

## Immunoregulatory peptides in bovine milk

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Bovine milk is known to contain a number of peptide fractions that can affect immune function. The vast majority of immunoregulatory peptides that have been characterised are hydrolysate derivatives of major milk proteins. Recent research has also indicated that the metabolic activity of probiotic lactic acid bacteria can generate *de novo* immunoregulatory peptides from milk, via enzymatic degradation of parent milk protein molecules. In contrast, relatively little is known of endogenous, preformed immunoregulatory peptides in milk that may be relevant to modulating human health. The natural *in vivo* role of preformed and enzymatically derived peptides is likely to be one of regulation of the neonatal (bovine) gastrointestinal tract immune system, in order to modulate immune function with respect to the development of immunocompetence and avoidance of undesirable immunological responses (e.g. tolerance, and hypersensitivity to nutrients). There is scope for the further characterisation of both the origin and function of milk-derived immunoregulatory peptides, so that their potential to influence human health can be fully appraised. This review highlights our current knowledge of milk-derived immunoregulatory peptides, and outlines areas that are of relevance for further research.

### Bovine milk: Immunomodulation: Immunoregulation: Peptides

#### Introduction

Enzymatic degradation of foodstuffs in the gut releases short-chain peptide sequences from intact proteins, glycoproteins and lipoproteins. In many cases, peptides liberated in this fashion can have important biogenic functions (Schlimme & Meisel, 1995). In order to effect a biological response, peptides must either transverse the intestinal epithelium and enter circulation, or else bind directly to specific intra-epithelial cell surface receptor sites. There is thus the scope for biogenic peptides, derived from the enzymatic hydrolysis of nitrogen-bearing foods in the gut, to directly affect physiological function through cellular signalling.

Bovine milk comprises a complex mixture of simple, globular and conjugated proteins. Most of these are susceptible to the degradative effects of gastric processing, and extensive hydrolysis takes place upon exposure to enzymes in the gut. These enzymes may be of endogenous origin (i.e. secreted by the digestive system) or exogenous origin (e.g. derived from actively metabolizing gut microflora). In either case, many of the peptides released by enzymatic hydrolysis in the gut have specific biological functions through their ability to bind to (and affect) cellular function (Meisel, 1997).

Several of the biologically active peptides released by enzymatic hydrolysis of milk proteins are known to affect cells of the immune system, and (as a consequence) to

affect downstream immunological responses and cellular functions (Coste & Tome, 1991; Kayser & Meisel, 1996). In addition, bovine milk contains some immunologically active peptides in preformed or near-formed state, i.e. those which occur naturally in milk and require no or minimal gastric modification to become biologically active (Stoeck *et al.* 1989, Guimont *et al.* 1997). The majority of research which has characterised immunologically active peptides has come from *in vitro* modelling of *in vivo* gastric processing of milk (Svedberg *et al.* 1985; Jolles & Migliore-Samour, 1986). Evidence for immunoregulatory activity has been subsequently derived from both *in vitro* studies of the direct effects of these peptides on cells of the immune system, and *in vivo* studies of the effects of peptides delivered by dietary or parenteral routes (Werner *et al.* 1986; Jolles *et al.* 1992; Schlimme & Meisel, 1995). There is now a substantial body of evidence to indicate that bovine milk contains a number of very potent immunoregulatory peptides. This review focuses on evidence for the presence of these peptides, their derivation from individual milk proteins, and describes their effects on the immune system.

#### Casein-derived peptides

##### $\alpha_{S1}$ -Casein

Peptide-rich hydrolysates of  $\alpha_{S1}$ -casein have been shown to

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**Table 1.** Various immunomodulatory activities of peptides derived from  $\alpha_{S1}$ -casein in bovine milk

Protein	Peptide	Digests	Immune effect	Reference
$\alpha_{S1}$ -casein		Pancreatin	↓ proliferation of lymphocytes	Otani & Hata (1995)
		Trypsin	↓ proliferation of lymphocytes	Otani & Hata (1995)
		Pepsin/chymotrypsin	No effect <i>in vitro</i> with mitogen-stimulated lymphocytes	Otani & Hata (1995)
		Pepsin/trypsin	↓ proliferation of human peripheral blood mononuclear cells	Kayser & Meisel (1996)
Isracidin	Thr-Thr-Met-Pro-Leu-Tyr (194–199)	Trypsin	↑ antibody formation, ↑ phagocytosis, ↓ <i>Klebsiella pneumoniae</i> infection	Jolles <i>et al.</i> (1992) Schlimme & Meisel (1995)
	N-terminal sequence 1–23	Chymosin	Protect mice against infection by <i>Staphylococcus aureus</i> . ↑ phagocytic response in mice infected with <i>Candida albicans</i> . Protect cows and sheep against mastitis	Lahov & Regelson (1996)
	Arg-Tyr-Leu-Gly-Tyr-Leu-Glu (90–96)		↑ lymphocyte proliferation, ↑ natural killer activity, ↑ neutrophil locomotion	Migliore-Samour & Jolles (1988) Elitsur & Luk (1991)
	Arg-Tyr-Leu-Gly-Tyr-Leu (90–95)		↑ lymphocyte proliferation, ↑ natural killer activity, ↑ neutrophil locomotion	Migliore-Samour & Jolles (1988) Elitsur & Luk (1991)
	Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys (23–34)		ACE inhibitor, ↑ phagocytosis, ↑ protective response to <i>Klebsiella pneumoniae</i> infection	Maruyama <i>et al.</i> (1987); Jolles <i>et al.</i> (1992); Fiat <i>et al.</i> (1993); Meisel (1997)

modulate immune function, with different modulatory effects attributable to varying enzymatic digestion regimes. For example Otani & Hata (1995) have shown that both pancreatin and trypsin digests of  $\alpha_{S1}$ -casein significantly inhibited the proliferative responses of murine splenic lymphocytes and rabbit Peyer's patch cells, whereas digests derived from pepsin and chymotrypsin treatment had no effect when included in *in vitro* culture with mitogen-stimulated cells. Peptide residues derived from the pepsin/trypsin hydrolysis of  $\alpha_{S1}$ -caseins were also found to significantly suppress mitogen-induced proliferation of human peripheral blood mononuclear cells *in vitro* (Kayser & Meisel, 1996). In contrast, trypsin treatment alone of bovine  $\alpha_{S1}$ -casein results in the release of Thr-Thr-Met-Pro-Leu-Tyr (residues 194–199 C terminal), which has been shown to promote antibody formation and accelerated phagocytosis *in vitro*, as well as reducing *Klebsiella pneumoniae* infection in mice *in vivo* following systemic (intravenous) administration (Jolles *et al.* 1992; Schlimme & Meisel, 1995). Since trypsin is a major proteinase in pancreatin, the substrate specificity of trypsin seems to be important for the formation of immunoenhancing or suppressing peptides from casein components (Otani & Hata, 1995).

Chymosin digestion of  $\alpha_{S1}$ -casein results in the immunoenhancing product isracidin (N-terminal sequence 1–23). This peptide has been found to protect mice against infection by *Staphylococcus aureus* when injected intramuscularly prior to being infected, and to correspondingly stimulate a phagocytic response in mice infected with *Candida albicans* when injected intravenously *in vivo* (Lahov & Regelson, 1996). It has also been found to protect cows and sheep against mastitis, following injection of the peptide into the udder (Lahov & Regelson, 1996).

Residues 90–96 (Arg-Tyr-Leu-Gly-Tyr-Leu-Glu) and 90–95 (Arg-Tyr-Leu-Gly-Tyr-Leu) of  $\alpha_{S1}$ -casein both have naloxone-inhibitible opioid-like properties. Opioid peptides, such as  $\beta$ -endorphins, have been demonstrated to exert *in vitro* and *in vivo* immunomodulating activities by

enhancing lymphocyte proliferative responses, natural killer cell activity and neutrophil locomotion (Migliore-Samour & Jolles, 1988; Elitsur & Luk, 1991). Opioid  $\mu$ -receptors have been demonstrated on T lymphocytes and human phagocytic leucocytes, and it is possible that absorbed milk peptides with opioid-like activities may be involved in the development of T cell functions and natural killer cell cytotoxicity (Samour & Jolles, 1988).

The  $\alpha_{S1}$ -casein dodecapeptide (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) (residues 23–34) has been shown to stimulate phagocytosis by murine peritoneal macrophages *in vitro* and consequently to exert a protective effect against *Klebsiella pneumoniae* challenge in mice after intravenous treatment *in vivo* (Meisel, 1997). This peptide is also an inhibitor of angiotensin I-converting enzyme (ACE). ACE inhibitors are well known for their antihypertensive properties, but have also been found to prevent cleavage of bradykinin by blocking the conversion of angiotensin I to angiotensin II by ACE (Jolles *et al.* 1992). Bradykinin acts as a mediator of the acute inflammatory process and is thus able to stimulate macrophages, enhance lymphocyte migration and to induce the secretion of lymphokines from lymphocyte cultures (Maruyama *et al.* 1987; Fiat *et al.* 1993).

The various immunomodulatory properties of  $\alpha_{S1}$ -casein-derived peptides are summarised in Table 1.

### $\beta$ -Casein

As with  $\alpha_{S1}$ -casein, pancreatin and trypsin digests of  $\beta$ -casein have been shown to significantly inhibit mitogen-stimulated proliferative responses of murine splenic lymphocytes and rabbit Peyer's patch cells when included in *in vitro* cell culture, although no effect has been observed with pepsin and chymotrypsin digests (Otani & Hata, 1995). Peptide residues derived from the pepsin/trypsin hydrolysis of  $\beta$ -caseins were also found to significantly suppress the mitogen-induced proliferation of human lymphocytes (Kayser & Meisel, 1996). In direct contrast,

**Table 2.** Various immunomodulatory activities of peptides derived from  $\beta$ -casein in bovine milk

Protein	Peptide	Digests	Immune effect	Reference
$\beta$ -casein		Pancreatin	↓ proliferation of lymphocytes	Otani & Hata (1995)
		Trypsin	↓ proliferation of lymphocytes	Otani & Hata (1995)
		Pepsin/ chymotrypsin	No effect <i>in vitro</i> with mitogen-stimulated lymphocytes	Otani & Hata (1995)
		Pepsin/trypsin	↓ proliferation of lymphocytes	Kayser & Meisel (1996)
	Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val (193–209)	Pepsin/chymosin	↑ proliferative responses in lymphocytes	Migliore-Samour & Jolles (1988)
	Pro-Gly-Pro-Ile-Pro-Asn (63–68)		↑ antibody formation and ↑ phagocytosis	Jolles & Migliore-Samour (1986); Jolles <i>et al.</i> (1988); Migliore-Samour & Jolles (1988)
	Leu-Leu-Tyr (191–193)		↑ antibody formation and ↑ phagocytosis, and ↑ antigen dependent T cell proliferation	Jolles & Migliore-Samour (1986); Jolles <i>et al.</i> (1988); Migliore-Samour & Jolles (1988)
$\beta$ -casomorphin	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu (60–70)	Trypsin	↓ lymphocyte proliferation, ↑ mice resistance to <i>Klebsiella pneumoniae</i> infection.	Elitsur & Luk (1991); Kayser & Meisel (1996)
$\beta$ -casomorphin-7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile (60–66)		↓ lymphocyte proliferation at low conc. ↑ lymphocyte proliferation at high conc.	Kayser & Meisel (1996)
$\beta$ -casokinin-10	Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg (193–202)		↓ lymphocyte proliferation at low conc. ↑ lymphocyte proliferation at high conc.	Maruyama <i>et al.</i> (1987); Kayser & Meisel (1996)
	Ala-Pro-Tyr-Pro-Gln-Arg (177–183)		ACE inhibitor	Maruyama <i>et al.</i> (1987)

the C-terminal sequence 193–209 of  $\beta$ -casein (obtained from a pepsin/chymosin digest of bovine casein) has been shown to directly stimulate proliferation of rat lymphocytes *in vitro* in the absence of extraneous mitogens or antigens (Meisel, 1997).

Hydrolysis of  $\beta$ -casein results in the hexapeptide Pro-Gly-Pro-Ile-Pro-Asn (residues 63–68) and the tripeptide Leu-Leu-Tyr (LLY, residues 191–193). Both peptides have been shown to promote antibody formation and accelerated phagocytosis by murine peritoneal macrophages *in vitro*, but LLY also enhances antigen-dependent T cell proliferation (Jolles & Migliore-Samour, 1986, 1988; Migliore-Samour & Jolles, 1988).

$\beta$ -Casomorphins are fragments of the  $\beta$ -casein sequence 60–70, which have opiate-like properties and have been shown to modulate the immune system.  $\beta$ -Casomorphins were originally isolated from human and bovine  $\beta$ -casein following *in vitro* trypsin hydrolysis (Elitsur & Luk, 1991). To be biologically active,  $\beta$ -casomorphin needs to be released from its precursor  $\beta$ -casein, and it is thought that this process takes place *in vivo* via enzymatic hydrolysis in the gut lumen (Elitsur & Luk, 1991). Indeed  $\beta$ -casomorphin has been found in the intestinal aspirates of human volunteers who had ingested bovine milk (Svedberg *et al.* 1985). Elitsur & Luk (1991) found a significant *in vitro* suppressive effect of  $\beta$ -casomorphins on human lamina propria-derived lymphocyte proliferation at concentrations as low as 10  $\mu$ M. This antiproliferative effect was reversed by addition to culture of the opiate receptor antagonist naloxone (Elitsur & Luk, 1991).

*In vivo*,  $\beta$ -casomorphins have been shown to have an opposite effect on immune function to the above *in vitro* work, in that they have been shown to enhance the resistance of mice to *Klebsiella pneumoniae*, probably

through a stimulation of peritoneal macrophages. However, as with the *in vitro* work, administration *in vivo* of an opioid antagonist to mice resulted in the suppression of this stimulatory effect, suggesting an active opioid receptor-binding site for the biologically active peptide (Parker *et al.* 1984; Elitsur & Luk, 1991).

Depending on the concentration,  $\beta$ -casomorphin-7 (residues 60–66 of  $\beta$ -casein) and  $\beta$ -casokinin-10 (residues 193–202 of  $\beta$ -casein) can exhibit bipolar modulatory effects on human peripheral blood lymphocyte proliferation. In *in vitro* culture with mitogen-stimulated T lymphocytes, both peptides have been shown to suppress proliferation at low concentrations but to enhance proliferation at high concentrations (Kayser & Meisel, 1996).

$\beta$ -Casein has ACE-inhibitory fragments that have been shown to stimulate phagocytosis by murine peritoneal macrophages and to exert a protective effect against *Klebsiella pneumoniae* infection in mice after intravenous treatment with doses as low as 0.5 mg/kg (Jolles *et al.* 1992; Schlimme & Meisel, 1995).  $\beta$ -Casokinin-10 and heptapeptide Ala-Val-Pro-Tyr-Pro-Gln-Arg (residues 177–183 of  $\beta$ -casein) have been found to inhibit ACE which, like residues 23–34 of bovine  $\alpha$ <sub>S1</sub>-casein, may act on the immune system by preventing the breakdown of bioactive bradykinin (Maruyama *et al.* 1987).

The various immunomodulatory properties of  $\beta$ -casein-derived peptides are summarised in Table 2.

#### $\kappa$ -Casein

Bovine  $\kappa$ -caseinoglycopeptide (residues 106–169, CGP) prepared from  $\kappa$ -casein digested with chymosin, has been shown to be a potent inhibitor of lipopolysaccharide (LPS)-induced and phytohaemagglutinin (PHA)-induced

**Table 3.** Various immunomodulatory activities of peptides derived from  $\kappa$ -casein in bovine milk

Protein	Peptide	Digests	Immune effect	Reference
$\kappa$ -casein				
$\kappa$ -caseinoglycopeptides	Residues 106–169 of $\kappa$ -casein	Chymosin	↓ proliferation of lymphocytes	Otani <i>et al.</i> (1995)
Fractionated CGP			↓ PHA-induced proliferative response on mouse splenic cells. Inhibition increases with increasing number of NANA residues (up to 5)	Otani <i>et al.</i> (1995)
Fractionated CGP			↓ LPS-induced proliferative response on mouse splenic cells. Most potent at 2 NANA residues	Otani <i>et al.</i> (1995)
Para- $\kappa$ -casein	Phe-Phe-Ser-Asp-Lys-(17–21) Tyr-Gly (38–39)	Trypsin	↑ antibody formation and ↑ phagocytosis <i>in vitro</i> ↑ proliferation of lymphocytes	Jolles & Migliore-Samour (1986); Jolles <i>et al.</i> (1988) Kayser & Meisel (1996); Meisel (1997)
		Pepsin/trypsin <i>Lactobacillus</i> GG strain enzymes + pepsin/trypsin	↑ proliferation of lymphocytes ↓ proliferation of lymphocytes	Sutas <i>et al.</i> (1996a) Sutas <i>et al.</i> (1996a)

proliferation of murine splenic lymphocytes *in vitro*, and also to suppress *in vitro* antibody production in murine spleen cell cultures (Otani *et al.* 1995). Otani *et al.* (1995) also confirmed that both LPS- and PHA-induced proliferative responses of rabbit Peyer's patch cells were suppressed by the presence of this peptide *in vitro*. It is known that LPS directly stimulates B lymphocytes, whereas PHA stimulates helper T lymphocytes, and it is therefore likely that CGP down-regulates the immune system by suppressing the proliferative responses of both lymphocyte subsets (Otani *et al.* 1995).

Otani *et al.* (1995) characterised seven fractions of CGP with zero to five *N*-acetylneuraminic acid (NANA) residues, which had different inhibitory effects from the unfractionated CGP on both LPS- and PHA-induced proliferative responses of murine spleen cells. The inhibitory effect on PHA-induced proliferative responses increased with increasing numbers of NANA residues, whereas inhibition of LPS-induced proliferation was highest when the CGP fraction had two NANA residues. Both inhibitory effects decreased significantly after neuraminidase digestion. These findings indicate that both the carbohydrate portions (particularly the NANA residues) and the polypeptide portions are essential for inhibitory effects on LPS- and PHA-induced proliferative responses of murine spleen cells (Otani *et al.* 1995).

In direct contrast to the work of Otani and colleagues, Sutas *et al.* (1996a) found that peptides derived from the pepsin–trypsin hydrolysis of  $\kappa$ -casein significantly enhanced the mitogen-induced proliferation of human lymphocytes. Furthermore, the  $\kappa$ -casein/trypsin-derived peptide Phe-Phe-Ser-Asp-Lys (residues 17–21, bovine para- $\kappa$ -casein [1–105 region of  $\kappa$ -casein]) has been shown to promote antibody formation and accelerated phagocytic activity of murine and human macrophages *in vitro* (Jolles & Migliore-Samour, 1986; Jolles *et al.* 1988). In addition, Tyr-Gly (residues 38 and 39 of  $\kappa$ -casein) is another immunomodulating peptide, which can in principle pass across the intestine in quantitatively significant amounts to reach local lymphocytes. The effects of Tyr-Gly on the proliferation of human peripheral blood

lymphocytes, activated with concanavalin A, have been examined *in vitro*, and Tyr-Gly was shown to significantly enhance cellular proliferation (Kayser & Meisel, 1996; Meisel, 1997).

#### *Lactic acid bacteria-derived bioactive peptides*

Although  $\kappa$ -casein has been shown to possess immunosuppressive properties against lymphocytes, a study by Sutas *et al.* (1996a) demonstrated that the metabolic activity of probiotic lactic acid bacteria (LAB) can assist in *de novo* formation of immunostimulatory peptides from  $\kappa$ -casein. When  $\kappa$ -casein was hydrolysed with *Lactobacillus* GG strain enzymes, followed by pepsin–trypsin treatment, the peptide derivatives were found to enhance the mitogen responsiveness of human lymphocytes, when included in *in vitro* cell culture. In contrast, similar treatment of  $\alpha$ <sub>S1</sub>- and  $\beta$ -casein failed to reverse the suppressive effects of  $\kappa$ -casein on human lymphocyte function. This study has highlighted the ability of LAB to influence the enzymatic generation of immunoregulatory peptides from milk (Sutas *et al.* 1996a).

Sutas *et al.* (1996b) further investigated whether bovine caseins, degraded by enzymes of an intestinal bacterial strain *Lactobacillus* GG, could regulate cytokine production by anti-CD3 antibody-stimulated human peripheral blood mononuclear cells, when included in *in vitro* cell culture. Purified casein derivatives up-regulated interleukin-4 (IL-4) and interferon- $\gamma$  (IFN- $\gamma$ ) production, whereas *Lactobacillus* GG-degraded casein down-regulated the IL-4 production with no effect on IFN- $\gamma$ . These results clearly demonstrate that intestinal bacteria may modify the immunomodulatory properties of native food proteins.

The various immunomodulatory properties of  $\kappa$ -casein-derived peptides are summarised in Table 3.

#### **Whey proteins**

##### *$\alpha$ -Lactalbumin*

Murine humoral immune responses to sheep and human red blood cells are known to be enhanced by a hydrolysed

**Table 4.** Various immunomodulatory activities of whey proteins and other milk-derived peptides

Protein	Peptide	Digests/fractions	Immune effect	Reference
Whey proteins				
$\alpha$ -Lactalbumin	Hydrolysed $\alpha$ -lactalbumin		$\uparrow$ mice immune response to SRBCs and HRBCs	Bounous & Kongshavn (1985)
$\beta$ -Lactoglobulin	$\beta$ -Lactoglobulin		Carries retinoic acid. $\downarrow$ lymphocyte blastogenesis	Guimont <i>et al.</i> (1997); Elitsur <i>et al.</i> (1997)
Lactoferrin	Residues 17–41 of lactoferrin (lactoferricin B)		$\downarrow$ IL-6 response when stimulated by LPS. $\uparrow$ release of IL-8 from leucocytes	Mattsby-Baltzer <i>et al.</i> (1996); Shinoda <i>et al.</i> (1996)
Glycophosphopeptide		Gel filtration, anion exchange	Strong mitogenic activity	Yun <i>et al.</i> (1996)
Milk growth factor		Cation exchange, HPLC, and size exclusion with HPLC	$\downarrow$ proliferation of lymphocytes	Stoek <i>et al.</i> (1989)

$\alpha$ -lactalbumin diet, and this effect involves the modulation of both B lymphocyte and T helper cell activities (Bounous & Kongshavn, 1985). Commercially available dietary hydrolysed  $\alpha$ -lactalbumin has also been shown to enhance immune responses to the T cell-independent antigen TNP-Ficoll, which can stimulate B lymphocytes in the absence of T lymphocyte co-operation (Bounous & Kongshavn, 1982). However, the biologically active peptides responsible for this immunomodulation were not identified further in these studies.

#### *$\beta$ -Lactoglobulin*

$\beta$ -Lactoglobulin is the most abundant of the whey proteins and is a carrier of small hydrophobic molecules, including retinoic acid, which is a potential modulator of lymphocyte responses (Guimont *et al.* 1997). Trans-retinoic acid has been found by Elitsur *et al.* (1997) to enhance mitogen-stimulated proliferation of human colonic lamina propria-derived lymphocytes (LPL). This effect was abolished when LPL preparations were depleted of macrophages, suggesting that trans-retinoic acid might affect accessory cells during lymphocyte proliferation.

#### *Lactoferrin*

Human lactoferrin has been shown to cause differentiation of CD4<sup>+</sup>/CD8<sup>-</sup> lymphocytes to CD4<sup>+</sup> helper T-lymphocytes *in vitro*. The lymphocyte receptor-binding region for human lactoferrin resides in an N-terminal peptide (4–52) released by the action of trypsin. The structural homology between bovine and human lactoferrin, together with the proven ability of native bovine lactoferrin to suppress lymphocyte blastogenesis *in vitro*, predicts a lymphocyte receptor-binding region for bovine lactoferrin similar to that of human lactoferrin (Schanbacher *et al.* 1997).

Bovine lactoferricin B (residues 17–41 of lactoferrin) has also been found to suppress interleukin-6 production by a human monocytic cell line in response to LPS stimulation *in vitro* (Mattsby-Baltzer *et al.* 1996). Both bovine lactoferrin and lactoferricin have the ability to stimulate the release of the neutrophil-activating chemokine interleukin-8 from human polymorphonuclear leucocytes (Shinoda *et al.* 1996). The fraction of bovine lactoferrin depleted of lactoferricin has no stimulatory effect (Miyachi *et al.* 1998).

#### *Glycophosphopeptide*

Yun *et al.* (1996) isolated a peptidic fraction from cheese whey protein concentrate, by gel filtration and anion exchange chromatography, which showed high natural mitogenic activity toward murine splenocytes *in vitro*. The fraction was found to contain calcium, inorganic phosphorus and carbohydrate, indicating the active component to be a glycophosphopeptide (GPP). The carbohydrate component of GPP containing N-linked oligosaccharide chains may be important in the strong mitogenic activity. None of the mitogenic factors that have been found in cheese whey or bovine milk have the same characteristics as seen in the GPP found by Yun *et al.* (1996). It remains uncertain whether GPP is formed during the course of cheese production or is an endogenous peptide which is contained already in raw milk and then concentrated in cheese whey.

The various immunomodulatory properties of whey protein-derived peptides are summarised in Table 4.

#### **Other peptides**

##### *Tuftsins*

Tuftsins is a tetrapeptide Thr-Lys-Pro-Arg (residues 289–292), which is derived from endopeptidase and leukokininase cleavage of the heavy chain F<sub>c</sub> region of IgG. Human Ig-derived tuftsins has a variety of immunoregulatory effects, including stimulation of leucocyte chemotaxis and phagocyte motility, enhancement of phagocyte oxidative metabolism and antigen processing, and increase in monocyte- and NK cell-mediated tumour cell cytotoxicity (Werner *et al.* 1986). Although the immunoregulatory role of bovine Ig-derived tuftsins remains to be determined, the high immunoglobulin content of bovine milk and colostrum would suggest that this might be a fruitful area for future study.

##### *Proline-rich polypeptide*

Proline-rich polypeptide (PRP) isolated from ovine colostrum whey has been found to possess regulatory properties that stimulate or suppress immune responses. A nonapeptide fragment Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro (VESYVPLFP) has been isolated from a chymotryptic digest of ovine PRP. VESYVPLFP along with the corresponding synthetic peptide and its C-terminal penta-

and hexapeptides, shows immunoregulatory activities in mice similar to native PRP, including enhancement of splenic antibody responses to foreign erythrocyte antigens (SRBC) when administered 3 h before immunisation with SRBC (Janusz *et al.* 1987). Ovine PRP has been shown to induce cytokine production by murine macrophages (Blach-Olszewska & Janusz, 1997) and also to induce growth and differentiation of resting B lymphocytes, but has not been shown to induce detectable Ig isotype switching in murine cells *in vitro* (Julius *et al.* 1988).

Bovine PRP has not yet been studied in any great detail, but if it is found to have similar properties to ovine PRP, then it will have great potential in the development of functional foods.

#### Milk growth factor

Milk growth factor (MGF) is a monomeric immunoregulatory peptide, which has been isolated from bovine milk and purified to a high degree by three-stage column chromatography (Stoeck *et al.* 1989). MGF shows complete N-terminal sequence homology with bovine TGF- $\beta$ 2 and has been found to universally suppress human T lymphocyte functions *in vitro*, including mitogen- and interleukin-2 stimulated cellular proliferation, recall proliferative responses to tetanus toxoid antigen, and proliferation of an antigen-specific human T cell clone (Stoeck *et al.* 1989).

#### Conclusions

The potential for bovine milk to generate immunoregulatory peptides is now well established. In many cases, both the bioactive molecules and their effects have been characterised via *in vitro* and *in vivo* studies of immune function (e.g. the work of Otani and colleagues on  $\kappa$ -casein-derived CGP). The majority of research so far has focused on the effect of immunoactive peptides *in vitro*, and in many instances the effects of these peptides *in vivo* has yet to be shown. The precise mode of action of most immunoactive peptides on the immune system also remains to be determined.

It is likely that the *in vivo* significance of immunomodulatory peptides is to regulate immune function in the neonatal bovine, such that the gastrointestinal tract immune system develops full functionality in the absence of unwanted effects, including tolerance to pathogen-derived antigens or hypersensitivity to nutrient molecules. However, the comparatively new field of *de novo* generation of milk-derived peptides via LAB biometabolism remains underexplored, and further research is required in this area to identify novel immunoregulatory peptides formed by probiota. Similarly, the notion that milk contains preformed peptides with immunoregulatory activity (e.g. GPP, MGF) is gaining momentum. Further technological advances in the field of low-molecular-weight peptide fractionation should facilitate the identification of endogenous immunoregulatory peptides in milk. Finally, immunoregulatory peptides have been demonstrated in milk or major milk proteins of other species (e.g. human tuftsin and ovine PRP), but the regulatory role of these molecules derived

from bovine milk remains an unexplored area. In all cases, there is scope for clinical scientists to prove that demonstrable immunoregulatory properties of milk-derived peptides can correspond to tangible improvements in human health.

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