Journal of Dairy Research (2005) **72** 217–225. © *Proprietors of Journal of Dairy Research 2005*
doi:10.1017/S0022029905000877 *Printed in the United Kingdom*

Effect of prior dietary exposure to cows' milk protein on antigen-specific and nonspecific cellular proliferation in mice

Susanne Brix, Orit H Magyar, Vibeke Barkholt and Hanne Frøkiær*

BioCentrum-DTU, Biochemistry and Nutrition Group, Technical University of Denmark, Kgs. Lyngby, Denmark

Received 26 June 2003 and accepted for publication 1 November 2004

The impact of dietary components on the immune system is gaining increased attention in the effort to develop safe food products, some even with health-promoting potential, as well as to improve the basic understanding of the immunomodulatory potential of common food components. In such studies, which are mainly based on experiments *in vitro*, it is important to be able to differentiate nonspecific activation of immune cells induced by dietary components from *ex vivo* restimulation of antigen-specific cells that might be present in cell cultures owing to prior dietary exposure to the antigens in cell donors. Focusing on the immunostimulatory potential of cows' milk proteins and peptides, we studied the impact of prior dietary exposure to cows' milk on proliferation of murine immune cells upon *ex vivo* stimulation with bovine milk proteins. Nonspecific proliferation induced by β-casein peptides was further assessed on cells from mice bred on a cows'-milk-free diet. Regarding the dietary effect, we found that prior oral intake of cows' milk proteins affected cell proliferation induced by culturing with cows' milk proteins *in vitro*, as spleen cells from mice fed a milk-containing diet showed a significantly greater proliferative response than did cells from mice bred on a cows'-milk-free diet. Studies of immune enhancing potentials of β-casein peptides showed that some peptides stimulate proliferation of immune cells nonspecifically. In conclusion, these findings stress the importance of employing immune cells from mice unexposed to cows' milk for studies of the immunomodulating capacity of cows' milk proteins and peptides, in order to rule out the interference caused by antigen-specific immune responses. By using such cells, we here show that some β-casein peptides possess the potential to induce proliferation in immune cells in a nonspecific manner.

Keywords: β-Casein, peptides, immunostimulation.

Milk proteins hold various specific and nonspecific activities including antimicrobial and immunomodulatory potentials (Clare & Swaisgood, 2000; Cross & Gill, 2000). The impact of such milk components is proposed to be significant for newborns in combatting infections and enhancing development of a functionally intact gut-associated and systemic immune system (Hanson, 1998; Goldman, 2000).

Certain milk proteins with immunostimulatory as well as immunoinhibitory activities have been identified. Lactoferrin, lactotransferrin, lactoperoxidase, α-lactalbumin and κ-casein all have immunoinhibitory capacities (Mincheva-Nilsson et al. 1990; Otani et al. 1992; Hakansson et al. 1995; Zimecki et al. 1996; Otani & Odashima 1997; Wong et al. 1997; Debbabi et al. 1998). Amongst the immunostimulatory proteins, most focus has so far been placed on peptides derived from one of the caseins, β-casein. Most of these studies have looked at the immunoenhancing effects of peptides from human and bovine β-caseins (Parker et al. 1984; Gattegno et al. 1988; Migliore-Samour et al. 1989; Shimizu et al. 1991; Coste et al. 1992; Kayser & Meisel, 1996; Otani & Futakami, 1996; Sutas et al. 1996; Wong et al. 1996; Hata et al. 1998; Meisel & Gunther, 1998; Otani et al. 2001; Sandre et al. 2001). Other common bovine milk proteins holding immunostimulatory potential are α_{s1}-casein (Migliore-Samour et al. 1989; Carr et al. 1990; Lahov & Regelson, 1996), and the whey protein β-lactoglobulin (Wong et al. 1998; Nagata et al. 2000).

Different approaches have been used to study the impact of milk proteins on cells of the immune system. However, one confounding factor relevant to all of these experimental settings, which has received only minor

*For correspondence; e-mail: hf@biocentrum.dtu.dk

attention, is the varying exposure to cows' milk through the diet of cell donors, both in man and experimental animals. Since dietary intake of cows' milk may induce milk-specific cells that are restimulated upon culturing *in vitro* with milk components, the above-mentioned studies do not provide data of the effect of milk proteins and peptides on immune cells completely naïve to milk proteins.

In the present study, we first examined in mice whether the presence of cows' milk proteins in the diet induces antigen-specific immune cells with the capacity to proliferate upon culturing *in vitro* with cows' milk proteins. As β-casein showed nonspecific stimulatory activity, and is a protein easily digested upon ingestion, we then tested the capacity of peptides resulting from *in vitro* digests of β-casein to induce *ex vivo* nonspecific proliferation.

Materials and Methods

Animals

BALB/c mice were raised on either a conventional milk-containing diet (40 g/kg skimmed cows' milk powder, Altromin 1314/1324, Altromin, Lage, Germany) or a commercially available milk-free diet (TRM 9608, Harlan Teklad, The Netherlands) for at least two generations, which in previous studies was found to be necessary to achieve mice completely non-responsive to milk proteins (Brix et al. 2005). All animal studies were approved by the Danish Animal Experiments Inspectorate and were carried out in accordance with the guidelines of 'The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purposes'.

Antigens

These were from the following sources: β-Casein (~90%, C-6905, Sigma-Aldrich (Sigma), St. Louis, MO, USA), β-lactoglobulin (>90%, variant A+B, L-0130, Sigma), κ-casein (>80%, C-0406, Sigma).

Cell proliferation assay

Single-cell suspensions of spleen and mesenteric lymph nodes (MLN) were prepared from BALB/c mice (10–12 weeks old). After three washings, cells were resuspended in culture medium (X-VIVO 10, serum-free medium, BioWhittaker, Verviers, Belgium) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all from Life Technologies, Paisley, UK). The cells were cultured at 5×10^5 viable cells/200 μl per well in 96-well flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark) with 25 μl antigen (in triplicates) diluted in phosphate-buffered saline (PBS, 20 mM-phosphate, pH 7·25) or in control cultures with 25 μl PBS alone (six replicates). The final concentration per well of antigen is indicated in the respective figure legends. Cell proliferation was determined by incorporation of [^{3}H]-thymidine (TdR, 1 μCi/ml, Amersham, UK) for 18 h after pre-incubation of cell cultures for 48 h at 37 °C in 5% CO_2. Cells were harvested onto fibreglass filters (Whatman, Maidstone, UK) using a 12-well cell harvester (Automash 2000, Dynex Technologies, Denkendorf, Germany) and the amount of incorporated TdR was determined by liquid scintillation counting (Tri-Carb®, Packard, Meriden CT, USA) as counts per min (cpm). Results are presented as a stimulation index (SI) calculated as the ratio of the mean cpm of antigen-stimulated and control cultures.

Immunization with β-casein

Mice were bred and maintained on either the milk-containing diet or the commercial milk-free diet. At 18 weeks, the mice were immunized intraperitoneally twice at 2 weeks' interval with β-casein (20 μg/mouse, Sigma) dissolved in PBS and mixed 1 : 1 (v/v) with Freund's incomplete adjuvant (Difco, Detroit MI, USA). Cell proliferation of spleen cells towards β-casein was performed one week after the last immunization, as described above.

Preparation of β-casein peptides

Peptides were prepared from β-casein using either digestion with trypsin (EC: 3.4.21.4, porcine, T-7409, Sigma) or a combination of pepsin (EC: 3.4.23.1, porcine, P-7012, Sigma) and pancreatin (porcine, P-1625, Sigma) after being dissolved in NH_4HCO_3 (2 mg/ml; 0·1 M). All proteins and buffer solutions were sterile filtered (0·22 μm, Millex, Millipore, Bedford MA, USA) prior to use. For pepsin pre-incubation, the pH was adjusted to 2·5 with HCl, and for digestion with pancreatic enzymes, pH was adjusted to 8·0 with NH_3. Digestions were carried out at 37 °C in a shaking water bath. Preliminarily, the incubation times were determined based on SDS-PAGE and analytical size-exclusion chromatography of samples taken at different times during hydrolysis. The enzyme/substrate (E/S)-ratios as well as the pH values and final incubation times for digestions was as follows: β-casein, trypsin digestion: E/S: 1/100, pH 8, 120 min; β-casein, pepsin/pancreatin digestion: pepsin, E/S: 1/100, pH 2·5, 60 min; pancreatin, E/S: 1/25, pH 8, 120 min. Hydrolysates were promptly frozen and lyophilized to terminate digestion.

Size-exclusion chromatography

Hydrolysates were dissolved in 2 ml cold (4 °C) NH_4HCO_3 (0·1 M, pH 8·0), and in order to make a fast separation of peptides from digestive enzymes, the hydrolysates were immediately subjected to size-exclusion chromatography at 4 °C using a 200-ml column (1·6 cm × 100 cm; APbiotech, Uppsala, Sweden) filled with Sephadex G-50 (fine, APbiotech). Absorbances were read at 226 nm and 280 nm. Elution was performed in NH_4HCO_3 (0·1 M,

https://doi.org/10.1017/S0022029905000877 Published online by Cambridge University Press

pH 8·0) at 10 ml/h, and 4-ml fractions were collected into autoclaved glass tubes. After finishing the separation, small samples were taken from each fraction for analysis of peptide profiles by capillary electrophoresis and to determine the peptide content by amino acid analysis. All fractions were then immediately frozen and lyophilized. To remove any residuals of eluting buffer, the peptides were twice evaporated with distilled water before inclusion in cell cultures.

Capillary electrophoresis

Capillary electrophoresis was used to analyse the peptide profile in each fraction, and pools were made of fractions containing similar peptide profiles. Before analysis, peptide samples were dried under vacuum, and then dissolved in the separation buffer (2 mg/ml; 0·1 M-Na_2HPO_4, 0·05 M-taurine, 0·035 M-cholate, pH 8·1) ten-times diluted, followed by centrifugation at 10 000 ***g*** for 10 min. The electrophoresis was accomplished during 20 min at 30 °C, 20 kV positive polarity using an uncoated fused-silica capillary (Hewlett-Packard, Birkerød, Denmark; ID 50 μm, length 614 mm, distance to detector 530 mm) connected to a high performance capillary electrophoresis apparatus (HP-CE^{3D}, Hewlett-Packard). Samples were run in separation buffer, and the capillary was rinsed in separation buffer between every four samples. Absorbance was recorded at 214 nm.

Amino acid analysis

The amino acid content in pooled fractions, unfractionated hydrolysates and intact protein was determined after acid hydrolysis as described previously (Barkholt & Jensen, 1989).

Mass spectrometry

The mass spectra of the peptide fractions from the β-casein digestions were recorded using a HP G2025A MALDI-TOF mass spectrometer (Hewlett-Packard, Palo Alto CA, USA) calibrated with an external peptide standard kit (HP G2052A, Hewlett-Packard). Before analysis, the peptide fractions were evaporated three times with distilled water to remove any residual salt, and then the matrix solution (100 mM-sinapinic acid (trans-3,5-dimethoxy-4-hydroxycinnamic acid, Sigma-Aldrich) in acetonitrile/methanol/water (15 : 9 : 2 (v/v)) was added and mixed. The peptide-matrix mixtures were stored for up to 24 h at 4 °C before analysis. A probe with 10 targets was used and 1 μl peptide-matrix solution was applied on each target. The mass spectra were recorded in a range up to 10^4 Da, in a linear positive mode with an acceleration voltage of 28 kV. For every fraction examined, more peaks were collected corresponding to heterogeneous samples containing two or more peptides. To calculate the molar concentrations of the β-casein peptide fractions, the molar weight corresponding to the largest peptide present in the sample was used. For hydrolysates an estimated molar weight of 1/5 of the molar weight of β-casein (23·6 kDa) was used.

Determination of endotoxin content

To determine endotoxin in antigen samples used for the cell proliferation experiments, the chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville MD, USA) was used. Endotoxin-spike recovery experiments were carried out for all samples.

Statistical analysis

Results were analysed for statistical significance (GraphPad Prism, version 3.02, GraphPad Software, San Diego CA, USA) using either one-way or two-way ANOVA followed by post tests as described in each figure; $P<0·05$ was considered significant.

Results

Influence of dietary cows' milk protein on ex vivo *proliferation towards milk proteins*

We examined in non-immunized mice whether the presence of cows' milk proteins in the diet influenced the proliferation of cells cultured *in vitro* with cows' milk proteins. The cell proliferation response towards three selected cows' milk proteins, β-casein, β-lactoglobulin, and κ-casein was evaluated on cells from mice fed either a cows'-milk-containing diet or a commercial cows'-milk-free diet (Fig. 1). For all three milk proteins tested, the diet influenced cellular proliferation giving rise to a significantly greater response of cells from mice bred on the milk-containing diet in contrast to those from mice fed the milk-free diet (β-casein, $P=0·02$, β-lactoglobulin, $P=0·04$ and κ-casein, $P<0·0001$). Interestingly, κ-casein inhibited proliferation of spleen cells from mice fed a milk-free diet. For β-lactoglobulin, the substantial stimulatory activity was recently found to be due to LPS contamination of the commercial preparation (Brix et al. 2003). No significant difference was found between the two dietary groups after culturing *in vitro* with the non-diet related egg white protein ovomucoid at 50 μg/ml ($P=0·25$, results not shown).

To examine whether the diet-related differences in responses might be due to induction of milk protein-specific cells upon feeding with cows' milk, mice fed either milk-containing or milk-free diets were immunized with β-casein before cultivation *in vitro* of spleen cells with β-casein. In contrast to the response observed with cells from non-immunized mice, immunization with β-casein expectedly revealed a significantly suppressed ($P<0·0001$) *ex vivo* β-casein-specific proliferation of splenocytes from mice fed cows'-milk-containing diet compared with those from mice fed a milk-free diet (Fig. 2). This reduced response was due to induction of antigen-specific regulatory

https://doi.org/10.1017/S0022029905000877 Published online by Cambridge University Press

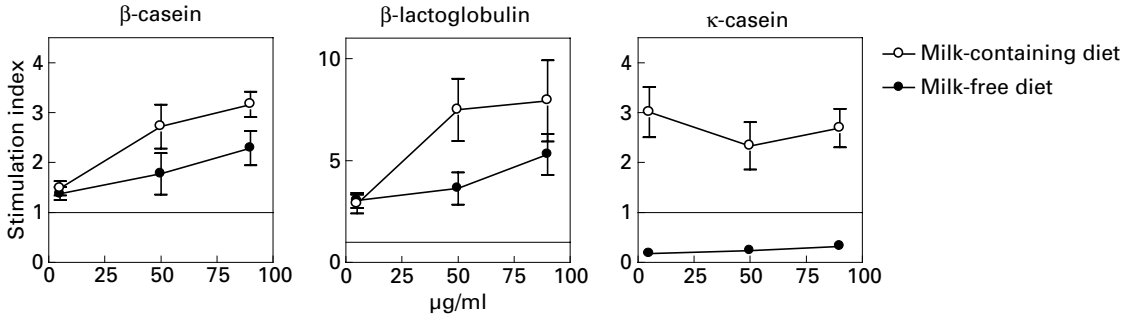

Fig. 1. Dietary exposure to milk proteins influences *ex vivo* proliferation of spleen cells in response to cows' milk proteins. Spleen cells from non-immunized BALB/c mice bred either on a milk-containing diet or a milk-free diet were cultured *in vitro* with different concentrations of bovine β-casein, bovine β-lactoglobulin or bovine κ-casein for 66 h. The cell proliferation results (mean ± SEM; $n=6$) are presented as a stimulation index calculated as mean counts per minute (cpm) of protein-stimulated cultures divided by that of control cultures. Differences between dose-response proliferations of the two dietary groups were tested using two-way ANOVA (*P* values for the diet effect are given in the text).

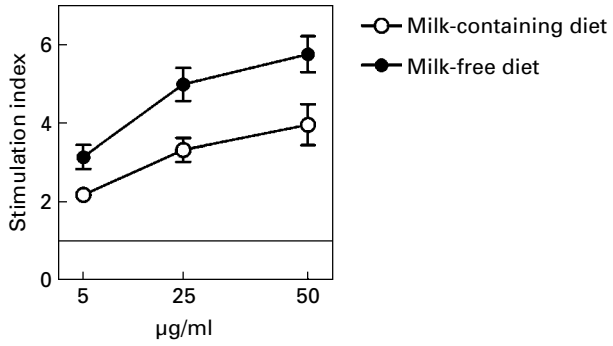

Fig. 2. Immunization with β-casein reveals the presence of β-casein-specific cells in mice fed milk-containing diet. BALB/c mice fed either a milk-containing or a milk-free diet were immunized with β-casein prior to *ex vivo* restimulation of spleen cells with different concentrations of β-casein. Values are means ± SEM ($n=5$). Differences between dose-response proliferations of the two dietary groups were tested using two-way ANOVA (*P* value for the diet effect is shown in the text).

cells, visualized as oral tolerance to β-casein, in the mice fed the milk-containing diet. Again, cell culturing with the non-diet related protein ovomucoid at 50 µg/ml revealed no significant difference between the two dietary groups ($P=0{\cdot}21$, results not shown).

Preparation of β-casein peptides

As β-casein showed non-LPS-induced stimulatory activity on cows'-milk-naïve cells, and is a protein naturally subjected to extensive digestion in the stomach, we aimed at testing the capacity of peptides resulting from *in vitro* digests of β-casein to induce *ex vivo* nonspecific proliferation. Peptides were generated after digestion of β-casein *in vitro* with either trypsin, or a combination of pepsin and pancreatic enzymes (pancreatin). To separate peptides in the hydrolysates, size-exclusion chromatography was carried out using a volatile elution buffer. The chromatograms from the separations of hydrolysates by size-exclusion chromatography, depicting the final pooling of eluted fractions, are shown in Fig. 3. The eluted fractions were finally pooled to reduce the number of samples for cellular analyses. To carry out the pooling of fractions, each eluted fraction from size-exclusion chromatography was analysed by capillary electrophoresis, and fractions containing similar peptide profiles were then pooled. An example to illustrate our principle of pooling according to capillary electropherograms is shown in Fig. 4.

Effect of β-casein peptides on nonspecific cellular proliferation

To evaluate the immunostimulatory potential of our prepared β-casein peptide fractions, we analysed the non-specific effect on proliferation of cells from a systemic lymphoid organ (spleen cells) as well as from a GI-tract related organ (MLN), both derived from mice bred on a milk-free diet. In general, the different fractions containing β-casein peptides had divergent effects on the two cell types (Fig. 5). Specifically, the tryptic peptide T-I (8 µM) significantly stimulated the proliferation of both spleen cells and MLN (Fig. 5B, C), whereas the T-II and T-VIII only stimulated significantly the proliferation of MLN (Fig. 5C). For the β-casein peptides resulting from pepsin/pancreatin digestion, the P-I stimulated proliferation of MLN (Fig. 6C). Both the unfractionated tryptic hydrolysate and the pepsin/pancreatin hydrolysate enhanced significantly the cell proliferation of spleen cells and MLN (Figs 5 and 6). Intact β-casein induced proliferation in spleen cells only (Figs 5 and 6).

Determination of endotoxin content in peptide fractions

Based on the assumption that the observed nonspecific immunostimulatory effect of some peptides may be due to

https://doi.org/10.1017/S0022029905000877 Published online by Cambridge University Press

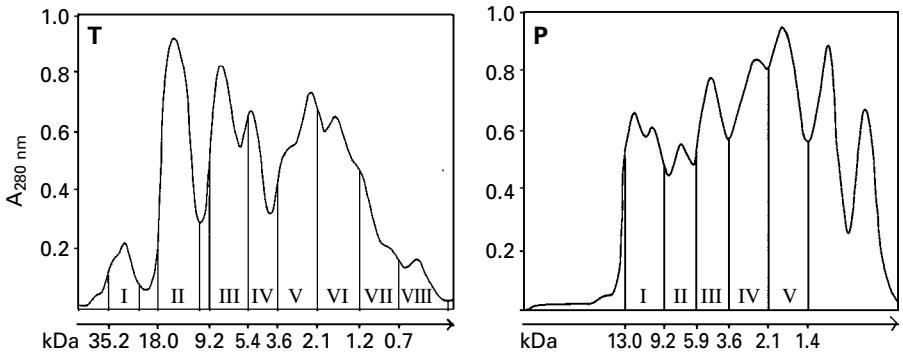

Fig. 3. Chromatograms from preparation of β-casein peptide fractions. Hydrolysates of bovine β-casein were subjected to size-exclusion chromatography on a Sephadex G-50 column, and fractions were collected in the molecular weight intervals given beneath each chromatogram. The molecular weights of peptide fractions were estimated based on chromatography of standard proteins. Absorbances were measured at 280 nm. The final pooling of fractions is shown as roman numerals in the chromatograms. Abbreviations: T: Trypsin digested, P: Pepsin/Pancreatin digested, H: hydrolysate.

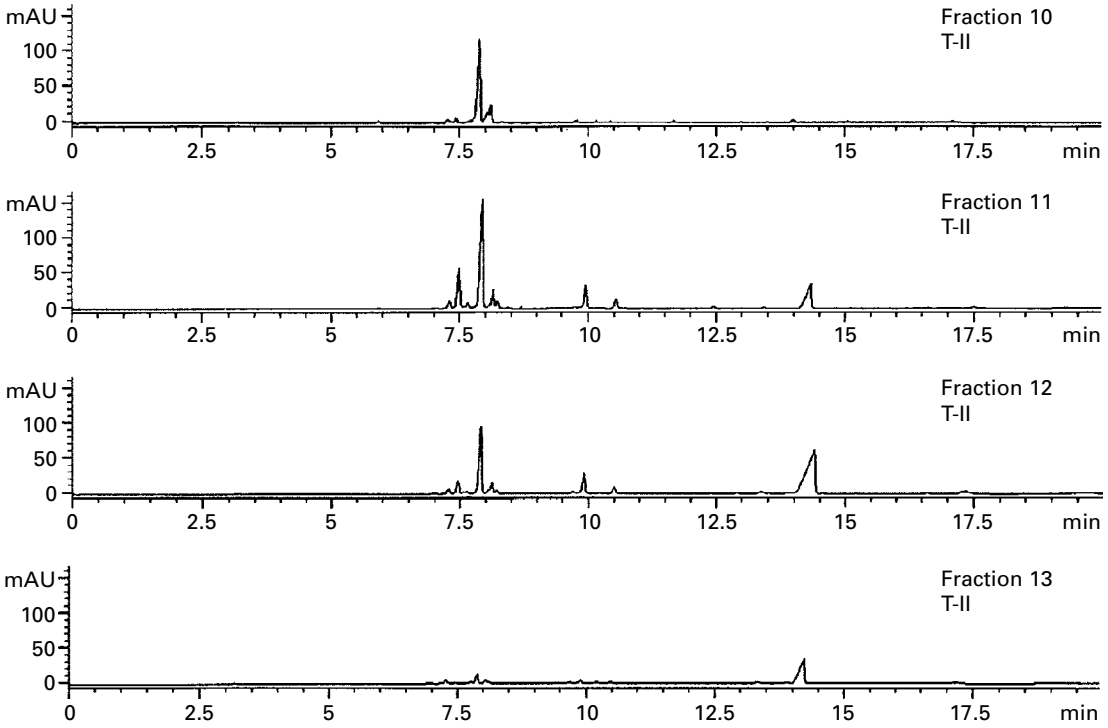

Fig. 4. Electropherograms from preparation of β-casein peptide fractions. Capillary electrophoresis was used analytically to separate peptides in the fractions arising from size-exclusion chromatography of β-casein digests. Fractions containing similar peptide profiles were subsequently pooled giving rise to the fractions designated with roman numerals (Fig. 3). The electropherograms show the peptide profiles in the fractions comprising T-II. Absorbance was read at 214 nm. Abbreviations: T: Trypsin digested, mAU: milli Absorbance Units.

presence of endotoxin, we used the *Limulus* amoebocyte lysate assay to test the stimulatory fractions for LPS contamination, since LPS seems to be a very likely mitogenic contaminant of cows'-milk preparations (Brix et al. 2003). Levels of endotoxin contamination were consistently below 200 pg/ml, and were therefore unlikely to have affected the cellular proliferative responses under investigation here.

Discussion

Various immunoregulatory effects of cows' milk proteins and peptides have been reported (Meisel 1998). However, whether these effects actually result from antigen-specific stimulation of cells or are due to nonspecific cellular activation is difficult to tell because most studies to date were performed *in vitro* on cells from individuals previously

https://doi.org/10.1017/S0022029905000877 Published online by Cambridge University Press

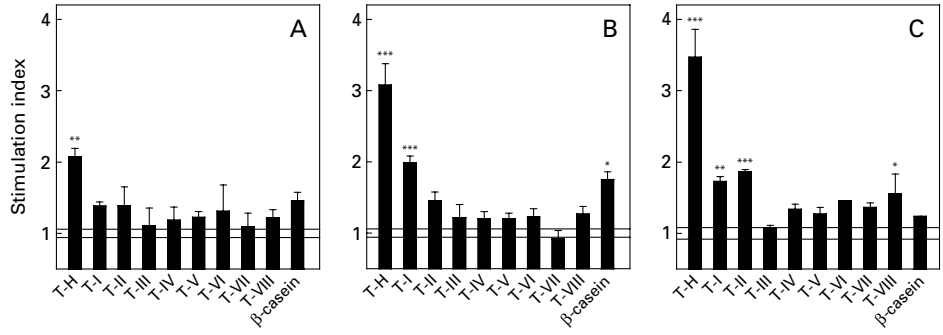

Fig. 5. Effect of peptides derived from trypsin digestion of β-casein on proliferation of cows'-milk-naïve immune cells. Spleen cells (**A, B**) and cells from mesenteric lymph nodes (**C**) from cows'-milk-naïve BALB/c mice were cultured *in vitro* with intact β-casein or β-casein peptides derived from a trypsin digestion as described in Materials and Methods. The final molar antigen concentration in each well was as follows: **A**: intact β-casein: 0·6 μM; unfractionated hydrolysate and peptide fractions: 4 μM, and for **B, C**, intact β-casein: 6 μM: unfractionated hydrolysate and peptide fractions: 8 μM. The molar concentrations were calculated from amino acid analysis and mass spectrometry. The molar weights of the hydrolysates were estimated as described in Materials and Methods. Results (means ± SEM; $n=3$) are shown as a stimulation index calculated as mean counts per minute (cpm) of antigen-stimulated cultures divided by that of control cultures, and are representative of three independent experiments. The two solid lines represent the proliferation of control cultures (mean+SEM and mean−SEM, respectively). Differences in proliferation between antigen-stimulated cultures and control cultures were tested using one-way ANOVA. Multiple comparisons between antigens and control were performed using the Bonferroni test. *$P<0·05$, **$P<0·01$, ***$P<0·001$ as compared with control. Abbreviations: T: Trypsin digested, H: hydrolysate.

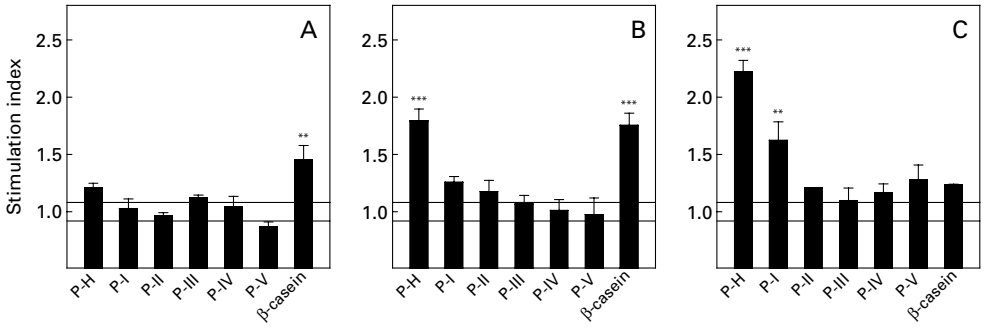

Fig. 6. Effect of peptides derived from pepsin/pancreatin digestion of β-casein on proliferation of cows'-milk-naïve immune cells. Spleen cells (**A**, **B**) and cells from mesenteric lymph nodes (**C**) from cows'-milk-naïve BALB/c mice were cultured *in vitro* with intact β-casein or β-casein peptides derived from a pepsin/pancreatin digest as described in Materials and Methods. Other parameters are as explained in Fig. 5. **$P<0·01$, ***$P<0·001$ as compared with control. Abbreviations: P: Pepsin/pancreatin digested, H: hydrolysate.

exposed to cows' milk proteins through the diet. Hence, at first, we focused on the impact of prior oral exposure to cows' milk on the subsequent proliferation induced *in vitro* by selected cows' milk proteins. Our results demonstrated that the presence of cows' milk in the diet significantly affected the *ex vivo* proliferation of murine immune cells induced upon culturing with cows' milk proteins. In fact, dietary exposure to cows' milk resulted in a significantly higher proliferation *in vitro* of splenocytes after incubation with bovine β-casein, β-lactoglobulin and κ-casein compared with the proliferation of immune cells from mice bred on a milk-free diet.

Upon immunization with β-casein to reveal diet-related differences in number of milk-specific immune cells, a reduced response was seen in cells from mice bred on the milk-containing diet compared with cells from mice bred on the milk-free diet, showing, as expected, that β-casein-specific immune cells are present in mice bred on a milk-containing diet owing to the β-casein content in the diet. The presence of milk-protein-specific cells in mice fed a milk-containing diet and the enhanced proliferation of splenocytes from non-immunized mice on milk-containing *v.* milk-free diet reflect induction of antigen-specific responses after *ex vivo* restimulation with cows' milk proteins. Dietary exposure to food proteins induces antigen-specific regulatory immune cells that are involved in the suppressed antigen-specific immune response observed in experimental animal models upon oral administration of food proteins (Miller et al. 1992; Thorstenson & Khoruts, 2001; Zhang et al. 2001). Although regulatory immune cells are believed to have a suppressive phenotype, it might be speculated that presence of a sufficient number of

https://doi.org/10.1017/S0022029905000877 Published online by Cambridge University Press

antigen-specific regulatory immune cells in mice fed on a milk-containing diet may give rise to the observed enhancement in antigen-specific *ex vivo* proliferation upon culturing with recall antigen, whereas only non-antigen-specific *ex vivo* proliferation towards cows'-milk proteins would be detected in cells from mice fed a milk-free diet, the latter possessing no antigen-specific cells. Our present results thus demonstrate that *ex vivo* restimulation of antigen-specific cells might interfere with results obtained in the search for immunostimulatory capacities of milk proteins and peptides in cases where the cell donors have been exposed to milk proteins via their diets prior to collection of cells. As a consequence, there is a call for studies of the immunostimulatory potential of bovine milk proteins or peptides where cows'-milk-naïve cells are used, which may reveal whether milk proteins or peptides are able to induce nonspecific activation of immune cells. Therefore, we aimed at investigating the nonspecific immunostimulatory potential of β-casein peptides, since β-casein in our initial studies stimulated proliferation of spleen cells from cows'-milk-naïve mice, and is a protein easily digested upon ingestion. The β-casein peptides were produced upon fractionation of *in vitro* digests prepared by using two combinations of digestion enzymes, resulting in different extents of hydrolysis.

When evaluating an *ex vivo* proliferative response, one important factor for detecting immunostimulatory peptides derived from milk proteins is the criterion used to define a positive response, i.e., a response significantly enhanced compared with the response in unstimulated cell cultures (control). As it seems nearly impossible to predict the extent of *ex vivo* stimulation needed to make it likely that peptides do have physiological relevance, we decided on a modest criterion where $P<0{\cdot}05$ was considered to be statistically significant, in order not to omit slightly stimulatory proteins/peptides.

In the discussion of our results, however, we use the criteria defined by Meisel & Gunther (1998), where proliferation towards β-casokinin-10 (residues 193 to 202) in mitogen (ConA)-activated PBMC resulting in a SI of 1·14 (at 10 μM) is reported to be slightly stimulatory, whereas a SI of 2·01 towards Tyr-Gly (κ-casein residue 38–39, at 100 μM) is reported to have a pronounced stimulatory effect. Using these criteria, our results with β-casein peptides revealed that only slightly immunostimulatory peptides appeared from trypsin and pepsin/pancreatin digestions of β-casein, none of these with SI >2. However, it should be noted that the significant effect of the β-casein peptides in the fractions T-I, T-II, T-VIII and P-I was on non-pre-activated MLN with SI ranging from $1{\cdot}56\pm0{\cdot}27$ to $1{\cdot}86\pm0{\cdot}03$. In addition, for T-I the proliferation-inducing capacity was also observed on spleen cells (SI $1{\cdot}99\pm0{\cdot}09$). Previously, the β-casein sequence β-CN(f192–209) was shown to stimulate non-pre-activated spleen cells (Coste et al. 1992), whereas other proliferation-enhancing effects of β-casein peptides have been found only on pre-activated cells that are either *in vivo*-primed (Coste et al. 1992) or ConA-stimulated (Kayser & Meisel, 1996). Pre-activation, such as *in vivo*-priming or mitogen stimulation might circumvent the already discussed effect of prior dietary exposure to cows' milk on immune cells. However, pre-activation obviously hampers identification of peptides that trigger primary cellular activation, i.e., nonspecific immune stimulation.

As regards our β-casein peptide fractions, the T-VIII peptide has been identified as residues 35–42 of κ-casein; present in the commercially available β-casein preparation owing to cross-contamination between caseins. A part of this sequence, the Tyr-Gly residue (κ-CN(f38–39)), has previously been shown to stimulate ConA-induced proliferation of human PBMC (Kayser & Meisel, 1996; Meisel, 1997). Our finding that κ-casein peptide (f35–42) induced proliferation in cows'-milk-naïve immune cells suggests that this peptide may possess an activation-inducing capacity on non-pre-activated immune cells as well.

The unfractionated tryptic β-casein hydrolysate showed a stimulatory effect on immune cells from cows'-milk-naïve mice, which may be explained by additive effects of the individual peptides simultaneously present in the tryptic β-casein hydrolysate. However, our result is in contrast to the findings of Otani & Hata (1995) showing an inhibitory effect of a trypsin β-casein hydrolysate on proliferation of spleen cells from mice bred on a conventional mouse chow. Factors that might contribute to these differences include different cell culture parameters such as incubation times and proliferation-detection method. Other important factors that may interfere with *in vitro* culturing systems may be contamination of protein preparations with cellular stimulants like LPS. Therefore, we tested the LPS content in all fractions possessing the capacity to stimulate the murine spleen cells or MLN. However, for the β-casein derived peptide fractions we found that the LPS-content was very low and therefore should be considered insignificant for the results. In accordance with this, we only observed an effect of intact β-casein on spleen cells, whereas the T-H, T-I and P-H, containing comparable LPS concentrations as intact β-casein, induced proliferation in MLN as well, thus suggesting differential non-LPS induced effects of the β-casein derived peptides on immune cells.

Taken together, the β-casein peptides showed non-specific proliferation-inducing potency when added in the highest concentration (8 μM), with most effects on MLN stimulation, whereas intact β-casein stimulated proliferation of spleen cells only. The nonspecific stimulatory effect of both β-casein and some peptides derived from it revealed that earlier reported effects of these are not exclusively caused by antigen-specific activation of immune cells. The observed differences in nonspecific effects of intact β-casein and peptides on spleen cells *v.* MLNs might be due to differences in B : T cell ratios in the two organs, with more T cells being located in MLN. Thus, intact β-casein and the peptides might act by different stimulatory mechanisms on different cell populations, possibly with

some of the peptides being T cell stimulants. In man, the presence of β-casein peptides in microenvironments in the gut may be physiologically feasible upon ingestion of milk products, and nonspecific regulation of immune cells could have an effect, most likely as a local activation of the gut-associated lymphoid tissue, including the MLN. Still, any effects of β-casein peptides on human gut-associated lymphoid tissue could depend on individual digestion capabilities. In this context, we observed that a more vigorous hydrolysis of β-casein, as achieved with the pepsin/pancreatin derived peptides resulted in a clear reduction in proliferation-inducing capacity compared with the peptides obtained upon trypsin digestion.

As regards milk allergy, β-casein peptides with non-specific activation potential on naïve immune cells seem to be of no concern, as allergic responses imply antigen-specific reactions. However, whether the presence of peptides possessing the capacity to induce nonspecific proliferation could play a role *in vivo* in maintaining allergy-related cell clones is a matter for further study.

In the present study, we also found that κ-casein inhibited proliferation of cells from mice on a milk-free diet, whereas this effect was not seen on cells from mice fed the milk-containing diet. This result further highlights that presence of milk-specific immune cells does interfere with results. In line with this, the inhibitory property of κ-casein has previously been revealed only after LPS-induced proliferation of splenocytes (Otani et al. 1992), suggesting that presence and proliferation of antigen-specific cells have to be overruled by mitogen-induced activation to reveal the nonspecific immunological effect of κ-casein.

In conclusion, the present results suggest that utilization of cells from cows'-milk-naïve animals may bring about new insights into the mechanisms involved in immune regulation by cows' milk proteins and peptides derived from these. Some fractions containing β-casein peptides were shown to significantly stimulate nonspecific proliferation of spleen cells and MLN, yet the physiological relevance of the slight *in vitro* immunostimulatory bovine β-casein peptides needs further investigation.

The technical assistance of Lillian Vile, Thea Gärtner and Nina Milora is much appreciated. We also thank Anne Blicher for performing the amino acid analysis, Marianne K Petersen for carrying out the mass spectrometry, and Hanne Risager Christensen, Tanja Kjær and Trine Mikkelsen for critical reading of the manuscript. Financial support by the Danish Research and Development Programme for Food Technology (FØTEK), The Danish Dairy Research Foundation and Centre for Advanced Food Studies, Copenhagen, Denmark is gratefully acknowledged.

References

Barkholt V & Jensen AL 1989 Amino acid analysis: determination of cysteine plus half-cystine in proteins after hydrochloric acid hydrolysis with a disulfide compound as additive. *Analytical Biochemistry* **177** 318–322

Brix S, Bovetto L, Fritsche R, Barkholt V & Frøkiær H 2003 Immunostimulatory potential of β-lactoglobulin preparations: Effects caused by endotoxin contamination. *Journal of Allergy and Clinical Immunology* **112** 1216–1222

Brix S, Christensen HR, Barkholt V & Frøkiær H 2005 Effect of maternal dietary cows' milk on the immune response to β-lactoglobulin in the offspring: A four-generation study in mice. *International Archives of Allergy and Immunology* **136** 250–257

Carr RI, Webster D, Sadi D, Williams H & Walsh N 1990 Immunomodulation by opioids from dietary casein (exorphins). *Annals of the New York Academy of Sciences* **594** 374–376

Clare DA & Swaisgood HE 2000 Bioactive milk peptides: a prospectus. *Journal of Dairy Science* **83** 1187–1195

Coste M, Rochet V, Leonil J, Molle D, Bouhallab S & Tome D 1992 Identification of C-terminal peptides of bovine beta-casein that enhance proliferation of rat lymphocytes. *Immunology Letters* **33** 41–46

Cross ML & Gill HS 2000 Immunomodulatory properties of milk. *British Journal of Nutrition* **84** S81–S89

Debbabi H, Dubarry M, Rautureau M & Tome D 1998 Bovine lactoferrin induces both mucosal and systemic immune response in mice. *Journal of Dairy Research* **65** 283–293

Gattegno L, Migliore-Samour D, Saffar L & Jolles P 1988 Enhancement of phagocytic activity of human monocytic-macrophagic cells by immunostimulating peptides from human casein. *Immunology Letters* **18** 27–31

Goldman AS 2000 Modulation of the gastrointestinal tract of infants by human milk, interfaces and interactions. An evolutionary perspective. *Journal of Nutrition* **130** 426S–431S

Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H & Svanborg C 1995 Apoptosis induced by a human-milk protein. *Proceedings of the National Academy of Sciences of the USA* **92** 8064–8068

Hanson LA 1998 Breastfeeding provides passive and likely longlasting active immunity. *Annals of Allergy Asthma & Immunology* **81** 523–537

Hata I, Higashiyama S & Otani H 1998 Identification of a phosphopeptide in bovine alpha s1-casein digest as a factor influencing proliferation and immunoglobulin production in lymphocyte cultures. *Journal of Dairy Research* **65** 569–578

Kayser H & Meisel H 1996 Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *FEBS Letters* **383** 18–20

Lahov E & Regelson W 1996 Antibacterial and immunostimulating casein-derived substances from milk: casecidin, isracidin peptides. *Food and Chemical Toxicology* **34** 131–145

Meisel H 1997 Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers* **43** 119–128

Meisel H 1998 Overview on milk protein-derived peptides. *International Dairy Journal* **8** 363–373

Meisel H & Gunther S 1998 Food proteins as precursors of peptides modulating human cell activity. *Nahrung* **42** 175–176

Migliore-Samour D, Floc'h F & Jolles P 1989 Biologically active casein peptides implicated in immunomodulation. *Journal of Dairy Research* **56** 357–362

Miller A, Lider O, Roberts AB, Sporn MB & Weiner HL 1992 Suppressor T-cells generated by oral tolerization to myelin basic-protein suppress both invitro and invivo immune-responses by the release of transforming growth-factor-beta after antigen-specific triggering. *Proceedings of the National Academy of Sciences of the USA* **89** 421–425

Mincheva-Nilsson L, Hammarstrom ML, Juto P & Hammarstrom S 1990 Human milk contains proteins that stimulate and suppress T lymphocyte proliferation. *Clinical and Experimental Immunology* **79** 463–469

Nagata S, McKenzie C, Pender SLF, Bajaj-Elliott M, Fairclough PD, Walker-Smith JA, Monteleone G & MacDonald TT 2000 Human Peyer's patch T cells are sensitized to dietary antigen and display a Th cell type 1 cytokine profile. *Journal of Immunology* **165** 5315–5321

Otani H & Futakami M 1996 Modification of nitrite production and phagocytosis of thioglycollate-elicited mouse peritoneal macrophages by bovine casein digests. *Food and Agricultural Immunology* **8** 59–69

Otani H, Monnai M & Hosono A 1992 Bovine kappa-casein as inhibitor of the proliferation of mouse splenocytes induced by lipopolysaccharide stimulation. *Milchwissenschaft* **47** 512–515

Otani H & Odashima M 1997 Inhibition of proliferative responses of mouse spleen lymphocytes by lacto- and ovotransferrins. *Food and Agricultural Immunology* **9** 193–201

Otani H, Watanabe T & Tashiro Y 2001 Effects of bovine beta-casein (1–28) and its chemically synthesized partial fragments on proliferative responses and immunoglobulin production in mouse spleen cultures. *Bioscience Biotechnology and Biochemistry* **65** 2489–2495

Parker F, Migliore-Samour D, Floc'h F, Zerial A, Werner GH, Jolles J, Casaretto M, Zahn H & Jolles P 1984 Immunostimulating hexapeptide from human casein: amino acid sequence, synthesis and biological properties. *European Journal of Biochemistry* **145** 677–682

Sandre C, Gleizes A, Forestier F, Gorges-Kergot R, Chilmonczyk S, Leonil J, Moreau MC & Labarre C 2001 A peptide derived from bovine beta-casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice. *Journal of Nutrition* **131** 2936–2942

Shimizu M, Kato M, Miwa K & Kaminogawa S 1991 L-cell growth stimulation by a beta-casein peptide: the importance of its phosphorylation site for activity. *Biochemistry International* **24** 1127–1133

Sutas Y, Soppi E, Korhonen H, Syvaoja EL, Saxelin M, Rokka T & Isolauri E 1996 Suppression of lymphocyte proliferation in vitro by bovine caseins hydrolysed with *Lactobacillus casei* GG-derived enzymes. *Journal of Allergy and Clinical Immunology* **98** 216–224

Thorstenson KM & Khoruts A 2001 Generation of anergic and potentially immunoregulatory CD25(+)CD4 T cells *in vivo* after induction of peripheral tolerance with intravenous or oral antigen. *Journal of Immunology* **167** 188–195

Wong CW, Seow HF, Husband AJ, Regester GO & Watson DL 1997 Effects of purified bovine whey factors on cellular immune functions in ruminants. *Veterinary Immunology and Immunopathology* **56** 85–96

Wong CW, Seow HF, Liu AH, Husband AJ, Smithers GW & Watson DL 1996 Modulation of immune responses by bovine beta-casein. *Immunology and Cell Biology* **74** 323–329

Wong KF, Middleton N, Montgomery M, Dey M & Carr RI 1998 Immunostimulation of murine spleen cells by materials associated with bovine milk protein fractions. *Journal of Dairy Science* **81** 1825–1832

Zhang XM, Izikson L, Liu LM & Weiner HL 2001 Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration. *Journal of Immunology* **167** 4245–4253

Zimecki M, Mazurier J, Spik G & Kapp JA 1996 Lactoferrin inhibits proliferative response and cytokine production of TH1 but not TH2 cell lines. *Archivum Immunologiae et Therapiae Experimentalis* **44** 51–56