Colostral whey concentrate supplement increases complement activity in the sera of neonatal calves

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Summary. We evaluated the effect of a commercial bovine colostral whey on the complement-mediated immune responses of calves. Two groups of neonatal calves were fed, in addition to whole milk (WM) and pooled colostrum (PC), different amounts of a commercial immunoglobulin concentrate made from pooled colostral whey (Ig-C) for the first two feedings *post natum*. The control group was fed WM and PC only. Serum samples were obtained at the ages of 2, 7, 14 and 30 d. Bacteriolytic activity against complement-sensitive *Escherichia coli* JM103 and opsonic activity against complement-lysis-resistant *E. coli* IH3080 strains were studied, as well as the levels of C3 complement component and *E. coli* JM103 specific antibodies in the sera.

Groups fed Ig-C had 2–3 times higher bacteriolytic activity than the control group of both the classic ($P < 0{\cdot}005$) and alternative pathways ($P < 0{\cdot}0001$) at days 2 and 7 *post natum*. This effect is obviously not caused solely by the antibodies ingested but also involves other unknown colostral factors, possibly lectins. The opsonisation capacities of the sera correlated well with the amounts of immunoglobulins ingested ($P < 0{\cdot}05$) at days 2–14. The levels of C3 component in sera did not differ between the groups. In the group fed the largest amount of immunoglobulins levels of *E. coli* JM103-specific antibodies were highest ($P < 0{\cdot}0001$). It can thus be concluded that the antibody independent complement activities of serum can be increased substantially by feeding colostral whey concentrate to calves during their first days of life.

Keywords: Colostral whey, complement, bacteriolysis, opsonization, calf.

Passive maternal immunoglobulin transfer via colostrum provides primary protection against infections in newborn calves, since the bovine placenta does not allow the transfer of macromolecules, including immunoglobulins (Ig), in significant amounts (Larson *et al*. 1980). Failure of this passive transfer of colostral antibodies is a well-documented cause of morbidity and mortality in calves (Staak, 1992; Besser & Gay, 1994; Butler, 1994). The most important factors influencing the passive transfer of antibodies are the age of the calf and the amount of Ig consumed (Stott

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& Fellah, 1983; Quigley & Drewry, 1998). The calf should get colostrum shortly after birth (Rajala & Castren, 1995; Hadorn *et al.* 1997). Maternal colostrum is sometimes not available, and colostrum pools or commercial substitutes have to be used. In those cases the amount of Ig ingested is critical for the survival and health of the calf (Nousiainen *et al.* 1994; Garry *et al.* 1996; Grongnet *et al.* 1996).

The most important functions of Ig are the activation of bacteriolytic reactions of complement, and augmenting the recognition and phagocytosis of bacteria by leukocytes (opsonisation). They are also capable of preventing the adhesion of microbes on surfaces, to inhibit bacterial metabolism, agglutinate bacteria, and neutralise toxins and viruses.

Complement activity is present in the serum of newborn calves and even in the fetal calf serum (Mueller *et al.* 1983). The antibody-complement system provides protection against infectious diseases by causing cytolytic membrane lesions on foreign cells, by opsonising microbes, and by generally enhancing the inflammatory response. Opsonisation by IgG or complement C3 fragments recognised by the specific surface receptors of phagocytic cells augments the phagocytosis of bacteria. The activation of the complement system occurs via the antibody dependent classical and/or the antibody independent alternative pathway in a sequential manner by proteolytic cleavages and the association of inert precursor molecules. Also some lectins can trigger complement activation (Turner, 1996).

This work is a continuation of the study of Nousiainen *et al.* (1994), who fed neonatal calves with a commercial Ig preparation to supplement low-quality colostrum and evaluated its effect on the absorption of Ig, serum Ig level, growth of the calves and the occurrence of diarrhoea. A commercial immunoglobulin concentrate made from pooled colostral whey (Ig-C) caused a linear increase in the serum IgG, IgM and IgA levels ($P < 0{\cdot}001$).

This study was conducted with the same serum samples of calves used in the study of Nousiainen *et al.* (1994). Its purpose was to evaluate the effect of Ig-C on complement-mediated bacteriolytic and opsonisation activity.

Materials and Methods

Diets and serum samples

The serum samples were from a previous experiment carried out by Nousiainen *et al.* (1994). Twenty-one calves were divided at random into three groups, seven calves in each, and given one of three feeding regimens on the first day of life (first feeding *post partum* and second 8–12 h later). The control group was fed normal pooled whole milk (WM) and pooled colostrum (PC) based on 1–5 milkings *post partum* (total Ig intake from the first and second feeding 19·5 g). The Treat 1 and Treat 2 groups were fed WM, PC and Ig-C giving a total Ig intake of 52·7 g and 119·0 g, respectively. On days 2–4, the calves were fed similarly by gradually decreasing the proportion of PC (Table 1). On days 4–56, all the calves were fed similarly with WM, hay and a commercial supplement. Blood samples were drawn from the *vena jugularis* with dry vacuum tubes on days 2, 7, 14 and 30 *post natum*. The sera were stored at −20 °C for analysis.

Measurement of complement dependent E. coli *bacteriolytic activity*

Bacteriolytic activity was measured by a method modified from that described by Virta *et al.* (1995, 1998). The assay is based on a complement-sensitive non-

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Table 1. *Feeding regimen during first 4 days of life (Nousiainen et al. 1994)*

Treatment	First feeding		Second feeding		Days 2–4	Ig intake‡
Control	1·0	l WM	1·0	l WM	3 l WM + PC-mixture†	19·5
	0·50	l PC	0·50	l PC		
Treat 1	0·50	l WM	2·0	l WM	3 l WM + PC-mixture†	52·7
	0·50	l PC	0·50	l PC		
	0·50	l Ig-C				
Treat 2	0·25	l WM	0·25	l WM	3 l WM + PC-mixture†	119·0
	0·50	l PC	0·50	l PC		
	0·75	l Ig-C	0·75	l Ig-C		

Ig-C, colostral Ig concentrate; PC, pooled colostrum; WM, whole milk.
† Gradually decreasing proportion of PC (similar in all groups).
‡ Total intake from first and second feeding.

encapsulated *E. coli* strain JM103 with plasmid pCSS962/pGB3 (Lampinen *et al.* 1992) containing a luciferase structural gene from a Jamaican click beetle *Pyrophorous plagiophthalamus* clone and expressed as described by Wood *et al.* (1989). The strain was kindly provided by Dr. Matti Karp of the University of Turku, Finland. In the presence of luciferine, luciferase converts the chemical energy associated with ATP into green light that can be measured with a luminometer. Thus, the amount of light emitted is proportional to the number of metabolically active cells (Griffiths, 1996). The survival of bacteria in the serum samples was calculated relative to that in heat-inactivated (56 °C, 30 min) fetal calf serum (FCS). The results were determined as a serum dilution with a chemiluminescence emission 50 % of that of blank samples containing a dilution buffer instead of a serum sample. This dilution represents a lethal dose 50 % value (LD_{50}) for the serum sample.

The lyophilised bacteria (2×10^9 cfu) were suspended in 22 ml of Hank's balanced salt solution (HBSS) without phenol red, pH 7·4. The serum samples were thawed and diluted serially with HBSS containing heat-inactivated FCS. Samples in duplicates were incubated with $3{\cdot}6 \times 10^6$ cells of *E. coli* JM103 in microtitre plate wells for 90 min at 37 °C. After that, 0·25 mM D-luciferine (Bio-Orbit, 20521 Turku, Finland) in 0·1 M disodiumcitrate (pH 5·0) was added and the bioluminescence emission was measured for 1 h with a Luminoskan EL1 luminometer (Labsystems, 00810 Helsinki, Finland).

The alternative pathway of complement was analysed by blocking the classical pathway with 10 mM EGTA in Ca^{2+} and Mg^{2+}-free HBSS. The inactivation of *E. coli* was measured as described above.

Opsonisation

Complement-insensitive *E. coli* strain IH3080, kindly provided by Prof. Martti Vaara of the University of Helsinki, Finland, was used as a model for serum opsonisation activity against bacteria. A mixture in HBSS containing 1 % serum sample and 2×10^9 *E. coli* was shaken at 220 rpm at 37 °C for 35 min. In preliminary tests, these concentrations were found to be relevant in testing the opsonisation capacity of serum. After centrifugation, the supernatant was removed, and the pellet was suspended in HBSS and stored at −20 °C. The control samples contained heat-inactivated FCS instead of serum samples.

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Leukocyte isolation

Fresh venous blood, anticoagulated with EDTA, was taken from a healthy calf. The leukocytes were isolated as described by Robinson (1993). Finally, the cells were suspended at the required concentration in HBSS containing 0·1% gelatin (gelatin from porcine skin, Sigma Chemical Co., St. Louis, MO 63178, USA).

Phagocyte activation

Phagocyte oxidative burst activity was measured as luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical Co., St. Louis, MO 63178, USA) enhanced chemiluminescence (CL) emission. The reaction mixture consisted of 25×10^3 leukocytes and 0·4 mM luminol in gelatin-HBSS. The CL reaction was stimulated with *E. coli* cells opsonised either with heat-inactivated FCS or with the serum samples ($2{\cdot}5 \times 10^6$ cells/well). The CL response was measured in four replicates at 1·5-min intervals for 1 h at 37 °C with a luminometer. The observed CL peak value (expressed in relative light units, rlu) was taken as the CL value. The results were calculated as the proportion of maximum CL compared to the CL of bacteria opsonised with inactivated FCS.

C3 content

The relative content of the complement C3 component in the sera was estimated by the radial immune diffusion method. In brief, anti-bovine C3 serum (Cappel, Organon Teknika, West Chester, PA 19380, USA) was diluted 1:80 in 2% SeaKem LE agarose (FMC BioProducts, Rockland, ME 04841, USA) in Mancini buffer and poured on Petri dishes. After cutting wells with a diameter of 3 mm, 4 μl serum sample was applied to each well. After 72 h incubation at 4 °C in a humid chamber, the precipitation rings were stained with PhastGel Blue R (Pharmacia LKB Biotechnology, 75184 Uppsala, Sweden) and the diameter of the precipitant was measured. FCS was used as a standard.

E. coli-*specific antibodies*

The relative amount of *E. coli* antibodies in the serum samples was assayed by an indirect ELISA. The wells of Combiplate (Labsystems, 00810 Helsinki, Finland) microtitre plates were coated with a 100-μl suspension of *E. coli* JM103 whole cells in phosphate buffered saline pH 7·3 (PBS) and the plates were incubated over night at 4 °C. The unbound antigen was removed by washing the wells three times with PBS containing 0·05% Tween 20 (Fluka Chemie AG, 9471 Buchs, Switzerland) and rinsed three times with distilled water. Serum samples (100 μl) diluted 1:50 in PBS containing 2% Tween 20 were added to the wells in duplicates. After incubation at 37 °C for 90 min, the wells were washed as previously and 100 μl alkaline phosphatase conjugated anti-bovine IgG (Sigma Chemical Co., St. Louis, Mo. 63178, US) (dilution 1:12000) was added, followed by incubation at 37 °C for 90 min. After washing, the plates were developed for 30 min with *p*-nitrophenyl-phosphate (Reagena Ltd., 70600 Kuopio, Finland) in diethanolamine-$MgCl_2$ buffer, pH 9·8 (2 mg/ml), and the absorbance at 405 nm was measured with the Labsystems Multiskan MCC/340 (Labsystems, 00810 Helsinki, Finland).

Statistical methods

For each calf there were repeated measurements at four points in time. Any two observations for a given calf were correlated and this correlation was taken into account in the selected models. The covariance structure of the repeated

measurements was chosen by comparing several potential structures using Akaike's information criterion (Wolfinger, 1996). The analyses were based on the following model:

$$y_{ijk} = \mu + \alpha_i + w_{j(i)} + \beta_k + \alpha\beta_{ik} + \epsilon_{ijk}$$

where y_{ijk} is the measured serum activity of calf j in group i, μ is the overall mean, α_i is the treatment, $w_{j(i)}$ is calf j in group i, β_k is the point in time and ε_{ijk} is the experimental error. The assumptions of the models were checked using graphical methods: a box-plot for the normality of the errors and plots of studentised residuals against fitted values for the constancy of the error variance (Neter *et al*. 1996). The parameters of the models were estimated by the restricted maximum likelihood estimation method. Comparisons between Treat 1 vs. controls and Treat 2 vs. controls were made at different points in time.

The analyses were performed using the SAS system for Windows release 6·12 by the MIXED procedure (SAS 1996).

RESULTS

Bacteriolytic activity of complement

At days 2 and 7 the alternative pathway of complement was two to three times more effective both in the sera of Ig-C-treated calves in the Treat 1 and Treat 2 groups than in the controls ($P < 0{\cdot}0005$ and $P < 0{\cdot}0005$, respectively; Fig. 1*a*). After day 7 the activity level decreased, being about 60% higher than that of the controls at day 14. At that time the P values were 0·06 and 0·03 for Treat 1 and Treat 2, respectively. At day 30 there were no differences between the groups. Thus, the change in the complement alternative pathway activity in the course of time was different in the treated groups from that in the controls ($P < 0{\cdot}01$).

Similarly, the classic pathway of complement at pooled days 2 and 7 was twice as active both in Treat 1 and Treat 2 groups than in the controls ($P < 0{\cdot}005$, and $P < 0{\cdot}0005$, respectively). After that, the activity level of the Treat 1 group decreased towards the control level, as in the alternative pathway activity. However, the activity of the classic pathway in the Treat 2 group was still higher at day 30 than in the controls ($P = 0{\cdot}03$; Fig. 1*b*).

Opsonisation

The *E. coli* opsonisation capacities of the sera are expressed as the relative increase in the oxidative burst activity of the phagocytising leukocytes evoked by opsonised *E. coli* IH3080 as compared to that of nonopsonised *E. coli* (Fig. 2). At days 2–14, there was a linear correlation between the opsonisation capacity and the logarithm of the amount of Ig ingested ($P < 0{\cdot}05$). Thus, the Treat 2 group had the highest serum opsonisation capacity. Treat 2 differed from the control group at pooled days 2–7 ($P = 0{\cdot}06$) and at day 14 ($P = 0{\cdot}03$). At day 30, the capacities of the control group had risen to the same level as those of the Treat 1 and Treat 2 groups.

C3 content

The results of the C3 content as relative values compared to fetal calf serum are presented in Fig. 3. No difference was observed between the groups. The C3 levels increased significantly in all groups during the study ($P < 0{\cdot}0001$). The increase in C3 concentration was highest from day 2 to day 7.

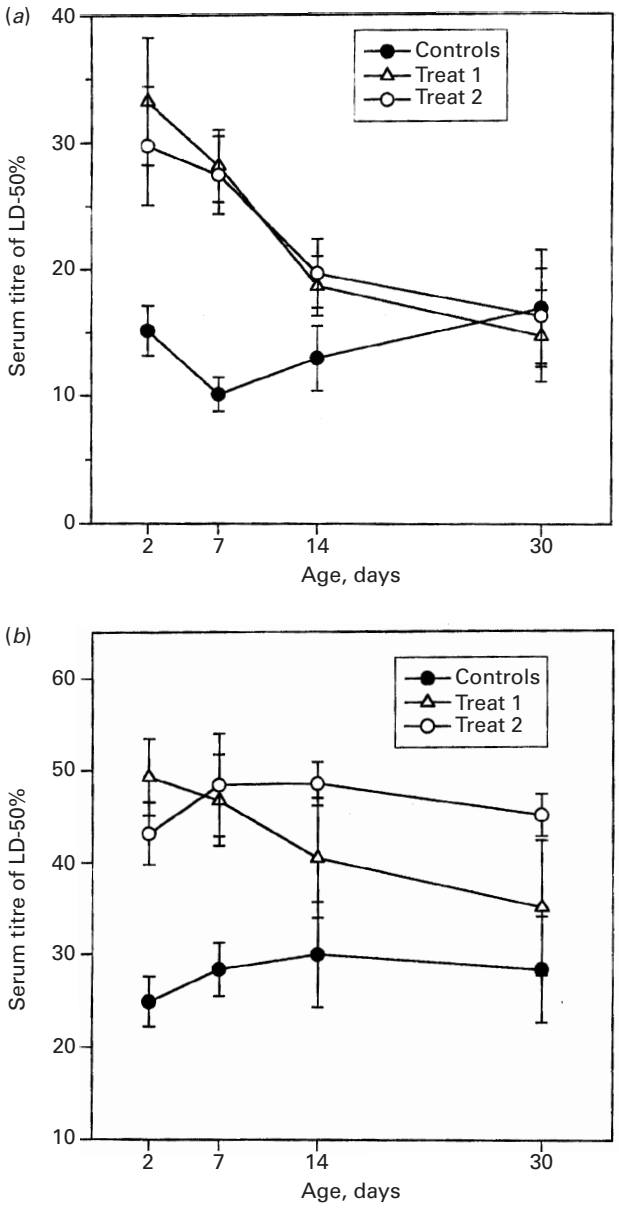

Fig. 1. Bacteriolytic activity of serum samples against *Escherichia coli* JM103: (*a*) alternative pathway activity and (*b*) classic pathway activity (10 mM EGTA used). The activities are represented as the serum concentration percent reducing 50% of the bacterial bioluminescence. Error bars represent ±SEM.

E. coli antibodies

The average OD-values of the serum samples in the groups fed different amounts of Ig are presented in Fig. 4. The Treat 2 group had a significantly ($P < 0{\cdot}0001$) higher level of *E. coli* JM103-specific antibodies than the control group. Treat 1 differed significantly from the control group only at pooled days 2–7 ($P = 0{\cdot}03$). The differences between Treat 1 and Treat 2 v. the control group diminished during

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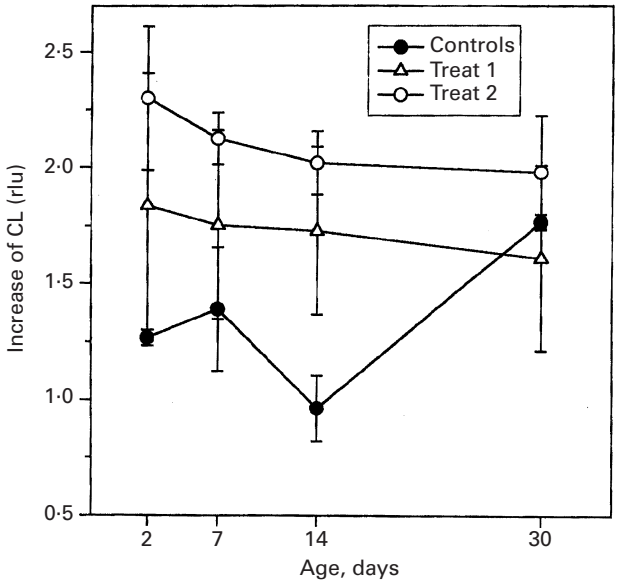

Fig. 2. Opsonisation activity of serum samples against *E. coli* IH3080 measured as an increase of phagocyte oxidative burst activity after opsonisation of the bacteria with 1% serum as compared to phagocyte activation with nonopsonised bacteria. CL = chemiluminescence, measured in relative light units (rlu). Error bars represent ±SEM.

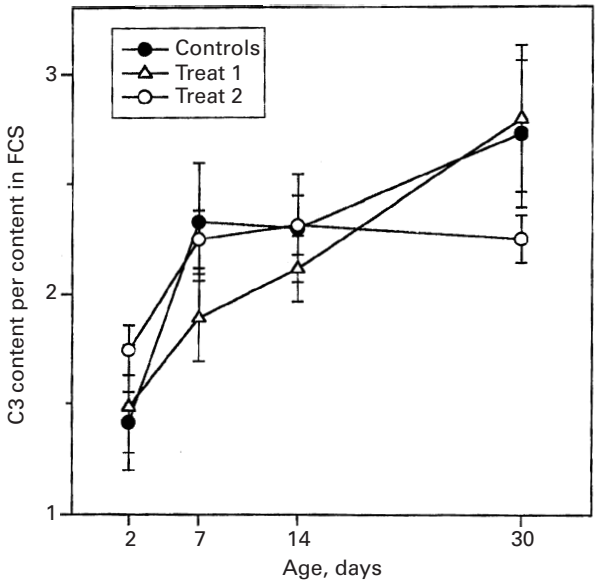

Fig. 3. Relative contents of complement component C3 in sera measured with the radial immune diffusion method. The C3 contents are represented as values obtained by dividing the precipitation area of the serum samples with that of a standard fetal calf serum (FCS). Error bars represent ±SEM.

the study ($P = 0{\cdot}06$ and $P = 0{\cdot}04$, respectively). In both Ig-C-treated groups the initial antibody levels were high and decreased towards the control values in the course of time.

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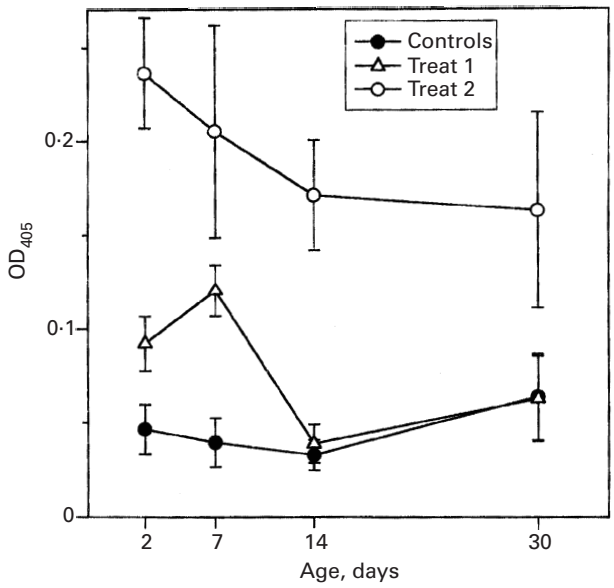

Fig. 4. Relative contents of *E. coli* JM103-specific antibodies in sera measured with an enzyme-linked immuno sorbent assay. The antibody levels are represented as obtained OD_{405} values. Error bars represent ±SEM.

DISCUSSION

Enterotoxigenic *E. coli* is a common cause of diarrhoea and other infectious diseases in calves during the first weeks of life. Under normal circumstances, the nonpathogenic types of *E. coli* are among the bacterial species to colonise the gastrointestinal tract and they are present throughout the gut by the end of the first day of life (Acres, 1985). The chances of colonisation by pathogenic bacteria have increased, e.g. for environmental reasons. Colostral Ig are an essential part of the protection against infections, but other antimicrobial factors such as lactoferrin, lysozyme and lactoperoxidase also contribute to this (Reiter, 1985; Pakkanen & Aalto, 1997).

Antibody dependent, naturally occurring bactericidal complement activity plays a significant role against coliform infections (Carroll & Crensaw, 1976). The occurrence of lytic complement in bovine colostrum has been demonstrated in several studies (Reiter & Brock, 1975; Korhonen *et al.* 1995). There is, however, very little information about the transfer of complement from colostrum to calves.

Fetal calf serum is essentially free of Ig but has an active complement system which, however, is not as active as in adult cows. Mueller *et al.* (1983) reported that the level of the C3 component in fetal calf serum is about 28% that of the adult cow. Our results show, like those of Mueller *et al.* (1983), that during the study as the calves grew older, the C3 concentration increased. Nevertheless, the C3 concentration was not higher in the Ig-C-supplemented animals (Fig. 3). The results of Mueller *et al.* (1983) concur with our conclusion that no considerable amounts of complement C3 protein are transferred from the cow to the calf by absorption from colostrum.

During days 2–7, both the Treat 1 and Treat 2 groups had higher levels of *E. coli* antibodies in their blood than the control group. In the Treat 1 group, *E. coli* antibody levels decreased to the control level by day 14. However, in the Treat 2 group, the antibody levels were at all time points significantly ($P < 0{\cdot}01$) higher

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than in the controls, although they were already decreasing towards the control level (Fig. 4). These *E. coli* specific antibody levels correlate with the total IgG concentrations in the sera as reported by Nousiainen *et al.* (1994).

The *E. coli* JM103 strain was chosen for use in this study as a model organism for monitoring the bacteriolytic activity of complement on the basis of its sensitivity to lysis. When microbes are resistant to complement-mediated lysis, the opsonophagocytosis remains the major antibacterial defence mechanism (Rautemaa & Meri, 1999). All Gram-positive but also several Gram-negative organisms are resistant to lytic activity (Hosea *et al.* 1980; Rautemaa & Meri, 1999). For this reason our opsonisation tests were carried out with an *E. coli* strain IH3080 resistant to complement lysis. Neither of these test organisms is known to be natural pathogens of calves but, nevertheless, the complement activity against these strains reflects well the functions of serum complement and antibodies against bacterial pathogens in general.

The results of this study indicate that the ingestion of Ig-C increases the complement and opsonisation activity of serum in calves during the first weeks of life as compared to the controls. Both the bactericidal activity of complement leading to lysis (Fig. 1) and the opsonisation activity (Fig. 2) were stronger in the sera of the experimental groups than in the control group.

The higher level of specific antibodies in the Treat 2 group caused higher complement classic pathway activity. Both the classic pathway activity and antibody levels were still high at day 30, whereas the opsonisation and alternative pathway activity had already returned to the same level as of the controls.

The cause for the 2- to 3-fold higher activity of the antibody independent alternative pathway in the Ig-C-treated groups remains obscure. It is possible that the transfer of some complement components other than C3 is crucial for serum bacteriolytic capacity. On the other hand, Ig-C may contain unknown transferred regulation factors, e.g. lectins, which increase the complement activity. Mannose-binding lectin is able to trigger the lectin pathway of complement activation by binding to sugar groups on the pathogen surface (Turner, 1996). Also, bovine conglutinin is known to show opsonising activity towards *E. coli in vitro* (Holmskov & Jensenius, 1996). Thus, lectins in colostral whey could be the cause of the increased complement bactericidal activity observed in the absence of the antibody dependent classical pathway. Riedel-Caspari *et al.* (1991) reported that the ingestion of colostrum increased the bactericidity of sera of calves against *E. coli*, but during the first week of life decreased the complement haemolytic activity. Lectin-boostered complement could also explain those results.

The trend of opsonisation capacity in our study (Fig. 2) was not similar to the *E. coli*-specific antibody levels (Fig. 4) but rather resembled the complement alternative pathway activity (Fig. 1*a*). This suggests that the effect which increased opsonisation at early points in time is mainly due to complement proteins and/or lectins, and antibodies are involved to a lesser extent.

Since the complement system plays a major role in resistance to microbial infections, the regulation of complement by colostrum is of vital importance for the health of a calf. The overall health of calves fed Ig-C was better than in the control group (Nousiainen *et al.* 1994), indicating that a sufficient intake of colostral immune components is important even in the long term.

In these experiments, we observed that the serum complement-mediated capacity to kill *E. coli* can be increased substantially by feeding colostral whey concentrate to calves during the first day of life.

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