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Review article

The complement in milk and defense of the bovine mammary gland against infections

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Abstract – The mammary gland of dairy cows, which is prone to infection by various bacteria, mobilizes local and systemic immune defenses to cope with pathogens. The complement system plays an important part in the innate immunity against microorganisms through its bactericidal, opsonic, and phlogistic functions. The amount of the complement in the milk of healthy glands of dairy cows is low. Moreover, the classical pathway of activation is not functional because of a shortage in C1q. By contrast, the alternative pathway is active, deposits C3b and C3bi on bacteria, and generates amounts of C5a which are highly variable among cows. A slight inhibition of the bactericidal/hemolytic activities, of the deposition of C1q on bacteria, and of the phlogistic activity of C5a makes milk a rather anti-inflammatory fluid. The inhibitory activity does not involve C3b/C3bi deposition on bacteria, nor the generation of C5a by the alternative pathway. When inflammation develops, the blood-derived complement components overcome the inhibitions and complement-dependent bactericidal, opsonic and phlogistic activities may be high in milk. Further research is necessary to evaluate the contribution of C5a to the recruitment of leukocytes in the mammary gland, and to specify the links between the complement system and the response of resident cells (leukocytes and mammary epithelial cells) to infection stimulus. This will help to define the contribution of the complement system to resistance against mastitis, and could help to differentiate animals more or less resistant to this frequent and costly disease.

complement / milk / mastitis / dairy cattle / inflammation

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1. INTRODUCTION

Defense against bacterial infection is mediated first by the recognition of the invading organism, then by the initiation of the inflammation involving the mobilization from the blood of circulating, motile, phagocytic and bactericidal leukocytes, such as neutrophils, to eliminate the infectious agent. This inflammatory response is part of an innate immunity, and represents the first line of the immune defense against infection. The complement system is central to innate immunity, because it is intimately involved in the process of inflammation and participates in the killing of microorganisms, either directly or through cooperation with phagocytic cells [110].

After its discovery, the complement system was mainly evaluated through its capacity to lyse microorganisms and erythrocytes. Nevertheless, it was also gradually appreciated that the complement promotes the recognition and ingestion of microorganisms by phagocytes, and is an important operator in the initiation and control of inflammation [29]. The coating of particles with fragments of the components C3 and C4 makes them prone to ingestion by phagocytic cells possessing receptors for these opsonins. The regulation of the inflammatory response by the component fragments C3a and C5a, which induce histamine release from mast cells and basophils, vasodilatation, increased vascular permeability, and the recruitment of phagocytes at sites of inflammation, are also important functions of the complement system [22]. More recently, it has become apparent that proteins of the complement system can influence the immune response and constitute a bridge between innate and acquired immunity [26, 93]. These functions of the complement have proven to be of broader consequence for host defense than is the complement-mediated lysis [29].

The major effector mechanisms of the complement system are of potential relevance to the defense of the bovine mammary gland against infections, which are usually due to pyogenic bacteria, which are mainly extracellular pathogens whose multiplication is controlled by phagocytic cells. An essential defense against mastitis is the phagocytosis of bacteria by polymorphonuclear neutrophils [20], and the complement can contribute to phagocytosis at three pivotal steps:

– The opsonization of bacteria, by deposition at the bacterial surface of fragments recognized by receptors on phagocytes.

– The attraction of phagocytes by phlogogenic fragments or complexes which exert a chemotactic activity.

– The priming or activation of ingestion or intracellular killing of pathogens by fragments of certain activated components.

The complement system is made up of at least 29 distinct plasma and membrane proteins. The activation of the system results in a sequence of biochemical reactions in which one component activates another component in a cascade fashion. Three activation systems have been described: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway of activation. Along these cascades, several biological functions are initiated. Few of the complement components have been identified and measured in bovine milk. In fact, the complement was generally not considered as a significant antimicrobial system in bovine milk [94, 106].

The bactericidal activity of the complement was first investigated in milk of dairy cows. Curiously, the more important function relating to phagocytosis has been

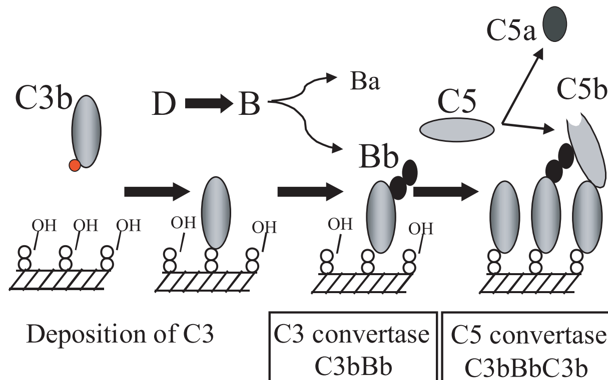

Figure 1. Major events in the activation of the alternative pathway of the complement at a bacterial surface. The proteolytic activation of C3 (spontaneous) forms metastable C3(H_2O), a molecule which has the functional properties of C3b. A fluid-phase C3 convertase generates the first molecules of C3b. Random deposition of C3b through covalent linkage (ester links with carbohydrates are represented) with a bacterial surface is followed by the interaction with the B factor. Factor D cleaves B, generating the C3 convertase. Deposition of C3b in the vicinity of the C3 convertase generates a new C3 convertase (amplification) and gives rise to the C5 convertase. Component C5 associates with C3b, and is cleaved in C5b and the chemotaxin C5a. Modified from [74].

neglected for a long time. Nevertheless, the opsonic activity of the milk complement may have been overlooked, and the inflammatory role of the complement in mastitis, commonly assumed as the auxiliary, may need reappraisal.

The idea that generally prevails is that the concentration of the complement in normal milk is not sufficient for chemotaxis or opsonization, and consequently that the biological significance of the complement is low if not negligible before higher amounts are mobilized from the blood by inflammation [63]. This opinion was not based on the direct measurement of the complement-dependent opsonic activity of milk or on the assessment of the contribution of complement-derived chemotactic fragments to the recruitment of neutrophils. The amount of the complement was considered low on the basis of the weak or undetectable hemolytic activity in normal milk (milk from a healthy uninflamed, uninfected gland). The pathways of the activation of the complement in milk were not determined, and the concentrations of individual components were unknown, with the exception of component C3 [63, 64]. More information is now available on the opsonizing and chemotactic activities of the complement in milk. This knowledge puts an emphasis on the AP (Fig. 1) and makes a better assessment of the contribution of the complement system to the defense of the bovine mammary gland possible.

2. CONCENTRATION OF THE COMPLEMENT COMPONENTS AND ORIGIN OF THE MILK COMPLEMENT

We have only a sketchy knowledge of the concentrations of the complement proteins in bovine milk (Tab. I). Data concern few complement components, and even for those which have been measured, all the physiopathological situations were not systematically studied. The most striking data are the relative deficiency of C1q, and the relative abundance of C3, as compared to blood serum values. Although the information on their concentration is lacking,

Table I. The concentrations of complement components in the serum and milk of dairy cows.

Component	Serum concentration	Mature normal milk		Mastitic milk	
		Concentration	(% of serum)	Subclinical	Clinical
BSA (mg/mL)	40–50	0.2–0.3	0.5	0.3–0.6	1–20
C1q (μg/mL)	200	0.2	0.1	ND	ND
C3 (% of serum)	100	ND	2.5	5	5–18
C5 (μg/mL)	ND	ND	0.2–1.3	ND	ND
C5a (ng/mL)	15.4[a]	0–0.12	NA	0.18	5.5–50
Potential C5a (ng/mL)[b]	4280	0.76–12.30	0.02–0.3	2.5	> 50
fB (μg/mL)	350	2.1	0.6	ND	ND

BSA is indicated as a marker of passive transfer from blood to milk.
ND: not determined. NA: not applicable.
[a] C5a is generated by the coagulation of blood. Value in EDTA-plasma was 0.58 ng/mL [91].
[b] Potential C5a is the amount of C5a that can be generated by activation of the complement with zymosan.
References: BSA [37, 39, 63, 79]; C1q [86]; C3 [63, 64, 88]; C5 [88]; C5a and potential C5a [88, 91, 104]; fB [83, 108].

many complement components are present in normal milk to operate the deposition of C3bi on bacteria and the functioning of the C5-convertase of the alternative pathway, such as functional concentrations of components D, H, I, and properdin.

Most of the complement proteins that circulate in the plasma are synthesized in the liver, and several are also produced in extrahepatic sites, mainly by tissue macrophages [18]. Evidence has also been obtained that other cell types, including the epithelial cells of the gastrointestinal and urinary tracts and skin fibroblasts, are capable of producing individual complement proteins. The regulation of complement gene expression is tissue specific: for example, the local regulation of fB, C2 or C4 production by macrophages is independent of the plasma concentrations of these proteins [18]. The local synthesis of several complement proteins is under the control of cytokines and growth factors. For example, proinflammatory cytokines like IL-1β, TNF-α or IL-6 regulate the synthesis of C3, C4 and fB by the intestinal epithelial cells [1].

The origin of the complement components found in bovine milk is essentially a matter of speculation. Transudation of complement proteins is likely to contribute to the complement being present in the milk of uninflamed glands. If this contribution was essential, the blood/milk concentration ratios would be the same for all the complement components. We know that C1q concentration is much less, and C3 concentration is much more than expected on a passive transudation basis in normal milk. Transudation is probably limited by the impermeability of the mammary epithelium, which may account for the lack of C1q, the largest component (900 kDa), in normal milk [86]. This physiological barrier is locally broken by the inflammatory response or the lesions developed during infection, and exudation of plasma proteins can take place. Exudation of the complement proteins is most likely to account for most of the enhancement of the complement activities (hemolytic, bactericidal, opsonic, chemotactic and activating) in mastitic milk during the acute phase. Apart from that particular situation, other mechanisms are probably major sources of the milk complement.

There is a poor correlation between C3 concentrations and BSA concentrations (r = 0.41) or between hemolytic activity and BSA concentrations (r = 0.29) in milk from normal glands and better correlations (r = 0.80 and 0.76, respectively) in milk from infected glands [63, 89]. C3 concentrations are much higher than expected both in normal milk (2.5% of serum concentration vs. 0.5% for BSA) and in mastitic milk after the acute phase of clinical mastitis induced with LPS: milk C3 concentrations remain high while BSA concentrations have returned to near baseline concentrations [64]. These observations suggest a local synthesis of C3, either by blood-derived monocytes and macrophages, or by mammary epithelial cells. In a recent study, cows with comparable C5 concentrations in plasma had concentrations of C5 in milk differing by a factor 10, suggesting the source of C5 in milk may be mainly local, and that the regulation of the synthesis of C5 differed widely among cows [88]. An alternative to the local production of the complement proteins by the epithelial cells and macrophages would be selective transcytosis or paracytosis by the epithelium, but this remains purely speculative.

As far as we know, the milk complement has partly different origins as a function of the health status of the mammary gland. Transudation and local synthesis of C3 may dominate in normal milk, whereas exudation and stimulated local synthesis are likely to dominate during infection.

3. EFFECTOR FUNCTIONS OF THE COMPLEMENT IN MILK

3.1. Bactericidal and hemolytic activities in normal milk

The complement in cow's milk was first evaluated by measuring its hemolytic and bactericidal activities. These activities involve the assembly of the terminal components of the complement system, namely the membrane attack complex (MAC), composed of the components C5b, C6, C7, C8 and several copies of C9. The MAC assembles when the whole cascade of complement activation has been developed. Consequently, killing is an end result of complement activation.

Bactericidal activity was found in colostrum only after dilution with bovine milk [96]. Undiluted colostrum inhibits the bactericidal activity of pre-colostral calf serum, a source of complement virtually devoid of antibodies (Ab), probably because of a prozone effect exerted by an excess of IgG1. In addition to an excess of antibodies, the prozone phenomenon involves an inhibitory or anti-complementary activity, independent of antibodies, which can be overcome by an excess of C3 [65]. Inhibition of the bactericidal activity of colostrum by chelation of Ca^{2+} ions with Mg-EGTA suggests that the activity of the colostral complement is entirely mediated by the classical pathway [8]. A bactericidal activity was found in the post-colostral milk of some cows [96], but it declined when the lactation progressed and became rapidly undetectable [96]. Conflicting results are reported over heat-labile bactericidal activity of normal milk, which has been found to be either bactericidal [12], or not [53, 89]. The different sensitivity to the complement of the test organisms used in these studies may account for the discrepant results. It is worthy of mention that for a long time the only virulence attribute identified among the strains of *Escherichia coli* isolated from mastitis cases was resistance to the complement [100].

A weak but significant hemolytic activity was found in the colostrum and in the end-lactation milk [24, 95, 96], but no activity was found in normal mature (mid-lactation) milk [24, 63]. The hemolytic tests made use of guinea pig red blood cells sensitized with Ab, so they are presumably able to detect the activation of both the AP and CP, since guinea pig erythrocytes activate the AP [7]. Nevertheless, the tests

may have been too insensitive to demonstrate hemolytic activity, because a sensitive microassay displayed an activity in most milk samples at every stage of lactation in normal milk [77]. In fact, the microassay did not prove that all the components were present in milk, since it was based on the effect of milk on a concentration of bovine serum giving 50% of ^{51}Cr release from guinea pig erythrocytes. What this complementation test did show is that milk contains the components which are at limiting concentrations in diluted serum. Complement titers of milk samples from uninfected quarters averaged 0.2 CH100 (complement hemolytic units inducing 100% hemolysis), compared to 40 CH100 in serum [77]. Some inhibition of hemolysis occurs in the milk samples, and this inhibitory activity has to be taken into account in the calculation of the hemolytic titers.

In the secretions of the non-lactating udder, the complement hemolytic titers increase somewhat at the beginning of the dry period, ranging from 0.3 CH50 (complement hemolytic unit inducing 50% hemolysis) at drying-off to 1.7 CH50 at 21 days after cessation of milking, but the inhibitory activity also increases, corresponding to 0.1 at drying-off to 0.6 CH50 21 days later [78]. Bactericidal activity to a serum-sensitive *E. coli* strain was found only in samples with more than 0.4 CH50, provided the inhibitory activity corresponded to less than this amount [78].

The studies on the complement-dependent bactericidal activity in milk did not address the question of the pathway involved. A study involving mastitis isolates of coliforms submitted to bovine serum showed that the CP appears to be the principal source of bactericidal activity toward *Klebsiella pneumoniae*, whereas both CP and AP were important for the killing of *E. coli* [47]. It is commonly admitted that Ab and the complement cooperate to kill serum-sensitive bacteria, and that very small concentrations of Ab confer a high degree of killing activity to the complement [109]. Low concentrations of Ab to most mastitis pathogens are usually found in milk, but it is not known if Ab may be a limiting factor for complement-dependent bactericidal activity. In most cases, the activation of the complement in the presence of Ab is by CP, but rather high concentrations of Ab can also induce the deposition of C3 on bacteria through AP, for example on type III *S. agalactiae* [25], or on certain strains of mastitis isolates of *E. coli* [44]. Depending on the strain, the complement alone was sufficient or the complement plus Ab were necessary to kill serum-sensitive *E. coli* [44]. Deposition and activation of C1q is possible in the absence of Ab through direct interaction with the surface of gram-negative serum-sensitive bacteria, via the deposition on bacterial components such as LPS [19, 61]. It is nevertheless assumed that many pathogenic bacteria become susceptible to the complement in the presence of a specific antibody, which may direct complement attack to critically sensitive sites of the bacterial surface membrane, or which provide a binding and/or protected site for C3b deposition [110]. IgM are particularly effective in activating the complement and inducing complement-dependent bactericidal activity [109]. Bactericidal activity of a serum-sensitive *K. pneumoniae* mastitis strain was shown to be associated with the antibodies present in the IgG1 and IgM isotypes, with the greatest activity in IgM [13]. The complement-dependent antibacterial activity of milk can be increased by systemic immunization of cows against a defined pathogen, even though Ab titres do not correlate well with the activity [58]. For example, after immunization with *Helicobacter pylori*, 43% of the milk samples of immune cows were bactericidal towards these bacteria, whereas the milk samples of control cows were inactive [57].

Resistance of bacteria to serum bactericidal activity is determined at the surface of bacteria, more specifically at the outer

membrane of gram negative bacteria [109]. It appears that serum resistance does not result from a block in the activation of the complement cascade, but rather on the impairment of the insertion of the MAC into the bacterial membranes. Lipopolysaccharides play a central role in determining the susceptibility of enterobacteria to serum, but capsular polysaccharides and some plasmid-determined factors also play an important role [109]. These considerations seem to apply to mastitis isolates of enterobacteria [113]. Modification of the bacterial surface by environmental conditions, such as those encountered by bacteria in the lumen of the mammary gland, may influence the sensitivity to bactericidal activity. Nevertheless, it was reported that iron deprivation of *E. coli* does not alter the expression of the capsule and has no effect on the susceptibility of isolates to the bactericidal activity of serum, although it decreases the susceptibility to phagocytosis by neutrophils [116].

3.2. Bactericidal and hemolytic activities in mastitic milk

In contrast to normal milk, milk from inflamed quarters frequently exhibits both bactericidal and hemolytic complement activities. Overall, the intensity of these activities is linked to the magnitude of inflammation. Most data are related to clinical mastitis induced with gram-negative bacteria or their surface lipopolysaccharide (LPS or endotoxin). For example, instillation of 50 μg LPS through the teat canal led to the appearance of hemolytic activity in milk, which reached a maximum after 4 to 12 h, then decreased and disappeared after 48 h [64]. The kinetics of hemolytic activity coincided grossly with the kinetics of concentrations of bovine serum albumin (BSA) in milk, but the kinetics of milk concentrations of C3 coincided only at the beginning of the inflammatory response. Concentrations of C3 remained elevated 48 h post-infusion, while SCC was still high, but BSA concentrations had markedly declined and hemolytic activity was low. This divergence between the C3 concentrations and hemolytic activity may reflect the lack of one or several complement components of the cascade of activation, or the appearance of some inhibitory activity. The divergence between C3 and BSA kinetics suggests that after one or two days of inflammation, the main source of C3 in milk is probably not a mere passive exudation from blood.

Following the infusion of viable *E. coli* through the teat canal, heat-labile bactericidal activity against a serum-sensitive *E. coli* strain appears at the onset of inflammation, concurrently with the increase in BSA concentrations in milk [43, 80]. In cases of severe mastitis, this heat-labile activity, most likely due to the complement, is followed by the appearance of a heat-resistant bactericidal activity of cell-free milk, probably due to other soluble bactericidal substances, whereas it recedes along with inflammation in cases of less severe masititis [80]. This suggests that, at the onset of infection, the complement is mobilized from the blood to the milk along with the exudation of plasma accompanying the inflammatory response of the gland.

Milk from chronic subclinical mastitis has also been shown to be hemolytic, provided the incubation time with sensitized erythrocytes is long enough [63]. Indeed, the incubation time has to be extended to an unusual length (more than 2 h, up to 16 h) in order to detect a hemolytic activity, and this technical requirement may have contributed to the negative results obtained in some studies. Nevertheless, all samples from subclinical infection are not bactericidal, probably depending on the degree of exudation of plasma [89]. The correlations between hemolytic titers and BSA concentrations ($r = 0.76$) or between C3 and BSA concentrations ($r = 0.80$) in whey from subclinically infected quarters support the idea that the bulk of the complement passes from the blood into the

milk during an inflammatory response in a passive manner, like BSA.

The pathways of complement activation responsible for the hemolytic and bactericidal activities in mastitic milk have not been specified. Nevertheless, it has been shown, with assays measuring the deposition of C3 and C4 on bacteria (see below), that the classical pathway is functioning in milk from inflamed glands, and that a mild inflammation, as associated with subclinical mastitis, is often sufficient to that end.

3.3. Opsonization of bacteria

Activation of the complement leads to the deposition of cleavage fragments of the C3 component, the key component of the system [59], on the surface of invading pathogens through a covalent linkage [49]. This deposition promotes the adherence of bacteria to phagocytic cells by interacting with receptors on the phagocytes, initiating the process of ingestion [29, 99].

A few data are available on the amount of C3 in milk. In the study by Mueller et al. [63], complement C3 concentrations, expressed as a percentage of the normal blood concentrations, varied between 1 to 4% in milk whey (obtained with rennin) from healthy quarters. The same range was found in skimmed or untreated milk from Holstein cows [88].

The capacity of normal milk to generate a C3-convertase was first investigated through conglutination. Information on the cascade of the complement in milk came from the search for conglutinogen activity, which demonstrates the presence of the first complement components of the classical pathway up to C3, and the cleavage of C3b in C3bi, an event which allows conglutinin, a tetravalent lectin rather abundant in bovine plasma, to bind C3bi and agglutinate opsonized particles [9]. Conglutinogen activity (conglutination of erythrocytes sensitized with Ab) was found in milk from uninflamed glands in mid-lactation [23]. This finding suggests that the classical pathway functions in mature milk in the absence of inflammation. On the contrary, although conglutinogen is regularly found in milk towards the end of the lactation period, it is not found in mid-lactation milk [95]. This discrepancy may arise from a technical detail: in the former study, it is not specified whether the serum used to sensitize the erythrocytes was heated. If not, C1q was not destroyed and could attach to the immune complexes on the cells, thus explaining why the classical pathway apparently operated in normal milk, since C1q is the only missing component (see below).

The capacity of milk to deposit C3 opsonic fragments on bacteria has been explored using a strain of *Streptococcus agalactiae* as a test organism [86]. This strain activates the alternative pathway in the absence of antibodies and activates both the alternative and classical pathway when bacteria are sensitized with antibodies [85]. The ELISA used enabled the testing of neat, undiluted milk, and thus measured the true intrinsic activity of the milk complement, operating in its natural environment, including Ca^{2+} and Mg^{2+} ions. All the milk samples tested effected a substantial deposition of C3 fragments on bacteria (Fig. 2). Normal milk is roughly equivalent to 2% serum of adult cows or to 5–10% serum of precolostral calf serum in this respect. The kinetics of C3 deposition was slow: deposition began after a prolonged lag period (30 to 45 min), and the maximum was reached in 2 h or more (Fig. 3). Such a sluggish activation was reproduced by diluting the serum, suggesting that it is a consequence of the limited concentration of the complement in milk (Fig. 3). However slow, the deposition of C3 was of a sufficient magnitude to yield maximal ELISA values, indicating that the bacteria were saturated with C3. By contrast, C4 was not deposited on the bacteria incubated in normal milk (Fig. 4). This indicates that the classical pathway was not operating. This did not result from a lack of antibodies (added Ab made no change),

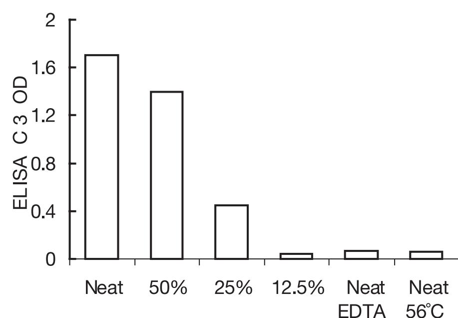

Figure 2. ELISA determination of C3 deposition on *S. agalactiae* effected by milk samples of uninfected, uninflamed glands (mean from 14 different cows). The bacteria were incubated for 2.5 h at 37 °C in neat or twofold dilutions of milk. Controls for complement activity involved the heat treatment of samples at 56 °C or the addition of 20 mM EDTA to chelate divalent cations. Data show that the capacity of milk to deposit C3 on bacteria vanishes rapidly upon dilution. Data from [86].

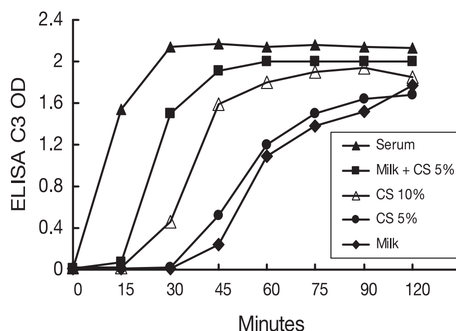

Figure 3. A comparison of the time course of C3 deposition on *S. agalactiae* effected by milk and serum, determined by ELISA with whole bacteria as the antigen. One feature of C3 deposition in milk is a prolonged lag phase, which is absent with pooled serum from adult cows (containing Ab to *S. agalactiae*, allowing the activation of the CP). Precolostral calf serum (CS), devoid of Ab to *S. agalactiae*, does not activate the CP, and the lag phase characteristic of the AP is present. Both the duration of the lag phase and the velocity of the deposition depend on the dilution of CS. Milk was equivalent to 5% CS. Deposition of C3 in normal milk is slow: more than 2 h are necessary for full deposition of C3 to take place in milk. Data from [86].

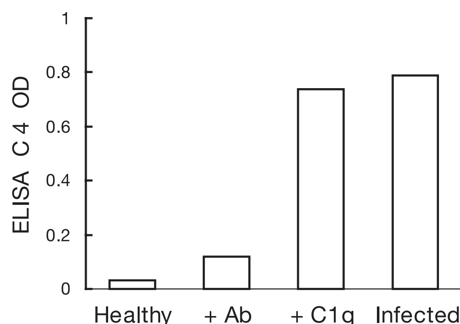

Figure 4. ELISA determination of the deposition of C4 on *S. agalactiae*. With milk of healthy glands, there is no deposition of C4, indicating that the deposition of C3 is not by the CP and suggesting that it is effected by the AP. Addition of Ab to *S. agalactiae* was without an effect, indicating that the lack of Ab is not responsible for the absence of activation of CP. The addition of purified bovine C1q (32 µg/mL) enabled the deposition of C4, indicating that the shortage of C1q precludes the activation of CP. In milk from infected glands, deposition of C4 takes place, suggesting that blood-derived C1q enabled the CP to operate. Data from [86].

but from the insufficient amount of C1q (only 150 to 250 ng/mL) available in milk [86]. The addition of purified C1q to milk accelerated the deposition of C3 and permitted the deposition of C4 on bacteria. Both C3b and C3bi were detected on bacteria: a proportion of deposited C3b on bacteria was cleaved in C3bi, as shown by the fixation of conglutinin and by the electrophoretic migration pattern of the C3 fragments eluted from the bacteria. Generation of C3bi on bacteria suggests that milk contains the regulatory complement components factor H, which displaces the fragment Bb of the C3-convertase, and factor I, which cleaves C3b in C3bi. The fragment C3b interacts with the receptor CR1 (CD35), which is present on bovine milk neutrophils [72], but the presence of C3bi is of consequence, because it interacts with another complement receptor, CR3 (the β_2 integrin CD11b/CD18), which is also important for phagocytosis by neutrophils [27].

Overall, these results suggest that the bacteria which activate the alternative pathway of the complement could be opsonized efficiently by normal milk. The consequence of deposition of C3 with regards to phagocytic efficiency was seldom checked in the case of opsonization with milk: it has been reported that killing of *S. agalactiae* in milk depends on the presence of immunoglobulin with the complement at the beginning of the inflammatory reaction (12 h post-infection), but that the heat-treatment of milk was without an effect later in the course of infection [62]. In serum, inactivation of the complement by heating was shown to decrease phagocytic killing by neutrophils for some of the mastitis isolates of *S. agalactiae* [90].

In mastitic milk, deposition of C4 on bacteria generally occurs (depending on the magnitude of inflammation), and deposition of C3 is quicker and higher than with normal milk, reflecting higher amounts of the available complement and the contribution of the classical pathway [86].

One of the specific components of the AP, factor B, was shown to be present in normal milk, at a low concentration (about 2 μg/mL) representing 0.6% of the concentration in the serum [83]. Factor B was apparently not a limiting factor for the functioning of the AP, since the addition of purified factor B to normal milk did not improve C3 deposition on *S. agalactiae*. Antibodies to factor B inhibited the deposition of C3 on bacteria, another indication that the AP is the sole pathway of complement activation in normal milk. There is no information on the lectin pathway of complement activation in milk, in particular on the presence of its specific components in milk. This pathway operates in the absence of C1q, whose function is taken over by the mannan-binding lectin (MBL). MBL is a C1q-like molecule which can activate C4, C2, and later acting complement components in the presence of serine proteases similar to but distinct from C1r and C1s via the lectin pathway [107]. Because C4 was not deposited on bacteria incubated in normal milk, the lectin pathway was not responsible for the activation of the complement and deposition of C3.

These results, obtained with *S. agalactiae*, have been shown to apply to *S. aureus*: in normal milk, C3 deposition takes place on staphylococci, whereas deposition of C4 does not, and the deposition of C3 can be blocked with Ab to factor B [2]. Opsonization with C3 by the AP in milk improved the oxydative burst of phagocytosing neutrophils, but ingestion and phagocytic killing were quite similar in heat-treated (56 °C for 1 h) and untreated milk samples, suggesting that milk Ab were the efficient opsonins and that the contribution of milk complement to opsonization was negligible.

3.4. Recruitment of phagocytes

Activation of the complement at a site of injury results in the production of the proinflammatory mediators C4a, C3a and C5a. As far as neutrophils are concerned, C5a is the most biologically significant peptide. It increases vascular permeability, is a potent chemoattractant for neutrophils, eosinophils, basophils, macrophages, and subpopulations of lymphocytes, and induces cellular responses from these cells such as the modulation of the phagocyte receptor for opsonins, increased oxidative metabolism, release of eicosanoids and degradative enzymes, and stimulation of cytokine synthesis (IL-1 and IL-6) [22, 29, 110]. Under natural conditions, serum carboxypeptidase N rapidly converts C5a to its derivative C5a-desArg by removal of the carboxy terminal arginine. In humans, the desArg form of C5a retains only a fraction of its activity [114]. It is not known if a carboxypeptidase is present in milk and which form of C5a predominates in normal or inflamed milk, but this may be of little consequence, because unlike human C5a, the

effects of C5a-desArg on PMN are practically identical to those of C5a [32]. The term C5a will be used below to engulf both C5a and C5a-desArg.

The inflammatory mediators responsible for the influx of neutrophils in milk have not been conclusively identified. The complement fragment C5a has been considered as a possible candidate, although the levels of the complement in milk were deemed too low to initiate chemotaxis [63]. Since then, C5a concentrations have been measured in milk [91]. Normal milk has been found to be virtually devoid of C5a (concentrations less than or equal to 0.1 ng/mL), but some C5a could be generated by activation of the complement with zymosan (yeast cell wall), indicating that a C5-convertase can be assembled in normal milk. In this study, only 1.1 ng/mL (range 0.68–2.17 ng/mL) of C5a could be generated. In a subsequent study [88], involving a greater diversity of cows, it appeared that a variability exists among animals, with generated C5a concentrations ranging from 0.6 ng/mL to 20 ng/mL in the milk from uninflamed, uninfected quarters. Concentrations of 25 and 50 ng/mL have been shown to induce the migration of bovine neutrophils through a cell culture system comprising a mammary epithelial cell monolayer [105], whereas 5 ng/mL were without an effect. These findings suggest that the amount of C5a which can be generated in the milk of certain cows could be of functional significance, whereas others would not be. Good producers of C5a had 6 times as much C5 in milk than low producers, indicating that C5 may be a limiting factor for C5a generation in normal milk [88].

Concentrations of C5a in the milk of quarters suffering from clinical mastitis can exceed 50 ng/mL [104]. Only part (around 10%) of the complement is consumed in vivo, since tenfold higher C5a concentrations can be generated in mastitic milk samples by ex vivo activation with zymosan [91, 98]. The early appearance of substantial amounts of C5a in mastitic milk after inoculation of the quarters with *Escherichia coli* was interpreted as an indication of its importance in the initial recruitment and activation of neutrophils to a developing *E. coli* mastitis [104]. On the contrary, milk samples from subclinical infections induced with *S. aureus* did not exhibit any increase in C5a concentrations [98].

The pioneering observation of O'Gairbhidhe et al. [68] is compatible with what we now know of the generation of C5a in milk. They showed that milk from mammary glands pretreated with endotoxin became chemotactic in vitro for bovine PMN when incubated with *S. aureus* (2×10^6 cfu/mL). This milk, although inflammatory, was not chemotactic by itself, probably because the complement had not been activated in vivo. The addition of bacteria in vitro enabled the activation of the complement and the generation of C5a. Heating milk at 56 °C for 30 min before incubation with bacteria halved the chemotactic activity, suggesting that the complement system was involved. The remaining activity may still be complement-dependent, because heat-inactivation of the complement in milk is complete only after one hour at 56 °C [83]. Finally, the heating of milk after incubation with bacteria did not reduce the chemotactic activity, in agreement with the heat stability of C5 and C5a [52, 111].

The infusion of purified bovine C5a into the teat cistern of cows induced a significant accumulation of leukocytes, mainly neutrophils [76]. This investigation demonstrated that C5a can act in vivo through an intact epithelium. A slight increase occurred as early as 1 h after the infusion of 65 ng of C5a. It must be noted that in this model, based on a surgical procedure separating the teat and udder cisterns [75], the agents introduced in the teat cistern are not in contact with the milk. The infusion of plasma activated with

zymosan (ZAP, a source of activated complement) into the mammary glands of non-lactating ewes or cows evoked a massive neutrophil influx [16, 17]. However, ZAP lacked an inflammatory activity in lactating ovine mammary glands [17]. Further investigations extended this observation to the bovine udder: ZAP induced a cell influx in the dried but not in the lactating mammary gland [17]. By mixing ZAP with whole ewe milk, the in vivo inflammatory activity was abrogated, whereas mixing with skimmed milk was without an inhibitory effect [17]. This result indicates that the components of whole milk inhibit the inflammatory activity of ZAP. One simple assumption is that the milk fat globules interfered with the inflammatory activity of C5a, but the investigations with bovine milk fat globules and purified bovine C5a showed that fat globules do not trap or inactivate C5a [84]. Whatever the underlying mechanism, the inhibition of the C5a recruiting activity by whole milk casts doubt on the contribution of the intrinsic milk complement in the initiation of the inflammatory reaction of the lactating mammary gland.

At the time being, whether complement factors are of importance for the triggering of cell migration in the bovine udder remains uncertain. On the one hand, the amount of C5a which is generated in the milk by low numbers of bacteria in a medium containing only limited amounts of complement may be insufficient to trigger inflammation. Also, milk may counteract the activity of the low amounts of C5a generated. On the other hand, levels of C5a measured in milk may be irrelevant (or only indirectly relevant) to the initiation of inflammation. The activation of the complement in the close vicinity of (at the surface, within, or beneath) epithelial cells may be of higher significance. It is possible that the intact epithelial barrier constitutes an obligatory relay for the inflammatory mediators generated in milk. This probably applies to bovine C5a which is an extremely charged molecule [32], even more cationic than human C5a, which may combine non-specifically with anionic sites on certain cells and heparin proteoglycan complexes [117]. Receptors for C5a have been demonstrated on human bronchial and alveolar epithelial cells, as well as in the tissue of the kidney and the intestine [40], but this information is lacking as far as mammary epithelial cells are concerned [69]. Apart from a direct chemotactic effect, C5a can also function as an autocrine activator to promote chemokine generation by macrophages or epithelial cells, thus acting through a cascade of activation to initiate or enhance the recruitment of leukocytes at the sites of infection [21, 30]. The occurrence of C5a receptors on mammary epithelial cells would lead to a reappraisal of the C5a capacity to start inflammation: the activation of the complement by bacteria in the vicinity of, or adhering to the epithelium would generate C5a molecules which could associate with receptors before being inactivated or trapped by some milk component or simply diluted out, and convey an inflammatory signal to the epithelium. Under these conditions, it is the local production of C5a at the sites of infection which matters, not the generation of C5a in the milk compartment, a simple indicator of the potential production of the activated complement.

Streptococci of human origin have been shown to interfere with the activity of C5a, and in particular group B streptococci (*S. agalactiae*) produce a surface-bound protease that specifically inactivates C5a [15, 46]. This C5a peptidase reduces the acute neutrophil response to group B streptococcal infections [6]. The inactivating activity of the C5a peptidase is restricted to certain animal species: the mouse, rat, guinea pig, rabbit, pig and sheep C5a preparations retain their chemotactic activity after exposure to group B streptococci C5a peptidase, but human and bovine C5a preparations are inactivated [5]. This finding raises the question of the production of C5a

peptidase by mastitis-causing strains of *S. agalactiae*, because the ability of this protease to inactivate bovine C5a could contribute to the pathogenesis of mastitis.

3.5. Priming and activation of phagocytes

In addition to recruiting phagocytes to sites of inflammation, C5a also stimulates other important functions of these leucocytes such as degranulation, activation of oxidative metabolism, and increased expression of CR1 and CR3 [29, 114, 117]. It has been hypothesized that phagocytosis by neutrophils is a recruited function at inflammatory sites, presumably to limit the amount of the damaging products secreted or generated during the migration to the site of inflammation [36]. According to this view, neutrophils phagocytose actively only after a contact with priming agents such as certain cytokines or inflammatory agents. Priming optimizes the activity of neutrophils in a two-step process, in which one stimulus "primes" the cells for more effective signal transduction by a second stimulus [31]. By deleting the gene for the C5a receptor in mice, the chemotactic-independent activity of C5a was illustrated: mice deficient in the C5a receptor were unable to cope with lung infection by *Pseudomonas aeruginosa*, despite an intense accumulation of neutrophils comparable to the influx occurring in mice with the normal C5a receptor [48]. It is likely that other couples of chemoattractants/receptors had compensated for the lack of C5a receptor, but not for the signals initiated by the activated C5a receptors, which ultimately enabled the neutrophils to kill the bacteria [42].

Unstimulated bovine neutrophils proved to be poorly efficient in phagocytosing opsonized *S. agalactiae* [87] or *S. aureus* [92]. Stimulating agents were necessary for effective phagocytic killing. The lack of stimulation of PMN becomes apparent when opsonized bacteria are washed free of serum before addition to neutrophils, because the serum contains non negligible amounts of C5a [91], thus causing inadvertent activation of PMN. Although less efficient than TNF-α, purified C5a dramatically accelerates the ingestion and intracellular killing [92]. The concentration of C5a which provides the highest activity (80 ng/mL) is of the order of magnitude of what can be found in milk during mastitis caused by *E. coli* [104], suggesting that the priming of neutrophils by inflammatory milk is of biological significance in cases of clinical mastitis. Neutrophils are probably not stimulated by C5a in milk during subclinical mastitis induced with *S. aureus*, because C5a remains undetectable [98].

Generation of C5a in milk could also stimulate both the recruitment and the bactericidal activity of neutrophils in an indirect way: it has been shown that macrophages, upon stimulation with C5a, react by secreting inflammatory mediators, such as TNF-α and IL-1β, and chemokines such as IL-8 [34, 51, 117]. The response of mammary macrophages to C5a is unknown in the bovine species.

Other complement fragments can activate phagocytes. Human Bb has been shown to stimulate the intracellular killing of *S. aureus* [60]. The activation of bovine monocytes and neutrophils by the Bb fragment of bovine factor B was also demonstrated [103]. In particular, the intracellular killing of *S. aureus* by bovine monocytes was improved in the presence of 25 μg/mL of the Bb fragment [102]. The concentration of fB in milk is about 2 μg/mL in normal milk [83], consequently a high concentration such as 25 μg/mL of Bb cannot be reached in normal milk by the activation of the intrinsic complement. The contribution of Bb to the activation of the neutrophil activities during clinical mastitis, when higher concentrations of the complement are available in milk, is possible but remains to be established.

4. LIMITATIONS OF CP ACTIVITY IN THE MILK BY INHIBITION AND DEFICIENCIES

4.1. Inhibition by bovine milk of the complement-dependent hemolytic and bactericidal activities

The complementation test used to detect hemolytic activity revealed that milk exerts an inhibitory activity which masks the low hemolytic activity present in the milk of healthy glands in full lactation and allowed the measurement of this activity [77]. The inhibitory activity was of the same order of magnitude as the complement hemolytic activity of milk or slightly higher (0.34 CH50 vs. 0.18 CH50) [89]. It vanished quicker than hemolysis on dilution, and consequently the samples were assayed at a 1/4 dilution of whey [77]. Such a limited inhibitory activity of milk had been mentioned previously, and heating milk (at 80 °C for 10 min) slightly increases inhibition [96].

The inhibitory activity of milk exerts itself also on the bactericidal activity [89]. Milk protected serum-sensitive *E. coli* from the killing effect of diluted bovine serum to a slight degree, but this protection was not detectable with a dilution less than 1/40. The protection was slightly better with milk heated at 56 °C for 30 min, either because of the inactivation of the milk complement, or because of an increase in the inhibitory activity of milk. Inflammation, in subclinically infected mammary glands, was associated with increases in hemolytic titers and heat-labile bactericidal activities [89]. Since the impairment of the complement-dependent bactericidal activity is overcome by concentrations of the complement corresponding to less than 1% of the serum values, it is not surprising that milk becomes bactericidal for serum sensitive strains in the course of clinical mastitis [11, 43, 80].

It is worth emphasizing that the adverse effect of milk on the hemolytic and bactericidal activities of the complement is inhibitory, and not, sensu stricto, anti-complementary. Anti-complementary compounds refer to substances that initiate the complement activation in the absence of any specific antigen-antibody reaction. In effect, complement activity, assessed through bactericidal activity, is stable in milk for more than 7 hours at 39 °C, demonstrating that the complement is not consumed by anti-complementary compounds intrinsic to normal milk [89]. In mastitic milk, the complement is not massively activated, although bacteria and soluble bacterial antigens are present in the milk along with Ab: although C5a was detected in sizeable amounts (20 ng/mL) in the milk of cows infected with *E. coli*, the in vitro activation of milk samples with zymosan yielded far higher amounts of C5a (900 ng/mL), demonstrating that most of the milk complement was still available for activation [98].

A possible milk inhibitor of the complement is lactoferrin (Lf), because Lf has been shown to inhibit C3 deposition on solid-phase bound immune complexes by modulating the classical C3 convertase [56]. Deposition of C4 is not affected, and the inhibition is probably taking place in the fluid phase, because Lf did not bind to the complexes. Several bacterial species associated with mastitis bind Lf at their surface, like coagulase-positive and negative staphylococci and *S. agalactiae* [66, 67, 81], which makes Lf a potential modulator of complement activation at the bacterial surface. In fact, activation of the classical pathway resulted from the binding of Lf to the surface of *S. agalactiae* [82]. Added at physiological concentrations in mature milk (0.01 to 0.04 mg/mL), Lf substituted for Ab for the binding and activation of C1q at the surface of *S. agalactiae*, thus activating the classical pathway. This activating rather than inhibitory activity may relate to the presentation of Lf, adsorbed on particulate activators of the complement as opposed to it being free in the fluid phase. Another study with Lf-binding

bacteria showed that the deposition of C3 on *S. aureus* in the absence of specific Ab was promoted by Lf at concentrations higher than 0.1 mg/mL, with an optimum at 1 mg/mL, whereas concentrations higher than 10 mg/mL are inhibitory [54]. The deposition of C3 followed the binding of Lf at the surface of the staphylococci, and resulted presumably from the activation of the alternative pathway. It may be that relatively high concentrations of Lf are necessary to activate the alternative pathway, whereas low concentrations of Lf may activate the classical pathway, and that the effect of Lf depends on the bacterial species and strains. In conclusion, Lf at physiological concentrations is unlikely to be responsible for the inhibitory activity of milk.

The comparison of the inhibitory activity of whole milk, skim milk, and whey from milk samples of uninfected quarters showed that defatting slightly reduces the inhibitory effect, but that the removal of casein (with rennin) is more efficient in this respect [77]. Milk whey retained a noticeable inhibitory activity, which was reduced to less than 20% of its initial value by a 1/4 dilution (Fig. 5). Another observation, that more complete clarification of the milk samples is obtained by precipitation of casein with rennin than by ultracentrifugation and that cloudy samples were devoid of hemolytic activity, on the contrary to clarified samples, is in favor of the contribution of casein micelles to the inhibitory activity of milk [24]. It is thus likely that fat globules, casein micelles, and soluble factors contribute to the inhibitory effect. Human milk is also endowed with a complement inhibitory activity [71]. The inhibitory activity was associated with fat globules, casein and soluble components, but on the contrary to bovine milk, the fat portion of human milk exerted a greater effect than whey did. The milk fat globule membrane, which is derived from the apical region of the mammary gland epithelial cells, may contribute to complement inhibition, because it is rich in

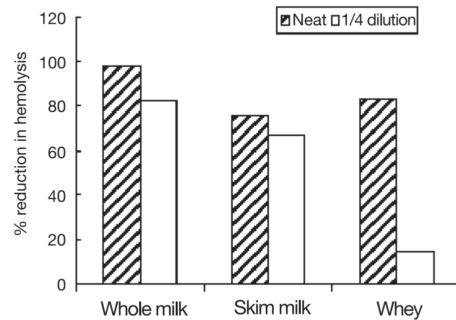

Figure 5. Inhibition of hemolysis by milk. One hemolytic unit (HU) 50% of bovine serum was incubated with either whole milk, skim milk or milk whey, and the % inhibition of hemolysis was calculated. Skim milk retained much of the inhibitory activity, but milk whey was less inhibitory as shown by its reduced inhibition when diluted. This suggests that casein plays a major part in the inhibition of hemolysis. Data from [77].

protectin (CD59), a cell-surface complement regulatory protein that binds and inactivates the membrane attack complex [38]. Protectin has also been found in a soluble form in human milk and colostrum [4, 38].

It is worth noting that the inhibitory effect of milk exerts itself on the hemolytic and bactericidal activities of the complement, not on the deposition of C3: the sluggish kinetics and the moderate magnitude of deposition can be accounted for by the limited concentration of the complement in mature milk. Generation of C5a does not seem subjected to an inhibition either [88]. This points to an inhibitory effect targeting the complement membrane attack complex.

4.2. Other inhibitions, deficiencies and peculiarities of the bovine milk complement

Besides impairment of the hemolytic and bactericidal activities, milk was shown to interfere with the deposition of C1q on streptococci: the C1q ELISA signal obtained after incubation of bacteria with

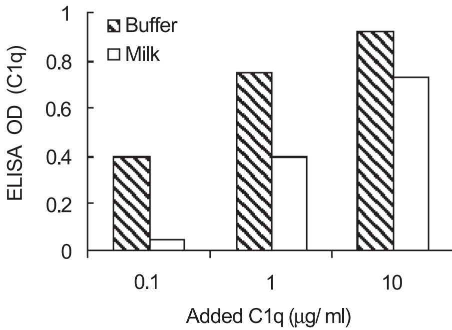

Figure 6. Deposition of C1q on Ab-coated *S. agalactiae*, measured by ELISA, as a function of added purified bovine C1q. When C1q deposition takes place in milk, ten times as much C1q is necessary to achieve the deposition occurring in the buffer. This shows that milk exerts a substantial inhibition on the interaction of C1q with Ag-Ab complexes. Data from [86].

increasing concentrations of purified C1q was different in milk as compared to the dilution in the buffer (Fig. 6), indicating that the deposition of C1q on bacteria was impaired [86]. In conjunction with the shortage of C1q, this interference may account for the impairment of the classical pathway in milk, at the first step of its activation.

Protein C5 is another component whose concentration is limiting, but only in the milk of certain cows [83]. This relative deficiency in C5 is associated with a negligible potential for generation of C5a, and may be of consequence for the initiation of the inflammatory response of the mammary gland.

Whole milk has been shown to interfere with C5a for the recruitment of PMN in the milk of lactating ewes and cows [17]. This inhibition, whose mechanism is unknown, may also be of consequence for the triggering of inflammation at the onset of infection.

There is no information on the concentrations of the components constituting the membrane attack complex (C6 to C9) in bovine milk. The possibility that the absence of hemolytic activity means mainly the shortage of the membrane attack complex components has to be considered.

The study of the complement in bovine milk is complicated by the low concentrations of its components, and also by peculiarities of the bovine milk complement. Two technical pitfalls may result from these idiosyncrasies. The unavailability of the commercial supply of purified components of bovine complement and of specific antibodies entails that most studies make use of indirect means, such as chelation of Ca and/or Mg cations or mild heating to preferentially inactivate certain complement components. Resorting to these indirect and loosely specific procedures is not devoid of shortcomings. In particular, experiments with mycoplasmas and *Escherichia coli* indicate that the effect of Mg-EGTA is not identical for bovine and human sera: killing by bovine serum is inhibited by Mg-EGTA, although it is mediated by the alternative pathway, whereas killing by human serum is not inhibited [50]. Mg-EGTA also inhibits the deposition of complement C3 on *Streptococcus agalactiae* in the absence of antibodies, although under these conditions, it was demonstrated that it is effected by the AP [85]. On the contrary, when Mg-EGTA in bovine serum was used to study the effect of lactoferrin (Lf) on the deposition of C3 on *S. aureus* or the bactericidal activity of serum on *E. coli*, C3b deposition or killing were not inhibited by this treatment [44, 54, 116]. It is likely that under at least certain experimental conditions, the effect of EGTA is the inhibition of both the CP and AP in bovine serum. This peculiarity is largely ignored, and in many publications reporting experiments with the bovine complement, Mg-EGTA is assumed to selectively block the CP, and the selective blockade is not checked. The method of choice with bovine serum as a source of the complement is desalting by gel filtration

and reconstitution with Mg or Mg/Ca cations [50, 85]. Unfortunately, this procedure suppresses all complement activity in normal milk.

Another commonly used procedure is mild heating of biological fluids to selectively inactivate the most susceptible components of the complement system. Usually the most heat-labile component is factor B. This is true in bovine serum, and heating at 56 °C for 3 min or at 50 °C for 45 min selectively depresses the alternative pathway [73]. In bovine milk nevertheless, factor B resists mild heating, and the alternative pathway remains active. Heating milk at 56 °C for at least 45 min is necessary to completely inhibit C3 deposition on bacteria [83].

5. THE BIOLOGICAL SIGNIFICANCE OF THE COMPLEMENT IN MILK AND AREAS FOR FUTURE RESEARCH

The biological relevance of complement-dependent bactericidal activity against serum-sensitive bacteria depends on both the host and the pathogen [109]. In the case of the bovine mammary gland, the bactericidal activity as an immune defense mechanism has been considered important because serum-sensitive bacteria were seldom isolated from mastitis cases. Mastitis-causing gram-positive bacteria are serum-resistant, and although *E. coli* of the environment are either serum-sensitive or serum-resistant, several studies showed that most mastitis isolates are serum-resistant [3, 10, 100]. In other studies nevertheless, serum resistance was not a prerequisite for coliform bacteria to cause naturally occurring intramammary infections, because both serum resistant and serum susceptible strains were isolated from the milk of infected quarters [28, 47]. This discrepancy may result from differences in the assessment of the degree of serum resistance, such as the percentage of serum used in the assay, or the technique used to assess the survival of bacteria. This is not the only explanation, because in one study, the percentage of serum-resistant isolates varied from high to low according to the geographical origin of the strains [55]. In the absence of a standardized assay, the degrees of resistance or susceptibility of bacterial isolates are impossible to compare from one study to another. Importantly, serum-susceptible strains, when inoculated into a healthy mammary gland, are unable to cause mastitis [3, 14], an observation which is probably linked to the early appearance of heat-labile bactericidal activity in milk at the onset of an inflammatory response to *E. coli* mastitis [43, 80]. Overall, it is likely that a certain degree of serum resistance is a necessary attribute of mastitis pathogens. In the case of coliform bacteria, serum-resistance is likely to contribute to the ability of inducing clinical mastitis. On the contrary, complement-dependent bactericidal activity may be considered of a limited scope, because bacteria commonly isolated from mastitis cases are serum-resistant. Other immune defenses are thus necessary to cope with these pathogens.

Phagocytosis by neutrophils is a major immune defense against serum-resistant mastitis pathogens. The first element which determines the importance of the milk complement to phagocytosis by neutrophils is the requirement of the complement for effective opsonization of the given bacteria. Unfortunately, this information is sparse concerning mastitis-causing pathogens. Many studies have shown that the complement is not indispensable for opsonophagocytic killing by neutrophils, as far as *S. aureus* [92], *S. agalactiae* [90], *E. coli* [45], or *S. uberis* [35] are concerned. Whether the complement improves the antibody-dependent opsonization is much less documented. It would seem that the generalisation of the results obtained with strains of medical interest to mastitis-causing strains is disputable: the complement is either required or useful for phagocytosis of

S. aureus or *S. agalactiae* of human origin [41, 60, 112], but it is only of secondary importance for mastitis isolates of *S. agalactiae* and possibly useless for *S. aureus* respectively [2, 90]. More research on opsonic requirements of mastitis-causing bacteria is required to delineate the contribution of the complement. Strain variation and the effect of culture conditions on the susceptibility to phagocytosis are likely to compound the issue. From the published literature, it undoubtedly appears that antibodies are the major opsonins in milk, and that the complement may play an auxiliary part in opsonization. The second condition that should be fulfilled for the complement to contribute to phagocytosis is its availability in a sufficient amount in milk. The amount of the complement or the capacity of the bacteria to activate the AP may be limiting in normal milk, but as soon as an inflammation develops and exudation of plasma begins, enough complement is available in milk to deposit C3 by both the alternative and classical pathways. The secretion of complement components by mammary epithelial cells, and the effect of inflammatory mediators, such as cytokines [1], on this local source of the complement would deserve investigations. Also, the amount of the complement and its activities in mammary secretion during the dry period have received little attention. Yet, many infections affecting the periparturient cow originate from this lactation stage, and a better knowledge of complement activity could help understand why certain bacteria are more likely to cause infection at or near calving.

The physiological significance of the inhibition of complement activities by milk is also to be considered. Overall, it appears that the pro-inflammatory activities of the complement system are dampened in bovine milk. Activation of the classical pathway by antibodies, cell recruitment by C5a, cell lysis by the membrane attack complex, all these events are weakened by milk. Inhibitory activities of human breast milk have also been reported [70, 71], and broad anti-inflammatory activities of human milk have been documented [33]. The activation of the complement has the potential of attacking membranes of neighboring cells. This unwanted event could take place either in the mammary gland during infection, or in the intestine of milk-fed newborns. Inhibition of the membrane attack complex may prevent such tissue damage, and thus contribute to protection of the mammary gland and the gastrointestinal tract of the newborn [70]. Inhibition of the complement membrane attack complex may operate at the expense of a reduced bactericidal efficiency, but other activities of the complement system, such as opsonization and presumably the modulation of cellular and humoral immune responses, are spared by the milk inhibitory mechanisms, allowing the complement system to play essential defense roles. Even the inhibitory effect of milk on the complement-mediated bactericidal activity is likely to be of a limited significance for the defense of the mammary gland, because a mild inflammation is sufficient to overcome this inhibition. Challenge experiments showed that mammary secretion displays bactericidal activity on serum-sensitive bacteria early in the course of infection [43, 80]. This is substantiated by the low pathogenicity of serum-sensitive bacteria for the bovine mammary gland [14]. It can be concluded that the inhibitory effect of milk on inflammation is selective and limited in magnitude.

The activation of the complement at the surface of the bacteria generates C5a, and the chemotactic and activating properties of this molecule towards neutrophils may be of major biological significance. Activation of neutrophils is required for intense phagocytosis and killing of bacteria, and C5a is required under certain conditions [42]. It remains to be determined if this is the case of bovine mastitis. At rather high concentrations (1 to 10 nM), which can be achieved next to the site of complement activation, C5a causes degranulation of neutrophils, and release of properdin, the

Table II. Characteristics of the complement in milk in relation to physiological and pathological status.

Properties	Colostrum	Mature milk			Dry secretion
		Normal	Subclinical	Clinical	
Amount	moderate	low	variable	moderate to high	moderate
Inhibitory effect	rather high	low	low	low	moderate
Hemolytic, bactericidal activities	low	no	low	moderate to high	moderate
Deposition of C3b	ND	slow, moderate AP	variable AP + CP	quick, intense AP + CP	ND
Generation of the C5a					
Potential	ND	variable among individuals	low to moderate	high	ND
Actual	ND	no	no or low	moderate	ND

ND: not determined.

positive regulator of the alternative pathway of complement activation [115]. This can initiate a positive feedback loop of complement activation, resulting in an enhanced AP activity at the proximity of interacting bacteria and neutrophils [101], all the more so since neutrophils store and secrete C3 and fB [115]. The role of C5a in the initiation of the inflammatory response in the mammary gland is still a matter of speculation. The answer to this question awaits the finding of receptors to C5a at the apical face of the mammary epithelial cells and the determination of their functional ability. At other epithelial surfaces such as the lungs of rats, the outcome of the exposure of alveolar epithelial cells to C5a is the release of proinflammatory cytokines and the enhanced expression of the C5a receptor, leading to an enhanced phlogistic response [97]. There is some indirect evidence that mammary epithelial cells react to C5a [76, 105]. If the contribution of C5a to the triggering of mammary inflammation proved true, it would be justified to address the origin of the variability of C5 concentration in milk (acquired or of genetic origin) [88], because it could have consequences on the cow'susceptibility to mastitis.

In conclusion, the contribution of the complement system to the defense of the mammary gland is very different before and after the inflammatory response, reflecting the variation of the amounts and activities as a function of the physiological and pathological states (Tab. II). In the healthy gland, selective deficiencies and inhibitions limit the scope of the possible contribution. When sizeable inflammation develops, larger amounts of the complement are mobilized from the blood, and the complement is able to play its normal part within the frame of innate and specific immune responses. The uncertain zone concerns the triggering of inflammation and the state, frequently met during subclinical long-lasting infections, characterized by very weak passive transfer of blood components. The local response of resident cells (macrophages and epithelial cells), and of recruited leukocytes, deserves to be investigated, since it could help differentiate animals more or less resistant to mastitis.

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