Immunomodulatory properties of milk

Martin L. Cross and H. S. Gill*

Milk and Health Research Centre, Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand

There is increasing commercial interest in the production of functional foodstuffs which have health-promoting properties. Over the last five to ten years, significant progress has been made in the identification and characterisation of bovine milk components that can affect the function of the immune system. This review outlines the major components of bovine milk that have been shown to modulate immune function, and discusses experimental approaches to the identification of various facets of the immune response that are known to be affected by milk-derived proteins.

Bovine milk: Immunomodulation: Lymphocytes: Antibody: Cytokines: NK-cells

Introduction

Ruminant milk and milk products have formed an integral part of human diets since the earliest domestication of livestock. Bovine milk, in particular, has long been associated with contributing towards a balanced nutrition, and as an important foodstuff in its own right. The nutritional benefits of milk-derived proteins, vitamins and minerals have been promoted extensively by commercial dairying enterprises, and the vast range of milk-based products now available owes a lot to the continued consumer image of milk as a 'healthy' product.

Research over the last twenty years, particularly that which has a commercial interest, has begun to identify milk as a product in terms beyond its nutritional value. The notion of milk as a functional food – one which has a direct and measurable influence on the health of its recipient – has gained increasing scientific credibility (Korhonen *et al.* 1998; Gill *et al.* 2000). From an objective viewpoint, it seems logical that a lactating animal, as well as providing vital early nutrition, would also protect the health of its offspring via the biochemical influences of its milk. In particular, the notion that components within milk can influence and direct the physiological development of the offspring, as its environmental exposure increases, is now widely accepted. The concept of bovine milk as a biologically active fluid is therefore not new (Newby *et al.* 1982), but the identification of factors within bovine milk that may be relevant to improving human health, and the potential development of bovine milk-containing preparations into products with proven health-promoting properties, certainly is.

In recent years, intense research interest has been focused on identifying biologically active components within bovine milk, and characterising the mode by which mammalian physiological function is modulated by these components. Not surprisingly, a significant proportion of this research has sought to characterise the potential of bovine milk, milk products or milk components to influence the immune system (Bounous *et al.* 1988, 1989; Wong & Watson, 1995). There is now a substantial body of evidence to suggest that major components of bovine milk, as well as several highly purified individual constituents or subfractions, can regulate immune function in non-ruminant as well as ruminant species. The most significant advances in this field have been made over the last five to ten years, and this review will focus primarily on the recent advances and current knowledge in this rapidly expanding field.

Scientific approaches to identifying immune modulating properties of milk

In order to address this issue, it may be instructive to consider the biological function of milk in the perspective of its producer, the dairy cow. Perhaps the fundamental question that should be asked is: why should an animal produce milk that is geared to regulate immune function? It should be borne in mind that neonatal ruminants enter the world with a poorly developed immune system (Goddeeris & Morrison, 1994). This naïve state is necessary, because the neonate requires a period of fine-tuning of the immune system suited to its individual requirements; in effect, the neonate enters the world with an immunological 'clean slate', and the subsequent development of its immune system can be regulated, to an extent, by maternal influence. This influence is manifested primarily via the biochemical effects of milk, and has one central aim: to provide the neonate with as comprehensive a defence system as possible, while at the same time avoiding the detrimental effects that an unbalanced immune system can

* **Corresponding author:** Professor H. S. Gill, fax +64 6 350 5446, email H.S.Gill@massey.ac.nz

generate (Roitt, 1997). If a researcher investigating the potential of bovine milk to influence human health via the immune system is willing to adopt this paradigm, then it should come as no surprise to them that the influences of bovine milk on the immune system are widely varied and subtle. There is no simple answer that milk promotes an enhanced immune function, rather that milk or milk components have the *potential* to *regulate* (or *modulate*) immune function.

This realisation brings into the fore one of the fundamental issues surrounding the demonstration of immune modulation by bovine milk: how to identify and characterise these effects? At its most fundamental level, this comes down to whether milk products (or their derivatives) are tested for their potential immunomodulatory effects in *in vivo* or *in vitro* experimentation. The simple answer is that both approaches have their strengths, and both have their weaknesses. Direct *in vivo* experimentation generally involves the controlled oral delivery of target components to small monogastric animal models (usually rodents) and monitoring longitudinal changes in their immune performance (Bounous *et al.* 1983; Wong & Watson, 1995); in comparison, *in vitro* experimentation involves the inclusion of test components with target cells (usually leucocytes obtained *ex vivo* or immortalised immune cell lines), and measuring the effects of changing treatment regimes on the function of these cells (Otani *et al.* 1992). Although a positive result from an *in vivo* study offers the strongest evidence that the test component will modulate immune function when used as a dietary inclusion, this approach may have missed some of the subtleties of the immune regulation. The researcher must be certain that an *in vivo* experimental design has covered as many variable permutations as possible (e.g. percentage of dietary inclusion, length of dietary regime, influence of other dietary factors); furthermore, the very immune parameters that are being measured must be investigated comprehensively, and preferably at a local (i.e. gastrointestinal) level as well as systemically, so that none of the possibly subtle localised immune modulatory effects are overlooked. In contrast, the *in vitro* testing of milk products or derivatives on immune cells or cell lines allows for a far wider manipulation of experimental variables (dose, time), and far more immune parameters can be measured, and at lower cost. However, a positive result from the *in vitro* approach merely provides the researcher with evidence that the component under test has the potential to modulate immune function; this is a long way away from proving (and defining) that the component truly affects immune function when included in dietary intake. In reality, the practical researcher utilises both methodologies in the approach to defining immune modulation. This dualistic approach is now most widely adopted by various research teams investigating the immunomodulatory potential of bovine milk, and forms the basis of research discussed in this review.

The objectives of research into milk products as modulators of immune function

To a large extent, research that has sought to characterise immune modulation by bovine milk has been product-driven, rather than esoteric. What this means at a practical level is that resources and effort are quite often directed toward a particular facet of the immune response which may be deemed commercially important; thus one observes a somewhat disproportionate level of research reported that deals with well-defined aspects of the specific immune response, for example antibody production (*in vivo*) and cellular proliferation (*in vitro*). The immune system comprises a complex interplay between myeloid and lymphoid cells, between cells and molecules, and between effector 'immune system' cells and remote signals from 'non-immune system' sources. Thus, it should never be considered that research which homes straight in on to an end-point effector mechanism will discern immediately whether a particular milk compound is or is not important in immune modulation. In this respect, it is enlightening to see that recent research has indeed begun to investigate the 'less glamorous' components of the immune system (Wong *et al.* 1997*a*).

In a similar vein, another important concept for the reader to comprehend is that the preferred term to describe the influence of milk on the immune system is *modulation*. This is a carefully chosen phrase, which encompasses the potential for both enhancement and suppression. Once again, looking at the situation from the perspective of the dairy cow producing milk with the potential to modulate its offspring's immune development, simply seeding the offspring's gastrointestinal immune system with potent immune-enhancing biochemicals would be a disaster. An overactive or unregulated immune system will quickly invoke aggressive and unwanted effects, such as atopic reactions and chronic inflammation. In this context, it should be realised that the potential for milk components to suppress immune function is just as important (at a physiological level) as the potential for immune enhancement. This has been demonstrated graphically by Kulkarni & Karlsson (1993), who showed that neonatal mice genetically deficient for the cytokine transforming growth factor beta (TGFβ, a potent immunosuppressant) developed chronic inflammation of the lower intestine, and remained viable only as long as they received maternal milk containing immunosuppressive TGFβ analogues. As will be seen, it tends to be only the curiosity-driven or esoteric research which characterises immunosuppressive qualities of milk, but it seems equally unusual that commercial research should disregard the potential for what is, after all, a very natural and necessary action of milk.

Components of the immune response that have been investigated

The most direct approach to a review of this kind is to first outline the individual components of the immune system which have been shown to be affected by bovine milk. Naturally, the majority of research attention has focused on the specific immune system, i.e. that which supports an antigen-specific response. Under this broad category, most research has been focused upon lymphocytes, particularly B lymphocytes, which produce antibody, and T lymphocytes, which direct and govern the character of antigen-

specific immune reactivity (Gill *et al.* 2000). The major functions of T cells, i.e. antigen recognition, proliferation and modulation of the immune response via the secretion of cytokines, have all been investigated. As an adjunct to this, several reports have also investigated the effects of bovine milk on other non-lymphoid components of the immune system which may interact with lymphocytes, such as expression of antigen-presenting molecules by macrophages.

In contrast, relatively little research attention has been afforded to those components of the immune system that may be considered as innate responses, i.e. those which are antigen non-specific and which are not directed primarily by lymphocytes. Within this category are responses involving polymorphonuclear cells (granulocytes), phagocytosis and cytotoxicity responses which may be undertaken by non-lymphoid cells (e.g. macrophages and natural killer (NK) cells), or the responses of such non-lymphoid cells to inflammatory stimuli. Of course, such distinctions between 'specific' and 'non-specific' components of the immune system are rather arbitrary, since inevitably during an *in vivo* reaction to an unwanted agent – which is what the 'immune system' is solely designed to deal with – there is a lot of interplay between these components. However, for the purposes of this review, it remains expedient to discuss evidence for modulation of immune function in terms of specific and non-specific immune responses.

Effects of bovine milk on lymphocyte responses

Lymphocyte proliferation

One of the most important steps in specific immune reactivity is clonal expansion (proliferation) to produce a pool of antigen-reactive lymphocytes. For experimental purposes, *in vivo* cellular proliferation is usually simulated *in vitro* by researchers via the addition to cell cultures of lectin mitogens, which are targeted toward B or T lymphocytes. Various milk components have been shown to modulate lymphocyte proliferation *in vitro*, including whole casein, α-, β- and κ-casein (and their derivatives), whole whey protein, lactoferrin, lactoperoxidase, milk growth factor and endogenous milk immunoglobulin G. Lymphocytes from a number of ruminant and non-ruminant species (including cattle, sheep, rabbits and mice) have been shown to be affected by bovine milk products. It seems, therefore, that the immunomodulatory potential of bovine milk is not phylogenetically restricted, although the precise effects of milk components may vary from species to species.

Carr *et al.* (1990) showed that α-$s_1$casein could enhance the mitogen-stimulated proliferation of murine splenic T lymphocytes, when included in *in vitro* cell culture at a concentration of 10^{-6} M, although no levels of significance were reported from their study. Wong *et al.* (1996) showed that β-casein significantly enhanced the mitogen-induced proliferation of ovine T and B lymphocytes in a dose-dependent manner, when included in *in vitro* cell culture; Otani & Hata (1995) found the opposite effect with kappa-casein, which was suppressive for murine and rabbit lymphocyte proliferation induced by a range of T and B cell mitogens. The major immunosuppressive effect of κ-casein has been shown by Otani *et al.* (1992) to be due to the glycomacropeptide component CGP (caseinoglycopeptide). Otani *et al.* (1995) have further demonstrated that different sized subfractions of CGP possessed different modulatory capabilities, with low carbohydrate-containing fractions showing no suppressive activity whereas others with higher sugar content were potent suppressants, especially those containing high *N*-acetylneuraminic acid (which showed particularly strong activity against T, but not B, lymphocytes). Furthermore, Otani & Monnai (1993) have demonstrated marked variation in the modulatory potential of non-size fractionated CGP, following its *in vitro* digestion with different commercial enzymes; in general, pepsin, chymotrypsin or neuraminidase tended to ablate the immunosuppressive potential of CGP, whereas trypsin, pancreatin and pronase had less of an effect, or even enhanced the degree of suppression in some of the enzyme-digested fractions. In contrast to the long list of immunosuppressive qualities of κ-casein and its derivatives, there is some evidence that certain subfractions of CGP can promote lymphocyte proliferation. Yun *et al.* (1996) isolated a subfraction from CGP by size-exclusion chromatography which promoted proliferation of murine spleen cells, in the absence of extraneous mitogens. The extensive research on κ-casein and its derivatives has clearly demonstrated the wide and cryptic range of *in vitro* immunomodulatory effects of a single milk product, in that whereas intact casein may only modulate B lymphocyte function (Otani *et al.* 1992), κ-casein (as well as subfractions and digestions thereof) has the potential to affect T or B lymphocytes, or both, or to have no effect.

In addition to caseins, bovine whey preparations have been shown to affect lymphocyte function *in vitro*. Torre & Oliver (1989) demonstrated suppression of bovine T lymphocyte mitogenesis by milk-derived whole whey protein, at a concentration of 1·1 ng/ml included in cell culture. Barta *et al.* (1991) also showed that whole whey derived from lactating dairy cattle could be a potent suppressant of bovine T and B lymphocyte proliferation *in vitro*, but that this activity was correlated with the prior history of inflammatory mastitis among the individual animals, thus indicating that the immunosuppressant may have been a mediator derived from localised tissue inflammation rather than a milk protein *per se*. Individual whey proteins have also been shown to affect lymphocyte function, in a variety of ruminant and non-ruminant species. Lactoferrin has been shown to suppress the proliferation of T cells from cows (Rejman & Oliver, 1992; Rejman *et al.* 1992*a*, 1993), mice (Otani & Odashima, 1997) and sheep (Wong *et al.* 1997*b*), when included in *in vitro* culture with mitogen-stimulated lymphocytes. Rejman & Oliver (1992) and Rejman *et al.* (1992*a*, 1993) found that lactoferrin could affect the proliferation of bovine lymphocytes in *in vitro* cell culture. These authors showed that lactoferrin suppressed the mitogen-stimulated proliferation of peripheral blood T lymphocytes, as well as the interleukin-2(IL-2)-stimulated proliferation of mammary gland lymphocytes. In contrast, lactoferrin was found to have a bimodal effect on the IL-2 dependent proliferation of a bovine T cell line, in that it

suppressed proliferation at high doses but enhanced proliferation at low levels in *in vitro* culture. Interestingly, there may be some species-restricted differences in the action of lactoferrin on different lymphocyte types, as Otani & Odashima (1997) showed that lactoferrin could suppress mitogen-induced proliferation of murine B and T lymphocytes, whereas Wong *et al.* (1997*b*) indicated that lactoferrin only suppressed T cell (not B cell) mitogenesis in sheep.

In addition to the immunomodulatory effects of lactoferrin, Wong *et al.* (1997*b*) also found that bovine lactoperoxidase suppressed ovine T cell mitogenesis *in vitro*, although similarly to lactoferrin, it had no measurable effect on B cell proliferation. It has also been shown that bovine milk-derived immunoglobulin G can suppress lymphocyte function, and levels as low as 0·3 mg/ml suppressed human lymphocyte proliferative responses to T cell and T/B cell mitogens when included in *in vitro* cell culture (Kulczycki *et al.* 1987). Since cow's milk frequently contains IgG at concentrations between 0·6 and 0·9 mg/ml, the authors concluded that endogenous bovine IgG might have the potential to modulate human immune function *in vivo*. Interestingly, this immune-modulating effect was unique to milk-derived IgG, and bovine serum IgG did not affect lymphocyte proliferation.

The ability of highly purified whey components to affect lymphocyte function has been demonstrated further by Stoeck *et al.* (1989), who isolated a growth factor from bovine milk by multi-step chromatography, which had a potent suppressive effect against human T lymphocyte proliferation *in vitro*. This milk growth factor was found to share partial amino acid sequence homology with the endogenous cytokine TGFβ, and could suppress human lymphocyte proliferation at picomolar quantities following extensive purification.

As a general rule, the immunomodulatory effect of individual milk components becomes more clearly defined (and the dose response more potent) as they are progressively purified from primary milk proteins. The corollary to this is that their effects may be diminished, or even remain undetectable, in the original parent milk product (Wong *et al.* 1997*b*). Thus, despite the pronounced immunomodulatory effects of β- and κ-casein on murine T and B lymphocyte proliferation *in vitro*, intact whole casein has only been shown to affect B lymphocyte proliferation (Otani *et al.* 1992). Similarly, although the purified whey proteins lactoferrin and lactoperoxidase have been shown to be potent modulators of ovine lymphocyte proliferation in isolation, when combined as a single fraction their effects were diminished (Wong *et al.* 1997*b*); furthermore, when the original intact whey protein concentrate (from which these proteins were purified) was tested, it had no modulatory effects on mitogen-induced proliferation of ovine lymphocytes (Wong *et al.* 1997*b*). Cross & Gill (1999) have recently shown that a modified whey protein concentrate (i.e. one which had been partially depleted of extraneous non-immunomodulating whey proteins) suppressed mitogen- and alloantigen-induced proliferation when included in *in vitro* culture with murine lymphocytes. This brings into focus the idea that the immunomodulatory action of primary milk proteins is well-balanced (as one might expect) and that certain components may serve to regulate the function of others, as suggested by Wong *et al.* (1997*b*). If this is the case, then future research which aims to identify novel immunomodulatory action of milk should consider that once something is taken out of a milk product, the residual material may become just as interesting as the isolate.

Many studies have also investigated the potential for milk proteins to affect lymphocyte function following *in vivo* exposure. In contrast to the majority of reports which have characterised milk-derived proteins as predominantly immunosuppressive in *in vitro* culture, most reports suggest a degree of immunoenhancement of lymphocyte function following *in vivo* exposure. Early research by Bounous *et al.* (1983) indicated that the inclusion of native α-lactalbumin in mouse diets could enhance *in vitro* proliferative responses of splenic lymphocytes to T cell mitogens, although this occurred only in mice that had first been primed by injection of *Mycobacterium bovis* BCG (a potent *in vivo* T lymphocyte stimulator) (Bounous & Kongshavn, 1985). The same authors also found that increasing concentrations of dietary lactalbumin hydrolysates promoted enhanced splenic lymphocyte responses to T and B cell mitogens in BCG-primed mice (Bounous & Kongshavn, 1982). More recently, Wong & Watson (1995) have shown that mice fed on whey protein-enriched diets had significantly elevated spleen-derived T and B cell proliferative responses to mitogens, compared to animals fed on control (soy- or wheat-based) protein diets. Debabbi *et al.* (1998) have demonstrated that oral delivery of lactoferrin could enhance murine antigen-specific lymphocyte responses in both Peyer's patch and spleen cell extracts, indicating that dairy products have the potential to affect local as well as systemic lymphocyte function. These reports have provided definitive evidence that bovine milk proteins, fed to monogastric animals, can modulate lymphocyte function. More recent research by Monnai *et al.* (1998) has shown that κ-casein-derived CGP can also affect lymphocyte function after *in vivo* exposure, in that mice fed a CGP-containing diet had enhanced T (but not B) lymphocyte responses to mitogens when subsequently tested *in vitro* (a somewhat surprising result, in that it was the opposite of that predicted from *in vitro* studies, where CGP has been shown to universally suppress lymphocyte proliferation). With the extensive demonstration of potent immunosuppressive activity by purified milk proteins *in vitro*, there is clearly scope for further progress with *in vivo* experimentation, to establish whether these effects are manifested when the proteins are included in diets.

Antibody production

In some cases, lymphocyte activation can be gauged by the *in vivo* production of specific antibody. There have been several reports that *in vivo* administration of bovine milk proteins to heterologous species can affect antibody responses to subsequent antigen challenge. Studies by Bounous *et al.* (1981, 1983) and Bounous & Kongshavn (1982) on mice demonstrated that dietary inclusion of the major whey protein α-lactalbumin could enhance antibody

responses to foreign systemically delivered antigens, and that the immunomodulatory effect could be achieved using α-lactalbumin in both the native and hydrolysed state. Indeed, Bounous and colleagues demonstrated that antibody responses in mice were enhanced by increasing concentrations of dietary α-lactalbumin hydrolysates (Bounous *et al.* 1981; Bounous & Kongshavn, 1982) and that this enhanced ability to produce antibody corresponded to enhanced resistance to challenge with *Salmonella typhimurium* (although whether this was a causal relationship was not investigated). Bounous & Kongshavn (1985) and Bounous *et al.* (1985) further demonstrated that dietary α-lactalbumin had a direct effect on B lymphocyte function, in that it suppressed antibody responses to T cell-independent as well as T cell-dependent antigens.

Wong & Watson (1995) and Monnai *et al.* (1998) have further demonstrated that dietary intake of crude whey protein concentrate (WPC) or κ-casein-derived CGP (which is a major constituent of WPC) can modulate specific antibody responses in mice. In both studies, antibody responses to heterologous systemically delivered antigens were affected, but in different ways; Wong & Watson demonstrated that IgG responses to ovalbumin were enhanced in whey-fed mice, whereas Monnai *et al.* showed that IgG (but not IgM, IgA or IgE) antibody responses to β-lactoglobulin were suppressed in mice fed CGP. Interestingly, Monnai *et al.* (1998) also showed a CGP-induced suppression of IgG antibody against orally delivered antigen, providing one of the rare documented cases of a bovine milk product directly affecting the gastrointestinal immune system *in vivo*. Recent research from our group (Gill & Rutherfurd, 1998) confirmed that localised immunomodulation occurs, in that a diet supplemented with WPC was shown to enhance localised (gut mucosal) antibody responses to heterologous orally delivered antigens in mice.

In addition to the earlier work of Bounous and colleagues, recent reports have also indicated that individual proteins isolated from bovine milk can affect antibody responses. Watson *et al.* (1992) reported that a bioactive fraction, isolated from bovine colostral whey by cation exchange and reverse phase chromatography, could suppress systemic IgE antibody responses in mice. Wong *et al.* (1996) also demonstrated that specific antibody responses to injected ovalbumin were suppressed in mice co-injected with β-casein, even in the presence of an immunopotentiating adjuvant. This latter study demonstrates clearly the potential potency of bovine milk components on the immune system.

In addition to *in vivo* studies of the effects of milk-derived proteins on immune function, some reports have demonstrated that bovine milk-derived immunoglobulin G can suppress antibody production by mitogen-stimulated cells *in vitro*. An early series of studies by Kulczycki & MacDermott (1985) and Kulczycki *et al.* (1987) demonstrated that milk-derived IgG could suppress the production of human IgG-, IgA- and IgM-class antibodies by human peripheral blood lymphocytes, when included in *in vitro* cell culture at concentrations as low as 40 μg/ml. Furthermore, these authors demonstrated that the immunosuppressive effect of milk-derived IgG was primarily due to the action of the Fc portion of the bovine immunoglobulin molecule.

Cytokine production and surface receptor expression

Once exposed to a stimulus (such as a mitogen or antigen) T lymphocytes can produce cytokines, which shape the ensuing character of the developing immune response, and in addition up-regulate the cell surface expression of a number of key receptor molecules that are important in the immune response. This process may be said to reflect a state of activation of the cell. Bovine milk components have been shown to affect lymphocyte cytokine production and surface marker expression in many cases. Wong *et al.* (1997*b*) showed that the whey proteins lactoferrin and lactoperoxidase, included in *in vitro* cell culture, could suppress mitogen-induced secretion of the cytokine γ-interferon by ovine lymphocytes; in contrast, β-casein had no effect on cytokine production (Wong *et al.* 1996). Otani *et al.* (1996) showed that κ-casein-derived CGP suppressed T cell surface expression of interleukin-2 receptor, in cultures of mitogen-stimulated murine lymphocytes. Cross & Gill (1999) have shown that a modified whey protein concentrate can suppress the mitogen-stimulated secretion of γ-interferon and surface expression of interleukin-2 receptor, when included in *in vitro* cell culture.

Hypersensitivity

The specific immune system can direct inflammatory processes, if there is a specific (i.e. antigen) basis to the response. Sometimes the system can become unbalanced and lead to overexacerbation of inflammation; the unwanted reaction is termed hypersensitivity. Otani & Yamada (1994) demonstrated that skimmed milk proteins could suppress antigen-induced immediate (Type I) skin hypersensitivity responses in guinea pigs, possibly by limiting local histamine release from sensitised mast cells when injected into the skin. The key proteins involved in this ameliorating effect were found to be κ-casein and lactoferrin. In contrast, neither protein was able to ameliorate immune-complex mediated (Type III) or delayed-cellular (Type IV) hypersensitivity reactions *in vivo* (Otani & Yamada, 1995). Dietary milk components have also been investigated for their potential ameliorating effects on *in vivo* hypersensitivity reactions. Bounous & Kongshavn (1985) failed to demonstrate an effect of dietary α-lactalbumin on *in vivo* delayed-type hypersensitivity responses in mice; however, Wong & Watson (1995) showed that mice fed a whey protein-enriched diet actually had significantly heightened hypersensitivity responses to antigen injected intradermally.

Effects of bovine milk on non-lymphoid cells

Macrophage function

Macrophages participate in many aspects of the innate (non-specific) immune system, including the phagocytosis of microbes; the generation of microbicidal reactive oxygen

and nitrogen intermediates; and the production of immune-modulating cytokines. In addition, macrophages also participate directly in the specific component of the immune response, by presenting antigen in association with cell surface major histocompatibility (MHC) class II molecules to responsive T cells. Quite naturally, research has looked at the effects of milk components on these responses. Otani & Futakami (1994) demonstrated heterogeneous effects of different milk proteins on phagocytosis by murine macrophages, both in terms of the numbers of cells that were actively phagocytic and the number of target microparticles phagocytosed per cell. α- and κ-casein, as well as lactoferrin, reduced phagocytic function, whereas β-casein and α-lactalbumin enhanced the responses, when these milk proteins were included in *in vitro* cultures of macrophages. In line with their earlier work on milk proteins that could modulate lymphocyte function, these authors also investigated the effect of *in vitro* enzymatic digestion on the ability of milk proteins to affect macrophage function (Otani & Futakami, 1996). Trypsin and chymotrypsin digests of α- and κ-casein suppressed macrophage phagocytosis, similar to the native molecules, whereas pepsin digestion had the opposite effect, in that digested fragments of both caseins enhanced the phagocytic uptake of non-specific targets (latex microbeads).

Otani & Futakami (1994, 1996) further demonstrated that α-casein, κ-casein and lactoferrin could suppress the production of nitrite (an index of reactive nitrogen intermediate production) by murine macrophages in a dose-dependent manner, when included in cell culture. Interestingly, the suppressive qualities of α-casein and κ-casein were reversed by treatment with pepsin, which formed modified components that enhanced nitrite production; in contrast, trypsin or chymotrypsin digestion had no effect on the suppressive qualities of α-casein but marginally revoked the suppressive effect of κ-casein. Native intact β-casein enhanced nitrite production, and this enhancement was elevated following pepsin digestion of beta-casein. The work of Otani and colleagues has clearly shown that the modulatory function of milk proteins on cells of the immune system can be radically altered by proteinase treatment; this may partially explain the often radical differences observed between the results of *in vitro* or *in vivo* studies of immune modulation.

In addition to the effects on phagocytosis and production of reactive nitrogen intermediates, bovine milk proteins have also been shown to affect the production of cytokines by activated macrophages. Otani & Monnai (1995) and Monnai & Otani (1997) studied the ability of κ-casein-derived CGP to modulate IL-1 family cytokines in murine macrophages and a macrophage cell line. They found that CGP induced production of a molecule which acted as an antagonist for the interleukin-1 receptor; this had the effect of blocking the immunopotentiating ability of IL-1 produced by macrophages following activation with bacterial lipopolysaccharide (LPS). This mechanism could partially explain the previously noted suppression of B lymphocyte mitogenesis by bacterial LPS in the presence of CGP, i.e. that CGP actually inhibits the immunopotentiating action of macrophage-derived IL-1 and that this (as much as a direct effect of CGP on B cell function) suppressed B cell mitogenesis. In contrast to the work of Otani and Monnai, Wong *et al.* (1996) demonstrated that β-casein could actually enhance the secretion of IL-1 by LPS-stimulated ovine macrophages, when the casein was included in *in vitro* culture. Furthermore, α-lactalbumin also enhanced IL-1 production in sheep macrophages (Wong *et al.* 1997*b*), but neither β-casein nor α-lactalbumin had any measurable effect on production of another key macrophage-derived cytokine, tumour necrosis factor (TNF).

So far, research has failed to demonstrate a measurable effect of milk proteins (β-casein, lactoferrin, lactoperoxidase or α-lactalbumin) on expression of MHC antigen-presentation molecules by macrophages (Politis *et al.* 1991; Wong *et al.* 1996, 1997*b*). However, this research has been far from exhaustive.

Granulocyte function

Granulocytes function as innate phagocytic and microbicidal cells in the immune system, and are important as migratory cells in localised tissue inflammatory responses. It has long been known that whole casein is a potent inflammatory mediator that induces the chemotactic migration of neutrophils, when injected into tissue (Metcalf *et al.* 1986). In contrast, Wong *et al.* (1996) have shown that purified bovine β-casein can actually suppress ovine neutrophil chemotaxis in response to a recombinant chemokinetic cytokine (IL-8), indicating once again that when a single complex milk protein source is fractionated, discrete and often complementary biological effects become apparent. Wong *et al.* (1997*b*) further demonstrated that whole bovine whey protein could inhibit neutrophil chemotaxis in response to serum-derived complement chemotaxins, but that fractionated whey proteins (lactoferrin, lactoperoxidase, α-lactalbumin) or whey protein concentrate had no effect on ovine neutrophils. This is one of the few examples where fractionated or concentrated proteins fail to replicate an immunomodulatory effect that is present in the parent (native) product. However, in this case, there may also be species-specific differences in the modulatory capacity of isolated bovine milk proteins, since it has recently been demonstrated that lactoferrin can affect the chemotactic function of human neutrophils *in vitro* (by inducing the release of IL-8 (Shinoda *et al.* 1996)).

Wong *et al.* (1996, 1997*b*) have further shown that bovine milk proteins have the ability to enhance neutrophil oxidative responses, predominantly in heterologous species, following inclusion in *in vitro* cell culture. β-casein, whey protein concentrate and lactoferrin/lactoperoxidase (mixed) were each able to enhance the ability of ovine neutrophils to generate superoxide anion. This is in contrast to the results of Cooray (1996), who demonstrated that whole intact casein could suppress bovine neutrophil respiratory burst responses and bactericidal capacity, when included in *in vitro* cell culture. Work by Miyauchi *et al.* (1998) has confirmed the ability of bovine milk-derived proteins to enhance *in vitro* neutrophil function in heterologous species, since human neutrophils showed enhanced phagocytic capacity following culture with

lactoferrin or lactoferricin (derived from pepsin hydrolysis of lactoferrin).

NK cell function

NK cells participate in cellular cytotoxicity against altered cells, such as those infected with virus or tumour cells. Wong *et al.* (1996, 1997*b*) have investigated the capacity of bovine β-casein, lactoferrin and lactoperoxidase to affect NK cell cytotoxicty against viral-infected target cells; no significant effects of any of these products were shown. In contrast, Shimizu *et al.* (1996) have demonstrated that lactoferrin can enhance T cell-mediated NK cell function in mice, following *in vivo* administration, and that this enhanced NK cell function corresponded to protection against challenge with cytomegalovirus. This study has provided one of the few documented cases of a milk-derived component having an impact on immune function which can be directly correlated with a measurable improvement in immune-mediated health.

Miscellaneous cell lines

Immortalised mammalian cell lines, such as tumour cells, are often used to demonstrate effects of milk proteins on functions related to the immune system. Yamada *et al.* (1991) studied a human fibroblast cell line, and found that several milk-derived proteins (including κ-casein, κ-casein-derived CGP and lactoferrin) could suppress production of the cytokine interferon-β; in contrast, α- and β-casein enhanced cytokine production, when included in cell culture. Research on bovine lactoferrin by Mattsby-Baltzer *et al.* (1996) has indicated that this protein can suppress lipopolysaccharide-induced secretion of the cytokine IL-6 by a human monocytic cell line (THP-1), when included in *in vitro* cell culture. Rejman *et al.* (1992*b*) further demonstrated that lactoferrin could inhibit the proliferation of a bovine mammary epithelial cell line (MAC-T) in *in vitro* culture. The same cell line was used by Zavizion *et al.* (1993) and Stelwagen *et al.* (1994) to characterize a 13 kDa protein from bovine milk (mammary-derived growth inhibitor, MDGI), which could inhibit cell proliferation at low picogram levels when included in *in vitro* cell culture.

Overview of the ability of milk proteins to modulate immune function

There is a great body of evidence to indicate that bovine milk-derived proteins have the potential to modulate immune function in a number of species (Gill *et al.* 2000). In many cases, exquisite *in vitro* experimental approaches have been able to pin-point the precise mode by which milk proteins affect immune function. However, definitive evidence is often lacking that the *in vitro* modulatory effects can be replicated *in vivo*. In fact, where a product has been tested both *in vitro* and *in vivo*, the evidence has often suggested a different modulatory effect in the two systems (Wong & Watson, 1995; Monnai *et al.* 1998). Such research should not be taken to imply that *in vitro* studies bear no relevance to the potential benefits of the component in question. On the contrary, it supports the case for further investigation as to how the defined *in vitro* modulatory effects might be maintained *in vivo* in a foodstuff product. As the subtleties of the gastrointestinal immune system become more clearly defined by contemporary research, so more advances should be made into delivering a proven immunomodulator and maintaining that beneficial effect to the recipient. Clearly, the work of Otani and colleagues has demonstrated that enzymatic modification of many bovine milk proteins can completely alter their biological properties, and this should be borne in mind when trying to correlate results of *in vitro* studies with those of *in vivo* trials. Similarly, the potential effects of modified complex milk components has hardly been investigated, but the commercial scale fractionation of proteins from milk (particularly whey) could generate the raw material to support important research into the immunomodulatory properties of residual or by-products.

The modes of action of milk-derived immunomodulatory components should be appraised for their potential benefits in human health improvement. Many of the immunosuppressive qualities of κ-casein and whey-derived proteins may be of enormous benefit to regulating intestinal inflammation, and it must be emphasised again that health promotion is not exclusively achieved via enhancement of immune function. The ultimate proof that a milk-derived product will or will not benefit human health will only come from clinical trials (Bounous *et al.* 1993), and the majority of the research discussed in this review is a long way from achieving that target. Nevertheless, important steps are being taken by several research teams to expand milk components with proven *in vitro* or *in vivo* modulatory abilities into health-promoting foodstuffs. In this field, it is important that complex milk-derived products (such as whey preparations) should be investigated thoroughly, in addition to individual fractions which have been purified to the highest degree. The subtle interactions of milk-derived proteins in regulating immune function should be considered when interpreting results from controlled laboratory-based studies, as has clearly been shown by the research of Wong and colleagues.

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