al., 2000). The NAb isotypes IgM, IgG1, IgG2 and IgA binding LTA were present in milk, showed significant variation among cows and were repeatable within cows over time. However, the very low titers of IgG2 may make this isotype less suitable to study its relationship with disease resistance in dairy cows. It remains to be established whether the contribution of different isotypes present in milk has a consequence for resistance to diseases, as suggested (Sordillo, 2005). Current studies are in progress to determine if NAb and their isotypes play a protective role in mastitis and other (infectious) diseases in cattle.

CONCLUSIONS

Natural antibodies binding LPS, LTA, PGN, and KLH are present in blood plasma and milk of clinically-healthy cows. NAb titers varied among cows and were repeatable over time within a period of 3 weeks. There was a high positive correlation between NAb titers in plasma and milk. NAb in milk binding LTA consisted of mostly isotypes IgM, IgG1, and IgA, but little IgG2. Variability and repeatability of the isotypes were similar to those of total NAb titers. Measuring NAb titers in milk, therefore, might provide an easily measurable parameter to investigate the role of humoral innate immunity to diseases in dairy cows.

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Laat me

(Ramses Shaffy)

Chapter 4

Genetic variation of natural antibodies in milk of Dutch Holstein-Friesian cows

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ABSTRACT

Defense mechanisms of dairy cows against diseases partly rest on their naturally present disease resistance capacity. Natural antibodies (NAb) form a soluble part of the innate immune system, being defined as antibodies circulating in animals without prior intentional antigenic stimulation. Genetic selection on NAb titers in milk, therefore, might improve disease resistance. We estimated genetic parameters of NAb titers binding lipopolysaccharide, lipoteichoic acid (LTA), peptidoglycan, and keyhole limpet hemocyanin, and titers of the NAb isotypes IgG1, IgM, and IgA binding LTA in milk of Dutch Holstein-Friesian heifers. Natural antibody titers were measured in 1 milk sample from each of 1,939 Holstein-Friesian heifers and used for estimating genetic parameters of NAb titers. The data show that phenotypic variation exists among heifers in NAb titers binding lipopolysaccharide, LTA, peptidoglycan, and keyhole limpet hemocyanin, and the NAb isotypes IgG1, IgM, and IgA binding LTA in milk. High genetic correlations among NAb (ranging from 0.45 to 0.99) indicated a common genetic basis for the levels of different NAb in bovine milk. Intra-herd heritability estimates for NAb ranged from 0.10 to 0.53. The results indicated that NAb levels have potential for genetic selection.

INTRODUCTION

Innate immunity plays an important role in preventing or combating infection. Knowledge of protection from intramammary infection or other infections of cattle by (components of) innate immunity is scarce. A potentially important humoral component of innate immunity is formed by natural antibodies (NAb) (Baumgarth et al., 2005; Elluru et al., 2008), being defined as antibodies present in nonimmunized individuals (Van Knegsel et al., 2007). In mice and human, NAb are preferentially derived from CD5+ B (B1) cells (Casali and Notkins, 1989). Natural antibodies are characterized by a broad-specificity repertoire, usually with low binding affinity (Casali and Notkins, 1989). In mammals, NAb are mostly of the IgM isotype class, but IgG and IgA NAb have been described as well (Boes, 2000), and are thought to provide a barrier to infection, and subsequently act as an adjuvant of specific immunity (reviewed by Kohler et al., 2003).

Natural antibodies are present in bovine milk and blood plasma (Van Knegsel et al., 2007; T. Ploegaert, unpublished data). Furthermore, increasing titers of NAb were found in blood and milk of aging cattle (Srinivasan et al., 1999; Van Knegsel et al., 2007). This suggests that NAb are the cumulative result of antigenic stimulation, by continuous polyclonal stimulation by exogenous microbes, or self-antigens, or both (Tomer and Shoenfeld, 1988; Avrameas, 1991; reviewed by Kohler et al., 2003). A high proportion of NAb (reviewed by Kohler et al., 2003) binds pathogen-associated molecular patterns that represent antigens shared by classes of microbes and serve as targets for identification of microbes by the innate immune system. Important pathogen-associated molecular patterns are LPS present on gram-negative (entero)bacteria, such as *Escherichia coli* or *Salmonella* spp.; lipoteichoic acid (LTA) present on gram-positive bacteria, such as *Staphylococcus aureus*; or peptidoglycan (PGN) present on gram-negative and gram-positive bacteria. A relationship was suggested between NAb titers binding keyhole limpet hemocyanin (KLH) or LPS and body condition, energy balance, milk yield, and plasma cholesterol concentration in the peripartum period (Van Knegsel et al., 2007).

Earlier, we found that NAb titers are repeatable over time and show variation among cows (T. Ploegaert; unpublished data). Natural antibody titers in milk are thus an easily measured parameter of humoral innate immunity that might be involved in dairy cattle disease resistance. Natural antibodies binding specific pathogens or antigens have been proposed to enhance protection against subsequent infection (reviewed by Kohler et al., 2003). Genetic variation in total levels of bovine serum isotype immunoglobulins (IgG1, IgG2, IgM, and IgA) have been published (Mallard et al., 1983). In poultry, heritability ($h2$) of total NAb was approximately 0.23 (Wijga et al., 2009). To our knowledge, information on h^2 of total NAb and their isotypes in dairy cattle, especially in milk, is scarce. Therefore, the first aim of this study was to estimate genetic parameters of total NAb titers binding LPS, LTA, PGN, and KLH in Dutch Holstein-Friesian heifers. Furthermore, because *Staph. aureus* is one of the important pathogens causing mastitis, the second aim was to estimate genetic parameters of antibody isotypes IgG1, IgM, and IgA in milk binding LTA from *Staph. aureus*.

MATERIALS AND METHODS

Animals

This study builds on samples collected in the Dutch Milk Genomics Initiative, a project that focuses on the genetic background of detailed milk composition. As part of this project, milk samples were collected from 1,953 first-parity cows in 398 commercial herds in the Netherlands during the first quarter of 2005 (Stoop et al., 2008). At least 5 cows per herd were present at the start of the experiment. Data collection was designed for estimating genetic parameters (many small families) and linkage analysis (some large families). The aim was to have 20 offspring from each of 50 young bulls and 200 offspring from each of 5 proven bulls. In total, 857 offspring from 50 young bulls and 909 offspring from 5 proven bulls were included. To obtain the minimum of 5 offspring per herd, 187 offspring from other proven bulls were included. Each cow was between 5 and 220 d in lactation at the start of the experiment. Cows were over 87.5% Holstein-Friesian. Pedigree and milk yield records of each of the 1,953 selected cows were provided by the cattle cooperative (CRV, Arnhem, The Netherlands). After data editing (cows with missing data were deleted), information on 1,939 milk samples remained, all of which were used in the analyses. Of those 1,939 milk samples, 1,450 had <80,000 somatic cells/mL.

Milk samples

Morning milk samples from 1,953 Dutch Holstein-Friesian cows in their first lactation (cows were milked twice a day), located in 398 herds, were collected. The samples were preserved with 0.03% (wt/wt) sodium azide, transported refrigerated, and stored frozen at −40°C within 1 d. Before testing the NAb titers, samples were aliquoted and stored at −20°C. Somatic cell count was determined as part of the regular milk recording at the certified laboratory of the Milk Control Station (Qlip, Zutphen, The Netherlands) and supplied to us by CRV. Somatic cell score was calculated by transforming SCC as follows: SCS = ln (SCC/1,000). In an earlier study we found a high repeatability (0.71 to 0.91) of NAb titers within cows over time (T. Ploegaert; unpublished data), indicating that it is sufficient to analyze a single milk sample of a cow. Repeatability was calculated from 10 repeated measurements over a time span of almost 3 wk as the between-cow variance expressed as fraction of phenotypic variance.

Total natural antibodies and antibody isotypes

In milk samples, titers of total NAb binding *Escherichia coli*-derived LPS (L2880, serotype O55:B5), *Staph. aureus*-derived LTA [L2515, both from Sigma-Aldrich Inc. (St. Louis, MO)], *Staph. aureus*-derived PGN (Bio-Chemika, Buchs, Switzerland), or *Megathura crenulata*-derived KLH from MP Biomedicals (Solon, OH) were determined. In addition, the isotypes IgG1, IgM, and IgA of antibodies binding LTA were measured. Titers of NAb were determined using an indirect ELISA procedure, essentially as described by Van Knegsel et al. (2007). In short, plates were coated with 100 μL/well of 1 μg of KLH, 4 μg of LPS, 5 μg of LTA, or 2 μg of PGN per mL of carbonate buffer (10.6 g/L Na2CO3, pH 9.6). After washing, plates were blocked with 100 μL/well of 2.5% rabbit serum in PBS with 0.05% Tween20 for at least 30

min at room temperature (21°C). Four serial dilutions of samples (1:4) in PBS, 0.05% Tween20, and 2.5% rabbit serum were added. Dilutions started at 1:30 for analysis of total antibodies and at 1:4 for analysis of isotypes. On each plate, the same positive sample was included with 8 serial dilutions (1:2) in duplicate. Plates were incubated for 1 h at room temperature (21°C). Binding of total antibodies to LPS, LTA, PGN, or KLH was detected using 100 µL/well of 1:15,000 diluted rabbit anti-bovine antibody (H + L) coupled to peroxidase (Sigma-Aldrich Inc.). Binding of isotype antibodies to LTA was detected using 1:16,000 diluted sheep anti-bovine IgG1 (Serotec, Dusseldorf, Germany), 1:16,000 diluted sheep anti-bovine IgM (Serotec), or 1:8,000 diluted sheep anti-bovine IgA (Serotec), all coupled to horseradish peroxidase. After washing, 100 µL/well of tetramethylbenzidine (71.7 µg/mL) and 0.05% H2O2 were added to the wells and incubated for 10 min at room temperature (21°C). The reaction was stopped with 50 µL/well of 2.5 *N* of H2SO4. Extinctions were measured with a Multiskan spectrophotometer (Flow, Irvine, UK) at a wavelength of 450 nm. Levels were calculated as titers, and titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of E*max*, where E*max* represents the highest mean extinction of a standard positive milk sample present in duplicate on every microtiter plate (Ploegaert et al., 2007; Van Knegsel et al., 2007; Star et al., 2009).

Statistical analysis

Variance components for genetic and herd effects were estimated with an animal model using ASReml (Gilmour et al., 2006). The animal model was the same as that used in the other studies on detailed milk composition on the same data set (Stoop et al., 2007, 2008; Schopen et al., 2008):

$$y_{ijklmn} = \mu + b_1 \times \mathrm{dim}_i + b_2 \times e^{-0.05 \times \mathrm{dim}} + b_3 \times afc_j + b_4 \times afc_j^2 + season_k + scode_l + herd_m + A_n + E_{ijklmn} \quad [1]$$

where y_{ijklmn} = dependent variable corresponding to milk NAb titer of cow n with a sire code l, age of first calving j during season k and at DIM (time between calving and date of sample) i; μ = general mean; dim_i = DIM, modeled with a Wilmink curve (Wilmink, 1987) with regression coefficients $b1$ and $b2$; afc_j = covariate describing the effect of age at first calving; $b3$ and $b4$ were regression coefficients for afc and afc^2; $season_k$ = 3 classes for season of calving: summer (June–August 2004), autumn (September–November 2004), and winter (December 2004–February 2005); $scode_l$ = fixed effect accounting for differences between groups of proven bull daughters and young bull daughters; $herd_m$ = random effect of herd m; A_n = random additive genetic effect of animal n; and E_{ijklmn} = random residual effect. Relationships between animals were taken into account. The pedigree included 26,300 animals (Stoop et al., 2007). Univariate analyses were used to estimate the intra-herd heritability, which was defined as

$$h^2 = \frac{\sigma^2_A}{\sigma^2_A + \sigma^2_E} \quad [2]$$

where σ^2_A is the additive genetic variance and σ^2_E is the residual variance. The intra-herd heritability expresses the proportion of phenotypic variance that can be explained by additive genetic variation between animals within herds. Univariate analyses were also used to estimate the across-herd heritability (h^2_{ac}) that was defined as

$$h^2_{ac} = \frac{\sigma^2_A}{\sigma^2_{herd} + \sigma^2_A + \sigma^2_E} \quad [3]$$

where σ^2_{herd} is the between-herd variance. The proportion of the total phenotypic variance due to variation between herds (h_{herd}) was calculated as

$$h_{herd} = \frac{\sigma^2_{herd}}{\sigma^2_{herd} + \sigma^2_A + \sigma^2_E} \quad [4]$$

Genetic correlation (r_g) between 2 traits (A1 and A2) was estimated from bivariate analyses using model [1] as

$$r_g = \frac{\sigma^2_{A1,A2}}{\sqrt{(\sigma^2_{A1} \times \sigma^2_{A2})}} \quad [5]$$

where σ^2_{A1} is the additive genetic variance for NAb titer A1, σ^2_{A2} is the additive genetic variance for NAb titer A2, and $\sigma^2_{A1,A2}$ is the additive genetic covariance between NAb titers A1 and A2. Correlations were calculated for the different total NAb titers and for the 3 NAb isotypes IgG1, IgM, and IgA binding LTA. Results of univariate analyses were used as starting values for variance structure in the bivariate analyses.

RESULTS

Natural antibody titers

Titers of NAb binding LPS, LTA, PGN, and KLH as well as titers of the isotypes IgG1, IgM, and IgA binding LTA in milk were determined. Table 1 shows the mean and standard deviation of titers of NAb binding LPS, LTA, PGN, KLH, and isotypes IgG1, IgM, and IgA binding LTA in milk. For the total antibodies, NAb titers binding LPS were, on average, lowest (3.22) and NAb titers binding PGN were highest (5.68). For the antibody isotypes, the IgA titers binding LTA were, on average, lowest (2.87), whereas IgM titers were, on average, highest (4.26). Substantial differences in titers between animals were found: the coefficient of variation ranged from 19% for NAb binding KLH to 41% for isotypes IgG1 and IgA binding LTA.

Table 1. Mean titer, standard deviation (SD), and coefficient of variation (CV) of total natural antibody titers[1] and natural antibody isotype (IgG1, IgM, IgA) titers binding lipoteichoic acid (LTA) measured in milk samples of 1,939 heifers

Antibody	Mean	SD	CV (%)
KLH	3.88	0.77	20
LPS	3.22	0.97	30
LTA	3.44	0.90	26
PGN	5.68	1.06	19
LTA-IgG1	3.40	1.39	41
LTA-IgM	4.26	0.89	21
LTA-IgA	2.87	1.18	41

[1]KLH = keyhole limpet hemocyanin; LPS = lipopolysaccharide; LTA = lipoteichoic acid; PGN = peptidoglycan.

Phenotypic variance, heritability, and herd effect of natural antibody titers

Table 2 shows estimates of intra-herd heritabilities, across-herd heritabilities, and herd effects for total antibody titers binding KLH, LTA, LPS, and PGN, and titers of antibody isotypes of IgG1, IgA, and IgM binding LTA in milk of all clinically healthy cows. Lowest intra-herd heritabilities were found for titers of IgG1 binding LTA (0.10) and highest intra-herd heritabilities were found for total NAb titers binding KLH (0.42), titers of IgM binding LTA (0.47), and IgA binding LTA (0.53). Across-herd heritabilities were also lowest for titers of IgG1 binding LTA and highest for total titers binding KLH and IgM and IgA titers binding LTA, but generally somewhat lower than intra-herd heritabilities. Herd effects explained between 11 and 29% of the phenotypic variance in the different traits (Table 2). The lowest contribution of herd effects was found for total NAb titers binding LPS (11%), and the highest value was found for total NAb titers binding PGN (29%). Table 3 shows estimates of intra-herd heritabilities, across-herd heritabilities, and herd effects of total NAb titers binding KLH, LTA, LPS, and PGN, and NAb isotype titers of IgG1, IgM, and IgA binding LTA in milk. This analysis included only cows that were considered clinically healthy and had SCC <80,000 cells/mL at the time of milk sampling, because this

cut-off value was earlier recognized to represent cows with healthy udders (Schepers et al., 1997). The lowest intra-herd heritability was found for NAb titers of IgG1 binding LTA (0.23) and the highest values for titers of IgM and IgA binding LTA (0.51). Across-herd heritabilities were also lowest for titers of IgG1 binding LTA and highest for IgM and IgA titers binding LTA, but generally somewhat lower than the intra-herd heritabilities. Herd effects explained 12 to 31% of the phenotypic variance found in the different traits (Table 3). The lowest value for herd effects was found for total NAb titers binding LTA and IgM binding LTA (12%), and the highest level was found for total NAb titers binding PGN (31%).

Table 2. Phenotypic variance (σ^2_P), intra-herd heritability (h^2), across-herd heritability (h^2_{ac}), and herd effect (h_{herd}) for total natural antibody titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), LPS, and peptidoglycan (PGN), and natural antibody isotype titers binding LTA (LTA-IgG1, LTA-IgM, LTA-IgA)

	σ^2_P (SE)	h^2 (SE)	h^2_{ac} (SE)	h_{herd} (SE)
KLH	0.58 (0.02)	0.42 (0.09)	0.36 (0.08)	0.16 (0.02)
LTA	0.83 (0.03)	0.32 (0.09)	0.27 (0.08)	0.13 (0.02)
LPS	0.94 (0.03)	0.15 (0.06)	0.14 (0.06)	0.11 (0.02)
PGN	1.12 (0.04)	0.13 (0.06)	0.09 (0.04)	0.29 (0.02)
LTA-IgG1	1.92 (0.07)	0.10 (0.06)	0.07 (0.04)	0.27 (0.03)
LTA-IgM	0.79 (0.03)	0.47 (0.10)	0.42 (0.09)	0.12 (0.02)
LTA-IgA	1.42 (0.06)	0.53 (0.11)	0.40 (0.09)	0.25 (0.02)

Table 3. Phenotypic variance (σ^2_P), intra-herd heritability (h^2), across-herd heritability (h^2_{ac}), and herd effect (h_{herd}) for total natural antibody titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), LPS, and peptidoglycan (PGN), and natural antibody isotype titers binding LTA (LTA-IgG1, LTA-IgM, LTA-IgA) estimated from cows (n = 1,450) that had a SCC <80,000 cells/mL at the time of milk sampling

	σ^2_P (SE)	h^2 (SE)	h^2_{ac} (SE)	h_{herd} (SE)
KLH	0.54 (0.03)	0.50 (0.11)	0.43 (0.10)	0.14 (0.03)
LTA	0.74 (0.04)	0.46 (0.11)	0.41 (0.10)	0.12 (0.02)
LPS	0.96 (0.04)	0.25 (0.09)	0.21 (0.08)	0.13 (0.03)
PGN	0.93 (0.04)	0.30 (0.10)	0.21 (0.07)	0.31 (0.03)
LTA-IgG1	1.74 (0.08)	0.23 (0.10)	0.18 (0.08)	0.22 (0.03)
LTA-IgM	0.75 (0.04)	0.51 (0.11)	0.44 (0.10)	0.12 (0.02)
LTA-IgA	1.26 (0.06)	0.51 (0.12)	0.37 (0.09)	0.27 (0.03)

Genetic and phenotypic correlations of natural antibody titers

Table 4 shows the genetic and phenotypic correlations between the total NAb titers binding LPS, LTA, PGN, and KLH. A high positive genetic correlation (0.99) was found between NAb titers binding LTA and LPS, and between NAb titers binding KLH and LTA (0.85). The lowest genetic correlation (0.45) was found between NAb titers binding PGN and KLH. As expected, phenotypic correlations were lower than genetic correlations. The phenotypic correlations ranged from 0.22 between NAb titers binding PGN and LPS to 0.50 between LTA and KLH. Table 5 shows the genetic and phenotypic correlation between the NAb isotype titers IgG1, IgM, and IgA binding LTA. The genetic correlations between IgG1 and total NAb titers binding LTA (0.97) and between IgM and total NAb titers binding LTA (0.93) were highest, with low standard errors. The genetic correlation between IgG1 and IgM titers binding LTA was 0.80 and between IgG1 and IgA titers binding LTA 0.71. These estimated genetic correlations had high standard errors (0.19 to 0.21). Phenotypic correlations varied from 0.30 between IgG1 and IgA titers to 0.60 between total NAb and IgM titers binding LTA.

Table 4. Genetic (above diagonal) and phenotypic (below diagonal) correlations between total natural antibody titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), LPS, and peptidoglycan (PGN)

	LPS	LTA	PGN	KLH
LPS	-	0.99 (0.06)[1]	0.52 (0.24)	NE[2]
LTA	0.42 (0.02)	-	0.51 (0.19)	0.85 (0.08)
PGN	0.22 (0.02)	0.43 (0.02)	-	0.45 (0.20)
KLH	NE	0.50 (0.02)	0.26 (0.03)	-

[1]Standard errors are in parentheses.
[2]NE = estimates of parameters did not converge.

Table 5. Genetic (above diagonal) and phenotypic (below diagonal) correlations between natural antibody isotype titers IgG1, IgM, and IgA binding lipoteichoic acid (LTA) and total natural antibody titers binding LTA

	LTA-IgG1	LTA-IgM	LTA-IgA	LTA-Total
LTA-IgG1	-	0.80 (0.19)	0.71 (0.21)	0.97 (0.00)
LTA-IgM	0.34 (0.02)[1]	-	NE[2]	0.93 (0.05)
LTA-IgA	0.30 (0.03)	NE	-	NE
LTA-Total	0.52 (0.02)	0.60 (0.02)	NE	-

[1]Standard errors are in parentheses.
[2]NE = estimates of parameters did not converge.

DISCUSSION

Total NAb titers binding KLH, PGN, LTA, and LPS, and the NAb isotype titers IgG1, IgM, and IgA binding LTA in milk samples of 1,939 heifers, considered clinically healthy without known deliberate immunization, were measured. Titers of total NAb binding to all 4 antigens and NAb isotype titers binding LTA varied between these first-parity cows. Variation in NAb titers among cows was observed previously (T. Ploegaert, unpublished data; Van Knegsel et al., 2007), but cows were from different parities in these studies. Titers of NAb increased with parity (Van Knegsel et al., 2007) and consequently more variation would be expected when cows are from different parities.

Natural antibodies are potential immune parameters for natural disease resistance of dairy cows. Previously, we showed that NAb titers varied among cows and were repeatable within individual cows over time (T. Ploegaert, unpublished data). Results of the present study indicate that a substantial part of the variation between cows in (total) NAb titers binding LPS, LTA, PGN, and KLH is heritable (Table 2). Differences between herds explained between 11 and 29% of the phenotypic variance between cows. This knowledge is important for evaluation of the potential of genetic and nongenetic strategies for modulating NAb titers aiming at improving health of dairy cows. Levels of intra-herd heritability, for all cows considered clinically healthy, of total and isotype NAb titers in the present study ranged from 0.10 for IgG1 binding LTA to 0.53 for IgA binding LTA. In poultry, heritability ranged from 0.09 to 0.23 for NAb titers binding LPS and 0.03 to 0.42 for NAb titers binding LTA depending on line and age (Siwek et al., 2006) and 0.23 for NAb titers binding rabbit red blood cells (Wijga et al., 2009). Douch et al. (1995) estimated heritabilities for concentrations of total antibody (ranging from 0.25 to 0.37) and IgG1 (being around 0.19) binding 4 nematode species in serum from Romney lambs after natural exposure. Mallard et al. (1983) estimated a heritability of 0.08 to 0.85 for total IgM levels in serum of Canadian Holstein-Friesian dairy cows. Heritability estimates for specific antibody levels in serum after natural exposure to *Mycobacterium avium* ssp. *paratuberculosis* in Holstein cows ranged from 0.05 to 0.13, depending on data set and model tested (Gonda et al., 2006; Hinger et al., 2008). Across-herd heritabilities of 0.36 for total NAb titers binding KLH, 0.42 for IgM titers binding LTA, and 0.40 for IgA titers binding LTA in the present study are among the highest reported for immune parameters in literature. The effect of udder health status on estimates of heritability of NAb titers was also studied. Estimates of intra-herd and across-herd heritability of total NAb titers and of isotype titers showed, in general, a slight increase when cows that were considered clinically healthy with SCC >80,000 cells/mL were excluded from analyses (Table 3), compared with all cows that were considered clinically healthy (Table 2). Cows with SCC <80,000 cells/mL are recognized as cows with a healthy udder (Schepers et al., 1997). These findings indicate that heritability of NAb is not negatively influenced by removal of cows with a potential subclinical intramammary infection, as indicated by the increased SCC values. Although possible sampling errors cannot be excluded totally, the results of the present study suggest that the estimates of NAb heritability are thus independent of the current intramammary

infection status. In line with this observation, NAb might not reflect a response to current infection (Kohler et al., 2003).

Estimates of genetic correlations between titers of total NAb binding different antigens ranged from 0.45 to 0.99 (Table 4). These results suggest that a common genetic basis exists for the level of NAb binding these different antigens. Consequently, breeding for NAb titers may not require antibodies binding to specific pathogens or antigens, but might be based on a model antigen such as KLH (that cows have never encountered before) as a parameter of innate humoral immune competence.

Previous analysis showed that heritability estimates for milk production traits found in the Dutch Milk Genomics population (Stoop et al., 2007); that is, the population that was used in this study, were similar to estimates for these traits in literature (Hayes et al., 1984; Ikonen et al., 1999; Wood et al., 2003). The across-herd heritability estimate for SCS in the present study based on almost 2,000 heifer milk samples was 0.07. This heritability estimate is well in the range of estimates for SCS reported (Arnould et al., 2009; Da et al., 1992; Soyeurt et al., 2007). These results suggest that our sample is representative of the Dutch dairy cow population.

Natural antibodies are usually IgM, but IgG and IgA NAb have been described as well (Boes, 2000). The presence of IgM, IgG, and IgA NAb might reflect different conditions generating these NAb and resulting in different effector functions of NAb (reviewed by Kohler et al., 2003; Korhonen et al., 2000). The present study also shows IgG1, IgM, and IgA NAb titers binding LTA in bovine milk. In these analyses (Table 2), estimates of the proportion of variance due to genetic factors (heritability) were substantially lower for IgG1 titers binding LTA (0.10) than for IgM titers binding LTA (0.47). In addition, the estimates of proportion of variation caused by environmental differences between herds (herd effect) were higher for IgG1 (0.27) than for IgM (0.12). This suggests that NAb of the IgG1 isotype might reflect earlier exposure to LTA. Earlier exposure could explain why the IgG1 titers are more affected by environmental than genetic factors of the cow, as reflected by the relatively high level of the herd effect and the relatively low heritability of IgG1 titers when compared with IgM titers (Table 2). On the other hand, IgM titers are naturally present (Casali and Notkins, 1989; Kohler et al., 2003), which could explain why the IgM titers, compared with IgG1 titers, were found to be more subject to genetic than environmental influences. High genetic correlations were estimated for the 3 isotypes binding LTA, which is in line with the dependency of IgG1 on the presence of IgM NAb, which do not increase due to infection (Kohler et al., 2003), but lower phenotypic correlations were estimated for the 3 isotypes binding LTA suggesting different (yet to be established) functions of the 3 NAb isotypes in bovine milk. Earlier we found high repeatability of IgG1 binding LTA in cows that were considered clinically healthy (T. Ploegaert, unpublished data), suggesting that IgG1 NAb levels do not represent exposure per se.

Higher levels of NAb binding KLH were positively correlated with survival and health status in poultry (Star et al., 2007) and fish (Kachamakova et al., 2006). Whether higher NAb levels or NAb isotypes in bovine milk are indicative of the health status of dairy cows is currently being studied.

CONCLUSIONS

This study showed variation among first-parity cows in total NAb titers binding LPS, LTA, PGN, and KLH, and the isotype titers of IgG1, IgM, and IgA binding LTA in milk. High genetic correlations indicate a common genetic basis for NAb titers in bovine milk. Heritability estimates demonstrate that total and isotype NAb levels have potential for genetic selection.

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Data! Data! Data! I can't make bricks without clay.

(Sherlock Holmes)

Chapter 5

Relation of natural antibodies in milk of Holstein Friesian heifers with the risk for high somatic cell count and clinical mastitis

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ABSTRACT

Innate immunity plays an important role in preventing and combating infection. Natural antibodies (NAb) are an important humoral component of innate immunity. In this study the relation between NAb titers, and the occurrence of high somatic cell count (SCC) and clinical mastitis (CM) later in lactation was investigated. Total and isotype (IgM, IgG1, and IgA) NAb titers binding lipopolysaccharide, lipoteichoic acid, peptidoglycan, and keyhole limpet hemocyanin were measured in a single milk sample from 1953 Holstein-Friesian heifers. From 1515 heifers information on CM and SCC of this first lactation were available. Two datasets were created. The first dataset contained data of all the, at the sampling moment clinically-healthy, heifers with information on CM and SCC (n = 1515); the all-heifers dataset. The second dataset contained data from, at the sampling moment clinically-healthy, heifers that did not show CM nor had high SCC (>150,000 cells/mL) during the SCC test-days before or at the sampling moment (n = 906); the healthy-heifers dataset. For all heifers, logistic regression analysis was performed on two traits in relation to NAb levels: 1) whether the heifer did or did not develop CM during the remainder of the lactation after the sampling moment, and 2) whether the heifer did or did not have high SCC during at least 1 SCC test-day during the remainder of the lactation after the sampling moment. In the all-heifers dataset nearly all NAb titers showed odds ratios (OR) > 1 for risk for high SCC that were statistically significant. In both datasets IgG1 NAb binding KLH decreased the risk for high SCC (OR < 1). Only in the healthy-heifers dataset higher total and IgG1 NAb binding LPS also significantly decreased the risk for high SCC (OR = 0.86 and 0.88 respectively). For IgG1 NAb binding LTA, in the all-heifers dataset, OR was 1.18 for the risk for CM. In the healthy-heifers dataset the OR for the risk for CM was below 1 for IgM and IgA binding all tested antigens, suggesting a protective relationship for these isotypes, albeit not statistically significant. We propose that NAb titers might play a role in the prevention of CM or high SCC depending on the NAb isotype and antigen-specificity but the CM and SCC history of the heifers affects NAb levels and needs to be taken into account.

INTRODUCTION

The innate immune system is crucially important in combating and preventing mastitis (Rainard and Riollet, 2006) that is a major health and economical (Halasa et al., 2007) problem in dairy farming. Natural antibodies (NAb) are an important humoral component of innate immunity (Baumgarth et al., 2005; Elluru et al., 2008). Natural antibodies are defined as antibodies present in non-immunized individuals (Avrameas et al., 1991) and have a broad specificity combined with mostly low affinity or avidity to the antigens they can bind (Casali and Notkins, 1989). In mammals, NAb are mainly of the IgM isotype class, but IgG and IgA NAb have been described as well (Boes, 2000). NAb are thought to provide an early barrier to infection, and may subsequently act as an adjuvant for the induction of protective immunity against infection as they are able to bind specific pathogens or antigens (Kohler et al., 2003).

Natural antibodies are present in bovine milk (Ploegaert et al., 2010; Van Knegsel et al., 2007) and plasma (Van Knegsel et al., 2007) with increasing titers in aging cattle (Srinivasan et al., 1999; Van Knegsel et al., 2007). This suggests that NAb are the cumulative result of antigenic stimulation, either through continuous polyclonal stimulation by exogenous microbes, self-antigens, or both (Tomer and Shoenfeld, 1988; Avrameas, 1991). A high proportion of NAb binds pathogen-associated molecular patterns (PAMP) (Kohler et al., 2003) that represent antigens that are shared by 'classes' of microbes and serve as targets for identification of microbes by the innate immune system. Important PAMP are lipopolysaccharide (LPS) present on gram negative (entero)bacteria, such as *Escherichia coli* or *Salmonella spp*; lipoteichoic acid (LTA) present on gram-positive bacteria, such as *Staphylococcus aureus*; or peptidoglycan (PGN) present on gram-negative and gram-positive bacteria. Natural antibodies of cows can also bind 'naïve' antigens that these animals have never encountered before, such as Keyhole Limpet Hemocyanin (KLH) (Van Knegsel et al., 2007), a glycoprotein from *Megathura crenulata* (a sea mollusk), illustrating the broad specificity that NAb can display. A relationship has been suggested between NAb titers binding KLH or LPS on the one hand, and body condition, energy balance, milk yield and plasma cholesterol concentration in the peripartum period on the other hand (Van Knegsel et al., 2007). Earlier, we found that NAb titers in milk (and blood) of healthy cows are repeatable over time, and vary among individual cows (Ploegaert et al., 2008). Thus, NAb titers in milk are an easily measured parameter of humoral innate immunity that might be involved in dairy cattle disease resistance (Ploegaert et al., 2008) that can be determined easily and non-invasively. However, little is known about the relation of total and isotype NAb titers in milk and the risk for mastitis in dairy cattle.

Therefore, the first aim of this study was to investigate if total and isotype (IgG1, IgM and IgA) NAb titers binding LPS, LTA, PGN, and KLH in Dutch Holstein-Friesian heifers are predictive for the risk for high somatic cell count (SCC) or clinical mastitis (CM) later in lactation. The second aim was to investigate if udder health status before and at the sampling moment affects the relation of NAb with risk for CM or high SCC.

MATERIALS AND METHODS

Animals

In February and March 2005 morning milk samples were collected from 1,953 heifers in 398 commercial herds as part of the Milk Genomics Initiative, a project that focuses on the genetic background of milk composition (Schennink et al., 2007). At least 5 heifers per herd were present in the herd at the start of the experiment. Each heifer was between day 63 and 263 d in lactation at the start of the experiment and milked twice a day. Heifers were over 87.5% Holstein-Friesian.

Milk samples, somatic cell count and clinical mastitis data

Milk samples were preserved with 0.03% wt/wt sodium azide, transported, refrigerated and stored frozen at -40°C within one day. Before testing the NAb titers the samples were aliquoted and stored at -20°C. Sampling date refers to the test-day on which samples were collected for NAb titer measurement. Information on SCC and milk yield for all test days during the first lactation were supplied by the cattle cooperative CRV (Arnhem, the Netherlands).

Somatic cell count (SCC) of the milk samples was determined with a Fossomatic at the certified laboratory of the Milk Control Station (Qlip, Zutphen, The Netherlands). Somatic cell score (SCS) of the milk samples was calculated as ln(SCC/1000). After milk samples were collected, dairy farmers were contacted again to supply CM data of the included heifers. In total 339 farmers responded to this request that resulted in 1515 heifers with information on clinical mastitis for the whole first lactation. Of these heifers, 906 had not shown signs of CM and did not have high test-day SCC (>150,000 cells/mL) prior to or at the moment of milk sampling. The chosen threshold for high SCC is currently used as threshold for heifers by the Dutch DHI program (Sampimon et al., 2010).

Total natural antibodies and isotype-specific antibodies

Titers of (total) NAb and isotypes IgG1, IgM, and IgA of NAb binding *Escherichia coli*-derived LPS (L2880, serotype O55:B5), *Staphylococcus aureus*-derived LTA (L2515), both from Sigma-Aldrich Inc. (St. Louis, MO), *S. aureus*-derived PGN (BioChemika, Buchs, CH), or *Megathura crenulata-derived* KLH from MP Biomedicals (Solon, Ohio) were determined using an indirect ELISA procedure as described before (Ploegaert et al., 2010). Briefly, 96-well microtiter plates (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) were coated with 100 µL/well of either 1 µg KLH, 4 µg LPS, 5 µg LTA or 2 µg PGN per mL of carbonate buffer (10.6 g/L Na_2CO_3, pH 9.6). After washing, plates were blocked with 100 µL/well 2.5% rabbit serum in PBS with 0.05% Tween20 for at least 30 min at room temperature. Four serial dilutions of samples (1:4) in PBS, 0.05% Tween20 and 2.5% rabbit serum were added. Dilutions started at 1:30 for analysis of total antibodies or at 1:4 for analysis of isotypes. On each plate the same positive milk sample was included with 8 serial dilutions (1:2) in duplicate. Plates were incubated for 1 h at room temperature. Binding of total antibodies to LPS, LTA, PGN or KLH was detected using 100 µL/well 1:15,000 diluted Rabbit anti-bovine antibody (H + L) coupled to peroxidase (Sigma-Aldrich Inc.). Binding of isotype-specific antibodies to LTA was detected using 1:16,000

diluted sheep anti-bovine IgG1 (Serotec, Düsseldorf, Germany), 1:16,000 diluted sheep anti-bovine IgM (Serotec), or 1:8,000 diluted sheep anti-bovine IgA (Serotec) all coupled to horseradish peroxidase. Binding of isotype antibodies to LPS was detected using 1:2000 diluted sheep anti-bovine IgG1 (Serotec), 1:16,000 diluted sheep anti-bovine IgM (Serotec), and 1:2,000 diluted sheep anti-bovine IgA (Serotec). Binding of isotype-specific antibodies to KLH was detected using 1:2,000 diluted sheep anti-bovine IgG1 (Serotec), 1:16,000 diluted sheep anti-bovine IgM (Serotec), and 1:4,500 diluted sheep anti-bovine IgA (Serotec). Binding of isotype-specific antibodies to PGN were detected using 1:2,000 diluted sheep anti-bovine IgG1 (Serotec), 1:8,000 diluted sheep anti-bovine IgM (Serotec), and 1:3,000 diluted sheep anti-bovine IgA (Serotec). After washing, 100 µL/well tetramethylbenzidine (71.7 µg/mL) and 0.05% H_2O_2 were added to the wells and incubated for 10 min at room temperature. The reaction was stopped with 50 µL/well 2.5 *N* of H_2SO_4. Extinctions were measured with a Multiskan (Flow, Irvine, UK) at a wave length of 450 nm. Natural antibody levels were calculated as titers expressed as Log_2 values of the dilutions that gave an extinction closest to 50% of Emax, where Emax represents the highest mean extinction of a standard positive milk sample present in duplicate on every microtiter plate (Ploegaert et al., 2010).

Statistical analysis

Two datasets were created for the traits CM and high SCC. The first dataset contained data on all heifers that were considered clinically-healthy at the sampling moment, but that could have had CM before the sampling moment or high SCC (>150,000 cells/mL) at test-days before or at the sampling moment; the all-heifers dataset. The second dataset contained data from heifers that were considered clinically-healthy at the sampling moment, and did not have high SCC at test-days before or at the sampling moment and did not show CM before or at the sampling moment: the healthy-heifers dataset. "Clinical mastitis" reflects whether the heifer did (1) or did not (0) have clinical signs of mastitis during the remainder of the lactation period after the sampling moment. "High SCC" reflects whether the heifer did (1) or did not (0) have SCC above 150,000 cells/mL at 1 or more test-days after the sampling moment during that same lactation.

To study the effect of NAb titers on the subsequent risk for CM and high SCC after the sampling moment, the logistic regression procedure Glimmix in SAS (SAS Institute Inc., Cary, NC; version 9.1) was used. This procedure was chosen because it can handle fixed as well as random factors in the model. The following model was used:

$$y_{ijkl} = \mu + b_1 \times \mathrm{dim}_i + b_2 \times e^{-0.05 \times \mathrm{dim}_i} + b_3 \times afc_j + b_4 \times afc_j^2 + b_5 \times NAb/SCS_k + herd_l + E_{ijkl}$$

where y_{ijkl} = dependent variable CM or high SCC for a heifer with age at first calving (afc) j and at days in milk (dim; time between calving and sampling date) i; modeled with a Wilmink curve (Wilmink, 1987) with regression coefficients b_1 and b_2; µ = general mean; afc_j = covariate describing the effect of age at first calving; b_3 and b_4 were regression coefficients for afc and afc^2; NAb/SCS_k = effect of antibody titer **or** SCS measured in the taken milk sample k, with regression coefficient b_4; $herd_l$ =

random effect of herd l; and E_{ijkl} = random residual effect (adapted from Ploegaert et al., 2010). The odds ratio (OR) was calculated as OR = $e^{estimate}$ where estimate reflects the regression coefficient estimate for the effect of NAb titer. Odds ratio shows the relative risk for CM or high SCC after the sampling moment if NAb titer rises 1 unit. If for instance OR = 0.85, the relative risk for CM decreases with 15% if the NAb titer rises 1 unit. If OR is higher than 1, the relative risk for CM increases with increasing NAb titers. NAb titers were divided in categories with 1 unit per category. For example, titer category 1 contains the data of all heifers with NAb titer between 0.5 and 1.5. For a given NAb titer category, predicted proportions of heifers that will develop CM or high SCC after the sampling moment were computed using model 1. The predictions were compared to the observed proportions of cows that developed CM or high SCC for that particular NAb titer category.

RESULTS

Description of datasets

In this study the relation between NAb titers in milk of Dutch Holstein-Friesian heifers and risk for CM and high SCC was investigated in two datasets. Number of farms, total number of heifers, and number of heifers with and without CM and high SCC after the sampling moment in both datasets are presented in table 1. The percentage of heifers that developed CM was 5.6% (85 cases) in the all-heifers dataset and 3.6% (33 cases) in healthy-heifers dataset. These CM incidences of both datasets are significantly different at the 5% level, when applying a Chi-square test.

Table 1. Number of farms, total number of heifers and number of heifers with and without clinical mastitis and high somatic cell count (SCC) after the sampling moment in the all-heifers dataset and in the healthy-heifers dataset

		Number of heifers (% of total number)				
Dataset	Farms	Total	Clinical mastitis		SCC > 150,000 cells/mL	
			Yes	No	Yes	No
All heifers[1]	339	1515	85 (5.6%)	1430	600 (39.6%)	915
Healthy heifers[2]	314	906	33 (3.6%)	873	231 (25.5%)	675

[1]All-heifers dataset contained data on all heifers with information on the traits, but not having clinical mastitis at the sampling moment.
[2]Healthy-heifers dataset contained data from healthy heifers, defined as heifers that did not have high SCC (more than 150,000 cells/mL) and did not show clinical mastitis before or at the sampling moment.

Effect of natural antibody titers on risk for high somatic cell count

In the all-heifers dataset the OR for high SCC was significantly higher than 1 for nearly all NAb (Table 2, left part). The highest OR (1.42) was estimated for total NAb binding PGN, indicating that when this antibody titer has increased with 1 unit, the relative risk to develop high SCC later in lactation increases with 42%. The

antibodies that revealed an OR slightly lower than 1 were IgG1 binding LPS (OR = 0.98) that was not significantly different from 1 (P = 0.63), and IgG1 binding KLH (OR = 0.93) that was significantly lower than 1 (P = 0.046).
In the healthy-heifers dataset OR for high SCC ranged from 0.85 to 1.10 for the different NAb antibodies, but were mostly not significantly different from 1 (table 2, right part). The only significant OR for high SSC were observed for total NAb (OR = 0.86) and IgG1 (OR = 0.88) binding LPS (P = 0.046 and 0.021 respectively) and IgG1 binding KLH (OR = 0.86, P = 0.007).

Table 2. Effect of natural antibody titers on risk for high somatic cell count. Estimate, standard error (SE), odds ratio (OR), and significance levels (P values) for the effect of natural antibody (NAb)[1] titers and somatic cell score at the sampling moment (SCS sample), on risk for subsequent high somatic cell count after the sampling moment, during the first lactation for the all-heifers dataset (left) and for the healthy-heifers dataset (right)

NAb[1]	All heifers				Healthy heifers			
	Estimate[2]	SE	OR	P value	Estimate[2]	SE	OR	P value
LTA-IgA	0.25	0.05	1.29	<.0001	0.01	0.07	1.01	0.946
LTA-IgG1	0.13	0.04	1.14	0.002	-0.05	0.06	0.95	0.377
LTA-IgM	0.27	0.06	1.31	<.0001	0.10	0.09	1.10	0.281
LTA-total	0.32	0.06	1.37	<.0001	-0.07	0.09	0.93	0.410
LPS-IgA	0.23	0.05	1.26	<.0001	-0.01	0.08	0.99	0.877
LPS-IgG1	-0.02	0.04	0.98	0.629	-0.13	0.06	0.88	0.021
LPS-IgM	0.16	0.05	1.17	0.004	-0.01	0.08	1.00	0.955
LPS-total	0.06	0.06	1.07	0.268	-0.16	0.08	0.86	0.046
KLH-IgA	0.25	0.06	1.29	<.0001	-0.03	0.09	0.98	0.780
KLH-IgG1	-0.08	0.04	0.93	0.046	-0.15	0.05	0.86	0.007
KLH-IgM	0.18	0.06	1.19	0.002	0.01	0.08	1.01	0.909
KLH-total	0.14	0.07	1.15	0.054	-0.17	0.11	0.85	0.112
PGN-IgA	0.32	0.06	1.38	<.0001	0.09	0.09	1.10	0.323
PGN-IgG1	0.17	0.04	1.18	<.0001	0.04	0.06	1.04	0.501
PGN-IgM	0.25	0.06	1.28	<.0001	0.09	0.09	1.10	0.304
PGN-total	0.35	0.06	1.42	<.0001	0.09	0.08	1.10	0.271
SCS sample	1.22	0.08	3.37	<.0001	0.87	0.12	2.39	<.0001

[1]NAb titers refer to total or individual isotype (IgA, IgM and IgG1) antibody titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), lipopolysaccharide (LPS) and peptidoglycan (PGN).
[2]Estimate refers to the regression coefficient estimate for the effect of NAb titer on risk for high somatic cell count.

Effect of natural antibody titers on risk for clinical mastitis

In the all-heifers dataset the OR for CM ranged from 0.94 to 1.18 for the different NAb (Table 3, left part), but was only significant (P = 0.026) for the IgG1 isotype binding LTA (OR = 1.18).

In the healthy-heifers dataset the OR for CM ranged from 0.75 to 1.09 for total NAb binding the different antigens (Table 3, right part). Comparison of the individual isotypes binding the different antigens revealed differences between the IgG1 isotype compared to the IgA and IgM isotypes. The OR for CM was on average close to 1 for IgG1, ranging from 0.95 to 1.09, while it was below 1 for the IgA and IgM isotypes, ranging from 0.74 to 0.85 (Table 3, right part). The lowest OR (0.74) was estimated for IgA binding LPS and KLH, and IgM binding LPS, suggesting that an increase with 1 antibody titer unit decreases the relative risk for CM later in lactation with 26%. Although none of the estimated OR for CM were significantly different from 1 in the healthy-heifers dataset, the OR were consistently below 1 for IgA and IgM isotypes binding all tested antigens.

Table 3. Effect of natural antibody titers on risk for clinical mastitis. Estimate, standard error (SE), odds ratio (OR), and significance levels (*P* values) for the effect of natural antibody (NAb)[1] titers and somatic cell score at the sampling moment (SCS sample), on relative risk for clinical mastitis after the sampling moment for the all-cows dataset (left) and for the healthy-cows dataset (right)

NAb[1]	All heifers				Healthy heifers			
	Estimate	SE	OR	*P* value	Estimate	SE	OR	*P* value
LTA-IgA	0.09	0.09	1.10	0.315	-0.17	0.15	0.85	0.263
LTA-IgG1	0.17	0.08	1.18	0.026	0.04	0.13	1.04	0.760
LTA-IgM	-0.02	0.12	0.98	0.888	-0.23	0.19	0.79	0.229
LTA-total	0.01	0.12	1.01	0.909	-0.22	0.20	0.80	0.269
LPS-IgA	0.09	0.09	1.09	0.317	-0.30	0.18	0.74	0.098
LPS-IgG1	0.04	0.07	1.04	0.602	0.08	0.11	1.09	0.462
LPS-IgM	-0.07	0.10	0.94	0.533	-0.31	0.18	0.74	0.085
LPS-total	-0.02	0.11	0.98	0.862	-0.08	0.17	0.92	0.627
KLH-IgA	0.16	0.10	1.18	0.106	-0.30	0.21	0.74	0.151
KLH-IgG1	0.01	0.07	1.01	0.868	-0.01	0.11	1.00	0.978
KLH-IgM	0.01	0.11	1.00	0.974	-0.23	0.17	0.80	0.187
KLH-total	0.07	0.14	1.07	0.606	-0.18	0.23	0.83	0.413
PGN-IgA	0.15	0.10	1.16	0.155	-0.26	0.22	0.78	0.256
PGN-IgG1	0.05	0.08	1.05	0.522	-0.05	0.13	0.95	0.705
PGN-IgM	0.05	0.11	1.05	0.659	-0.28	0.21	0.76	0.176
PGN-total	0.06	0.10	1.06	0.560	-0.28	0.19	0.75	0.133
SCS sample	0.40	0.09	1.49	<.0001	-0.27	0.24	0.76	0.248

[1]NAb titers refer to total or individual isotype (IgA, IgM and IgG1) antibody titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), lipopolysaccharide (LPS) and peptidoglycan (PGN).
[2]Estimate refers to the regression coefficient estimate for the effect of NAb titer on risk for clinical mastitis.

Isotype NAb titers in relation to risk for clinical mastitis in the healthy-heifers dataset

The observation that the estimated OR for CM later in lactation was consistently below 1 in the healthy-heifers dataset for IgA and IgM binding all the different antigens, suggests a protective role for these isotypes, albeit not statistically significant. To examine this relation more closely, the estimated and observed percentages of heifers that developed CM were determined for heifers that were grouped by their NAb titer category to the different antigens. For all the antibody titer categories with more than 2 heifers developing CM after the sampling moment, the observed percentage of heifers that developed CM was within the 95% confidence interval of the predicted percentage. For all the IgA and IgM isotypes binding the different antigens, heifers within the lowest NAb titer categories had a considerably higher estimated and observed risk for CM later in lactation than heifers in the highest antibody titer categories (figure 1). For instance, heifers in IgM binding LPS antibody titer category 0 have predicted CM incidence of 5.2% and heifers within the antibody titer category 4 have 1.6% predicted CM incidence. The data for the IgG1 isotype titers were different. Heifers in the highest IgG1 antibody titer categories did have a similar estimated risk for CM (IgG1 binding KLH) or even an increased risk (IgG1 binding LPS and LTA) compared to heifers within lower antibody titer categories. Only for IgG1 binding PGN a somewhat reduced risk for CM was predicted for heifers in the highest IgG1 NAb titer category (3.2% in category 6) compared with heifers in the lowest NAb titer category (3.8% in category 2).

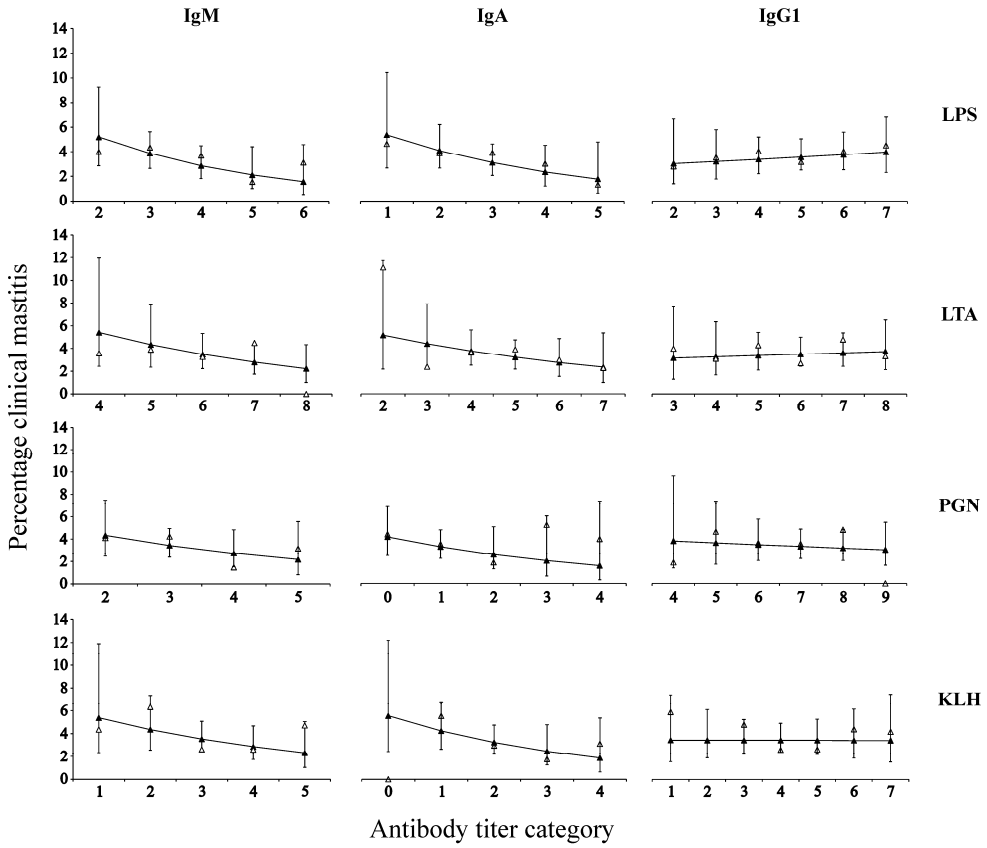

Figure 1. Isotype NAb titers in relation to risk for clinical mastitis in the healthy-heifers dataset. Predicted (filled triangles) and observed (open triangles) proportion of heifers that developed CM. NAb titers refer to total or individual isotype (IgA, IgM and IgG1) antibody titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), lipopolysaccharide (LPS) and peptidoglycan (PGN).

DISCUSSION

Concerning the first aim of the study, our findings suggested that heifers without CM or high SCC history, but with high NAb titers of the IgM and IgA isotypes (and total NAb) in their milk show a tendency towards a reduced risk to develop CM later in lactation. The lowest OR (0.74) was found for IgA binding LPS and KLH, and IgM binding LPS, suggesting that an increase with 1 unit of these NAb titers decreases the relative risk for CM later in lactation with 26%, if significant. This tendency to a protective effect of the IgM and IgA isotypes was not observed in the all-heifers dataset. For high SCC in the all-heifers dataset, OR were generally higher than 1 (up to 1.42) and significant for nearly all these antibodies (table 2), except for IgG1 binding KLH (OR = 0.93, P = 0.046). This indicates that heifers with higher NAb titers have an increased risk to develop high SCC later in lactation, but for IgG1 binding the naive antigen KLH a decreased risk. In the healthy-heifers dataset the OR for high SCC was generally lower (ranging from 0.85 to 1.10) than in the all-heifers dataset, but only significantly lower than 1 for total and IgG1 NAb binding LPS (OR = 0.86 and 0.88 respectively) and IgG1 binding KLH (OR = 0.86). This suggests that these NAb decrease the risk for high SCC in the healthy-heifers dataset later during lactation.

The second aim of this study was to investigate if udder health status before and at the sampling moment affects the relation of NAb with risk for CM or high SCC. Odds ratios of all NAb for risk for CM or high SCC were lower in the healthy-heifers dataset than in the all-heifers dataset (except for IgG1 binding LPS for risk for CM). This difference is probably due to (subclinical) infections previous to or at the sampling moment that resulted in increased antibody titers. Infections can result in increased antibody titers due to general enhancement of antibody production (Saeij et al., 2008), an increased transfer of antibodies from blood to milk by higher vascular permeability in reaction to infection (Harmon et al., 1976; Kohler et al., 2003; Korhonen et al., 2000), or production of antigen-specific antibodies. Previous udder infections are known to be a risk factor for developing CM later on (Steeneveld et al., 2008) and could be an indication of a less effective (innate) immune system.
Especially heifers with previous CM or high SCC, probably did encounter LPS, LTA or PGN before that could have resulted in an increase in NAb titers binding these PAMP. We still use the word natural for LPS, LTA or PGN binding antibodies according to the definition that NAb are present without intentional or controllable antigenic stimulation. Heifers probably did not and will not encounter KLH, so NAb binding KLH reflect the innate immune capacity (Star et al., 2007). Disease history did, however, affect also NAb binding KLH, except for IgG1.

The all-cows dataset is less suitable than the healthy-cows dataset to determine the protective effect of preexisting NAb. Although NAb could be protective in these cows, their relation might be masked by increased titers as a result of previous or current (intramammary) infections, as explained above. Thus, for evaluating the protective value of NAb we need to concentrate on the results obtained in the healthy-heifers dataset. In this healthy-heifers dataset different effects of NAb titers on the risk for CM were observed for the different isotypes. Odds ratios of IgM and

IgA (and total) NAb for CM in the healthy-heifers dataset were consistently well below 1 (0.74 - 0.85) for all antigens, whereas OR of IgG1 ranged from 0.95 to 1.09. Results were not significant, possibly due to the low incidence of CM that decreased the power to detect a significant effect on risk for CM. The low number of heifers with subsequent CM in our dataset is in line with the lower incidence of CM in heifers than in older cows (Fox, 2009) and with the fact that the highest rate of CM is observed in the first 6 weeks of lactation while the heifers in our datasets were later in lactation at the sampling moment. The suggested protective effect of the IgM and IgA isotypes can also be observed in figure 1, in which the risk for CM is shown for different NAb titer categories in the healthy-heifers dataset. For IgM and IgA binding all different antigens, the predicted risk for CM was consistently lower for heifers in the highest compared to the lowest NAb titer categories. For example, for IgM binding LPS between titer categories 0 and 4, the predicted percentage CM changed from 5.2% to 1.6% that represents a reduction of approximately 70%. In contrast to IgM and IgA NAb, heifers in the highest IgG1 NAb titer categories showed a similar or increased risk for CM compared to animals within the lower antibody categories.

The data presented above indicate that NAb of the IgM and IgA isotypes might play a role in the prevention of CM. We anticipated that heifers with relatively high NAb titers of the IgM and IgA isotypes to a particular antigen will be more likely to have also higher IgM and IgA titers against other antigens. To examine this, Pearson correlation coefficients between the different NAb titers were determined in the uncorrected data. The correlation between for instance antibody titers binding KLH and the other antigens ranged from 0.72 to 0.81 for IgM, from 0.59 to 0.70 for IgA and from 0.15 to 0.29 for IgG1 (data not shown). Thus, IgM and IgA NAb titers had indeed higher correlations between the different antigens, where IgG1 NAb titers showed lower correlations between the different antigens.

A previous study (Ploegaert et al., 2010) showed that across-herd heritability in heifers was substantially lower for IgG1 titers binding LTA (0.07) than for IgM (0.42) or IgA (0.47) titers binding the same antigen. This suggests that in heifers IgG1 titers are much more influenced by the environment (e.g. earlier infection or exposure to LTA) than the titers of the more “innate” IgM and IgA isotypes. This is in line with results of the present study, where OR of IgM and IgA for risk for CM were consistently below 1 for all antigens in the healthy-heifers dataset, suggesting an ‘innate’ protective effect of these isotypes, although not significant.

In mammals, although NAb are mostly of the IgM isotype class, IgG1 and IgA NAb have also been described (Boes, 2000; Lacroix-Desmazes et al., 1998). Isotypes might reflect different effector functions that include barrier functions (innate immunity) and enhancing antigen-specific protective immune responses (adaptive immunity) (Korhonen et al., 2000; Zinkernagel, 2003). The exact mechanism of the potential relation between NAb titers in milk and CM or SCC in heifers remains to be elucidated.

CONCLUSIONS

The results of the present study suggest a possible protective role of NAb in prevention of CM (IgM and IgA to all antigens) and high SCC (IgG1 to KLH and LPS) in healthy heifers, but udder health history should be taken into account. Further studies with more clinical mastitis cases are needed to obtain more insight in the relations of NAb with risk for CM and high SCC.

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Chapter 6

Relation of natural antibodies with risk for mastitis and high somatic cell count in Dutch Holstein Friesian cows

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ABSTRACT

An important component of the immune-mediated disease resistance consists of natural antibodies (NAb). In this study the relationship of NAb titers in milk of dairy cows and the risk to develop high somatic cell count (SCC) and clinical mastitis (CM) later in lactation was investigated. In single milk samples from 1998 cows of 60 farms the isotypes IgG1, IgM and IgA of NAb that bind to lipopolysaccharide, lipoteichoic acid, peptidoglycan and keyhole limpet hemocyanin were determined in a dataset with primi- and multiparous cows. From these cows information on diseases and test-day SCC of the present lactation were used to create 2 datasets. The first dataset contained data of all the, at the sampling moment clinically-healthy, cows (n = 1998); the all-cows dataset. The second dataset contained data from the clinically-healthy cows that did not show CM nor had high SCC (defined as >150,000 cells/mL for primiparous cows, >250,000 cells/mL for multiparous cows during the SCC test-days) before or at the sampling moment (n = 1260); the healthy-cows dataset. Logistic regression was used to evaluate the effect of NAb titers on risk for CM and high SCC. Results indicated IgG1 binding keyhole limpet hemocyanin in the healthy-cows dataset to be protective against CM (odds ratio 0.8), but not against high SCC. Furthermore, NAb titers appear to be affected by udder health history. Finally, heifers appear to differ from older cows in the relation of NAb titers with risk for CM or high SCC. This study reveals the putative importance of NAb in prevention of mastitis in dairy cows of different parities.

INTRODUCTION

The innate immune system is crucially important in combating and preventing mastitis (Rainard and Riollet, 2006) that is a major health and economical problem in dairy farming (Halasa et al., 2007). The humoral innate immune system consists, among others, of natural antibodies (NAb) that are defined as antibodies present in non-immunized individuals that are able to bind to different antigens (Avrameas et al,. 1991). Natural antibodies have a broad specificity, with usually low binding affinity (Boes, 2000; Casali and Notkins, 1989; Ochsenbein et al., 1999). Natural antibodies were shown to be present in bovine milk and blood plasma (Ploegaert et al., 2010; Van Knegsel et al., 2007). Natural antibodies are thought to provide an early barrier to infection, and may subsequently act as an adjuvant of specific immunity (Kohler et al., 2003). A relationship has been suggested between NAb titers binding keyhole limpet hemocyanin (KLH) or lipopolysaccharide (LPS) on the one hand, and body condition, energy balance, milk yield and plasma cholesterol concentration in the peri-partum period on the other hand (Van Knegsel et al., 2007). In mammals, NAb are mainly of the IgM isotype class, but IgG and IgA NAb have been described as well (Boes, 2000). Bacteria are often responsible for intra-mammary and other infections in dairy cows. Certain pathogen-associated molecular patterns (PAMP) are shared by 'classes' of bacteria and serve as targets for identification of microbes by the innate immune system (Kohler et al., 2003). A high proportion of NAb can bind these PAMP. Therefore, several PAMP were chosen as a model for these bacteria in this study. Important PAMP are LPS present on gram-negative (entero)bacteria, such as *Escherichia coli*; lipoteichoic acid (LTA) present on gram-positive bacteria, such as *Staphylococcus aureus*; and peptidoglycan (PGN) present on gram-negative and gram-positive bacteria. Furthermore, NAb can bind antigens that cows likely did not and will not encounter, such as KLH (Van Knegsel et al., 2007), a glycoprotein from the sea mollusk *Megathura crenulata*. Natural antibodies binding KLH could mirror the innate humoral immune capacity of an animal, and KLH is consequently chosen as an antigen in this study.

Results of our previous study (Chapter 5) suggested that the NAb isotypes IgM and IgA binding LPS, LTA, PGN and KLH can be protective against the development of clinical mastitis (CM) in a dataset with clinically-healthy primiparous cows without previous CM and high test-day somatic cell count (SCC; >150,000 cells/mL) cases. In a dataset with 'all' heifers, which contained also heifers that experienced previous cases of CM or high SCC, such a protective effect was not found, underscoring the importance of taking udder health history into account. Furthermore, the titer of IgG1 binding KLH was suggested to be protective against the development of high SCC, but more profoundly in 'healthy' heifers. The dataset contained only heifers. To the best of our knowledge, the effect of NAb on risk for the development of CM and high SCC in cows of different parities has not been described before.

Previously, increasing NAb titers were found in blood and milk of ageing cattle (Srinivasan et al., 1999; Van Knegsel et al., 2007), suggesting that NAb are the cumulative result of antigenic stimulation, either by continuous polyclonal stimulation by exogenous microbes, or self-antigens, or both (Avrameas, 1991;

Kohler et al., 2003; Tomer and Shoenfeld, 1988). The effect of NAb on the risk for CM or high SCC could therefore be different in multiparous cows compared to primiparous cows. The aim of the present study was to investigate if NAb of isotypes IgG1, IgM and IgA binding LPS, LTA, PGN or KLH have an effect on the risk for subsequent high SCC or CM, in a dataset with primi- and multiparous cows. Isotypes of NAb were determined to obtain better insight in the behavior of different parts of the antibody 'section' of the humoral innate immune system, than would be possible with total NAb only. For comparison SCC level at the sampling moment was also analyzed.

MATERIALS AND METHODS

Animals and farms

From 2100 Holstein Friesian cows from 60 Dutch commercial dairy farms single milk samples were collected between June and October 2009. Farms participated in the 4 weekly milk recording of individual cows via the cattle cooperative CRV (Arnhem, The Netherlands), including SCC measurement. The average number of cows per farm that met the inclusion criteria (i.e. clinically-healthy at the sampling moment and between 5 - 90 days in lactation) was 34 with a minimum of 8 and a maximum of 112. All cows were milked twice a day and were from first to eleventh lactation.

Samples

Milk samples were preserved with sodium azide and bronopol, transported, refrigerated, aliquoted and frozen at -20°C. Samples were refrigerated up to 14 d, because it was practically not feasible to aliquot and freeze them within 1 d. However, previous tests demonstrated that refrigerated storage (4°C) for up to 4 weeks did not affect NAb titers (T. Ploegaert, unpublished data). Milk samples were a mixture of afternoon and morning samples taken for the 4-weekly milk recording. Sampling date refers to the milk recording test-day on which samples were collected for NAb titer measurement.

Mastitis and somatic cell count data

Somatic cell count was measured with a Fossomatic 5000 (Foss A/S, Denmark) at the certified laboratory Qlip (Zutphen, the Netherlands) within 1 day after sampling. Information on SCC was provided by CRV (Arnhem, The Netherlands). Somatic cell score was calculated as ln of SCC/1000. Somatic cell count was considered high if >150.000 cells/mL for primiparous cows and >250.000 cells/mL for multiparous cows at 1 or more test-days after the sampling moment. The chosen thresholds for high SCC are currently used as threshold for heifers and older cows by the Dutch Dairy Herd Improvement program (Sampimon et al., 2010). Clinical mastitis cases were recorded by the participating farmers. Finally, from 1998 cows CM and SCC data were obtained.

Natural antibody isotypes

Titers of NAb isotypes IgG1, IgM, and IgA of NAb binding *Escherichia coli*-derived LPS (L2880, serotype O55:B5), *Staphylococcus aureus*-derived LTA (L2515) both from Sigma-Aldrich Inc. (St. Louis, MO), *S. aureus*-derived PGN (BioChemika, Buchs, CH), or *Megathura crenulata-derived* KLH from MP Biomedicals (Solon, Ohio) were determined using an indirect ELISA procedure as described before (Ploegaert et al., 2010). Briefly, plates were coated with 100 µL/well of either 1 µg KLH, 4 µg LPS, 5 µg LTA or 2 µg PGN per mL of carbonate buffer (10.6 g/L Na_2CO_3, pH 9.6). After washing, plates were blocked with 100 µL/well 2.5% rabbit serum in PBS with 0.05% Tween20 for at least 30 min at room temperature. Four serial dilutions of samples (1:4) in PBS, 0.05% Tween20 and 2.5% rabbit serum were added. Dilutions started at 1:4. On each plate the same positive milk sample was included with 8 serial dilutions (1:2) in duplicate. Plates were incubated for 1 h at room temperature. Binding of isotype-specific antibodies to LTA was detected using 1:16,000 diluted sheep anti-bovine IgG1 (Serotec, Düsseldorf, Germany), 1:16,000 diluted sheep anti-bovine IgM (Serotec), or 1:8,000 diluted sheep anti-bovine IgA (Serotec) all coupled to horseradish peroxidase. Binding of isotype antibodies to LPS was detected using 1:2000 diluted sheep anti-bovine IgG1 (Serotec), 1:16,000 diluted sheep anti-bovine IgM (Serotec), and 1:2,000 diluted sheep anti-bovine IgA (Serotec). Binding of isotype-specific antibodies to KLH were detected using 1:2,000 diluted sheep anti-bovine IgG1 (Serotec), 1:16,000 diluted sheep anti-bovine IgM (Serotec), and 1:4,500 diluted sheep anti-bovine IgA (Serotec). Binding of isotype-specific antibodies to PGN were detected using 1:2,000 diluted sheep anti-bovine IgG1 (Serotec), 1:8,000 diluted sheep anti-bovine IgM (Serotec), and 1:3,000 diluted sheep anti-bovine IgA (Serotec). After washing, 100 µL/well tetramethylbenzidine (71.7 µg/mL) and 0.05% H_2O_2 were added to the wells and incubated for 10 min at room temperature. The reaction was stopped with 50 µL/well 2.5 *N* of H_2SO_4. Extinctions were measured with a Multiskan spectrophotometer (Flow, Irvine, UK) at a wave length of 450 nm. Natural antibody levels were calculated as titers. Titers were expressed as Log_2 values of the dilutions that gave an extinction closest to 50% of Emax, where Emax represents the highest mean extinction of a standard positive milk sample present in duplicate on every microtiter plate (Ploegaert et al., 2010).

Datasets and statistical analysis

Two datasets were created for the traits CM and high SCC. The first dataset contained data on all cows with information on CM and high SCC: the all-cows dataset. These cows were considered clinically-healthy at the sampling moment, but could have had CM in that lactation before the sampling moment, or high SCC at test-days in that lactation before or at the sampling moment. To study if the effect of NAb titers is changing with udder health history a second dataset was created: the healthy-cows dataset. The cows in this dataset were considered clinically-healthy at the sampling moment, and in that lactation did not have high SCC at test-days before or at the sampling moment and did not show CM before or at the sampling moment. For the two datasets, CM and SCC data after the sampling moment were analyzed. To study the effect of NAb titer on the risk for CM or high SCC later in

lactation, logistic regression analysis was performed. The procedure Glimmix in SAS (SAS Institute Inc., Cary, NC; version 9.1) was used with the following model:

$$y_{ijklm} = \mu + b_1 * (afc_i * maturity_j) + parity_k + lactst_l + monthsampling_m + b_2 * NAb/SCS_n + herd_o + E_{ijklmno} \quad [1]$$

where y_{ijklm} = dependent variable CM or high SCC for a cow with age at first calving (afc) i combined with state of maturity (primiparous or multiparous cow) j with regression coefficient b1, parity (parity 1, 2, 3, 4 or >=5) k, lactation stage (lactst, 1 (d 5 - 30), 2 (d 31 - 60) or 3 (d 61 - 90)) at sampling moment l, monthsampling (month of the year in which milk sample was taken (June, July, August or September/October) m; μ = general mean; afc_j = covariate describing the effect of age at first calving; NAb/SCSn = effect of NAb titer **or** SCS measured in the taken milk sample n, with regression coefficient b2; $herd_o$ = random effect of herd o; and $E_{ijklmno}$ = random residual effect. The odds ratio (OR) was calculated as OR = $e^{estimate}$ where estimate reflects the regression coefficient estimate for the effect of NAb titer or SCS. Odds ratio shows the relative risk for CM or high SCC after the sampling moment if NAb titer rises 1 unit. If for instance OR = 0.85, the relative risk for CM or high SCC decreases with 15% if the NAb titer rises 1 unit. NAb titers were divided in categories, where category 1 contained the data of all cows with NAb titer 0.5 to 1.5, category 2 titer 1.5 to 2.5 and so on. For a given NAb titer category, the predicted proportion of cows that will develop CM after sampling was computed using model (1). The predictions were compared to the observed proportions of cows that developed CM after sampling for that particular NAb titer category.

RESULTS

Description of datasets

Total number of cows and number of cows with and without CM and high SCC after the sampling moment in both datasets are presented in table 1.

Table 1. Total number of cows, and number of cows with and without clinical mastitis (CM), and high somatic cell count (HSCC) after the sampling moment in the all-cows dataset and the healthy-cows dataset

Dataset	Number of cows (% of cows) (after sampling[1])				
	Total	CM		HSCC	
		Yes	No	Yes	No
All-cows	1998	156 (7.8%)	1842	523 (26.2%)	1475
Healthy-cows-CM	1260	66 (5.2%)	1194	200 (15.9%)	1060

[1]Period from sampling moment until December 2009, reflecting on average 145 d within the lactation period after sampling.

The percentage of cows that developed CM was 7.8% (165 cases) in the all-cows dataset and 5.2% (66 cases) in the healthy-cows dataset. Also the percentage of cows that developed high SCC was higher in the all-cows dataset (26.2%) than in the healthy-cows dataset (15.9%).

Effect of natural antibody titers on risk for high somatic cell count

In milk NAb isotypes IgG1, IgM and IgA titers binding LTA, LPS, PGN and KLH were measured. Logistic regression analysis was performed on the all-cows dataset and the healthy-cows dataset to estimate the relation of the NAb titers with the risk for developing high SCC after sampling. Table 2 shows OR, standard error and *P* values of all NAb related to the risk for high SCC. In the healthy-cows dataset OR for NAb were all nearly equal to or above 1 (ranging from 0.96 to 1.16), but were lower than OR of NAb in the all-cows dataset (ranging from 1.07 to 1.31). The only OR that were (nearly) significant in the healthy-cows dataset were those of IgG1 binding LTA (OR = 1.11, *P* = 0.056) and PGN (OR = 1.16, *P* = 0.01). In the all-cows dataset OR of NAb were all above 1 and significant, except for IgG1 binding KLH. The OR of SCS at the sampling moment was above 1 and significant for both datasets.

Table 2. Effect of NAb titers on risk for high somatic cell count. Estimate, standard error (SE), odds ratio (OR) and significance levels (*P* values) for the effect of natural antibody (NAb)[1] titers and somatic cell score at the sampling moment (SCS sample), on relative risk for high somatic cell count after the sampling moment for the all-cows dataset (left) and for the healthy-cows dataset (right)

	All cows				Healthy cows			
NAb	Estimate	SE	OR	*P* value	Estimate	SE	OR	*P* value
LPSIgA	0.18	0.04	1.20	<.0001	0.02	0.06	1.02	0.743
LPSIgM	0.19	0.05	1.21	<.0001	0.09	0.07	1.10	0.181
LPSIgG1	0.07	0.04	1.07	0.049	0.06	0.06	1.07	0.286
LTAIgA	0.22	0.04	1.24	<.0001	0.06	0.06	1.06	0.321
LTAIgM	0.21	0.04	1.23	<.0001	0.07	0.06	1.07	0.244
LTAIgG1	0.23	0.03	1.26	<.0001	0.10	0.05	1.11	0.056
KLHIgA	0.21	0.05	1.23	<.0001	-0.01	0.07	0.99	0.902
KLHIgM	0.19	0.05	1.21	<.0001	0.03	0.07	1.03	0.717
KLHIgG1	0.07	0.04	1.07	0.072	-0.04	0.06	0.96	0.498
PGNIgA	0.18	0.03	1.20	<.0001	0.04	0.05	1.04	0.419
PGNIgM	0.19	0.04	1.20	<.0001	0.04	0.06	1.04	0.580
PGNIgG1	0.27	0.04	1.31	<.0001	0.15	0.06	1.16	0.010
SCS sample	0.74	0.05	2.10	<.0001	0.58	0.11	1.78	<.0001

[1]NAb titers refer to antibody isotype (IgA, IgM and IgG1) titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), lipopolysaccharide (LPS) and peptidoglycan (PGN).

Effect of natural antibody titers on risk for clinical mastitis

Logistic regression analysis was performed on the all-cows dataset and the healthy-cows dataset to estimate the relation of the NAb titers with risk for CM after sampling. The OR for IgG1 binding KLH in the healthy-cows dataset was significantly (P = 0.013) below 1 (0.80) suggesting a protective effect of this NAb against CM. Also OR of IgA binding LPS, IgG1 binding LTA, and IgA and IgM binding KLH and PGN in the healthy-cows dataset were below 1 for CM (ranging from 0.83 to 0.92), but not significant (Table 3). Furthermore, in the all-cows dataset OR of IgG1 binding KLH for CM was not significantly just below 1, and all the other OR for CM were around or above 1, ranging from 1.03 to 1.18 (Table 3). However, of these only OR of IgG1 binding PGN (1.18) was significant (P = 0.001). Odds ratios for SCS at the sampling moment were above 1 for both datasets, but only significant (P < 0.001) for the all-cows dataset. Odds ratios were in general higher for the all-cows dataset, than for the healthy-cows dataset.

Table 3. Effect of NAb titers on risk for clinical mastitis. Estimate, standard error (SE), Odds ratio (OR) and significance levels (P values) for the effect of natural antibody (NAb)[1] titers and somatic cell score at the sampling moment (SCS sample), on relative risk for clinical mastitis after the sampling moment for the all-cows dataset (left) and for the healthy-cows dataset (right)

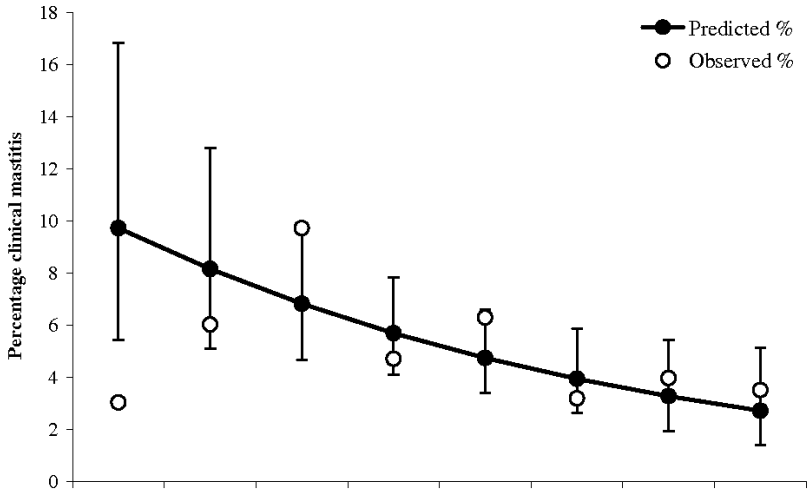
	All cows				Healthy cows			
NAb	Estimate	SE	OR	*P* value	Estimate	SE	OR	*P* value
LPSIgA	0.04	0.06	1.04	0.513	-0.09	0.10	0.92	0.354
LPSIgM	0.04	0.07	1.04	0.607	0.03	0.10	1.04	0.737
LPSIgG1	0.03	0.06	1.03	0.606	-0.01	0.09	0.99	0.936
LTAIgA	0.11	0.05	1.11	0.051	0.05	0.09	1.06	0.528
LTAIgM	0.05	0.06	1.05	0.443	0.00	0.09	1.00	0.980
LTAIgG1	0.05	0.05	1.05	0.342	-0.11	0.09	0.89	0.188
KLHIgA	0.05	0.07	1.05	0.507	-0.18	0.11	0.83	0.100
KLHIgM	0.04	0.07	1.04	0.550	-0.09	0.11	0.91	0.397
KLHIgG1	-0.02	0.06	0.98	0.785	-0.22	0.09	0.80	0.013
PGNIgA	0.08	0.05	1.08	0.106	-0.02	0.08	0.98	0.829
PGNIgM	0.07	0.06	1.08	0.246	-0.03	0.10	0.97	0.792
PGNIgG1	0.17	0.05	1.18	0.001	0.08	0.09	1.08	0.376
SCS sample	0.34	0.06	1.40	<.0001	0.04	0.16	1.04	0.781

[1]NAb titers refer to antibody isotype (IgA, IgM and IgG1) titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), lipopolysaccharide (LPS) and peptidoglycan (PGN).

Higher IgG1 titers binding KLH reduce risk for CM in the healthy-cows dataset

The observation that the estimated OR for CM later in lactation was below 1 in the healthy-cows dataset for IgG1 binding KLH, indicates a protective role for this isotype. To examine this relation more closely, the estimated and observed

percentages of cows that developed CM were determined for cows that were grouped by their IgG1 titer category binding KLH (figure 1). For all the antibody titer categories with 2 or more cases of CM, the observed percentage of cows that developed CM was within the 95% confidence interval of the predicted percentage. Cows in the lowest IgG1 titer category binding KLH had a considerably higher estimated risk for CM later in lactation (9.7%) than cows in the highest antibody titer category (2.7%) (Figure 1).



KLH-IgG1 category	0	1	2	3	4	5	6	7
Number of cows with CM	1	5	14	13	20	6	5	2
Total number of cows	33	83	144	276	318	188	126	57

Figure 1. Percentage of clinical mastitis (CM) per titer category of IgG1 binding keyhole limpet hemocyanin (KLH-IgG1) in the healthy-cows dataset. Error bars represent 95% confidence intervals.

DISCUSSION

This study investigated the effect of NAb in milk of dairy cows on the risk for future development of CM and high SCC. In the healthy-cows dataset OR for CM were below 1 (0.79 – 0.91) for all NAb isotypes binding KLH, but only significant for the IgG1 isotype (OR = 0.8, $P = 0.013$). In this healthy-cows dataset the estimated risk to develop CM later in lactation was more than 3 times lower for cows within the highest IgG1-KLH titer category compared to cows in the lowest titer category (2.7% vs. 9.7%). For the all-cows dataset no protective effect of NAb against CM was found. Results of the healthy-cows dataset suggest a protective effect of IgG1 NAb binding KLH measured in milk of clinically-healthy dairy cows against subsequent development of CM. In a previous study, where milk samples and CM and SCC data

were available from 1515 Holstein Friesian heifers (Ploegaert et al., 2010), OR of risk for CM consistently suggested a protective but not significant effect for the IgM and IgA isotypes binding all 4 tested antigens (LPS, LTA, PGN and KLH) (Chapter 5). In the present study, this suggestive protective effect of the NAb against antigens other than KLH was not found. However, the previous study only contained primiparous cows and the present study mostly multiparous cows (about 75%). Unfortunately, a reliable estimation of OR for CM for only primiparous cows in the current study was not possible due to the low number of CM cases (n = 9 in the healthy-cows dataset) found in the heifers after sampling. Estimation of OR for risk for high SCC for only primiparous cows in the current study was possible (70 cases in healthy-cows dataset), and revealed a similar protective effect of IgG1 binding KLH for heifers in this study (OR = 0.88) (results not shown) and the previous study (OR = 0.86) (Chapter 5). In contrast to the previous study, the effect was not significant in this study that could be caused by the much smaller number of heifers (n = 381).

In the present study parity was taken into account in the analysis, and showed a significant effect; the higher parity, the higher risk for CM (data not shown). This finding underscores the difference between the previous study (Chapter 5) with only primiparous cows and the present study with a large percentage (75%) of multiparous cows, with a large variation in parity. The latter may in these cows reflect their infectious history that, naturally, is much more extensive in this population than in the previously tested heifers. Considerable differences between primiparous and multiparous cows have been reported by others. Van Knegsel et al. (2007) showed increasing total NAb titers binding KLH and LPS in plasma and milk of dairy cows during ageing. Furthermore, the incidence of CM (Fox et al., 2009; Sargeant et al., 1998; Valde et al., 2004; Van den Borne et al., 2010) and prevalence of high SCC is generally higher in multiparous cows than in primiparous cows (Van den Borne et al., 2010). Besides, cellular immune analysis showed that phagocytic and killing activity and radical production of blood and milk polymorphonuclear leukocytes (PMNL) against *S. aureus*, and PMNL viability in milk was higher in primiparous cows than in multiparous cows (Mehrzad et al., 2009). Thus, at least several immune parameters and CM are different in primiparous cows and multiparous cows. The mechanism behind this difference awaits further studies.

Odds ratios of all measured NAb for CM and high SCC were lower in the healthy-cows dataset than in the all-cows dataset. This is in line with the previous study on heifers (Chapter 5). This difference between the all-cows dataset and the healthy-cows dataset is probably a consequence of the difference in udder health history of cows in the all-cows dataset versus the healthy-cows dataset. Also in the previous study on primiparous cows we found that previous or current (subclinical) infections could affect the relation between NAb and CM or SCC. This effect could be due to increased antibody titers in the milk as a result of production of antigen-specific antibodies, general enhancement of antibody production (Saeij et al., 2003), or increased transfer of antibodies from blood to milk by higher vascular permeability, in reaction to infection (Harmon et al., 1976; Kohler et al., 2003; Korhonen et al., 2000). For investigation of CM and high SCC it is very important to take into account at least CM and SCC history of cows. For evaluating the protective value of NAb we need to concentrate on the results obtained in a healthy-cows dataset.

It is likely that most multiparous cows did encounter the general PAMP LPS, LTA and/or PGN that are present on many bacteria, during their life. We nevertheless still consider these antibodies NAb as they were not intentionally induced according to the definition. However, they will not have encountered the naive antigen KLH. Therefore, NAb binding KLH reflect the innate immune capacity in these multiparous cows. Indeed, all the isotypes binding KLH had OR for CM lower than 1 (range 0.80 to 0.91). This is in line with the study of Star et al. (2007) that reported OR of 0.80 for total NAb binding KLH for survival of laying hens.

In conclusion, our analyses suggest that NAb titers of the isotype IgG1 binding KLH protect against CM in healthy cows. Udder health history affects NAb titers. Additionally, primiparous cows appear to differ in NAb levels and their relation to CM and high SCC from multiparous cows. Further research is needed to unravel the possible protective effect of NAb against several important diseases in dairy cows of different parities.

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Chapter 7

General Discussion

T.C.W. Ploegaert

The main aims of this research were to identify immune parameters that are indicative for the susceptibility of individual cows for development of mastitis and possibly other diseases, and to determine their prospects as biomarkers to improve (udder) health by selective breeding. For future routine screening of dairy cows it is important that the parameters can be determined in samples that can be obtained easily and in a non-invasive way. In this respect it is important that these parameters can be measured in milk.

Immune parameters that could potentially be related to mastitis susceptibility were selected based on literature (*Chapter 1*). Their suitability to study was investigated in *Chapters 2 and 3*, and will be further discussed in this chapter. Natural antibodies (NAb) determined in milk were found to show a sufficient repeatability over time within individual cows and variation among cows. Natural antibodies were, therefore, chosen as parameters for subsequent studies. Genetic variation for different NAb titers was estimated in samples of the Milk Genomics data (*Chapter 4*). In the Milk Genomics data also the relation of NAb with risk for clinical mastitis (CM) and high somatic cell count (SCC) was studied (*Chapter 5*). Finally, a field study was performed (*Chapter 6*) that was designed to investigate the relation of NAb with risk for CM, high SCC and other diseases. Furthermore, in this chapter the relation of NAb with risk for other diseases, implications and future perspectives will be discussed.

DESIGN OF THE STUDY

In order to study disease resistance (at least) 3 approaches can be conducted. The first approach studies the type of immune response induced by the different pathogens. For example, chemokine gene expression was different after *ex vivo* stimulation of bovine mammary cells with *E. coli*-derived LPS or *S. aureus*-derived LTA plus PGN (Mount et al., 2009). Furthermore, cytokine responses, among which tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), transforming growth factor (TGF)-β1, TGF-α, interleukin (IL)-1β, IL-8 and IL-10, to mammary infections, were different for different pathogens, among which *E. coli, S. aureus, M. bovis, S. uberis,* and *P. aeruginosa* (Bannerman, 2009). *Staphylococcus aureus* is known to cause more often subclinical and chronic mastitis while *E. coli* more often causes CM (Yang et al., 2008). Furthermore, LTA or heat-killed *S. aureus* did not activate the transcription factor NF-κB, whereas LPS or heat-killed *E. coli* did (Yang et al., 2008). In addition, even different strains of *Streptococcus agalactiae* differed in their requirement of opsonisation by antibodies or complement to be phagocytosed (Rainard et al., 1988).

The second approach studies variation among cows in specific adaptive humoral and cellular immune responses to an administered antigen, in relation to cows' susceptibility to diseases. In this approach, as used by Begley et al. (2009), Heriazon et al. (2009) and Wagter et al. (2000), the induced specific antibody titers and delayed-type hypersensitivity reaction against an injected model antigen, like ovalbumin or hen-egg white lysozyme, was used to determine the specific humoral and cell-mediated immune response capacity of individual cows. Subsequently, cows

were classified into low, medium and high responders and their responsiveness to the model antigen was related to the risk for subsequent development of certain diseases like mastitis. This approach usually does not involve experimental infections with pathogens.

The third approach relates variation in components of the innate immune system among cows to their susceptibility to diseases. This is the approach that was followed for the observational research described in this thesis. We did not study the response to specific pathogens, but studied the overall effects of selected immune parameters on risk for diseases in clinically-healthy cows. In our study, we did not determine immune responses to specific pathogens, but we studied the relation of the levels of (humoral) immune parameters in individual cows to their susceptibility for mastitis and several other diseases. Generally, such a study is less controlled and therefore more animals are needed than in an experimental study, and more external factors can influence the outcome. However, only samples are taken and no further treatments need to be given to the animals, avoiding pain, discomfort or stress. Furthermore, in such a study animals are usually kept in their familiar surroundings, receiving their usual feed. This allowed us to study these immune parameters in cows under husbandry field conditions.

POTENTIAL PARAMETERS AND PREREQUISITES FOR APPLICATION

Literature searches yielded different immune parameters that could potentially be protective against different diseases and be detectable in bovine milk and blood. These parameters need to meet certain criteria before they can be used to study their protective value with respect to susceptibility to develop disease. First they need to be measurable in bovine milk, blood or both. Secondly, they need to be present in detectable basal levels in the majority of cows. Thirdly, they need to show variation among individual cows. Fourthly, they need to be repeatable over time in an individual cow given similar husbandry conditions. Furthermore, it is preferred that parameters have a good correlation between levels in milk versus blood, to study if they can reflect not only local but also systemic immunity. These prerequisites were studied for several suggested immune parameters as described below. In literature, however, very few studies were found where all of these steps were followed. This makes it difficult to compare results from different studies and we recommend that this sequence of criteria would become a standard procedure for new parameters. Once it is determined that parameters meet these 4 criteria, the predictive value of the parameter for future health status needs to be determined.

Reactive oxygen species production and types of cells

The innate immune system in mammals is one of the first lines of host defense to infection as it has the capacity to immediately recognize and respond to the earliest signs of infection. The first line defense mechanism, after physical barriers, against mastitis development caused by infection invading the mammary gland, is based on the resident phagocytes, mainly mononuclear cells like neutrophilic granulocytes (neutrophils) and macrophages. After a complicated cascade releasing biological

substances and activation of the endothelium, neutrophils migrate into the mammary gland and finally also appear in the milk, if the infection cannot be cleared by resident phagocytes. It is this massive influx of neutrophils that will resolve the infection through sequestration and killing of the invading bacteria. Both acute and chronic mastitis conditions are associated with a dramatic increase in SCC in milk, with neutrophils being the predominant leukocyte found in both the infected mammary quarter and milk.

Encompassing important populations of immune cells, white blood cells (leukocytes) were isolated from blood and milk and several cell surface (CD) markers and the production capacity of reactive oxygen species (ROS) were analyzed. Using our well designed human protocols based on the use of Ficoll flotation, the isolation of peripheral blood mononuclear cells from cows remained highly variable in the contamination with red blood cells that was difficult to control. An alternative method for isolation of leukocytes was hypotonic lysis of red blood cells with water (Zerbe et al., 1996) that worked much more consistent, rapid and easier and this was routinely used in further studies.

The cellular fraction in milk contains primarily leukocytes and mammary epithelial cells (Rainard and Riollet, 2006) and these are commonly called somatic cells. These cells could be easily recovered from milk by dilution in RPMI medium and subsequent centrifugation and washing steps. However, the yield of milk cells is very variable between individual samples and the somatic cell count (SCC) is variable within a single cow over time. Repeatability for SCC was 0.25 and for SCS 0.55 in the study described in *Chapter 2 and 3* (data not shown). A reason for the variability between individual samples could be that milk cells are usually more terminally differentiated than peripheral blood cells, since they migrate selectively by chemotactic factors and homing receptor expression as mature cells to milk. In addition, also physiological mechanisms or latent infections can be involved, but this is not yet completely understood (Rainard and Riollet, 2006). After having exerted their role in immune function neutrophils die by senescence in the absence of pro-inflammatory agents prior to their removal by macrophages, hereby preventing the release of their cytotoxic content. Necrotic cells are not always promptly cleared by phagocytes and their chromatin proteins may then boost prolonged inflammation.

In the ROS test for milk cells more dead cells were detected with propidium iodide than for blood cells (data not shown). Additionally, (much) more milk is needed than blood to obtain similar numbers of cells for subsequent analysis due to the generally much lower level of cells in milk compared to blood.

The forward and sideward scatter picture obtained with a flow cytometer of isolated milk cells appears to be different from that of peripheral blood cells. The flow cytometric profile of peripheral blood cells showed different populations of cells (granulocytes, monocytes and lymphocytes), but no clear cell populations could be distinguished for milk cells by this method (Figure 1). This could be due, in part, to the activation state of milk cells, because of which some changes in shape and size of the cells occur.

Staining of cells with surface (CD) marker specific antibodies to detect specific types of cells present in milk and blood by flow cytometry was performed to

determine their suitability for further use in this study concerning the potential relation between cell types and their numbers and the disease resistance capacity of cows. Surface staining for several CD markers worked well for blood cells (CD4, CD8 and γδ-T cells, B cells and NK cells), but some (CD4 and γδ-T cells) appeared to be unreliable for milk cells due to high background staining of negative controls (*Chapter 2*).

Furthermore, ROS production was measured by stimulating cells with peptidoglycan (PGN) from *S. aureus* and using dihydrorhodamine 123 (DHR) for detection (*Chapter 2*). The stimulation experiments were performed with cells, present in milk and blood of clinically-healthy cows, reflecting the reactivity potential towards actual infections. While the test worked well for both blood and milk cells, it required large amounts of milk to obtain sufficient numbers of cells for subsequent analysis. Given the required number of milk cells, this test was not feasible for use in our subsequent studies.

Cytokines: TNF-α and IFN-γ

During mastitis cytokines, such as IL-1, IL-6, IL-8 and TNF-α, are released locally. In milk, an increase in TNF-α is observed. Many of the biological activities of LPS are mediated by TNF-α. It was anticipated that differences among cows in basal levels of cytokines in milk and blood plasma might have a relation with differences among these cows in their natural disease resistance. A sandwich ELISA was optimized to determine the levels of TNF-α and IFN-γ and for most samples these were under or around minimal reliable detection levels (approximately 40 pg/mL) of the tests, making these cytokines insufficiently distinctive to determine variation between the majority of cows. However, one cow consistently, also upon retesting, showed higher TNF-α (496 – 931 pg/mL) and IFN-γ (163 – 422 pg/mL) levels in milk (and in plasma also, higher than in milk) than other cows over the entire period of the experiment (*Chapter 2*). Surprisingly, this cow did not show any clinical signs of disease. It is likely that cytokines, like TNF-α and IFN-γ, are secreted in the event of an ongoing infection, only triggered after recognition of a pathogen, and therefore these cytokines are not present in measurable basal levels in healthy animals. Therefore, these cytokines in milk do not appear useful immune parameters for large scale monitoring, determination of heritability, and establishing predictive relationships with risk for clinical diseases, including mastitis.

Nitric oxide

During mastitis, factors like LPS and different cytokines stimulate the synthesis of nitric oxide (NO) that is a vasodilator and a diatomic free radical. It is lipid soluble and diffuses easily through the cell membrane. It is short lived and usually degrades or reacts within a few seconds. NO is synthesised from L-arginine. An inducible NO synthase (E.C. 1.14.13.39, iNOS) is expressed by a variety of cells, especially monocytes, as a result of triggering by substances of microbial origin such as LPS and host factors, like TNF-α. Nitric oxide is produced by macrophages in response to invading pathogens and acts as an antimicrobial agent (Rainard and Riollet, 2006). It is also cytotoxic and can induce apoptosis if produced in excessive amounts.

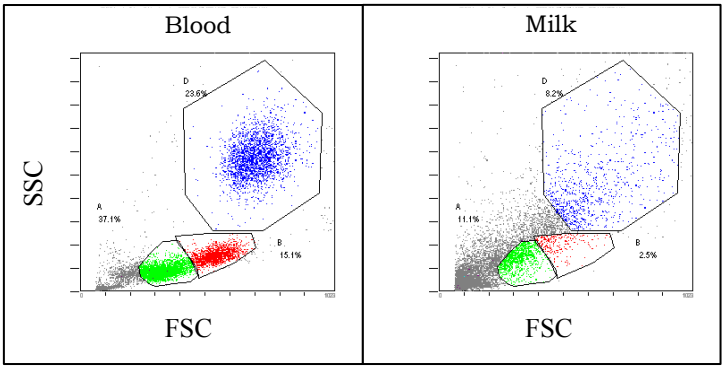

Figure 1. Forward (FSC) and sideward (SSC) scatter from flow cytometry of bovine white blood cells (left) and bovine milk cells (right). Gates represent mostly lymphocytes (green), monocytes (red) and granulocytes (blue) in bovine blood. The same gate settings in milk do not distinguish separate cell populations.

As an important radical produced by different immune cells and able to kill bacteria and fungi, the production capacity of NO by milk or blood cells was considered an important and potentially useful parameter reflecting natural disease resistance. Cells were isolated from milk and blood and stimulated with various concentrations of PGN, LPS, LTA, or ConA. However, no detectable NO levels could be measured in these stimulated samples with the test that is routinely used (Griess reaction, data not shown). Furthermore, the test could not be applied to full milk due to high background values, by the white color of milk. The reason for the observed lack of NO detection of isolated cells remains elusive, but perhaps cells would have needed co-stimulation from cytokines such as IFN-γ. Further optimization and increased sensitivity are necessary for this test to be applicable in this type of research. Thus, NO could not be used in our subsequent analyses.

Natural antibodies

For the analysis of natural antibodies (NAb) a previously developed indirect ELISA (Van Knegsel et al., 2007) was adapted for the detection of total and isotypes of NAb present in bovine milk and plasma. Blood plasma contained higher NAb titers than milk as described previously (Van Knegsel et al., 2007) and shown in our study (*Chapter 3*). The titers of NAb in blood and milk were repeatable over time and they showed detectable variation among individual cows (*Chapter 3*). Beside total NAb, isotypes binding LTA were first tested, because LTA is derived from *S. aureus* that is considered a major mastitis pathogen. Subsequently, also the isotypes IgM, IgA and IgG1 binding LPS, PGN and KLH were tested to analyze their potential in binding these same antigens, but these NAb were only determined in milk. Natural antibody isotype titers ranged from 1.05 for IgG1 binding KLH to 4.13 for IgG1 binding PGN and were present in all analyzed milk samples. All NAb isotype titers were significantly different among individual cows implying that variation in NAb titers exists among individual cows. Repeatability ranged from 0.84 (for IgA and IgG1 binding PGN) to 0.95 (for IgG1 binding LPS); hence all isotype titers are repeatable over time (Appendix Table 1). Therefore, all measured total and isotype NAb, except

IgG2, fulfilled the criteria for repeatability within individual cows over time and variation among cows and were present in reasonable levels in milk samples. Based on these results, they were selected for further analysis to determine their potential protective effect on risk for CM, high SCC and possibly other diseases. This was also based on the notion that natural occurring antibodies, particularly IgM class, display an important physiological function by opsonisation of pathogens, likely leading to enhanced phagocytosis and efficient clearance but also, facilitating a persistent and highly amplified antigen-specific adaptive immune response (Delves and Roitt, 2010; Kohler et al., 2003). Synergy between the Fc and C3b receptors activity and neutrophil ROS production ensures the rapid and efficient killing of these pathogens opsonised by these antibodies (Burvenich et al., 2003).

Different functions of antibodies, including NAb, for neutralization of viral and bacterial infections and toxins are illustrated in figure 2.

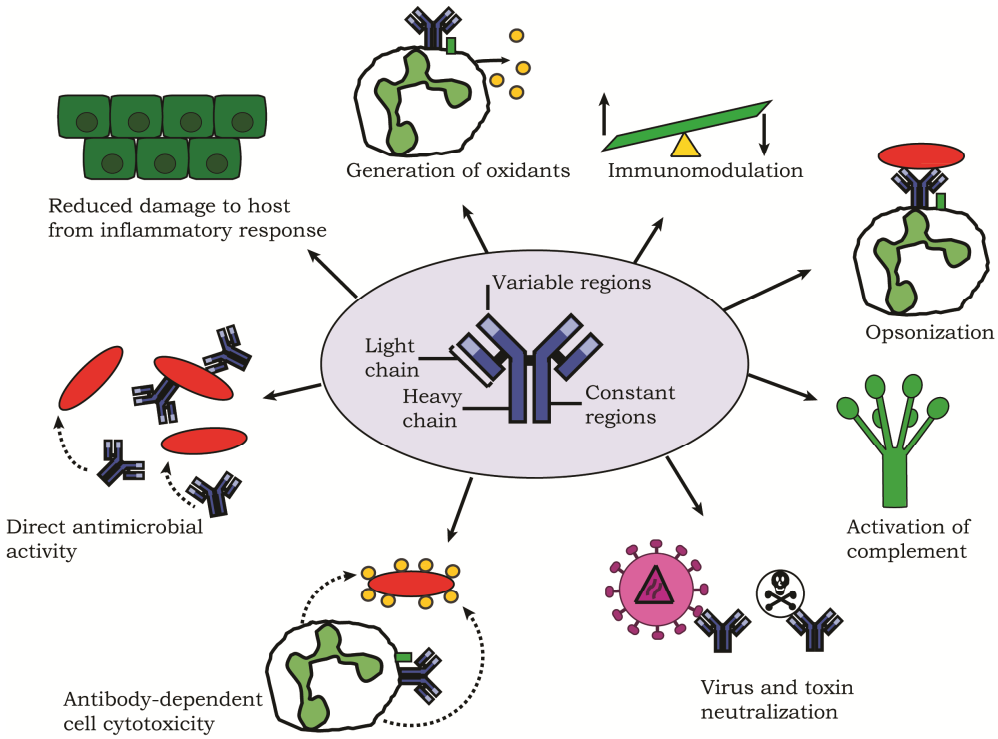

Figure 2. Virus and toxin neutralization, complement activation and direct antimicrobial functions such as the generation of oxidants are independent of other components of the host immune system, whereas antibody-dependent cellular cytotoxicity and opsonization depend on other host cells and mediators. Adapted from: Casadevall et al. (2004).

For establishing their use in this study, total NAb titers were tested for their binding to LPS, LTA, PGN and KLH, and the isotypes IgM, IgA, IgG1 and IgG2 binding LTA as

these NAb could be detected in milk and blood plasma. The levels of IgG2, however, were on average low, and therefore IgG2 was excluded from further analysis.

With milking also the antibodies present in the milk will be removed. The presence of antibodies in milk suggests that there are continuous sources of B cells producing antibodies and selective transport systems exist that continuously transfer antibodies into the newly produced milk. Jerne (1984) hypothesized that roughly 1% of B cells are responsible for this continuous antibody production. Östensson and Lun (2008) found that transfer of antibodies through mammary endothelium is mostly by diffusion, although the molecule size can impede diffusion. For example IgM antibodies are larger than IgG1 antibodies (900 versus 160 kDa, respectively) that makes diffusing more difficult for IgM. Transfer of antibodies through mammary epithelium and thus concentrations in milk are likely affected by selective transport and local synthesis. These authors also indicated that IgG1 is transferred selectively through epithelium, possibly by the FcRn receptor as indicated in transgenic mice expressing bovine FcRn that was pH dependent (Lu et al., 2007). Östensson and Lun (2008) indicated, in addition to blood, a local source of IgM producing cells in the mammary tissue. They also indicated IgA to be produced by plasma cells present in mammary tissue and diffusing from tissue fluid into the milk. In mice, IgA producing B cells were dependent on VCAM-1 and alpha4 integrins to be able to migrate into the lactating mammary gland and IgA was transferred passively into the milk (Low et al., 2010). IgG2 appeared to be only transferred to milk by passive diffusion (Östensson and Lun, 2008). So far, it is not completely clear by what mechanisms antibodies are transferred to the milk, but different and continuous ways exist for different isotypes. How infections modulate the production and transport mechanisms of the various NAb and thereby affect also their relation to disease development (e.g. mastitis) or increased levels of SCC, has not been fully elucidated yet.

Binding of plasma and milk NAb to KLH, to which animals have not been primed, probably reflects true innate humoral immune competence. Natural antibodies binding LPS, LTA, or PGN, in the current study of clinically-healthy cows, may also represent active humoral innate immune competence. In this respect, these NAb do not reflect a specific antibody response to a current infection with specific microbes, in which specific antibody titers usually rise within 7 to 14 days. This study showed that, in the absence of an observed alteration in the health status of the cow, titers of NAb were relatively constant over time (*Chapter 3*). It should be kept in mind that a division between innate and specific immunity is strongly artificial, and specificity is rather relative of nature. Formation of antibody idiotypes is largely an *at random* process via gene recombination or conversion, where many antibodies are randomly made. There are more idiotypes made (10^{20}) and present within the body than antigenic structures (10^{14}) present. Survival of a B cell clone rests on interactions with its antigenic ligands, whether obtained from external or internal origin (Graham et al., 2010). Determination of affinity constants of the tested antibody populations could have provided more information on their selective specificity. Specific immunity implies that certain B cell clones survive a competition for the antigen, based on higher affinity for the antigen than other B cell clones that determines their 'relative specificity'. Many antibodies are directed to auto-antigens. Since many

molecular structures are shared between host and pathogens, this phenomenon explains the presence of NAb (directed to self), but as a consequence also binding pathogenic determinants. Natural antibodies, in particular IgM with low affinity, thus represent the continuous presence of *at random* produced antibodies that fit an antigen either exogenous or autologous of nature.

Antibody titer measurement
The rate of binding between antibody and antigen is dependent on the affinity constant. The affinity constant can be affected by temperature, pH, and buffer constituents. Varying the relative concentrations of antibody and antigen in solution can also control the extent of antibody-antigen complex formation. As it is not usually possible to change the concentration of antigen, therefore, the optimal working concentration (dilution) of each individual antibody must be determined for each application and set of experimental conditions. For instance, a milk sample containing antibodies is diluted serially (1:2, 1:4, 1:8, 1:16... and so on). Using an ELISA, which is a common means of determining antibody titers, each dilution is tested for the presence of detectable levels of antibody. The assigned titer value is indicative of the last dilution in which the antibody was detected. For example, if antibody was detected in each of the tubes listed above, but not in a 1:32 dilution, the titer is said to be 16. If it is detected in the 1:2 and 1:4 dilutions, but no others, the titer is said to be 4. This titer of 16 reflects 4-fold more interacting antibody compared to the sample with titer 4. Therefore the titer is the degree to which the antibody-milk solution can be diluted and still contain detectable amounts of antibody. The titer is thus an estimate of the combined effects of affinity and concentration of the whole polyclonal antibody pool present in the milk or in the plasma sample. In the current experiments no estimations of affinities of NAb and their relation with mammary diseases were made. A higher affinity of antibodies binding the tested antigens might indicate additional antibody maturation and therefore an active specific immune process.

ANALYSIS OF MILK GENOMICS DATA

Genetics
To determine the prospects for selective breeding, total NAb titers were determined in single milk samples of nearly 2000 heifers, obtained during the previous Milk Genomics study (*Chapter 4*). This study was designed for analysis of genetic variation in fat and protein composition of milk (Heck et al., 2009; Schopen et al., 2009a; Schennink et al., 2008; Stoop et al., 2008). The data were also used to estimate genetic (and non-genetic) variation in milk urea nitrogen (Stoop et al., 2007), and concentration of minerals selenium, calcium, potassium, zinc, magnesium, and phosphorus in milk (Van Hulzen et al., 2009). In *Chapter 4*, estimates of genetic variation for NAb titers in milk for levels of total NAb binding LPS, LTA, PGN and KLH, and the isotypes IgM, IgA and IgG1 binding LTA were reported. Meanwhile, also genetic variation for these isotypes binding the other antigens LPS, PGN and KLH was estimated. Table 2 (Appendix) shows phenotypic

variance, intra-herd and across-herd heritability, and herd effect for the different isotypes binding to these 3 antigens. In literature across-herd and intra-herd heritability are reported. Across-herd heritability for IgG1 ranged from 0.10 for PGN to 0.27 for KLH, for IgM from 0.35 for PGN to 0.41 for KLH, and for IgA from 0.30 for PGN to 0.55 for KLH. Intra-herd heritability was generally somewhat higher than across-herd heritability. Comparing these data with each other and with those from isotypes binding LTA and total NAb for all 4 antigens, NAb binding KLH have highest heritabilities and NAb binding PGN have lowest heritabilities. Heritability of IgM and IgA is higher than that of IgG1, for all tested antigens, thereby more reflecting the innate part of the immune system. For the dataset with only cows that had SCC lower than 80,000 cells/mL (Chapter 4), heritabilities generally indicated on average a slight decrease (-0.03) for IgA and a slight increase for IgM and IgG1 (+0.05) (Appendix Table 3). Using only cows with SCC lower than 80,000 cells/mL for the genetic analysis, did not dramatically decrease heritability of isotypes (IgG1, IgM and IgA) binding LPS, PGN or KLH, compared to using all cows, in line with the data of isotypes binding LTA and total NAb from *Chapter 4*. Thus, heritability of NAb is not likely to be related with SCC levels. The measured NAb have been suggested to be among the highest reported heritabilities for humoral immune parameters (*Chapter 5*). Heritability estimates for neutrophil response to chemotactic factors and bactericidal activity were generally very low (reaching 0 and some higher, but with high standard errors) (Detilleux et al., 1994). Also heritability estimates for SCC or somatic cell score (SCS) and CM are generally low (*Chapter 1*). Thus, levels of NAb reflect a genetically determined immune capacity, although they are also externally influenced. Isotypes of NAb are potentially important, because they can have different functions in clearing infections, and individual isotypes are therefore more informative than total NAb. As for all total NAb and isotypes binding LTA (*Chapter 5*), also for the isotypes binding LPS, PGN and KLH there is potential for genetic selection. If NAb titers are conclusively proven to reduce the risk for mastitis or other diseases, genetic selection on NAb titers can improve disease resistance of dairy cows. However, in order to be applicable for genetic selection it should be studied if there are no negative relations with other traits, and if so the most optimal breeding strategy should be developed to improve resistance to mastitis or other diseases, meanwhile not diminishing other traits.

Effect of natural antibodies on relation with clinical mastitis and high somatic cell count

For most of the heifers that participated in the Milk Genomics study, SCC and CM data during their first lactation period were available (n = 1515). Data analysis was therefore performed to determine the relation of the different NAb titers with risk for developing CM and high SCC later in lactation (*Chapter 5*).

In the Milk Genomics study for primiparous cows IgM and IgA NAb consistently suggested a protective effect of NAb titers for all 4 tested antigens against CM in the healthy-cows dataset, albeit not significant. In particular, the IgM antibodies binding to KLH reflect true natural occurring antibodies (NAb), and these KLH binding IgM NAb titers correlate strongly to e.g. LTA (and also LPS and PGN) binding IgM antibody titers corroborating that these latter antibodies are in this dataset to a

large extend also reflecting true NAb. Total NAb, but not IgG1, gave comparable results, suggesting that in these data on heifers with healthy udder history total NAb are mostly a reflection of IgM and IgA NAb. Data from the all-cows dataset did not suggest any protection against CM, indicating that NAb are also affected by previous or current infections and thus the population of so called NAb in milk will also contain a varying percentage of antibodies that are induced upon exposure to infections and thus probably reflect also antigen-specific antibodies. The effect of infections consists of a general enhancement of antigen-specific and NAb production, the latter of which will be induced by so called bystander stimulation based on the release of growth factors, like cytokines, in the vicinity of activated B cells (Saeij et al., 2003). The currently used techniques for antibody titer measurements do not segregate between these two populations of antibodies. The advantage of this study is that heifers generally have lower levels of specific antibodies or a limited repertoire of antibodies in their milk than older cows, just because they had less time to encounter antigens, therefore displaying lower levels of memory B cells resulting in lower levels of peripheral antibodies and a larger contribution of real NAb. In particular a high SCC at the moment of sampling may obscure the identification of a potential protective effect of NAb present in milk. An increased SCC represents inflammation and will coincide with an increased transfer of antibodies from blood to milk due to higher vascular permeability of inflamed tissue (Harmon et al., 1976; Kohler et al., 2003; Korhonen et al., 2000). Cows with an ongoing (chronic) infection will therefore have considerably higher NAb levels in their milk than would be normally present in a healthy udder. High SCC is a known risk factor for high SCC or CM later in lactation. In hindsight it is therefore not surprising that in the All-cows datasets the OR of NAb for these risks is considerably higher than 1 (*Chapters 5 and 6*). Thus, to study effects of NAb on resistance, NAb should be measured in healthy cows without previous or current infections.

In the healthy-cows dataset total and IgG1 NAb titers binding LPS and KLH appeared to be protective against high SCC, while IgM and IgA binding all the different antigens were suggested to be protective only against CM in heifers. The mechanism behind such a protective effect of NAb against CM could be the opsonization and probable other immune responses that are enhancing the properties to fight pathogens and prevent development of disease. The mechanism behind the protective effect of NAb against high SCC is as yet unclear. A high SCC could reflect the cellular immune response against an ongoing infection that may or may not be accompanied by clinical signs of a disease. Additionally, a high SCC could be a reflection of an increased cellular immune capacity which upon challenge by infection will result in a rapid and effective induction of a protective immune response and thus this high SCC will not be accompanied by signs of an ensuing disease. Possibly, NAb can aid cells in clearing infections more effectively, but this remains to be elucidated.

Finally, it cannot be excluded that the 'protective' effect of high NAb levels or their isotypes does not represent a direct functional relation between NAb and infectious agents, as well illustrates a level of immunocompetence that encompasses an as yet to be determined immune mechanism underlying resistance to infection. In this respect, it is noteworthy that levels of NAb binding autoantigens were

strongly correlated with humoral immune responsiveness in a mammal and indicative for longevity (Graham et al., 2010).

TOWARDS A FIELD STUDY

Several genetic and environmental factors are known to influence antibody levels in the milk. Besides genetic variation between breeds for total IgG content in milk (Król et al., 2010; *Chapter 4*) also environmental factors might influence the level of antibodies, possibly including NAb of different isotypes and specificities, in cows. Such factors comprise dietary components, stress and welfare conditions, and exercise. Van Knegsel et al., (2007) reported that cows fed a glucogenic diet were characterized by higher total NAb titers binding KLH in plasma and lower SCC, whereas cows fed a lipogenic diet were characterized by lower NAb titers binding KLH and a higher SCC. Cows fed whole flaxseed as fat supplementation had higher anti-ovalbumin (OVA) IgG antibody levels in blood plasma after subcutaneous injection with OVA than cows fed microencapsulated fish oil or a control diet (Caroprese et al., 2009). Furthermore, IgG concentrations in colostrum were not affected by natural (RRR {alpha}-tocopherol acetate) or synthetic (all-rac{alpha}-tocopherol acetate) vitamin E supplementation (Horn et al., 2010). Also, stress reduces immune system functionality. In addition, glucocorticoids caused lower IgM levels in mammary secretions (Burton and Erskine, 2003). Antibody responses in serum and in milk whey of periparturient cows to intramuscularly administered OVA were positively correlated with growth hormone concentration and negatively associated with insulin-like growth factor 1 (Mallard et al., 1997, 1998). Also, antibody responses to intramuscularly administered J5 antigen from *E. coli* in serum were positively correlated with growth hormone concentration (Mallard et al., 1997). Total IgG1 levels in serum were negatively correlated with growth hormone and positively correlated with insulin-like growth factor 1, and reverse relations were found in whey (Mallard et al., 1997). Serum concentrations of IgG1 decreased and concentrations of IgG2 increased around parturition (Detilleux et al., 1995). The immune system is impaired during the functional changes of the mammary gland, especially around calving (Sordillo, 2005). Thus, features around parturition affect antibody levels.

All of the above mentioned factors are related to management and husbandry factors that are (partly) controlled by the farmer. This could explain the herd variance of NAb in our study. We found the herd variance generally being smaller than the genetic variance (*Chapter 4*). The Milk Genomics study (*Chapter 5*) was not suitable to determine the relation of immune parameters with health disorders, other than mastitis, because these data were not collected. Therefore, a field study was conducted that was specifically designed to validate if NAb titers present in milk samples taken during the first 3 months of lactation (i.e. the period with most health problems) had a prognostic value for susceptibility to CM, high SCC or other diseases later in that lactation (*Chapter 6*), and to assess differences between primiparous and multiparous cows. Additionally, such a field study can help to

explain differences among farms and help to identify strategies for immuno-modulation, when farm factors are evaluated.

FIELD STUDY

A field study was conducted that was designed to test if NAb titers present in milk samples taken during the first 3 months of lactation (i.e. the period with most health problems) had a prognostic value for the susceptibility to mastitis and high SCC (*Chapter 6*) or other diseases later in that lactation, in primiparous and multiparous cows.

The results of the field study suggested, for cows in the healthy-cows dataset, a protective effect against CM for all NAb isotypes binding KLH (OR = 0.80 – 0.91), but this was only significant for the IgG1 isotype (OR = 0.80, $P = 0.013$). In this dataset the estimated risk to develop CM later in lactation was more than 3 times lower for cows within the highest IgG1 binding KLH titer category compared to cows in the lowest IgG1 binding KLH titer category (2.7% vs 9.7%). In the previous study on Holstein Friesian heifers (*Chapter 5*), OR of risk for CM consistently suggested a protective effect for the IgM and IgA isotypes binding all the tested antigens (LPS, LTA, PGN and KLH) in the healthy-heifers dataset, although these were not statistically significant. In the field study, this suggestive protective effect of the IgM and IgA isotypes was not as clear. This could be due to the fact that the previous study only contained heifers and the present study mostly multiparous cows (75%). The very low number of CM cases (n = 9) that developed in the heifers in the field study made it unreliable to compare the results in this subgroup with the previous study that used heifers only. The field study also did not show a protective effect against development of high SCC for IgG1 binding KLH when all cows were analyzed, in contrast with analysis of Milk Genomics data that did show a significant protective effect of this NAb. However, in heifers of the field study a protective effect against high SCC of IgG1 binding KLH (OR = 0.88) was found, which is similar to the effect found in the Milk Genomics study data (OR = 0.86) (*Chapter 5*). This protective effect was only significant in the Milk Genomics data, presumably due to the much larger number of high SCC cases (n = 231) compared to the field study (n = 70).

Heifers are younger than multiparous cows, thereby strongly reducing the chance that they have encountered the PAMP candidates LPS, LTA or PGN or more of them that are present on many bacteria (as explained before in this Chapter). This could explain why in the Milk Genomics Healthy-heifers dataset the OR for CM was only consistently below 1 for the more “innate’ isotypes IgM and IgA directed against these PAMP, that only contained heifers, but not in the field study when all cows (mostly multiparous) were included. For these multiparous cows specifically induced antibodies against the PAMP might obscure a potential protective effect of innate NAb titers. However, cows will not have encountered the naïve antigen KLH. Therefore, NAb binding KLH also reflect the innate immune capacity in multiparous cows. Indeed, all the isotypes binding KLH had OR for CM lower than 1 (range 0.80 to 0.91), although this was only significant ($P = 0.013$) for the IgG1 isotype binding KLH.

In the field study the effect of NAb titers on other health problems was also analyzed. Only for genital and fertility problems (GFP) and leg and claw problems (LCP) enough cases were reported after sampling to do a risk analysis similarly as described in *Chapter 6* for CM and high SCC. Besides the previously described all-cows dataset and a dataset of cows with healthy udder history (in appendix Tables 4 and 5 referred to as healthy-cows-CM) a third dataset was included. This consisted of the health-cows-CM dataset cows, from which cows that had experienced GFP or LCP prior to or at the moment of sampling were also excluded: the health-cows-All dataset. The OR for GFP of IgG1 NAb binding KLH were below 1 for all 3 datasets (ranging from 0.79 to 0.91), and lowest and most significant for the healthy-cows-CM dataset (Table 4, Appendix), indicating that IgG1 NAb binding KLH in milk also reduced the risk for GFP, in all 3 datasets. IgG1 binding LPS was also below 1 for both healthy-cows datasets (0.86 – 0.91), but not significant (Table 4, Appendix). Odds ratios of IgG1 binding KLH for LCP were also below 1 for all 3 datasets, suggesting a protective effect of IgG1 binding KLH for LCP, but not statistically significant (Table 5, Appendix). Odds ratios for SCS of the sampling moment were also below 1, but not significant.

For both healthy-cows datasets the results consistently suggest a protective effect of IgG1 NAb binding KLH in the milk for subsequent development of CM, GFP and a tendency for protection against LCP. Also in the all-cows dataset IgG1 NAb binding KLH were suggested to have a protective effect against GFP. This is in line with the study of Star et al. (2007) that reported OR of 0.80 for total NAb binding KLH for survival of laying hens. These data suggest that selective breeding for higher IgG1 titers binding KLH might not only have potential for reducing CM, but might also reduce certain other health problems. All the measured NAb specificities in this study correlate with each other, and thus, future studies could reveal the possibility of measuring NAb of several isotypes binding 1 antigen only, e.g. KLH. Since it is unlikely that NAb in milk perform a protective function in preventing GFP, it can be suggested that milk NAb levels reflect (passively) transferred levels of NAb from the periphery and illustrate a humoral immune competence.

CONCLUSIONS

- Our data suggest that NAb titers in milk can reflect higher resistance to diseases such as CM, but also non-mammary related diseases (GFP).
- Heifers differ from multiparous cows in the relation of selected NAb with resistance to mastitis.
- Udder health history affects the relation of selected NAb with resistance to mastitis.
- NAb titers have potential for genetic selection.
- The high correlations between NAb, phenotypically as well as genetically, offer the opportunity to limit estimation of NAb effects to a limited set of antigens (ultimately 1, like KLH).
- Collectively our data show that genetic selection for NAb titers offers a prospect to improve disease resistance of dairy cows.

IMPLICATIONS AND FUTURE PERSPECTIVES

The aim of our study was to investigate the relation of suitable immune parameters with risk for mastitis and possibly other diseases. The data suggest a protective effect of NAb titers against mastitis, but different effects for heifers and multiparous cows. The levels of NAb measured in milk did not seem protective against high SCC, except for (mainly) IgG1 binding KLH in heifers. Our studies did not investigate causes and consequences. Further research will be needed to determine if and which NAb (specificities and isotypes) can best be used in practice for prevention of diseases including mastitis. If so, resistance of cows to diseases can be increased and fewer antibiotics and other medicine will be needed.

For future use it is recommended to develop the ELISA test from a titer determination further into the detection of the absolute amounts of antibodies present in several sample matrices, especially in milk. Pure antibody of the different immunoglobulin isotypes (IgM, IgG1, IgA) obtained by affinity purification against the antigen of interest would therefore be required as a standard.

Other possible interesting immune parameters are complement factors for their pathogen killing activities certainly in connection with several antibody classes that induce complement activation (*Chapter 1*). In addition, mucins have been suggested interesting immune parameters worth to be studied more extensively as they potentially bind pathogens and prevent them from adhesion to cells (Pallesen et al., 2002; Patton et al., 1995).

To improve NAb titers, factors possibly affecting NAb titers can be investigated, too. Some data concerning factors affecting antibody levels were discussed, but most were on total Ig levels or specific antibodies, and not on NAb binding specific antigens. For the field study, besides collection of disease and SCC data, and NAb measurements, a farmer oriented survey was organized in the form of a detailed questionnaire. The survey contained questions concerning general farm information (numbers of cows etc.), breeding choices, diseases and therapy, milking machine, milking process, feed, housing conditions and calve management. While these data have been collected and processed, the data await future analysis. With these data farm-associated factors potentially affecting NAb titers and diseases can be investigated more extensively in relation to disease resistance.

Genetics

Our results showed potential for genetic selection for NAb. Based on the results presented in *Chapter 4* selection based on NAb measured in clinically-healthy animals holds promise to reduce the risk for mastitis in cows. It should, however, be investigated if selection for improved NAb levels has unintentionally also negative relations with other selection traits. Changes in levels of NAb titers with an increase in age of cows were observed (Van Knegsel et al., 2007). It would be interesting to study the genetic relationship between NAb titers measured at different ages to increase our understanding of the effect of ageing of cows. Furthermore, a QTL study could help to increase our understanding of the genes that contribute to genetic variation and thereby the mechanisms involved in disease resistance. A QTL study was performed using 1,341 SNP covering all 29 bovine autosomes. The same

set of markers was used by Schennink et al. (2009a, b), Schopen et al. (2009b), and Stoop et al. (2009), in QTL linkage studies on milk fatty acids and proteins. For NAb binding LTA and KLH (BTA 3) and LPS (BTA 6) QTL were detected (Sell-Kubiak et al., 2010). In addition 1 QTL possibly pleiotropic for milk yield was found on BTA 9 for IgM binding LTA. For IgA binding LTA a QTL was detected on BTA23, in the vicinity of the bovine MHC and close to the IL-17 gene. In total 6 QTL regions were found affecting IgM NAb binding LTA. Further studies investigating genetic associations for total and isotype NAb can give insight about regions (and genes) in the bovine genome possibly related to resistance to diseases. Such association studies can help us understanding the genetic mechanism of resistance to (udder-related) diseases.

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APPENDIX TABLES TO GENERAL DISCUSSION

Table 1. Natural antibody (NAb) isotype (Log_2) titers IgM, IgA and IgG1 binding lipopolysaccharide, peptodiglycan or keyhole limpet hemocyanin in milk. Means, overall standard deviations, standard deviations among cows (SD among cows), standard deviations within cows (SD within cows), significance for variation among cows and repeatability are given

NAb[2]	Mean	Overall SD	SD among cows	SD within cows	Variation among cows (*P* value)	Repeatability
LPS-IgG1[1]	1.79	1.21	1.46	0.35	0.003	0.95
LPS-IgM[1]	2.18	0.94	0.86	0.32	0.002	0.88
LPS-IgA[1]	2.12	1.11	1.02	0.38	0.002	0.88
PGN-IgG1[1]	4.13	1.12	0.96	0.41	0.003	0.84
PGN-IgM[1]	2.10	1.05	0.97	0.34	0.002	0.89
PGN-IgA[1]	1.74	1.21	1.08	0.47	0.003	0.84
KLH-IgG1[1]	1.05	1.05	1.01	0.25	0.002	0.94
KLH-IgM[1]	3.05	0.83	0.77	0.26	0.002	0.90
KLH-IgA[1]	2.03	1.02	0.92	0.36	0.002	0.86

[1]Titers (Log_2) from 10 milk samples from 19 individual cows were analyzed.
[2]Titers (Log_2) refer to isotype antibodies (IgM, IgA and IgG1) binding lipoteichoic acid (LTA), lipopolysaccharide (LPS), peptidoglycan (PGN) and keyhole limpet haemocyanin (KLH).

Table 2. Phenotypic variance (σ^2_P), intra-herd heritability (h^2), across-herd heritability (h^2_{ac}), and herd-effect (h_{herd}) for natural antibody (NAb) isotype titers IgG1, IgM and IgA binding keyhole limpet hemocyanin (KLH), lipopolysaccharide (LPS) and peptidoglycan (PGN)

NAb	σ^2_P (SE[1])	h^2 (SE)	h^2_{ac} (SE)	h_{herd} (SE)
LPS-IgG1	2.12 (0.08)	0.24 (0.08)	0.21 (0.07)	0.15 (0.02)
LPS-IgM	1.07 (0.04)	0.40 (0.09)	0.36 (0.09)	0.10 (0.02)
LPS-IgA	1.36 (0.06)	0.55 (0.11)	0.50 (0.10)	0.09 (0.02)
PGN-IgG1	2.00 (0.07)	0.13 (0.06)	0.10 (0.05)	0.21 (0.02)
PGN-IgM	0.85 (0.03)	0.39 (0.09)	0.35 (0.09)	0.10 (0.02)
PGN-IgA	1.00 (0.04)	0.34 (0.09)	0.30 (0.08)	0.13 (0.02)
KLH-IgG1	2.16 (0.09)	0.34 (0.09)	0.27 (0.07)	0.21 (0.02)
KLH-IgM	1.01 (0.04)	0.46 (0.10)	0.41 (0.09)	0.10 (0.02)
KLH-IgA	1.03 (0.05)	0.63 (0.12)	0.55 (0.11)	0.14 (0.02)

[1]SE = standard error

Table 3. Phenotypic variance (σ^2_P), intra-herd heritability (h^2), across-herd heritability (h^2_{ac}), and herd-effect (h_{herd}) for natural antibody (NAb) isotype titers IgG1, IgM and IgA binding keyhole limpet hemocyanin (KLH), lipopolysaccharide (LPS) and peptidoglycan (PGN) estimated from cows (n = 1450) that had a somatic cell count lower than 80,000 cells/ml at the time of milk sampling

NAb	σ^2_P (SE[1])	h^2 (SE)	h^2_{ac} (SE)	h_{herd} (SE)
LPS-IgG1	2.09 (0.09)	0.31 (0.10)	0.26 (0.09)	0.17 (0.03)
LPS-IgM	1.03 (0.05)	0.48 (0.12)	0.42 (0.10)	0.12 (0.02)
LPS-IgA	1.16 (0.06)	0.50 (0.12)	0.45 (0.11)	0.09 (0.02)
PGN-IgG1	1.83 (0.08)	0.26 (0.10)	0.20 (0.08)	0.20 (0.03)
PGN-IgM	0.76 (0.04)	0.43 (0.11)	0.37 (0.10)	0.14 (0.03)
PGN-IgA	0.68 (0.03)	0.34 (0.10)	0.30 (0.09)	0.11 (0.03)
KLH-IgG1	2.09 (0.09)	0.36 (0.10)	0.28 (0.08)	0.21 (0.03)
KLH-IgM	0.95 (0.05)	0.47 (0.11)	0.43 (0.10)	0.10 (0.02)
KLH-IgA	0.80 (0.04)	0.58 (0.13)	0.52 (0.12)	0.10 (0.02)

[1]SE = standard error

Table 4. Effect of natural antibody titers on risk for genital and fertility problems[2]. Estimate (Est), standard error (SE), Odds ratio (OR) and significance levels (*P* values) for the effect of natural antibody (NAb)[1] titers and somatic cell score at the sampling moment (SCS sample), on relative risk for genital and fertility problems after the sampling moment for the all-cows dataset (left) and for the healthy-cows-CM dataset (middle) and for the healthy-cows-All dataset (right)

	All cows				Healthy cows CM				Healthy cows All			
NAb	Est	SE	OR	*P* value	Est	SE	OR	*P* value	Est	SE	OR	*P* value
LPS-IgA	0.09	0.05	1.09	0.096	0.17	0.07	1.18	0.025	0.06	0.09	1.06	0.539
LPS-IgM	0.15	0.06	1.16	0.014	0.16	0.08	1.18	0.042	0.02	0.10	1.02	0.802
LPS-IgG1	0.04	0.05	1.04	0.365	-0.09	0.07	0.91	0.184	-0.15	0.09	0.86	0.088
LTA-IgA	0.04	0.05	1.04	0.456	0.09	0.07	1.09	0.204	0.09	0.08	1.09	0.300
LTA-IgM	0.05	0.05	1.05	0.351	0.06	0.07	1.06	0.407	-0.03	0.09	0.97	0.722
LTA-IgG1	0.10	0.04	1.10	0.023	0.14	0.06	1.15	0.025	0.19	0.08	1.21	0.012
KLH-IgA	0.09	0.06	1.10	0.136	0.09	0.08	1.10	0.280	0.05	0.10	1.05	0.610
KLH-IgM	0.15	0.06	1.16	0.018	0.19	0.09	1.21	0.025	0.13	0.10	1.14	0.192
KLH-IgG1	-0.10	0.05	0.91	0.046	-0.24	0.07	0.79	0.000	-0.20	0.08	0.82	0.011
PGN-IgA	0.02	0.04	1.02	0.659	0.08	0.06	1.09	0.159	0.05	0.07	1.06	0.446
PGN-IgM	0.12	0.06	1.13	0.027	0.16	0.07	1.18	0.026	0.08	0.09	1.09	0.346
PGN-IgG1	0.09	0.05	1.09	0.062	0.14	0.07	1.15	0.036	0.19	0.08	1.21	0.017
SCS sample	-0.03	0.05	0.97	0.626	-0.08	0.13	0.93	0.549	-0.20	0.15	0.82	0.184

[1]NAb titers refer to antibody isotype (IgA, IgM and IgG1) titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), lipopolysaccharide (LPS) and peptidoglycan (PGN).
[2]Genital and fertility problems consisted of (endo)metritis, pyometra, retaining placenta after 24 h, purulent metrorrhagia, abortion, cystic ovary, anoestrus, and no heat shown.

Table 5. Effect of natural antibody titers on risk for leg and claw problems[2]. Estimate (Est), standard error (SE), Odds ratio (OR) and significance levels (*P* values) for the effect of natural antibody (NAb)[1] titers and somatic cell score at the sampling moment (SCS sample), on relative risk for leg and claw problems after the sampling moment for the all-cows dataset (left) and for the healthy-cows-CM dataset (middle) and for the healthy-cows-All dataset (right)

	All-cows				Healthy-cows-CM				Healthy-cows-All			
NAb	Est	SE	OR	*P* value	Est	SE	OR	*P* value	Est	SE	OR	*P* value
LPS-IgA	-0.09	0.05	0.91	0.086	-0.05	0.07	0.95	0.463	0.04	0.08	1.04	0.586
LPS-IgM	-0.04	0.06	0.96	0.513	0.01	0.08	1.01	0.908	0.08	0.09	1.08	0.389
LPS-IgG1	0.01	0.05	1.01	0.872	0.08	0.07	1.08	0.222	0.15	0.08	1.16	0.051
LTA-IgA	-0.02	0.05	0.98	0.632	0.06	0.06	1.06	0.341	0.13	0.07	1.14	0.076
LTA-IgM	0.03	0.05	1.04	0.518	0.11	0.07	1.11	0.129	0.12	0.08	1.13	0.141
LTA-IgG1	0.02	0.04	1.02	0.574	0.01	0.06	1.01	0.908	0.03	0.07	1.03	0.674
KLH-IgA	-0.02	0.06	0.98	0.784	0.05	0.08	1.06	0.495	0.16	0.09	1.17	0.081
KLH-IgM	0.01	0.06	1.01	0.850	0.11	0.08	1.12	0.184	0.21	0.10	1.23	0.031
KLH-IgG1	-0.06	0.05	0.94	0.209	-0.11	0.06	0.89	0.081	-0.04	0.07	0.96	0.536
PGN-IgA	0.00	0.04	1.00	0.944	0.06	0.06	1.06	0.327	0.10	0.07	1.10	0.145
PGN-IgM	0.10	0.06	1.10	0.081	0.17	0.07	1.19	0.019	0.15	0.08	1.16	0.073
PGN-IgG1	0.08	0.04	1.09	0.066	0.09	0.07	1.10	0.146	0.11	0.07	1.11	0.150
SCS sample	-0.03	0.05	0.97	0.565	0.08	0.12	1.08	0.509	0.13	0.14	1.13	0.361

[1]NAb titers refer antibody isotype (IgA, IgM and IgG1) titers binding keyhole limpet hemocyanin (KLH), lipoteichoic acid (LTA), lipopolysaccharide (LPS) and peptidoglycan (PGN).
[2]Leg and claw problems consisted of laminitis, swollen/inflamed heel, joint or leg, (inter)digital dermatitis, sole ulcer, Mortellaro's disease, foot rot, interdigital necrobacillosis, toe necrosis, white line disease and lameness.

There is nothing impossible to those who will try

(Alexander the Great)

Summary

Samenvatting

Populaire samenvatting

SUMMARY

Mastitis is one of the most important health problems of dairy cattle and is responsible for reduced animal welfare, the large scale use of antibiotics and considerable economic losses. Resistance against mastitis and many other diseases is partly based on the naturally present disease resistance capacity: the innate immune system. The project described in this thesis therefore aimed to identify new immune parameters and determine their relation with the susceptibility of the individual animal for mastitis and possibly also other health problems. Ideally these immune parameters can be easily determined in milk that could make routine screening of dairy cows possible. Determining the heritability of promising immune parameters was another important aim, because this would reveal the prospects of selective breeding to improve (udder) health of dairy cows.

Based on literature a list was composed of immune parameters that could potentially be related to mastitis susceptibility (**Chapter 1**). To be an adequate marker of natural resistance, such immune parameters should show variation among cows and repeatability within cows over time. To test variation and repeatability 10 milk and blood samples were taken over a 17-day period from each of 20 cows. In **Chapter 2** repeatability and variation of the cytokines tumor necrosis factor α (TNF-α and interferon γ (IFN-γ in milk and blood, and reactive oxygen species (ROS) production and different milk and blood cell types were reported. The TNF-α and IFN-γ levels showed repeatability within (0.84 – 0.99) and variation among cows (P < 0.01), although 1 cow had much higher TNF-α and IFN-γ levels than the other cows. When this cow was excluded from the analysis, repeatability of these cytokines decreased to 0.57 - 0.66, except for TNF-α in milk that became 0.05. Levels of TNF-α and IFN-γ of the majority of cows appeared to be below the minimal reliable detection level of the tests, and they were therefore considered to be not suitable for further study to assess their relation with natural resistance. Reactive oxygen species production by stimulated leukocytes, and quantity of different milk and blood cell types were mostly not repeatable or variable, and were therefore also not suitable for further study. In **Chapter 3** repeatability and variation of total natural antibodies (NAb) titers binding the Pathogen Associated Molecular Patterns (PAMP): lipopolysaccharide (LPS) from *Escherichia coli,* lipoteichoic acid (LTA) and peptidoglycan (PGN) from *Staphylococcus aureus*, and the truly naive antigen keyhole limpet hemocyanin (KLH) from *Megathura crenulata* were studied. Furthermore, titers of NAb isotypes IgG1, IgG2, IgM and IgA binding LTA in milk were investigated. Total NAb titers binding all tested antigens showed repeatability within cows over time (0.60 – 0.93) and variation among cows (P < 0.005). Invividual isotypes (IgM, IgA, IgG1 and IgG2) in milk binding LTA also showed variation among cows (P < 0.005), and repeatability within cows over time (ranging from 0.84 to 0.92). Also a considerable correlation between NAb titers in milk and blood was observed for total NAb binding all tested antigens (from 0.69 to 0.91). Based on these results, NAb titers binding the tested antigens (LPS, LTA, PGN and KLH), and isotypes IgG1, IgM and IgA binding LTA were considered suitable to further study their relation with natural resistance of dairy cows. Although isotype IgG2 was repeatable and variable, it was only present at low titers and was not used for further study.

To study heritability, total NAb titers binding LPS, LTA, PGN and KLH, and isotypes binding LTA were determined in milk samples taken from nearly 2000 heifers, from the Milk Genomics study, with known genetic background. Low to moderate heritability's (ranging from 0.10 to 0.53) for total NAb titers binding all tested antigens (LPS, LTA, PGN and KLH) and NAb isotypes IgG1, IgM and IgA binding LTA in milk were found (**Chapter 4**). High genetic correlations among NAb (ranging from 0.45 to 0.99) indicated a common genetic basis for the titers of different NAb in bovine milk. Results indicated that NAb titers have potential for genetic selection.

For 1515 heifers that participated in the Milk Genomics study, information on clinical mastitis (CM) and somatic cell count (SCC) of this first lactation was available. Data analysis could therefore be performed to determine the relation of the different NAb titers with risk for developing CM and high SCC later in lactation (**Chapter 5**). Two datasets were created. The first dataset contained data of all the heifers; the all-heifers dataset (n = 1515). The second dataset contained data from heifers that did not show CM nor had high SCC (>150,000 cells/mL) during the SCC test-days before or at the moment of NAb measurement (n = 906); the healthy-heifers dataset. For all heifers, two traits were analyzed in relation to NAb titers; 1) whether the heifer did or did not develop CM during the remainder of the lactation after the sampling moment, and 2) whether the heifer did or did not have high SCC during at least 1 SCC test-day during the remainder of the lactation after the sampling moment. In the all-heifers dataset nearly all NAb showed odds ratio (OR) > 1 for risk for high SCC that was statistically significant. In both datasets IgG1 NAb binding KLH significantly decreased the risk for high SCC (OR = 0.93 (all-heifers) and 0.86 (healthy-heifers)). Only in the healthy-heifers dataset higher total and IgG1 NAb titers binding LPS also significantly decreased the risk for high SCC (OR = 0.86 resp. 0.88). For IgG1 NAb binding LTA, logistic regression with the all-heifers dataset revealed a significant OR of 1.18 for the risk for CM. In the healthy-heifers dataset the OR of IgM and IgA binding all antigens for the risk for CM was below 1 (OR = 0.74 – 0.85), suggesting a protective effect of these isotypes against CM, albeit not statistically significant.

The Milk Genomics study had been designed to determine heritability of fat and protein composition of milk. Although additionally collected udder health data made it possible to identify parameters that are related to mastitis susceptibility, the Milk Genomics study was not primarily designed for this. The Milk Genomic Project is also not suitable to determine the relation of immune parameters with health disorders, other than mastitis, because these data were not collected. Therefore, a field study was conducted that was specifically designed to validate if NAb titers present in milk samples taken during the first 3 months of lactation (i.e. the period with most health problems) had a prognostic value for the susceptibility to CM, high SCC (>150,000 cells/mL for primiparous cows and >250,000 cells/mL for multiparous cows) or other diseases later in that lactation (**Chapter 6**). In single milk samples from 1998 cows of 60 farms the isotypes IgG1, IgM and IgA of NAb that bind to LPS, LTA, PGN and KLH were determined in a dataset with heifers and multiparous cows. From these cows information on diseases and test-day SCC of that lactation were used to create 2 datasets. The first dataset contained data of all

the, at the sampling moment clinically-healthy, cows (n = 1998); the all-cows dataset. The second dataset contained data from the, at the moment of sampling clinically-healthy, cows that did not show CM nor had high SCC before or at the moment of sampling (n = 1260); the healthy-cows dataset. Logistic regression analysis indicated IgG1 binding KLH in the healthy-cows dataset to be protective against CM (odds ratio 0.8), but not against high SCC. Results also indicated that CM and SCC history affects NAb titers. Furthermore, differences in the relation between NAb and udder health became evident between heifers and multiparous cows. This study reveals the putative importance of NAb in prevention of CM and high SCC in dairy cows of different parities.

Our data suggest that NAb titers in milk can reflect higher resistance to diseases such as CM, but also non-mammary related diseases (genital and fertility problems) (**Chapter 7**). Heifers differ from multiparous cows in the relation of selected NAb with resistance to mastitis. Furthermore, udder health history affects the relation of selected NAb with resistance to mastitis and NAb titers have potential for genetic selection. The high correlations between NAb, phenotypically as well as genetically, offer the opportunity to limit estimation of NAb effects to a limited set of antigens (ultimately 1, like KLH). Collectively our data show that genetic selection for NAb titers offers prospect to improve disease resistance of dairy cows.

SAMENVATTING

Mastitis, of uierontsteking, is één van de belangrijkste gezondheidsproblemen in de melkveehouderij en is verantwoordelijk voor verminderd dierenwelzijn, grootschalig gebruik van antibiotica en aanzienlijke economische verliezen. Weerstand tegen mastitis en vele andere ziekten is gedeeltelijk gebaseerd op de natuurlijk aanwezige weerstand tegen deze ziekten: het aangeboren deel van het immuunsysteem, vroeger ook wel het niet-specifieke deel genoemd. Naast het aangeboren deel, bestaat het immuunsysteem ook uit een verworven (adaptief) deel. Beide delen zijn er op gericht infecties te bestrijden. Zij verschillen echter in de wijze van herkenning, de celtypen en moleculen die hierbij betrokken zijn en de manier waarop het micro-organisme onschadelijk wordt gemaakt, maar beide delen van het immuunsysteem hebben wel een sterke interactie met elkaar. Het project beschreven in dit proefschrift beoogde nieuwe immuunparameters voor natuurlijke weerstand te identificeren en daarbij hun relatie met de gevoeligheid van het individuele dier voor de ontwikkeling van mastitis en misschien ook andere gezondheidsproblemen vast te stellen. Idealiter kunnen deze immuunparameters eenvoudig in melk gemeten worden, zodat het mogelijk wordt om de weerstand van melkkoeien routinematig te monitoren. Een ander belangrijk doel was om de erfelijkheid van veelbelovende immuunparameters te bepalen, omdat dit de mogelijkheden aan het licht kan brengen om via fokkerij de (uier)gezondheid van melkkoeien te verbeteren.

Op basis van literatuurgegevens is een lijst samengesteld van immuunparameters, die mogelijk een relatie kunnen hebben met mastitisgevoeligheid (**Hoofdstuk 1**). Om geschikt te zijn als parameter voor natuurlijke weerstand, zouden zulke immuunparameters variatie tussen koeien en herhaalbaarheid binnen koeien in de tijd moeten tonen. Om deze variatie en herhaalbaarheid te testen, zijn geselecteerde immuunparameters gemeten in 10 melk- en bloedmonsters van 20 melkkoeien, gedurende een periode van 17 dagen. In **Hoofdstuk 2** worden cytokinen, zuurstof radicaal productie en verschillende melk- en bloedceltypen beschreven. De niveaus van de cytokinen interferon γ (IFN-γ) en tumor necrosis factor α (TNF-α) vertoonden herhaalbaarheid binnen (0,84 – 0,99) en variatie tussen koeien (P < 0,01). Tumor necrosis factor α en IFN-γ niveaus bleken bij de meerderheid van de koeien rond of onder het minimale betrouwbare detectieniveau van de testen te liggen. Daarom werden ze niet geschikt bevonden om hun relatie met natuurlijke weerstand verder te onderzoeken. Zuurstofradicaalproductie van met peptidoglycaan (PGN) gestimuleerde bloedcellen en aantallen van verschillende melk- en bloedceltypen bleken merendeels een lage herhaalbaarheid te hebben en geen significante variatie tussen de verschillende koeien te vertonen. Daarom werden ook deze parameters niet geschikt bevonden om verder te onderzoeken. In **Hoofdstuk 3** werden de herhaalbaarheid en variatie van de titers van totale natuurlijk voorkomende antistoffen (NAb) onderzocht, waarbij deze antistoffen in staat waren te binden aan moleculaire structuren op het oppervlak van bacteriën, de Pathogen Associated Molecular Patterns (PAMP): zoals lipopolysaccharide (LPS) van *Escherichia coli,* lipoteichoic acid (LTA) en PGN van *Staphylococcus aureus* en het naïeve antigeen keyhole limpet hemocyanin (KLH) van het zeeweekdier *Megathura crenulata.* Verder werden de NAb titers van de isotypen IgG1, IgG2, IgM en IgA

gericht tegen LTA en voorkomend in melk onderzocht. De totale NAb titers tegen de geteste antigenen toonden een herhaalbaarheid aan binnen individuele koeien in de tijd (0,60 – 0,93) en een variatie tussen koeien ($P < 0,005$). De isotypen titers in melk tegen LTA vertoonden ook variatie tussen koeien ($P < 0,005$) en een herhaalbaarheid binnen individuele koeien in de tijd (0,8 – 0,92). Ook werd een aanzienlijke correlatie tussen de NAb niveaus in melk en bloed voor totale NAb tegen alle geteste antigenen (0,69 - 0,91) gevonden. Deze resultaten geven aan dat van de geteste humorale en cellulaire immuunparameters, de totale NAb titers tegen de geteste antigenen (LPS, LTA, PGN en KLH) en van diverse isotypen (IgG1, IgM en IgA) voldoen aan de gestelde criteria voor herhaalbaarheid en variatie. Hoewel isotype IgG2 tegen LTA herhaalbaar en variabel was, waren de titers laag en is dit NAb isotype niet voor verdere studie gebruikt.

Om de erfelijkheid te bestuderen zijn in **Hoofdstuk 4** de NAb titers tegen LPS, LTA, PGN en KLH en isotypen tegen LTA bepaald in melk van bijna 2000 vaarzen van de Milk Genomics studie waarbij van deze dieren de genetische achtergrond bekend is. De resultaten van de analyse van de genetische variatie van NAb titers in melk laten een lage tot middelmatige erfelijkheid zien (h^2 tussen 0,10 en 0,53) voor de gemeten NAb titers. Hierbij hebben isotypen IgM en IgA hogere erfelijkheid dan IgG1 en NAb tegen KLH hogere erfelijkheid dan NAb tegen de andere antigenen. Daarnaast werden van de NAb in de monsters van deze vaarzen de fenotypische en genetische correlaties berekend. Hoge onderlinge genetische correlaties tussen de NAb van verschillende isotypen en bindings-specificiteiten (antigenen) (tussen 0,45 en 0,99) suggereren een gemeenschappelijke genetische basis voor de titers van de verschillende NAb in rundermelk. Uit deze resultaten kan worden geconcludeerd dat NAb titers inderdaad de potentie hebben voor genetische selectie.

Van 1515 vaarzen die aan de Milk Genomics studie deelnamen was informatie over het voorkomen van klinische mastitis (KM) en verhoogd celgetal (SCC) van deze eerste lactatie beschikbaar. Voor SCC werd de in Nederland veel gebruikte afkapwaarde (150.000 cellen/ml) voor vaarzen gebruikt. Hiermee kon een logistische regressieanalyse uitgevoerd worden om de relatie van de verschillende NAb titers met het risico op het ontwikkelen van KM en een hoge SCC later in lactatie te onderzoeken (**Hoofdstuk 5**). Hiervoor werden twee datasets gecreëerd. De eerste dataset bevatte gegevens van alle vaarzen; de alle-vaarzen dataset. De tweede dataset bevatte gegevens van vaarzen die KM noch hoog SCC (>150.000 cellen/mL) op een SCC testdag vóór of op het moment van monstername hadden (n = 906); de gezonde-vaarzen dataset. Voor alle vaarzen werden twee eigenschappen (traits) geanalyseerd in relatie tot NAb titers; 1) of de vaars al of niet KM ontwikkelde in de rest van de lactatie na het moment van monstername en 2) of de vaars al of niet een hoog SCC had tijdens tenminste 1 SCC testdag in de rest van de lactatie na het moment van monstername. In de alle-vaarzen dataset vertoonden bijna alle NAb een odds ratio (OR) > 1 voor het risico op een hoog SCC wat statistisch significant was. In beide datasets bleken de IgG1 NAb tegen KLH het risico op een hoog SCC te verlagen (OR = 0,93 (alle-vaarzen) en 0,86 (gezonde-vaarzen)). Enkel in de gezonde-vaarzen dataset verlaagden ook hogere totale en IgG1 NAb titers tegen LPS het risico op hoog SCC (OR = 0,86 resp. 0,88). Voor IgG1 NAb tegen LTA, werd in de alle-vaarzen dataset een OR van 1,18 voor het risico op KM gevonden. In de gezonde-

vaarzen dataset waren de OR van IgM en IgA NAb tegen alle geteste antigenen voor het risico op KM lager dan 1. Deze bevinding suggereert een beschermende werking voor deze NAb isotypen tegen KM, hoewel het verband niet statistisch significant was.

De Milk Genomics studie was ontworpen om de erfelijkheid van melk eiwit- en vetsamenstelling vast te stellen. Hoewel de verzamelde uiergezondheidsgegevens het mogelijk maakten om parameters te identificeren die betrekking hebben op mastitisgevoeligheid, was de Milk Genomics studie hier niet primair voor ontworpen. De Milk Genomics studie was ook niet geschikt om de relaties tussen immuunparameters en gezondheidsstoornissen, anders dan mastitis, vast te stellen, omdat deze gegevens in dit project niet waren verzameld. Daarom werd een veldstudie opgezet en uitgevoerd die specifiek ontworpen werd om te valideren of NAb titers aanwezig in melkmonsters genomen tijdens de eerste 3 maanden van de lactatie (d.w.z. de periode met de hoogste gevoeligheid voor het ontwikkelen van gezondheidsproblemen) een voorspellende waarde hadden voor bijvoorbeeld de gevoeligheid voor het ontwikkelen van KM, een hoog SCC (>150,000 cellen/mL voor vaarzen en >250,000 cellen/mL voor oudere kalfskoeien) en andere ziekten later in die lactatie (**Hoofdstuk 6**). In een enkel melkmonster van 1998 vaarzen en oudere kalfskoeien van 60 melkveebedrijven werden de isotypen IgG1, IgM en IgA van NAb tegen LPS, LTA, PGN en KLH gemeten. Van deze koeien werd de beschikbare informatie over ziekten en testdag SCC in die lactatie geregistreerd en gebruikt om twee datasets te creëren. De eerste dataset bevatte gegevens van alle, op het moment van monstername klinisch gezonde, koeien (n = 1998); de alle-koeien dataset. De tweede dataset bevatte gegevens van de, op het moment van monstername klinisch gezonde, koeien die KM noch hoog SCC hadden vóór of op het moment van monstername (n = 1260); de gezonde-koeien dataset. Logistische regressieanalyse op deze gegevens toonde aan dat IgG1 NAb tegen KLH in de gezonde-koeien dataset beschermend is tegen KM (OR = 0,8), maar niet tegen een hoog SCC. De resultaten toonden ook aan dat de geschiedenis van voorkomen van KM en SCC de gevonden NAb titers beïnvloeden. Daarnaast werd duidelijk dat er verschillen zijn in de relatie tussen NAb en uiergezondheid, tussen vaarzen en oudere kalfskoeien. Deze gegevens laten zien dat NAb van belang (kunnen) zijn bij de preventie van KM en hoog SCC in melkkoeien van verschillende pariteiten. Het is interessant en relevant om nader onderzoek te verrichten naar deze relaties.

In de Algemene discussie zijn de verkregen resultaten geïntegreerd. Onze resultaten suggereren dat NAb titers in melk een hogere weerstandscapaciteit weergeven die beschermend kan werken tegen de ontwikkeling van ziekten zoals KM, maar ook tegen niet-uier gerelateerde ziekten, zoals geslachtsorgaan- en vruchtbaarheidsproblemen (**Hoofdstuk 7**). Vaarzen verschillen van oudere kalfskoeien in de relaties van de geselecteerde NAb met hun weerstandsvermogen tegen de ontwikkeling van KM en hoog SCC. Daarnaast heeft de uiergezondheidsgeschiedenis van koeien invloed op de relatie van de geselecteerde NAb met het weerstandsvermogen tegen de ontwikkeling van KM of hoog SCC. Natuurlijke antistof titers hebben daarbij de potentie voor genetische selectie op een verhoogd weerstandsvermogen. De hoge correlatiegraad tussen NAb, zowel fenotypisch als genotypisch, maakt het mogelijk om de schatting van NAb effecten te

beperken tot een gelimiteerde set van antigenen, misschien uiteindelijk maar een antigeen, zoals KLH. Concluderend laten de data die in dit proefschrift worden beschreven zien dat er, door genetische selectie op NAb titers, perspectief is om de weerstand tegen (bepaalde) ziekten bij melkkoeien te verbeteren.

POPULAIRE SAMENVATTING

Net als bij mensen is de gezondheid van dieren van groot belang. Vanwege het dierenwelzijn, maar ook voor het welzijn en de portemonnee van de boer. Gezonde koeien met een goede weerstand zitten immers minder vaak in de ziekenboeg, kosten de boer minder werk en zullen meer melk voortbrengen tegen lagere kosten. Ontsteking van de uier, ofwel mastitis, is één van de belangrijkste gezondheidsproblemen bij melkkoeien. Niet alleen zorgt mastitis voor verminderd welzijn voor de koe door pijn, maar het zorgt ook voor grootschalig gebruik van antibiotica en aanzienlijke kosten voor de boer.

Weerstand tegen mastitis en vele andere ziekten is gedeeltelijk gebaseerd op de natuurlijk aanwezige weerstand tegen deze ziekten: het 'aangeboren' deel van het afweersysteem. Het project beschreven in dit proefschrift beoogde nieuwe indicatoren voor natuurlijke weerstand te vinden en daarbij ook hun relatie met de gevoeligheid van melkkoeien voor de ontwikkeling van mastitis en misschien ook andere gezondheidsproblemen vast te stellen. In het ideale geval zouden de te vinden weerstandsparameters eenvoudig in melk gemeten kunnen worden, zodat het mogelijk wordt om de weerstand van melkkoeien routinematig te bepalen. Bijvoorbeeld op vergelijkbare wijze als het meten van de temperatuur bij een ziek dier. Een ander belangrijk doel was om de erfelijkheid van veelbelovende weerstandsparameters te bepalen, omdat dit de mogelijkheden aan het licht kan brengen om via fokkerij de (uier)gezondheid van melkkoeien te verbeteren.

Bij aanvang van het onderzoek is eerst de bestaande wetenschappelijke literatuur bestudeerd. Op grond hiervan is een lijst opgesteld met mogelijk bruikbare weerstandsparameters. Om geschikt te zijn als indicator voor natuurlijke weerstand, zouden zulke weerstandsparameters verschillen tussen koeien moeten vertonen (variatie) en over de tijd voor individuele dieren redelijk stabiel moeten zijn (herhaalbaarheid). Daarnaast moeten ze goed meetbaar en aanwezig zijn in melk of bloed. Om de variatie en herhaalbaarheid te testen is een eerste proef gedaan, waarvoor gedurende bijna 3 weken van elk van 20 gezonde melkkoeien 10 melk- en bloedmonsters zijn genomen. In deze monsters zijn de geselecteerde weerstandsparameters gemeten om de variatie en herhaalbaarheid te bepalen. De gemeten weerstandsparameters waren: verschillende typen cellen, zuurstofradicaal productie van cellen (stoffen voor het doden van ziekteverwekkers), signaalstoffen en antistoffen. Alleen de antistoffen bleken variatie tussen koeien te vertonen, herhaalbaar te zijn en in goed meetbare hoeveelheden aanwezig te zijn in melk en bloed van koeien. Daarom zijn in het verdere onderzoek alleen de antistoffen gebruikt om hun relatie met natuurlijke weerstand tegen ziekten te onderzoeken. De gemeten antistoffen zijn antistoffen tegen 3 stoffen die op het oppervlak van veel bacteriën voorkomen, kort gezegd heten ze: LPS, LTA en PGN. Daarnaast zijn antistoffen tegen een stof gemeten die alleen voorkomt op een zeeweekdier (zeeslak), kort gezegd heet deze stof KLH. Het afweersysteem van koeien zal nooit met deze stof in aanraking geweest zijn, omdat KLH alleen in die bewuste zeeslak voorkomt. Binding van antilichamen aan deze stof wordt als een indicatie gebruikt van het vermogen om direct aan een vreemde stof te kunnen binden die het lichaam binnendringt en waarmee het afweersysteem voor de allereerste keer in contact

komt. Er bestaan verschillende subtypen van antistoffen bij koeien: IgG1, IgG2, IgM en IgA. Deze subtypen kunnen verschillende functies hebben. Daarom is er zowel naar het totaal aan antistoffen als ook naar de subtypen gekeken die aan LPS, LTA, PGN of KLH binden.

Van de antistoffen tegen LPS, LTA, PGN en KLH is vervolgens voor elk de erfelijkheid berekend om vast te stellen of eventueel op hogere niveaus van deze antistoffen gefokt zou kunnen worden. Hiervoor zijn de genoemde antistoffen gemeten in melk van bijna 2000 eerste kalfskoeien (vaarzen). Deze melkmonsters waren verzameld gedurende een eerder onderzoek: de Milk Genomics studie. Deze studie was ontworpen om de erfelijkheid van melk eiwit- en vetsamenstelling vast te stellen. Van deze vaarzen was daarom de genetische achtergrond (familierelaties) bekend, waardoor de erfelijkheid berekend kon worden. De resultaten van de analyse van antistofniveaus in melk laten een lage tot middelmatige erfelijkheid zien voor de gemeten antistoffen. Uit deze resultaten kan worden geconcludeerd dat de niveaus van deze antistoffen door middel van gerichte fokkerij verhoogd kunnen worden.

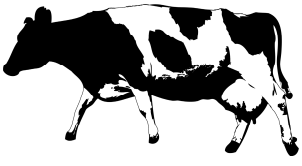
Van 1515 vaarzen die aan de Milk Genomics studie deelnamen was voor de eerste lactatie informatie over het vóórkomen van klinische mastitis (KM) beschikbaar. Daarnaast was bekend hoeveel cellen er in de melk zaten. Dit wordt het “somatic cell count” (SCC) genoemd. Deze SCC is een belangrijke maat voor een (nog niet zichtbare) infectie. Bij een infectie van de uier zullen er namelijk witte bloedcellen vanuit het bloed naar de melk gaan om daar de infectie te bestrijden. Dit noemen we dan een ontsteking en is te zien door een hoog aantal cellen (SCC). In Nederland gaat men er meestal vanuit dat vaarzen met een SCC van 150.000 cellen of meer per milliliter een uierinfectie hebben. Met de beschikbare gegevens kon een analyse uitgevoerd worden om de relatie van de verschillende antistofniveaus met het risico op het ontwikkelen van KM en een hoog SCC later in de lactatie te onderzoeken. Hiervoor werden twee gegevensbestanden gecreëerd. Het eerste bestand bevatte gegevens van alle vaarzen; het alle-vaarzen bestand. Het tweede bestand bevatte gegevens van vaarzen die eerder geen KM en geen hoog SCC (meer dan 150.000 cellen per ml) hadden (aantal koeien: 906); het gezonde-vaarzen bestand. Voor de 2 bestanden werd gekeken of de vaarzen wel of niet KM of hoog SCC ontwikkelden in de rest van de lactatie na het moment van monstername. In het alle-vaarzen bestand leken hogere niveaus van bijna alle antistoffen het risico op een hoog SCC te verhogen. In beide bestanden bleken hogere IgG1 antistofniveaus tegen KLH (van de zeeslak) echter beschermend te zijn tegen hoog SCC. Enkel in het gezonde-vaarzen bestand waren ook hogere totale en IgG1 antistoffen tegen LPS beschermend tegen hoog SCC. In het gezonde-vaarzen bestand leken IgM en IgA antistoffen tegen alle 4 geteste stoffen beschermend te zijn tegen KM, maar dit verband was niet significant, dus nog niet helemaal duidelijk. In het alle-vaarzen bestand was dit verband niet te zien. De verschillen in resultaten tussen de 2 bestanden wijzen erop dat de geschiedenis van het vóórkomen van KM en hoog SCC de antistofniveaus beïnvloedt.

Hoewel de verzamelde gegevens over KM en (hoog) SCC het mogelijk maakten om de voorspellende waarde van antistoffen voor het risico op mastitis te bepalen bij vaarzen, was de Milk Genomics studie hier oorspronkelijk niet voor ontworpen. De

Milk Genomics studie was ook niet geschikt om de relaties tussen weerstandsparameters en andere problemen dan mastitis vast te stellen, omdat deze gegevens in dit project niet waren verzameld. Daarom werd een praktijkproef opgezet en uitgevoerd om te onderzoeken of antistofniveaus in melkmonsters een voorspellende waarde hadden voor de weerstand tegen KM, hoog SCC (meer dan 150.000 cellen per ml voor vaarzen en meer dan 250.000 cellen per ml voor koeien die al vaker gekalfd hadden (dit worden oudere kalfskoeien genoemd)) en andere ziekten, bij vaarzen en oudere kalfskoeien. Hiervoor zijn melkmonsters genomen tijdens de eerste 3 maanden van de lactatie (d.w.z. de periode met de hoogste gevoeligheid voor het ontwikkelen van gezondheidsproblemen). In een melkmonster van 1998 vaarzen en oudere kalfskoeien van 60 melkveebedrijven werden de subtypen IgG1, IgM en IgA van antistoffen tegen LPS, LTA, PGN (van bacteriën) en KLH (van de zeeslak) gemeten. Van deze koeien werd de beschikbare informatie over ziekten en SCC in die lactatie verzameld en gebruikt om twee bestanden te creëren, vergelijkbaar met de 2 bestanden gebruikt voor de Milk Genomics gegevens: Het alle-koeien bestand en het gezonde-koeien bestand. Voor de 2 bestanden werd geanalyseerd of de antistofniveaus voorspellend waren voor het optreden van KM en hoog SCC later in die lactatie. Deze analyses toonden aan dat hogere IgG1 antistofniveaus tegen KLH in het gezonde-koeien bestand beschermend zijn tegen KM, maar niet tegen een hoog SCC. De resultaten toonden ook aan dat, net als bij de Milk Genomics gegevens, de geschiedenis van vóórkomen van KM en hoog SCC de gevonden antistofniveaus beïnvloeden. Daarnaast werd duidelijk dat er verschillen zijn in de relatie tussen antistoffen en uiergezondheid, tussen vaarzen en oudere kalfskoeien. Naast KM en hoog SCC konden ook geslachtsorgaan- en vruchtbaarheidsproblemen en been- en klauwproblemen geanalyseerd worden. Hogere niveaus van subtype IgG1 tegen KLH bleek niet alleen beschermend te zijn tegen KM, maar ook tegen geslachtsorgaan- en vruchtbaarheidsproblemen (en er leek een zelfde tendens te zijn voor been- en klauwproblemen). De invloed van ziektegeschiedenis op antistoffen was hier minder duidelijk dan bij KM en SCC.

Deze resultaten laten zien dat antistoffen van belang (kunnen) zijn bij de preventie van bepaalde ziekten in melkkoeien van verschillende lactatienummers. Vaarzen verschillen van oudere kalfskoeien in de relaties van de geselecteerde antistoffen met hun weerstandsvermogen tegen KM en hoog SCC. Daarnaast heeft de uiergezondheidsgeschiedenis van koeien invloed op de relatie van de geselecteerde antistoffen met het weerstandsvermogen tegen KM of hoog SCC. Verder hebben natuurlijke antistofniveaus de potentie voor fokken op een verhoogd weerstandsvermogen. Er moet echter nog wel onderzocht worden of er geen negatieve relatie is met andere eigenschappen waarop gefokt wordt. Daarnaast is relevant om nader onderzoek te verrichten naar de invloed van lactatienummer en ziektegeschiedenis op de relaties van antistoffen met de weerstand tegen ziekten.
Concluderend laten de gegevens die in dit proefschrift worden beschreven zien dat er, door fokken op antistofniveaus, perspectief is om de weerstand tegen (bepaalde) ziekten bij melkkoeien te verbeteren.

2000 x



Dankwoord

(Acknowledgements)

List of abbreviations

DANKWOORD

Het was niet niks, maar het ligt er nu wel, mijn proefschrift!
Vier jaar, bijna 4000 ELISAplaten, zo'n 4200 melkmonsters, ruim 200 bloedmonsters, liters buffers, hopen potjes testantistoffen, antigenen en andere reagentia, niet te vergeten de harkjes en tankjes van de elektrische pipet (geweldige uitvinding), een ELISAplaat-wasapparaat (ook superhandig, Eric bedankt), 4 studenten, bergen ziekte-, MPR- en labtestgegevens, data-analyses en literatuur, bijeenkomsten, presentaties, frustraties, stress, blije, vervelende, gezellige en onvoorstelbare momenten verder, is het gelukt.
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Tosca

LIST OF ABBREVIATIONS

BTA	Bos taurus autosome (bovine chromosome)
CD	cluster of differentiation (cluster of designation) (cell surface markers)
CM	clinical mastitis
CV	coefficient of variation (SD/mean)
d	day(s)
DHR	dihydrorhodamine 123
DIM	days in milk (lactation stage)
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
Fc	fragment crystallizable (receptor)
FcRn	neonatal Fc receptor
g	gram
g	gravity
GFP	genital organ and fertility problems
h	hour(s)
h^2	heritability
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
IFN-γ	interferon γ
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
KLH	keyhole limpet hemocyanin
ln	natural logarithm
LBP	LPS binding protein
LCP	leg and claw problems
LPS	lipopolysaccharide
LTA	lipoteichoic acid
n	number of samples
N	normal (concentration)
NAb	natural antibody/ies
NFκB	nuclear factor κB
NK cell	natural killer cell
NO	nitric oxide
OH	hydroxyl
OR	odds ratio
PAMP	pathogen associated molecular pattern(s)
PBS	phosphate buffered saline
PGN	peptidoglycan
pIgR	polymeric Ig receptor
PRR	pattern recognition receptor
QTL	quantitative trait locus/loci
r	correlation coefficient
ROS	reactive oxygen species

σ^2	variance
sCD14	soluble CD14
S. aureus	*Staphylococcus aureus*
SCC	somatic cell count
SCS	somatic cell score (ln(SCC/1000))
SD	standard deviation
SE	standard error
SNP	single nucleotide polymorphism
S. uberis	*Streptococcus uberis*
TGF	transforming growth factor
TLR	Toll like receptor
TNF-α	tumor necrosis factor α
UCDB	universal casein diluents/blocker

About the author

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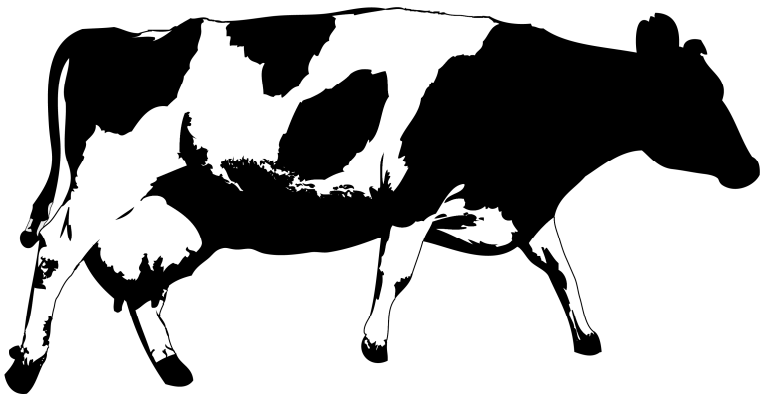

CURRICULUM VITAE (ENGLISH)

Tosca C. W. Ploegaert was born in Castricum, The Netherlands on 24 August 1980. After graduating from the Bonhoeffercollege high school in Castricum, The Netherlands, she studied Animal Sciences at Wageningen University. During this study she did a minor MSc thesis on the behavior of Przewalski horses in Hustai National Park, Mongolia. In 2003-2004 she organized the General Introduction Days 2004 for the new students of Wageningen University, together with 5 other students. For her major MSc thesis she studied the modulation of the antibody immune response of laying hens by pathogen associated molecular patterns. For this thesis she was awarded with the second NZV (Dutch Zootechnical Association) thesis prize 2006. For her first intership she studied risk factors for introduction of avian influenza at Dutch poultry farms at the Department of Knowledge of the Ministry of Agriculture, Nature and Food quality in Ede, The Netherlands. She studied the effect of different feeds against a parasite infection in lactating rats for her second intership at the Scottish Agricultural College in Edinburgh, Scotland. After her graduation she started her PhD project in September 2006. During her PhD she organized the Wageningen Institute of Animal Sciences (WIAS; graduateschool) Science Day 2007, where PhD students can present their research. Furthermore she was member of WIAS associated PhD Students Council, for 2 years, that received the Team of the year prize 2007. Currently, she studies Veterinary Medicine at Utrecht University, following a long lasting dream and the opportunity to expand her knowledge and skills on animals and diseases.

CURRICULUM VITAE (NEDERLANDS)

Tosca C. W. Ploegaert is geboren op 24 augustus 1980, in Castricum, Nederland. Na haar VWO (Gymnasium) diploma behaald te hebben aan het Bonhoeffercollege in Castricum, studeerde ze Zoötechniek (Dierwetenschappen) aan Wageningen Universiteit. Tijdens deze studie deed ze een 'klein' afstudeervak (minor) waarvoor ze het gedrag van Przewalski paarden bestudeerde in Hustai Nationaal Park, Mongolië. In 2003-2004 organiseerde ze samen met 5 andere studenten de Algemene Introductie Dagen 2004 voor de nieuwe studenten van Wageningen Universiteit. Voor haar 'grote' afstudeervak (major) heeft ze gekeken naar de modulatie van de antistof immuunreactie van leghennen door moleculen afkomstig van pathogenen. Voor dit afstudeervak kreeg ze de tweede NZV (Nederlandse Zoötechnische Vereniging) scriptieprijs 2006. Voor haar eerste stage keek ze naar risicofactoren voor de insleep van aviaire influenza op Nederlandse pluimveebedrijven bij Directie Kennis van het Ministerie van LNV in Ede. Ze bestudeerde het effect van verschillende voeding op een parasitaire infectie in lacterende ratten tijdens haar tweede stage bij Scottish Agricultural College in Edinburgh, Schotland. Na haar afstuderen startte ze haar AIO project in september 2006. Tijdens haar AIO project organiseerde ze de Wageningen Institute of Animal Sciences (WIAS; graduateschool) Science Day, waar AIO's hun onderzoek kunnen presenteren. Verder was ze 2 jaar lid van de WIAS Associated PhD Students council, die de Team of the year prize 2007 kreeg. Op dit moment studeert ze Diergeneeskunde aan Universiteit Utrecht, een lang gekoesterde droom en een mogelijkheid haar kennis en vaardigheden over dieren en ziekten uit te breiden.

LIST OF PUBLICATIONS

Peer reviewed papers

Ploegaert, T.C.W., Wijga, S., Tijhaar, E., Van der Poel, J.J., Lam, T.J.G.M., Savelkoul, H.F.J., Parmentier, H.K., Van Arendonk, J.A.M. 2010. Genetic variation of natural antibodies in milk of Dutch Holstein-Friesian cows. J. Dairy Sci. 93(11), 5467-73.

Ploegaert, T.C.W., De Vries Reilingh, G., Nieuwland, M.G., Lammers, A., Savelkoul, H.F.J., Parmentier, H.K. 2007. Intratracheally administered pathogen-associated molecular patterns affect antibody responses of poultry. Poult Sci. 86(8), 1667-76.

Ploegaert, T.C.W., Tijhaar, E., Lam, T.J.G.M., Taverne-Thiele, A., Van der Poel, J.J., Van Arendonk, J.A.M., Savelkoul, H.F.J., Parmentier, H.K. Natural antibodies in bovine milk and blood plasma: variability among cows, repeatability within cows, and relation between milk and plasma titers. Provisionally accepted in Vet. Immunol. Immunopathol.

Submitted paper

Ploegaert, T.C.W., Ducro, B.J., Oosterik, L.H., Van der Poel, J.J., Lam, T.J.G.M., Parmentier, H.K., Van Arendonk, J.A.M., Savelkoul, H.F.J., Tijhaar, E., Relation of natural antibodies in milk of Holstein Friesian heifers with the risk for high somatic cell count and clinical mastitis**.**

Conference proceedings papers

Ploegaert, T.C.W., Tijhaar, E., Parmentier, H.K., Van der Poel, J.J., Van Arendonk J.A.M., Lam, T.J.G.M., and Savelkoul, H.F.J., Immune parameters in milk as potential predictive parameters for mastitis resistance, Proceedings of international conference "5th IDF Mastitis Conference" 21 – 24 March 2010, Christchurch, New Zealand, p. 308 – 314.

Sell-Kubiak, E., **Ploegaert, T.C.W.**, Schopen, G.C.B., Parmentier, H.K., Tijhaar, E., Van Arendonk, J.A.M., Bovenhuis, H., and Van der Poel, J.J. 2010. QTL affecting innate immunity in Dutch dairy cattle, Proceedings 9th World Congres on Genetics applied to Livestock Production, Leipzig, paper 0177.

Ploegaert, T.C.W., Tijhaar, E., Parmentier, H.K., Van der Poel, J.J., Van Arendonk J.A.M., and H.F.J. Savelkoul, Parameters for natural resistance in bovine milk Proceedings of international conference "Mastitis control, from science to practice". 30 September – 2 October 2008, The Hague, the Netherlands, p. 103-109.

Posters

Ploegaert, T.C.W., Tijhaar, E., Parmentier, H.K., Van der Poel, J.J., Van Arendonk, J.A., Lam, T.J.G.M., Savelkoul, H.F.J. 2010. Natural antibodies, parameters for udder health in dairy cows?; International Dairy Federation Mastitis Conference, 21-24 March 2010, Christchurch, New Zealand.

Ploegaert, T.C.W., Tijhaar, E., Parmentier, H.K., Van der Poel, J.J., Van Arendonk, J.A., Savelkoul, H.F.J. 2009. Antibodies, parameters for natural resistance in bovine milk?; WIAS Science Day, 12 March 2009.

TRAINING AND SUPERVISION PLAN

The Basic Package

WIAS Introduction Course, Wageningen, The Netherlands	2007
WIAS Course Broaden your Horizon, Wageningen	2007
Subtotal Basic Package:	***3 credits****

Scientific exposure

International conferences

Heifer Mastitis Conference, Ghent, Belgium	2007
Congress Mastitis Control, Den Haag, The Netherlands	2008
NVVI[1] congress, annual meeting, Noordwijkerhout, The Netherlands	2008
IDF[2] Mastitis Conference, Christchurch, New Zealand	2010

Seminars and workshops

WIAS seminar Macrophages and their role in the immune system, Wageningen	2006
Symposium UGCN[3], De Eemlandhoeve, Bunschoten, The Netherlands	2006
Symposium Science meets society; robustness, Wageningen	2007
NVVI course, Lunteren, The Netherlands	2007, 2009
Symposium Mastitis Control, from Science to Practice, Ede, The Netherlands	2008
Workshop Mastitis Control, Den Haag, The Netherlands	2008
WIAS seminar Regulation of the immune response in Teleost Fish, Wageningen	2008
WIAS seminar Immunomodulation and allergy, Wageningen	2008
Seminar: Genetics of Milk Quality, Wageningen	2009
WIAS science Day	2007-10

Oral presentations

Parameters for natural resistance in cow's milk; DMRW[4] meeting, Wageningen	2007
Parameters for natural resistance in bovine milk; Congress Mastitis Control, Den Haag, The Netherlands	2008
DMRW annual meeting: Parameters for natural resistance in cow's milk, Utrecht, The Netherlands	2007
DMRW annual meeting: Parameters for natural resistance in bovine milk, A potential for antibodies?, Deventer, The Netherlands	2008
DMRW annual meeting: Immuun parameters in rundermelk als potentiële parameters voor natuurlijke (mastitis) weerstand, Wageningen	2009
WIAS Science Day: Immune parameters as potential parameters for bovine disease resistance	2010
IDF Mastitis Conference: Natural antibodies as potential parameters for bovine mastitis resistance, Christchurch, New Zealand	2010

Poster presentations

WIAS Science Day: Antibodies, parameters for natural resistance in bovine milk?	2009
IDF Mastitis Conference: Natural antibodies, parameters for udder health in dairy cows?	2010
Subtotal Scientific Exposure:	***15.8 credits***

In-Depth Studies

Disciplinary and interdisciplinary courses

Mastitis course, Deventer, The Netherlands	2006
Study of Resistance Mechanisms in Animal Infectious Diseases, Liège, Belgium	2006
Infection and Immunity, Utrecht, The Netherlands	2007
Fish Immunology Workshop, Wageningen	2007
ELISA: basic understanding and trouble shooting, Wageningen	2007

Advanced statistics courses

WIAS Advanced Statistics Course: Experimental Design	2007
WIAS Course: Statistics for the Life Sciences	2008
Subtotal In-Depth Studies:	***8.3 credits***

Skills

Professional Skill support courses

Personal Efficacy, Wageningen	2006
WIAS course: Science meets Society, Wageningen	2006-2007
Career coaching workshop, Wageningen	2007
Project- and Time Management, Wageningen	2007
NWO talent day, Utrecht, The Netherlands	2009
Techniques for Writing and Presenting Scientific Papers, Wageningen	2010

Didactic skills

Supervising practicals during Fish Immunology Workshop	2007
Supervising practicals Cell Biology I course	2008
Supervising 4 major MSc theses	2008-2010

Management skills

Organisation of the WIAS Science Day	2007
Membership of WIAS Associated PhD Students council	2007-2008
Subtotal Skills:	***19.2 credits***
Total number of credit points:	***46.3***

Herewith the WIAS Graduate School declares that the PhD candidate has complied with the educational requirements set by the Edcational Committee of WIAS which comprises of a minimum total of 30 credit points.

***** According to the ECTS (European Credit Transfer and Accumulation System), 1 credit point represents a normative study load of 28 hours of study.

[1]Nederlandse Vereniging Voor Immunologie (Dutch Society for Immunology)

[2]International Dairy Federation

[3]Uiergezondheidscentrum Nederland (Dutch Udder Health Center)

[4]Dutch Mastitis Research Workers

Doe je best en vergeet de rest, dan komt alles op zijn pootjes terecht

(Anna Sewell, vertaald uit het Engels)

COLOPHON

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