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Severity of *E. coli* mastitis is mainly determined by cow factors

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Abstract – Intramammary infections of dairy cows with Gram-positive bacteria such as *Staphylococcus aureus* (major cause of mastitis) have received a lot of attention because of their major economic impact on the dairy farm through production losses induced by an increase in somatic cell count. Management strategies, including greater awareness for efficient milking and hygienic measures, have limited the spread of Gram-positive bacteria and resulted in a significant decrease of proportion of *S. aureus* isolates and subclinical mastitis worldwide. Other organisms such as coliform subspecies and *Streptococcus uberis*, both environmental bacteria that cause clinical mastitis, have received less attention. *Escherichia coli* causes inflammation of the mammary gland in dairy cows around parturition and during early lactation with striking local and sometimes severe systemic clinical symptoms. This disease affects many high producing cows in dairy herds and may cause several cases of death per year in the most severe cases. It is well known that bacterial, cow and environmental factors are interdependent and influence mastitis susceptibility. Many studies, executed during the last decade, indicate that the severity of *E. coli* mastitis is mainly determined by cow factors rather than by *E. coli* pathogenicity. During *E. coli* mastitis, the host defense status is a cardinal factor determining the outcome of the disease. Today, we know that the neutrophil is a key factor in the cows' defense against intramammary infection with *E. coli*. Effective elimination of the pathogen by neutrophils is important for the resolution of infection and the outcome of *E. coli* mastitis. This review is a compilation of some major findings over the last 15 years concerning mainly host factors that modulate and influence neutrophil function and the mammary inflammatory reaction. The individual chapters address: virulence factors of *E. coli* strains, how neutrophils kill *E. coli*, connection between endotoxins, tumor necrosis factor- α and nitric oxide, severity classification of *E. coli* mastitis, lifespan of neutrophils, host factors that influence severity, tissue damage and production loss.

dairy cow / *Escherichia coli* mastitis / neutrophil / severity

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1. INTRODUCTION

Escherichia coli causes infection and inflammation of the mammary gland in dairy cows mainly around parturition and during early lactation with striking local and sometimes severe systemic clinical

symptoms. This so-called environmental type of mastitis may affect productivity of high producing cows in dairy herds. It may also cause several deaths per year in the most severe cases. *E. coli* invades the udder through the teat canal where it grows and initiates a prompt inflammatory reaction,

with increasing numbers of neutrophils appearing. This neutrophil infiltration is one of the first steps of the inflammatory reaction because they have to prevent escape and multiplication of the pathogens.

It is well known that bacterial, environmental or management, and cow factors may change susceptibility for mastitis. These factors are interdependent and especially the relative impact of each factor depends on the type of pathogen [234].

1.1. Pathogen factors

The pathogenesis of coliform mastitis is completely different from diseases such as colibacillosis in calves and pigs. For example, it is well known that in so-called enterotoxigenic *E. coli* (ETEC) strains, the colonisation of intestinal mucosa is related to the expression of several different proteinaceous surface structures, i.e. colonisation factors, which recognise specific receptors on the intestinal epithelial surface [47, 206]. The pathogenicity in these animals mainly depends on expression of particular virulence factors. For coliform mastitis this is not the case, and it is generally accepted that the type of *E. coli* strain does not play a major role in the severity classification [24]. A non-specific but potent factor that is important during the pathogenesis of *E. coli* is the endotoxin or lipopolysaccharide (LPS) [64]. At the one hand it induces defence mechanisms and at the other hand it is a toxic compound. Pathophysiological effects of endotoxins in ruminants are dose-dependent and elicit a variety of metabolic and clinical signs [143, 144].

1.2. Management factors

Management factors that influence the cow's condition during late pregnancy, mainly with respect to energy, in order to achieve a fast and high peak of milk production, might be the cause of metabolically immunocompromised cows that have lower resistance to stress induced by partu-

rition and early lactation. Once adapted, i.e. after peak lactation, it seems that cows are less susceptible for environmental pathogens.

The organised control of bulk and cow milk samples by specialised milk quality centres has resulted, especially in Europe, in a significant reduction in normal somatic cell count (SCC). There is a tendency to believe that low milk SCC fails to protect the udder from environmental pathogens (reviewed by [227]). This finding is mainly based on epidemiological data and there are only a few experimental data that can confirm these findings (e.g. [210]). Finally, higher stock densities have resulted in a greater pasture contamination with easier access for environmental pathogens to the udder through their open teats during calving and early lactation. Management factors fall beyond the scope of this review.

1.3. Cow factors

The ability of neutrophils to sequester and kill the pathogens is a critical step in the defence reaction against *E. coli*. The progress of the inflammatory reaction is determined by a constitutive balance between forces opposing pathogen growth and production of virulent factors, and the forces favouring it. Neutrophils are very beneficial to the udder following *E. coli* infection, and form the most important body defence mechanism. It is essential that they arrive in the lumen of the udder promptly if they are to be totally effective. Since 1990, numerous reviews have been written summarising and updating the progress toward the understanding of the mechanisms of the neutrophil response into udder tissue and cistern during *E. coli* mastitis [31–33, 177, 179]. More recently, excellent reviews have been published in connection with the immune mechanisms in the mammary gland [119, 180, 220]. It is well recognised that clinical *E. coli* mastitis cases occurring during established lactation show a minimum of systemic signs (such as fever, discomfort, temporary milk

production drop, etc.). Clinical signs are moderate, subside very rapidly and the animal cures itself. Under these conditions, the inflammatory reaction has to be considered as a 100% physiological reaction. It seems that at parturition and during early lactation, high producing cows are more sensitive for infection with common environmental pathogens such as *E. coli*. Many factors may have contributed to this phenomenon but the animals' physiological pre-infection status also influences the outcome of coliform mastitis.

From many studies performed during the last 15 years, it is evident that the severity of bovine *E. coli* mastitis is mainly determined by cow factors rather than by the pathogenicity of the invading pathogen. During coliform mastitis, the host defence status is a cardinal factor determining the pathogenicity and the clinical outcome of this disease, depending on its interaction with the pathogen and its LPS. In this review some aspects will be covered of LPS in relation with severity of coliform mastitis.

Initiation and resolution of inflammation is controlled by a complex network of biological substances that belong to the homeostatic armamentarium of the host. Since 1990 a long list of original papers, dealing with the impact of the physiology of the cow (parturition, lactation stage, age, metabolism, pro- and anti-inflammatory endogenous substances, growth factors, platelet activating factor, etc.) on the pathogenesis of mastitis, have been published. Each imbalance in initiation, tuning and resolution results in host and/or tissue damage and severe prognosis of the disease.

As a consequence of neutrophil infiltration into the invaded area of the mammary gland, changes in blood cell composition occur, due to bone marrow stimulation and alteration of the normal venular equilibrium that controls attachment, rolling and diapedesis. Next to the changes in the cellular blood picture, composition of extracellular water (internal sea; [37]) also

changes. The concept of a well-balanced inflammatory response where pro- and anti-inflammatory local mediators, such as cytokines, regulate the outcome of the inflammatory process, is also applicable to systemic factors [34]. The inflammatory reaction is a rapid and mainly a non-specific defence that is accompanied by the release and/or "de novo synthesis" of specific and non-specific molecules, such as systemic and tissue hormones and (poly)peptides. The metabolism of the animal is also changed. Hormones, metabolites and acute phase proteins, may influence the outcome of mastitis.

Curing (the final goal of the inflammatory reaction) of the mammary gland is only successful if minimal host and tissue damage occurs. Therefore, the inflammatory reaction has to be fast, short lasting and regulated. After elimination of the invading pathogens, neutrophil reaction subsides to promote a predominant mononuclear cell picture. Macrophages do not only replace neutrophils but also actively participate in their removal [181]. This last step represents the resolution of the inflammatory reaction. Neutrophil infiltration and resolution during *E. coli* mastitis are necessary to reset the composition of the "internal sea" towards the pre-infection status of the organism and this is accompanied by specific and non-specific systemic events. This is in agreement with the general concepts in physiology [37].

This chapter is a compilation of some major findings over the last 15 years concerning mainly host factors that modulate and influence neutrophil function and the inflammatory reaction of the lactating cow. The individual chapters are dealing with virulence factors of *E. coli* mastitis strains, how neutrophils sequester and kill *E. coli*, the connection between endotoxins, tumor necrosis factor- α (TNF- α) and nitric oxide (NO), severity classification of coliform mastitis, lifespan of neutrophils (from maturation to senescence), host factors that

influence severity, tissue damage and production loss.

2. VIRULENCE FACTORS OF *E. COLI* MASTITIS STRAINS

2.1. *E. coli* as pathogen

E. coli is a member of the family of Enterobacteriaceae. Of primary importance is their ability for fermentation of lactose. Over 700 antigenic types or serotypes of *E. coli* have been recognised based on O, H, and K antigens. Two classes of coliforms have to be distinguished: strains that are harmless (non-pathogenic strains) and strains that cause a wide variety of typical clinical infections (pathogenic strains, [47]). Millions of non-pathogenic *E. coli* bacteria are living in the normal intestinal microflora of both humans and animals. *E. coli* adheres to the mucus overlaying the large intestine where they constitute a useful microbial barrier because of their antagonism to pathogenic bacteria. *E. coli* is massively excreted with the faeces in the environment. *E. coli* is ubiquitous in the cow's environment. It is obvious that challenge of *E. coli* udder infection will remain so long as cows produce faeces [112]. They behave as opportunistic pathogens. There has been a substantial rise in the incidence of *E. coli* mastitis since 1960 and it is accepted to be the most common cause of fatal mastitis [163]. In contrast to the enteropathogenic and bacteraemic strains, which are caused by a relatively low number of *E. coli* serotypes [47], the isolates from bovine coliform mastitis belong to a very large number of serological groups. *E. coli* serotypes in mastitic milk are similar to the faecal isolates. There are a very large number of *E. coli* serotypes as classified by surface antigens but the inner layer of the cell wall is common to all serotypes of *E. coli* and Enterobacteriaceae in general [65].

2.2. Adhesion and colonisation

Following experimental intramammary (I.M.) challenge with *E. coli*, attachment to mammary epithelium was not observed [136]. Electron microscopic research on the pathogenesis of coliform mastitis never revealed attachment of bacteria to the epithelium [24]. Attachment of *E. coli* to mammary epithelial cells seems not to be essential for the pathogenesis of clinical coliform mastitis [76, 175]. Most of mastitis pathogen adhesion studies have been performed with cell cultures for which several models have been described. Lammers et al. [132] used *E. coli* strain HB101 as negative control to test adhesion and invasion of udder pathogens. In a recent study, the specificity and efficiency of adhesion of 11 different *E. coli* strains was studied. The cell tropism of the tested *E. coli* strains was strongly strain-dependent and very variable from very low to high efficiency [133]. Binding can also occur to molecules of the cell matrix and Ditcham et al. [58] emphasised the need for an epithelial model that reflects as closely as possible the in vivo situation i.e. intact and unstimulated epithelium with no expression of extracellular matrix.

Fibronectin, a glycoprotein of high molecular weight, is produced by mammalian cells in tissue culture [170]. Faris et al. [72] tested the ability of 70 *E. coli* strains, isolated from bovine mastitis in Australia, Denmark, Norway and the USA, to bind fibronectin in vitro. Fifty-three strains (76%) interacted with iodinated fibronectin at a level exceeding 5% of the total radioactivity added. In a recent study of Lammers et al. [133] 11 *E. coli* mastitis strains bound fibronectin to approximately the same extent as a fibronectin binding *Staphylococcus* strain with impaired fibronectin-binding protein expression (20% of the wild type). None of the tested *E. coli* strains was able to bind fibronectin efficiently. It is known that in the normal mammary gland fibronectin is not present at the apical side of the epithelium. In the gland it is a normal

component of the basement membrane of the epithelium where it is present as an insoluble matrix. It is also present at the surface of myo-epithelial cells and fibroblasts. LPS increases cell surface-associated fibronectin in fibroblasts in vitro [127]. Frost et al. [78] showed that two hours after I.M. infection with *E. coli*, epithelial lesions occurred in the teat sinus. The basement membrane was exposed [26] but lesions never went beyond the basal membrane.

It is clear that *E. coli* is primary an extra-cellular pathogen that is involved in I.M. infection [97]. Most studies dealing with the capability of *E. coli*, isolated from clinical mastitis, to adhere to mammary epithelia, do not indicate any important role for adherence. The organisms generally do not invade mammary tissue but remain in the lumen of the teat canal and lactiferous sinus. According to our current knowledge it is clear that the pathogenicity of coliforms isolated from acute mastitis cases is not related to invasion and/or colonisation. The role of these virulence factors is less clear in cases of chronic coliform mastitis [66]. Binding of *E. coli* to fibronectin in vivo, remains questionable. The greater residual number of bacteria in the gland after milking when compared to the previous milking, indicates that infection has progressed and the faster the bacteria multiply, the higher their numbers will be, but colonisation is not necessarily involved [23]. In the field, it is generally accepted that frequent milkout of *E. coli* infected cows is helpful for the treatment of coliform mastitis. In a recent study, the effect of frequent milkout was tested on the outcome of experimentally induced *E. coli* mastitis in cows. However, under these conditions, frequent milkout did not appear to be an efficacious treatment for moderate to severe *E. coli* mastitis [137], indicating that the bacteria replicate very fast.

During clinical coliform mastitis it can not be excluded that temporary reversible attachment of the *E. coli* pathogen occurs

on the intact epithelium of the lactiferous sinus. This can not be defined as adhesion because this association is not followed by any specific receptor-mediated binding and colonisation as seen in the gut with enteropathogenic *E. coli* strains. Most studies show that adherence of *E. coli* pathogens to the epithelium does not exist or is at very least weak. However, such a reversible binding could eventually trigger secondary mediator release of cytokines that attract inflammatory cells. MAC-T cells (a non-differentiated bovine mammary epithelial cell line, [107]) secrete IL-1 in response to endotoxin stimulation and IL-1 is an important mediator for the release of the bovine IL-8 by MAC-T cells [20]. NO release from mammary epithelial cell lines was also measured in response to endotoxin [21].

2.3. Endotoxin as local mediator

Virulence of coliforms and the induction of an inflammatory reaction depend on their ability to replicate in the organism and to destroy cellular structures. During multiplication, destruction and lysis, they release an outer-membrane component, the endotoxin or LPS [68], a thermostable molecule that is a unique structural component of the cell wall of the Gram-negative pathogens. Two phenotypic forms of coliforms with slightly different LPS can be distinguished: smooth with complete LPS side-chains and rough with incomplete LPS side-chains [65, 154, 169]. Many studies have shown that the pathogenicity of *E. coli* in the mammary gland is associated with LPS. LPS is considered to be a key molecule in the pathogenesis of this disease [41]. This is based on the following facts: (1) LPS is released as a result of the lysis and growth of *E. coli* in the lactiferous sinus [68]. Once the bacteria reach the teat cistern they will grow very fast, 8–10 fold increase every two hours, releasing a lot of LPS. (2) I.M. administration of LPS induces the same local signs as observed

during *E. coli* mastitis [53, 176]. (3) Pre-treatment with polymyxin B (an LPS neutralising agent) or its simultaneous administration reduces and delays the febrile and leukopenic responses to I.M. LPS, and prevents the reduction in plasma zinc [262].

LPS is responsible for many pathophysiological signs observed during Gram-negative bacterial infections in ruminants such as fever, changes in the number of circulating leukocytes (leukopenia, leukocytosis), complement activation, activation of macrophages, increase of venular permeability, changes in plasma levels of metabolites, minerals, acute phase reactants and hormones, etc. LPS induces a defence response, especially in low doses. Intravenous administration of high doses of LPS may produce lethal shock [143, 144].

Although the pathogenic potential of purified bacterial LPS is well documented in general, the role of LPS in the pathogenesis of coliform mastitis has not been completely resolved. At the one hand there is no doubt that locally released LPS may play an important role in many of the local inflammatory signs during coliform mastitis [31]. However, it seems that its influence is short lasting and that it does not necessary cover all inflammatory changes. For example, the pre-treatment with polymyxin B only marginally influenced the degree of udder inflammation and it had no effect on the increase in SCC [262]. Twenty years ago Brooker et al. [26] already showed that two toxins were involved in experimentally induced *E. coli* mastitis: (1) the endotoxin, and (2) a heat labile cytotoxic compound that appears in a filtrate of *E. coli* culture. At that time Brooker et al. [26] already mentioned that the generally accepted concept that I.M. infusion of LPS produces a reaction indistinguishable from *E. coli* is not justified.

2.4. Endotoxin as systemic mediator

I.M. infusion of polymyxin B at 30 or 60 min after LPS only prevented the

increase in plasma lactate dehydrogenase (E.C.1.1.2.3) activity and moderated the decrease in plasma zinc concentration. This treatment failed to alter the clinicopathologic course of endotoxin-induced acute mastitis [262]. The possible absorption of LPS from the inflamed udder during coliform mastitis into the general circulation, and its significance at systemic level is not so clear and has been discussed in literature during the last decades (see e.g. [144]). LPS was detected in the plasma of 3 out of 12 cows during the first 10 h after I.M. *E. coli* challenge. Thereafter, only low LPS concentrations were detected in blood plasma. LPS concentrations of 55, 134 and 93 pg/mL were reached for a very short lasting period [64]. This short lasting event might be due to a fast clearance of LPS in plasma or to a limited release of LPS from the mammary gland into the circulation. The values corresponded with those obtained from naturally occurring gangrenous *E. coli* mastitis [89], in which 85 ± 68 pg LPS/mL was detected in blood plasma. The sporadic detection of limited LPS amounts in plasma contrasts with the massive LPS amounts that are released in the mammary gland during *E. coli* mastitis [246]. In the LPS model, the inflammatory reaction is induced by "bolus injection"; in the *E. coli* infection model, LPS is released during several hours [64, 93, 97, 124, 161, 167, 210, 244, 245]. Most LPS mastitis models described in literature are performed after peak lactation. In these "bolus" LPS mastitis studies, LPS seems to be effectively detoxified, and low levels of cytokines, produced during the inflammatory process, are the primary cause of the moderate systemic effects. The defence mechanism of the animal is not compromised at that moment. Milk production loss is less pronounced during LPS mastitis than during *E. coli* mastitis. Milk production losses in the uninflamed quarters are negligible in LPS mastitis, but substantial during *E. coli* mastitis (more pronounced systemic effects). Reticulorumen motility is only inhibited during *E. coli* mastitis

and changes in plasma haptoglobin are more pronounced during *E. coli* mastitis, although they occurred sooner during LPS mastitis [143, 144].

In a recent study, LPS mastitis was induced in high yielding Holstein cows, 5–12 weeks after parturition, by I.M. infusion of 500 µg into one quarter. The first milk sample was taken 4 h after I.M. LPS challenge and contained elevated LPS levels. These LPS levels gradually decreased over the entire course of the experimental period [258]. The data of I.M. increase of LPS are in agreement with those obtained by Ziv et al. [261] and Hartman et al. [92], who also found an increase of LPS in blood plasma. However, in the study of Yagi et al. [258] no LPS could be detected in serum obtained from the milk vein or the jugular vein, which is more compatible with current concepts. The discrepancies between the results obtained in the study of Ziv et al. [261] in the '70s and the recent study of Yagi et al. [258] are not inexplicable. Under field conditions, coliforms have been isolated in blood samples from diseased animals [253]. To our best knowledge, this has not been confirmed in experimentally induced coliform mastitis [190, 208] but this finding certainly needs more attention in the future. Especially the interplay between mediators from bacterial origin, such as endotoxins and non-LPS toxins, and the release of endogenous mediators (e.g. TNF-α) during coliform septicaemia in the cow should be focused.

2.5. Endotoxin clearance

In the study of Dosogne et al. [64] *E. coli* counts were about 2×10^5 colony forming units (cfu)/mL milk. The LPS release from *E. coli* during a 6 h growth period was 16.8 µg (inoculum size of 10^5 cfu/mL, [235]). The calculated amount of LPS in milk from infected quarters was approximately 10^5 times higher than the highest LPS value observed in blood. This suggests that a very large proportion of LPS is detoxified locally inside the inflamed mam-

mary gland during *E. coli* mastitis, probably by neutrophils. A significant detoxification system is acyloxyacyl hydrolase (AOAH), which is present in bovine neutrophil granules [154]. AOAH hydrolyses two acyl chains of the lipid A core of the endotoxin, resulting in a decreased toxicity whereas the immunogenic properties of LPS are largely maintained [169]. In some cows, a decreased blood neutrophil AOAH activity was observed immediately after calving [60]. The fact that LPS was only detected in the plasma of animals with the highest cfu counts in milk suggests that LPS absorption is probably to a high extent dependent on the number of bacteria within the mammary gland and can therefore also be prevented by bacterial growth inhibition [64]. In the case LPS is absorbed from the udder into the systemic circulation (outside the portal hepatic system), it will reach the lung circulation first and activate the pulmonary alveolar macrophages. In this way the effective inactivation capacity by the von Kupffer cells of the liver is evaded. The same phenomenon occurs when the ability of the liver to eliminate LPS fails due to liver disease [36]. The general phrase that animals suffering from coliform mastitis show signs of endotoxemia should be avoided. In fact they rather show signs of toxinemia caused by TNF-α. Endotoxemia is probably more the exception than the rule. Many animals however, may rather suffer from a mediator shock. The highest amounts of blood plasma TNF-α are found in severely diseased cows [98, 102]. There are various indications that LPS might not be released from the mammary gland into the general circulation during LPS mastitis (discussed in [102, 143, 144]).

3. HOW NEUTROPHILS SEQUESTER AND KILL *E. COLI*

The first line defence mechanism against *E. coli* invading the mammary gland is based on the resident phagocytes, mainly

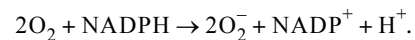
mononuclear cells. Most chemotactic factors are produced by the host but also some bacterial factors act as chemoattractant (e.g. the LPS of *E. coli*). However, unlike human neutrophils, bovine neutrophils lack the formyl-methionyl-leucyl-phenylalanine (fMLP) receptor [228]. After a complicated cascade of release of biological substances and activation of the endothelium, neutrophils migrate into the mammary gland and finally also appear in the fluid of the lactiferous sinus [31]. Several antimicrobial systems exist in the mammary gland [31, 119, 178]. However, it is the massive influx of neutrophils that will resolve the infection through sequestration and killing of the invading bacteria [31, 180].

Immunological recognition is mainly accomplished by specific antibodies (IgG₂ and IgM) which recognise the bacterium through Fab-regions and bind to PMN via Fc-receptors on the PMN plasma membrane [177]. There is a synergy between the Fc and C3b receptors activity and PMN reactive oxygen species (ROS) production [171]. Targowski and Niemialtowski [229] observed an increased Fc receptor expression and phagocytosis after in vitro migration of bovine PMN through membranes. This was confirmed in further studies by Berning et al. [12] and Worku et al. [257]. During migration of PMN into milk in response to *E. coli*, increased binding of C3b was observed [54]. Following adherence of *E. coli* to surface receptors on the PMN, phagocytosis and respiratory burst are triggered. The process of opsonisation, though not essential for phagocytosis, certainly promotes the uptake of *E. coli* by PMN, which is energy-dependent and requires the presence of a functional cytoskeleton [103]. The cytoskeleton machinery, sequentially activated following receptor stimulation, is thought to envelope the micro-organism in a "zipper mechanism" [86]. Thus, PMN are fully armed to confront invading *E. coli*. Generally, there are two different microbicidal mechanisms within

the PMNs: the oxidative and the non-oxidative system.

3.1. Oxidative mechanism of *E. coli* killing

Within seconds after the recognition of *E. coli*, PMN become activated and generate powerful reactive oxygen species, such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), halogen reactive species and hydroxyl radicals ($^{\circ}OH$) by partial reduction of O_2 [138]. This process is linked to the respiratory burst and associated with the PMN's cell membrane and granules (e.g. [8, 13, 196, 221]). Many different mechanisms of oxidation-reduction reactions are involved in intracellular bacterial destruction [194, 252]. The oxygen-dependent mechanism is the most prominent mechanism for *E. coli* destruction [222, 228]. Baldrige and Gerard [9] reported that an increase in O_2 consumption by PMN takes place when phagocytosis is triggered. This coincides with the increase of the hexose monophosphate shunt activity and glucose consumption. The respiratory burst is based on the activation of a membrane-bound oxidase (NADPH-oxidase), which catalyses the following reaction [7, 198, 199]:



The oxygen molecule is central in this phenomenon [3, 7, 8]. Its importance was highlighted by inefficient PMN bactericidal activity under anaerobic conditions [150]. For each molecule of O_2 consumed 4 O_2^- ions are generated, resulting in an intravascular O_2^- release of about 4 mol/L [194].

Another O_2 -dependent microbicidal system uses halides and the enzyme myeloperoxidase (MPO, E.C. 1.11.1.7). MPO and/or eosinophil peroxidase catalyses the reaction between H_2O_2 and Cl^- , I^- , or Br^- , with formation of the highly toxic hypohalides and the subsequent oxidation of amines [7, 198, 199]. MPO is localised in the azurophilic granules of the PMN and

reaches the micro-organisms when the granule content is released into the phagolysosome [44], providing a destructive environment for phagocytosed *E. coli*. Among these ROS are also a variety of oxidised halogens, including hypohalite ions [4, 196, 233] and a variety of chloramines [232].

Root and Cohen [196] have suggested several possible direct sites of action for ROS, related to their microbicidal activity. These include: (1) unsaturated carbon bonds that may lead to toxic lipid peroxidation; (2) sulphhydryl groups leading to the destruction of sulphhydryl containing enzymes; (3) amino group and possible peptide bond breakage; and (4) nucleic acids. In vitro studies with O_2^- generating systems such as xanthine oxidase [197] suggest that O_2^- are far more toxic for *E. coli* if they operate in the MPO- H_2O_2 -halide system. This leads to the production of powerful halogenated oxidising agents such as ClO^- ions, which have a bactericidal effect by halogenating the bacterial proteins. The halogenated oxidising agents are produced inside the phagosome when PMN ingest bacteria [44]. It is also generally agreed that ROS boost oxygen-independent microbicidal activity [194].

Nitrogen oxide also acts as an antimicrobial agent in the presence of ROS [130]. It synergises bactericidal activity of H_2O_2 against *E. coli* by damaging the *E. coli* DNA and declining bacterial glutathione contents [182]. In human, NO release is a part of the integrated response of PMN stimulated by phorbol-myristate-acetate (PMA, an ester that activates the NADPH-oxidase in the cell membrane). NO together with O_2^- produces other oxidants, such as peroxynitrite (ONOO⁻; [40]).

3.2. Non-oxidative mechanisms of *E. coli* killing

Non-oxidative mechanisms of bacterial killing by bovine PMN are less well defined, but they heavily rely on the ability

of PMN's enzymes and proteins to alter the membrane permeability of *E. coli* [191, 207]. Studies have demonstrated that a cationic bactericidal/permeability-increasing protein (BPI) present in both rabbit and human PMN is the basic non-oxidising bactericidal agent of these PMN toward several strains of *E. coli* bacteria [70, 251]. Bovine lactoferrin has also considerable inhibitory effect on *E. coli* growth [15]. Moreover, extracellular matrix proteins such as laminin promote neutrophil motility and attachment to *E. coli*, enhance phagocytosis activity of neutrophils, substantially prime neutrophil ROS production and stimulate lysozyme release into the phagosome [188, 230]. Lysozyme has the capacity to neutralise and strongly interact with *E. coli* LPS [173]. Laminin also enhances the neutrophil bactericidal activity against *E. coli*, the effect being larger with increasing IL-1 β and IL-8 concentrations. Also fibronectine enhances the bactericidal activity, synergised by TNF- α [213]. The cytosin-dependent cell-mediated bactericidal activity of PMN is also crucial for killing of *E. coli*, especially during the early phase of *E. coli* mastitis.

Another non-oxidative antimicrobial agent is PMN elastase. Elastase degrades the outer membrane protein A of *E. coli*, which is located on the surface of the bacteria [11, 250]. Mice lacking PMN elastase and other granule-proteases are more sensitive to *E. coli* infection [10] and other bacterial and fungal infections [194]. Activation of proteases provokes the influx of a large amount of ROS into the phagolysosome, resulting in a strong anionic charge. This leads to the release of cationic granule proteins, facilitating the bactericidal process. The anionic charge is compensated by a K^+ flux from cytosol into the phagosome [194]. The large amount of K^+ in the phagosome is a crucial factor for the non-oxidative bactericidal activity by its effect on proteases and pH homeostasis [111, 153, 194].

Extracellular ROS

Intracellular ROS

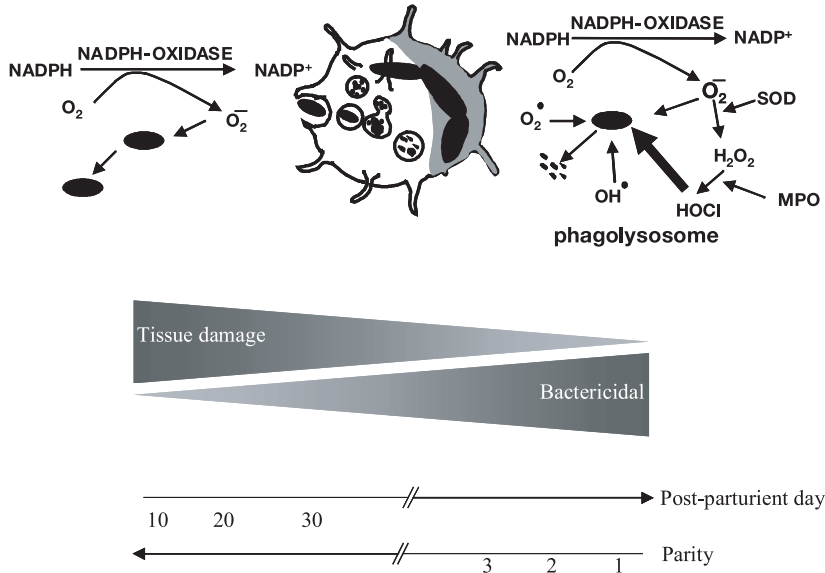


Figure 1. Diagram illustrating the hypothesis that during severe *E. coli* mastitis extracellular production of reactive oxygen species (ROS) by polymorphonuclear leukocytes may be impaired and contribute to the tissue damage. Normally, ROS for bactericidal activity is produced intracellularly for effective destruction of bacteria with a minimum of tissue damage. This hypothesis is based on the study of the kinetics of chemiluminescence: (1) there is a difference between extracellular and intracellular ROS production of both blood and milk PMN during different stages of lactation, and (2) in blood and milk, extracellular ROS production by PMN is more pronounced in older cows. This may lead to an impaired bactericidal capacity of resident milk PMN and boost tissue damage in older cows [158, 161].

The osmotic forces in the vacuole must be considerably high to enable the shrinkage of ingested bacteria (about 50% of their original volume). Osmotic forces also activate PMN granule proteases [194]. High extracellular osmolarity decreases the killing capacity of PMN against *E. coli* [90]. Many other factors such as calcium ions [139], granulocyte colony-stimulating factor (G-CSF) [134], growth hormone and insulin-like growth factor (IGF)-I [108] enhance PMN killing capacity against *E. coli*.

In short, successful intracellular killing of *E. coli* depends on ROS production. Bovine blood and milk PMN ROS production can be measured by chemiluminescence (CL; see [157–159]), which is associated with the bactericidal capacity of PMN [3, 84, 187, 249]. Studies on CL kinetics in PMN revealed extra- and intracellular events during ROS production [25, 49, 69, 73]. Figure 1 provides a scheme of a hypothesis showing extra- and intracellular ROS production of PMN in relation to possible tissue damage or effective killing

depending on some physiological parameters (age and lactation stage). This hypothesis is based on findings from studies performed on healthy and experimentally infected cows [158, 161]. A low ROS production of resident milk PMN is observed during early lactation. This is more pronounced in older cows where most of ROS is produced extracellularly. This may compromise milk PMN bactericidal capacity and boost tissue damage. Our preliminary findings indicate that during *E. coli* mastitis both extracellular and intracellular ROS substantially increase from 6 to 48 h post-infection. The studies may suggest that in moderate responders, ROS is mainly produced intracellularly whereas in severe responders it is extracellular ROS.

The above explanation is a general basic knowledge about killing of *E. coli* and is not the whole explanation about PMN-*E. coli* interactions during and after phagocytosis. There are interspecies differences as well as inter-subspecies differences on oxidative and non-oxidative PMN bactericidal mechanisms [225, 228].

4. THE LPS, TLR 4, TNF- α AND NO CONNECTION

Peptidoglycan monomers and LPS are unique to all Gram-negative cell walls. They are elements of the pathogen associated molecular patterns (PAMP) and bind to pattern-recognition receptors (PRR) that are present on a variety of defence cells of the body causing them to synthesise and release a variety of cytokines. The LPS interacts with LPS-binding protein and CD14, which in turn promotes the ability of the Toll-like receptor, TLR4 (a PRR) on macrophages to respond to the LPS with the release of various pro-inflammatory cytokines. These inflammatory mediators bind on target cells via specific receptors and initiate inflammation. Toll-like receptor proteins, homologues of the *Drosophila* protein Toll, have been detected on the sur-

face of mammalian cells [14]. They are important in the responses of macrophages to bacterial, viral, and fungal antigens. TLR2 and TLR4 have candidate genes for resistance to several diseases as they recognise broad classes of PAMPs, such as peptidoglycans and LPS [254]. Very little TLR4 is present on the surface of human neutrophils. TLR2 is important for response to Gram-positive bacteria, bacterial peptides and zymosan and is more abundant on the neutrophil. TLR4 activates the inflammatory gene expression through nuclear factor κ B (NF κ B) [156]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) dramatically up-regulate TLR2 and CD14 expression [129]. Recent in vitro studies have shown that mammary epithelial cells may participate in the immunoregulation during mastitis via cytokine production. A MAC-T cell line was utilised to investigate the expression of interleukin-1 mRNA and its subsequent secretion after *E. coli* LPS stimulation and it showed that these cells secrete IL-1 in response to LPS. IL-1 also appeared to be an important mediator for the release of the IL-8 [20, 107].

During coliform and LPS mastitis in ruminants, IL-1, IL-6 and IL-8 [210], platelet-activating factor [184], prostaglandins [5, 27, 186], activated complement C5 (C5a; [211]) and NO [16, 19], are released locally. In milk, an increase in TNF- α , a cytokine of particular interest in the pathogenesis of *E. coli* mastitis, is observed [98, 102, 211, 219]. The increase in TNF- α occurs faster during LPS mastitis than during *E. coli* mastitis but it peaks higher during I.M. *E. coli* infection. TNF- α remains unchanged in non-challenged quarters [16, 102]. Absorption of this cytokine into the systemic circulation is highest during *E. coli* mastitis. Plasma levels are much lower than in the milk and correspond with maximal pyrexia [102]. Several studies show that the amount of TNF- α released in

milk correlates with the severity of the disease [16, 98, 102, 210, 219].

Many of the biological activities of LPS are mediated by TNF- α . LPS and cytokines stimulate the synthesis of NO, which is a vasodilator. NO is a diatomic free radical. It is lipid soluble and diffuses easily through cell membrane. It is short lived and usually degrades or reacts within a few seconds. The natural form is a gas that reacts with a variety of molecules and mediates a large spectrum of biological effects. 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 with substances of microbial origin such as LPS and TNF- α . Bovine monocytes produce NO in response to LPS [1, 114, 223]. NO is produced by macrophages in response to invading pathogens and acts as an antimicrobial agent. It is also cytotoxic and can induce apoptosis if produced in excessive amounts [6, 166].

Several studies on ruminants have shown a relationship between LPS, TNF- α and production of NO. The increase in TNF- α is followed by a delayed increase in NO_x (NO₂ + NO₃) [16, 19]. The NO_x production lasts longer in *E. coli* challenged quarters. A causal relationship between TNF- α and NO_x production was observed in studies in which *E. coli* LPS was injected intravenously [115]. It seems that severe cases of coliform mastitis are accompanied by the highest increase in blood plasma concentrations of both TNF- α and NO_x [16, 98]. The increase in NO_x and TNF- α during *E. coli* mastitis can't be inhibited by antibiotic treatment [16] indicating that its release is LPS mediated. Initially called endothelium derived factor (EDRF), NO causes vasodilation. It is released after the fever peak, that is, at the moment of the delayed phase of vasodilation occurring during LPS induced mastitis in lactating goats and cows [53]. It is not impossible that NO is involved in this delayed phase of hyperemia.

5. SEVERITY CLASSIFICATION OF COLIFORM MASTITIS

5.1. Clinical severity classification

Mastitis is a multifactorial disease and its risk factors can be classified into three groups of disease determinants: the pathogen, the environment and the host [234]. To combat mastitis, sophisticated models, such as the five-point-plan, have been adopted worldwide and have resulted in significant reduction of subclinical mastitis. However, despite classical efforts for control and prevention, the susceptibility of cows to coliform infection is increasing [131, 205, 224]. Moreover, from both epidemiological and experimental studies it can be concluded that severity of coliform mastitis is of much more concern than its incidence. Many high producing cows are extremely sensitive for *E. coli* infections around parturition and during early lactation. Clinical mastitis with grave systemic symptoms occurs at parturition and during early lactation, whereas during mid and late lactation signs are mild to moderate [97]. Estimation of the sensitivity of a cow for I.M. *E. coli* infection refers to its clinical expression. In the field, these signs are ranked from mild, to moderate and severe, and resistant animals are insensitive. Severe clinical mastitis is characterised by sudden onset with grave systemic signs: diarrhoea, inhibition of rumen motility, paralysis, hypothermia, very high pulse rate and hypersalivation. It is well recognised that the severity of coliform mastitis determines the outcome of the disease as well as the degree of production loss. Therefore, accurate evaluation of the severity in the field is needed to determine appropriate treatment and management measures. However, there is no simple on-farm evaluation system to characterise disease severity that relates accurately to the outcome of the disease. For example, bacteraemia was never observed in field cases by Powers et al. [189] whereas Cebra et al. [42] reported bacteraemia in 1/3 of the cows that were

suffering from severe clinical mastitis. Wenz et al. [253] isolated bacteria from the blood of some cows that were scored as “mild reaction”.

5.2. Scoring severity under field conditions

Despite appreciable efforts to introduce clear definitions and scoring for mild, moderate and severe based on systemic signs [253], there is still some confusion in the field. In the field, oversimplifying the term mastitis may lead to wrong interpretation of the terms mild, moderate and severe. The terms mild and moderate are sometimes (mis)used to describe the local symptoms; e.g. mild is often used to describe appearance of clots or flakes in the milk with little or no signs of swelling of the mammary gland. In the field this is usually associated with infections by minor pathogens [128] such as coagulase negative *Staphylococci* (CNS) and *Corynebacterium bovis*. Moderate and severe are often used to refer to visibly abnormal milk accompanied by swelling in the affected mammary quarter. Generally, a correlation exists between the local clinical signs and the severity of systemic symptoms. However, discrepancies have been noticed, especially during the onset of severe *E. coli* mastitis around parturition where slow progressing and less visible local swelling is clinically misleading because this mild local reaction will be rapidly succeeded by severe symptoms of shock [93, 95, 245]. The dilemma for farmers and veterinarians is that the lack of inflammation and clinical symptoms in the udder often makes rapid diagnosis difficult. By the time a diagnosis can be made, it is too late to save the animal. On the other hand, during mid and late lactation the local symptoms of the disease may be so short lived and mild, that it is difficult for a milker to recognise this self-curing condition [97]. It is clear that under field conditions there is a need for exact ranking of severity through application of clear and generally accepted definitions. Mis-

classification through misuse or use of less specific definitions may lead to dubious conclusions. Agreement on definitions would also be in favour of comparing field and experimental studies. The terms mild, moderate and severe that are used by Wenz et al. [253] under field conditions are only based on systemic parameters such as rectal temperature, hydration status, rumen contractility and attitude of the animal. The principle to relate severity to systemic factors is in agreement with the severity classification used under experimental conditions (e.g. [31, 59, 93, 98, 124–126, 210, 242, 245]).

5.3. Experimental severity classification

Studies of Hill et al. [95] with *E. coli* mastitis demonstrated that those animals which displayed a pronounced local acute inflammatory reaction showed fewer systemic symptoms than those that did not. Occasionally, and only during early lactation, bacterial multiplication in the mammary gland induces only a very slow acute phase response. The progress made by the invading pathogen is so fast, or the acute inflammatory reaction by the host so slow, that the cows show very grave systemic signs because of the lack in local control of the pathogen. This clinical type of mastitis was defined by Schalm et al. [203] as a peracute mastitis and corresponds with the severe responders in the study of Vandeputte-Van Messom et al. [245]. Over the years, several attempts have been made to estimate severity under experimental conditions. It was suggested by Heyneman et al. [93] to distinguish between moderate and severe responders during early lactation. Although this method has been shown to be useful at many occasions, one of the problems is that in some cases the severity response is very gradual, especially if the whole lactation period is considered. In this case severity classification as proposed by Hirvonen et al. [98] into mild, moderate and severe responders is

preferred. Parameters that can be used to estimate severity under experimental conditions can be divided into local and systemic disease parameters.

5.4. Local disease parameters

Fast and uncontrolled growth of I.M. *E. coli* with large numbers of bacteria in the udder cistern can occur. This is especially related with the period of parturition and early lactation [26, 41, 59, 79, 93, 97, 125, 126, 145, 167, 210, 241, 244, 245] and this explosive growth of I.M. *E. coli* is less apparent during established lactation [16, 97, 98, 102, 195, 210]. Also older cows exhibit higher cfu during coliform mastitis [190, 241]. In these studies the number of bacteria that grow in the gland cistern (estimated as cfu) is linked to the severity of coliform mastitis. Cows in the severe group have significantly more bacteria in the gland cistern than cows in the moderate groups. Under experimental conditions this can be quantified by measuring the area under the curve (AUC) of bacterial growth. AUC has been successfully used in many experimental studies [59, 125, 126, 145, 241, 242]. LPS may increase dramatically at sites of bacterial replication especially during fast and uncontrolled growth of *E. coli* in the udder cistern. Although methods have been developed to measure the LPS content of milk [92, 258, 261] no attempt has been made to estimate severity on basis of the endotoxin dosage in the udder cistern. A good severity indicator may be TNF- α in milk, because of its typical release during acute *E. coli* mastitis and not during *S. aureus* mastitis [195]. Generally, most studies indicate that the amount of TNF- α that is released in milk is closely associated with peracute signs of coliform mastitis [98, 210, 211, 219] but practically no difference in local TNF- α response was found between *E. coli* and LPS mastitis [16, 102]. Exact quantification of the expression of mRNA for TNF- α in cells derived from infected mammary glands through reverse

transcription polymerase chain reaction might be helpful in the future.

During a balanced I.M. inflammatory reaction, bacterial growth will be under control and only a few bacterial cfu will develop in the udder cistern of the cow. Low levels of LPS will be released leading to moderate and specific cytokine pattern production by the macrophages which will promote optimal body innate defence mechanisms and minimal systemic signs such as fever and leukopenia. Animals reacting in this way were called moderate responders [245]. They typically show a steep increase in SCC, due to fast migrating neutrophils that inhibit bacterial growth effectively. There is experimental evidence that these neutrophils might also destroy the endotoxin in the mammary gland [60]. Slow neutrophil influx is observed in severe responders (see Fig. 2, [245]) and they might start releasing their proteases and ROS in the mammary tissue before reaching the pathogens. For some reason, neutrophils are stacked in the tissue. However, this phenomenon could not be confirmed by Shuster et al. [210]. As *E. coli* population can double every 20 min, a delay of just 20 min in neutrophil arrival in lacteal secretions has a significant effect. A 1-h delay in neutrophil recruitment into the mammary gland could result in an 8-fold larger number of *E. coli* to kill and that much more endotoxin to detoxify [119].

In a recent study, in vitro growth of *E. coli* in milk was studied to explore the hypothesis that milk from individual cows differs in its growth medium properties for *E. coli*, and whether possible variation could be related to specific milk constituents. The growth differed substantially between individual cows within an incubation period comparable with the early phase of I.M. infection, suggesting that the growth medium properties of milk could be of importance in the pathogenesis of *E. coli* mastitis and subsequent outcome of disease [123].

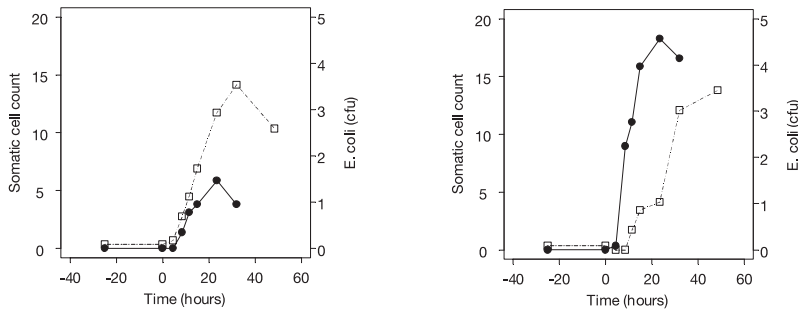


Figure 2. Multiplication rate of *E. coli* ($\times 10^6$ /mL; \bullet) in inoculated mammary glands and pattern of leukocyte influx ($\times 10^5$ /mL; \square) into milk in (a) periparturient moderate responders compared with (b) those in severe responders during experimentally induced *E. coli* mastitis. (Figure taken from the original paper [245], with permission.)

5.5. Systemic disease parameters

During experimental studies, differences in the fever response were observed that were related to severity [102, 210, 245]. Under field conditions, rectal temperature is one of the disease parameters used to classify severity [253]. Careful interpretation of local TNF- α release is needed because it is known that at low doses, TNF- α has a protective role during invasive infections so that increase in TNF- α may be a normal response to bacterial invasion. For example, although practically no difference in local TNF- α response was found between the *E. coli* and LPS mastitis model, the increase in plasma TNF- α peaked highest after I.M. *E. coli* infection [16, 102]. TNF- α might have been absorbed from the *E. coli* infected udder and/or secreted from activated mononuclear cells such as monocytes and macrophages in the body [102]. The subsequent grave systemic signs may be due to abnormal regulation of a normal TNF- α -mediated response. The decreased rumen motility that has been observed during coliform mastitis under both experimental [102, 143, 245] and field conditions [253] may be related to the increase of TNF- α in the blood plasma. The pivotal role of TNF- α in this host reaction is suggested by four lines of evidence: (1) the

decrease in rumen motility is not observed or at least very mild during experimentally induced endotoxin mastitis [102, 143]; (2) TNF- α is one of the earliest mediators secreted by the host in response to I.M. *E. coli* infection [195]; (3) TNF- α in blood plasma is higher after I.M. *E. coli* infection than during endotoxin mastitis [102] and (4) administration of TNF- α to goats elicits inhibition of rumen contractions [240]. One of the criteria used by Wenz et al. [253] under field conditions is rumen contractility. Clinical mastitis induces a non-specific acute phase response (APR) and the initiation of APR is also thought to be caused by the production of TNF- α [102]. Changes in plasma haptoglobin occurred sooner in endotoxin mastitis than during *E. coli* mastitis. However, the total response was practically similar in both models [102]. These observations are in accordance with the findings in the study of Hirvonen et al. [98] where serum haptoglobin did not correlate with the severity of the disease. Serum amyloid-A (SAA, another acute phase protein) rose gradually in the severely affected cows, and significant differences were found between severely vs. moderately or mildly affected cows at day 4. Serum TNF- α concentrations only increased in the severely affected cows. During severe clinical coliform mastitis a decrease in total milk yield occurs. It

is not surprising that milk production was also reduced in uninfected quarters during coliform mastitis [59, 93, 99, 100, 102, 152, 167, 210, 245]. The magnitude of this reduction is the result of: (1) a local effect induced by the invading pathogen and in which inhibition of milk synthesis and tissue damage is involved, and (2) the degree of systemic disease; e.g. decrease in gastro-intestinal motility and subsequent reduction in availability of milk precursors [30, 93, 143, 144]. The loss in milk production in the uninfected contralateral quarters on day 2 after infection, has been used as a criterion to classify cows as moderate or severe responders to experimental *E. coli* mastitis after parturition [93, 245]. Milk production losses in uninflamed quarters are negligible in endotoxin mastitis but substantial during *E. coli* mastitis, probably due to pronounced systemic effects [102]. Harmful effects are first seen systemically, due to excessive release of cytokines. The animals are depressed, have ruminal stasis and diarrhoea, cardiovascular collapse resulting in shock and multiple system organ failures which in its most extreme form may lead to death [97]. The infected gland shows extensive tissue destruction [97] and decrease in milk secretion [152]. In the uninfected glands milk production is depressed for several days [93, 245].

5.6. Is severity predictable?

Severity classification and prediction under experimental conditions have received a lot of attention over the past 15 years, especially in connection with treatment regimens where sham treated animals are compared with specific drug treated animals (e.g. [244]). Nowadays we know that several host determinants influence the sensitivity for coliform mastitis, i.e. stage of lactation, number and function of peripheral PMN, SCC, age of the host, genetic resistance, nutritional status, other periparturient diseases, and the vaccination status [180]. The variability in severity to *E. coli*

Table I. Correlations between the phorbol myristate acetate (PMA) and zymosan induced respiratory burst of bovine neutrophils expressed as O_2^- generation/cell (nmoles O_2^- /cell) and O_2^- O_2^- generation capacity (nmol O_2^- × neutrophil number) prior to the inoculation of *Escherichia coli* and the severity of mastitis¹. (Table taken from the original paper [93], with permission.)

Characteristic of blood neutrophil	Decrease in production of	
	Milk	α -Lactalbumin
Neutrophil number	-0.58*	-0.48*
PMA - induced		
O_2^- generation / cell	-0.16	-0.22
H_2O_2 generation / cell	-0.18	-0.26
Zymosan - induced		
O_2^- generation / cell	-0.80**	-0.67*
PMA - induced		
O_2^- generation capacity	-0.77*	-0.72**
Zymosan - induced		
O_2^- generation capacity	-0.90***	-0.75**

¹ Severity of mastitis was estimated as the percentage decrease in milk and α -lactalbumin production on day +1 and +2 after inoculation of *E. coli* into the udder.

² Correlation coefficients with probabilities * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$ for 14 cows.

mastitis during early lactation can be so large that extremely high numbers of animals per group have to be included in the study. Several prediction markers have been tested in relation to severity of *E. coli* mastitis under experimental conditions, among them: total number of circulatory leukocytes and PMN (see Tab. I; [93]), the oxidative burst of PMN (see Tab. I), PMN chemotaxis and neutrophil alkaline phosphatase (NAP, E.C. 3.1.3.1). The number of circulating peripheral leukocytes just before bacterial inoculation also predicted the severity estimated by the AUC of bacterial counts and residual milk production (reviewed by [33]). Variation in severity could not be explained by pre-infection

NAP levels. Of the pre-infection tests for leukocyte function, chemotaxis of PMN isolated from the peripheral blood at day -2 and -1, and immediately prior to infection best predicted the outcome of the induced *E. coli* mastitis [125]. Predictions were most significant within 24 h before challenge (see Tab. I). Predictive significance was rapidly lost when samples were taken earlier (from day -2 to -6, [125]). It was concluded that PMN functions could not be used to predict severity over longer periods, probably due to the short half-life of these cells. On the other hand, very short significant predictions can be made (within two days) which may be useful for some experimental purposes.

5.7. Modulation of severity

Activation of innate immune responses by LPS can be inhibited by various soluble molecules that bind LPS and neutralise its activity. However, LPS is not a classic virulence factor but it contributes to the pathogenic potential of Gram-negative bacteria by activation of host defensive systems which ultimately results in both, clinical signs and tissue damage. The pro-inflammatory nature of LPS plays a critical role in the successful elimination of the infecting pathogen [75]. Furthermore, in many LPS-models the effect on several biological systems seems to be dependent on the dose [151]. Therefore, modulation rather than complete inhibition of the action of the LPS molecule is preferable.

Modulation of inflammation and tissue damage in coliform mastitis can be obtained through antibodies directed against the cell wall [65] or through LPS scavengers such as polymyxin B [262]. Nowadays, the tools, techniques and products to study and modulate host-pathogen interactions are made available by advances in molecular biology. The cloning and expression of proteins such as recombinant bovine soluble (rbos) CD14, may provide ways of minimising damaging effects of LPS during acute coliform mastitis. CD14 binds and neutralises

LPS and causes local recruitment of neutrophil after binding of CD14-LPS complexes to mammary epithelial cells [180].

6. LIFESPAN OF PMN: FROM MATURATION TO SENESCENCE

6.1. Proliferation, differentiation and maturation of PMN

During postnatal life hematopoiesis is restricted to the bone marrow, whereas the liver and the spleen are inactive but retain their hematopoietic potential. All blood and immune cells originate from a self-renewing small population of pluripotent stem cells (CFU-S) that can replicate themselves, or can become committed to a particular development pathway. PMN are formed through the multi-step process of granulopoiesis, from the colony-forming unit of granulocytes (CFU-G) through myeloblasts, promyelocytes, myelocytes, metamyelocytes, and band cells. Precursor cells undergo striking morphologic, biochemical and functional changes during granulocytic maturation. These changes are associated with significant changes in cell size and nuclear shape, and with the development of stage-specific proteins essential for phagocytosis and bacterial killing. Glasser and Fiederlein [83] provided knowledge about the acquisition sequence of functional properties during human granulopoiesis. Data were frequently extrapolated to the bovine species, although species variation in neutrophil function has been clearly demonstrated [225]. Because alterations in the granulopoiesis may underlie the significant changes in the function, number and maturity of circulating PMN observed during coliform mastitis, attention was drawn to the niche of these cells in the bovine. A prerequisite for studies on the maturity of cells is a reliable separation method, in order to obtain enriched cell fractions of different maturation stages. Recently, Van Merris et al. [238] developed

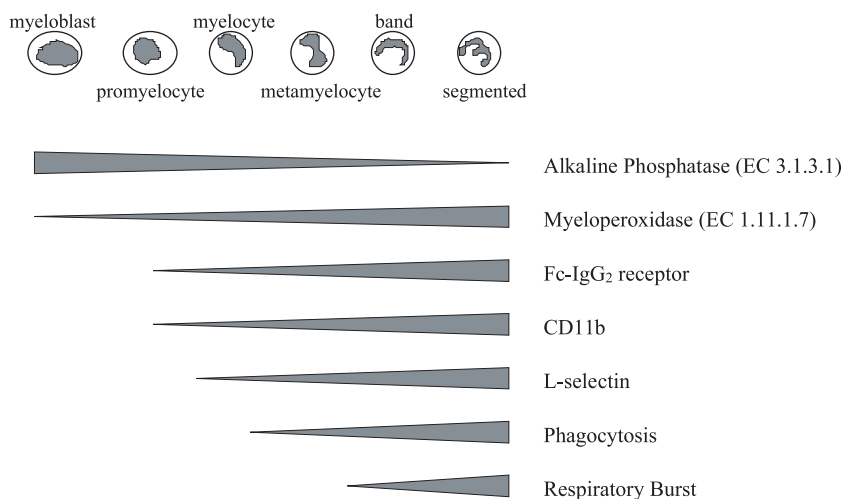


Figure 3. Stepwise acquisition of functional properties in bovine bone marrow cells during granulopoiesis. (Figure adapted from the original paper [239], with permission.)

a discontinuous Percoll gradient on which centrifugation of bone marrow resulted in three maturation-dependent cellular fractions. In a subsequent study, the functional maturation sequence during bovine granulopoiesis was established and proposed to be the following: myeloperoxidase activity – Fc-IgG₂ receptor expression – CD11b expression – L-selectin – phagocytosis – respiratory burst activity (Fig. 3) [239]. In analogy to human hematopoiesis [83], the acquisition of various biochemical and functional properties in bovine PMN was found to take place in different stages of maturation in the bone marrow. The efficiency of PMN against invasion of the mammary gland was previously shown to be highly dependent on the rate of diapedesis into the infection site [95] and on the ability of these PMN to generate ROS [93]. Although the immature cells expressed already the membrane adhesion molecule CD11b, they were not capable to rapidly migrate to the infected mammary gland, and ingest and kill efficiently the invading bacteria. The limited chemotactic and phagocytic ability in immature cells could be due to membrane rigidity with poor cel-

lular deformability, in association with a large nucleus [141]. The incapacity to produce ROS was attributed to the absence of membrane-bound NADPH-oxidase activity, as myeloperoxidase was already present in the rare azurophilic granules at the promyelocytic stage [239]. These findings were in general agreement with previous observations of Silva et al. [212]. Thus, when maturation is impaired due to an increased proliferation rate, a higher number of immature cells will appear in the circulation. These findings support the hypothesis postulated by Guidry and Paape [88], namely that the presence of myelocytes, metamyelocytes and band cells (left-shift) observed during acute coliform mastitis in early lactation may compromise the cows' resistance by supplying more cells that are morphologically immature and functionally inadequate.

Alkaline phosphatase is present in substantial amounts in the cytoplasm of PMN [248]. In human, NAP activity fluctuates in a variety of physiological (pregnancy, stress) and pathological conditions (mononucleosis, myeloproliferative disorders) (summarised in [174]). Conflicting data

resulted in an unclear and still controversial relationship between cell maturity and NAP activity. Some authors observed the NAP activity mostly in functionally and chronologically older neutrophils [17, 118]; others assessed highest NAP in immature cells [120, 256]. The latter indicated that elevation of NAP activity of circulating PMN may coincide with the increased release of precursor cells from the bone marrow. A significant increase in NAP activity was observed during experimental *E. coli* mastitis [94]. Van Werven et al. [242] demonstrated that the increase was a result of increased enzyme activity per neutrophil, rather than an increase of the number of neutrophils exhibiting NAP activity. The NAP-inducing factor was shown to be identical to G-CSF [200], the growth factor thus being responsible for starting the synthesis of the enzyme in the neutrophil. Therefore, the enhanced NAP activity after onset of mastitis was believed to be induced by G-CSF, reflecting an increased proliferation and differentiation of bone marrow granulocytes [94].

It has been postulated that cells leave the bone marrow more-or-less by a pipeline mechanism, the older cells being released first [149]. The mechanisms that control the release of mature PMN from the bone marrow into the circulating pool are poorly understood. Anatomical localisation of the hematopoietic cells close to vascular spaces, and the development of transient holes in the venous sinuses to allow egress of marrow cells have been suggested as mechanism. Also the developmental changes in the nucleus and the cytoplasm of the maturing cells that increase their motility, deformability, and chemotactic ability could attribute to the release of terminally differentiated PMN from the hematopoietic compartment into the efferent vascular space [140]. Van Eeden et al. [236] assigned a possible role to L-selectin in the release of PMN from the bone marrow in human, as L-selectin was highly expressed on mature PMN in the post-mitotic pool in the bone marrow [231].

With active bone marrow release there was an increase in the number of PMN expressing high levels of L-selectin in the circulation [236]. However, levels of L-selectin on circulating PMN never reached levels expressed in the bone marrow, suggesting that L-selectin is shed as PMN enter the circulation. Recently, Monfardini et al. [168] provided evidence for a maturation-dependent increase in L-selectin expression during granulopoiesis in the bovine. The findings were consistent with previous reports in human that a high number of circulating bovine PMN expresses CD62L but at a lower level than mature bone marrow granulocytes. PMN can shed L-selectin from their surface as a part of a de-adhesive event during recruitment into a site of inflammation [46]. The loss of L-selectin when crossing the endothelial barrier in the bone marrow upon release into the blood stream could be a similar process. Alternatively, high levels of L-selectin on mature PMN might keep them attached to the endothelium of the bone marrow sinusoids, and stimuli for release may activate the proteolytic cleavage of L-selectin [236].

6.2. Regulation of the granulopoiesis

The process of granulopoiesis is strictly controlled by regulatory growth factors, comprising cytokines and colony-stimulating factors (CSF), to ensure the maintenance of a steady state. The hematopoietic growth factors have pleiotropic effects on proliferation, differentiation and functional activation of precursor cells [164]. They interact at various levels of the differentiation cascade, from unipotent stem cell to mature PMN. The biologic effects of the growth factors are mediated through specific receptors on the surfaces of target progenitor cells.

Hoeben et al. [101] investigated the effect of hormones and metabolites, of which the concentration changes substantially around parturition in the bovine. Using an optimised cell culture assay for the bovine [237], it was demonstrated that

β -hydroxybutyric acid (BHBA) and acetoacetic acid (AcAc) induced significant inhibitory effects on the proliferation of hematopoietic cells at concentrations observed after parturition. In a study of Van Werven et al. [243] a high pre-infection BHBA concentration was strongly negatively correlated with the pre-infection number of circulating PMN. Bovine pregnancy-associated glycoprotein (bPAG) also reduced the proliferative activity of bovine progenitor cells. Hydrocortisone-21-acetate (HCAc) inhibited the hematopoietic activity of bovine bone marrow at concentrations of 10 to 100 ng/mL [101]. At calving, a short lasting cortisol peak of approximately 20 ng/mL is observed, whereas during mastitis increases up to 100 ng/mL are possible [215]. The inhibitory effects of BHBA, AcAc, bPAG, and HCAc may be involved in the changes in the number, differentiation and maturity of circulating PMN after calving [101], as the circulating pool is largely depending on the proliferative capacity of the bone marrow.

The action of growth factors, inflammatory mediators, metabolites and hormones on the proliferation, differentiation, and function of PMN is provided in Figure 4.

6.3. Ageing of PMN

After having exerted their role in immune function PMN die by senescence [255]. Aged PMN undergo spontaneous apoptosis in the absence of pro-inflammatory agents prior to their removal by macrophages [201], thus preventing the release of their cytotoxic content. One of the dominant theories of senescence, the soma theory [122], suggested that replicative cells accumulate genetic damage, resulting in functional decline. Arguing against this proposal in the case of ageing of PMN, were the findings of Chatta et al. [45] and Born et al. [18] indicating that the proliferative and differentiation capacity of hematopoietic progenitor cells was largely unaffected by ageing in humans. However, the ability of progenitors to proliferate and differenti-

ate in response to hematopoietic factors does not necessarily guarantee that the resulting PMN are competent [35]. Inflammation and infection do increase the rate of PMN production, shortening the maturation time and leading to the release of immature immune cells in the circulation. Schalm and Lasmanis [204] reported that during mastitis the myeloid/erythroid ratio in bovine bone marrow increased above 1.0, reflecting an intensified granulopoiesis, probably to ensure replenishment of the PMN reserve and the return to homeostasis.

7. HOST FACTORS THAT INFLUENCE SEVERITY

7.1. Stage of lactation

Defense of the bovine mammary gland is continually challenged by environmental exposure to bacteria, and many factors affect the outcome of this challenge. On average 1% of mammary quarters in the dairy cow population will have I.M. infection with Gram-negative bacteria compared to an infection rate of 35–50% for Gram-positive bacteria [68]. The rate of clinical mastitis during the first 2, 4, and 8 weeks after parturition increases from 25%, to over 45% and to 60%, respectively [109]. A high percentage of I.M. infections present at parturition also develop into clinical mastitis during the first 60 to 70 days of lactation [104]. The early post-partum period is a period of high risk for severe clinical mastitis because of extreme sensitivity for a number of animals for I.M. infection with ordinary environmental bacteria such as coliforms [93, 124, 125, 245]. Severe clinical mastitis with grave systemic symptoms typically occurs around parturition and during early lactation. Coliform mastitis after peak lactation is a moderate and self-curing disease. In some cases (during mid and late lactation) the symptoms of the disease are so mild and so short lasting, that it is difficult for a milker to recognise this self-curing condition

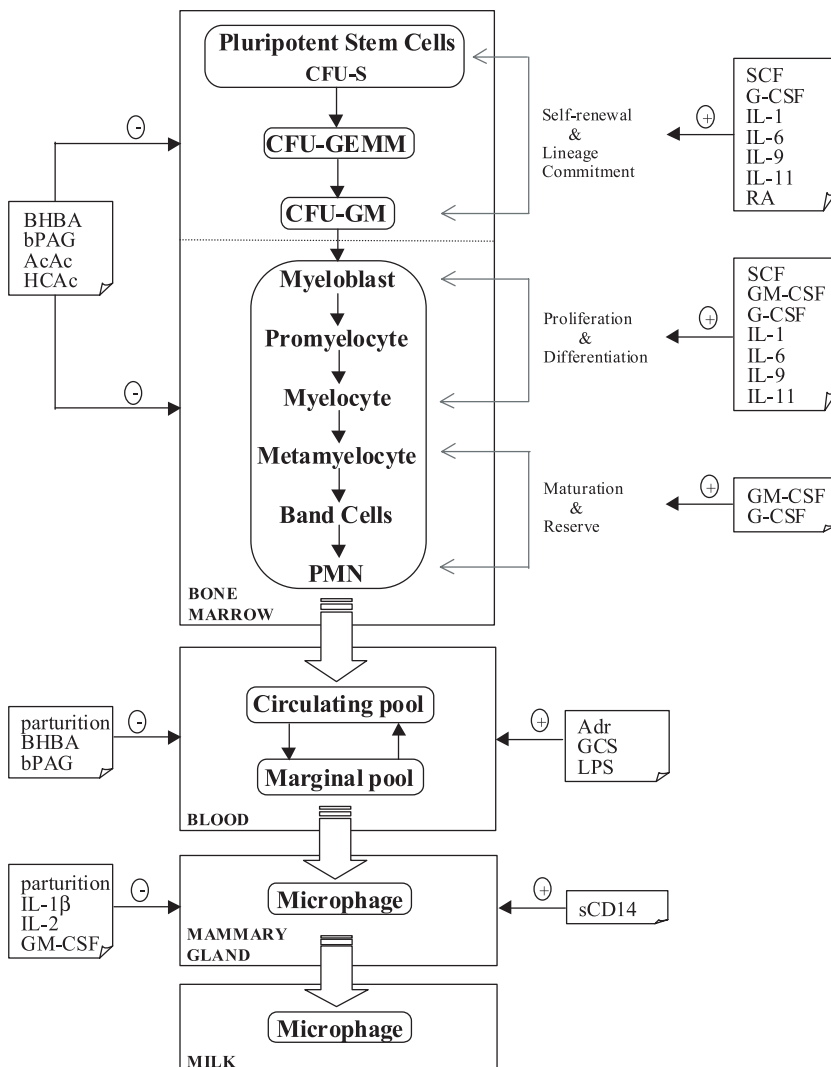


Figure 4. Action of growth factors, inflammatory mediators, metabolites and hormones on the proliferation, differentiation, and function of PMN in the bovine. CFU-S, colony-forming units spleen; CFU-GEMM, colony-forming units of granulocyte, erythrocyte, monocyte and megakaryocyte; CFU-GM, colony-forming units granulocyte and monocyte; SCF, stem cell factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; BHBA, β -hydroxybutyric acid; AcAc, acetoacetic acid; bPAG, bovine pregnancy-associated glycoprotein; IL, interleukin; RA, retinoic acid; Adr, adrenaline; GCS, glucocorticosteroid.

[97]. Today, we know that severity of bovine *E. coli* mastitis is mainly determined by cow factors rather than by the pathogenicity of the invading pathogen. For example, it is well recognised that the

bovine immune system is less capable of battling pathogens during the periparturient period and early lactation. Although exact causes for a compromised immune system are not fully understood, they are

believed to be at least influenced by hormonal and metabolic changes associated with pregnancy, parturition, and onset of lactation [57, 61, 62, 87, 126, 147, 148, 162, 185, 226].

7.2. Number and function of circulating PMN

The PMN population represents a major line of defence against infection in the bovine mammary gland. Indeed, the speed of PMN influx into the udder tissue can determine the severity of the mastitis [96, 245]. However, during the periparturient period the number and the different functions of circulating PMN, such as diapedesis, phagocytosis, and bacterial killing are altered [31, 57]. Major variations in PMN number and function occur within a fairly small period of about one week after parturition (Fig. 5). Interestingly, studies performed during early lactation (up to two months after parturition), indicate that the number of circulating PMN correlates with the severity of Gram-negative mastitis [93, 126, 243, 245]. Different characteristics of peripheral PMN, such as stimulated CL activity, cytochrome c reduction, chemotaxis, and stimulated L-selectin and CD11b expression, have also been related to the severity. In addition, other pre-infection metabolic parameters in blood, such as β -hydroxybutyric acid, non-sterified fatty acids, glucose, Mg^{2+} , Ca^{2+} , cortisol, and cholesterol concentration, as well as in milk, such as SCC, albumin, fat, and casein production, have also been found to be associated with the severity of *E. coli*-induced mastitis [52, 59, 93, 116, 121, 126, 210, 245, 259]. Other studies have compared the functionality of PMN between early and later stages of lactation [158–160]. The basal adhesion molecule levels of CD62L and CD18 at the surface of circulating PMN and the phagocytic activity of blood and milk PMN have been described to be similar during early lactation as compared to later stages of lactation [63, 210]. Interestingly, ROS production and killing

activity of blood and milk PMN were significantly decreased in early lactation [63, 158, 159, 161]. Because PMN functions are markedly altered around parturition (Fig. 5), detailed studies have been done during the last years in our laboratory. CL of stimulated blood and milk PMN was lower during early lactation as compared to mid or late lactation. Moreover, the ratio of stimulated milk to blood PMN CL was lower immediately after calving [158]. The low responsiveness of milk PMN towards PMA during early lactation could have resulted from a weaker stimulation of protein kinase C and NADPH-oxidase [117]. In addition, differences were observed in the kinetics of the PMN CL curve. Luminol-enhanced blood PMN CL kinetics showed a biphasic course during mid and late lactation. However, within the first ten days after parturition, only the immediate first peak was observed. CL in milk PMN was always monophasic with minimal intensity and durability during early lactation [158]. The luminol-dependent CL system requires H_2O_2 [73, 142] and both intra- and extracellular events contribute to this CL [69]. It is likely that the intracellular H_2O_2 production and MPO- H_2O_2 -halide activity is higher in blood than in milk PMN and their impairment is maximal during early lactation (Fig. 6; [158]). The decrease in ROS production in early lactating cows seems to be related with the diminished viability of milk-resident PMN detected in this period.

7.3. SCC and age of the host

There is evidence to suggest that minor pathogens increase milk cell counts and can help to protect the udder against mastitis [128]. In addition, herds with low SCC have a higher incidence of environmental mastitis compared to herds with high SCC [85, 205, 227, 247]. However, the correlation between SCC in the milk and the immune response of the udder to infection is not clear. Indeed, mastitis incidence and SCC levels are both higher in older cows. This paradoxical finding could be well

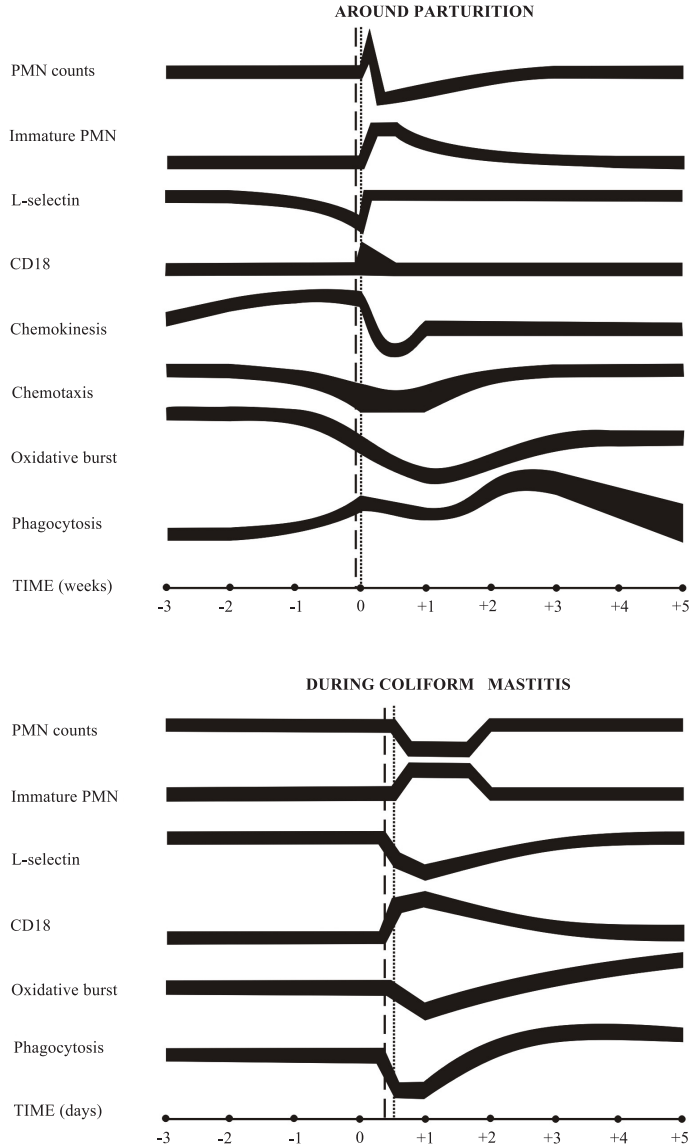


Figure 5. Schematic illustration showing major dynamic changes in circulating PMN numbers and functions around parturition and during coliform mastitis. The thickness of the curves reflects the degree of observed experimental variability with respect to a given parameter. The increase of catecholamines (dashed lines) and glucocorticoids (dotted lines) levels in blood occurs concomitantly with calving or shortly after coliform mastitis. (Figure taken from the original paper [57], with permission.)

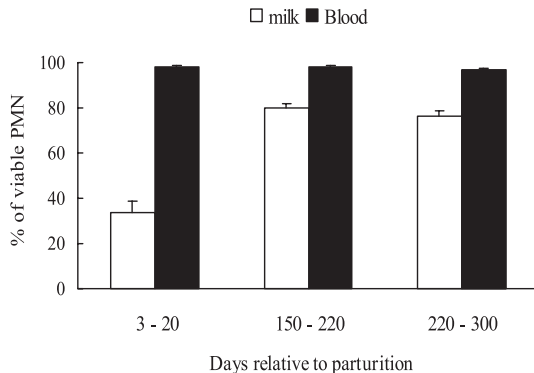


Figure 6. Viability of milk and blood PMN during early, mid and late lactation in dairy cows. Values are means \pm SEM of 10 cows. (Figure taken from the original paper [158], with permission.)

related to the functionality of the resident milk cells. Indeed, milk PMN in primiparous cows have been found to have a higher viability and ROS production as compared to older animals [161]. Therefore, primiparous and older high-yielding cows should be evaluated in different categories.

7.4. Nutritional and metabolic status

The peripartum period represents a period of significant decrease in energy, protein, and trace element concentrations in blood that may influence the outcome of the disease. Vitamin E and Se are essential nutrients that share common biological activities. Deficiencies in either of these micronutrients have been related in increased incidence and severity of mastitis. They are integral components of the antioxidant defence of tissues and cells [105, 214]. Elevated plasma concentration of ketone bodies in blood, due to the enormous increase in demand for energy by the onset on lactation at calving, has also a negative effect on PMN function and is associated with susceptibility to mastitis [226].

7.5. Genetic resistance

Several significant genetic associations have been found between the severity of the mastitis and several parameters, such as the

number and different functions of circulating PMN in early lactation, the serum concentration of Ig, and the SCC. For details we refer to specialised reviews [51, 180].

8. INFLAMMATORY PARAMETERS AND SEVERITY

8.1. Local signs

Udder abnormalities (increased firmness, swelling, watery milk with clots or flakes) are often a first indication of mastitis. However, these udder and milk changes do not predict the overall severity of the disease. The severity of *E. coli* mastitis has been directly related to the level of bacterial counts in milk [59, 98, 145, 210, 245] and the loss of milk production, especially in the non-infected quarters [59, 93, 245].

8.2. Circulating leukocytes

PMN in blood and milk are important components of the mammary gland defence against coliforms [31]. Number and function of bovine PMN respond differently to *E. coli* experimental infection depending on the severity of the mastitis. It is likely that the more severe disease course of *E. coli* mastitis during early lactation is due to a delay in mobilisation rate of PMN into the udder [96, 210, 245] and a reduced capacity of PMN to generate ROS [93],

resulting in failure to eliminate the bacteria from the infected quarters. The efficient migration of circulating PMN to the mammary gland depends on the correct expression and activation of several adhesion molecules. Bovine leukocyte adhesion molecules such as L-selectin (CD62L) and β_2 -integrins play a key role in the initial contact and in the subsequent strong adhesion to the vessel endothelium, respectively [56, 218]. Interestingly, in high-yielding cows infected with *E. coli* during early lactation, a higher increase of CD11b expression and a more pronounced decrease of CD11a and CD62L levels have been reported in severely diseased animals [55, 59]. These molecules are known to be indicators for the activation status of the cells. Therefore, the difference in expression level is probably related to the presence of bacteria in milk for a longer period in severely affected cows, which in turn release LPS and thereby extend the period of the activation status of blood PMN. During the first stages of experimental mastitis, the chemotactic response of leukocytes decreased in both, moderately and severely infected animals. The recovery of the chemotactic response of the leukocytes occurred more rapidly in moderately than in severely diseased cows [124]. It has been observed that early after challenge phagocytosis decreases in circulating PMN, but the initial decrease occurred faster in severely affected cows. From day 6 on after challenge, values increased over pre-infection levels and remained higher in severely affected animals [59]. Heyneman et al. [93] found a correlation between ROS production during the inflammatory response and the severity of mastitis.

In moderately affected cows, the initial neutropenia was followed by a return to basal levels after 2 to 3 days. However, one week after infection severely diseased animals developed a neutrophilia during which metamyelocytes and myelocytes appeared frequently in the blood, and even became the predominant leukocyte types [94]. Possibly, the larger proportion of

immature PMN partially accounts for the lower chemotactic response of the circulating PMN during experimental mastitis in the severely diseased cows [125, 245].

Neutrophil alkaline phosphatase activity was significantly higher in severe cases of mastitis during the 2nd week after inoculation. Basal NAP activity was regained after about two weeks in moderately diseased animals but lasted for several weeks in severely affected cows [94, 242]. The severity of the infection has been correlated with lower plasma Zn and Fe concentrations during the acute phase [145]. In addition, in severe mastitis highly persistent levels of cortisol were measured as compared with mild or moderate situations [98].

It seems that circulating PMN of severely diseased animals are in a status of increased activation for a longer period of time than moderately affected cows. Indeed, it has been observed that the clinical condition of severe cases only starts to deteriorate after one or two days post-infection, which is much later than the onset of clinical mastitis [98]. This idea is in agreement with the concept that severity of coliform mastitis can be estimated on basis of the milk loss in the uninfected quarters, 48 hours after infection [59, 93, 125, 244, 245].

8.3. PMN viability

A crucial aspect of mammary gland non specific defence status is milk PMN viability, which is influenced by (patho)physiological conditions of the gland [157, 158, 161, 187]. Apoptotic PMN are removed through phagocytosis by macrophages. Necrotic cells are not promptly cleared by phagocytes and their chromatin proteins may boost prolonged inflammation [202]. A low viability of resident milk PMN is observed during early lactation and this may compromise the total phagocytic and bacteriostatic capacity of the teat cistern. This could facilitate the growth of *E. coli*, especially during their lag phase. However, it is far more efficient to clear the

bacteria from the teat cistern with newly attracted viable PMN that engulf and kill the bacteria effectively. The increased milk PMN viability during LPS and *E. coli* mastitis that has been observed recently [157, 160] can be considered as a physiological homeostatic mechanism that could prevent inappropriate and prolonged inflammation. Mammary tissue damage may also be more important if viability is not optimal during *E. coli* mastitis. It is conceivable that milk PMN viability contributes to the pathophysiology and the outcome of *E. coli* mastitis.

9. TISSUE DAMAGE AND PRODUCTION LOSS DURING *E. COLI* MASTITIS

9.1. Pathologic findings during *E. coli* mastitis

General pathology books describe necrosis of the mammary epithelium during severe, naturally occurring clinical coliform mastitis [113]. Epithelial lesions appear very rapidly in experimental models where *E. coli* is infused I.M. with variable doses (200 to 10^9 cfu). Frost et al. [77] showed that after I.M. infection (approximately 10^9 cfu) *E. coli* multiplied very rapidly in the teat sinus, lactiferous sinus and large ducts. Within one hour, epithelial lesions were seen on these places, especially in the teat sinus. After two hours epithelial lesions were extensive and there was microscopical evidence of inflammation. However, lesions never went beyond the basal membrane. At four hours post-infection, inflammation was seen in the ducts and secretory tissue and there was massive infiltration of the lesions with PMN. Especially in moderate cases, there was a minimum of alveolar involvement. The same sequence of events was observed when smaller numbers of pathogens were infused I.M. [26]. In very severe cases of coliform mastitis there is a limited inflammatory reaction with an extensive involvement of

the secretory tissue. A rapidly ascending infection, spreading via the ductular system, was observed [79]. In its most severe form, with totally uncontrolled bacterial multiplication and all lactiferous sinus epithelia lost, interstitial tissue became totally haemorrhagic and the animal died of toxæmia within a few hours of infection [97]. In the early stages of experimentally induced *E. coli* mastitis, when lesions develop in the epithelium of the teat and lactiferous sinuses, neutrophils are in the subepithelial tissue. They first appear as mounds over the lesions but finally move through these lesions into the lumen of the gland into the milk [97]. Experimentally induced *E. coli* mastitis also promotes apoptosis and cell proliferation. During the later stages of moderate *E. coli* mastitis, apoptosis has been observed in the mastitic tissue. Expression of Bcl-2 associated x protein (Bax, a proapoptotic protein) and IL-1 β converting enzyme increased in the tissue at 24 h and 72 h post-infection. At 24 h Bcl-2 expression decreased. Induction of matrix metalloproteinase-9, stromelysin-1 and urokinase-type plasminogen activator was also observed in the mastitic tissue. Next to this, cell proliferation was also increased in the inflamed mammary gland [146].

9.2. Tissue damage and milk loss vs. high SCC

I.M. infection is the primary reason for increased SCC for all types of mastitis. A negative correlation exists between SCC and milk yield [50, 192], indicating that a great part of the mammary epithelium may be damaged by the invading bacteria and/or the phagocytes that were sent out by the defence system of the host. Decreased or lost milk production is an important aspect of clinical mastitis. High-producing cows might never achieve the total lactational output that would have been expected. Permanent blunting of milk production may also occur for the remainder of that lactation. This effect is very damaging when

mastitis occurs before the cow reaches its peak lactation. Phagocytes, and more especially neutrophils, are necessary to eliminate the invading bacteria. PMN migrate through mammary tissue as a result of infection of the udder. Once within the cisterns, ducts and alveolar lumen of the gland, phagocytosis by PMN and subsequent degranulation may account for the negative correlation between SCC and milk production. Chemical compounds, such as ROS released from neutrophils can cause mammary secretory cell damage and reduced milk yield. Substances secreted by the pathogen may also cause damage. Although degranulation of neutrophils ensures the potentiation of the inflammatory reaction, it is usually undesirable in the very sensitive mammary gland [97]. In order to minimise mammary tissue damage and milk production loss, elimination of invading bacteria should proceed fast, probably rather because of the side effects of the activated phagocytes and their ROS than because of a direct effect of bacterial LPS.

9.3. Do trafficking neutrophils cause damage?

The mechanism by which tissue damage occurs during coliform mastitis is not yet fully understood. Tissue damage caused by neutrophils may originate from two sites: intratissular or intraluminal. The damage may originate from the inside of the parenchyma (intratissular), i.e. starting at the basal side of the epithelium. Theoretically, neutrophils trafficking in mammary tissue towards the luminal side of the udder might cause cell and tissue injury, especially when it occurs massively, and mainly through the release of mediators helping them to traverse the different obstacles (basal membranes, matrix and cells). It is not clear whether transcellular epithelial migration, as described for postcapillary venular endothelial migration, occurs during coliform mastitis [74]. Transcellular endothelial neutrophil migration mainly occurs through all or non-diaphragmed

fenestrated endothelia (to our best knowledge, in the goat mammary gland, no such fenestrations were found, [28]). Because fenestrations are absent in mammary epithelium this mechanism is not expected in the mammary gland. However, a comparable mechanism could occur. In the inflamed gland some (damaged) epithelial cells can be expelled by neutrophils into the luminal surface of the ducts and cisterns to create “a gap” [97] through which neutrophils could traverse massively into the lumen under influence of a chemotactic gradient that starts in the postcapillary venules and increases toward this gap and the site of the nidus of infection. The process of venular migration starts around two hours after infection and an additional two to four hours is needed to reach the epithelium of the teat sinus. In mammary parenchyma, where postcapillary venules are closer to the milk alveolus, shorter times could be reached [183].

If LPS is used to induce the inflammatory response in the mammary gland, no lesions develop, and the PMN move between the epithelial cells and the tight junctions to get into the lumen of the gland [97]. This is in agreement with the finding that the drop in milk production during LPS mastitis is short lasting and that it is completely self-curing within 2–3 days depending on the dose [53]. We have the experience that primary cultured bovine mammary epithelial monolayers have variable and underdeveloped intercellular junctions compared to the *in vivo* situation. However, neutrophil migration induced by C5a did not change electric resistance over the cell layer over a short period. Moreover, cell lysis or epithelial detachment were not observed [216–218]. The above mentioned findings are in agreement with the basic concept that paracellular migration of neutrophils is locally modulated and that release of proteolytic enzymes in the matrix (e.g. elastase) does not occur. Instead, elastase is only activated in the cytoplasm-membrane at the end of the pseudopodia [43, 106], probably to affect

the cell junctions protein complex, cadherin/catenin. Boulanger et al. [22] demonstrated activation of gelatinase activity in isolated bovine neutrophils stimulated with LPS and PMA. Nevertheless, no epithelial cytotoxicity could be detected. This may explain why aprotinin, a serine proteinase inhibitor which inhibits the proteolytic activity, had no effect on epithelial damage in a culture of MAC-T cells. However, in other models, tissue lesions have been observed following neutrophil diapedesis. For example, neutrophil-mediated injury to gut epithelium leading to disruption of the epithelial barrier function with consequent organ dysfunction was studied *in vitro*, using a colonic epithelial cell line. In this model it was demonstrated that neutrophil-derived proteases can mediate spatially delimited disruption of epithelial apical junctions during transmigration [82].

9.4. Intraluminal induced damage

Intraluminal induced damage originates from the luminal part of the gland, in cisterns and ducts where cells and chemical mediators of the host are released to eliminate the invading bacteria around the nidus of infection. As the inflammatory cascade progresses and spreads to ducts and alveoli, it will extend over the gland and the lumina. As a result, alveoli will be filled up with harmful exudate. This is apparently the most plausible explanation for the tissue damage observed during *E. coli* mastitis. Gelatinases, especially milk matrix metalloproteinases (MPP), that are known to be involved in the degradation of the blood-milk barrier, increase massively in the exudate during LPS-induced mastitis. The major source of MPP originates from the neutrophils and the MPP changes are concordant with the increase in SCC during mastitis [193]. I.M. infusion of 10^8 intact or lysed PMN caused palpable signs of inflammation for 24 h and leukocytosis for 5 days [110]. A mammary explant model was developed to assess mammary tissue damage caused by normal PMN

function [38]. Tissue from the udder of lactating Holsteins was cultured in the presence of intact or lysed PMN. PMN that were allowed to phagocytose opsonised zymosan were used. The observed cytological damage included cell sloughing from the basement membrane, pyknosis, cell debris in luminal areas and epithelial vacuolation. The damage to mammary epithelium caused by these treatments was ranked as follows: phagocytosing PMN > lysed PMN > intact PMN.

To study the underlying pathway of tissue damage caused by migrating neutrophils during coliform mastitis (intratissular vs. intraluminal) *in vitro* studies with primary cell lines and MAC-T cells have been used. Although the *in vitro* studies are challenging and promising for detailed research, careful interpretation is needed not to oversimplify the phenomenon when extrapolating the results to the *in vivo* mastitis situation where massive neutrophil diapedesis occurs and where many local physiological homeostatic mechanisms are drastically altered. For example, from both field and experimental studies where *E. coli* was infused I.M. in cows, there is the well-known experience that sometimes red blood cells can appear in the milk of the inflamed quarters as a result of the dramatic alteration in mammary blood flow and microvascular integrity during mastitis [245].

9.5. Cytotoxic mediators

Three groups of mediators have been studied and attempts were made to dissociate these factors to assess their individual potential for damaging the mammary epithelium. (1) Antibacterial defence involves activation of neutrophils generating ROS capable of killing bacteria [91]. It was observed that neutrophils promote tissue injury and disturb mammary function via ROS production. Host lipid peroxidation, initiated by enzymes such as NADPH oxidase and myeloperoxidase, occurs [260]. (2) Neutrophilic degranulation, during which

release of proteases occurs as a result of neutrophil trafficking or as a consequence of their phagocytic activity, was also shown to be cytotoxic [2, 22, 38, 135, 165, 172]. During phagocytosis lysosomes migrate toward the particle being internalised and lysosomal enzymes are released before fusion of the pseudopodia is completed, resulting in the release of lysosomal contents to the outside of the PMN [81]. (3) Brooker et al. [26] postulated that at least two bacterial toxins are involved in *E. coli* mastitis. I.M. administration of purified commercial LPS (Boivin extraction) produced an intense inflammatory reaction without any epithelial lesion. Neutrophils appeared all over the surface but were not associated with lesions. A fresh filtrate of the medium in which *E. coli* was growing during 18 h, elicited a mild inflammatory response but scanning electron microscopic examination showed that there was extensive damage of the lining epithelium. Both layers of the epithelium of the lactiferous sinus were lost four hours after infusion. The basement membrane was exposed at that time and by four hours neutrophils also started to appear at the lesions. The postulated cytotoxic agent was not identified but was found to be heat labile. The extensive damage to the ductular epithelium is presumably induced by the unknown cytotoxic compound, produced by *E. coli*. It was further postulated that the damage to the alveolar epithelium may not have the same genesis [79]. For the most severe cases, where all lactiferous sinus epithelia are lost and where interstitial tissue became totally haemorrhagic [97] it was postulated that the LPS molecule might be responsible for the profound circulatory disturbances [79].

In a recent study, LPS and PMA were not found to be toxic to MAC-T cells, whereas the amino acid histidine (≥ 6 mg/mL) was found to be toxic to mammary secretory epithelial cells. Together, histidine and 500 000 activated PMN/mL were also toxic [135]. As a consequence, the effect of anti-oxidants was investigated on the neutrophil

induced epithelial damage in vitro. The model consisted of a co-culture of MAC-T cells with isolated bovine neutrophils. PMA-activated neutrophils in combination with *E. coli* endotoxins caused release of superoxide. Epithelial damage was also observed [22, 135]. Melatonin that scavenges hydroxyl radicals and peroxynitrite reduced neutrophil-induced cytotoxicity dose-dependently [22].

9.6. Effect of rbST on milk loss during mastitis

The above mentioned findings inspired investigators to evaluate the potential protective effects of recombinant bovine somatotropin (rbST) during coliform mastitis, because during this disease the neutrophil plays a pivotal role in its outcome [244]. In a first study, 19 cows, classified as moderate or severe responders (based on the respiratory burst activity of blood neutrophils before challenge), were treated with rbST or vehicle, 48 h after I.M. inoculation of *E. coli*. Clinical status and changes in milk production and composition were compared in the four groups. In a second study, 8 cows received rbST or vehicle 7 days before bacterial challenge. The decrease in milk synthesis was accompanied by increased milk sodium and chloride and decreased potassium and lactose concentrations (Fig. 7, [29]). The latter indicates the opening of tight junctions between mammary epithelial cells, which occurs during inflammation of the mammary gland of ruminants [28]. The decrease in lactose concentration is also the result of its altered synthesis which is, in part, due to the decreased synthesis of α -lactalbumin [93]. Milk production of rbST cows recovered better than that of placebo cows. Recovery of milk components, such as lactose (Fig. 7), was accelerated in severe responders treated with rbST, but not in moderate responders. Pre-treatment of severe responders with rbST enhanced milk production before infection, protected the mammary glands from excessive loss

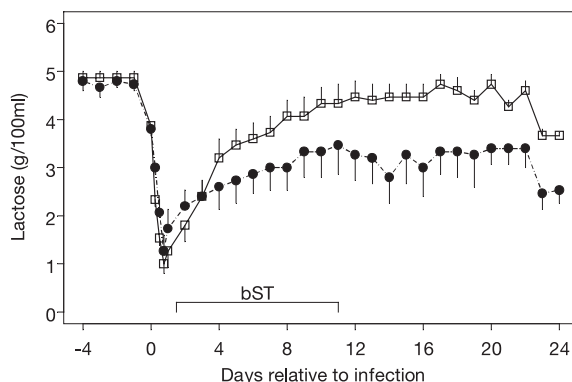


Figure 7. Milk lactose concentrations in infected quarters of rbST treated (●, $n = 7$) and control (□, $n = 10$) cows with acute *E. coli* mastitis in early lactation. (Figure taken from the original paper [29], with permission.)

of milk during the subsequently induced coliform mastitis, and accelerated normalisation of milk composition. It was concluded that: (1) the beneficial effects of rbST upon normalisation of milk production and composition was restricted to severe responders; (2) the beneficial effects of the treatment with rbST was most pronounced on milk composition in infected glands. The acceleration of the tightening of the blood-milk barrier was spectacular; the gain in milk production was less pronounced. This might suggest that rbST (or IGF-I) increases milk production in the diseased gland, partly by inhibiting epithelial cell loss and/or restoring cellular differentiation. In a T84 cell monolayer model it was demonstrated that IGF-I and IGF-II regulate the paracellular permeability through a receptor-mediated process that probably involves changes in protein synthesis and cytoskeletal structure [155]. (3) In severe responders that had been treated with rbST, changes observed during mastitis resembled those in moderate responders treated with the placebo. The exact mechanism of how rbST affects the recovery of the secretory epithelium during mastitis remains unknown. During coliform mastitis bST concentration is higher

in challenged cows, IGF-I increases in whey of the infected glands [34, 209], and IGF binding proteins also increase in the whey of infected glands [209]. The endogenous release of bST and IGF-I during mastitis might be considered as a homeostatic mechanism involved in the resolution of the inflammatory response. It is also possible that rbST increased the rate of cell renewal in the lactating mammary gland as it does during normal lactation [39]. The rbST or its intermediary IGF that increases in milk whey during mastitis, may have interfered with apoptotic factors that are released during mastitis and that induce cell death. It is also not excluded that rbST may have had an indirect effect on mastitis through enhancing the defence system of the cow [34]. Elvinger et al. [71] showed that administration of rbST to cows increased lymphocyte blastogenesis but not phagocytosis and killing of *E. coli* by PMN. Recombinant human growth hormone primes human neutrophils for enhanced superoxide anion secretion [80].

The possible effect of rbST was also studied during *S. uberis* mastitis, another environmental mastitis type. Cows were treated with long acting rbST 7 days before

and after infection and compared with control cows that received the excipient. Comparable results were obtained. Somatotropin protected the mammary gland from excessive production losses. It also significantly improved the normalisation of composition, indicating a beneficial effect on the restoration of the integrity of the blood-milk barrier [100]. Also in this model, rbST treatment induced significantly higher IGF-I and rbST concentrations in plasma. Despite these positive effects on milk production and composition, no effect on some defence parameters such as chemiluminescence, rate of diapedesis and the expression of adhesion receptors of circulating PMN could be detected [99].

9.7. Lesions in rare cases of atypical *E. coli* mastitis

The pathogenesis of atypical cases of *E. coli* mastitis falls beyond the scope of this review. It is however interesting to focus on some particular characteristics. Sometimes a delayed PMN infiltration results in a failure to eliminate the bacteria. Pathogens and PMN co-exist for many days, with a gradual increase in the proportion of mononuclear cells in the secretion, which are themselves less efficient at killing bacteria [48]. In these cases dramatic epithelial hyperplasia occurs. The secretory function of the gland is lost or severely impaired. Two strains of *E. coli*, isolated from the milk of two different cows suffering from persistent *E. coli* mastitis were tested for adhesion to and invasion of three primary mammary epithelial cell cultures derived from mammary biopsies of the two infected cows. Here, intracellular *E. coli* were detected during five days post-infection in vitro. Histology of mammary tissue revealed chronic inflammatory changes in quarters that were persistently infected by *E. coli* but in contrast to the in vitro situation, intracellular bacteria were not detected in mammary tissue sections [67].

10. CONCLUSION

From both epidemiological and experimental studies it can be concluded that severity of coliform mastitis is of much more concern than its incidence. Moreover, from many recent studies it appears that the severity of *E. coli* mastitis is mainly related to "host factors" rather than to the pathogen itself. The periparturient period, the early stage of lactation, parity, hormonal and metabolic alterations, and negative energy balance may affect PMN function and bactericidal efficiency in blood and the mammary gland of the dairy cow. The PMN dysfunction influences the balance between the inflammatory reactions, bactericidal capacity and tissue damage, boosting the severity of *E. coli* mastitis. This dysfunction may originate at the level of the bone marrow, the blood circulation and/or the mammary gland. In dairy cows the severity of *E. coli* mastitis is more likely during early lactation and more pronounced in older cows. Any management strategy that aims to influence the severity of *E. coli* mastitis in dairy cows should be focused on both the dry and periparturient period. Moreover, this strategy should be directed towards minimizing energy and environmental stress. Some genetic factors might also be involved temporarily. It is possible that as a result of parturition, negative energy balance, etc., certain genes are (or are not) expressed during the beginning of lactation. Optimisation of the bactericidal capacity of PMN and diminishing tissue damage during *E. coli* mastitis is a challenge for research in the near future.

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