

Review

Twenty-five years of research on bovine lactoferrin applications

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Abstract

Lactoferrin (LF) was identified as a milk protein in 1960. Large-scale manufacturing of bovine LF (bLF) was established more than 20 years ago. Using this commercially available material, research for bLF applications has advanced from basic studies to clinical studies, and bLF has been applied to commercial food products for the last 25 years. During this period, it was found that LF is digested by gastric pepsin to generate a multi-potent peptide, lactoferricin. It was also demonstrated that oral administration of bLF augments host protection against infections via antimicrobial action and immunomodulation of the host. In addition, researchers have demonstrated that oral administration of bLF prevents cancer development. In this review, we look back on 25 years of bLF research and development.

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1. Introduction

Lactoferrin (LF) was first fractionated as an unknown “red fraction” from cow’s milk by Sørensen and Sørensen in 1939 [1]. In 1960, the red protein from both human and bovine milk was defined as a transferrin-like glycoprotein [2–4]. Studies of the antimicrobial activities by Arnold et al. [5], studies of the immunomodulatory activities by Broxmeyer et al. [6], and structural studies by Spik et al. [7] have been recognized as early, pioneering basic research on LF. Following this research with human LF (hLF), research on bovine LF (bLF) progressed from basic studies to clinical studies to find good applications and supporting evidence. In this review, we look back on 25 years of research and development of bLF. Table 1 summarizes 25 years of achievement in research and development of both hLF and bLF. Since many review articles on basic LF research have been published, we cite some of them here [8–11]. We would like to also recommend our comprehensive reviews of

LF research and technologies for application [12] and research on the LF-derived peptide lactoferricin (LFcin) [13].

2. Twenty-five years of bLF research and development

2.1. Manufacturing of bLF, bLF-containing infant formula and other products

LF can be isolated from cow’s milk by various purification methods [12]. A cation-exchange chromatography system was selected for large-scale production of bLF by manufacturing companies. The pioneering production of bLF on an industrial scale was started by Oleofina Company in Belgium in 1985. Then, MILEI GmbH in Germany started to produce bLF from cheese whey or skim milk using manufacturing technology developed by Morinaga Milk Industry Co., Ltd. in 1989 [14]. Presently, bLF is produced by many companies including MILEI in Germany, DMV International in the Netherlands, DOMO Food Ingredients in Belgium, Tatura Nutritionals and Fonterra in New Zealand, MG Nutritionals in Australia, and Armor Proteins in France. The recent production scale of bLF worldwide is assumed to be over 60 t/year.

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Table 1
Twenty-five years of LF research and development worldwide

Year	Research and development ^a
1984	Determination of primary structure of hLF [61]
1985	Commercial production of bLF by Oleofina Company
1986	Marketing of bLF-containing infant formula by Morinaga Milk Industry [14]
1987	Cloning and sequencing of mLF gene [62] Determination of the 3D structure of hLF [63]
1988	Determination of the glycan-chain structure of bLF [64]
1989	Large-scale production of bLF by MILEI GmbH [14]
1990	Cloning and sequencing of hLF and bLF genes [65,66]
1992	Discovery of LFCin B and LFCin H [54] Identification of LRP as a liver LF receptor [67,68] Large-scale production of rhLF by Agennix Incorporated [69]
1993–1995	Inhibition of overgrowth and translocation of intestinal bacteria by oral bLF and bLFhyd [25,26]
1995	Identification of hLF as a transcriptional activator [70]
1997	Determination of the 3D structure of bLF [71] Marketing of bLF-containing yogurt by Morinaga Milk Industry [12]
1997–Present	Cancer-preventive effects and mechanism of oral bLF and bLFhyd in animal models [34,35]
1998	Determination of the 3D structure of LFCin B [72]
1999–Present	Anti-infective effects of oral bLF in humans [31,32]
2000–Present	Anti-infective effects and mechanism of oral bLF in animal models [12,27]
2001	Identification of intelectin as an intestinal LF receptor [47]
2003	Generation of an LF knockout mouse [73]
2003–Present	Identification of associations between hLF SNPs and diseases [74]
2005	Determination of the 3D structure of LFCin H [75]
2006	Completion of a clinical study of oral bLF on colorectal adenomas in humans [37]

^a Abbreviations used in this table are as follows: hLF, human lactoferrin; rhLF, recombinant hLF; bLF, bovine lactoferrin; bLFhyd, bLF-pepsin-hydrolyzate; mLF, mouse lactoferrin; LFCin, lactoferricin; 3D, three-dimensional; LRP, LDL receptor-related protein; SNP, single nucleotide polymorphism.

In the 1980s, we had little knowledge about the effects of orally administered LF. However, there was some basic research on LF, as described in Section 1, suggesting its importance in breast milk and the potential benefits of supplementing bLF in infant formula. In 1986, marketing of a bLF-containing infant formula, “BF-L dry milk”, was started by Morinaga Milk Industry in Japan, the first such formula in the world [14]. Later, several evidences indicating benefits of bLF supplementation in infant formula were reported, including an improvement in intestinal microbial flora [15,16], enhanced serum ferritin [17] and hematocrit [18] levels, and reduced lower respiratory tract illnesses [18]. Addition of bLF also inhibits lipid oxidation of infant formula [19]. Currently, bLF-containing infant formulas are sold in Indonesia and Korea, as well as in Japan. Other bLF-containing products include yogurt, skim milk, milk-type drinks, supplemental tablets, pet food, and cosmetics [12]. The beneficial effects of these bLF-containing products on the health have been proved in clinical and animal studies. The effect of yogurt on rotaviral gastroenteritis and the effect of tablets on chronic hepatitis C, rotaviral gastroenteritis, and *Helicobacter pylori* gastric infection have been reported [20–22]. The therapeutic effect of pet food on dermatitis in dogs and cats was also shown [23]. In Sections 2.2 and 2.3, we review the beneficial

effects of orally administered bLF on intestinal flora, infections, and cancers, which support the usefulness of bLF-containing products.

2.2. Effect of oral bLF on intestinal microflora

In the late 1980s, researches to evaluate whether orally administered bLF has beneficial effects were started. First, intestinal microflora was targeted, because LF has antimicrobial activity and was expected to influence the bacterial composition in the intestine. Administration of infant formula containing 1 mg/ml of bLF increased the ratio of *Bifidobacterium*, but decreased that of Enterobacteriaceae and *Clostridium*, in the feces of low-birth weight infants, while there was no comparison of the effect with control infant formula without added bLF [15]. In a study comparing infant formula containing 2.8 mg/ml of bLF and basic infant formula, there was no significant difference in the effects of the two formulas on the fecal microflora of normal infants [24]. Infant formula containing 1 mg/ml of bLF established a *Bifidobacterium*-dominant fecal flora in normal infants, whereas infant formulas containing 0 or 0.1 mg/ml of bLF did not [16]. The effect of bLF on human intestinal microflora may be influenced by the population of subjects and the detection methods. It is necessary to assess the effect of bLF on human intestinal microflora more accurately and comprehensively by modern molecular biological methods.

In the early 1990s, effects of bLF on intestinal flora were analyzed using laboratory animals and clear evidence was obtained. When mice are fed only cow’s milk, the number of Enterobacteriaceae and other pathogenic organisms increases in the intestine. In this environment, oral administration of bLF suppressed the overgrowth of Enterobacteriaceae, *Streptococcus*, and *Clostridium*, and translocation of intestinal bacteria, including Enterobacteriaceae [25,26]. bLF-pepsin-hydrolyzate (bLFhyd) also showed effects similar to those of bLF.

2.3. Anti-infective and cancer-preventive effects of oral bLF

In order to elucidate the anti-infective activities of oral bLF, research was started to evaluate the effects of bLF feeding in a guinea pig model of dermatophytosis. It was found that bLF facilitates the cure of dermatophytosis and decreases fungal abundance in the skin [27]. Research also demonstrated the beneficial effects of oral bLF in other animal infection models, including herpes virus skin infection, oral candidiasis, and influenza virus pneumonia [28–30]. Other research reported the anti-infective effects of oral bLF in animals with *H. pylori* gastric infection, *Staphylococcus aureus* systemic infection, and *Escherichia coli* urinary tract infection [12].

Since a report on chronic hepatitis C was published by Tanaka et al. in 1999 [31], many clinical studies of the effects of oral bLF on human infections have been performed. Ingesting bLF facilitates cure of tinea pedis, a type of dermatophytosis [32], a finding that has been confirmed in animal models [27]. bLF also improves some symptoms of *H. pylori* gastric

infection [22] and increases the eradication rate of triple therapy against *H. pylori* in the stomach [33]. Recently, the beneficial effects of bLF on rotaviral gastroenteritis were shown [20].

In 1997, Tsuda's group reported the preventive effects of bLF feeding on colon carcinogenesis in rats [34]. This group then clarified the cancer-preventive effects of bLF and bLFhyd in various organ-specific cancer models [35]. They also showed that bLF, bLFhyd, and LFcIn B have anti-metastatic effects [36]. Recently, their group found in a randomized, double-blind, placebo-controlled study that oral bLF may inhibit progression of colorectal polyps. Administration of bLF at 3 g/day for 1 year showed a tendency to suppress colorectal adenomas of less than 5 mm in diameter compared with that of placebo administration [37].

The metabolism and mechanism of action of orally administered bLF have been investigated. It was shown that fed bLF is not completely degraded in the gastrointestinal tract, but is retained to some degree, as LFcIn-containing peptides [38]. If bLF is transferred to the body, it may cause undesirable reactions such as allergenicity. Research data suggest that ingested bLF is generally not absorbed in the blood [39], but acts on the intestinal immune system and influences the systemic host-protective system as illustrated in Fig. 1 [12,40]. Orally administered bLF enhanced production of interleukin (IL)-18

in intestinal epithelial cells and IL-10 and interferon (IFN)- γ in intestinal intraepithelial lymphocytes and mesenteric lymph node cells [41,42], and increased the numbers of CD4⁺ cells, CD8⁺ cells, and natural killer (NK) cells in the intestinal mucosa of mice [43]. bLF also enhanced gene expressions of NOD-2, IFN- β , and IL-12p40 in the small intestine of mice [44]. In the systemic immune system, oral bLF increases the numbers of cells in lymph nodes and the spleen, enhances the activity of peritoneal macrophages and splenic NK cells, and enhances the production of Th1 type cytokines (IL-12 and IFN- γ) [28,34,45,46]. The circulation of secreted cytokines or other humoral factors and the migration of immune cells may be signals bridging the intestinal immune system and the diseased sites in the action of oral bLF.

However, the detailed molecular mechanisms, including interaction between bLF/bLF-digested peptides and LF receptors such as intelectin [47], internalization of bLF/peptides into cells, and intracellular signaling, must be elucidated in future studies. Dendritic cells may be important target cells of ingested bLF/peptides, because recent papers reported that recombinant hLF can induce the maturation of dendritic cells to prime naïve T cells [48,49]. DC-SIGN, a C-type lectin, is a candidate of bLF receptors on human dendritic cells, because bLF and its C-lobe bind strongly to this protein [50]. Although it is reported that hLF and peptides overlapping the LFcIn H

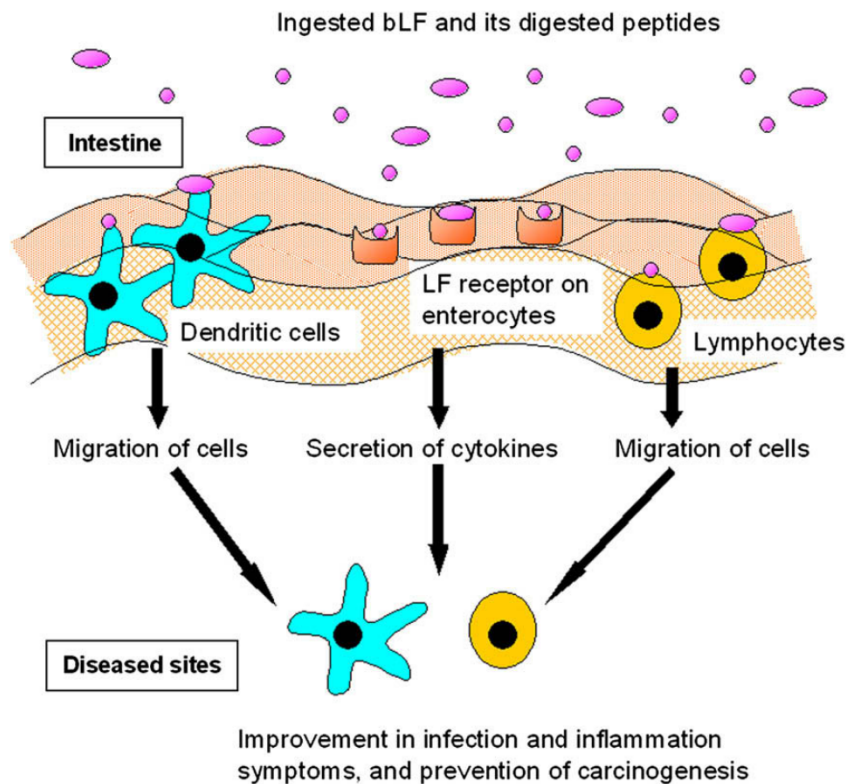


Fig. 1. Deduced mechanism of action underlying the host-protective effects of orally administered bLF. After ingestion, bLF is partially digested to peptides by proteases in the stomach and intestine. In the small intestine, bLF and its digested peptides bind to receptors on enterocytes and immune cells such as dendritic cells and lymphocytes residing in the intestinal epithelium. bLF/peptides may be internalized into the cells and/or trigger intracellular signaling to activate transcription of genes. Humoral factors like cytokines are secreted from these cells by bLF/peptides stimulation and reach diseased sites through the circulation. Otherwise, the stimulated immune cells in the intestine migrate to diseased sites. These humoral factors and immune cells then act to improve infection and inflammation symptoms, and to prevent carcinogenesis.

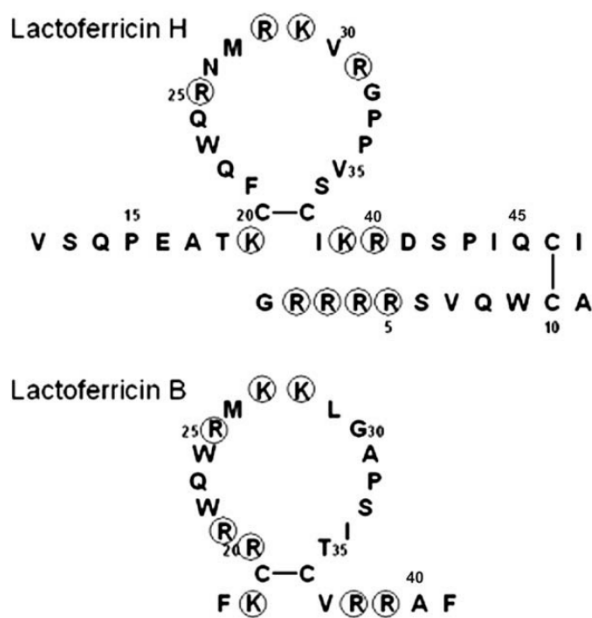


Fig. 2. Amino acid sequences of lactoferricin (LFcin) H and B. Basic amino acids are represented by circles. Modified from [54]. LFcin H and B are 47 and 25 amino acid peptides derived from the homologous N-terminal region of hLF and bLF, respectively, by pepsin digestion.

region bind to a receptor of activated human lymphocytes [51], another paper indicated that bLF and hLF, but not bLFhyd, bind to proteins on the Jurkat human lymphoblastic T cell line [52]; therefore, similarities and differences in the actions of bLF and bLF peptides have to be carefully examined.

2.4. Discovery of LFcin

To explore the activities of LF after digestion, one study attempted to hydrolyze LF with various proteases and evaluate their biological activities. It was found that LF hydrolyzed by pepsin shows potent antimicrobial activity [53]. Next, the active peptides were isolated and the amino acid sequences of the peptides were determined. The peptides were named LFcin B and LFcin H, indicating their bovine and human origins, respectively, and were reported in 1992 (Fig. 2) [54]. These peptides are derived from the N-terminal region of LF and have characteristics of typical cationic antimicrobial peptides. Later, LFcin B was also found to be generated in the stomach after ingesting bLF [55]. The generation of LFcin H or similar peptides in the stomach or other sites in humans is an interesting issue, but it has not been reported yet.

Because LFcin B is more potent than LFcin H, our research focused on LFcin B. A comparison of the antimicrobial spectra of LFcin B and bLF indicated that LFcin B has strong antimicrobial activities, especially against bacteria and yeasts [13]. Electron micrography of bacteria treated with LFcin B showed loss of membrane integrity and alterations of cytoplasmic structures [56]. We have also been investigating antimicrobial mechanisms of the peptides, the synergism between LFcin B and other antimicrobial compounds, and synthetic LFcin derivatives including *N*-acylated and *D* enantiomer core peptide (RRWQWRMCK) [13]. To date, research on

LFcin-related peptides, which are various short-length and amino acid-substituted peptides, has been conducted by many researchers and has widened to fields of three-dimensional structures, structure–activity relationships, mechanisms of action, and *in vivo* efficacies [13,57,58]. Overall, 168 articles were found by searching for “lactoferricin” in PubMed in June 2008.

Although LFcin B and H were first isolated by HPLC [54], LFcin B can be purified by hydrophobic chromatography from bLFhyd, and this procedure could be scaled up [59]. Currently, the applications of LFcin are limited. However, bLFhyd containing LFcin B is supplemented in some peptide-based infant formulas. AM-Pharma B.V. in the Netherlands announced that they have completed phase I clinical trials with N-terminal 11-mer portion of LFcin H (GRRRRSVQWCA) and have suggested that they will develop it as an antibacterial and antifungal drug [60].

3. Conclusion

In the past 25 years, bLF research has achieved remarkable advances which have expanded the value of bLF for health promotion. It is expected that researches in this field will lead to further progress. In the future, novel effects of orally administered bLF and mechanisms of action will be shown and these may include improving symptoms of anaemia and periodontitis. Results of these studies will open up new application opportunities for bLF.

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