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Inhibitors of complement activity in human breast-milk: a proposed hypothesis of their physiological significance

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Several natural components abundant in the fluid phase of human breast-milk have been shown to be inhibitors of complement activation *in vitro*, particularly the classical pathway. These include lysozyme, lactoferrin, lactalbumin alpha and other ligand chelators, complement regulator proteins and other specific soluble inhibitors of complement activation. Their physiological significance probably resides in their ability to restrict *in vivo* complement activation to specialized (compartmentalized) sites on the cellular membrane structures in human milk, represented by the abundant surface area of the milk fat globule membranes. This would serve to prevent inflammatory-induced tissue damage of the delicate immature gastrointestinal tract of the newborn as well as the mammary gland itself. A number of recognized and potential inhibitors of complement activity in human milk and other biological fluids are hereby reviewed, with a proposal of their physiological significance.

Abbreviations: HBM, human breast-milk; APC, alternative complement activation pathway; MAC, membrane attack complex (C5b-9); MFGM, milk fat globule membrane

Key words: Human breast-milk, complement system, inhibitors, milk fat globule membrane

Introduction

The serum complement (C) system consist of at least 19 proteins, mostly in pre-activated enzymatic forms, activated in a multi-step cascade reaction via either the classical or alternative pathways (Figure 1). The classical pathway is activated mainly by antigen–antibody complexes (IgG or IgM mostly) starting with C1q.[1] The alternative pathway (APC) utilizes active sites (such as are present on zymosan, yeast, cobra venom, Gram-negative bacteria, sheep erythrocytes and human cells deficient in the expression of regulatory molecules) in the presence of properdin, serum factors B and D, to activate C3. The two pathways proceed uniformly after C3 activation to the formation of (C5b-9) membrane attack complexes (MAC), capable of inserting into biological membranes and producing cell lysis and death.[2]

The immunochemical levels of certain complement components in the human colostrum (C3, C4) have been found to approach that of normal serum levels.[3–5] However, only a small fraction of the serum complement activity is measurable using *in vitro* assays. A number of factors might be accountable for this effect. Firstly, there is apparently a relative deficiency of some of the essential components of the complement cascade system. For example, properdin, a stabilizer of fluid-phase alternative pathway convertases, has been reported to be either absent in human breast-milk (HBM), or only present in minute quantities less than 1 μg/ml.[6] It is also possible that some of the identifiable complement components, though native, might be haemolytically and physiologically inactive.[7] Thirdly, there is a wide array of natural human breast-milk components which have been shown to inhibit complement activity *in vitro*.

It has been observed that cow's milk contains some inhibitory effect on the activity of serum complement. This inhibitory effect in bovine milk has been partly ascribed to a prozone phenomenon of excessive antibodies in undiluted milk, and other unexplained factors in heated milk, particularly in casein micelles.[8,9] A similar inhibitory effect of breast-milk on the serum complement activity has also been

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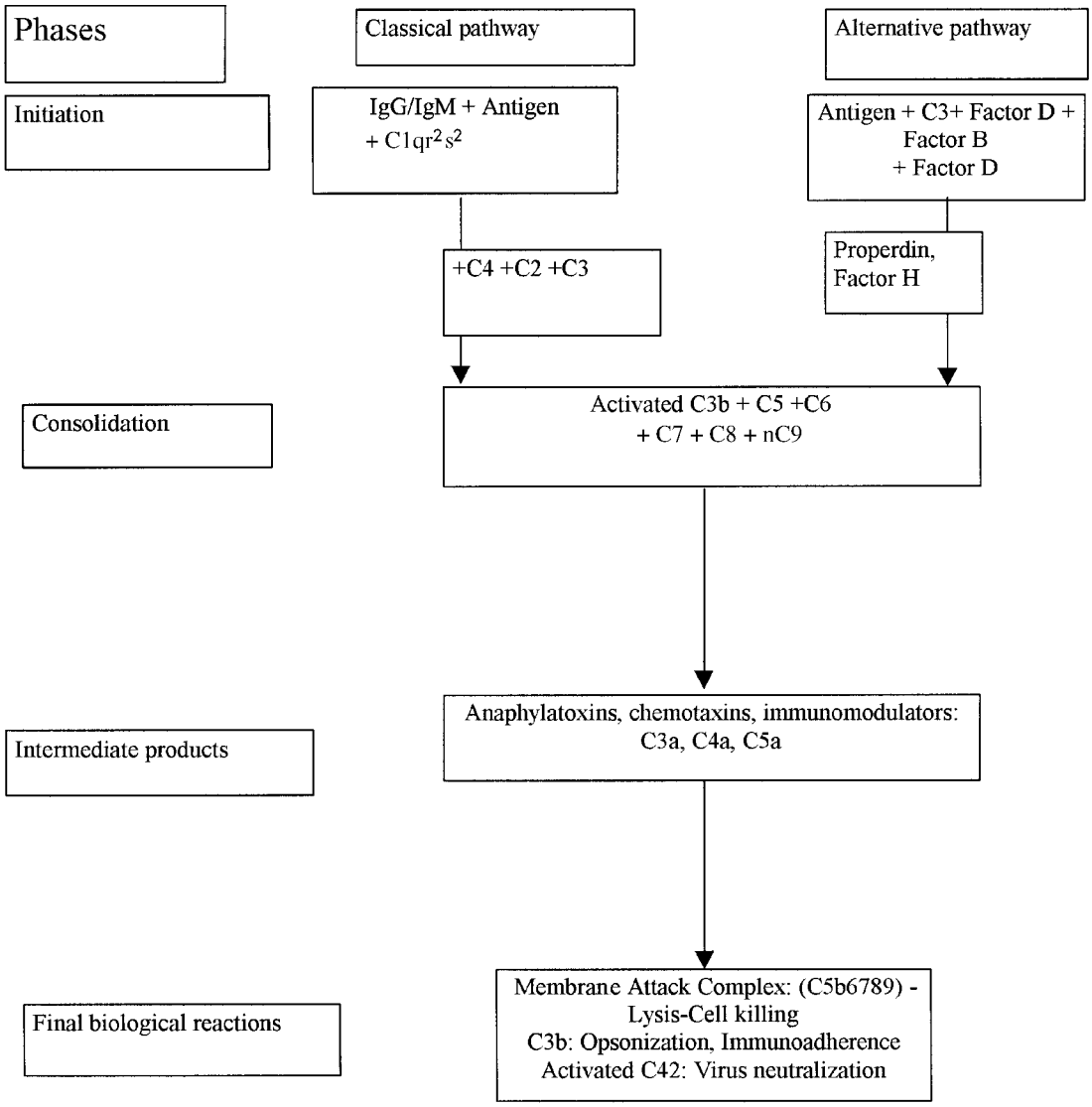

FIG. 1. Diagrammatic representation of the classical and alternative pathways of complement activation.

observed in human milk, although the inhibitory effect appears to reside mainly in its fat component.[10] This inhibitory activity presents unequivocal evidence for the presence of anti-complement factors in human milk (Table 1).

Soluble Inhibitors of Complement Activation in Human Breast-Milk

α-Lactalbumin and ligand ion chelators

Some milk proteins, such as α-lactalbumin, and low-molecular weight ligands, such as citrates and phosphates, are known to have high affinity binding sites for calcium.[11] They may therefore act as inhibitors of complement indirectly by chelating the divalent ions required for complement activation.[12]

Lactoferrin

Lactoferrin is a glycoprotein with an approximate MW of 80 kDa. It is produced by epithelial cells, neutrophils and monocyte macrophages. It is present as a major antimicrobial component of many body fluids, including tears, saliva, seminal fluid and pancreatic secretions. The highest concentration of lactoferrin (up to 6 mg/ml) is found in the colostrum and this gradually falls, as lactation proceeds, to a constant value in mature milk (approx. 1 mg/ml) after about 4 months of lactation. It is resistant to acid denaturation during its passage through the intestinal tract.[3,4]

Lactoferrin in tears from humans and several animals has been shown to inhibit the classical pathway of complement activation, by preventing the formation of C3/C5 convertase. It competitively

Table 1 List of proven and possible inhibitors of complement activity in human breast-milk

Breast-milk component	Effect on complement activity	Ref.
Lactoferrin	Inhibits the assembly of the classical C3/C5 convertase	13,14
Fat globule membrane	Non-specific target surface for active C fragments	10
Free lipids in solution	Chelation of calcium and magnesium, non-specific binding of lipophilic C fragments	12,34
Soluble protectin (CD59)	Inhibits MAC formation	21,22
Poly IgA and antigen-bound IgA	Inhibits IgG-mediated complement activation	18,19
Proteases	Non-immune complement consumption	18,27,28
Bacteria and other pathogenic contaminants	Consumption of classical or alternative path C components	30,31
Citrates, phosphates and proteins	Binding of ionic calcium required for optimal C activity	11,12
Lysozyme	Binding of ionic calcium required for optimal C activity, Hydrolysis of native C components	11,12,15
α-Lactalbumin	Binding of ionic calcium required for optimal C activity	11,12
Soluble IgG-, IgM-antigen complexes	Fluid-phase complement consumption	
Other unidentified factors	Non-specific C consumption	

inhibits the binding of C2 to EA14 cells. This inhibitory effect is dose-dependently reversed by the addition of Fe^{3+} ions.[13,14]

Lysozyme

Lysozyme is an acid- and heat-stable enzyme that is abundant in the breast-milk and in most other mucosal body fluids. The concentration of lysozyme in the colostrum is approximately 40–100 μg/ml and gradually increases as the lactation progresses.[3,4]

Lysozyme catalyzes the hydrolysis of the β(-1,4-) linkage between *N*-acetylglucosamine and *N*-acetylmuramic acid in the bacterial cell wall. The enzyme lyses mostly Gram-positive and a few Gram-negative bacteria, or induces their aggregation. Lysozyme interacts with other immunoprotective components of human milk in exerting its antimicrobial actions. It is bacteriocidal for *E. coli* and *Salmonella* spp. in the presence of sIgA and C3 complement component.

Lysozyme has been found to inhibit the classical pathway of serum complement activity in a dose-dependent fashion, especially within the range of pathological concentrations. The inhibitory effect of lysozyme seems to be minimal under physiological conditions in the absence of inflammation, since only a minimal inhibition was produced within this physiological range. The inhibitory effect of lysozyme might be related to its ability to degrade certain glycoprotein components of native complement factors.[15]

Native and aggregated immunoglobulins

IgA is the most abundant immunoglobulin in the colostrum and breast-milk, and constitutes the major protein content in the colostrum.[3,4] It has been long argued that IgA activates neither the classical nor the alternative complement pathway. Subsequent experiments, however, have shown that it is capable of limited activation of the complement system. IgA myeloma has been shown to activate the classical pathway.[16] Aggregated IgA and IgA coated onto plastic (possibly denatured), but not antigen-bound IgA have also been shown to activate the alternative pathway of complement (APC).[17] Fab fragments of IgA have also been previously discovered to be capable of activating the APC.[20]

In a solid-phase antigen-dependent C3b-binding ELISA system, IgA antibodies were unable to activate complement by either pathway. IgA antibodies were found to inhibit significantly the activation of complement initiated by antigen-bound polyclonal or mixed monoclonal IgG antibodies. This inhibition was found to be independent of the ability of the IgA antibodies to compete against the IgG antibodies in binding to either antigen or C1q.[18,19]

IgA Fab fractions have also been found to inhibit the activation of complement system. The invasive pathogenicity of certain mucosal bacteria has been postulated to be related to their possession of IgA1 proteases, which cleave secretory IgA1 antibodies to antigen-binding Fab-a fragments. These fragments are not only defective in mucosal defence properties, but also protect the organisms from other immune effector systems, such as the classical pathway of complement activation.[18]

Soluble forms of membrane complement regulatory proteins

Protectin (CD59), a cell surface complement regulatory protein, that binds and inactivates the

membrane attack complex (MAC), has recently been discovered to be secreted and embedded in milk fat globules.[21] It is also present in soluble forms in human milk.[22] The soluble forms could have been shed from the milk fat membrane, and possibly could also partly arise from aging neutrophils in breast-milk.[23] The extent of the inhibitory effect of this protein under physiological conditions, however, remains unknown. Decay-accelerating factor (DAF) has been detected on most epithelial cells at sites of mucosal immunity, and its soluble form has been found in various body fluids.[24] This complement regulatory molecule has, however, not yet been specifically reported in breast-milk or on mammary gland epithelial cells. Vitronectin, which has also been detected in human tears, is yet to be described in human breast-milk.[25]

Other specific complement cascade inhibitors

Factor H has been measured immunochemically in the human milk,[26] its serum level being approximately 0.233 μg/ml (0.12%). It is a specific inhibitor of the APC, and its relatively low level in human milk might raise doubts as to its physiological significance on mucosal complement activity. In our laboratory, C1-INH could not be detected in human milk, using a single radial immunodiffusion method with a detection limiting value of 0.048 g/l. Factor I (C3 inactivator) has also not been reported previously in human breast-milk.

Milk proteases as possible inhibitors of complement activation

Heat-stable milk proteases, including plasmin, are present in breast-milk. They have been shown to be capable of splitting β-casein in milk,[27,28] and could contribute to the complement-inhibitory activity of human milk by non-immune splitting of native complement components.

Other potential inhibitors

There is no conclusive evidence to suggest that all the possible inhibitors of complement system in milk have been identified. Further research might yet discover many other unidentified natural components or contaminants, which directly or indirectly contribute to the observed diminution of inflammatory reactions on the mucosal surfaces, in the presence of active potentially inflammatory protective mechanisms. This would be in line with the mucosal anti-inflammatory hypothesis proposed by Goldman and co-workers.[29] However, their suggestion of insignificant functionality of complement, based on the assumption of minimal levels in human milk, would not be supported by the discovery of levels of certain complement components approaching those available in serum. The presence of such a wide array of inhibitors, apparently serve teleologically to minimize the undesirable inflammatory processes, while permitting the infant to benefit from the other non-inflammatory activities of the complement.

Bacterial and other pathogenic contaminants of human milk might indirectly constitute a source of complement depletion, by wasting the potentially useful native components, and diminishing the levels effectively delivered to the nursing infant. This is particularly so, based on the observation that human milk is rarely sterile, often moderately contaminated with non-pathogenic normal skin flora.[30] It also sometimes contains potential pathogens, which however seem to produce no ill effects on the suckling infant.[31]

Similarly, soluble IgG or IgM complexes, with or without antigens, secreted into the milk from the mother's blood circulation, could potentially activate and deplete the mucosal complement.

Milk Fat Globule Membrane as an Inhibitor of Breast-Milk Complement

Human milk consists mainly of a protein, sugar and cellular suspension in 95% water and 2–5% lipid fraction. The cellular elements consist of macrophages, neutrophils, lymphocytes and mammary gland epithelial cells. The lipid fraction consists mainly of triglycerides enveloped in a complete trilaminar unit of biological cell membranes, the milk fat globule membrane (MFGM). This membrane is derived from the apical region of the mammary gland epithelial cells, and is budded off around the milk lipids as they are being secreted by the cells.[32] The MFGM is similar to any other cell membrane of eukaryotic cells[33]

The inhibitory effect of human milk has been found to be progressively diminished with increasing speed of centrifugation and the degree of de-fattening.[10] Two possible explanations have been proposed for these observations. The MFGM might be either activating the complement reaction cascade, and thereby depleting serum complement components, or acting as an alternative reaction site, preventing the assembly of the active complement components on the target sheep red blood cells (SRBC).

Free fatty acids (FFA) in milk are also capable of forming soaps with calcium and magnesium ions, thereby preventing optimal activation of the complement system reaction cascade.[12,34] They could also bind to the more lipophilic components of the complement system, preventing them from participating in activation reactions by making them chemically inactive.

Physiological Significance of Complement Activities and their Soluble Inhibitors in Human Breast-Milk

Despite the levels of some components comparable to those in serum, activation of human milk complement in the fluid phase has been found to be less than optimal under physiological conditions, requiring additional divalent cations. However, *in vitro* activities of human milk complement have recently been demonstrated, including haemolysis of sensitized SRBC and bacteriolysis of a serum-sensitive *E. coli, S. aureus* and *S. epidermidis*. Furthermore, non-immune mechanisms of complement activation have been demonstrated in the mucosal secretions of the lachrymal gland,[35] suggesting that the inhibition of classical pathways of complement activation at these mucosal sites could still be circumvented, to enable some of the physiological roles of the complement to be realized.

Apart from the complement and free fatty acids (FFA), most of the available antimicrobial agents in human milk are bacteriostatic. Since bactericidal FFA are also present in the artificial formula feeds, and they do not seem to contribute to the protection of formula-fed infants,[36] the complement system should be regarded as potentially the source of a significant contribution to the increased resistance of breast-fed infants against infection;[37] Furthermore, clinical studies have shown that a deficient secretion of complement components in human milk constitutes a higher risk factor for the development of mastitis in the lactating mother.[38] Breast-fed infants are also known to possess higher levels of complement components and activities compared to their formula-fed counterparts.[44] Human milk, therefore, constitutes an important source of either native components, absorbed from the infantile intestines, or some other humoral factors capable of enhancing the synthesis and activities of the complement system *in vivo*.

The levels of both mucosal lysozyme and most other soluble inhibitors tend to increase during periods of infection in parallel with the complement components.[38] Their inhibitory effects under physiological conditions might, therefore, be expected to be minimal, and sharply increase with the onset of full-blown infections. The protective functions of the various body defence factors at the mucosal surfaces, including those in the human milk, have been proposed to be carried out mainly with the exclusion of inflammatory processes.[29] Increased overall physiological activities of these mucosal factors would be an indication for a greater need for the suppression of inflammatory components of their activities, hence the need for increased inhibitory activities.

The observation of specific physiological functions attributable to the complement system, as listed above, in the presence of soluble inhibitors, would suggest that the mucosal complement might be actively involved in such activities as bacteriolysis, opsonization and immuno-adherence, neutralization of certain viruses, as well as modulation of immune responses at mucosal surfaces (see Figure 1), in the absence of, or with the suppression of inflammatory processes.[39] This would serve to protect the body against the constant threat of foreign invaders. The presence of secretory anti-complementary components would then serve to protect the tissues against secondary damage during acute overwhelming infections, where excessive inflammatory reactions of the complement might be undesirable.

An Hypothetical Viewpoint of the Physiological Mechanisms of Human Breast-Milk Complement Activation and the Role of Milk Fat Globule Membrane

The presence of such a wide variety of potent soluble inhibitors of complement activity in human breast-milk, particularly those of the classical pathway, would prevent their optimal physiological activities in the fluid phase. However, clinical and experimental evidence suggest that the complement system is physiologically active *in vivo*.[38,44] The activation of the complement might, therefore, be expected preferentially to take place outside the fluid phase, on the surface of any widely available solid phase, such as milk fat globule membranes, with the alternative pathway being favoured over the classical pathway.

In vivo, MFGM appears to be the most suitable template for complement activation, where all the products of complement activation could be segregated together to obtain a relatively high local concentration and maximal effect on the membrane-bound antigens. A number of observations and assumptions point to the possible role of the MFGM as the major site of complement activation *in vivo*.

Firstly, the MFGM possesses independent mechanisms for the sequestration and trapping of certain pathogenic bacterial antigens by attachment to its surface glycoproteins.[40] Secondly, our recent studies have provided evidence that many native and activated products of complement are closely bound to the MFGM. For example, in a modified CH50 assay, where non-specific haemolytic effects of FFA have been inhibited, up to 50% of complement activity is lost by de-fattening of whole-milk through centrifugation. The unidentified components of cow's milk capable of preferentially binding to C1q might be represented in human milk by the MFGM.[41] Thirdly, protectin (CD59), a membrane surface complement regulatory molecule, has been found to be expressed on the MFGM.[21] Homologous cells are protected from the lytic effect of complement through the expression of surface membrane regulatory molecules, such as decay-accelerating factors (DAF, CD 55), membrane

cofactor protein (MCP), homologous inhibitor of reactive lysis (protectin, CD 59), C3 receptors 1, 3 and 4 (CD35, CD11b, c/18). This would suggest a physiological mechanism by which the MFGM is protected *in vivo*, at least by protectin, in the presence of ongoing antigen-induced complement activation, without leading to a secondary damage of the MFGM. Lastly, most milk-borne macrophages are found to be laden with fat vacuoles which they have ingested *in vivo*.[42,43] The explanation for this observation would seem to be based on the deposition of C3 fragments or IgG on the MFGM, which opsonizes them for phagocytosis by macrophages.

The abundant supply of MFGM in human milk and its ability to bind to both native and activated complement components, as well as particulate antigens, which are also able to independently activate the alternative pathway, even in the absence of specific antibodies, makes MFGM the most likely site for physiological activation of human milk complement. While pathogens to which the mother is already sensitized might be disposed of by the large amounts of secretory IgA (sIgA) in breast-milk, newly acquired antigens from the environment, which might not be able to immediately stimulate the production of specific antibodies at the mucosal site, could be attacked by the breast-milk complement through the aid of the MFGM, thereby protecting both the mammary gland and the suckling infant against infection.

The MFGM might assume particular significance when the level of particulate antigens, such as bacteria, overwhelm the inhibitory ability of the sIgA and the other bacteriostatic components of human milk fluid-phase. They are then available to be bound on the surface of the MFGM where they could activate the complement system and subsequently be killed by lysis, without significant interference of the wide variety of inhibitors present in the fluid phase.

The ability of the MFGM to bind C3 opsonin fragments, thereby enhancing its phagocytosis and ultimate degradation by macrophages, might account for at least one of the mechanisms by which it is ultimately destroyed along the intestinal tract of the suckling infant.

Conclusion

Research in the field of complement system reactivity in human breast-milk is at a relatively early stage. Further efforts are likely to lead to the discovery of many interesting interactions between complement components and other natural components of human milk. The physiological significance of the complement in protecting the mammary gland and the nursing infant can no longer be denied. The presence of a wide range of inhibitors of complement probably serve to limit the activation of the complement system in the fluid-phase, avoiding the consequent risk of widespread damage to soft tissues by associated inflammatory processes, while promoting the solid-phase activation of the reaction cascade. The milk fat globule membrane thereby acts as a most suitable template to support the solid-phase reactivities.

Understanding the actual mechanisms of the complement reaction cascade and interaction with such a wide variety of inhibitors present in the aqueous phase of human milk, is the real challenge for investigators in the coming decades.

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