

Bovine colostrum modulates immune activation cascades in human peripheral blood mononuclear cells *in vitro*

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SUMMARY

Bovine colostrum (BC) is the thick yellow fluid a lactating cow gives to a suckling calf during its first days of life to support the growth of the calf and prevent gastrointestinal infections until the calf has synthesized its own active immune defense system. BC contains a complex system of immune factors and has a long history of use in traditional medicine. In an approach to evaluate the effects of bovine colostrum (BC) on the T-cell/macrophage interplay, we investigated and compared the capacity of BC containing low and high amounts of lactose and lactoferrin to modulate tryptophan degradation and neopterin formation in unstimulated and mitogen-stimulated human peripheral blood mononuclear cells (PBMC). The present study shows significant immunomodulatory effects of these BC preparations in human PBMC, either by enhancing or suppressing the occurrence of a Th-1 type immune response. The amount of lactose present in BC seems to diminish the activity of BC in our test system, since BC with higher amounts of lactose attenuated the stimulatory as well as the suppressive activity of BC.

KEY WORDS: Bovine colostrum, Peripheral blood mononuclear cells, Indoleamine 2,3-dioxygenase, Tryptophan, Neopterin

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INTRODUCTION

Colostrum is the first milk produced by the mammary glands of mammalia within the first days after giving birth. Bovine colostrum (BC) is homologous to human colostrum, although the protein content of BC is about twenty-, and the amount of IgG about ten-times higher than that of the human analog (Pakkanen and Aalto, 1997; Solomons, 2002). Since the availability of human colostrum is limited, BC, which can easily be produced in large quantities, has been accepted for use in humans (Pakkanen and Aalto, 1997). BC

contains large quantities of immunological factors to support the growth and immune maturation of the digestive tract and to provide passive immunity until the newborn calf has synthesized its own active immune defense system (Kaushik *et al.*, 2002; Pakkanen and Aalto, 1997).

The composition of BC comprises, beside nutrients such as carbohydrates, fat, vitamins, amino acids and minerals also immunoglobulins (Ig) including IgG, IgM and IgA, which may provide a defense in both the treatment and prevention of viral and bacterial infections (Elfstrand, 2002; Struff and Sprotte, 2007, 2008).

Furthermore, BC contains growth factors such as insulin like growth factor (IGF) I and II, transforming growth factor- β (TGF- β) or epidermal growth factor (EGF), and pro-inflammatory cytokines such as interleukin-1 β (IL- β), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) (Floren *et al.*, 2006; Pakkannen and Aalto, 1997; Playford *et al.*, 2000; Tripathi

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and Vashishtha, 2006). In addition, also anti-inflammatory factors such as IL-1 receptor antagonist- α (IL-1 α), type II soluble IL-1 receptor (sIL-1RII), sCD14 and high amounts of lactoferrin, a 78 kD protein that has been shown to transport essential iron to haematopoietic cells and to prevent harmful viruses and bacteria from getting iron needed for growth, are present in BC (Conneely, 2001; Filipp *et al.*, 2001; Hagiwara *et al.*, 2000, 2005; Viljoen, 1995; Yamauchi *et al.*, 1993).

Although colostrum has only received widespread attention as a dietary supplement since the late 1990s, it has a long history of medicinal use. In India, Ayurveda practitioners have used colostrum as a treatment for various diseases for thousands of years and in the USA it was used as an antibacterial agent until the discovery of antibiotics. In the 1950s, colostrum was applied to treat rheumatoid arthritis (RA) and the first oral vaccine for poliomyelitis, developed by Albert Sabin, was also prepared from BC (Sabin and Fieldsteel, 1962). Colostrum also exerts beneficial effects in various gastrointestinal disorders e.g. in the treatment of bacterial and viral diarrhea (Antonius, 2000; Bogstedt *et al.*, 1996; Davidson *et al.*, 1989) or to stop the adhesion activity of *Helicobacter pylori* and *Helicobacter mustelae* (Bitzan *et al.*, 1998) and to protect from gut injury induced by non-steroidal anti-inflammatory drugs (NSAIDs) (Sigthorsson *et al.*, 1998).

Furthermore, colostrum was reported to be effective in tissue repair, to reduce the incidence of self-reported symptoms of upper respiratory tract infection and to enhance immunity (Boesman-Finkelstein and Finkelstein, 1989; Brinkworth and Buckley, 2003; Ogra and Ogra, 1978; Stephan *et al.*, 1990).

However, despite a large amount of literature concerning the properties of human or bovine colostrum, there is only sparse data on the effects of colostrum on the human immune system. In human peripheral blood mononuclear cells (PBMC) natural BC was shown to induce IL-12 and to enhance IFN- γ production after weak antigenic stimulation (Biswas *et al.*, 2007).

Furthermore, a commercially available BC protein concentrate was shown to stimulate secretion of interferon- γ (IFN- γ), interleukin-10 (IL-10), and interleukin-2 (IL-2) in PBMC cultures, thereby promoting a Th-1 type immune response.

In contrast, pretreatment of PHA-stimulated PBMC with BC, enhanced the secretion of IL-10 and IL-12 and suppressed production of IFN- γ and TNF (Shing *et al.*, 2009).

During Th-1 type immune response, activated T-cells release large amounts of cytokines such as IL-2 or IFN- γ , which mediate pro-inflammatory functions critical for the development of cell-mediated immune responses.

Besides other pathways, T-cell derived IFN- γ induces also activation of the enzyme indoleamine 2,3-dioxygenase (IDO) in macrophages, that converts tryptophan into N-formylkynurenine, which subsequently is deformed to kynurenine (Wirleitner *et al.*, 2003). Accelerated tryptophan degradation manifests in decreased serum tryptophan concentrations and increased kynurenine to tryptophan ratios (kyn/trp), which may serve as a measure of IDO activity. IDO plays a central role in the suppression of intracellular bacteria and viruses during an antimicrobial immune response, as ongoing tryptophan degradation limits protein biosynthesis due to deprivation of this essential amino acid (Ozaki *et al.*, 1988; Pfefferkorn, 1986).

In parallel to tryptophan degradation, IFN- γ also stimulates formation of neopterin, via induction of the enzyme guanosine-triphosphate-(GTP)-cyclohydrolase, representing another marker for the activation of the T cell-macrophage axis in humans (Huber *et al.*, 1984; Wirleitner *et al.*, 2003). Increased tryptophan degradation and neopterin production were found in patients during diseases which are associated with Th1-type immune activation such as infections, autoimmune diseases, malignant disorders, and during allograft rejection episodes (Murr *et al.*, 2002).

Due to the short half-life of cytokines whose measurement is often challenging, the measurement of IDO activity and formation of neopterin provides a robust approach to evaluate the modulation of a Th-1 type immune response in human PBMC.

The *in vitro* model of activated PBMC has been well established in clinical immunology for several decades and allows standardization of T-cell activation and T-cell/macrophage interactions, which is highly relevant in the pathogenesis of immunological disorders.

The aim of this study was to assess the influence of BC containing low and high amounts of lac-

tose and lactoferrin on Th-1 type immune response in terms of tryptophan degradation and neopterin formation in unstimulated and phytohaemagglutinin (PHA)-stimulated human PBMC.

MATERIALS AND METHODS

BC preparations

BC preparations investigated in this study were obtained from New Zealand marketed by Neovite (London, United Kingdom). BC was processed from fresh chilled material, which was pasteurised, defatted, and then either dried immediately (BC containing high amounts of lactose =37%), or lactose reduced (BC containing low amounts of lactose =9%) and then dried. Lactoferrin was a kind gift from Biopole (Gembloux, Belgium).

Isolation and stimulation of human PBMC

PBMC were isolated from whole blood of healthy donors of whom informed consent for the use of their donated blood for scientific purposes, unless otherwise used, has been obtained. Separation of blood cells was performed using density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway).

After isolation, PBMC were washed three times in phosphate buffered saline containing 0.2% ethylenediaminetetraacetic acid (0.5 mM, Sigma Aldrich, Vienna, Austria). Cells were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated foetal calf serum (Biochrom, Berlin, Germany), 1% of 200 mM glutamine (Serva, Heidelberg, Germany) and 0.1% of gentamicin (50 mg/ml, Bio-Whittaker, Walkersville, MD, USA) in a humidified atmosphere containing 5% CO₂ for 48 hours.

This procedure was observed earlier to reveal best reproducible results when applied for testing of anti-inflammatory effects of compounds or drugs (Widner *et al.*, 1997).

Average tryptophan content in the supplemented RPMI 1640 medium was 31.5 µM. For each of three experiments run in duplicates, PBMC were freshly prepared from individual donors. Isolated PBMC were plated at a density of 1.5 x10⁶ cells/well in supplemented RPMI 1640, preincubated for 30 minutes with or without BC prepa-

rations or lactoferrin and stimulated or not with 10 µg/ml PHA for 48 hours.

Measurement of tryptophan, kynurenine, and neopterin concentrations

After incubation of cells for 48 hours, supernatants were harvested by centrifugation and tryptophan and kynurenine concentrations were measured by high performance liquid chromatography (HPLC) using 3-nitro-L-tyrosine (Sigma Aldrich, Vienna, Austria) as an internal standard (Widner *et al.*, 1997). To estimate IDO activity, the kynurenine to tryptophan ratio (Kyn/trp) was calculated and expressed as µmol/mmol (Widner *et al.*, 1997). Neopterin concentrations were determined by enzyme-linked immunosorbent assay (ELISA; Brahms, Hennigsdorf/Berlin, Germany), according to the manufacturer's instructions with a detection limit of 2 nM.

Measurement of cell viability

After incubation of PBMC with BC preparations or lactoferrin for 48 hours, cell viability was measured by reduction of the tetrazole 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, Vienna, Austria), which is reduced to purple formazan in living cells (Mosmann, 1983) and by trypan blue (Sigma Aldrich, Vienna, Austria) exclusion method in three experiments done in triplicates. No toxicity could be observed at the concentration range applied (data not shown).

Statistical analysis

For statistical analysis, the Statistical Package for the Social Sciences version 14.0 (SPSS Inc., Chicago, Ill, USA) was used. Because not all data sets showed normal distribution, for comparison of grouped data non-parametric Friedman test and Wilcoxon signed ranks test were applied. *P*-values below 0.05 were considered to indicate significant differences.

RESULTS AND DISCUSSION

Effect of BC preparations on tryptophan metabolism and neopterin formation in unstimulated PBMC

The supernatants of unstimulated PBMC, cultivated for 48 hours under standard cultivation

conditions, contained $30.4 \pm 0.8 \mu\text{M}$ tryptophan and $0.9 \pm 0.04 \mu\text{M}$ kynurenine resulting in a kynurenine to tryptophan ratio (kyn/trp) of $28.1 \pm 1.5 \mu\text{mol}/\text{mmol}$, as a measure of spontaneous IDO activity. In the same supernatants, neopterin concentrations of $3.6 \pm 0.2 \text{ nM}$ were detected (Table 1).

Treatment of PBMC with 0.2 mg/ml BC, containing low amounts of lactose, enhanced IDO activity about two-fold ($199.7 \pm 29.2\%$), whereas a significant reduction of IDO activity to $72.2 \pm 5.9\%$ could be observed at a dosage of 20 mg/ml (Figure 1A). Higher amounts of lactose present in BC attenuated the stimulatory, as well as the suppressive capacity in unstimulated PBMC, since BC containing high amounts of lactose did not influence tryptophan metabolism significantly (Figure 1A).

Treatment with antimicrobial lactoferrin (Caccavo, *et al.*, 2002), of which large quantities are present in BC ($1.5\text{-}5.0 \text{ g/L}$), suppressed IDO activity not until a dosage of 20 mg/ml ($54.7 \pm 5.0\%$; Figure 1A). Measurement of neopterin formation within the same supernatants revealed that also BC with low amounts of lactose bore the strongest capacity to induce neopterin production at doses of 0.2 mg/ml ($258.5 \pm 30.0\%$). Unlike the observed inhibition on

TABLE 1 - Concentrations of tryptophan, kynurenine, kynurenine to tryptophan ratio (kyn/trp) and neopterin in the supernatant of unstimulated PBMC and in cells stimulated with 10 g/ml phytohaemagglutinin (PHA) for 48h. Results shown are the mean values \pm S.E.M. of three independent experiments run in duplicates ($*p < 0.05$, compared to unstimulated cells).

	Unstimulated	PHA ($10 \mu\text{g/ml}$)
Tryptophan [μM]	30.4 ± 0.8	$8.2 \pm 0.8^*$
Kynurenine [μM]	0.9 ± 0.04	$9.8 \pm 0.5^*$
Kyn/trp [$\mu\text{mol}/\text{mmol}$]	28.1 ± 1.5	$1312 \pm 187^*$
Neopterin [nM]	3.6 ± 0.2	$13.2 \pm 1.5^*$

tryptophan degradation after treatment with 20 mg/ml of BC containing low amounts of lactose, neopterin formation in the same samples was enhanced ($158 \pm 11.4\%$). Furthermore, in contrast to the influence on tryptophan metabolism, BC with high amounts of lactose showed a stimulatory capacity on neopterin formation, which, at doses of 2 mg/ml ($235 \pm 22.3\%$), was comparable to the effect of BC containing low amounts of lactose

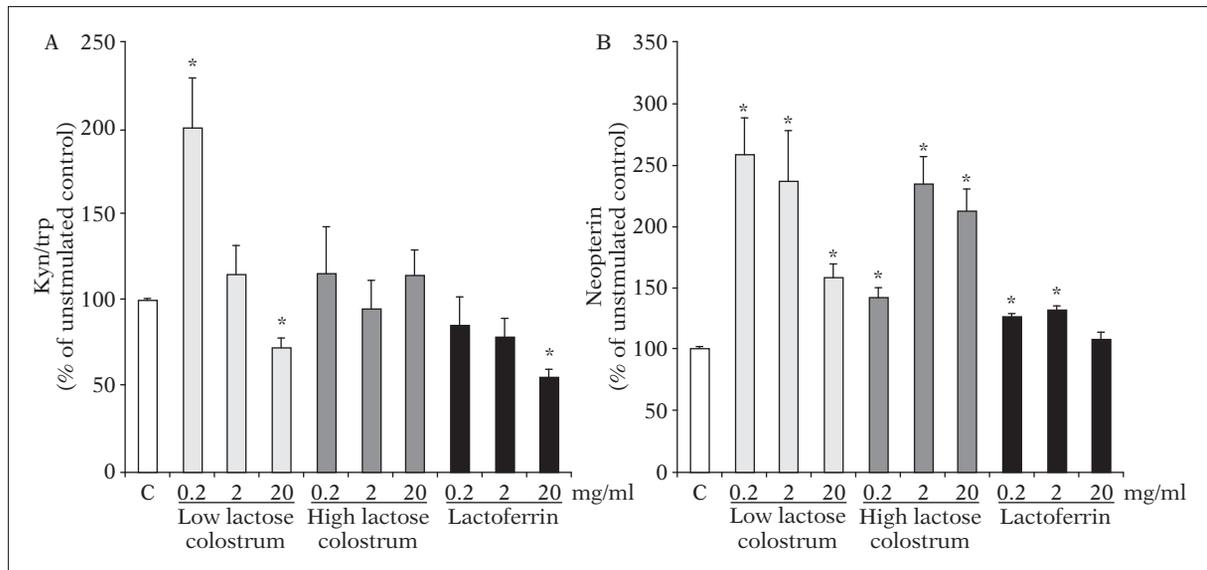


FIGURE 1 - (A) Kynurenine to tryptophan ratio and (B) neopterin formation expressed as % of unstimulated control (C) in PBMC treated or not with increasing concentrations of bovine colostrum with low and high amounts of lactose or lactoferrin for 48 hours. Results shown are the mean values \pm S.E.M. of three independent experiments run in duplicates ($*P < 0.05$).

($237 \pm 41.4\%$; Figure 1B). The addition of BC containing high amounts of lactose at doses of 0.2 mg/ml seem to attenuate the stimulatory capacity ($142 \pm 8.3\%$) of BC containing low lactose ($259 \pm 30\%$), whereas at doses of 20 mg/ml neopterin production was even stronger ($213 \pm 18.3\%$) as compared to the effect of low lactose containing BC ($158 \pm 11.4\%$). Treatment of unstimulated PBMC with lactoferrin induced only a moderate increase of neopterin at 0.2 or 2 mg/ml to $125 \pm 2.8\%$ or $132 \pm 4.1\%$, respectively (Figure 1B).

These results correspond well with data of Shing *et al.*, 2009, who reported a stimulatory capacity of a BC protein concentrate on IFN- γ secretion in unstimulated human PBMC, which is the main inducer of tryptophan degradation and neopterin formation in human macrophages. Earlier experiments of Biswas *et al.*, 2007 revealed also a stimulatory effect of BC on interleukin (IL)-12 production in human PBMC. However, although IL-12 is regarded as a main inducer of IFN- γ , in this study BC was unable to stimulate IFN- γ production, which is possibly the result of the lower BC concentration applied (≤ 10 $\mu\text{g/ml}$). Accordingly, also the findings of our study imply that colostrum at doses even lower than the 0.2 mg/ml may have an effect on unstimulated or mi-

togen stimulated degradation of tryptophan or formation of neopterin, two biochemical pathways, which are well known to be induced by IFN- γ . However, such low doses were not tested in this system.

Effect of BC preparations on tryptophan metabolism and neopterin formation in PHA-stimulated PBMC

Upon treatment of PBMC with PHA (10 $\mu\text{g/ml}$) for 48 hours, tryptophan content in the supernatant decreased to 8.2 ± 0.8 μM whereas kynurenine concentrations increased concomitantly to 9.8 ± 0.5 μM , indicating an approximately 47-fold increase of IDO activity (Kyn/trp: 1312 ± 187 $\mu\text{mol/mmol}$). Within the same supernatants, neopterin concentrations raised about 3.6-fold to a level of 13.2 ± 1.5 nM (Table 1).

Pre-treatment of PHA-stimulated PBMC cultures with BC preparations containing low or high amounts of lactose or lactoferrin, revealed a strong and dose dependent capacity to suppress PHA-induced tryptophan degradation. BC with low amounts of lactose showed the strongest inhibitory effect on IDO enzyme activity (0.2 mg/ml: $25.4 \pm 5.3\%$) followed by the effect of lactoferrin (0.2 mg/ml: $34.0 \pm 9.7\%$) and BC with higher amounts of lactose ($46.0 \pm 16.6\%$; Figure 2A).

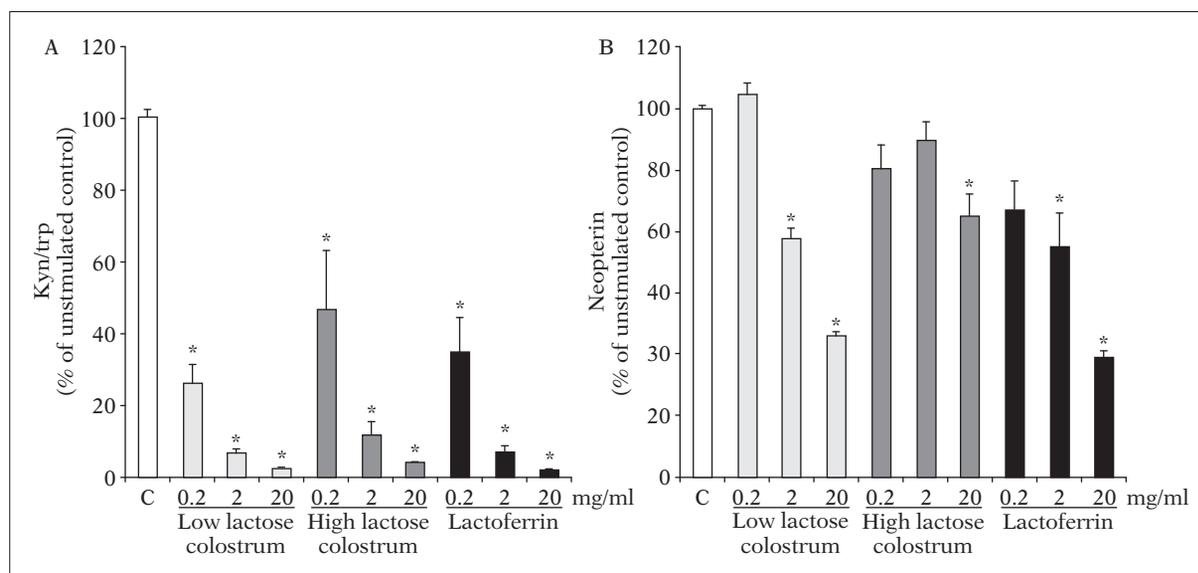


FIGURE 2 - (A) Kynurenine to tryptophan ratio and (B) neopterin formation expressed as % of phytohaemagglutinin (PHA, 10 $\mu\text{g/ml}$) stimulated control (C) in PBMC cotreated or not with increasing concentrations of bovine colostrum with low and high amounts of lactose or lactoferrin for 48 h. Results shown are the mean values \pm S.E.M. of three independent experiments run in duplicates (* $P < 0.05$).

Similar to the results obtained in unstimulated PBMC, higher amounts of lactose present in BC seem to attenuate also the inhibitory activity of BC in PHA-stimulated cells. At higher concentrations of 2 or 20 mg/ml, all preparations almost completely counteracted PHA-stimulated tryptophan degradation in the same rank order of activity (Figure 2A).

Mitogen induced neopterin formation was also diminished by these BC preparations and lactoferrin, although with lower potency as compared to the effects on PHA stimulated tryptophan degradation (Figure 2B). Again, BC with higher amounts of lactose showed the weakest inhibitory effect on PHA-stimulated neopterin formation, exerting a significant inhibition to $70.5 \pm 7.2\%$ only at a dosage of 20 mg/ml.

The potency of BC with low amounts of lactose, taking effect at 2 mg/ml ($64.0 \pm 4.8\%$) and 20 mg/ml ($37.1 \pm 3.0\%$), were comparable to the effect of lactoferrin (2 mg/ml: $61.3 \pm 11.7\%$; 20 mg/ml: $31.1 \pm 3.9\%$).

These results are also in accordance with data of Shing *et al.*, 2009, who showed that co-treatment of PHA-stimulated PBMC with BC, suppressed production of proinflammatory IFN- γ . However, no significant inhibitory effect of BC on antigen-induced IFN- γ production could be detected by Biswas *et al.*, 2007, which may result from the lower concentration range applied in this study (0.1-10 $\mu\text{g/ml}$), in comparison to the concentrations used by us (0.2-20 mg/ml) or Shing *et al.*, 2009 (12.5-50 mg/ml).

The present study shows that BC exerts a biphasic mode of action by promoting a Th-1 type immune response in unstimulated PBMC, as well as a strong suppressive capacity on biochemical pathways stimulated by a Th-1 type immune response in PBMC.

The suppressive potential of BC on PHA-induced tryptophan degradation and neopterin formation may be related to the content of lactoferrin, which was shown to be effective in the same range of activity. Since BC containing high amounts of lactose attenuated the stimulatory capacity in unstimulated PBMC as well as the suppressive activity in mitogen stimulated PBMC, we speculate that high amounts of lactose may interfere with cytokine signaling due to complexation of immune factors present in BC, which results in reduced bioavailability. Therefore, the use of lac-

tose reduced BC may be more reasonable to down-regulate a Th-1 type immune response. Although this effect of BC to suppress a Th-1 type immune response has been gathered from *in vitro* experiments only and at comparatively high concentrations, we assume that at least when topically applied for the treatment of skin lesions of e.g. patients with atopic dermatitis or psoriasis, all components of BC are present at effectual concentrations to down-regulate the activity of infiltrated T-cells, macrophages and other mononuclear cells. Still further studies are needed to confirm this assumption for a potential beneficial therapeutic effect of BC in patients suffering from inflammatory dermatoses.

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