Veterinary Immunology and Immunopathology 171 (2016) 21–27

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Bovine natural antibodies in antibody-dependent bactericidal activity against *Escherichia coli* and *Salmonella* Typhimurium and risk of mastitis

S.E.C. van Altena[a,*], M.A. Peen[a], F.H. van der Linden[a], H.K. Parmentier[b], H.F.J. Savelkoul[a], E.J. Tijhaar[a]

[a] Cell Biology and Immunology Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, Wageningen, the Netherlands
[b] Adaptation Physiology Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, Wageningen, the Netherlands

ARTICLE INFO

Article history:
Received 14 December 2015
Received in revised form 29 January 2016
Accepted 31 January 2016

Keywords:
Antibody-mediated complement killing
Natural antibodies
Dairy cattle

ABSTRACT

Natural antibodies (NAbs) are mostly IgM antibodies produced without antigenic stimulation and serve as a first line of defence of the immune system. As both natural and specific antibodies are present in animals, NAbs are studied by determining the IgM response to naïve antigens like keyhole limpet hemocyanin (KLH). In this study, we selected cows based on high and low anti-KLH IgM titers, reflecting high and low NAb titers, and determined if the anti-KLH IgM titers were indicative for the recognition of common microbial structures (lipopolysaccharide, lipoteichoic acid and peptidoglycan) and intact bacteria (*Escherichia coli* and *Salmonella* Typhimurium). Sera with high NAbs titers showed more IgM and IgG binding to common microbial structures and *S.* Typhimurium bacteria than sera with low NAbs titers. The same association was observed for IgM binding to *E. coli*, but not for IgG binding to *E. coli*. Antibody-mediated complement killing of *E. coli* and *S.* Typhimurium in a newly developed bactericidal test was equal between high and low NAb cows. However, relating the outcome of the bactericidal test to the development of mastitis within one and even four years after sampling showed a significant negative correlation implying cows that were less potent in bacterial killing had a higher chance on developing mastitis. In conclusion, sera with high NAbs titers had more antibodies binding to common microbial structures and intact bacteria. Furthermore, the bactericidal test might provide a useful prognostic tool for the development of mastitis.

1. Introduction

Natural antibodies (NAbs) are polyreactive antibodies that are produced without prior antigenic stimulation and mostly directed to auto-antigens and common microbial structures like Pathogen-Associated Molecular Patterns (PAMP) (Avrameas, 1991). In mammals, NAbs are mostly IgM (Baumgarth et al., 2005) and are produced by B1 B-cells (Baumgarth, 2013; Baumgarth et al., 2005), performing a first line of defence against invading pathogens (Ochsenbein and Zinkernagel, 2000). In contrast, specific antibodies (SpAbs) are produced by B2 B-cells in response to antigen exposure, and by consequence, are far more restricted in their epitope recognition compared to NAbs. Mouse studies showed that NAbs have a unique role in the immune system. NAbs can act in similar ways as SpAbs by initial recognition and opsonisation of pathogens, resulting in subsequent complement-dependent killing or phagocytosis (Ehrenstein and Notley, 2010; Hangartner et al., 2006). Moreover, NAbs play a homeostatic role e.g. by clearing apoptotic cells and neo-epitopes (Grönwall et al., 2012; Lutz et al., 2009). High serum NAb levels were associated with increased survival of laying hens (Sun et al., 2011) and sheep (Graham et al., 2010) and NAbs were essential in the survival of mice suffering from an induced acute peritonitis (Boes et al., 1998). In cows, NAb levels in milk were suggested to be positively related with resistance to mastitis (Ploegaert et al., 2008).

Binding of bovine serum antibodies to PAMP makes it hard to discriminate the role of NAbs from that of SpAbs. In other species, NAbs binding the naïve antigen Keyhole Limpet Hemocyanin (KLH) were studied. Dairy cows unlikely will have SpAbs to KLH, since KLH is derived from the Californian Pacific coast sea mollusc *Megathura crenulata* (Harris and Markl, 1999), but dairy cow do have NAbs

* Corresponding author at: De Elst 1, 6708 WD, Wageningen, the Netherlands.
E-mail address: christine.vanaltena@wur.nl (S.E.C. van Altena).

http://dx.doi.org/10.1016/j.vetimm.2016.01.009
0165-2427/

binding KLH in milk and serum (Ploegaert et al., 2011; van Knegsel et al., 2007).

The aim of the present study was threefold. First, it was studied whether NAbs in bovine serum, represented by anti-KLH IgM antibodies, were related with the levels of IgM and IgG serum antibodies binding lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan (PGN) representing PAMP from Gram negative and Gram positive bacteria, and intact *Escherichia coli* (*E. coli*) and *Salmonella enterica* subspecies *enterica* serovar Typhimurium bacteria (*S.* Typhimurium). These bacteria were chosen, as *E. coli* is a major pathogen in bovine infectious diseases in the udder (Olde Riekerink et al., 2008; Verbeke et al., 2014) and *S.* Typhimurium is an opportunistic bacterium that emerges when a cows' health status is compromised. The level of antibodies binding to *S.* Typhimurium is chosen as an indicator for natural immune competence. Such antibody-based indicator of natural immune competence was previously described in chickens (Star et al., 2007, 2009). Second, it was determined if high serum NAb titers resulted in higher microbial killing via antibody-mediated complement killing in a newly developed bactericidal test. Third, NAb titers and bactericidal activity of bovine serum were related with the future development of mastitis.

2. Materials and methods

2.1. Animal selection

Bovine serum samples were selected from the "Resilient Cattle" (Weerbaar Vee) biobank containing serum, blood and milk samples collected on 29 conventional, Dutch farms between 2011 and 2015. In total, 2032 and 3109 serum samples were collected during winter 2011 and spring 2012, respectively. Disease registration and milk recording data were collected. The disease registration data cover the type and numbers of disease, the date when disease occurred and treatments per cow.

Serum samples were tested for IgM and IgG antibodies binding to Keyhole Limpet Hemocyanin (KLH). Since the majority of NAbs are IgM antibodies (Baumgarth et al., 2005), cows were selected on anti-KLH IgM titers and the following criteria: exclusion of cows with parity 0, cows between 0 and 30 days in lactation and cows with elevated somatic cell counts (SCC) at the moment of sampling. Maximum SSC were < 150,000 cells/mL for primiparous cows and < 250,000 cells/mL for multiparous cows, respectively. Exclusion of cows by the aforementioned criteria resulted in 1494 and 2384 cows in winter 2011 and spring 2012, respectively (Fig. 1A and B).

2.2. IgM and IgG antibodies binding KLH

IgM and IgG antibody titers to KLH were determined using an enzyme-linked immunosorbent assay (ELISA) as described (de Klerk et al., 2015). IgM and IgG levels binding to KLH were expressed as titer where the titer represented the log2 value of the dilution closest to 50% of E_{max}. E_{max} is the highest value of the standard curve (Ploegaert et al., 2010).

2.3. IgM and IgG antibodies binding LPS, LTA and PGN

IgM and IgG antibodies in serum binding to LPS, LTA and PGN were determined by ELISA. High-binding 96-wells plates were coated overnight at 4 °C with 4 μg/mL LPS (from *E coli* O55:B5, Sigma–Aldrich Inc., Missouri, US), LTA or PGN (from *Staphylococcus aureus*, Sigma) in PBS. Plates were blocked using Casein (Casein Diluent, Stereospecific Detection Technologies, Baesweiler, Germany) for 1.5 h at room temperature (RT). Serum samples were diluted 10×, 25× and 1000× in 1:3 diluted Casein in PBS for PGN, LPS and LTA, respectively. Diluted sera were incubated for 1 h at RT. Plates were washed with PBS containing 0.05% Tween-20 and incubated with either sheep-anti-bovine IgM HRP or IgG HRP (Bethyl Laboratories Inc., Montgomery, US) 1:5000 diluted in 1:3 Casein/PBS for 1 h at RT. After washing, plates were incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) (SDT, Germany) for 20 min at RT and stopped using 2% hydrogen chloride (HCl). OD-values were measured at 450 nm and blanc corrected using the Filtermax F5 plate reader (Molecular Devices, Sunnyvale, California).

2.4. IgM and IgG antibodies binding E coli and Salmonella Typhimurium

E. coli (JM109) and *S.* Typhimurium (SL3261) bacteria were grown overnight at 37 °C in LB medium. The next day, bacteria were transferred to fresh LB medium and grown to log-phase (OD_{600nm} = 0.6–0.8). Bacteria were washed three times in 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.6).

Medium-binding plates were coated with 2 × 108 bacteria/mL in 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.6) and incubated overnight at 4 °C. Bacteria were fixed using 4% paraformaldehyde for 2 h at RT. Plates were blocked using Casein for 1 h at RT. Serum samples were diluted 100 times in 1:3 Casein/PBS and incubated for 1 h at RT. After washing with PBS containing 0.05% Tween-20, IgM and IgG antibodies binding to *E. coli* or *S.* Typhimurium were detected as previously described (See IgM and IgG antibodies binding to LPS, LTA and PGN).

2.5. Bactericidal activity of bovine serum

Serum samples containing high or low NAb titers were selected and tested for antibody-mediated complement killing. Bacterial killing was expressed as the minimal inhibitory dose (MID), the highest serum dilution that inhibited bacterial growth (Supplementary Fig. 1), and was measured in a newly developed bactericidal test based on (Szabo et al., 2010). Bacteria (*E. coli* JM109 or *S.* Typhimurium SL3261) were inoculated in LB medium and grown to log-phase. Serum samples were heat-inactivated (HI) for 30 min at 56 °C. The HI sera were diluted with PBS and titrated with two-step dilutions starting at 1:10 in 80 μL/well in flat-bottom 96-wells culture plates (Greiner Bio-One, Frickenhausen, Germany). Next, 20 μL/well untreated foetal calf serum (FCS, Thermo Fischer Scientific, MA) was added as fixed source for complement with low levels of endogenous antibodies. Bacteria were diluted to 4 × 106 bacteria/mL in LB medium and 100 μL bacteria were added to the diluted sera. Plates were measured at 595 nm using the Filtermax F5 microplate reader before and after overnight incubation at 37 °C while shaking at 250 rpm.

The majority of NAbs in mice were shown to be IgM antibodies and only a minor part was IgG (Baumgarth et al., 2005). To minimize the influence of specific IgG antibodies in the test, cows were selected with low IgG levels to *E. coli* or *S.* Typhimurium in combination with high or low IgM antibody levels against these bacteria. This selection resulted in 20 high and 20 low cows for *E. coli* and for *S.* Typhimurium, which were compared in the bactericidal test.

2.6. Statistical analysis

Differences in IgM and IgG levels between cows with high- and low anti-KLH IgM titers were determined using the Mann–Whitney *U* test since the samples were not normally distributed (GraphPad Prism 5). Normality was tested using the Shapiro–Wilk normality test.