Immunomodulatory effects of bovine colostrum in human peripheral blood mononuclear cells

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SUMMARY

Human and bovine colostrum (BC) contain a remarkable amount of bioactive substances, including antibodies towards many common pathogens of the intestinal and respiratory tract as well as growth factors, vitamins, cytokines and other proteic, lipidic and glucidic factors. In this study we investigated whether BC had any immunomodulatory effect on human peripheral blood mononuclear cells (PBMC) from healthy donors. To this aim we focused on the production of IL-12 and IFN-γ, cytokines involved in the Th1 polarization required for a successful immune response towards intracellular pathogens, such as bacteria and viruses. BC induced a dose-dependent production of IL-12 by CD14+ monocytes, but was unable to induce IFN-γ production. However, BC differentially affected stimuli-induced IFN-γ production: it enhanced IFN-γ in response to weak antigenic stimulation and it inhibited IFN-γ in response to strong antigenic stimulation. These effects were not dose-dependent. We also measured PBMC proliferation, which was substantially unaffected by BC. Our data suggest that the Th1-promoting activity of BC could contribute, together with the antibodies, to the protective effect of BC on the offspring. BC could also represent an inexpensive therapeutic tool in prevention and treatment of several human microbial infections, including influenza.

KEY WORDS: Bovine colostrum, Human peripheral blood mononuclear cells, Cytokines, IL-12, IFN-γ, Immune modulation, Th1 response

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INTRODUCTION

Colostrum is the pre-milk yellowish fluid produced by female mammary glands in late pregnancy and within 48-72h after giving birth. Colostrum greatly favors growth and conveys protection to the offspring of many mammalian species, including humans, against several microbial pathogens (Henderson DR, Mitchell D, 1999). The protein content of bovine colostrum (BC) is three to four times higher than in regular cow's milk and about twenty times higher than that of human colostrum (Solomons, 2002). Bovine colostrum indeed contains a large number of growth factors (EGF, FGF, IGF-I, IGF-II, TGF-α and TGF-β), antimicrobial (lactoferrin) and immunomodulatory substances, such as colostrinin or proline-rich-polypeptide, and nutrients (vitamin A, E, B12) (Playford *et al.,* 2000), but, most of all, it is rich in γ-globulins (or immunoglobulins or antibodies) (Kelly, 2003; Korhonen *et al.*, 2000b). While the human infant receives antibody protection via the placenta and colostrum, the calf has no placental transfer of antibodies and relies totally on colostrum (Lilius and Marnila, 2001). The antibodies contained in BC are against many common microbes, particularly those that

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affect the gastrointestinal and respiratory tract (Lilius and Marnila, 2001; Rump *et al.*, 1992). Of interest, Sabin *et al.* recognized that the neutralizing activity against type 2 poliovirus found in human and BC was linked to the antibody fraction (Sabin and Fieldsteel, 1962). It is expected that antibodies and other factors contained in BC would be destroyed by the acidic milieu of the stomach and via proteolysis by intestinal proteases. Nevertheless, a study has documented a large recovery of functionally active antibodies in the ileal fluid of volunteers who had ingested immunoglobulin-concentrated BC (Warny *et al.*, 1999). Protection from degradation appears to rely mainly on trypsin inhibitors, oligosaccharides and glycoconjugates, which are also found in BC (Gopal and Gill, 2000; Harpaz and Schachter, 1980; Tsuji *et al.,* 1982).

Immunity encompasses a humoral arm, mainly represented by antibodies produced by differentiated B lymphocytes and an equally important cellular arm composed by cells belonging to innate (neutrophils, monocytes, NK cells) and adaptive immunity (T and B lymphocytes). T lymphocytes can be functionally distinguished into cytotoxic (Tc) and helper (Th) cells. The latter are embodied by CD4+ T lymphocytes which orchestrate essentially all phases of an immune response, mainly through producton of soluble factors, called cytokines (or interleukins, IL). Th lymphocytes can be divided into two major functional subsets, in both mice and humans, according to cytokine secretion: Th1 primarily produce IL-2 and interferon (IFN)-γ, whereas Th2 produce IL-4, IL-5 and IL-10 (Mosmann *et al.,* 1986; Paul and Seder, 1994). The dimeric proinflammatory cytokine IL-12 produced by dendritic cells and monocytes is the principal inducer of IFN-γ and thus represents a fundamental cytokine in the development of a Th1 response (Trinchieri, 1995).

Due to the capacity of BC to convey protection towards many intracellular pathogens, we hypothesized that this could occur not only by means of passive transfer of specific antibodies, but also by promoting a Th1 response. Thus, in this study we investigated the immunomodulatory potential of BC in favoring a Th1 response through the evaluation of the production of IL-12 and IFN-γ in peripheral blood mononuclear cells (PBMC). We also assessed whether BC had a proliferative effect on PBMC.

MATERIALS AND METHODS

BC preparation

The BC used in our study is de-fatted and freeze-dried, containing less than 1% of fat, 85-90% of protein of which about 85% are IgG (Colexan, Colostrum Technologies, GmBH). We dissolved 1 g of BC powder in 1 liter of culture medium RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) yielding a stock solution of 1 mg/ml. According to the *in vitro* activity range of several immunomodulatory factors, including microbial derivatives, mitogens, differentiating agents and cytokines, we chose to use the following final concentrations of BC in our assays: 10, 1 and 0.1 µg/ml.

Donors

Five healthy individuals (4 females, 1 male) were recruited from the laboratory personnel and donated blood upon informed consent on the nature of the study. Whole blood was withdrawn in EDTA-containing tubes. PBMC, consisting of monocytes and lymphocytes, were obtained by density gradient purification (Lymphoprep nycomed, Axis-Shield, Oslo, Norway) and resuspended in RPMI 1640 medium supplemented with 2 mM of l-glutamine, 10 U of penicillin-streptomycin/ml, and 10% heat-inactivated fetal calf serum (FCS; BioWhittaker) (complete medium).

IL-12 production

PBMC were seeded in flat-bottom 96-well microtiter plates at $5x10^6$/ml, 200 µl/well in the presence of medium alone or stimuli or BC or stimuli plus BC and cultured at 37°C in a 5% CO_2 incubator for 18 hours in the presence of a transport inhibitor (Golgi Stop, BD, 2 µM). In regard to the stimuli, PBMC were primed for 2 h with interferon (IFN)-γ (10 ng/ml, R&D Systems, Inc.) prior to addition of lipopolysaccharide (LPS, 100 ng/ml, Sigma, St. Louis, Mo.) (Hayes *et al.,* 1995), or fixed *Staphylococcus aureus* (SAC, 10 µg/ml, Calbiochem) plus re-addition of IFN-γ (10 ng/ml). IL-12 production by monocytes was evaluated by flow cytometry via intracellular staining of IL-12 and surface staining of CD14. PBMC were stained with FITC-conjugated mouse anti human CD14 monoclonal antibody (Mab) (BD, clone Mϕ P9), fixed, permeabilized, and then stained with 0.125 µg/well of PE-conjugated mouse anti human IL-

12 Mab (BD, clone C11.5) following PharMingen's staining protocol. According to the manufacturer this antibody recognizes the IL-12 p40 monomer and the p70 heterodimer, but not the p35 monomer. Flow cytometric analyses were carried out on a FACSCalibur instrument (BD) equipped with CELLQuest software (BD). In acquisition 5000 events were collected in a gate drawn on the monocyte morphologic parameters. In analysis an analogic gate was built integrating the morphologic gate with a fluorescence gate drawn on CD14-brightly positive cells. Data are expressed as percent of double positive cells (CD14+1L-12+).

IFN-γ production

IFN-γ production was assessed via the ELISPOT assay (Enzyme Linked Immunospot). Ninety-six-well polyvinylidene difluoride-bottom plates (MAIPS4510; Millipore, Bedford, Mass.) were precoated with anti-IFN-γ capture monoclonal antibody (MAb B-B1; Diaclone, Besançon, France) and kept at 4°C overnight. PBMC were seeded in duplicate at $1x10^5$ cells/well and cultured with the different Ags, BC and Ags plus BC, at the indicated concentrations. Negative controls were represented by PBMC in medium alone. Phytohemagglutinin (PHA) (Sigma, St. Louis, Mo.) was used as positive control at 5 µg/ml. After 24 h incubation at 37°C in air plus 5% CO_2, biotinylated anti-IFN-γ detection MAb (B-G1; Diaclone) was added. After 2 h of incubation, streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) was added for 1 h. After a washing step, a chromogenic substrate (nitroblue tetrazolium-BCIP [5-bromo-4-chloro-3-indolylphosphate]) was added for 10-15 min; the plates were then washed with tap water and dried. Single spots were counted in an ELISPOT reader equipped with an automated image analysis system (AID-GmbH, Strassberg, Germany). Data are expressed as spot forming cells (SFC) x 10^5 PBMC and each spot corresponds to an IFN-γ-producing cell. Antigens (Ag) used in the assay: *Candida albicans* Ag (Ca) (Sanofi Diagnostic Pasteur, Marnes la Coquette, France), used as a recall Ag, and tested at 25 µg/ml; CMV (BioWhittaker) tested at a final 1:2000 dilution; IPP (Sigma Chem. Comp., St. Louis, MO), tested at 12 µg/ml, is a synthetic phosphoantigen highly cross-reactive with naturally-derived phosphoantigens from mycobacteria which are specific activators of peripheral Vγ9δ2 T lymphocytes (Biswas *et al.,* 2003).

Cellular proliferation

PBMC were plated in duplicate in round-bottom 96-well plates in complete medium alone, or with BC at the three different concentrations, or with PHA (5 µg/ml), used as positive control, or with PHA plus BC. After 48 h incubation at 37°C in air plus 5% CO_2 cells were pulsed overnight with ^{3}H-thymidine (GE Healthcare) at 1 µCi/well, harvested with a 96-well plate harvester (Filtermate, Packard Instrument Co., Meriden, CT) and filters were counted in a microplate scintillation counter (TopCount, Packard). Data of ^{3}H-thymidine uptake are expressed as counts per minute (cpm).

RESULTS

IL-12 production

Data of IL-12 production by monocytes present in PBMC are shown in Figure 1. In PBMC cultured in medium alone there is less than 1% of IL-12-producing CD14+ monocytes. A modest, although consistent, dose-dependent increment of IL-12-producing CD14+ monocytes was observed when BC was added to the cultures (Figure 1). By

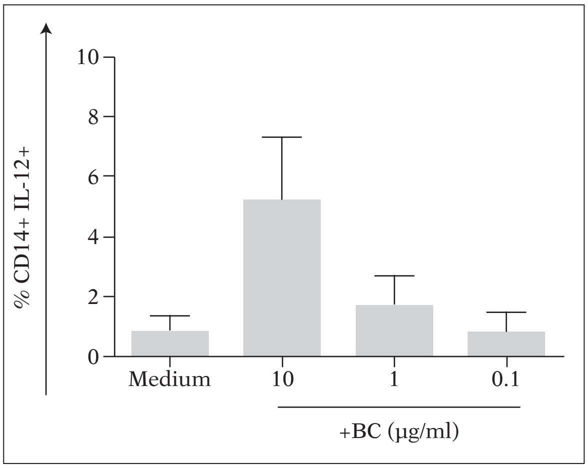

FIGURE 1 - *IL-12 production. IL-12-producing monocytes in untreated and BC-treated cultures were assessed by flow cytometric analyses. Mean values (± SD) of the percentage of CD14+IL-12+ cells from the five donors are depicted.*

TABLE 1 - *Effects of BC on stimuli-induced IL-12 and IFN-γ production and cell proliferation.*

Assay	*Medium*	*Stimulus*	*Stimulus + BC*		
			10 μg/ml	*1 μg/ml*	*0.1 μg/ml*
IL-12 production - % CD14+IL-12+	0.8 (±0.3)	LPS 34 (±10) SAC 40 (±14)	35 (±7) 42 (±13)	38 (±10) 45 (±11)	33 (±9) 34 (±14)
IFN-γ production - SFC x 10^5 PBMC	0.5 (±0.4)	PHA 231 (±49)	192 (±32)	224 (±38)	228 (±54)
Proliferation - cpm x 10^3	0.2 (±0.07)	PHA 26 (±8)	17 (±5)	21 (±7)	22 (±6)

Data are mean values from the five donors; numbers in parenthesis represent SD.

means of the paired parametric Student' s T test the increase was statistically significant when BC was used at 10 μg/ml (p=0.007), as well as when it was used at 1 μg/ml (p=0.033). Conversely, the lowest concentration of BC (0.1 μg/ml) showed no substantial effect. The IL-12-inducing capacity of the highest concentration of BC was approximately seven-fold lower than that of strong *in vitro* stimuli represented by LPS and SAC (Table 1).

IFN-γ production
BC presented no substantial effect on IFN-γ production (IFN-γ SFC) at all three concentrations used (Figure 2A). In this ELISPOT assay a response is empirically scored as positive if the test wells contain a mean number of SFC higher than the mean value plus two standard deviations (SD) in negative control wells and when the number of SFC per million PBMC in stimulated wells (subtracted of the values of negative control wells) is >20. By plating $2x10^5$ PBMC we previously observed that negative control wells from 128 healthy subjects yielded a mean SFC of 4.88±7.82 (Scarpellini *et al.*, 2004). Two donors presented borderline responses to IPP and one to Candida Ag (Figure 2B). In these cases the addition of BC enhanced the IFN-γ production, but there was no clear correlation with the concentration used (Figure 2B). Finally, two donors presented clear positive Ag-specific responses, one to CMV and one to IPP (Figure 2C). In this circumstance, two concentrations of BC presented no substantial effect, whereas one concentration presented an inhibitory effect. However, there was no concordance between the two donors: BC at 10 μg/ml resulted in a 50% inhibition in CMV-induced IFN-γ response in SB, whereas BC at 1 μg/ml resulted in about 40% inhibition in IPP-induced IFN-γ response in PM (Figure 2C).

Cell proliferation
The effect of BC on proliferation of PBMC was then evaluated. No substantial effects were observed. At 1 μg/ml there was a slight increase of cell proliferation, which, however, did not reach statistical significance (Figure 3).

Lack of effect of BC on maximal stimulation
Finally, Table 1 summarizes the results of experiments in which BC was used in addition to known maximal *in vitro* stimuli: LPS and SAC for IL-12 production, PHA for IFN-γ production and proliferation. The highest concentration of BC, 10 μg/ml, slightly inhibited PHA-induced IFN-γ production and proliferation; however, in both cases the difference was not statistically significant. Thus, overall BC did not show any substantial modulation of the stimuli-induced responses.

DISCUSSION

We here assessed a potential immunomodulatory effect of BC, focusing on the production of cytokines that mediate a Th1 response which is generally triggered upon intracellular microbial infections.
Two facts must be considered:
1) we used BC on human PBMC;
2) we used a preparation of natural, not immune, BC.

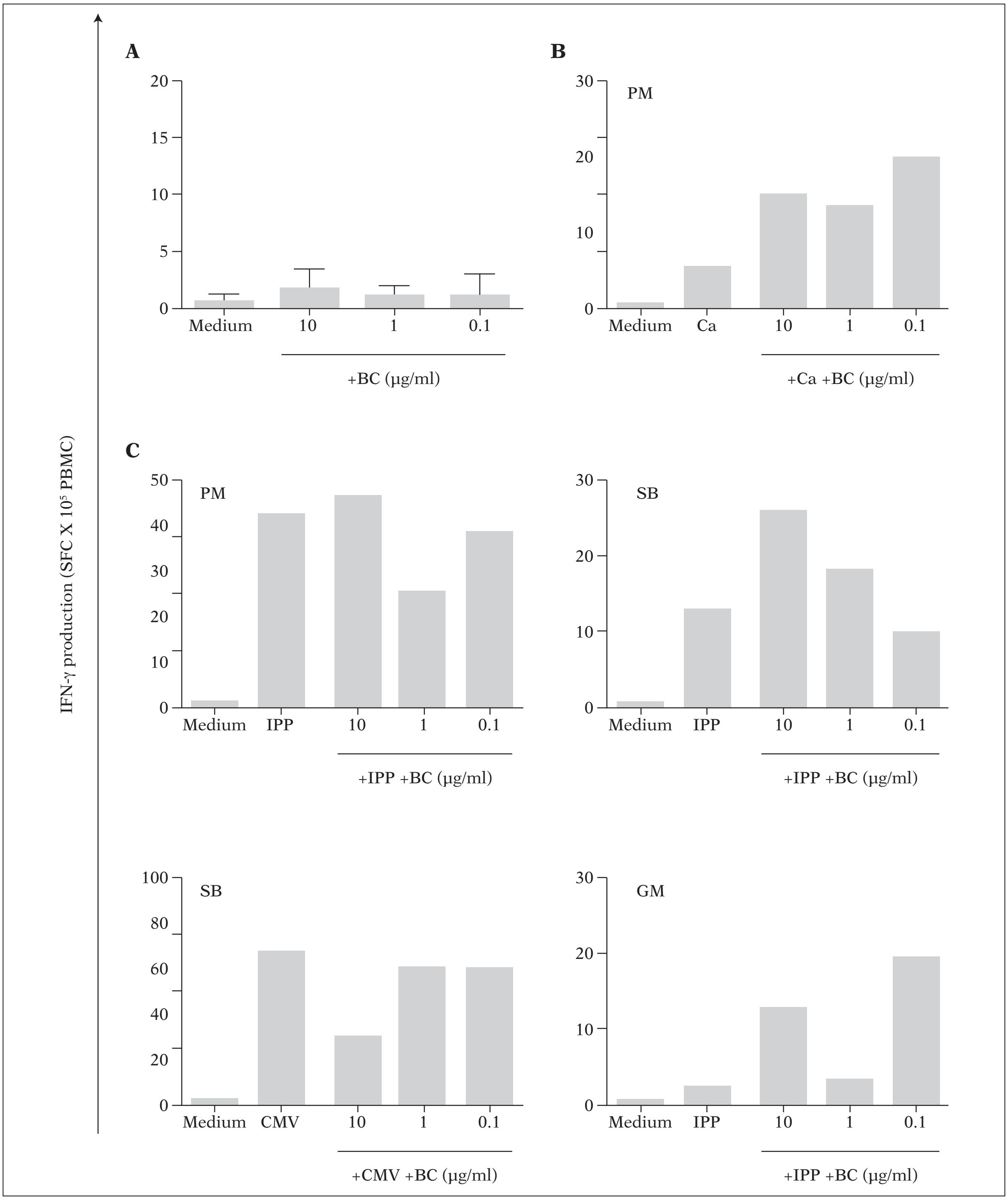

FIGURE 2 - *IFN-γ-production. IFN-γ-producing cells (SFC x 10^5 PBMC) were evaluated through the Elispot assay. Cells were untreated (medium) or treated with Ags alone (Ca, IPP, CMV) or with Ags plus BC at the three indicated concentrations. 2A: the mean value (± SD) of SFC from the five donors is shown. 2B: the three vertical panels show individual IFN-γ production by three donors with weak responses to Ca Ag or IPP; 2C: the two vertical panels show the IFN-γ production in two donors who had a strong antigenic response, PM to IPP and SB to CMV. 2B and 2C depict mean values of the duplicate cultures for each donor.*

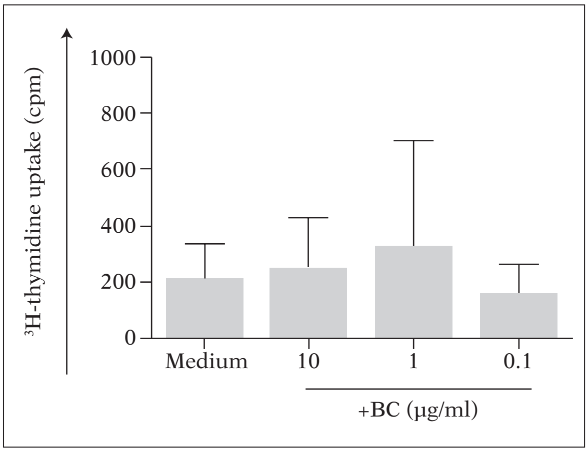

FIGURE 3 - *Cellular proliferation. Proliferation was assessed by an overnight ^{3}H-thymidine uptake by PBMC cultured for 48 h in complete medium in the presence or absence of BC at the indicated concentrations. Data are mean (± SD) values from the five donors.*

Regarding the first, any effect we observed should be due to bovine components with enough homology to human counterparts in order to act on human cells. This occurs among mammalians; for example, human TNF-α and IL-2 are active on murine cells and BC has been found to increase proliferation of canine skin fibroblasts (Torre *et al.,* 2006). Concerning the second fact, immune or hyperimmune BC is prepared from cows previously immunized with specific antigens or whole inactivated pathogens (Kelly, 2003). Passive immunity with immune BC has been assessed for prophylaxis and therapy against selected mucosal infections in humans (Davidson *et al.,* 1989; Korhonen *et al.,* 2000a; Weiner *et al.,* 1999) and this approach has shown particular importance in conveying protection towards enteric pathogens in immunocompromised individuals, such as AIDS patients (Greenberg and Cello, 1996; Nord *et al.,* 1990). In our study we used natural BC because we were more interested in the immunomodulatory potential of other components of BC, rather than in the antibodies themselves.

The main finding of our study is a dose-dependent induction of IL-12 by BC in human PBMC. Noteworthy also is the enhancing effect of BC on IFN-γ production in the case of weak antigenic stimulation. PBMC proliferation was substantially not affected by BC; nevertheless, the slight inhibition of PHA-induced proliferation could be linked to a previously described factor present in human and BC which inhibited IL-2 production (Mandalapu *et al.,* 1995).

The enhancing effect on weak IFN-γ production could be a direct consequence of the capacity of BC to induce IL-12. Indeed, although BC is not capable of directly inducing IFN-γ, it could synergize with weak antigenic stimuli, most likely through the induction of IL-12, which is an inducer of IFN-γ. In this regard, strong IL-12-inducing stimuli, such as LPS and SAC, are also used *in vitro* together with IFN-γ (as reported in the Material and Methods section). The mechanism by which BC is capable of inducing IL-12 in human PBMC is not known and could be a matter of future studies.

Several cytokines have been found in BC, including both pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, IFN-γ (Goto *et al.,* 1997; Hagiwara *et al.,* 2000) as well as anti-inflammatory components, such as IL-1ra, sIL-1RII and sCD14 (Filipp *et al.,* 2001; Hagiwara *et al.*, 2000; Hagiwara *et al.*, 2005).

This is intriguing and suggests that BC may provide the infant not only with cytokines to promote activation/inflammation, but also the means to reduce it, if perhaps too much is achieved. In our hands certain concentrations of BC inhibited robust antigen-specific IFN-γ responses. IL-1ra or sIL-1RII and sTNFRI or sTNFRII are established soluble feedback molecules for IL-1 and TNF-induced functions, respectively.

However there are no known IFN-γ soluble feedback molecules. It has recently been suggested that indoleamine 2,3-dioxygenase (IDO), induced by IFN-γ, could represent a counter regulatory mechanism for IFN-γ (Muller and Prendergast, 2007). It is not known whether bovine or human colostrum contains IDO. The difficulty in establishing a dose-dependency of both the enhancing and the inhibiting effect of BC on IFN-γ production could rely on the fact that cytokine concentration is not always in a linear correlation with function. Indeed high cytokine concentrations also induce counter regulatory molecules which in turn inhibit the function mediated by the cytokine.

Finally, when maximal stimulation was achieved *in vitro*, BC was unable to significantly inhibit any of the functions tested, probably because the concentrations used were too low to allow for the induction of counter-regulatory mechanisms.

By virtue of the many bioactive substances contained in BC, it has been proposed as a therapeutic tool to treat as well to prevent certain diseases (Thapa, 2005).

However, there are very few placebo-controlled trials to prove its efficacy. In this regard, a recent large study compared BC and anti-influenza vaccination in the prevention of flu episodes in two cohorts: normal healthy subjects (n=137) and high-risk cardiovascular subjects (n=60). The results are indeed remarkable: in both groups BC was at least 3 times more effective than vaccination (Cesarone *et al.,* 2007). In subjects treated with BC the average number of flu-related episodes in 2 months (0.33) was significantly lower than the average number of episodes registered in untreated subjects (1.3) or treated with anti-flu vaccine (1.1) (Cesarone MR *et al.,* 2007).

This positive outcome appears to fit well with an induction of a Th1 response along with neutralizing antibodies and with our findings that BC is capable of inducing IL-12, which promotes a Th1 response upon antigenic challenge. Along these lines, an *in vivo* study in mice has shown that oral administration of BC stimulated a Th1 polarization in intestinal intraepithelial lymphocytes (Yoshioka *et al.*, 2005).

In conclusion, owing to increasing evidence of the beneficial effects of BC and to its relative low cost, the therapeutic potential of BC should not be underestimated, especially in low-income countries.

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