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Bovine Lactoferrin Enhances Proliferation of Human Peripheral Blood Lymphocytes and Induces Cytokine Production in Whole Blood Cultures

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation;
D – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Lactoferrin belongs to the immunoregulatory milk proteins involved in iron metabolism as well in providing innate immunity to newborns. The protein has been the subject of numerous clinical studies.

Objectives. The aim of this investigation was to evaluate the effects of bovine lactoferrins (bLF), differing in source and iron content, on spontaneous proliferation of human peripheral blood mononuclear cells (PBMC) and cytokine production by human whole blood cultures.

Material and Methods. The following bLF preparations were used: partially iron saturated or devoid of iron bLF from milk and bLF from colostrum. The study was conducted on 12 healthy volunteers (men, 20–24 years old). The effects of bLFs on the proliferation of PBMC in four-day cultures was studied at 50–0.6 µg/mL concentration range and the rate of proliferation was determined using the MTT colorimetric method. TNF α and IL-6 levels, induced by the bLFs in 24 h whole blood cultures, were measured by ELISA.

Results. The lactoferrins stimulated autologous proliferation of human peripheral blood mononuclear cells (PBMC) in a dose-dependent manner, with a comparable efficacy. This stimulation occurred both in the constant presence of bLFs in the cultures and also upon preincubation of PBMC with bLFs with subsequent exhaustive wash of cells. Only bLF from colostrum induced production of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in cultures of whole blood cells. This phenomenon took place predominantly at concentration of 50 µg/mL.

Conclusions. The results showed potent stimulation of the proliferative response of PBMC by bovine lactoferrin, associated with the induction of proinflammatory cytokines only in the case of colostral bLF. This observation may be of importance when high doses of bLF are used in therapy and by designing diet supplementation with this protein (**Adv Clin Exp Med 2014, 23, 6, 871–876**).

Key words: IL-6, proliferation, lactoferrin, TNF-α, autologous PBMC culture.

Lactoferrin is a protein abundant in colostrum and milk as well in the excretory fluids of mammals, constituting an important element of innate immunity [1]. The protein, which is also contained in secondary granules of neutrophils [2], plays a role in iron metabolism [3].

Lactoferrin affects the immune system by promotion of lymphocyte maturation [4, 5] and stimulation of mucosal and systemic immune response [6]. Lactoferrin interacts with a wide spectrum of receptors, distributed in virtually all organs and cell types [7]. They include, among others, mannose receptors [8], toll-like receptors [9], heparan sulfate [10], CD14 [11], nucleolin [12], intelectin [13], sialoadhesin [14] and CD22 [15].

Lactoferrin has been shown to inhibit the *in vitro* mitogen-induced [16, 17] or alloantigen-induced [18] proliferative response of lymphocytes in several experimental models. The proliferative response *in vivo* may, however, be stimulated [6]. Our more detailed studies revealed that the effects of bLF on mitogen-induced PMBC proliferation

are, in fact, immunoregulatory and depend on the individual immune reactivity of healthy volunteers and status of the patient [19–21].

Bovine lactoferrin has been the subject of numerous clinical studies (for review, see [22]) and is used as an ingredient in infant food [23]. It was, therefore, of importance to investigate the effect of lactoferrin on the proliferation of autologous human lymphocytes, a model which has not been studied yet. In addition, the ability of bLF to induce production of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in whole blood cell cultures was determined. For the studies, partially iron saturated and iron-free milk bLFs and colostrum-derived bLF, were applied.

Material and Methods

Reagents

RPMI medium, Hanks' medium with and without Ca^{+2} and Mg^{+2} and LSM Lymphoprep separation medium (1.077 g/mL) were from Cytogen GmbH (Germany). MTT-93-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide derived from Sigma-Aldrich (USA). Fetal calf serum was purchased from Gibco (USA). The cytokines (TNF-α and IL-6) were measured using ELISA kits manufactured by RD Systems (USA & Canada).

Lactoferrins

The following forms of bovine lactoferrin were used: (1) bLF from Tatua Co-Operative Dairy Company, New Zealand; (2) Apolactoferrin (apo-bLF) prepared from bLF (Tatua) as originally described by Mansson; 1% bLF solution in distilled water (100 mg of protein/10 mL water), dialyzed against 20 × volume of 0.1 M citrate acid for 36 h, then dialyzed against distilled water for 3 days at 4°C (3 times in 20-fold water volume) and finally lyophilized. (3) Colostral bovine lactoferrin (cbLF) from Sigma (no. L4765).

Preparation of Peripheral Blood Mononuclear Cells

Venous blood from each of the donors was withdrawn into syringes with sodium citrate and diluted twice with PBS. PBMCs were isolated by centrifugation on a Lymphoprep gradient (density 1.077 g/mL) and centrifuged at 800 × g for 20 min at 4°C. Cells from the interphase, consisting of lymphocytes (20%) and monocytes (80%), were then washed 3 times with Hanks' medium and re-suspended in a culture medium, referred to below as the culture medium, consisting of RPMI-1640 supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics, at a density of 2×10^6 cells/mL.

Preincubation of PBMC with Lactoferrins

PBMCs resuspended in the culture medium, were transferred to a 6-well culture plate (3×10^6 cell//mL) and the respective concentrations of the studied lactoferrins were added (50 μg/mL). The cells were incubated for 1 h at 37°C in a cell culture incubator. After the incubation, the cells were harvested in cold Hanks' medium devoid of Ca^{++} and Mg^{++}. Next, the cells were resuspended in Hanks' containing Ca^{+2}, Mg^{+2} and washed 5 times (centrifuged for 10 min at 1.200 rpm at 4°C). Then, the cells were resuspended in the culture medium at a density of 2×10^6 cells/mL and transferred to a 96-well plate.

Determination of Autologous Cell Proliferation

The PBMCs were distributed into 96-well flat-bottom plates in 100 μL aliquots (2×10^5 cells//well). The lactoferrins were added at a concentration range of 50–0.6 μg/mL. Alternatively, the cells were pulsed with lactoferrin and washed as described above. After a 4-day incubation in a cell culture incubator, the proliferative response of the cells was determined using the colorimetric MTT method. The data is presented as a mean OD value from quadruplicate wells ± SE.

Induction of Cytokines in the Human Blood Culture

Human whole blood from healthy males, 20–24 years old, was diluted 5 × with RPMI 1640 medium and distributed to 24-well culture plates in 1 mL aliquots. After an overnight incubation, the supernatants were harvested and frozen at –80°C until cytokine determination.

Colorimetric MTT Assay for Cell Growth and Kill

Briefly, 25 μL of MTT (5 mg/mL) stock solution was added per well at the end of the cell incubation period and the plates were incubated for an additional 3 h in a cell culture incubator. Then, 100 μL of the extraction buffer (20% SDS with 50% DMF, pH 4.7) was added. After an overnight incubation, the optical density was measured at 550 nm with the reference wavelength of 630 nm in a Dynatech 5000 spectrophotometer.

Statistical Analysis

The results are presented as mean values ± standard error (SE). Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of the difference between groups. Nonparametric data was evaluated with the Kruskal-Wallis analysis of variance, as indicated in the text. Significance was determined at $p < 0.05$. Statistical analysis was performed using STATISTICA 7 for Windows.

Results

Effects of Lactoferrins on Spontaneous Proliferation of Human PBMC

The lactoferrins were added at a concentration range of 50–0.6 μg/mL to the PBMC cultures, incubated for 4 days and the rate of proliferation was measured. The results, presented in Fig. 1, show a representative example of 12 determinations in individual subjects with very similar results. All bLFs displayed similar, dose-dependant stimulatory effects on the proliferation of cells in an autologous system. The strongest effect was observed at 50 μg/mL whereas 2.5 μg/mL was not stimulatory.

In order to check whether pulsing of PMBC with bLFs is sufficient to induce the stimulatory effect on cell proliferation, PBMCs were incubated for 1 h with 50 μg of lactoferrins and washed 5 times. The results (Fig. 2) indicate that only in the case of colostral bLF was the 1 h exposure of PBMC to bLFs sufficient to exert the stimulatory effects on cell proliferation. However, the stimulatory effects were lower in comparison to cultures with constant presence of lactoferrins (Fig. 1).

Effects of Lactoferrins on Production of TNF-α and IL-6 in Whole Blood Cell Cultures

BLF has been shown in the past to induce cytokine production *in vivo* and *in vitro* [19, 24, 25]. Here we determined the effects of colostrum-derived lactoferrin on the production of TNF-α and IL-6 in the whole blood cell cultures of 4 representative individuals (Table 1). The results show that cbLF, at 50 μg/mL induced production of TNF-α

Table 1. Induction of TNF-α and IL-6 by cbLF in human whole blood cell cultures

Donor	TNF-α (pg/mL)		IL-6 (pg/mL)	
	cbLF (μg/mL)		cbLF (μg/mL)	
	50	10	50	10
1	404	–	1761	99
2	154	–	896	30
3	90	–	653	8
4	264	–	1742	80

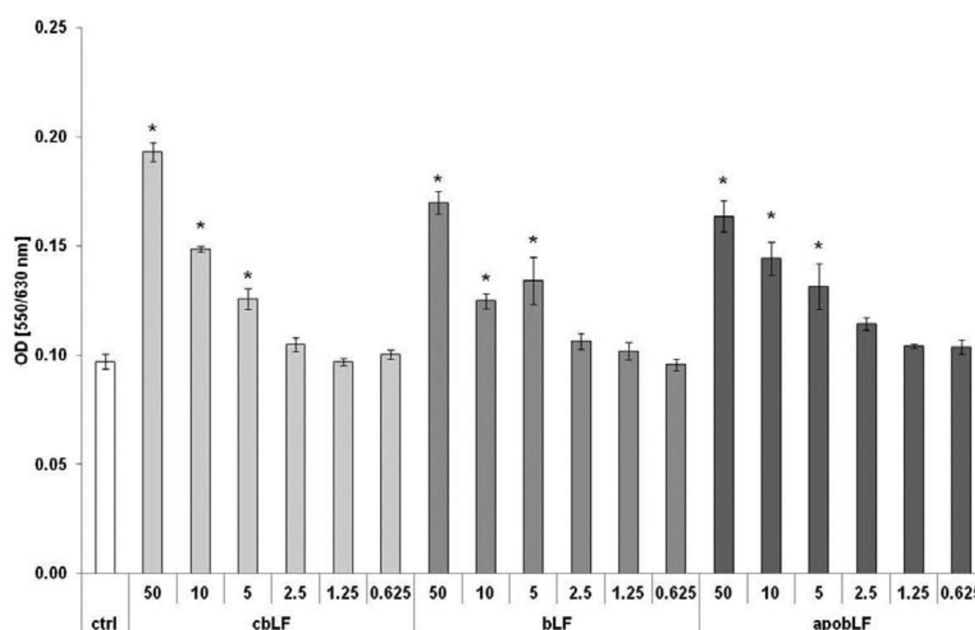

Fig. 1. Stimulatory effects of lactoferrins on proliferation of peripheral blood mononuclear cells (*, $p < 0.05$, when compared with control cultures)

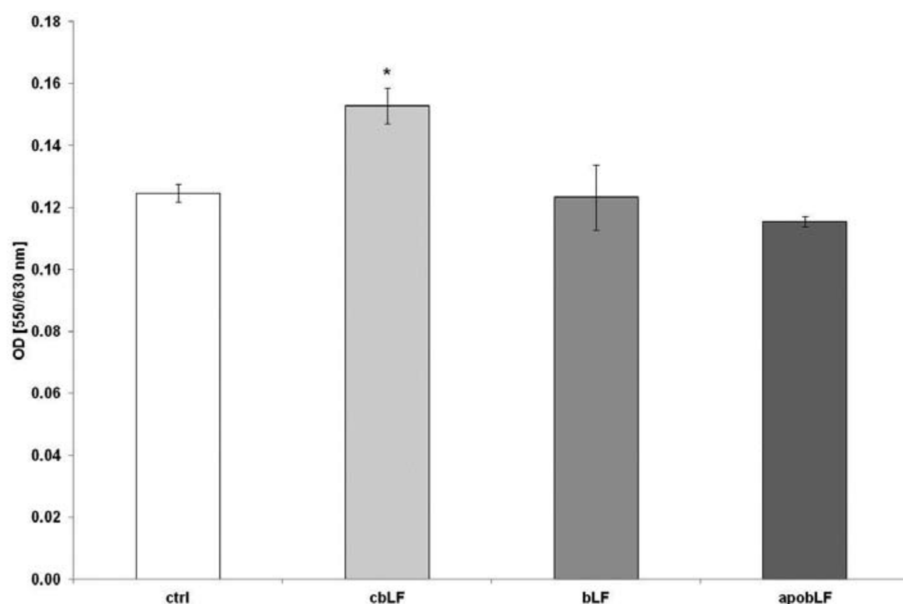

Fig. 2. Effects of preincubation with lactoferrins on lymphocyte proliferation in autologous culture
(* $p < 0.05$, when compared with control cultures)

and IL-6 to various degrees. However, at the concentration of 10 μg/mL only some level of IL-6 was induced. Other lactoferrins were not effective in cytokine induction (not shown).

Discussion

In the present investigation, we showed that bovine lactoferrins, irrespectively of origin and iron saturation, were capable of significantly stimulating the proliferation of PMBCs in autologous cultures. This action was distinctly uniform (stimulatory) in all tested individuals in contrast to the phytohemagglutinin A-induced proliferation of PBMC [20], where the effects of bLF were differential and strongly depended on the individual reactivity of blood donors. The responding cell in the autologous mixed lymphocyte reaction is the $CD4^+$ T cell [26]. Therefore, all effects exerted by lactoferrin on T cells, mainly described as phenotypic changes and induction of signaling pathways, are relevant in this phenomenon. These effects include induction of the CD4 antigen [4] and lymphocyte adhesion molecule-1 [27], which may have significance in interaction with ICAM-1 receptors on accessory cells. Lactoferrin is also able to increase expression of the zeta chain in the CD3 complex, responsible for transmission of the stimulatory signal in T cells [28]. More importantly, lactoferrin was shown to activate mitogen-activated protein kinase in Jurkat cells [29], the action revealing a triggering of the signaling pathway leading to activation of T cells. In addition, other actions of lactoferrin described may contribute to increased cell proliferation, involving inhibition of apoptosis [30] and the property of scavenging harmful hydroxyl radicals [31]. Effective stimulation of cell proliferation in the case of pulsing of the cell with cbLF (Fig. 2) supports the notion that a short time signal delivered by lactoferrin may be sufficient for T cells to initiate proliferation. It also appeared that the presence of iron in the lactoferrin molecule did not affect the degree of cell proliferation, indicating that the conformation of the lactoferrin molecule was without effect on the interaction of a bLF ligand with the respective cell receptor. In this particular case, the receptor for bLF may bear a resemblance to that present on the surface and inside Jurkat and stimulated human lymphocytes [32].

The results also confirmed our previous findings on the induction of TNF-α and IL-6 in whole blood cultures [25]. The induction of TNF-α could represent an additional stimulus for cell proliferation as reported elsewhere [33] and account for a stronger induction of cell proliferation. Of interest, the induction of cytokines was only observed in the case of colostrum-derived lactoferrin. This phenomenon could be associated with a different pattern of glycosylation in cbLF as compared with milk-derived lactoferrin. Generally, colostral proteins are highly sialylated as compared to milk-derived proteins [34]. Lactoferrin isolated from bovine colostrums is a glycoprotein carrying complex and high-mannose type glycans [35]. Therefore, a putative candidate for the interaction of bLF is siglec7, present on monocytes. This receptor is responsible for activation of cells for cytokine production, including TNF-α and IL-6, by ligands terminated by sialic acid. Nevertheless, it seems that the signals leading to cell proliferation and cytokine production are separate since milk--derived lactoferrins, not able to induce cytokine production, were equally effective in inducing cell proliferation.

In summary, this is the first demonstration of a potent stimulation of the proliferative response of PBMC in autologous culture by bovine lactoferrin. This observation should be taken into account when high doses of bLF are used in therapy and by designing diet supplementation with this protein. The results of this study may also explain the strong stimulatory effects of oral bLF on the response of the gut-associated lymphoid system.

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