Milk immunoglobulins and complement factors

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The importance of colostrum for the growth and health of newborn offspring is well known. In bovine colostrum, the antibody (immunoglobulin) complement system provides a major antimicrobial effect against a wide range of microbes and confers passive immunity until the calf's own immune system has matured. Bovine serum and lacteal secretions contain three major classes of immunoglobulins: IgG, IgM and IgA. The immunoglobulins are selectively transported from the serum into the mammary gland, as a result of which the first colostrum contains very high concentrations of immunoglobulins (40–200 mg/ml). IgG1 accounts for over 75 % of the immunoglobulins in colostral whey, followed by IgM, IgA and IgG2. All these immunoglobulins decrease within a few days to a total immunoglobulin concentration of 0·7–1·0 mg/ml, with IgG1 representing the major Ig class in milk throughout the lactation period. Together with the antibodies absorbed from colostrum after birth, the complement system plays a crucial role in the passive immunisation of the newborn calf. The occurrence of haemolytic or bactericidal complement activity in bovine colostrum and milk has been demonstrated in several studies. This review deals with the characteristics of bovine Igs and the complement system to be exploited as potential ingredients for health-promoting functional foods.

Milk: Colostrum: Immunoglobulins: Complement: Antimicrobial activity

Introduction

The unique significance of milk and colostrum to the health and growth of newborn mammals is well documented (Brambell, 1969; Butler, 1974, 1986, 1994; Larson, 1992; Quigley & Drewry, 1998). In the 1890s, Paul Ehrlich proposed that colostrum was a vehicle for transporting immune factors, 'antikörpern', from the mother to her offspring (Ehrlich, 1892). Since then, the species-specific differences in the mechanism of the transfer of passive immunity to the newborn have been clearly demonstrated. In humans, for example, maternal immunoglobulins (Ig) with specific antimicrobial activity are transferred via the placenta to the newborn infant, and can confer a degree of passive immunity (Goldman, 1993). On the other hand, in other species such as pigs, horses, sheep and cows, Igs are only transferred postnatally via colostrum, as the placenta does not allow significant transfer of macromolecules (Butler, 1994). A selective accumulation of Igs from the blood circulation into the colostrum starts several weeks before parturition. In a newborn calf, the Igs are absorbed from the colostrum into the circulation within 24–36 h after birth via a non-selective macromolecular transport system (McFadden *et al.* 1997). This knowledge has been utilised for the development of commercial supplements containing Igs and complement factors concentrated from

bovine colostrum or whey (Haines *et al.* 1990; Nousiainen *et al.* 1994; Mee & Mehra, 1995). In addition, the possibility of isolating bovine colostral Igs from cows, following immunisation against pathogens, has been investigated as a potential strategy for the prevention and treatment of gastrointestinal diseases in humans. This article reviews the physico-chemical, biochemical, immunological and technological characteristics of bovine Igs and the complement system.

Characteristics of immunoglobulins

Bovine serum and lacteal secretions contain three major classes of Igs: IgG, IgM and IgA. The basic structure of all Igs is similar, and is composed of two identical light chains (23 kDa) and two identical heavy chains (53 kDa). These four chains are joined together with disulphide bonds. The complete Ig or 'antibody' molecule has a molecular weight of about 180 kDa. The two identical antigen-binding sites are formed by the N-terminal part of one heavy and one light chain. The bovine IgG molecule occurs predominantly in two subclasses: IgG1 and IgG2. Monomeric IgM and IgA have a similar basic structure to IgG except for the addition of a C-terminal octapeptide to the heavy chains. IgA occurs as a monomer or dimer, the latter comprising

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two IgA molecules joined together by a J-chain and a secretory component. This complex is called secretory IgA (SIgA) and has a molecular weight of about 380 kDa. Except for ruminant lacteal secretions, IgA is the dominating Ig in all external secretions of the body. IgM consists of five subunits, similar to monomeric IgA, which are linked together in a circular mode by disulphide bonds and a J-chain; the molecular weight of pentameric IgM is approximately 900 kDa.

The concentration of the various bovine Igs in serum and in lacteal secretions varies according to the breed, age, health status, and stage of lactation of the animal (Butler, 1986, 1994; Larson, 1992; McFadden *et al.* 1997). In colostrum, Igs make up 70–80 % of the total protein content, whereas in mature milk, immunoglobulins account for only 1–2 % of the protein (Larson, 1992). In serum, both IgG subclasses are present at about equal concentrations (IgG1 11·2 mg/ml, IgG2 9·2 mg/ml), whereas IgM and IgA occur at concentrations of about 3·0 mg/ml and 0·4 mg/ml, respectively (Butler, 1994). After the cessation of lactation, the IgG1 accumulates selectively from the blood circulation into the colostrum by an active receptor-mediated transfer across the mammary gland secretory epithelium. This process results in a 5- to 10-fold increase in the concentration of IgG1 in colostrum compared to maternal serum (Butler, 1983; Besser & Gay, 1994). It has been suggested that approximately 50–100 g per day or up to 500 g per week of IgG1 may be transferred from the blood into the mammary secretions during colostrum formation (Brandon & Lascelles, 1971). The concentration of Igs can vary considerably, from 30 to 200 mg/ml, in the first colostrum (Kruse, 1970; Korhonen *et al.* 1977; Stott *et al.* 1981; Hancock 1985; Larson, 1992; Korhonen *et al.* 1995). IgG1 comprises over 75 % (46·4 mg/ml) of the Igs in colostral whey, followed by IgM (6·8 mg/ml), IgA (5·4 mg/ml) and IgG2 (2·9 mg/ml) (Butler, 1994). The total Ig levels in milk decline rapidly following parturition to around 0·7–1·0 mg/ml. IgG1, however, remains the predominant Ig subclass in these secretions.

The Igs are produced by B lymphocytes. All Igs exhibit one or more effector function in addition to antigen binding. Whereas one part of an antibody (Fab) binds to antigen, other parts (mostly the Fc region) interact with other elements. In effect, antibodies function as flexible adaptors linking various parts of the immune system. The immunological function mediated by the Igs depends on the Ig class. IgG antibodies have a multitude of functions, the most important of which is possibly the activation of complement-mediated bacteriolytic reactions. Another vital function of Igs is their ability to augment the recognition and phagocytosis of bacteria by leucocytes (opsonisation). Igs are also able to prevent the adhesion of microbes to surfaces, inhibit bacterial metabolism, agglutinate bacteria, and neutralise toxins and viruses. IgM antibodies, although produced in smaller amounts than IgG, are considerably more efficient than IgG with regard to most of the above activities, especially complement-mediated lysis. IgA, in contrast, does not fix complement or opposing bacteria, but agglutinates antigens, neutralises viruses and bacterial toxins, and prevents the adhesion of enteropathogenic bacteria to mucosal epithelial cells. The

secretory piece component of the IgA molecule makes it resistant to the activities of proteolytic digestive enzymes. In humans, this property is manifested by the passage of a considerable proportion of undigested active IgA from human colostrum through the gut of the neonate baby (Goldman, 1993). In comparison, many *in vitro* studies have shown that bovine IgG is also relatively resistant to proteolysis by digestive enzymes and is not inactivated by gastric acid (de Rham & Isliker, 1977; Brock *et al.* 1978; McClead & Gregory, 1984); e.g. about 10 % of intact IgG and virus neutralising F(ab)$_2$ fragments were detected in the faeces of newborn babies who were given bovine Ig concentrates orally (Zinkernagel *et al.* 1972; Hilpert *et al.* 1987). Using ^{15}N-labelled Igs isolated from bovine colostrum, Roos *et al.* (1995) demonstrated that about 19 % of ingested IgG and IgM was found to retain immunological activity in the ileum of healthy human adults. Kelly *et al.* (1997) measured the survival of orally administered bovine immunoglobulin concentrate against *Clostridium difficile* toxins in the human gastrointestinal tract. Without encapsulation the mean faecal bovine Ig content of three-day stools was 1·6–3·8 % of the administered Ig, whereas Ig administered in enteric capsules resulted in a 32·7 % recovery in faeces. Thus, an appropriate delivery system and the controlled release of Igs in a desired part of the gastrointestinal tract are likely to play a crucial role in the pharmacological efficacy of the Igs ingested.

Commercial isolation of Igs

Since the 1980s, a number of patented methods have been developed for the isolation and purification of Igs from colostral or cheese whey, based on ultrafiltration (UF) or a combination of UF and chromatography (Kothe *et al.* 1987; Abraham, 1988; Stott & Lucas, 1989; Korhonen *et al.* 1998). For the commercial production of crude Ig preparations, a combination of different membrane technologies seems to be the most cost-effective approach. However, for the improvement of the recovery rate of Igs from whey and to increase the Ig concentration of the final preparation, specific chromatographic techniques need to be applied. For example, the separation of IgG from UF-treated whey has been successful using immobilised metal chelate chromatography (Al-Mashikhi *et al.* 1988; Fukumoto *et al.* 1994*a*), whereas the most suitable process for the isolation of bovine IgG subclasses IgG1 and IgG2 is an immunoaffinity chromatography process using immobilised egg yolk antibodies (Akita & Li-Chan, 1998).

The effects of processing and storage conditions on the stability of purified Igs or Ig concentrates have been the subject of many recent studies. During processing, the stability of the Ig activity in colostrum or milk is influenced by thermal treatment (Lindström *et al.* 1994; Li-Chan *et al.* 1995; Dominguez *et al.* 1997). Following high temperature/short time (HTST) pasteurisation (72°C/15 s) only 10–30 % of the Ig activity is lost, whereas ultrahigh temperature (UHT) treatment (138°C/4 s) and evaporation processing destroys the majority of the specific immune activity of milk (Kummer *et al.* 1992; Li-Chan *et al.* 1995). The rapid heat inactivation of IgG starts at temperatures

higher than 65°C, and at 81°C, as much as 90 % of the virus neutralisation activity is lost in less than two minutes (Mainer *et al.* 1999). However, bovine IgG added to UHT milk has been shown to retain its specific immune activity for over several months (Fukumoto *et al.* 1994*b*; Virtanen *et al.* 1998). Also, Ig molecules seem to retain their specific activity well in milk powder, irrespective of the storage temperature; the storage of freeze-dried anti-*Campylobacter jejuni* Igs at 4, 20 and 37°C has been found to have little effect on the immune specificity for up to 12 months of storage (Husu *et al.* 1993).

Characteristics of the complement system

The complement system plays a major role in the host-defence mechanisms against infectious microbes, as it is involved both in specific and non-specific immunity. It is a complex system of over 20 different proteins that can be activated by antigen–antibody complexes (classical pathway), by certain carbohydrates (lectin pathway), or by a variety of surfaces that are not protected by natural inhibitors (alternative pathway). Killing of micro-organisms, clearing of immune complexes, and induction and enhancement of antibody responses are the major biological functions of complement (Brown *et al.* 1983; Tabel, 1996; Fearon, 1998). The classical pathway is initiated by the activation of the first, and the alternative pathway by the third complement protein in a cascade order. Because of the enzymatic capabilities of several components of this cascade, such activation leads to a strong amplification of the reactions. Activation by the classical pathway is mainly initiated by binding of the first component to antigen–antibody complexes or directly to certain microbes. In the absence of antibodies the complement activation occurs by lectins bound to pathogen surfaces via the lectin pathway (Holmskov & Jensenius, 1996; Turner, 1996) or the alternative pathway, which is initiated in the presence of bacterial cell membrane components such as lipopolysaccharides or β-glucan. Activation by any of the pathways leads to the enzymatic cleavage of the third (C3b) and the fifth (C5) components of complement, generating the cleavage products C3a, C3bi, C5a and C5b. These products are responsible for most of the biologically important functions of the system (Janeway, 1997). For example, C3b, C3bi, C4b and perhaps also C1q function as opsonins augmenting phagocytosis, thus helping the clearance and destruction of bacteria and immune complexes. The small anaphylatoxins C3a, C4a and C5a recruit inflammatory leucocytes to the sites of an inflammation and activate their effector mechanisms. They are also involved in mast cell and basophil-mediated inflammatory reactions such as vascular dilation. In the latter part of the complement reaction, cascade C5b associates with components C6, C7, C8 and multiple C9. Polymeric C9 forms a channel (membrane attack complex, MAC) in the bacterial cell membrane, leading to the leakage of electrolytes, collapse of metabolism and subsequent death of the cell (Born & Bhakdi, 1986). Most Gram-negative bacteria are sensitive to the lytic action of complement, but all Gram-positive and some Gram-negative bacteria are resistant and, therefore, more virulent (Rautemaa & Meri, 1999). However, both Gram-positive and Gram-negative bacteria can become opsonised by C3b and C3bi, and opsonophagocytosis is thus the main defence mechanism against bacteria resistant to complement lysis (Rautemaa & Meri, 1999).

Apart from its direct antimicrobial effects, complement maintains the antibodies in soluble form by limiting the formation of harmful immune complexes and inhibiting the precipitation of Igs. The Ig-complexes are opsonised with C3b protein following complement activation, and removed by the tissue macrophages mainly in the liver and spleen. In effect, the ability to keep immune complexes soluble by reducing the number of antigen epitopes that the antibodies can bind is one of the main tasks of the classical complement pathway. Complement can also rapidly resolubilise the precipitated Ig complexes through the alternative pathway. The resolubilisation occurs by the insertion of C3b and C3d into the complexes (Walport, 1996).

Complement activity is known to occur in the serum of fetal calves (Triglia, 1980; Mueller *et al.* 1983). Together with the antibodies absorbed from colostrum after birth, the complement system plays a crucial role in providing passive immunity to the newborn calf (Butler, 1986; Staak, 1992). Apart from serum, the complete complement system can be found in bovine colostrum, and components of the system are present in milk. Several studies have demonstrated the occurrence of haemolytic or bactericidal complement activity in bovine colostrum (Brock *et al.* 1975; Reiter & Brock, 1975; Eckblad *et al.* 1981; Korhonen *et al.* 1995). Compared to normal milk whey, the concentration of C3 and haemolytic activity are elevated in the milk whey from mastitic cows, but the values do not reach the level present in blood serum (Muller *et al.* 1982; Rainard *et al.* 1984). Also, conglutinating activity, indicating the presence of complement components C1,4,2,3 has been found irregularly in milk of mid and late lactation (Reiter & Oram, 1967; De Cueninck, 1979). It is doubtful whether the active complement system found in colostrum is effective *in vivo* against bacterial infections in the gut of the newborn calf, since the bactericidal activity of colostrum can be readily destroyed by trypsin or pancreatic juice. However, it has been demonstrated experimentally that the loss of complement bioactivity due to trypsin can be prevented by the addition of excess bovine colostral trypsin inhibitor (Brock *et al.* 1975). This enzyme is known to occur naturally in colostrum (Pineiro *et al.* 1975).

Antibody-related antimicrobial activity of colostrum and milk

Bovine colostrum and milk are known to contain a large number of naturally occurring antimicrobial substances (Reiter, 1985; IDF, 1991; Pakkanen & Aalto, 1997; Regester *et al.* 1997). Among them, the antibody-complement system is considered as the major agent of the antimicrobial activity of colostrum. The antibody-augmented bactericidal activity of complement has been demonstrated in normal or immune colostrum and serum but not always in milk samples against a great number of Gram-negative bacteria, e.g. *Aerobacter aerogenes* (Carroll & Jain, 1969), coliform (Carroll, 1974), enterotoxigenic

Escherichia coli (Reiter & Brock, 1975), *Campylobacter jejuni* (Husu *et al.* 1993; Syväoja *et al.* 1994) and *Helicobacter pylori* (Korhonen *et al.* 1995). The mode of the antimicrobial action in colostrum is not clearly defined. In a study by Korhonen *et al.* (1995), all colostrum samples derived from normal healthy cows were naturally bactericidal against *H. pylori*, whereas none of the milk samples from the same animals showed bactericidal activity. In contrast, bactericidal activity was detected in 43 % of the milk samples obtained from cows hyperimmunised pre-partum with a *H. pylori* vaccine. The latter fact clearly demonstrates that the antibacterial activity of milk can be increased by systemic immunisation of cows against a defined pathogen, although in that case the bactericidal activity did not correlate with titres of specific Ig or with total IgG concentrations. Previous reports had demonstrated that the bactericidal property of immune colostrum against complement-sensitive pathogens is associated with a rise in the titre of pathogen-specific Igs (Korhonen *et al.* 1994; Syväoja *et al.* 1994). In addition, certain clinical conditions may predispose the cow to produce enhanced endogenous antimicrobial activity. For example, when compared with milk from healthy cows, the milk from mastitic cows has been shown to possess increased bactericidal activity against *Aerobacter aerogenes* (Jain & Jasper, 1967) and udder-pathogenic *E. coli* (Korhonen, 1973; Rainard *et al.* 1984). In these studies, heating at 56°C for 30 minutes destroyed the bactericidal effect (a treatment regime that is known to inactivate the complement system).

A number of earlier studies have indicated that so-called agglutinins may inhibit the growth of certain dairy starters and cause the flocculation of milk fat globules (IDF, 1991). Later, it was shown that these agglutinins were mainly associated with IgM. The natural agglutinating activity of bovine colostrum preparations has been demonstrated against a variety of pathogenic bacteria (Stephan *et al.* 1990; Loimaranta *et al.* 1998a).

Raw and pasteurised milk from non-immunised cows has been shown to contain specific antibodies to human rotavirus (Yolken *et al.* 1985). Also, raw milk from non-immunised cows contains specific Igs to lipopolysaccharides from major human pathogenic bacteria, e.g. *E. coli*, *Salmonella enteriditis*, *S. typhimurium* and *Shigella flexneri* (Losso *et al.* 1993). Further, natural antibodies to a colonisation factor antigen (CFA-1) of human enterotoxigenic *E. coli* have been found in normal colostrum and milk (Facon *et al.* 1995).The specific antibodies in the colostrum of cows immunised with human pathogens have also been found to exert a synergistic effect on the activity of non-specific antimicrobial factors such as lactoferrin and lysozyme (Takahashi *et al.* 1992) as well as lactoperoxidase (Loimaranta *et al.* 1998b).

Conclusions

The biological function of cow's milk, and especially that of colostrum, is not only to give nourishment to the offspring but also to provide it with an immune protection against environmental pathogens. Cow's colostrum and milk contain virtually all compounds of bovine cellular and humoral immune defence, including antibodies and complement proteins. Commercial whey or colostral antibody preparations, which may also contain complement proteins, have been used for a long time as feed supplements or substitutes for farm animals, mainly calves and piglets, in order to prevent contagious diseases. These preparations have proven effective especially in the prevention of diarrhoeal diseases. Moreover, commercial products for human use have appeared on the market and a lot of scientific research work is currently under way for the application of milk antibodies in the prevention or treatment of microbial diseases in humans. Specific antibodies can be produced also in cell cultures or isolated from other body fluids than milk for use in laboratory experiments. However, thus far and in the near future those preparations are too expensive or not available for the scale of production required for commercial products. Milk and colostrum are ideal sources of these defence molecules for industrial-scale production because of their ready availability and safety as compared, for example, with blood-derived analogues. The main limitation of milk antibodies in human use is that they are derived from a foreign species and can thus be used only against oral and gastrointestinal pathogens or for topical applications. In order to overcome this limitation, it may be possible in the future to produce human antibodies and complement proteins in transgenic cows.

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