



Lactoferrin Increases the Output of Neutrophil Precursors and Attenuates the Spontaneous Production of TNF- α and IL-6 by Peripheral Blood Cells

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Abstract. The aim of this report was to investigate the effects of bovine lactoferrin (BLF) taken orally (*per os*) by healthy individuals, on selected immune parameters. Three groups of volunteers (7 persons per group) were taken daily for 7 days, one capsule containing 2, 10 or 50 mg of BLF. A control group has taken placebo only. Venous blood was taken for tests a few hours before the first dose of BLF, one day and 14 days after the last dose of the preparation. For the evaluation of BLF action on the immune response system we have chosen 3 parameters: content of neutrophil precursors in the peripheral blood (in percentage), spontaneous production of interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α) by unstimulated blood cell cultures. We found that oral treatment of volunteers with BLF caused a transient (one day after last dose) increase of immature forms of neutrophils in the circulating blood. That increase was more than 2-fold in the case of 10 mg dose. However, statistically significant increases in the percentage of neutrophil precursors were also registered at doses of 2 and 50 mg of BLF. No change in the immature cell content was observed in the placebo group. The treatment with BLF also resulted in a profound decrease of the spontaneous production of IL-6 and TNF- α by cultures of peripheral blood cells. This decrease was significant (10 mg/dose) one day following the last dose of BLF and persisted for additional 14 days. These results confirmed our earlier data on the effects of *per os* treatment with a nutritional preparation containing BLF. Furthermore, we were able to closer establish the optimal dose of BLF affecting selected immune indices.

Key words: bovine lactoferrin; blood cells; neutrophil precursors; interleukin 6; tumor necrosis factor α .

Introduction

Despite enormous progress in modern medicine, immunology and pharmacology, serious problems associated with high mortality and morbidity in intensive care units remain unsolved. Postoperative complications usually result from an inadequate function of the immune system caused by certain immune abnormalities.

Lack of the immune response to surgery, or its excessive values are usually undesirable^{2, 21}. Attempts to alleviate uncontrolled immune response in patients by application of antipolysaccharide or anticytokine treatments proved unsuccessful^{10, 12}. We confront another challenge in autoimmune patients, including rheumatoid arthritis¹⁴ where the balance between activities of two major T helper cell subsets is disturbed. Synthetic

antiinflammatory compounds can diminish clinical manifestation of the disease, however often significant toxicity associated with such a therapy, prevents from common use of those compounds.

It is, therefore, a great interest to develop a new modality that would apply natural immunomodulators which are constitutive parts of our physiological system. In our laboratory we have studied potential clinical application of an extract from calf thymus¹⁸ and a proline-rich polypeptide from ovine colostrum^{16, 23}. Recently, we also turned our attention to lactoferrin (LF), an iron-binding protein contained in milk and secretory fluids of mammals (for review see⁵). Specific receptors for LF have been described on many cell types including brush border cells¹⁵ and monocytes¹⁷. LF was shown to exhibit antibacterial, antiviral, antifungal, antiparasite and antitumor properties^{3, 22, 24, 25, 29}. We and others have demonstrated its effects on maturation of lymphocytes^{32, 35} and cytokine production^{5, 7, 36}. More recently we concentrated our research on potential application of LF in prevention and therapy. BLF was found to attenuate surgery-elicited cytokine production in mice³⁹, and modified selected immune parameters in surgery, trauma and septic patients *in vitro*^{1, 30}. BLF is frequently found as an ingredient of commercially available nutritional products. These products are recommended in cases of malnutrition, impaired iron metabolism or improper colonization of the intestinal flora^{6, 20}. Using one of those nutritional products, Nutrifemme, containing in addition to BLF a number of antioxidants, we found that the preparation, given orally (*per os*), affected several immune parameters in healthy volunteers³⁸. Two parameters were preferentially altered – the output of neutrophil precursors into circulation and the ability of blood cell cultures to spontaneously produce IL-6 and TNF- α . Although we regarded BLF to be solely responsible for the observed immunoregulatory effects, a possible influence of other antioxidants such as selenium, dismutase, vitamins E and C could not be excluded.

Therefore, the aim of this study was to confirm the role of BLF, taken orally, in modifying selected immunological parameters. In addition, by using 3 different doses of BLF, we attempted to establish a dose of BLF changing most profoundly the level of neutrophil precursors and spontaneous cytokine production.

Materials and Methods

Bovine lactoferrin was provided by FerroDynamics Inc. (Houston, TX 77042, USA). It was essentially LPS-free as determined by *Amebocyte limulus* assay.

The BLF was admixed in capsule with lactose. Placebo capsules contained lactose only.

Treatment of volunteers with BLF. Twenty seven healthy volunteers (11 men and 17 women, age 25–55 years) were divided into 4 groups consisting of 7 individuals each. They were taken *per os* 1 capsule daily, for 7 days, containing 1) placebo (lactose), 2) 2 mg BLF, 3) 10 mg BLF, and 4) 50 mg of BLF. Venous blood was withdrawn into heparinized tubes (5 ml) from each patient at: 1) time 0 (a few hours before the first dose), 2) 1 day after taking the last dose and 3) 14 days following the last dose of BLF.

Preparation of blood cell cultures. Heparinized blood was diluted with RPMI-1640 culture medium to achieve a concentration of 10^6 cells/ml. The cells were distributed in 2 ml aliquots to 24-well culture plates and cultured overnight in a cell culture incubator. The supernatants were harvested and used for cytokine determination.

Determination of IL-6 activity. The assay was performed according to VAN SNICK et al.²⁸. Briefly, 7TD1 indicator cells were washed 3 times with Hanks' medium and resuspended in Iscove's medium supplemented with 10% FCS, HEPES buffer, glutamine and antibiotics to a density of 2×10^4 cells/ml. Then, the cells were distributed in 100 μ l aliquots into 96-well flat-bottom plates containing 100 μ l serially diluted plasma or supernatant in triplicate. After 72 h of culture the proliferation of 7TD1 cells was determined using the MTT colorimetric method¹⁶. The results of IL-6 activity are presented in pg per ml – such concentration of IL-6 corresponds to the activity of IL-6 expressed in U/ml²⁸. One unit of IL-6 activity was calculated as the inverse dilution of a plasma sample where a half-maximal proliferation of 7TD1 cells was registered.

*Determination of TNF- α activity*⁹. For determination of TNF- α activity the indicator clone WEHI 164.13 was used. The cells were washed 3 times with Hanks' solution and resuspended in RPMI 1640, supplemented with 10% FCS, glutamine and antibiotics at a concentration of 2×10^5 /ml. The cells were then distributed into 96-well, flat-bottom plates (2×10^4 /well). Serially diluted plasma or supernatant samples were prepared on separate plates and transferred to microtiter plates containing WEHI 164.13 indicator cells. The medium contained in addition 1 μ g/ml of actinomycin D to increase sensitivity of the assay. After an overnight incubation the survival of cells was determined using MTT colorimetric assay¹³. The results of TNF- α activity are presented in pg/ml where 10 pg of TNF- α correspond to 1 U of activity when tested a recombinant human TNF- α (kindly provided by Prof. W. Stec,

Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Łódź, Poland). One unit of TNF- α was calculated as an inverse dilution of a given plasma sample where 50% survival of WEHI 164.13 cells took place.

Colorimetric determination of cells proliferation/death. The assay was performed according to HANSEN et al.¹³. Briefly, MTT solution, 5 mg/ml in 0.9% NaCl, was added in a volume of 25 μ l/well and incubated for 2–4 h. Then, 100 μ l of a lysing buffer was added (20% SDS, 50% DMF, pH 4.7). After on overnight incubation at 37°C the optical density (OD) was measured using ELISA reader Dynatech 5000, at the wavelength of 550 nm and reference wavelength of 630 nm.

Morphology of blood cells. A drop of heparinized blood was applied onto a microscopic slide and a smear was made. After drying, the preparation was treated with May-Grünwald reagent and then with Giemsa reagent. The cells were counted (differentiated) at a magnification of 800 $\times$ in an immersion oil. Up to 200 cells were counted. The results are presented as a percentage of immature forms of neutrophils.

Statistics. The results were evaluated using Student's *t*-test. The results are presented as a mean values from 7 determinations (individuals) $\pm$ SE. The differences were regarded significant when $p > 0.05$.

Results

Elevation of immature neutrophil number in the peripheral blood of individuals treated with BLF

Previous study on volunteers, taking a nutritional preparation containing BLF orally, showed that such a treatment resulted in a 2-fold increase in the level of neutrophil precursors in circulation³⁸. In this study we presented (Table 1) that treatment of volunteers with various concentration of BLF caused a transient, statistically significant increase in immature neutrophil levels. The highest output of neutrophil into circulation was achieved for patients taking 10 mg BLF/dose. No significant changes in the levels of this cell type were registered in persons receiving placebo only (lactose-containing capsules).

Effect of BLF treatment on the spontaneously produced IL-6 and TNF- α by blood cell cultures

Our recent study on volunteers, taking the nutritional preparation containing BLF orally, showed that following that treatment the ability of peripheral blood cells to spontaneously produce cytokines in culture was

Table 1. Percentage of immature neutrophil forms in the peripheral blood of healthy volunteers taking orally bovine lactoferrin (BLF)

Group	Before treatment	1 day after last dose	14 days after last dose
Placebo	4.0 $\pm$ 0.0	4.7 $\pm$ 0.55 NS	4.1 $\pm$ 0.2 NS
BLF 2 mg	3.7 $\pm$ 0.5	6.4 $\pm$ 0.6 $p < 0.02$	4.1 $\pm$ 0.05 NS
BLF 10 mg	3.9 $\pm$ 0.5	8.6 $\pm$ 0.53 $p < 0.01$	5.6 $\pm$ 0.4 NS
BLF 50 mg	3.4 $\pm$ 0.3	5.7 $\pm$ 0.4 $p < 0.05$	4.7 $\pm$ 0.7 NS

The volunteers were taking BLF capsules daily for 10 days. The percentages of immature neutrophil forms were determined in blood smears before, 1 day, and 14 days after last dose of BLF. The number of individuals per group = 7.

NS – not significant.

Table 2. Spontaneous production of TNF- α by peripheral blood cultures of healthy volunteers taking orally bovine lactoferrin (pg/ml)

Group	Before treatment (1)	1 day after last dose (2)	14 days after last dose (3)	p
Placebo	16.8 $\pm$ 2.8	14.4 $\pm$ 2.27	19.3 $\pm$ 2.9	NS
BLF 2 mg	20.5 $\pm$ 1.2	15.7 $\pm$ 0.91	14.7 $\pm$ 3.5	NS
BLF 10 mg	26.1 $\pm$ 2.03	4.6 $\pm$ 1.4	3.0 $\pm$ 0.9	1:2 <0.001 1:3 <0.001
BLF 50 mg	14.5 $\pm$ 3.7	9.0 $\pm$ 3.0	5.4 $\pm$ 1.5	1:2 NS 1:3 <0.001

Spontaneous TNF- α production was determined in 24 h whole blood cultures using the indicator cell line WEHI 164.13.

The significance of the BLF effects after 1 day and 14 days following the last dose was calculated as compared to the initial TNF- α values (1:2 or 1:3)

Table 3. Spontaneous production of IL-6 by peripheral blood cultures of healthy volunteers taking orally bovine lactoferrin (pg/ml)

Group	Before treatment (1)	1 day after last dose (2)	14 days after last dose (3)	p
Placebo	24.6 $\pm$ 6.9	29.3 $\pm$ 1.6	34.1 $\pm$ 3.7	1:2:3 NS
BLF 2 mg	32.5 $\pm$ 3.5	26.3 $\pm$ 5.2	19.5 $\pm$ 7.6	1:2 NS 1:3 NS
BLF 10 mg	60.5 $\pm$ 6.2	5.8 $\pm$ 3.5	2.1 $\pm$ 0.2	1:2:3 <0.001
BLF 50 mg	25.5 $\pm$ 5.2	16.9 $\pm$ 6.8	1.6 $\pm$ 0.3	1:2 NS 1:3 <0.001

Spontaneous IL-6 production was determined in 24 h whole blood cultures using the indicator cell line 7TD1.

The significance of the BLF effects after 1 day and 14 days following the last dose was calculated as compared to the initial IL-6 values (1:2 or 1:3)

decreased. The results depicted in Table 2 and 3 of this paper demonstrate that the treatment of individuals with BLF alone also significantly diminishes the ability of cells to produce IL-6 and TNF- α . The most pronounced

inhibitory effect correlates with the highest output of neutrophil precursors in the group taking 10 mg BLF/day. The dose of 2 mg caused some, not statistically significant, decreases in the spontaneous production of cytokines. The response to BLF in persons taking 50 mg/day seems to be similar to 10 mg/dose.

Discussion

One of the great advances of modern immunology is the recognition that normal immune homeostasis depends on co-ordinated interactions among the various immune cells. The balance is achieved largely through intracellular communication mediated by a network of cytokines. The production of this highly diverse group of small molecular weight proteins is further controlled by many constituents of the phagocytic cells. Lactoferrin is one of those constituents that is released from the activated neutrophils and plays an important role in a feedback mechanism of inflammatory responses¹⁹. Although, lactoferrin is often discussed as a mediator of various insult-induced metabolic imbalances, its potential immunoregulatory function has been severely underestimated. In general, lactoferrin is considered as an antimicrobial factor rather than a systemically acting immunomodulator.

In this communication we demonstrated that BLF, taken orally, can significantly alter the immune responses of persons by elevating the percentage of immature neutrophil forms and decreasing the ability of blood cells to spontaneously produce IL-6 and TNF- α . The ability of BLF to increase the turnover of neutrophils shown, in this study, was comparable to that described previously for Nutrifemme, a nutritional supplement containing BLF³⁸.

It has been suggested that a more rapid turnover of neutrophils is triggered by LF released from degranulating neutrophils following infection or after treatment with LF which simulates infection^{4, 19}. This is associated with a transient decrease in neutrophil number^{4, 38}. Our unpublished observations showed that in the circulation of septic, non-surviving patients, the level of neutrophil precursors may attain as much as 30%. At the same time the level of released lactoferrin was up to 10 times higher as compared with the physiological concentration. Therefore, it seems logical that ingestion of LF by healthy subjects will lead to an increased turnover of neutrophils. Also, this is indirectly, a confirmation of the finding that about 10% of orally ingested LF penetrates the circulation in an intact (not digested) form^{25, 27}.

The main observation of this report was, however, a very profound inhibition of the spontaneous cytokine

production by cells isolated from BLF-treated individuals. The ability of BLF to suppress spontaneous production of cytokines was much higher when compared with the Nutrifemme study³⁸. This could be attributed to the different regiment treatment, type of BLF (different source) or final composition of the medication.

The spontaneous production of cytokines may be elicited by minor non-specific¹¹ and specific stimuli³⁷. It is usually 10–20 $\times$ lower as compared with LPS-induced cytokine production in PBMC cultures^{1, 30}. Explanation of the mechanism, by which orally taken BLF causes suppression of the spontaneously released cytokines, may be at present only speculative. Most likely, BLF by induction of TNF- α ^{1, 30} and other cytokines⁵ may modulate expression of cell receptors responsible for recognition³⁷, adhesion³³ and activation³⁴ of cells. In addition, LF is able to activate cells in a manner similar to that of mitogens⁸. Thus, repeated treatment with BLF may induce a state of hyporeactivity. However, when we studied the LPS-induced cytokine production in BLF-treated individuals we only observed a regulatory effect of BLF³⁸. These differences may result from the nature of eliciting signals (cell-to-cell, MHC-restricted interactions versus mitogen action).

Nevertheless, the resultant effect of BLF treatment seems to be beneficial for the function of the immune system since it increases by several fold the ratio between LPS-induced versus spontaneous cytokine production³⁸. In other words, the relative cell response to exogenous stimuli is stronger. Such a property of the immune system cells would be particularly relevant in an adequate immune response to surgery which is essential to combat potential infections and to accelerate the wound healing process.

In summary, the data presented herein provide essential information with regard to optimal treatment for clinical patients who would benefit from the immunoregulatory properties of lactoferrin. Since only isolated studies described lactoferrin oral effects on phenotype and activity of blood cells³¹ or preventive effects in neutropenic patients²⁶, further studies are clearly needed to elucidate the mechanism by which lactoferrin modulates the immune system.

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