



Review

Lactoferrin and Immunologic Dissonance: Clinical Implications

“True teaching is not an accumulation of knowledge;
it is an awaking of consciousness which goes through successive stages”

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Abstract. Homeostasis is the maintenance of equilibrium in a biological system by means of positive and negative feedback control mechanisms that counteract influences tending toward physiological dissonance. At the molecular level, homeostasis is controlled by the network of the neuro-endocrine-immune system, in which lactoferrin (LF) plays a central role. The purpose of this review is to provide a comprehensive summary of a collaborative study established between the Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland) and the University of Texas Health Science Center (Houston, USA) regarding LF and its role in homeostasis. In our studies we focused on the immunoregulatory functions of LF, both *in vitro* and *in vivo*. We investigated the immune status of individuals subjected to different insults, including experimental endotoxemia in mice and surgery in humans. We also studied a LF-dependent delayed type hypersensitivity (DTH) response to evaluate some of the mechanisms by which LF can effectively substitute an adjuvant in vaccine.

Key words: lactoferrin; inflammation; immunomodulation; oxidative stress; anti-aging.

Introduction

Homeostasis is normally dependent upon coordinated interactions among the various lymphoid, phagocytic and somatic cells that comprise the immune system. In general, these interactions are tightly regulated to obtain a balance between the mechanisms necessary to eliminate harmful pathogens, and to control overaggressive responses which can lead to the destruction of

host tissue. A systemic response to insult is mediated via the macrophage-derived cytokines that target end-organ receptors in response to injury or infection. Cytokines are primary or secondary mediators. Primary mediators are tumor necrosis factor α (TNF- α), interleukin 1 (IL-1), IL-6 and IL-8. These mediators stimulate the release of secondary mediators, including prostaglandin E₂ (PGE₂), platelet-activating factor, vasoactive peptides, such as bradikinin, angiotensin, and

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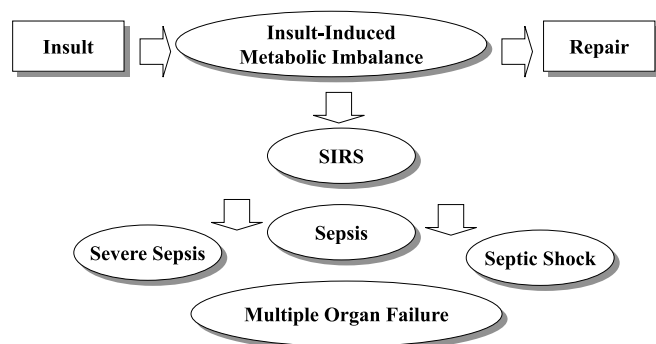


Fig. 1. Progression of insult-induced metabolic imbalance. The clinical picture of sepsis is a progression through 4 syndromes developing from an initial insult-induced metabolic imbalance: systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock. The septic conditions directly link the multiple organ failure

vasoactive intestinal peptide, and various complement-derived products. Through complex signaling, a positive feedback circuit is established, which in normal conditions, amplifies and sustains the activity of the inflammatory responses^{9, 17, 47, 67}. However, if these responses are activated in an uncontrolled fashion with dissemination via the circulation, organs distant from the initial insult can be affected over a period of time. Endothelial damage by endotoxemic shock and sepsis results from persistent and repetitive inflammatory insults. Eventually, these insults produce so much damage that down-regulation can no longer occur. This leads to a state of metabolic anarchy, known as multiple organ failure, in which the body can no longer control its own inflammatory responses, progressing into a self-perpetuating, generalized state of hyper- or hypo-activity⁹ (Fig. 1).

The role of cytokines in septic shock has been investigated in several animal species including mice, rats, rabbits, pigs and dogs. It has been proposed that gut-barrier failure and the subsequent translocation of enteric bacteria and endotoxin is a major contributing factor in the development of sepsis^{16, 41, 62}. Thus, the gastrointestinal tract may provide a source of inflammatory mediators for release into systemic circulation, stimulating other inflammatory cells, such as neutrophils, to become activated as well. Cytokines clearly play an important role in sepsis, and there is evidence that in the systemic cascade of inflammatory responses to lipopolysaccharide (LPS), the gut-associated lymphoid tissue produces and liberates more pro-inflammatory cytokines which further stimulate the translocation of enteric bacteria to distant sites^{15–17, 48}. How these agents interact is far from understood, but it is clear that

they create a complex, often paradoxically counteracting network of processes. Some interactions seem to augment host responses to infection. Interferon γ (IFN- γ) and TNF- α , for example, can enhance the phagocytic activity of neutrophils⁹. Other interactions appear to limit inflammation or restore homeostasis. The cycle in which TNF- α prompts prostaglandin inhibitor 2 (PGI₂) release and then PGI₂ suppresses further TNF- α synthesis is one example⁵². Another is the process by which IL-8 decreases neutrophil adhesion to the endothelium¹⁸. The inflammatory mediators have multiple overlapping effects which together create an elaborate web of reactions designed to limit new damage and ameliorate whatever damage has already occurred. Therefore, it is not surprising that clinical trials aimed at downregulating individual mediators have proved to be disappointing²⁹. Specific therapies against TNF- α involving antibodies^{1, 12, 13, 19, 24, 55} or soluble TNF receptor²² were as unsuccessful as blocking IL-1^{23, 25, 49} or other mediators that have been recognized as significant players in a pro-inflammatory cascade⁵. Indeed, most trials addressed the pro-inflammatory phase of systemic inflammation in the belief that the anti-inflammatory mediators would automatically be adjusted upon modulation of their immunologic response precursors. However, evidence indicates that the immunologic dissonance arising from the development of systemic inflammation is so complex that careful modulation of both pro- and anti-inflammatory mediators is required^{63, 74, 83, 84}. Moreover, it requires an accurate measure of immune organ function in an individual subjected to the immune therapy; thus, treatment should be “custom made”, only for those patients who respond inadequately to an insult.

LF and Insult-Induced Immunologic Dysregulation

Lactoferrin (LF), an iron-binding glycoprotein, is considered an important mediator in the host defense against environmental insults in mammals. Lactoferrin is produced continuously by the secretory epitheliums, where it functions as an antimicrobial agent, and the granulocytic cells, namely neutrophils, where it plays an important role as a mediator of inflammatory responses. Because of its high concentration in human colostrum, LF has been studied extensively in host defense responses in infants^{10, 28}. It is theorized that LF within human milk provides protection against pathogens during a newborn's adaptation to non-uterine life and plays a role in rendering breast-fed infants more

resistant to the development of microbe-induced gastroenteritis (compared to formula-fed babies)¹⁰. The significance of LF in health and disease has been the subject of several reviews^{39, 59}. Lactoferrin has well-defined, direct antimicrobial activity, including an iron-dependent bacteriostatic property and bacteriocidal action on LPS-bearing Gram-negative bacteria^{8, 63, 70, 71}. The ability of LF to bind large quantities of iron may also provide protection against pathogens and their metabolites by enhancing phagocytosis and cell adhesion and controlling the release of pro-inflammatory cytokines^{3, 4}. Lactoferrin has a profound modulatory action on the immune system^{75, 79, 81, 82}, it promotes the maturation of T cell precursors into immunocompetent helper cells and the differentiation of immature B cells to efficient antigen-presenting cells⁷⁷. Lactoferrin upregulates the expression of leukocyte function-associated-1 (LFA-1) antigen on human peripheral blood lymphocytes⁷⁵. In addition, LF augments the delayed type hypersensitivity (DTH) response to specific antigens and is capable of inducing cell-mediated immunity (CMI) in mice^{2, 73}. Finally, LF is an integral part of the cytokine-induced cascade during insult-induced metabolic imbalance^{30–34, 72} (Fig. 2).

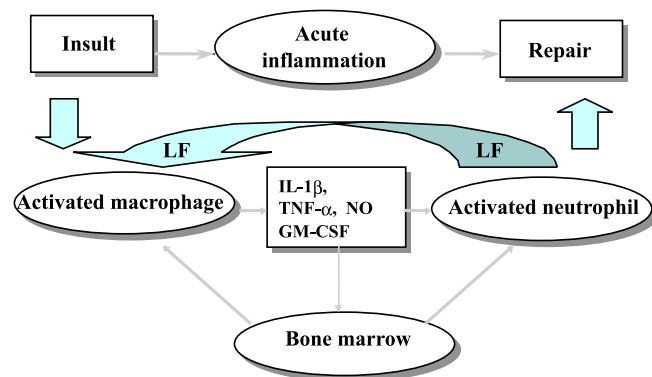


Fig. 2. Modulatory effects of lactoferrin (LF) on outcomes of acute inflammation. Insult, defined by infection, toxic mediators (LPS) or trauma, leads to activation of the monocyte/macrophage system and stimulates the production of IL-1 β , IL-6, TNF- α , GM-CSF and NO, which in turn activates circulating neutrophils and stimulates the production of fresh neutrophils and monocytes/macrophages from the bone marrow. Activated neutrophils degranulate at the site of injury and release massive amounts of secondary mediators, including LF. By binding to the specific receptors on monocytes, LF attenuates the production of cytokines, which reduces the production and activation of fresh monocytes and neutrophils

So far, several cell surface receptors have been implicated in lactoferrin's unique properties, including those on macrophages⁶⁶, lymphocytes⁴⁴, platelets³⁷, hepatocytes⁵⁶ and intestinal cells¹⁴. The characteristics of LF specific cell surface receptors appear to vary among

different organs⁶⁰. Unlike transferrin, LF and its receptor are not internalized by the cell⁵⁷. Transepithelial transport of LF occurs either through a degradative pathway⁴⁵, in which most of the LF is degraded and iron is released intracellularly, or the immunoreactive LF passes through the epithelium with its iron still bound. Recently, LF has been associated with the onset of Alzheimer's disease (AD), demonstrating an active role in the clearance of amyloid β through the low-density lipoprotein receptor-related protein (LRP)⁵¹. The biological role of LF and its specific receptors in modulating both acute and chronic inflammation is still under active investigation.

Although, LF is considered an important component of the nonspecific host defense system against various pathogens, its concentration in blood is normally low (0.2–0.6 $\mu\text{g/ml}$), and increases only transiently upon insult-induced activation of neutrophils²⁰. In fact, a high level of LF in plasma has been suggested as a predictive indicator of sepsis-related morbidity and mortality⁶. In addition, progression in chronic inflammatory disorders, such as AD, or autoimmune disorders, such as multiple sclerosis; is not interrupted by LF elevation in various physiological fluids^{27, 50}. Although the endogenous production of LF is increased in these disorders, it is either not sufficient or does not trigger the pathway(s) of molecular events to aid a defense system against the disorder. It is hypothesized that the initial up-regulation of LF during acute inflammation is directed at capturing free iron, which would be a protective response against the damaging function of free radicals. In chronic inflammations, such as AD and other neurodegenerative diseases, it is suggested that LF, accumulating around specific lesions, reduces the neurotoxic effects of such lesions or deposits (e.g. amyloid β -plaques). A question that arises is why this abnormal LF level often exacerbates and amplifies local inflammation²¹. Again, we have to take into consideration that chronic disorders develop over a long period of time, during which LF successively protects against oxidative stress by scavenging free iron. When for any reason (e.g. natural aging, environmental conditions or genetic make-up) metabolic imbalance progresses, LF is not able to compensate the growing immunologic dissonance, but it can reduce some of the toxic effects associated with such chronic conditions. This problem perhaps best illustrates the fact that, although LF is certainly at the center of these overlapping effects, it is not the only one to be involved in the regulation of homeostasis. Therefore, it would be difficult to postulate LF as a single-agent therapy for any chronic inflammatory disorder.

LF and Iron-Dependent Oxidative Homeostasis

As mentioned before, by virtue of iron sequestration, LF can control the physiological balance between reactive oxygen species (ROS) production and the rate of their elimination, which naturally protects against oxidative cell injury⁶⁴. 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 defense against infection^{43, 46, 58}. Although it is known that oxidant species are produced during metabolic reactions, it is largely unknown which factor(s), of physiological or pathophysiological significance modulate their production *in vivo*. Under normal physiological conditions, the rate and magnitude of reactive oxidant formation is dependent on the availability of the transition metals. In particular, traces of iron can be detrimental to physiological processes under reactive oxygen conditions. Indeed, iron is at the center of ROS control. It has the ability to catalyze the two step process known as the Haber-Weiss reaction (Fig. 3). In the first reaction a superoxide molecule reacts with ferric (Fe^{3+}) salt to form ferrous (Fe^{2+}) salt and ground-state oxygen. The second reaction is known as the Fenton reaction. In this reaction ferrous (Fe^{2+}) salt reacts with hydrogen peroxide to form ferric (Fe^{3+}) salt, the hydroxyl radical and alcohol. The formation of the hydroxyl radical via the iron-dependent Haber-Weiss reaction has been implicated in phagocyte microbicidal activity and lipid peroxidation. Reactive oxygen species, in particular the hydroxyl radical, can react with all biological macromolecules (lipids, proteins, nucleic acids and carbohydrates). Among the more susceptible targets are polyunsaturated fatty acids. Abstraction of a hydrogen atom from a polyunsaturated fatty acid initiates the process of lipid peroxidation, and the intermediates, such as hydroxyalkenals (HNE), become new radicals, which have the ability to induce functional changes in many biologically important macromolecules⁵⁴. These changes, often toxic to an organism^{7, 38}, can be reduced by glutathione transferase¹¹, an enzyme that offers a major detoxication pathway which protects cells and tissues (Fig. 3).

In normal physiologic conditions, the production and neutralization of these ROS depend on the efficiency of key enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). In the presence of Fe^{2+} , hydrogen peroxide (H_2O_2), which is the major ROS intermediate, is converted in a spontaneous reaction to the highly reactive hydroxyl radical ($\cdot\text{OH}$). The Fe^{3+} produced in this pro-

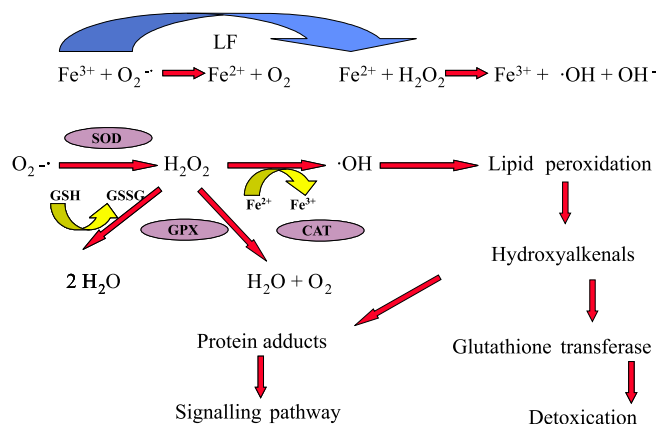


Fig. 3. Reactive oxygen species (ROS) in iron-dependent oxidative stress. At normal physiologic conditions superoxide will spontaneously dismutate and produce hydrogen peroxide. In the presence of free iron, hydrogen peroxide can be reduced to highly reactive hydroxyl radical. These reactions are regulated not only by metal chelators, such as lactoferrin (LF), but also by the oxidative stress-controlling enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). A fine balance between ROS and antioxidants is required to maximize the often beneficial effects of ROS production (e.g. the microbicidal effect of neutrophils)

cess is coordinated by LF and is safely transported to the macrophages of the reticulo-endothelial system, where it can be stored in ferritin.

The production and control of reactive oxidants are integral life processes very important for species survival. If the process of neutralization of ROS is not efficient, it can contribute to development of oxidative stress. As shown above, endogenous LF participates in these processes at the cellular level, though it is not understood how exogenous LF contributes to these molecular events (Fig. 3). We can only speculate that exogenous LF can transduce signaling pathways different from those of the endogenous molecule. Consequently, the therapeutic end effects could be different.

LF and Gut Mucosal Integrity

The clinical recognition that the gut may be a reservoir for bacteria causing systemic infections in intensive care unit patients prompted researchers to propose that the gut is the “motor” of multiple organ failure^{16, 41}. In this hypothesis, enteric bacteria or endotoxin serve as triggers to initiate, perpetuate or exacerbate the septic state. Consequently, several investigators have attempted to reduce the incidence of systemic infections in critically ill patients by oral antibiotic therapy, known as “selective gut decontamination”⁴⁰. Although the results of these studies are encouraging in some

patients (low to moderate risk), in others the therapy failed to improve survival. It is possible that the failure of such therapy in high-risk patients was due to the profoundly immunocompromised state of these patients, whose intestinal barrier function was lost to endotoxin and bacteria as well. In a way this was an inspiration for us to test lactoferrin's ability as both an antibiotic as well as accessory immunomodulator.

In our studies we initially examined the protective role of LF during the development of endotoxemia in mice. It was our goal to understand the molecular mechanisms underlying LF-induced survival during endotoxemia. Although no reports have shown toxicity to LF⁶⁹, we undertook baseline studies to investigate the influence of orally administered LF on intestinal function and structure in the absence of systemic inflammatory response syndrome (SIRS). Naive mice were gavaged daily for 21 days with human LF (5 mg, morning administration); control counterparts were given saline. The mice were monitored for food consumption and cumulative weight gain (Fig. 4), with no significant differences between the LF groups and the controls. Identical results were obtained using doses ranging 1–5 mg (not shown).

Histological analysis of jejunal epithelium showed that LF was able to protect against tissue damage occurring during LPS-induced endotoxemia. As published elsewhere³¹, the intestinal epithelium of mice injected with LPS exhibited severe vacuolar degeneration with shortening and swelling of the villi and elongation of the crypts. In the LF-treated mice the vacuolar degeneration was less pronounced, with the epithelium resembling the highly polarized absorptive epithelium. The reduction in LPS-induced gut injury in mice treated with LF suggests that the protection of intestinal structures may play an important role in the survival of septic mice. The jejunal segments were also examined for changes to mucin production as related to LF treatment. Administration of LF during LPS-induced endotoxemia caused a dramatic increase in the number of mucin producing cells compared with mice given LPS (Fig. 5). Quantitation of stained mucin also revealed a greater production of mucin per cell upon treatment with lactoferrin. Of interest, LF alone (in the absence of LPS-endotoxin signal) caused enhanced mucin production compared with saline intraperitoneal injections. In these initial studies we also evaluated the effects of intraperitoneal administration of LF on behavioral changes due to endotoxemia. LPS-treated mice quickly become hypothermic, whereas those pretreated with LF showed a less pronounced drop in body temperature. Development of hypothermia in LPS-treated mice was

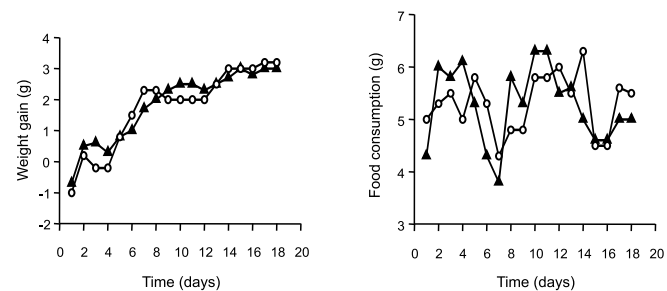


Fig. 4. Cumulative food consumption and body weight. No adverse effects in cumulative food consumption and body weight gain following oral gavage with LF. Mice were orally administered 5 mg LF (circle), or saline as a control (triangle), for 21 days. Body weight (left) and food consumption (right) were monitored. No significant differences were demonstrated from the control saline groups, and no difference due to dosage ranging 1–5 mg LF. Mean results are shown

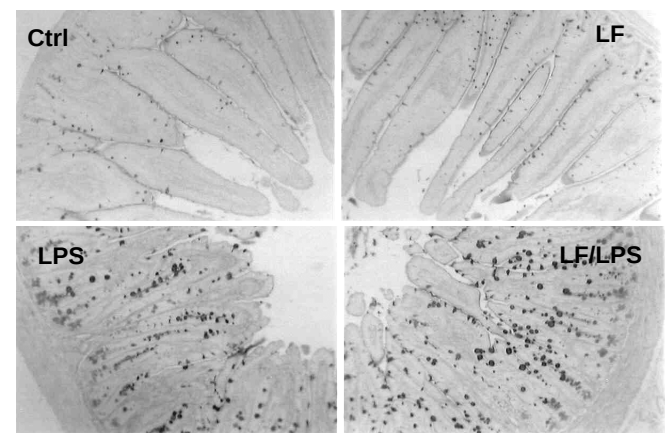


Fig. 5. Increased mucin production by lactoferrin (LF). Sections were stained with hematoxylin and eosin and with periodic acid/Schiff to visualize the brush border and epithelial mucin. Quantitation of stain used software controlling a Nikon Optiphot microscope. The number of positive cells in parentheses Ctrl = saline (242); LF = 5 mg lactoferrin (595); LPS = LPS-induced endotoxemia (895); LF/LPS = lactoferrin (5 mg) given 1 h prior to LPS (1101)

correlated with severe lethargy. LPS-treated mice became lethargic as early as 30 min after LPS injection. On the other hand, LF-treated mice expressed normal behavioral activities, including eating and drinking, in the presence or absence of LPS. Moreover, as presented elsewhere³¹, LF administered 1 h before LPS injection significantly increased the survival of mice. Overall, the mortality rate was 16.7% in the LF-treated mice and 83.3% in the saline control group. Collectively, these results indicate that LF has the ability to maintain physiological homeostasis through the protection of the intestinal structure in mice.

In addition, we investigated the effects of intraperitoneal administration of LF on the LPS-induced release

of TNF- α , IL-6, IL-10 and nitric oxide (NO) in mice. Lactoferrin was administered as a prophylactic, concurrent or therapeutic event relative to endotoxic shock by intravenous injection of 100 μ g LPS. Inflammatory mediators were measured in serum at 2, 6 and 18 h post shock induction. Administration of LF 1 h before LPS resulted in a rather uniform inhibition of all mediators: TNF by 82%, IL-6 by 43% and IL-10 by 47% at 2 h following LPS injection, and a reduction in NO (80%) 6 h post shock. Prophylactic administration of LF 18 h prior to LPS injection resulted in similar decreases in TNF- α (95%) and inducible NO synthase (iNOS) (62%), but no statistical reduction in IL-6 or IL-10. Similarly, when LF was administered as a therapeutic post induction of endotoxic shock, significant reductions were apparent in TNF- α and iNOS in serum, but no significant effect was seen on IL-6 and IL-10. The results of these studies published elsewhere³², suggest that the mechanism of action for LF contains a component for the differential regulation of cellular immune responses during *in vivo* models of sepsis.

LF in Cell-Mediated Immunity Dissonance

We have previously established that LF is able to promote the differentiation of both T⁷⁶ and B⁷⁷ cells from their immature precursors. Moreover, the acquirement of the phenotypes, typical for mature cells, was accompanied by the acquirement of immunocompetence. Subsequent studies revealed that LF can strongly inhibit the effector phase of cellular immunity determined in the model of delayed type hypersensitivity (foot pad test)⁷⁷. As demonstrated later, this phenomenon could, in part, be associated with a selective inhibition of T helper 1 (Th1) function, since LF decreased IL-2 receptor (IL-2R) expression on a Th1 cell line, whereas expression of IL-4R on a Th2 cell line was not affected⁷⁸. Furthermore, LF decreased the proliferative response of the Th1 but not the Th2 cell line in the antigen presentation assay. The finding that LF can also inhibit nonspecific immunological reactions, such as carrageenan-induced inflammation in rats, provided additional evidence that LF can limit excessive inflammatory reactions. New trends in the search for safe immunological adjuvants turned our attention to LF as a potential agent for the augmentation of vaccine effectiveness. The rationale for using bovine LF was that the protein has abundant sugar residues with affinity to the mannose receptor. Such receptors are expressed on antigen-presenting cells in the skin and skin epithelium as well as in the gut epithelium⁶¹. Lactoferrin

was given to mice orally or intraperitoneally at the time of immunization, or subcutaneously in a mixture with the immunizing doses of the antigens: sheep red blood cells (SRBC), bacillus Calmette-Guérin (BCG) or ovalbumin (OVA). A DTH reaction was determined 24 h after the administration of an eliciting dose of antigen as a specific increase in foot pad swelling. Lactoferrin enhanced DTH reaction to all the studied antigens in a dose-dependent manner. Lactoferrin given to mice in conjunction with antigen administered in an incomplete Freund's adjuvant induced the DTH response at the level of control mice given antigen in a complete Freund's adjuvant. In addition, LF remarkably increased DTH response to a very small, otherwise non-immunogenic SRBC dose. The increase in DTH response was less pronounced by orally administered LF than by any other route of administration; however, statistically significant augmentation was demonstrated for each antigen studied. Although the costimulatory action of LF was accompanied by the appearance of bovine LF-specific cellular responses in mice, it is very unlikely that such responses will be generated in humans, since bovine LF is a dietary antigen to which a tolerance has been acquired. Considering the involvement of LF in the generation of stimulatory signals during the induction phase of the antigen specific immune responses, we suggest that LF may be useful in the development of safer and more efficacious vaccination protocols.

LF in Human Studies

The animal study results prompted us to initiate human studies and look for similar clinical benefits in some human disorders. Since there is no commercial source for human LF, we used high-quality bovine milk-derived LF in all our studies. Although good for nutritional supplementation, bovine LF cannot be used for parenteral applications in humans. The sequence homology between human LF and its bovine counterpart is only 69%⁵³. Therefore, to avoid the anaphylactic reaction, a species-specific, recombinant LF may be required for future parenteral applications in humans. We postulated that oral administration of bovine LF would be a reasonable compromise to obtain the medical benefits, at least for some human disorders. In fact, orally administered LF has been implicated in protection against sepsis in piglets³⁶, prevention of colon carcinoma in rats⁶⁵ and suppression of experimental metastasis in mice³⁵. As for the protective effects against sepsis in piglets, the benefits of orally administered LF

are likely associated with its absorption via the gastrointestinal tract and systemic dissemination, as it was demonstrated that newborns (up to 3 days after birth) can adsorb high-molecular-weight macromolecules. However, there is no evidence that in normal physiological conditions LF can cross the gut-blood barrier and exerts its immunologic function in adult animals or humans. Therefore, the mechanisms of action of orally administered LF in adults must be different from those reported for infants. With this possibility in mind, we have focused on analyzing systemic immunity in both *in vitro* and *in vivo* experiments.

***In vitro* Studies**

Initially, we studied the *in vitro* effects of LF on the mitogen-induced proliferation and cytokine secretion of peripheral blood mononuclear cells (PBMC) of patients subjected to cardiac surgery. PBMC were tested before, during, and on day 1 and day 8–10 following surgery. In control donors, low spontaneous and phytohemagglutinin (PHA)-induced proliferation of PBMC as well as LPS-induced TNF- α secretion were stimulated by LF, but high production of the cytokine was inhibited. In patients, the proliferation of PBMC and the ability to produce IL-6 and TNF- α by these cells underwent characteristic changes depending on the preoperative immune reactivity of the patients. In general, a low preoperative reactivity of PBMC showed a tendency to increase within the monitoring period whereas moderate/high responsiveness diminished. Lactoferrin had, in the majority of cases, a down-regulatory effect on the proliferative response, best pronounced in patients with high/moderate preoperative response. Similarly, LF exhibited an inhibitory effect on LPS-induced IL-6 production. In terms of TNF- α production, the considerable up-regulatory effect of LF, particularly in low-responding patients, was of special interest. In summary, we suggested that LF may play a role in lowering the immune response of patients to surgery and promoting tissue regeneration⁸⁰.

Lactoferrin has also been investigated for its potential antitumor activities in various models. It has been shown to activate natural killer (NK) cells, induce colony-stimulating activity, stimulate NK cells and augment macrophage cytotoxicity (reviewed in⁶⁵). Therefore, we attempted to study the immunoregulatory effects of LF on ζ -chain expression in peripheral blood T lymphocytes from patients with cervical cancer. By quantitative flow cytometry analysis we demonstrated that the mean ζ -chain expression was significantly

higher in freshly isolated T lymphocytes from healthy donors (69%) compared with the patients (38%). We compared the effects of addition of human LF in a 3-day culture of PBMC with the effects of immobilized anti-CD3 monoclonal antibodies (MoAb) on ζ -chain expression. Human LF significantly enhanced ($p = 0.002$) ζ -chain expression compared with control PBMC (47–66%). That effect was slightly higher than that of anti-CD3 MoAb (64%). The addition of LF to the anti-CD3 MoAb cell cultures resulted in an even higher stimulation of the ζ -chain expression. In summary, we demonstrated, that LF alone and in combination with anti-CD3 MoAb can significantly upregulate the expression of ζ -chain, indicating that the pathological defects in ζ -chain could be corrected by the therapeutic application of LF²⁶.

***In vivo* Studies**

Of particular interest to us was to reveal the effects of LF given to healthy volunteers, anticipating that the ultimate goal would be to develop a clinical protocol for the prevention or treatment of certain disorders in humans. In our first trial we investigated the effects of LF, taken orally (*per os*) by healthy individuals, on selected immune parameters. Three groups of volunteers (7 persons per group) were given one capsule containing 2, 10 or 50 mg of LF daily for 7 days. A control group has given placebo only. Venous blood was taken for tests a few hours before the first dose of LF, at day 1 and at 14 days after the last dose of the preparation. For evaluation of LF action on the immune response system we have chosen 3 parameters: the content of neutrophil precursors in the peripheral blood (in percentage) and the spontaneous production of IL-6 and TNF- α by unstimulated blood cell cultures. We found that oral treatment of volunteers with LF caused a transient (one day after the last dose) increase of immature forms of neutrophils in the circulating blood. That increase was more than 2-fold in the case of the 10 mg dose (Table 1). However, statistically significant in-

Table 1. Percentage of immature neutrophil forms (bands) in healthy subjects treated orally with lactoferrin (LF) for 10 days

Groups n = 7	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
LF 10 mg	3.9 $\pm$ 0.5	8.6 $\pm$ 0.13 p<0.01	5.6 $\pm$ 0.4

NS – not significant.

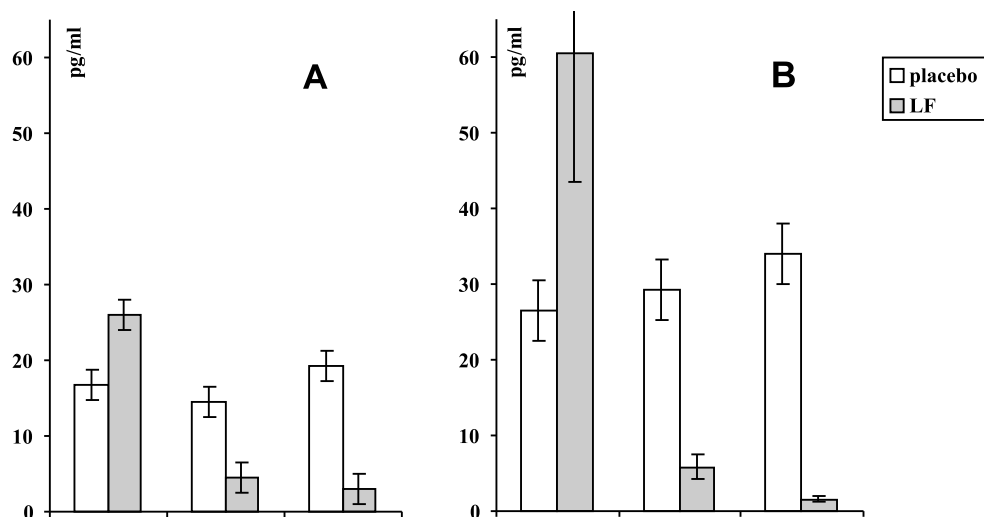


Fig. 6. Spontaneous production of TNF- α (A) and IL-6 (B) by peripheral blood cultures of healthy volunteers taking lactoferrin (LF) orally for 10 days. Spontaneous TNF- α and IL-6 production were determined in 24 h whole blood cultures using the indicator cell line WEHI 164.13 for TNF- α and the 7TD1 cell line for IL-6

creases in the percentage of neutrophil precursors were also registered at doses of 2 and 50 mg of LF. No change in the immature cell content was observed in the placebo group. The treatment with LF also resulted in a profound decrease in the spontaneous production of IL-6 and TNF- α by cultures of peripheral blood cells (Fig. 6). This decrease was significant (10 mg/dose) one day following the last dose of LF and persisted for an additional 14 days. These results confirmed our earlier data on the effects of *per os* treatment with a nutritional preparation containing LF. Furthermore, we were able to establish more closely the optimal dose of LF affecting selected immune indices.

Having established the immunoregulatory effects of orally administered LF, we decided to introduce it to patients subjected to minor surgery. Clinical insult, depending on its severity, is associated with a transient stimulation, followed by hyporeactivity, of the immune system. We expected that LF, given orally before operation, could modify the post-surgical immune response. The action of LF was evaluated in 18 LF-treated patients vs 28 placebo counterparts. Patients (women and men, mean age 50 years) were given daily oral doses (20 mg each) of LF for 5 consecutive days prior to thyroid surgery. The following immune response parameters were determined in blood samples taken from the patients 1 day before, 1 day after, and 5–7 days following surgery: cell morphology, the proliferative response of PBMC to PHA, and the spontaneous and LPS-induced production of TNF- α and IL-6. As a consequence of the thyroid surgery, the total leukocyte count increased on the post-operative day by about 50% in all patients and the percentage of lymphocytes fell by 26 and 35% in the control vs LF-treated group. The content of neutrophils, on the other hand, elevated on

day 1 post-operation by 51 and 68%, respectively. The percent of neutrophil precursors was markedly higher in LF-treated patients, particularly on the day before and the day after surgery (4.1 and 4.8 vs 2.5 and 3.7%, respectively). The post-surgical values were, however, comparable in both groups for neutrophils. The proliferative response of lymphocytes showed a slight decrease in the control group and an increase in the LF-treated patients on day 5 post-operation (20% over the control group). LPS-induced TNF- α production was higher in LF-treated patients both one day before and one day following surgery (28 and 24%, respectively; Fig. 7). LPS-induced IL-6 production was comparable in both the placebo and LF-treated patients before surgery; however, on day 1 and 5 following surgery the production of IL-6 was higher in LF-treated patients by 65 and 27%, respectively (not shown). Taken together, the data presented in this study revealed an increased immune responsiveness in all patients treated with LF and subjected to thyroid surgery. This suggests that treatment with lactoferrin could constitute an effective protective measure against post-surgical complications.

Conclusions

In summary, our extensive research on the immunotropic activities of LF in various *in vitro* and *in vivo* models leads to the following conclusions: LF given orally appeared to be absolutely safe and well tolerated for a prolonged period of time. Spectacular effects were demonstrated in mice of lactoferrin's ability to protect the gut structure and function during experimental endotoxemia. Moreover, in this mouse model of sepsis,

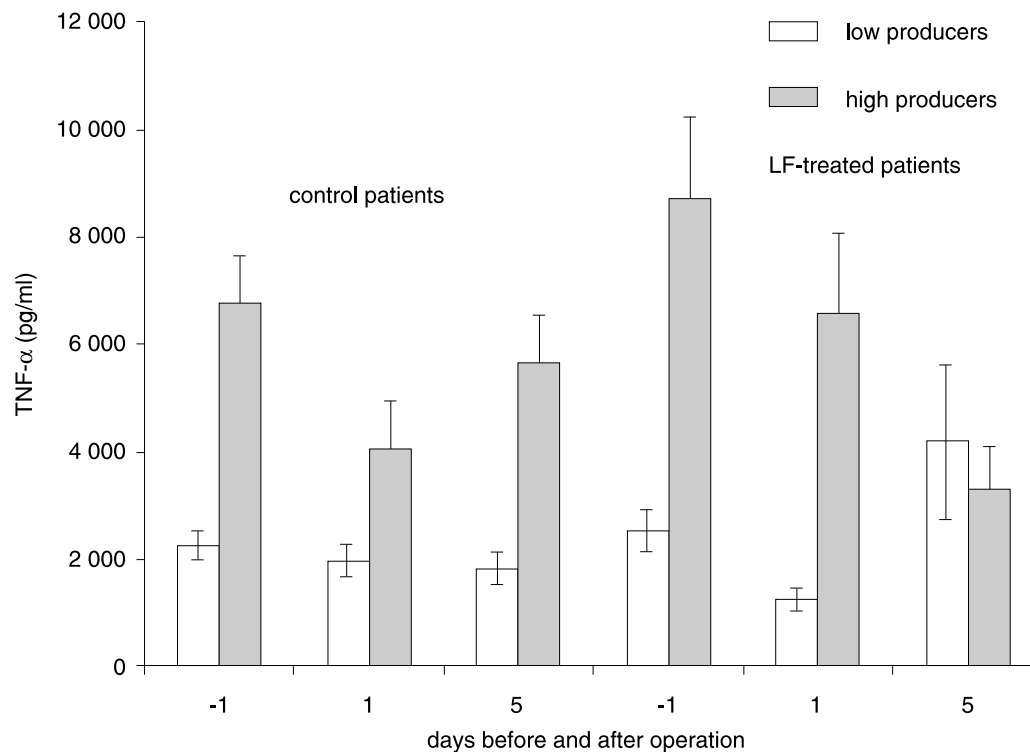


Fig. 7. The production of LPS-inducible TNF- α by PBMC of low and high cytokine producers. LPS-inducible TNF- α was determined in 24 h whole blood cultures using the indicator cell line WEHI 164.13

LF effectively suppressed the LPS-induced hypothermia and reduced production of proinflammatory mediators such as TNF- α and NO. Lactoferrin was also shown to promote maturation of T and B cells and to inhibit delayed type hypersensitivity reactions when given together with the eliciting dose of antigen. The discovery of the adjuvant properties of LF in the generation of DTH opens a new perspective for LF as a potential adjuvant for vaccination protocols. *In vitro* studies showed that LF may be an interesting immunomodulator for the diminished reactivity of blood cells from patients after major surgery or trauma or in septic shock. In turn, the trials performed in healthy volunteers revealed that oral pretreatment with LF resulted in immunoregulatory actions on several parameters, such as the proliferative response of PBMC to mitogens and cytokine production. Most characteristic, however, was the induction of recruitment of neutrophil precursors. Interestingly, the immunoregulatory effects of orally administered LF were long-lasting and persisted for several weeks. The results of the first clinical trial met our expectations, since patients pretreated with LF exhibited higher immune reactivity post-operation and, which may be equally important, they were armed with an increased pool of the most efficient phagocytes – the circulating neutrophils. Other *in vitro* data indicate that LF can upregulate expression of ζ -chain in the CD3 T cell receptor, typically downregulated in the circulatory and tumor-infiltrating T cells in cervical cancer

patients. Finally, a fundamental question for us concerned how LF given orally can influence immune responses during the development of an acute oxidative stress. Although more studies will be necessary to answer this question, we postulate that lactoferrin's ability to control lipid peroxidation may be a common mechanism underlying cytokine induction, cell differentiation, apoptosis, and signal transduction in general.

Our goal is to develop LF further as a prophylactic and/or therapeutic agent to protect against or ameliorate events of systemic inflammatory response syndrome in humans.

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