



Effect of milk proteins and their hydrolysates on *in vitro* immune responses[☆]

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ARTICLE INFO

Keywords:

Caprine
Bovine
Milk protein
Hydrolysis
Cell proliferation
Immunomodulation

ABSTRACT

The aim of this study was to perform a screening of various milk protein samples of both cow and goat origin to study their *in vitro* immunomodulating properties on human peripheral blood mononuclear cells (PBMC).

The protein content in the milk of the two different species varies most notably in the amount of α_{s1} -casein. A high degree of genetic polymorphism is related to the goat α_{s1} -casein genes resulting in a variable amount of total protein in the goat milk.

The milk proteins were hydrolysed using human gastric and duodenal juice or commercial pig derived enzymes to simulate *in vivo* digestion. Although different immunomodulating effects caused by various milk protein components have been observed, the mechanisms underlying these effects are not always known. In addition, most studies on the immunomodulating properties of milk protein digests have used a wide variety of commercial enzymes to simulate *in vivo* digestion. Exploring the difference in immunomodulating properties of milk protein-derived peptides produced by the aid of enzymes from human gastric secretions, compared to those produced by commercial enzymes, is a novel approach that may be of great importance. It could help to explore which peptides are actually produced during *in vivo* early digestion of milk and how they influence the immune system.

Especially the whey protein concentrates from goat and cow showed a dose-dependent inhibition of human PBMC proliferation *in vitro*. This effect could neither be explained by a toxic effect on the PBMCs as shown by a standard viability test, nor by induction of apoptosis caused by the same milk protein samples. We suggest that intact or hydrolysed components in the milk protein samples affect the production of activation signals thereby inhibiting lymphocyte proliferation.

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1. Introduction

Even though milk is considered highly nutritious, the consumption of milk on a world basis is decreasing (Boland et al., 2001). In contrast, the number of health-conscious

individuals is increasing. These individuals are often interested in consuming food that, in addition to providing the necessary nutrients, also could reduce the risk of disease (Roberfroid, 2000). This has led to a growing awareness in the dairy industry for developing so-called functional foods. Milk and other dairy products seem to have potentially health-promoting constituents which are unique to this type of food. The result is an increase in research, attempting to discover hidden bioactivities within the structures of the milk proteins.

The composition of caseins in goat's milk is strongly influenced by the genetic polymorphism at the casein loci (Tziboula-Clark, 2003; Haenlein, 2006).

[☆] This paper is part of the special issue entitled 5th International Symposium on The Challenge to Sheep and Goats Milk Sectors Guest Edited by Antonio Pirisi, André Ayerbe, Giovanni Piredda, George Psathas and Yvette Soustre.

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The total content of caseins in goat's milk is somewhat lower than in cow's milk (Jenness, 1980; Miranda et al., 2004). This is primarily due to the high degree of genetic polymorphism of the α_{s1} -casein genes resulting in different concentrations of α_{s1} -casein in the goat milk ranging from 0 to 3.6 g/l (Grosclaude et al., 1987). The Norwegian goat breeds have a particularly high frequency of goats producing milk with no α_{s1} -casein, the so-called "null" type milk (Vegarud et al., 1999). In bovine milk, α_{s1} - and β -caseins are the main caseins whereas β -casein is the main casein in caprine milk. The α_{s2} -casein and κ -casein content in goat's milk is higher than in cow's milk (Jenness, 1980; Haenlein, 2006).

The content of the major whey proteins β -lactoglobulin (β -LG) and α -lactalbumin (α -LA) in goat and cow milks does not vary to the same extent as the caseins. The level of α -LA is approximately the same, whereas there is a slightly higher amount of β -LG in cows milk compared to goats milk (Park et al., 2007; Martin and Grosclaude, 1993; Miranda et al., 2004; Moatsou et al., 2005).

In addition to β -LG and α -LA, caprine and bovine milks contain serum albumin, lactoferrin, lactoperoxidase and immunoglobulins as well as proteose-peptone (Park et al., 2007).

Cow's milk frequently causes allergy in small children and goat's milk is believed to be a good replacement food. Lara-Villoslada et al. (2004) reported that when goat's milk was used as the first source of protein after a period of breastfeeding in mice, it was less allergenic than cow's milk. On the other hand, Ah-Leung et al. (2006) showed that goat's milk allergy occurred in children of higher age compared to infants of cow's milk allergy, and in addition a higher sensitivity of the casein fraction compared to the whey proteins was observed.

During the last decade various milk proteins and their enzymatically derived peptides have demonstrated modulating capacities on *in vitro* lymphocyte activation and proliferation (Cross and Gill, 2000). The modulating ability of the whey proteins and peptides also includes cytokine secretion, antibody production, granulocyte and natural killer (NK) cell activity and phagocytic activity (Gauthier et al., 2006).

Cross and Gill (1999) demonstrated a dose-dependent inhibitory effect of a modified whey protein concentrate on both T- and B-cell proliferation *in vitro*. They also observed that the same whey protein concentrate was able to suppress mitogen-induced T-cell secretion of γ -interferon (IFN- γ). In 2004, Mercier et al. (2004) demonstrated that a microfiltered whey protein isolate significantly increased *in vitro* lymphocyte proliferation. However, after digesting the whey protein isolate with trypsin and chymotrypsin, the stimulatory effect decreased. The same study also showed that by fractionating the hydrolysed whey protein isolate, some of the resulting peptide fractions stimulated lymphocyte proliferation even at reduced protein concentrations.

Individual whey protein fractions such as lactoferrin and lactoperoxidase are also reported to have suppressive effects on mitogen-activated lymphocyte proliferation (Wong et al., 1997; Mercier et al., 2004). Both proteins also inhibited the production of IFN- γ (Wong et al., 1997). However, this inhibitory effect was reduced

when the two proteins were used together in a cell culture. The same study (Wong et al., 1997) also found that neither α -LA, nor bovine serum albumin (BSA) had any effect on T- and B-cell responses to mitogen activation.

Purified β -LG has been shown to induce proliferation of resting murine spleen cells, and the effect was amplified after digestion with pepsin, trypsin, chymotrypsin or pancreatin (Mahmud et al., 2004). Wong et al. (1998) also demonstrated that purified β -LG could promote *in vitro* proliferation of mouse spleen cells.

Reviews on bioactive milk-derived peptides (Meisel, 1998; Clare and Swaisgood, 2000) showed that many of the casein-derived peptides significantly stimulated lymphocyte proliferation. However, a study by Kayser and Meisel (1996) revealed that β -casomorphin-7 and β -casokinin-10 (both derived from β -casein) appeared immunostimulating at high concentrations and immunosuppressive at low concentrations. Since it is not yet known how much of a certain peptide can be released during enzymatic digestion, this could imply that there is a great amount of uncertainty related to what the *in vivo* effect of milk-derived peptides would be. There is nevertheless some evidence pointing in the direction of the actual formation of these peptides *in vivo*. Peptides such as the β -casomorphins have been identified in the juice of the small intestine of adult humans after they had consumed 1 l bovine milk (Svedberg et al., 1985).

The present study was performed as a screening of different milk protein samples to establish their possible immunomodulating properties on human peripheral blood mononuclear cells. Furthermore, the intention was to reveal whether a different effect would be induced by goat milk proteins compared to their cow milk analogues. In addition, we wanted to know whether the caseins and whey proteins together could induce a different modulating response on peripheral blood mononuclear cells compared to the whey proteins alone.

2. Materials and methods

2.1. Raw materials

Whey protein concentrate from cow's milk (WPC-C) was produced from pasteurised cheese whey at TINE (Norwegian Dairies), Norway.

Cheese whey was also the basis for the whey protein concentrate from goat's milk (WPC-G) which was produced at the Department of Chemistry, Biotechnology and Food Science, the Norwegian University of Life Sciences, UMB (Ås, Norway). Both whey protein concentrates had a final protein content of about 80% (w/w).

Freeze-dried BSA was obtained from Integen Company (NY, USA), and bovine skim milk (SK) was produced at TINE (Norwegian Dairy), Norway.

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats and from whole blood collected from healthy donors.

Pepsin from porcine stomach mucosa came from Sigma (St. Louis, MO, USA) whereas Corolase PP (CPP) from pig pancreas was delivered by Röhm GmbH (Darmstadt, Germany). CPP is a mixture of trypsin, chymotrypsin and several amino- and carboxypeptidases.

Human gastric (HGJ) and duodenal juice (HDJ) obtained from one individual were kindly donated by the Department of Nutrition, the University of Oslo, Norway (Almaas et al., 2006).

As cell culture medium complete RPMI 1640 (cRPMI) was used unless otherwise is specified: RPMI 1640 (Gibco, Invitrogen Corporation, Paisley, UK) was supplemented with L-glutamine, 45 µmol/l monothioglycerol, 18 mg/l garamycin (Schering-Plough, Brussels, Belgium), 9 ml/l non-essential amino acids (Invitrogen Corporation, USA), 9 ml/l sodium pyruvate (Invitrogen Corporation, Carlsbad, CA, USA) and 10% heat inactivated foetal calf serum (FCS), (Gibco, Invitrogen Corporation, Paisley, UK). All cell culture plates were flat bottom 24-/48- and 96-well Cell Culture Clusters from Costar (Corning, Acton, MA, USA).

All incubations of cells were performed at 37°C in a humidified 5% CO₂ incubator.

2.2. Milk protein hydrolysis

Hydrolysis of milk proteins was carried out using either the commercial enzymes pepsin and CPP, or by using HGJ and HDJ. The reason for applying two different enzyme systems was to investigate the possibility of differences between the resulting hydrolysates causing different immunological responses.

A modified *in vitro* protein digestibility assay was performed in two steps according to Almaas et al. (2006). The first step used HGJ or pepsin (~4 mg/g protein in solution), and the second step used HDJ or CPP (~4 mg/g protein in solution) to mimic the digestion in the human stomach and duodenum. BSA and WPCs were hydrolysed at a concentration of 5% and the skim milk had a protein concentration of 3.3% (w/v). BSA was only hydrolysed by the commercial enzymes as it was only included as a negative control according to a previously published study (Wong et al., 1997).

Samples collected after the first step will be termed either HGJ or pepsin and the samples collected after the second step will be called HDJ or CPP in the following discussion.

A total of 17 different samples were freeze dried (Heto Drywinner 6–85) for further use in immunological assays.

2.3. SDS -polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was executed using Pharmacia PhastSystem™ (Pharmacia Laboratory Separation Division, Amersham Biosciences AB, Uppsala, Sweden) with the separation medium, PhastGel™ Homogenous 20 (Amersham Biosciences)

The electrophoresis was performed according to a standard protocol (Laemmli, 1970) and the gels with the separated proteins were stained with Coomassie blue using PhastSystem™ Development Technique with peptide fixa-

tion. A low molecular weight standard kit (LMW Calibration kit, Amersham Biosciences) was used as molecular mass markers.

2.4. Isolation of human peripheral blood mononuclear cells (PBMC)

Human peripheral blood mononuclear cells (PBMC) were isolated from a buffy coat and from whole blood by Lymphoprep™ (Nycomed Pharma, Asker, Norway) density gradient centrifugation. The isolated PBMCs were adjusted to the proper cell concentration in cRPMI. Cells isolated from the buffy coat were frozen in liquid N₂ in freezing medium (cRPMI containing 20% dimethylsulphoxide (DMSO)). Freshly isolated PBMCs were used for the flow cytometric test whereas the other experiments were carried out using cells that had previously been frozen.

2.5. Viability test

100 µl 10, 5 or 2.5 mg/ml of WPC-G start, WPC-G HDJ or WPC-G CPP was added to 100 µl 10⁶ cells/ml in cRPMI and incubated for up to four days. 10 µl of each sample was then mixed with 10 µl acridine orange/ethidium bromide mixture on a microscopic slide. Living (green fluorescence) and dead (orange fluorescence) cells were counted using a fluorescence microscope (Leitz Wetzlar, Germany).

2.6. In vitro lymphocyte proliferation assays

2.6.1. Mitogen-induced in vitro lymphocyte proliferation

Intact and hydrolysed milk protein samples were tested for their effects on lymphocyte proliferation. Each protein sample was diluted two-fold down to 0.125 mg/ml in cRPMI.

100 µl 10⁶ cells/ml was added to each well in a 96-well cell culture plate. BSA was used as a negative control, and the positive cell control was without milk protein. Cells were stimulated with the polyclonal activators phytohemagglutinin (PHA), concanavalin A (Con A) or monoclonal anti-CD3 antibodies (anti-CD3) at optimal concentrations. 20 µl of PHA, Con A or anti-CD3 was added to the appropriate wells in triplicates before adding 100 µl of each concentration of the milk protein samples.

The plates were incubated for 72 h. 20 µl [³H]-thymidine (0.1 µCi) was added to each well, for the last 24 h. The cells were harvested using a Filtermate harvester (Packard Bioscience, Groningen, The Netherlands), and [³H]-thymidine incorporation into the cellular DNA was measured using TopCount NXT™ (Packard Bioscience, Groningen, The Netherlands).

2.7. In vitro generation of antigen experienced T cells

PBMCs were stimulated with anti-CD3/anti-CD28 coated Dynabeads (Invitrogen, Oslo, Norway) to activate the T cells. 1 ml 0.5 × 10⁶ cells/ml and approximately 10 µl 1.5 × 10⁸ beads/ml anti-CD3/anti-CD28 coated beads were added to each well in a 24-well plate. After 5 days incuba-

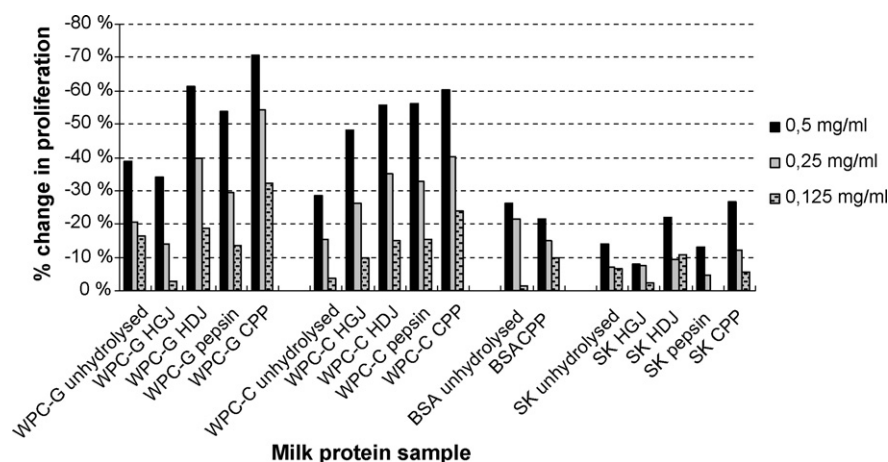


Fig. 1. Dose-dependent inhibition of anti-CD3 stimulated PBMC proliferation by different milk protein samples. Abbreviations: WPC-G: whey protein concentrate from goat milk, WPC-C: whey protein concentrate from cow's milk, BSA: bovine serum albumin, SK: skim milk, HGJ: human gastric juice, HDJ: human duodenal juice and CPP: Corolase PP.

tion, the beads were removed using a magnetic device and more cRPMI was added to sustain their growth.

2.7.1. Restimulation of antigen experienced T cells

The activators used were (1) soluble anti-CD3 at a final dilution of 1/75,000 (v/v), (2) anti-CD3 coated Dynabeads (5 beads per cell), and (3) anti-CD3/anti-CD28 coated Dynabeads (5 beads per cell) dissolved in cRPMI.

100 μ l 0.5×10^6 cells/ml were added to each well in a 96-well plate. Next 20 μ l of either of the three activators were added before adding 100 μ l 0.5 mg/ml of each milk protein fraction in triplicates to the appropriate wells. Incubation and [^{3} H]-thymidine incorporation was as described for the mitogen-induced lymphocyte proliferation assay.

2.7.2. Flow cytometric apoptosis test

To detect whether the protein samples induced apoptosis in lymphocytes, a flow cytometric apoptosis test (APOPTESTTM-FITC, Nexins Research, Kattendijke, The Netherlands) was employed.

400 μ l of each milk protein sample (0.5 mg/ml) was added to 400 μ l 10^6 cells/ml (freshly isolated) in a 24-well plate and incubated for 24 h. Then 200 μ l of each sample (about 10^6 cells) was centrifuged for 3 min before removing the cRPMI medium from the cells. The labelling of the cells was carried out according to the manufacturer's instructions. Within 5 min after finishing the incubation, the cell samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and the CellQuestTM software (Becton Dickinson, San Jose, CA, USA). A total of 5000 cells were counted per sample, and the percentage of annexin V positive (apoptotic cells) versus propidium iodide (PI) positive (necrotic cells) cells was determined.

3. Results

3.1. Effect of *in vitro* enzymatic digestion of milk proteins

Hydrolysis of the different milk protein samples resulted in a gradual degradation illustrated by a reduced pH due to

an increased amount of degradation products. In addition, the proteolysis was confirmed by SDS-PAGE clearly showing the degradation of the original protein samples after treatment with both enzyme systems. As expected, the amount of degradation products increased after treatment with HDJ or CPP when compared with the first hydrolysis step (data not shown).

3.2. Effects of milk protein samples on *in vitro* mitogen-induced lymphocyte proliferation

To reveal any effect on *in vitro* lymphocyte proliferation, graded amounts of milk protein preparations were added to cultures of PBMCs and stimulated with optimal concentrations of PHA, Con A and anti-CD3. Addition of the milk protein samples resulted in a dose-dependent inhibition of lymphocyte proliferation. Fig. 1 depicts data from a representative experiment carried out with anti-

Table 1

Amount of viable cells compared to the control sample after up to four days incubation. Only WPC-G hydrolysed using pepsin and CPP seemed to be cytotoxic and the effect was only detected after four days incubation. Control cells were incubated without milk protein samples

	% viable cells		
	Concentration (mg/ml)	After 24 h incubation	After four days incubation
WPC-G unhydrolysed	10	91	80
	5	97	94
	2.5	94	94
WPC-G HDJ	10	94	82
	5	92	89
	2.5	88	88
WPC-G CPP	10	88	n.d.
	5	88	89
	2.5	90	90
Control	0	87	72

WPC-G: whey protein concentrate from goat milk, HDJ: human duodenal juice and CPP: corolase PP.

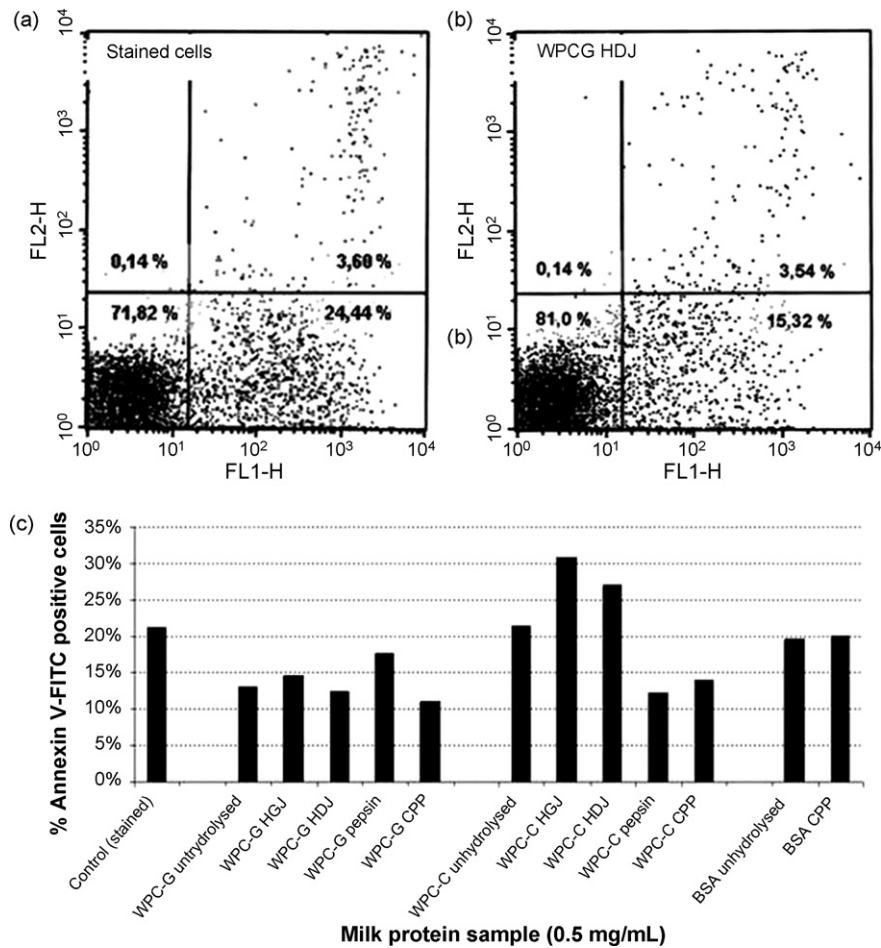


Fig. 2. Testing for annexin V/propidium iodide positivity by flow cytometry. FL1-H denotes the number of annexin V-FITC positive cells, while FL2-H denotes PI positive cells. (a) Control: annexin V-FITC and PI labeled cells without addition of WPC (b) Cells added WPC-G digested with both HGJ and HDJ. A representative picture of the distribution of annexin V-FITC and PI labeled cells added different milk protein samples. (c) Annexin V-FITC positive lymphocytes in the cell samples (average of two representative experiments). No dramatic increase in the number of annexin V-FITC positive (early sign indicative of apoptosis) cells was detected. See Fig. 1 for key to abbreviations in addition to FITC = fluorescein isothiocyanate. Control cells were incubated without milk protein samples.

CD3 stimulation. At a concentration of 0.5 mg/ml WPC-G and WPC-C significantly suppressed the lymphocyte proliferation whereas a concentration of 0.125 mg/ml had a much weaker effect. Data obtained with the other activators were similar.

BSA and skim milk preparations appeared to be less inhibitory than the other milk protein samples with only a slight suppressive effect at the highest concentration used.

The caprine WPC seemed to be somewhat more inhibitory to PBMC proliferation compared to the bovine WPC, but the major difference was, however, found between the two WPCs and the skim milk.

For all the milk protein samples, except for BSA it was evident that the most highly hydrolysed samples had the greatest inhibitory effect.

3.3. Viability test

To study whether the observed effects could be due to the protein samples being toxic to the cells, a standard acridine

dine orange/ethidium bromide viability test was carried out. After 24 and 96 h incubation, viable and dead cells were counted in a fluorescence microscope. The results of this test are shown in Table 1.

As can be observed, WPC-G samples hydrolysed with pepsin and CPP at a concentration of 10 mg/ml seemed to have a negative effect on the number of viable lymphocytes, but only after four days of incubation. The other protein samples did not negatively affect the amount of viable cells compared to the control. Some preparations even seemed to increase cell viability. This preliminary test indicated that the milk protein samples were not toxic to the cells.

3.4. Effect of milk protein samples on apoptosis

Although we did not detect any significant toxic effect of the milk protein fractions on the cells by a standard viability test, we wanted to ascertain that the inhibition of proliferation was not due to an increased tendency to apoptosis among the cells. Testing for annexin V/propidium iodide

positivity by flow cytometry (Fig. 2) allows the discrimination between apoptotic and necrotic cells. Depicts an average of two representative experiments revealing that the milk protein samples in general did not greatly increase the number of annexin V positive cells. The number of necrotic cells (PI positive cells) was low and did not increase significantly when adding any of the milk protein samples (data not shown).

3.5. Effect of milk protein samples on *in vitro* T lymphocyte proliferation in the absence of accessory cells

Since the milk protein-induced inhibition of cell proliferation did not seem to be caused by a toxic effect on the cells, other options had to be checked. When cultures of PBMCs are stimulated by polyclonal activators, most responding cells are T cells. T-cell activation by mitogens and monoclonal anti-CD3 antibodies also depend upon the presence of accessory cells like dendritic cells and monocytes. Thus, an effect of milk protein samples on cell proliferation in cultures of PBMC could be due to an effect on T cells, accessory cells or both. To address this question, we first generated a population of activated T cells. After an *in vitro* expansion period these cells were for all purposes devoid of accessory cells. When restimulating such “antigen experienced” T cells with anti-CD3/anti-CD28 coated beads, the milk protein samples appeared to be slightly stimulating on T-cell proliferation. These results also demonstrated that the milk protein samples were not toxic to the cells. On the other hand, restimulation with anti-CD3 coated beads followed by the addition of the protein samples led to a decrease in [³H]-thymidine incorporation and thus suppression of T lymphocyte proliferation. Restimulation of the T cells with soluble anti-CD3 antibodies which is highly dependent on accessory cells, did not induce proliferation at all. The effect was the same for WPC of both goat and cow origin.

4. Discussion

The effect of different milk protein samples on the *in vitro* proliferation of human peripheral blood mononuclear cells was investigated. Previous research has revealed both suppressive and stimulating modulatory effects on *in vitro* lymphocyte proliferation, including whole casein, α -, β - and κ -casein, whole whey protein, lactoferrin, lactoperoxidase, milk growth factor and endogenous milk immunoglobulin G (Cross and Gill, 2000).

In this study intact and hydrolysed samples of WPC-C, WPC-G, BSA and SK were investigated for their effect on *in vitro* PBMC proliferation. The proteins were hydrolysed by two sets of enzymes, either human HGJ and HDJ or commercial pepsin and CPP. No apparent difference in the immunological response induced by the two enzyme systems was observed.

A standard lymphocyte proliferation assay revealed that the incorporation of [³H] thymidine was suppressed to various degrees by the different milk protein samples when stimulated with Con A, PHA and anti-CD3. The inhibition of the lymphocyte proliferation was dose-dependent for all the proteins samples, but the whey protein concen-

trates seemed to be more suppressive than SK and BSA. WPC from goat's milk seemed slightly more suppressive than the bovine WPC, although the major difference was found between the two WPCs and the skim milk. This could indicate that the skim milk could contain one or more immunostimulating components, such as the caseins, masking the suppressive effect of the whey proteins.

Both individual milk proteins and their enzymatically derived peptides have been demonstrated to have various effects on immune cell functions (Gill et al., 2000). The milk protein samples applied in this study consisted of a complex mixture of different proteins and peptides.

A homogenous protein or peptide fraction may be expected to have defined immunomodulating effects. However, a mixture of different fractions might not result in any modulation of immune cell functions at all. The more purified the individual milk proteins or their peptides get, the more clearly defined their immunomodulating effects would be (Cross and Gill, 2000; Wong et al., 1997). Thus, the original milk product, consisting of variable amounts of immunomodulating proteins and peptides, may possess altered immunomodulating properties compared to individual and highly purified peptides. SK for example may contain proteins/peptides that diminish or suppress the effect of the inhibitory whey protein components. For instance, research has found immunostimulating effects of caseins and casein-derived peptides (Fiat et al., 1993; Kayser and Meisel, 1996).

Both immunostimulating and immunosuppressive peptides of α_{s1} -casein origin have been observed (Gill et al., 2000). However, it is not known which of these peptides will dominate in milk after *in vivo* digestion or how they might affect each other.

Because no purification of the different milk protein constituents were performed in the present study, it is impossible to tell which of the protein components may have inflicted the suppressive effect on the lymphocytes. Instead an attempt to identify a possible mechanism for the inhibition was investigated.

The mitogen-induced lymphocyte proliferation assay is a frequently applied test system that does not distinguish between inhibition of early and late phases of the T-cell activation process.

Suppression of [³H] thymidine incorporation could therefore have several possible explanations. It could either be caused by an inhibitory effect on early events in lymphocyte activation, or it could be caused by protein samples blocking the cell cycle progression at a certain stage. Other possible explanations could be that the milk protein samples could induce apoptosis or be directly toxic to the cells.

The inhibitory effect was found not to be caused by toxic effects on the lymphocytes as addition of the milk protein samples did not influence the amount of viable cells in a sensitive viability test. In addition the samples proved to have a stimulating effect on T cells when these were also stimulated with anti-CD3/ant-CD28 coated beads. This would not have been the case had the samples been toxic.

In addition, the milk protein samples did not seem to increase the amount of apoptotic cells in a culture as shown by the flow cytometric apoptosis test. This test offers a

possibility for differentiating between viable, necrotic and apoptotic cells (Vermes et al., 1995). Binding of annexin V to phosphatidylserine (PS) without the binding of propidium iodide (PI) indicates that the cell is apoptotic. Nevertheless, the binding of annexin V to PS is only indicative of an early event in the apoptotic process taking place. Whether the milk protein samples would induce apoptosis after prolonged incubation is not known.

Since the milk protein samples did not seem to induce apoptosis or be cytotoxic to the cells, other possible explanations for the inhibition of proliferation had to be investigated. Given that the majority of the cells that were activated by the activator substances were T cells, the inhibition of [^{3} H]-thymidine incorporation would most likely primarily have affected T-cell proliferation. To induce proliferation of resting, naive T cells, accessory cells expressing important costimulatory molecules are required. The inhibitory effect was therefore assumed to be caused by a direct effect on the T lymphocytes, their accessory cells or on both cell types.

As shown in Fig. 3 the milk protein samples induced significantly different modulating effects on the proliferation of antigen experienced T lymphocytes stimulated with either anti-CD3/anti-CD28-coated beads or anti-CD3 coated beads.

Whereas the proliferation of the T lymphocytes stimulated with the anti-CD3 coated beads was markedly decreased, the proliferation of T cells activated by the anti-CD3/anti-CD28 coated beads slightly increased. When the cells were stimulated with anti-CD3 coated beads the inhibition even seemed stronger than what was observed for the mitogen-activated proliferation assay. Even SK appeared to be equally inhibitory as the whey protein concentrates. The difference in the immunomodulating effect caused by the milk protein samples may be due to the fact that the anti-CD3/anti-CD28 coated beads provide different and more potent activating signals compared to anti-CD3

coated beads. When stimulating with anti-CD3/anti-CD28 coated beads both activation signal 1 and 2 are conferred to the T lymphocytes (Trickett and Kwan, 2003).

When stimulating antigen experienced T cells with anti-CD3 coated beads, the test system relies on internal activation signals to sustain proliferation. Our finding that milk protein samples inhibited anti-CD3 induced proliferation could imply that the protein samples were somehow able to block the intracellular signals in the T-cell activation process. This could further imply that the initial suppression of the PBMCs could be caused by milk protein samples affecting the signalling involved in activation of proliferation

Cross and Gill (1999) demonstrated an inhibitory effect of a modified whey protein concentrate (WPC) on both B and T lymphocyte proliferation. The same modified WPC also inhibited the production of IFN- γ in a Con A stimulated cell culture and caused a suppression in the percentage of activated T cells. This indicates that some of the components of the modified WPC alone or together were able to suppress *in vitro* lymphocyte activation. When treating the modified WPC with the digestive enzymes pepsin and pancreatin, the digested filtrate showed no immunomodulating properties against the lymphocytes. The present study on the other hand, revealed a somewhat increased immunosuppressive effect of the digested protein samples on PBMCs. This indicates that the conditions during *in vitro* digestion, as well as the enzymes applied could influence the formation of immunomodulating components.

Both inhibition and stimulation of the immune system could in a certain context be positive. By enhancing the proliferation of immune cells, potential pathogens invading the body may be defeated more quickly (Meisel and Bockelmann, 1999).

On the contrary, a suppressive effect on lymphocyte proliferation might be of great importance when ensuring a state of tolerance towards non-self sub-

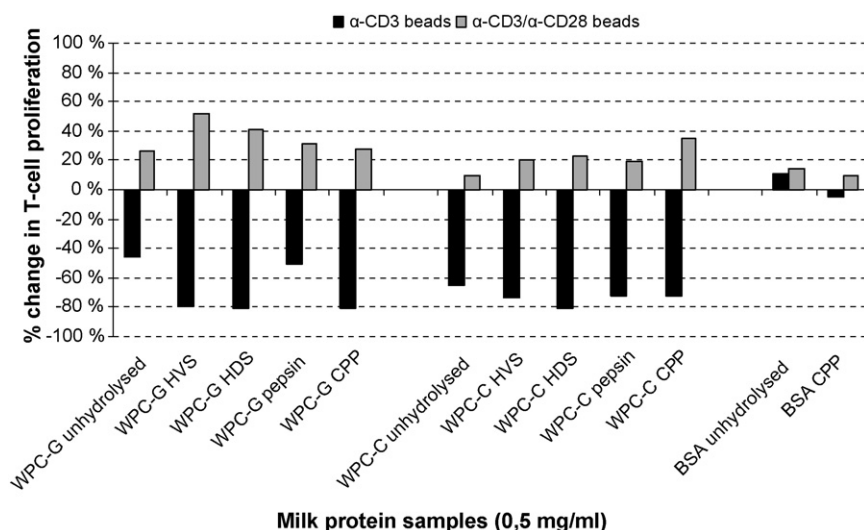


Fig. 3. Effect of whey protein samples on the proliferation of T lymphocytes restimulated using either anti-CD3/anti-CD28 coated beads or anti-CD3 coated beads. The samples seemed to augment T-cell proliferation when stimulated with the anti-CD3/anti-CD28 beads. On contrary, the stimulation by anti-CD3 coated beads in the presence of the milk protein samples seemed to inhibit lymphocyte proliferation. See Fig. 1 for key to abbreviations.

stances such as food molecules entering the body. Milk is originally intended as food for the newborn. Different milk protein-derived immunomodulating components may therefore somehow exhibit a regulatory effect on each other to avoid an unfavourable stimulation or suppression of the neonatal immune system (Cross and Gill, 2000).

In vivo studies have demonstrated that some immunomodulating peptides formed during digestion can be found in the juice of the small intestine of adult humans after consumption of bovine milk (Svedberg et al., 1985). Other studies have revealed conflicting results both between various *in vitro* assays and between *in vitro* and *in vivo* analysis of the same protein components (Gauthier et al., 2006). The review by Gauthier et al. (2006) pointed out how different raw materials and methodological approaches applied in the diverse immunological assays reviewed, could lead to conflicting results for many of the whey proteins preparation tested. There is still much left to do to confirm that results obtained *in vitro* could occur *in vivo*, however, using human digestive secretions to simulate digestion could give a better understanding of what actually occurs in the gastrointestinal tract. The peptides produced with human enzymes versus those produced by enzymes from other species could be different depending on the specificity of the enzymes.

While the majority of the *in vitro* analysis of milk-derived proteins has found immunosuppressive effects, the *in vivo* tests have revealed immunostimulating effects (Cross and Gill, 2000). It is moreover not possible to predict whether these molecules are absorbed through the epithelial cells lining the intestinal mucosa. For this reason, results from *in vitro* assays are only to be taken as indications of what may happen when potentially immunomodulating molecules are consumed. *In vitro* assays are nonetheless interesting for the identification of potential bioactive peptides in food.

Further analyses are needed to determine their relevance in the regulation of the immune response, both systemically and throughout the intestine. It is of great importance to establish if a protein preparation has the same biological function *in vivo* as it has *in vitro* before using it as an ingredient in a functional food.

5. Conclusion

The results revealed that the whey protein samples both from goat and cow showed a dose-dependent inhibition of T lymphocyte proliferation *in vitro*. This inhibitory effect is suggested to be due to interference with the generation of the important T-cell activating signals, caused by one or several milk protein-derived components. This would hinder further lymphocyte proliferation.

Acknowledgement

I would like to thank research technician Ellen Solum Karlstrøm at the Institute of Immunology, for excellent technical assistance and helpful advice during these studies.

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