Influence of whey and purified whey proteins on neutrophil functions in sheep

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(*Received 22 February 1996 and accepted for publication 28 November 1996*)

Summary. The effects of ruminant whey and its purified fractions on neutrophil chemotaxis and superoxide production in sheep were studied. Both colostral whey and milk whey were found to inhibit chemotaxis regardless of whether they were autologous or homologous, but the inhibitory effects were abolished by washing neutrophils with culture medium before their use in the chemotaxis assay. Colostral whey and milk whey also inhibited the chemotactic activity of zymosan-activated serum. Whey fractions of various degrees of purity such as lactoferrin, lactoperoxidase, lactoferrin–lactoperoxidase, α-lactalbumin, bovine serum albumin and whey protein concentrate were then studied. While none of these proteins showed any effects on chemotaxis, lactoferrin–lactoperoxidase and whey protein concentrate were found to have an enhancing effect on superoxide production in a dose-dependent manner. Our results provide information on the modulatory role of ruminant milk proteins in inflammatory responses and warrant future investigation.

Milk and colostral whey consist of a complex mixture of proteins such as γ-globulin, β-lactoglobulin, α-lactalbumin (α-la) serum albumin, lactoferrin (LF) lactoperoxidase (LP, EC 1.11.1.7) and various growth factors. Most of these proteins possess important immunobiological activities (Duncan & McArthur, 1981; Newby *et al.* 1982; Goldman *et al.* 1986; Mincheva-Nilsson *et al.* 1990; Politis *et al.* 1991; Fiat *et al.* 1993). In particular, the inflammatory activities of phagocytes are modulated in milk by some of these milk proteins or their peptides (Goldman *et al.* 1986; Fiat *et al.* 1993).

During the last decade there has been a growing interest in finding new biomedical applications for whey proteins. This has stimulated many studies on the immunoregulatory and anti-inflammatory properties of whey components (Bounous *et al.* 1989; Watson *et al.* 1992; Wong & Watson, 1995). Recently, the prophylactic potential of whey proteins against initiation of colon cancer has been reported (Bounous *et al.* 1988; Bounous & Gold, 1991; McIntosh *et al.* 1995) and IgE suppressor factors have been discovered in human colostrum (Sarfati *et al.* 1986). Suppression of IgE responses by ruminant colostrum has also been noted in this laboratory (Watson *et al.* 1992).

Neutrophils play a major role in the innate immune response. Their functional properties include adherence to microvascular endothelium and extracellular matrix

components, chemotactic migration, ingestion and production of oxygen-derived free radicals to destroy microorganisms, and many others. These functions are subject to complex regulation by endogenous and exogenous mediators. The experiments described in this paper explored the effects of whey fractions of increasing purity on chemotaxis and superoxide production by ovine neutrophils.

Materials and methods

Animals

Female and castrated male Merino sheep, weighing 40–50 kg, were obtained from the CSIRO Pastoral Research Laboratory, Armidale and used as the source of blood neutrophils.

Neutrophil isolation

Jugular venous blood was collected from sheep into tubes containing ACD solution (sodium citrate dihydrate 1·37 g, anhydrous citric acid 0·44 g and hydrous dextrose 1·47 g in 100 ml pyrogen-free water) at the rate of one part of ACD to four parts of blood. The samples were dispensed into 50 ml tubes and centrifuged at 1600 ***g*** for 20 min. The plasma and the buffy coat layers were carefully aspirated and discarded; neutrophils were in the red cell pellet. Pyrogen-free water (15 ml at 37 °C) was added to the pellet in each tube and the cell suspension was agitated in a water bath at 37 °C for 60 s to lyse the red blood cells. Then 1·5 ml 10-fold concentrated Tyrodes solution (sodium chloride 8·0 g, potassium chloride 0·2 g, sodium dihydrogen phosphate monohydrate 0·05 g, sodium bicarbonate 0·5 g and D(+)-glucose 0·5 g in 100 ml pyrogen-free water) was added, followed by 25 ml Hanks Balanced Salt Solution (HBSS; Trace Biosciences Ltd, Castle Hill, NSW 2154) containing no Mg^{2+} and Ca^{2+} but with gelatin (1 g/l). The neutrophils were centrifuged at 250 ***g*** for 10 min, washed twice in HBSS, then resuspended at required concentrations in HBSS with Mg^{2+} and Ca^{2+} for use in assays.

Whey protein fractionation

Colostrum and milk were expressed manually from ewes immediately post partum and 3 weeks post partum respectively. These were defatted after centrifugation at 4 °C, after which whey was prepared by addition of rennet and saturated $CaCl_2$ solution followed by incubation at 37 °C for 2 h. The whey was filtered through 5 μm Acrodisc filters (Gelman Sciences, Ann Arbor, MI 48016, USA) and stored at −20 °C until required.

For preparing the LP and LF fractions from bovine Cheddar cheese, the method used was that detailed by Francis *et al.* (1995). Briefly, 10 l microfiltered cheese whey at 4 °C was adjusted to pH 6·5 with 0·1 M-NaOH and then pumped over a strong cation-exchange resin pre-equilibrated in 50 mM-sodium citrate. After loading, the resin was washed with four column volumes of 10 mM-sodium citrate and eluted stepwise using firstly 400 mM-NaCl (LP) and then 1 M-NaCl (LF). Both LP and LF eluates (~ 1 l) were adjusted to pH 7·4 with 0·1 M-NaOH, then concentrated and desalted using an Amicon DC-10 ultrafiltration unit fitted with a spiral wound cartridge having a 3 kDa exclusion membrane (Amicon, Beverly, MA 01915, USA). The concentrated and desalted LP and LF were then freeze dried. The approximate recoveries of proteins were 22·7 and 160 mg/l for the LP and LF fractions respectively. These fractions were confirmed to have *in vitro* growth promoting activities for three mammalian cell lines.

The α-la fraction was prepared from Cheddar cheese whey (bovine) using a thermal precipitation method at low pH as described by Pearce (1983). Bovine serum albumin was purchased from Sigma (St Louis, MO 63178, USA). Whey protein concentrate (WPC; United Milk Tasmania, Devonport, TAS 7310) was commercially prepared by ultrafiltration, diafiltration and spray drying of bovine Cheddar cheese whey.

All whey fractions were passed through polymyxin B gel columns (Detoxi-Gel™, Pierce Chemical Company, Rockford, IL 61105, USA) to remove endotoxin before use in the study.

Reagents

Zymosan-activated serum (ZAS) was prepared by incubating 15 mg zymosan (Sigma) in 1·0 ml fresh sheep serum at 37 °C for 1 h. After inactivation at 56 °C for 30 min, the serum was centrifuged at 1000 ***g*** and 4 °C for 15 min and the supernatant stored at −70 °C until use. Recombinant ovine interleukin-8 (roIL-8, batch 13) was kindly provided by Dr Heng F. Seow (CSIRO Division of Animal Health, Parkville, VIC 3052; Seow *et al.* 1994). Either ZAS at 1:30 dilution in HBSS with Mg^{2+} and Ca^{2+} or roIL-8 at a dose of $10^{-7·5}$ M was used as a chemoattractant agent (Seow *et al.* 1994).

Phorbol myristate acetate (Sigma) was prepared at a concentration of 2 mg/ml in dimethyl sulphoxide (Sigma) before being stored at −70 °C. For stimulating neutrophils, a final concentration of 20 ng/ml was prepared in HBSS with Mg^{2+} and Ca^{2+}. Nitroblue tetrazolium (grade III, Sigma) was freshly made at 1 mg/ml in HBSS with Mg^{2+} and Ca^{2+} for use.

Experimental design

The study was first carried out to assess the effects of crude colostral whey and milk whey on neutrophil chemotaxis. Whey fractions of various purities including LP, LF, LP–LF, α-la and WPC were then tested. Bovine serum albumin was also used as a control protein. The details are further described in the results.

Chemotaxis assay

The assay was performed in six replicates for each animal using a 48 well Micro Chemotaxis Chamber (Neuro Probe Inc., Bethesda, MD 20034, USA) with 3 μm polycarbonate filter membranes and a 20 min incubation period at 37 °C. Neutrophils suspended in HBSS with Mg^{2+} and Ca^{2+} at 5×10^6 cells/ml with or without whey proteins were placed into the upper well, and the chemoattractant (either ZAS or roIL-8) with or without whey proteins was placed in the lower well. Cells were incubated for 20 min. Cells migrating to the lower surface of the filter membrane were counted with or without the aid of an image analyser (Quantimet 500E Image Processing and Analysis System; Leica Cambridge Ltd, Cambridge CB1 3QH, UK) in five fields from each well at 200× magnification under a light microscope.

Superoxide production assay

An enzyme immunomicroassay based on quantitating nitroblue tetrazolium reduction was employed (Pick, 1986). Briefly, 5×10^5 neutrophils in phenol red-free HBSS with Mg^{2+} and Ca^{2+} containing different concentrations of whey proteins were dispensed into 96-well microplates (Immulon II; Dynatech Laboratories Inc., Chantilly, VA 22021, USA) and incubated at 37 °C with phorbol myristic acetate and nitroblue tetrazolium solution for 15 min. The amount of formazan formed within the cells was quantitated at 550 nm in an ELISA plate reader.

Table 1. *Effects of ovine colostral whey and milk whey on ovine neutrophil chemotaxis*

(Values are expressed as percentage of the corresponding control without whey, means ± SEM for $n = 5$)

	Whey concn, g/l		
Colostral whey	10	100	1000
Not washed	124·5 ± 22·7	84·6 ± 14·5	16·7 ± 6·8*
Washed†	100·6 ± 8·7	96·8 ± 12·9	125·9 ± 13·6
Milk whey			
Not washed	124·5 ± 27·3	103·8 ± 15·6	58·2 ± 6·8*
Washed†	94·5 ± 4·5	97·8 ± 5·8	80·6 ± 2·1

† Experiments in which neutrophils were washed after incubation with whey prior to assays.
* Values were significantly different from those with washed neutrophils: $P < 0·01$.

Statistics

The results for each experiment are expressed as means ± SEM of either absolute values or percentages of the relevant control value. For dose–response testing, repeated measurement of variance analysis was performed and, if the dose effect was significant, Bonferroni's t test was used to compare each concentration with the control group. Analysis of variance (ANOVA) and least significant difference (LSD) were employed for Fig. 2.

RESULTS

Effects of crude whey

The effects of colostral whey and milk whey on chemotaxis of neutrophils were first studied by incorporating them with the cell preparation in the upper well and using ZAS as chemoattractant in the lower well. Table 1 shows that the highest concentration of colostral whey (1000 g/l, i.e. undiluted) significantly inhibited neutrophil chemotaxis ($P < 0·05$). Similarly, undiluted (1000 g/l) milk whey also inhibited chemotaxis ($P < 0·05$).

To determine whether the above inhibitory effects were reversible, after neutrophils had been incubated with various concentrations of colostral whey or milk whey for 20 min at 37 °C, they were centrifuged at 250 ***g*** for 10 min and washed twice with HBSS prior to their use in the chemotaxis assay. As shown in Table 1, the inhibitory effects were abolished by the washing step. Neutrophils were also treated with autologous and homologous whey and used in the chemotaxis assay. Both colostral whey and milk whey at a concentration of 1000 g/l exhibited strong inhibitory effects on neutrophil chemotaxis regardless of their sources (Table 2).

Effects of colostral whey and milk whey on the chemotactic activity of ZAS were then studied by incorporating whey into the ZAS preparation prior to tests. Inhibitory effects on the chemotactic activity of ZAS were found in a dose-dependent manner when either colostral whey or milk whey was incorporated (Fig. 1).

Effects of purified whey fractions

Neutrophil chemotaxis and superoxide production were employed to compare whey protein fractions of different purity. Recombinant ovine IL-8, which was found to have potent chemotactic activity for ovine neutrophils, was used in the chemotaxis assay (Seow *et al.* 1994). As shown in Table 3, there were no significant effects of any of these fractions on roIL-8-induced chemotactic migration of neutrophils. However, two fractions, LP–LF and WPC, exhibited enhancing effects

Table 2. *Effects of either autologous or homologous whey at 1000 g/l on ovine neutrophil chemotaxis*

(Values are means ± SEM for $n = 5$)

Treatment	Neutrophils per 200 × magnification field
Control	127·4 ± 4·4
Autologous ovine colostral whey	35·3 ± 15·9*
Autologous ovine milk whey	31·4 ± 5·7*
Homologous ovine colostral whey	21·0 ± 5·6*
Homologous ovine milk whey	31·6 ± 2·2*

* Values were significantly different from the control: $P < 0{\cdot}01$.

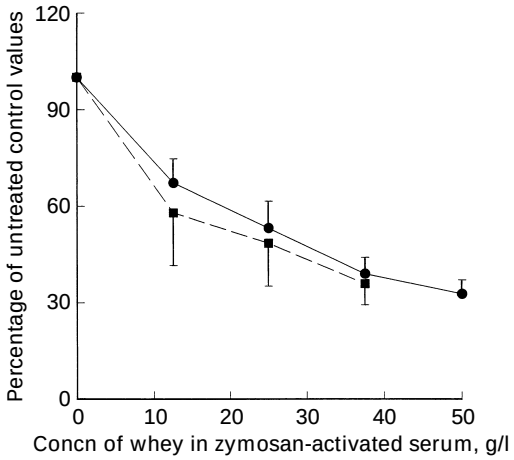

Fig. 1. Effects of ●, colostral whey and ■, milk whey on chemotactic activity of zymosan-activated serum in ovine neutrophils. Both whey preparations had dose-dependent inhibitory effects ($P < 0{\cdot}05$). Results are expressed as percentage of untreated control values, and are means for $n = 4$ with SEM indicated by vertical bars.

Table 3. *Effects of whey fractions on chemotaxis of neutrophils induced by recombinant ovine interleukin-8*

(Values are expressed as percentage of the corresponding control value, means ± SEM for $n = 4$)

	Concn, μg/ml						
Fraction	3·125	6·25	12·5	25	50	100	200
Lactoferrin (1)†	119·4 ± 8·6	102·6 ± 9·8	100·3 ± 12·6	114·6 ± 12·1	121·0 ± 4·2	119·3 ± 7·9	110·0 ± 10·7
Lactoferrin (2)	45·7 ± 15·0	ND	59·0 ± 10·0	ND	76·5 ± 24·7	ND	90·9 ± 14·1
Lactoperoxidase (1)	132·2 ± 12·7	157·4 ± 20·0	125·8 ± 16·8	125·4 ± 20·0	154·3 ± 20·0	150·5 ± 15·3	146·6 ± 28·0
Lactoperoxidase (2)	92·0 ± 9·5	ND	105·1 ± 33·1	ND	89·5 ± 12·4	ND	88·1 ± 17·3
Lactoferrin–lactoperoxidase	115·6 ± 16·9	ND	112·6 ± 14·1	ND	119·5 ± 5·3	ND	95·8 ± 6·2
α-Lactalbumin	74·2 ± 6·2	ND	61·5 ± 7·3	ND	78·0 ± 18·6	ND	71·9 ± 5·4
Whey protein concentrate	88·8 ± 19·8	ND	83·0 ± 20·0	ND	80·4 ± 8·4	ND	86·4 ± 8·2
Bovine serum albumin	100·6 ± 10·6	ND	114·2 ± 6·8	ND	95·7 ± 3·7	ND	87·2 ± 14·8

ND, not determined.
† Numbers in parentheses are batch nos.

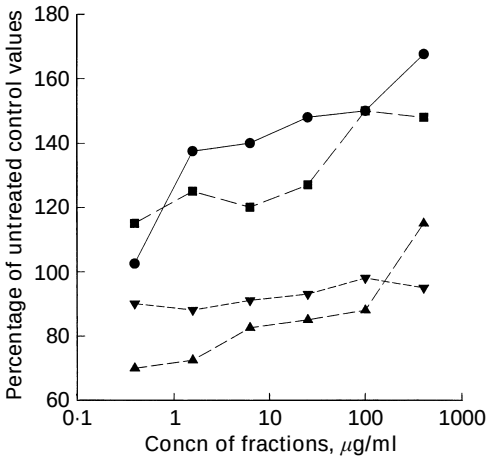

Fig. 2. Effects of bovine whey fractions on ovine neutrophil superoxide production. Results are expressed as percentage of untreated control values and are means for $n = 4$. ●, Lactoferrin–lactoperoxidase ($P < 0{\cdot}05$); ■, whey protein concentrate ($P < 0{\cdot}05$); ▲, α-lactalbumin; ▼, bovine serum albumin.

on superoxide production ($P < 0{\cdot}05$); α-la had a similar but not statistically significant enhancing effect (Fig. 2). As neutrophils treated with bovine serum albumin exhibited a stable pattern of superoxide production response regardless of the concentrations used, these results served as a control.

DISCUSSION

The present study shows the presence of anti-inflammatory factors in both ruminant milk whey and colostral whey by demonstrating their capacity to inhibit chemotactic migration of neutrophils. The mechanism involved in inhibition by whey of neutrophil chemotaxis is unclear. Colditz & Maas (1987) found that activated complement lost its *in vivo* inflammatory potential when present in whole ruminant milk, but not when it was present in skimmed milk (without milk leucocytes and fat). It was suggested that proteolytic activity might be responsible for the inactivation of complement by components of whole milk (Colditz & Maas, 1987). In contrast, our *in vitro* results (Fig. 1) showed that activated complement significantly lost its chemotactic activity in the presence of various concentrations of whey free from leucocytes and fat. However, as this study shows that the chemotaxis of neutrophils could be restored by washing the cells free of whey, it is more likely that the effect was due to the physical environment provided by the whey. A recent report (Buescher, 1991) has supported this interpretation by showing that exposure to human cell-free colostrum significantly reduced the locomotor response of neutrophils by decreasing their deformability and increasing their content of cytoskeleton-associated actin. This hypothesis is also consistent with our results in that no significant effects on neutrophil chemotactic migration were found when various purified whey fractions were used. However, the possibility that whey might exert inhibition through the impairment of the interaction of ZAS with receptors specific to, for example, C5a/C5a-des-Arg at the neutrophil surface cannot be excluded.

LF is mainly synthesized in cells of either the myeloid series or secretory epithelia. While the antimicrobial activity of lactoferrin has been described for years, its role

in inflammation is still somewhat controversial. Its antioxidant role is believed to be responsible for the anti-inflammatory effects of LF through at least two different properties: iron-binding and lipopolysaccharide-binding (Miyazawa *et al.* 1991; Britigan *et al.* 1994). However, Gahr *et al.* (1991) have shown that the exposure of human polymorphonuclear leucocytes to human LF could enhance their chemotaxis and superoxide production. Despite being the second most abundant protein in human milk whey, LF of epithelial source is only present as a minor protein in ruminant milk whey (Masson & Heremans, 1971). It is notable that the antimicrobial peptide in bovine lactoferrin was found to be more powerful than that in human LF (Bellamy *et al.* 1992). Despite this, the present study showed that a highly purified bovine LF fraction did not exert any effect on chemotaxis of ovine neutrophils. Unlike LF, LP has not received much attention regarding its immunomodulatory activity even though it is also thought to have antibacterial properties (Schanbacher & Smith, 1974; Pruitt *et al.* 1991). The contribution of myeloperoxidase to peroxidase activity in milk, which is still controversial, seems to obscure the search in this area. Our results show that bovine LP had no effect on chemotaxis of ovine neutrophils. However, a mixed whey fraction containing both bovine LF and LP had a strong effect on promoting neutrophil superoxide production. It is shown that the presence of hydrogen peroxide is favourable to the native antimicrobial properties of LP (Pruitt *et al.* 1991). Therefore, the subsequent increase in hydrogen peroxide resulting from exogenously enhanced production of superoxide in neutrophils may provide a synergic effect on their microbicidal activity.

Dietary whey protein concentrate has been reported to have immunoenhancing effects in laboratory species (Bounous *et al.* 1988, 1989; Bounous & Gold, 1991; McIntosh *et al.* 1995; Wong & Watson, 1995), and the present study has further endorsed this view by demonstrating its enhancing effect on the superoxide production of neutrophils. Whether the presence of LF and LP in WPC would have a role in its modulatory effect remains to be clarified. However, without exposure to any metabolic process, the WPC and LF–LP fractions exhibited significant *in vitro* enhancing effects on neutrophil superoxide production in this study, suggesting that LF and LP do play a role in the immunoenhancing properties of whey.

In conclusion, these experiments showed that pro- and anti-inflammatory factors exist in ruminant whey. Although purified whey fractions did not seem to be responsible for the inhibitory effect of crude whey on neutrophil chemotaxis, some of them enhanced neutrophil superoxide production in this study. It is believed that the various factors in whey may counteract each other, and thus this study not only provides relevant information on the modulatory role of ruminant milk proteins in inflammatory responses but also warrants future investigation of the immuno-inflammatory applications of purified whey proteins.

The authors thank Chris Leger for her excellent technical assistance. This work was funded by the Dairy Research and Development Corporation in Australia.

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