Milk and colostrum are rich in proteins and peptides which play a crucial role in development of the immune system in mammalian offspring. Immunotrophic properties of these compounds prompted investigators to search for their utility in prevention and therapy of various disorders in humans. The following constituents of milk are of particular interest: 1) Lactoferrin (LF) - exhibits antibacterial, antifungal, antiviral, antiparasite and antitumor activities. It is protective with regard to intestinal epithelium, promotes bone growth and accelerates recovery of the immune system function in immunocompromised animal; 2) A Proline-Rich Polypeptide (PRP) - shows a variety of immunotrophic functions, including promotion of T-cell maturation and inhibition of autoimmune disorders. PRP was recently found to improve or stabilize the Instrumental Activity of Daily Living status in Alzheimer's disease patients. 3) Casein – has been protective in experimental bacteremia by eliciting myelopoiesis. Casein hydrolyzates were also protective in diabetic animals, reduced the tumor growth and diminished colicky symptoms in infants. Casein-derived peptides have been found to have antihypertensive effects. Glycomacropeptide (GMP) - a peptide derived from kappa casein, exhibits antibacterial and antithrombotic activities. 4) Alpha lactalbumin (LA) - demonstrates antiviral, antitumor and anti-stress properties. LA-enriched diets were anxiolytic, lowered blood pressure in rats, prevented diarrhea and led to a better weight gain in malnourished children. 5) Lysozyme – is effective in treatment of periodontitis and prevention of tooth decay. Milk enriched in lysozyme was used in feeding premature infants suffering from concomitant diseases. 6) Lactoperoxidase – shows antibacterial properties. In conclusion, milk-derived proteins and peptides are bio-accessible and safe for the prevention and treatment of numerous disorders in humans.

**Key words:** milk, colostrum, lactoferrin, proline-rich polypeptide, glycomacropeptide, casein, lactalbumin, lysozyme

**INTRODUCTION**

Milk is the specific diet of mammalian neonates and is rich in immunoglobulins, antimicrobial peptides, and growth factors. Nutritional value of milk, especially colostrum is inseparably associated with its role in enhancing the innate immunity of offspring during the early period of neonatal life. It has long been recognized that breast-feeding offers pronounced enhancement of passive immunity and promotes infantile gut
immunity (1, 2, 3, 4). Thus the constituents of milk not only ensure adequate resistance to pathogens by delivery of maternal immunoglobulins and other non-specific, protective factors, but also play a crucial role in promoting maturation of the immune system. Milk proteins are often precursors of different biologically active peptides which are inactive within the sequence of the precursor protein but can be released by enzymatic proteolysis. Many milk protein-derived peptides, such as proline-rich polypeptides or caseinophosphopeptides, reveal multifunctional bioactivities. Immunotropic properties of these proteins and peptides have been extensively studied, predominantly in animal experimental models. The results are very promising and have already led to a broad application of milk constituents as supplements to products of the dairy and pharmaceutical industry. The proteins have also been introduced in clinical trials and proved valuable in prevention and therapy of autoimmune and neoplastic diseases, immunodeficiencies, recovery of the immune system function after chemotherapy, and sepsis or endotoxemia. The aim of this article is to review properties, experimental data and results of clinical trials of several proteins and peptides from milk and colostrum, used alone or in combination with other conventional therapeutics, with a particular attention paid to lactoferrin.

Milk is composed of water (87%), fat (4%) and non-fat solids (9%) including protein, lactose, minerals and vitamins. The most prevalent protein in milk is casein. It comprises 80% of the total milk protein. The proteins that remain in solution after removal of casein are by definition termed whey proteins. Whey of bovine milk consists of beta-lactoglobulin, alpha-lactalbumin, immunoglobulins, serum albumin and minor proteins accounting for the remaining 20% of total milk protein (Table 1).

The composition of milk progressively changes postpartum to meet specific requirements at different stages of neonatal development (5, 6). During the first few days postpartum, in addition to normal nutrients such as proteins, carbohydrates, fats and minerals, colostrum contains high levels of growth factors, antimicrobial compounds and immune-enhancing constituents (7). Generally, the transition of colostrum to mature milk is associated with a substantial decrease in total protein concentration and an increased concentration of lactose. Thus only an approximate composition of milk can be given at specific lactation time.

Evidence continues to accumulate that many milk-derived products provide a variety of health benefits including antimicrobial, anti-inflammatory, anticarcinogenic, hypcholesterolemic or hypertension controlling effects. Recently, significant progress has been made in the identification and characterization of milk and/or whey components that can be utilized in health and disease in humans. The following proteins are potential candidates for clinical testing to obtain a specific health benefit, such as enhancing body functions or reducing the risk for certain diseases:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-lactoglobulin</td>
<td>52.5</td>
</tr>
<tr>
<td>Alpha-lactalbumin</td>
<td>22.5</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>12.5</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>7.5</td>
</tr>
<tr>
<td>Glycomacropeptide</td>
<td>3.0</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>1.5</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>0.5</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Proline-Rich Polypeptides</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>
LACTOFERRIN

Lactoferrin (LF) is an iron binding protein found in secretory fluids of mammals (8) and the secondary granules of circulating neutrophils (9). Colostrum and subsequently mature milk is very rich in LF (10). A variety of biological functions have been ascribed to lactoferrin (11).

**Antibacterial properties.** Lactoferrin exhibits bacteriostatic activity against a wide range of bacteria due to its ability to sequester iron, which is essential for microbial growth. In addition, lactoferrin exhibits non-iron-dependent bacteriocidal activity. By virtue of binding to specific bacteria, namely Gram-negative species, LF changes the permeability of bacterial walls and their metabolism. It was demonstrated that human LF can bind to OmpC and PhoE porins of *Escherichia coli* (*E. coli*) via 1-5, 28-34 and 39-42 residues and shows the antibacterial effect (12, 13). In addition, the effects of LF on smooth versus rough *Salmonella typhimurium* (*S. typhimurium*) mutants, were found to be affected by the polysaccharide moiety of lipopolysaccharide (LPS, also known as endotoxin) which prevents interaction with porins. In another report (14) a direct effect of LF on the outer membrane of enteric Gram-negative bacteria was associated with a significant release of LPS from the bacterial wall. A direct antibacterial action in vitro was also shown for enzymatic hydrolysates of bovine LF, particularly when LF was degraded by porcine pepsin (15). That effect was at least eightfold greater than that of undigested LF. The authors also identified the bactericidal domain of LF which, in case of bovine lactoferrin, consists mainly of a loop of 18 amino acid residues formed by a disulfide bond between cysteine residues 19 and 36 (16). The peptide, called lactoferricin B, binds rapidly to the surface of *E. coli* and *Bacillus subtilis* (*B. subtilis*) (>10⁶ peptide molecules per cell) (17). Lactoferricin B inhibited bacterial uptake of 3H proline with effectiveness similar to polymyxin B, a known membrane-disruptive agent. In the search for new antimicrobial molecules, others identified the minimal sequence of human LF-derived fragment (residues 1-33) designated LF-33 (18). LF-33 appeared to be more potent than polymyxin B at suppressing endotoxin-induced LAL coagulation and tumor necrosis factor alpha (TNF-α) secretion by RAW 264.7 cells under serum-free conditions. Very recently, a novel antibacterial peptide in the N1-domain of bovine LF (residues 268 - 284) was identified and termed lactoferrampin (19). The peptide exhibits candidacidal activity higher than native LF and also was active against *B. subtilis*, *E. coli* and *Pseudomonas aeruginosa*. In contrast, lactoferrampin, was not effective against the fermenting bacteria *Actinomyces naeslundii*, *Porphyromonas aeruginosa*, *Streptococcus* mutants and *Streptococcus sanguis*.

Interestingly, LF may act synergistically with lysozyme in destruction of the bacterial wall (14). Both proteins are present in high concentration at mucosal sites and neutrophil granules, contributing to a higher resistance to infections. LF and lysozyme alone appeared to be bacteriostatic in relation to *Vibrio cholerae*, *S. Typhimurium* and *E. coli*, whereas together they show a bactericidal effect. The transmission electron microscopy revealed that bacteria exposed to both agents enlarged and showed a hypodense structure, suggesting the killing by osmotic damage.

Another example of a direct antibacterial action of LF may be the prevention of bacterial attachment to target cells. In one study bovine LF was shown to inhibit colonization of enterotoxigenic *E. coli* to human epithelial cells in vitro and to intestinal mucosa of germ-free mice in vivo (20). Others found inhibition of the adhesion of enteropathogenic *E. coli* to enterocytes – HeLa cells (21). Free secretory component had a similar activity. To get a better insight into the mechanism of that phenomenon, the influence of LF on the *Haemophilus influenzae* IgA protease and Hap adhesin, the proteins facilitating colonization of the bacteria, was studied (22). The results demonstrated that human milk LF efficiently extracted the IgA protease precursor from the outer bacterial membrane and specifically degraded the Hap adhesin, preventing Hap-mediated adherence. Suppression of these effects by serine protease inhibitors suggested that a fragment of LF molecule in the N-lobe exhibited a serine protease activity. An important antibacterial mechanism, initially proposed for LF, is the iron withholding property (23). Such a mechanism could also be relevant to antitumor properties of LF, since quickly dividing cells require more iron for growth. The ability of LF to bind free iron could be of significance to control the physiological balance between reactive oxygen species (ROS) production and the rate of their elimination, which naturally protects against oxidative stress cell injury (24). Oxidative stress has been implicated in a variety of pathological and chronic degenerative processes including the development of cancer, atherosclerosis, inflammation, aging, neurodegenerative disorders and also in defense against infection (25, 26, 27).

The indirect antimicrobial action of LF is associated with the mobilization of the immune system. LF was shown to accelerate the clearance of *E. coli* from the blood, as well as the killing rate in the liver, lungs, spleen and kidney (28). The authors concluded that the defense system, generated by LF in mice in vivo, is
primarily a bacteria-killing one. In mice with alloxan-induced diabetes LF was not able to protect mice against lethal dose of E. coli, however, it significantly enhanced the killing rate of bacteria in the organs (29). The protective activity of LF was demonstrated also in Staphylococcus aureus (S. aureus) kidney infection, when LF was given in drinking water (2%) and the viable bacterial counts were reduced 5-12 fold (30). It appeared that the oral LF treatment of experimental E. coli urinary tract infections in mice, was also effective in reducing infection at a distant site, the urinary tract, possibly through transfer of LF to the site of infection via renal secretion (31). Others demonstrated that the protective mechanism of LF against E. coli infection may result from both an accelerated neutrophil recruitment and down-regulation of TNFα (32). Also it was shown that the injection of homologous LF into mice, resulted in an increase in the colony stimulating factor (CSF) production by 12 hours and prior to the increase in bone marrow granulocyte-macrophage progenitor cells (GM-CFC) at 48 hours (33). The utility of LF was further confirmed in a clinical trial, showing reduction of infection and its severity in neutropenic patients caused by chemotherapy (34). LF has a property to selectively act on the gut microflora. The protein inhibits growth of E. coli and other pathogenic bacteria (mainly from Enterobacteriacea) but not the Gram-positive probiotic bacteria such as Bifidobacterium (35). It is well established that proper colonization of newborn gut microflora ensures efficient digestion processes, elaboration of some vitamins, protection against pathogenic bacteria and enhanced resistance. In germ-free mice fed sterile milk, the addition of 2% LF resulted in a significant decrease in concentrations of lactose, glucose and galactose in the cecal contents, which led to a bacteriostatic effect in relation to Enterobacteriacea (36). Therefore, it was suggested to include LF in the neonate diet and later in the infant formula.

Lactoferrin can also increase susceptibility of bacteria to some antibiotics, such as vancomycin (a polypeptidic antibiotic) (37), penicillin (38) and cefodoxim (cephalosporin) (39). LF decreased by two-fold the needed therapeutic concentration of vancomycin toward Staphylococcus epidermidis (37). A combination of penicillin and LF increased up to four-fold the antibiotic activity against S. aureus (38). LF applied with cephalexin prolonged the life of mice infected with a lethal dose of Klebsiella pneumoniae and lowered the effective dose of the antibiotic (39).

Some studies indicated that human LF may serve as a source of iron for some bacteria, mainly Helicobacter pylori (H. pylori) (the etiologic factor of gastric and duodenum ulcers) (40) and Neisseria species (41). However, the above mentioned bacteria were unable to use iron from the bovine LF. Since gaining an access to iron plays an important role in the virulence of bacteria, the utility of human LF as an anti-H. pylori or anti-Neisseriacea needs to be carefully re-examined.

**Antifungal and antiparasitic properties.** There are a great number of studies on antifungal properties of LF, however, the results are not always consistent and more importantly not conclusive. Fungicidal effect of LF on Candida albicans and Candida crusei was dose-dependent and only with iron-free lactoferrin (apolactoferrin). Electron microscopy indicated cell surface alteration such as leakage of proteins and formation of surface blebs (42). Killing of Candida albicans by iron-free LF was time-dependent and higher at pH 7.0 than at pH 5.5 (43). However, since both phosphate and bicarbonate ions blocked the antifungal effect it seems unlikely that LF belongs to the major host defense factors against candidiasis (43).

Studies designed to establish the region of human lactoferrin responsible for the candidacidal activities established that the 1-11 cationic domain of LF N-terminus was more effective than the second, 21-31 domain and the whole LF molecule (44). Others (45) found that human milk, in vitro, showed a potent inhibitory effect on fungal growth and that most of this action was caused by LF via its iron-binding capacity. The authors concluded, based on the viability test and electron microscopy, that the growth inhibitory effect of human milk, mediated by LF, is fungistatic rather than fungicidal. Nevertheless, application of LF in vivo proved effective against fungi in several animal and human studies. In guinea pigs, infected on the back with Trichophyton mentagrophytes, daily administration of bovine LF (per os) did not prevent development of symptoms during the early phase of infection but facilitated clinical improvement of skin lesions after the peak of the symptoms (46). In the same model (47) the authors showed that LF stimulated proliferation of splenocytes induced by concanavalin A or inactivated Trichophyton mentagrophytes. Also, the culture supernatants from splenocytes enhanced the ability of macrophages to kill the fungi. In a placebo-controlled, double blind study (48) oral doses (600 or 2000 mg daily, for eight weeks) were effective in the treatment of tinea pedis. Mucosheesive, LF-containing tablets for buccal application were also developed for the treatment of Candida albicans infections (49), the effectiveness of such still needs to be proved. Promising results from the in vitro studies revealed that LF-derived peptides may act in cooperation with all azole antifungal agents (50). The investigations demonstrated that the candidacidal activity was initiated by the LF-derived
peptide while fluconazole was required during the effector phase of the Candida albicans killing (51).

Lactoferrin may also be inhibitory for parasite growth. Mice given orally 5 mg of lactoferrin, or intraperitoneally 0.1 mg, were protected against lethal infection with Toxoplasma gondii (35 day survival) and the number of cysts in the brain was significantly lower than that in untreated animals (52). Studying the mechanism of Toxoplasma gondii inhibitory activity in murine macrophages by LF, the authors showed it was not mediated by reactive oxygen intermediates, (53) but instead LF promoted the phosphorylation of tyrosine residues in macrophage proteins of approximately 30 kDa (54). LF was also shown, similar to iron chelators, to inhibit the growth of Plasmodium falciparum (55). The authors suggest that the LF/iron complex generates ROS which may cause membrane damage to both infected erythrocytes and parasites. The involvement of ROS was also demonstrated in LF stimulation of Trypanosoma cruzi phagocytosis and killing by human blood monocytes (56). On the other hand, LF may serve as an iron source for Trichomonas vaginalis (57) and Leishmania chagasi (58), which could be an undesirable phenomenon.

Antiviral and anticancer properties. The role of LF in viral disorders has been elucidated mostly in in vitro experiments. In a series of studies (59-63) LF was found to exhibit inhibitory activity against hepatitis C virus infection. Binding of LF to the virus, although not via the N-terminal loop, was suggested as one of the mechanisms of LF antiviral activity (59). It was subsequently shown (62) that the carboxyl region of LF, which partially shows amino acid sequence homology to human CD81, specifically binds to the HCV E2 envelope protein. In addition, cysteine in position 628 was critical for binding to the E2 protein (61). The antiviral activity of LF was also exhibited by a 33 amino acid peptide from LF. That was the first identification of a natural protein-derived peptide that specifically binds to HCV E2 protein and prevents HCV infection. It appears that interference with virus binding to target cells is the main mechanism of antiviral activity of LF as suggested in the model of HIV-1 cultured with C8166 T cell line (63) or with dendritic cells (64). More detailed studies showed that LF can block human deficiency virus type-1 (HIV-1) variants that use either CXCR4 or the CCR5 co-receptor (65). One of the best examples of LF antiviral action is the competitive binding of LF and human papillomavirus to the common cellular receptor heparan sulphate (66). Interference with heparan sulphate or chondroitin sulphate receptor binding of Herpes simplex virus was also demonstrated for bovine LF (67). Heparin-interacting sites of LF molecule were also involved in anti-adenovirus activity through competition for common glycosaminoglycan receptors (68). The anti-adenovirus activity of LF seems to be mediated via the cluster of positive charges at the N-terminus of the molecule. Interestingly, the entry of human cytomegalovirus was inhibited both by iron-depleted LF and heparin (69), however, the mixtures of LF and heparin mutually blocked each other’s antiviral activities. Modification of LF structure, like binding of metals (manganese or zinc), decreased the antiviral activity compared to apo- or holo-LF. In addition, removal of sialic acid enhanced the anti-rotavirus activity of LF (70). The antiviral activity of LF may have, however, another basis than interference with the viral receptor binding. In one study (71) administration of bovine LF before murine cytomegalovirus (MCMV) infection completely protected mice from death. Also, a significant increase in NK cell activity was found in these mice but not in the antigen-specific cytolytic T cells. No such LF-mediated effect was found in athymic nude mice although it could be restored by a transfer of splenic T cells from LF-treated donors. These results suggest that T lymphocytes potentiate the antiviral effect. Similarly, as in the case of antibacterial and antifungal drugs, LF exhibited synergy when combined with acyclovir in the Herpes simplex 1 (HSV-1) and HSV-2 infection (72). LF was also proved effective in inhibition of canine herpesvirus in Madin-Darby canine kidney cells (73) and of feline calicivirus and poliovirus as models for enteric viruses (74).

The antitumor activity of LF and LF-derived peptides has recently been established in several, mostly in vivo models. Peptide analogs, derived from the N-terminal alpha-helical region of lactoferricin showed antitumor activity against Meth A, HT-29 and MT-1 cell lines. The peptides, that had all cationic residues concentrated in one sector of the helical structure, were most active against the tumor cell lines (75). Cytotoxic effects of lactoferricin B, demonstrated against Meth A fibrosarcoma, B16F10 melanoma and C26 colon carcinoma, were evidenced in vitro by membrane disruption and in vivo by extensive hemorrhagic necrosis (76). LF, LF hydrolyzate and lactoferricin, given orally to mice bearing subcutaneous implants of highly metastatic colon carcinoma 26 (Co26Lu) inhibited lung metastasis (77). The content of asialo GM1+ and CD8+ cells in the peripheral blood of LF-treated mice was elevated indicating importance of these cells in the protective action of LF. A major role of NK cells was also demonstrated in the control of colonization of lungs by B16-F10 melanoma cells in syngeneic mice (78). In another study (79), using B16-BL6 melanoma and L5178Y-MI25 lymphoma cells, bovine apolactoferrin
and lactoferricin B were shown to inhibit tumor metastasis through different mechanisms. Both agents inhibited the number of tumor-induced blood vessels and suppressed tumor growth on day 8 after tumor inoculation. However, in a long-term analysis of tumor growth for up to 21 days after tumor inoculation, a single administration of apo-LF significantly suppressed the growth of B16-BL6 cells throughout the examination period, whereas lactoferricin B showed inhibitory activity only during the early period. The antitumor activity of LF via inhibition of angiogenesis was also confirmed in a dorsal air sac assay (80). In that model both oral and intraperitoneal administration of LF significantly suppressed 3LL-induced angiogenesis. Oral administration of LF also markedly elevated IL-18 concentration in serum and induced IL-18 in peritoneal macrophages. The results suggest that LF participates as a regulator of angiogenesis, possibly by blocking endothelial cell function and inducing IL-18 production. In conclusion, stimulation of NK activity (79), iron withholding (23) and inhibition of angiogenesis (79, 80) could represent the major anticancer activities exhibited by LF.

**Immune regulatory and antiinflammatory properties.** LF demonstrates interesting stimulatory effects on maturation of lymphocytes and initiation of the immune response. LF was shown to directly act on T-cell precursors in the thymus, driving them to cells expressing the helper cell phenotype (CD4+CD8-) (81). The enhancement of the humoral immune response in mice to sheep red blood cells (SRBC) was comparable to that elicited by IL-1. Similarly, LF promoted maturation of B cells from neonatal mice (82) and enabled B cells from normal newborn mice and adult immunodeficient CBA/N mice to present antigen to T cell lines (82). LF also exhibited adjuvant properties in the generation of delayed type hypersensitivity (DTH) in mice (83) and, given orally, significantly augmented both local and systemic nonspecific immune response (84). On the other hand, LF, administered with the eliciting dose of antigen, showed immunosuppressive activity in the DTH reaction (85). Likewise, the effector activity of a TH1 cell line was inhibited by LF (86). LF was also studied in several animal models corresponding to human diseases and clinical situations. The protein, administered for a prolonged time to autoimmune-prone New Zealand Black mice (87) decreased the frequency of positive Coomb’s reaction. LF was also shown to accelerate renewal of the immune function after administration of a sublethal dose of cyclophosphamide (CP). Given orally, for 14 days, LF reconstituted DTH response which was accompanied by an increase of the spleen cellularity, peritoneal and alveolar macrophages and CD4+ T cell levels (88). LF was also able to substantially restore the number of antibody-forming cells in the spleens of mice after five weeks following CP administration. That was correlated with a significant restoration of the proliferative response of splenocytes to ConA and PWM, as well as with the level of CD3+ and Ig+ cells (89). In experimental colitis induced in rats by sodium dextran sulphate (90), LF given by gavage for the whole lifetime attenuated the colitis as reflected by several parameters such as improvement in clinical disease activity index, white blood cell count, hemoglobin concentration, macroscopic and histological scores and myeloperoxidase activity. LF also proved effective in reducing histopathological changes in the liver and in regulating cytokine production in rats with inducible obstructive jaundice (91). Interestingly, LF may exhibit antinociceptive action with morphine when co-administered with subeffective dose in the formalin test (92). The authors showed that the antinociceptive activity of LF was mediated by nitric oxide. The same mediator was involved in the suppressive effect of LF on psychological stress in rats (93). The reversal of LF action by naloxone, an opioid receptor antagonist, indicated that LF activates an endogenous opioidergic system. Very recently, LF was also shown to increase bone formation in vivo (94) that opened a possibility of using LF as a therapeutic agent in osteoporosis. The immune modulatory function of LF has been well described at various stages of inflammatory processes. It is postulated that LF can control the cytokine-induced cascade during the development of systemic inflammatory response syndrome (SIRS) (95, 96). Any insult or trauma leads to activation of the monocyte/macrophage system and stimulates the production of IL-1β, IL-6, TNF-α, GM-CSF, and NO, which in turn activates circulating neutrophils and stimulates the production of fresh cells from the bone marrow. Activated neutrophils degranulate at the site of injury and release massive amounts of the secondary mediators, including lactoferrin. By binding to the specific receptors on monocytes, lactoferrin attenuates the production of proinflammatory cytokines, by which lactoferrin can control the development of SIRS (97, 98). Also, freshly released apo-LF can bind free iron and reduce the production of reactive oxygen species (ROS), one of the most important factors in propagation of inflammatory responses. The anti-inflammatory activity of LF was recently linked to its anti-allergic property. It was shown in mice that LF decreases allergic airway inflammation induced by ragweed pollen grains extract (RWE). Specifically, it was shown that LF decreases inflammatory cell accumulation in the airways induced by the redox-
Lactoferrin in clinical trials. The utility of LF in treatment of various disorders has been tested in a handful of clinical trials. The most recent report includes patients with progressive advanced solid tumors who had failed conventional chemotherapy, who were taken oral human recombinant lactoferrin at doses from 1.5 to 9 g/day, using a 2 weeks on, 2 weeks off schedule (105). Patients were evaluated for drug toxicity, tumor growth rate, lactoferrin pharmacokinetics and cytokine markers. The treatment was well tolerated and no hematological, hepatic, or renal toxicities were reported. Of the eight patients who were radiologically evaluable, seven patients (88%) had a decrease in their tumor growth rate. The three patients with non-small cell lung cancer (NSCLC) all survived for at least one year following the start of lactoferrin therapy. In other studies six patients with multiple sclerosis were taken 50 mg/day of bovine milk-derived LF for seven days (106). The effects of LF and placebo treatment on inducible IL-10 and IFNγ production were determined. Before treatment the placebo group showed a higher, initial IL-10 and lower IFNγ production as compared to LF-treated patients. Cells from treated and untreated groups were further stimulated with PHA and LPS. The effects of LF and placebo treatments in the respective groups were differential. In the LF-treated patients the ability of cell to produce IL-10 rose very strongly (7-day determination), whereas it was diminished in the placebo group. On the other hand, IFNγ determination revealed a gradual drop in production of that cytokine in LF treated group, whereas in the placebo group it was transiently enhanced with a subsequent reduction. By virtue of differential regulation of IFNγ and IL-10 production, LF proved to control the pathogenesis of MS, which is due, in part, to a significant imbalance of TH1/TH2 immune responses. These results also suggest a possible utility of LF in patients suffering from other stress-related neurodegenerative disorders.

Initial results from in vitro experiments on hepatitis C virus led to the evaluation of the relationship between the dose of bovine LF and its effect on serum alanine aminotransaminase (ALT) and HCV RNA levels in patients with chronic hepatitis C (CH-C). Forty-five patients entered at each of the three dose levels (LF of 1.8, 3.6, and 7.2 g/day) received orally an 8-week course of LF. There was no significant relation between the dose of LF and the effect of LF on serum ALT or HCV RNA levels. Biochemical (a 50% or greater decrease in the serum ALT level) and virological (a 50% or greater decrease in HCV RNA level) responses were observed in two and four patients, respectively, but all responders relapsed during the follow-up period after LF treatment. The LF treatment was generally well tolerated, and no patient had any serious adverse event (107). In other trials LF...
was given to healthy volunteers orally for seven days (50 mg/day). The treatment significantly increased the percentage of neutrophil precursors in the peripheral blood and reduced the spontaneous production of IL-6 and TNFα by cultured peripheral blood mononuclear cells (PBMC). The effects of LF administration persisted for additional 14 days (108). It was also shown (109) that oral administration of LF (50 mg daily for five days) before minor surgery, enhanced surgery-elicited suppression of the immune response as determined by proliferation rates of PBMC to PHA, and by TNFα and IL-6 production by PBMC cultures. Again, pretreatment of patients with LF led, in addition, to a significant increase of neutrophil precursors in circulation. It also appeared that a decreased zeta-chain expression in peripheral blood T lymphocytes from cervical cancer patients could be elevated following incubation with human LF to a similar degree as upon incubation with anti-CD3 antibodies (110). Addition of LF to the cultures stimulated with anti-CD3 antibody resulted in even higher stimulation of that marker expression. The authors suggest therapeutic value of LF in patients with cervical cancer patients. In an open, randomized, single center study (111) conventional therapy of H. pylori infection was supplemented with bovine LF. The preliminary results revealed a better effect of such a therapeutic strategy in comparison with antibiotic therapy alone. Other trial showed benefits of LF in the intestinal form of Graft vs Host (GvH) disease in children (112).

**PROLINE-RICH POLYPEPTIDE**

A proline-rich polypeptide (PRP), also known as Colostrinin (CLN), was first isolated from ovine colostrum and characterized as a first milking component of colostrum. It contains about 22% proline, a high proportion of non-polar amino acids, a low percentage of glycine, and no alanine, arginine, histidine, tryptophan, methionine and cysteine residues. Colostrinin is not phosphorylated or glycosylated and the hydrophobic amino acids constitute 51% of the total protein (113). It was suggested that blocks of proline residues could render it resistant to proteolytic degradation. However, in recent studies (114) PRP was shown to consist of a mixture of more than 32 peptides (HPLC). The authors revealed a significant homology of the peptides to beta casein and a hypothetical beta-casein homolog. They postulated that PRP represents a diverse group of peptides produced by the mammary gland of mammals for the early development of infant’s immune system and the protection of newborns against environmental shocks. Early studies on the activity of PRP showed that it stimulated the humoral immune response to SRBC, increased the skin permeability in guinea pigs (115) and enabled induction of GvH reaction by cortisone-sensitive (less mature) thymocytes (116). Subsequent studies demonstrated that products of chymotrypsin digestion were active in the regulation of the humoral immune response, delayed type hypersensitivity and maturation effects on thymocytes (117). PRP exhibited a co-stimulatory activity in the proliferation of thymocytes to ConA, similarly as IL-1 (118) and, at higher doses, induced proliferation of lymph node cells. It appeared that PRP acted on very early T-cell precursors (Thy-, H2+, CD4-, CD8-, CD3-) by inducing expression of CD4, CD8, CD3 and alpha/beta TCR (119). The ability of PRP complex and its active fragments to induce several cytokines such as IFNγ, TNFα, IL-6 and IL-10 was also demonstrated (120). In a model of experimental autoimmune response in thymectomized mice, immunized with rat red blood cells, PRP was shown to decrease the anti-erythrocyte autoantibody production (121). The peptide also lowered the incidence of positive Coomb’s reaction and prolonged the mean age of New Zealand Black mice (122). Also, it has been suggested that the function of PRP in mothers’ milk is to protect against excessive oxidative stress in the respiratory and intestinal track of the newborn infant. In more recent studies it was shown that CLN reduces intracellular level of ROS, inhibits 4-hydroxynonenal- (4HNE)-mediated glutathione depletion and reduces 4HNE-induced activation of c-Jun in vitro (123). These findings suggest that CLN appears to down regulate 4HNE-mediated lipid peroxidation and its product-induced signaling that otherwise may lead to pathological changes at the cellular and organ level. There is increasing consensus that the production and accumulation of beta amyloid (Aβ) peptide is central to the pathogenesis of Alzheimer’s disease (AD). It has been shown that CLN prevents the aggregation of Aβ in vitro. Moreover, the reduction of fibrils of Aβ peptides by CLN has been concomitant with the reduction of the cytotoxic effects of Aβ on SHSY-5Y neuroblastoma cells (124). Thus, the neuroprotective effect of CLN is due to reduced aggregation of Aβ. Also, it was demonstrated that upon treatment with CLN, medullary pheochromacytoma cells ceased to proliferate and extend neuritis (125). The arrest of CLN-treated cells in G1 phase of the cell cycle was due to an increase in the phosphorylation of p53. Recently, the cognitive enhancing effect of CLN has been demonstrated in day-old domestic chick model (126). The study confirmed CLN effects on retention of memory for single one-trial learning paradigm – avoidance of bitter tasting substance. A dose response
curve indicated that CLN exhibited potency as a cognitive enhancer over a 100-fold concentration range. Moreover, the differential impact of CLN has been shown on the respiratory activity of mitochondria derived from the inbred mice strains consisting of accelerated senescence-prone (SAMP1) and accelerated senescence-resistant (SAMR1) mice. The SAMP1-derived cells produced more ROS and exhibit severe mitochondrial dysfunction and have a decreased life-span compared to cells from SAMR1 mice. Addition of CLN to SAMP1 cell cultures significantly decreased ROS production, comparable to levels in SAMR1-derived cell cultures (127). These data suggest that CLN could ameliorate age-associated increase in mitochondrial ROS generation thereby may have therapeutic significance in preventing age-related degenerative pathologies in the central nervous system, skin and immune system.

**PRP in clinical trials.** The effectiveness of oral administration of PRP was tested in several human trials (128-130). Initial studies utilizing low dose regimen of PRP (100 mg/day) showed improvement in cognitive and behavioral abilities of AD patients (128, 129). In the most recent trial, consisted of 105 AD patient, the efficacy of PRP (trademarked as ColostrininTM) was tested for 15 weeks in double-blind phase (comparing active with placebo), followed by a second 15 week open labeled phase when all patients received PRP (130). The dosage of Colostrinin was 100 µg on alternate days for three weeks followed by two weeks drug-free. This cycle was repeated three times for each phase. The primary efficacy parameters used were Alzheimer’s Disease Assessment Scale-cognitive portion (ADAS-cog) and Clinical Global Improvement (CGI). Secondary efficacy parameters were Instrumental Activities of Daily Living (IADL); Mini-Mental State Examination (MMSE); ADAS-non cognitive test (ADAS-non cog); and overall Patient Response. The FSA analysis at week 15 showed a stabilizing effect of Colostrinin on cognitive function in ADAS-cog (p = 0.02) and on daily function in IADL (p = 0.02). The overall patient response was also in favor of the active (p = 0.03). Patients graded as mild on entry also showed a superior response of ADAS-cog compared with more advanced cases (p = 0.01). Evidence from this study indicates an early beneficial effect on cognitive symptoms and daily function.

**CASEINS**

Among milk proteins, caseins are the most heterogenous structures with a little evidence of direct physiological role. However, many of casein-derived peptides or components (e.g. alpha s1 or beta subunits) have been shown to express significant biological effects. The N-terminal segment (1-23) of alpha s1-casein B, named “isracidin”, was significantly effective in vivo at concentrations that were competitive with known antibiotics, as seen in the protection of mice against lethal infection by *Staphylococcus aureus* strain (131). Field trials showed that injection of isracidin into the udder gave protection against mastitis in sheep and cows. Isracidin was both therapeutic and prophylactic and responses to its therapeutic effect produced long-term immune resistance. Isracidin protected mice against *Candida albicans*, by stimulation of both phagocytosis and immune responses. Casein was shown to prevent enamel demineralization in a modified intra-oral caries model (132). Intact alpha s1-casein and tryptic peptides were incorporated into the inter-enamel plaque. The incorporation of casein and its breakdown products in plaque did not produce a significant change in the amount or composition of plaque bacteria. A mouthrinse solution containing casein derivatives gave promising results as a caries-preventing agent in a 124 participant trial with dry mouth syndrome, as compared with a mouthrinse solution containing 0.05% sodium flouride (133). In a model of post-menopausal bone loss in aged ovariectomized rats, dietary Ca-bound casein phosphopeptides were tested (134). Control groups included rats fed control diets (CaCO3 and KH2PO4) and rats that were sham-operated. During a 17-week period there was little change in femoral bone mineral densities of ovariectomized rats fed casein phosphopeptides, whereas the value of that parameter in the respective control rats decreased with time. The authors conclude that the inhibitory effect on bone loss in aged ovariectomized rats could be due to the effects of dietary calcium-bound casein phosphopeptides on phosphorus and calcium metabolism. Casein was found to exhibit protective activities in experimental bacteremia and endotoxemia. When casein was administered subcutaneously (s.c.) 24 hours before lethal infection of mice with Gram-positive and Gram-negative bacteria, it prevented animals from lethal effects of bacteremia (135). That was accompanied by enhanced early clearance of bacteria, greater phagocytosis, and oxidative burst. Casein-induced inflammation was also associated with increased concentrations of G-CSF in serum. The effect of s.c. injection was similar to that achieved by injection of recombinant murine G-CSF between 3 and 24 hours before casein-induced acute phase response. The casein-induced acute phase response did not affect serum concentration of TNFα, IL-1β and IL-6 after *E. coli* injection. In a model of mice given orally LPS from *Salmonella typhimurium* and fed a commercially available casein preparation, consisting mainly of...
bovine alpha s2-casein (1-32) and beta casein (1-28), the authors found fecal and intestinal anti-LPS IgA and total IgA significantly higher than in those fed a control diet (136). The results suggest that dietary casein phosphopeptide may protect a host from invasion of the intestinal mucosa by food-born pathogens. Others found a protective effect of a diet containing casein hydrolyzate on diabetes. In one report (137) the hydrolyzate protected non-obese, diabetic mice against diabetes. That effect was not, however, associated with the formula on T cells. In diabetes-prone rats fed a hydrolyzed-casein-based semi-purified diet the incidence of diabetes was 2-3 fold lower compared with rats fed cereal-based diets (138). In a model of dimethylhydrazide-induced intestinal tumors in rats, several diets were tested, including those enriched in whey, casein, soybean or red meat (139). The results showed that whey and casein diets were more protective against the development of intestine tumors than other diets taking into account tumor burden, mean index and number of rats affected. Also, the intracellular concentration of glutathione, an antioxidant and anticarcinogenic tripeptide, measured in liver, was highest in whey protein and casein-fed rats. In another study (140) bovine milk proteins were investigated for their activities on melanogenesis in B16 cells. Whey proteins and casein exhibited depigmenting properties. Among the major milk proteins only kappa casein demonstrated that kind of effect. The effect of 50% casein diet on iodine goiter was studied by surveying the changes of thyroid weight, serum thyroid hormones and TSH concentration in mice fed excess iodine water (141). The investigators showed that casein could reduce the excess of iodine entrance into the thyroid cells and decrease the excess of colloid formation in the follicular lumen. It also appeared that hydrolysis of sodium caseinate could result in isolation of fourteen peptides with antihypertensive activity (142). The diets enriched in casein also appeared to be effective in managing colicky symptoms associated with protein sensitivity in infants (143). The nutritive values of casein have to be however reevaluated due to the fact that peptides from gluten and casein may have a role in the origins of autism and that the physiology and psychology of autism might be explained by excessive opioid activity linked to these peptides. Research has reported abnormal levels of peptides in the urine and cerebrospinal fluid of persons with autism. Therefore, diets free of gluten and/or casein should reduce the symptoms associated with autism.

Glycomacropeptide (GMP), a peptide derived from kappa casein, has attracted considerable interest in recent years (reviewed in 144). GMP contains a high proportion of sialic acid, which may vary among ruminants (145). The biological activities of GMP, such as enhancing effects on phagocytosis and proliferation of human macrophage cell line U937, was particularly increased in GMP rich in sialic acid (146). Likewise, the inhibitory effect of GMP on binding of cholera toxin to chinese hamster ovary cells (CHO)-K1 seemed to be attributed to their terminal sialic acid (147). In another study, however, both native and desialylated variants of the same bovine GMP totally prevented the adhesion of Actinomyces viscosus Ny1, Staphylococcus sanguis OMZ9 and Staphylococcus mutants OMZ176 to polystyrene surfaces (148). Such results suggest that GMP may interact with cells via different types of receptors, i.e. recognizing sialic acid or an amino acid sequence in the GMP molecule. Besides GMP, an inhibitory activity was also exhibited by other mucin-type glycoproteins carrying short O-linked carbohydrate chains. The authors concluded the GMP prevention of oral bacterial adhesion to polystyrene tubes takes place with no species specificity and can be compared to nonspecific inhibition exhibited by various polymers. In a model of arterial thrombosis, triggered by laser-induced intimal injury in the guinea pig, GMP, the undecapeptide (residues 106-116), and the pentapeptide (residues 112-116) were anti-aggregating peptides and exerted a significant antithrombotic activity (149). That activity was achieved in vivo for doses less than one could predict from in vitro results. Most promising results derived from studies on infant rhesus monkeys. GMP-enriched formula given to the monkeys from birth to five months reduced the degree of diarrhea elicited by administration of enteropathogenic Escherichia coli (150). In a similar model (151) monkeys were breast-fed, fed control, alpha lactalbumin, or GMP-enriched formulas. The infants fed GMP had higher food intake than did other formula-fed infants. In addition, they had higher plasma zinc and zinc absorption than did breast-fed infants. In experimental bacteremia and endotoxiaemia our data indicate protective effects of GMP, surpassing the effects of LF. The protective effects of GMP were recently tested in experimentally induced endotoxiaemia or bacteremia in mice (152). The results showed that BALB/c mice, given intraperitoneally GMP, 24 hours before intravenous injection of a high dose of LPS from E. coli, strongly inhibited serum levels of TNFα and IL-6, measured 2 hours later by bioassays. In addition, GMP, administered 24 hours before infection of CBA mice with a sublethal dose of E. coli, significantly lowered the number of bacterial cells in the spleen. The analysis of main blood cell types in mice pretreated 24 hours prior to infection with GMP revealed significant increase in the content of granulocytes and immature neutrophils. The induction of myelopoiesis by GMP
may be a primary cause of the increased clearance of bacteria during the development of bacteremia in mice.

**Caseins in clinical trials.** Casein-derived peptides seem to be very effective in the reduction of systolic blood pressure (SBP). In the most recent study a total of 131 volunteers with high-normal blood pressure and mild hypertension were randomly divided into four groups (n 32 or 33 in each group) (153). Each volunteer was given two tablets containing one of the major angiotensin-I-converting enzyme inhibitory peptides Val-Pro-Pro (VPP) or Ile-Pro-Pro (IPP). A significant decrease in systolic blood pressure was observed at 6 weeks in the active group receiving 1.8 mg VPP and IPP; in the active groups receiving either 2.5 mg or 3.6 mg, systolic blood pressure was decreased at both 3 weeks and 6 weeks compared with systolic blood pressure measured before treatment. Changes in the systolic blood pressure after 6 weeks of treatment were dose dependent. The antihypertensive effect was greater in mildly hypertensive subjects (n 20 or 21 in each group) than in any of the other subjects. No significant change of diastolic blood pressure was observed for all the test groups, and no differences in diastolic blood pressure in the test sample groups compared with the placebo group were observed during the test period.

In another study (154) 10 hypertensive subjects were evaluated to determine whether a single dose of a hydrolysate of bovine milk protein (designated C12 peptide; low and high dose), either alone or combined with alginic acid (low and high dose), reduced daytime blood pressure. A significant reduction of 9.2 +/- 3.2 mm Hg in systolic blood pressure was reported on the higher dose of alginic acid (1754 mg) combined with C12. The C12 peptide with the higher dose of alginic acid also showed a significant reduction of 6.0 +/- 2.0 mm Hg in diastolic blood pressure. Although the preliminary data are encouraging, much larger and longer treatment trials will be required to confirm further clinical utility of C12 peptide.

**ALPHA-LACTALBUMIN AND LYSOZYME**

These proteins may be related, since they have diverged from a common ancestor as reflected in the striking relationship between their amino acid sequence and high conservation of disulfide bridges, intro-exon organization and three-dimensional structures (155). Alpha-lactalbumin (LA) was found to exert antiviral activity against HIV-1 by inhibiting HIV-1 protease and integrase, but not HIV-1 reverse transcriptase (156). Beta-lactalbumin and casein exhibited similar properties. A majority of data on the protective activity of LA derive from the in vivo studies, thus most valuable ones, suggesting potential application of LA in prevention and therapy. In two rat models, such as gastric mucosal injury by ethanol/HCL and water-immersion restrain stress, LA given 30 min before the induction of gastric injury, demonstrated a marked protective effect with the same potency as the typical antiguyster agent, selbex (157). Pretreatment of rats with indomethacin resulted in a significant reduction in the protective effect of LA indicating an involvement of prostaglandins in that protective effect. In another study involving the same model, LA caused elevation of PGE2 in gastric tissue, increase of the gastric mucin content, and prostaglandin-independent responses such as elevation of gastric luminal pH, increase in gastric fluid volume and delay in gastric emptying (158). The effect of a diet enriched in LA, as compared with a diet enriched in sodium caseinate, was tested in highly stress-vulnerable and stress-invulnerable subjects (159). A hypothesis to be tested was that LA, rich in serotonin precursor-tryptophan, may improve the ability to cope with stress. The results showed that in the group of stress-vulnerable patients the LA-enriched diet, as compared with the respective control group, led to an increase of the ratio of tryptophan to other large, neutral aminoacids, higher prolactin concentration, decrease in cortisol and reduced depressive feelings. LA-enriched diet was shown to enhance serotonin release and consequently to induce anxiolytic-like and rewarding action in rats (159). LA and a tetrapeptide lactorphin (Tyr-Gly-Leu-Phe), derived from LA, also improved arterial function in hypertensive rats (160, 161). LA lowered blood pressure in a dose-dependent manner without affecting heart rate. Naxolone, a specific opioid receptor antagonist, abolished that effect, which provides evidence for an involvement of opioid receptors in the depressive action of LA. Alpha-lactorphin improved vascular relaxation in adult, spontaneously hypertensive rats in vitro (161). The beneficial effect of alpha-lactorphin was directed towards endothelial function. A tryptic digest of beta LA (lactokinin) was shown to reduce (by 29%) the release of vasoconstrictor endothelin-1 (162). Also, beta-lactosin B (Ala-Leu-Pro-Met) from beta LA, decreased systolic blood pressure in spontaneously hypertensive rats (163). A milk-derived antihypertensive peptide was expressed in E. coli (164) and produced at high yield (500 mg/l culture), opening a possibility of production of the peptide on an industrial scale. The protective activity of LA was also shown in experimentally-induced diarrhea and diarrhea in children. In infant rhesus monkeys which were breast-fed, fed control formula or a formula supplemented with LA from birth to five months of age, breast-fed or LA-supplemented
formula-fed monkeys had no diarrhea following administration of $10^8$ CFU E. coli, whereas in infants fed control formula, the diarrhea was acute. A diet with a high content of LA hydrolyzate, given to severely malnourished children, led to a better average weight gain when compared with other formulas or cow’s milk (165). However, another study (166) has not confirmed a superiority of such a diet over three other formulas (rehydration solutions) in children with acute diarrhea. HAMLET®, a complex of LA and oleic acid, in a model of human glioblastoma xenograft implanted into nude rats, reduced the intracranial tumor volume and delayed the onset of pressure symptoms (167). HAMLET® also proved effective in patients with cutaneous papillomas that were resistant to conventional therapies (168).

Lysozyme is another milk-derived ingredient, which found application in food preservation, infant feeding formulas, as well as clinically in the treatment of periodontitis and prevention of tooth decay (reviewed in 169). Milk enriched in lysozyme was used in feeding premature infants suffering from concomitant diseases (170). The control group was fed artificial product and milk without lysozyme. A favourable effect was shown, comparable with that of breast milk, taking into account the following parameters: increase in the body weight, more rapid sanation of the infectious inflammatory foci, normalization of the stool, stabilization of lysozyme levels in the coprofiltrates, and increase of lysozyme in the blood serum (170). It appeared, however, that the antibacterial action of lysozyme is associated with the presence of LF in the model of neonatal rats infected intestinally with E. coli (171). Lysozyme was also found to possess an interesting new antiinflammatory action, i.e. it inhibited the hemolytic activity of serum complement when tested within the levels present in normal and inflamed breast milk samples. Of note, there are also analgesic properties postulated for lysozyme (172).

LACTOPEROXIDASE

Lactoperoxidase (LCP) has a potential to be applied as an antimicrobial agent (173). In rats fed a cariogenic diet and inoculated with Streptococcus sobrinus, liposome-encapsulated LCP given for a period of 35 days significantly lowered the caries incidence (174). The lactoperoxidase system was also tested in calves infected with Salmonella typhimurium in parallel to calves fed heated milk (175). In that case the clinical findings and Salmonella excretion pattern were similar in both groups. That was in contrast to the results from the in vitro experiments, where in acidified raw milk, addition of LCP and exogenous H$_2$O$_2$ led to a rapid decrease of salmonellas. However, in a later study on calves (176) experimentally infected with E. coli and given a preparation containing LCP system and LF, the mortality occurrence and duration of diarrhea were significantly lower in these animals as compared with nontreated calves. As in the case of lysozyme, the antibacterial action of LCP requires cooperative action with LF.

In conclusion, evidence continues to accumulate that many milk-derived products provide a variety of health benefits including antimicrobial, antiinflammatory, anticarcinogenic, hypocholesterolemic or hypertension controlling effects. This emphasizes the potential for prevention of many age-related disorders by timely utilization of such clinical nutrition. More importantly, milk-derived proteins and peptides are fully bio-accessible and safe for utilization in humans.

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Milk-derived proteins and peptides of potential therapeutic and nutritive value


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