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Immunoregulatory function of lactoferrin in immunosuppressed and autoimmune animals

Wpływ laktoferryny na status immunologiczny zwierząt poddanych farmakologicznej supresji oraz z indukowaną chorobą autoimmunologiczną

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Summary

In this article we review our recent results on the effects of lactoferrin (LF), given orally, on the immune status of mice subjected either to chemotherapy or immobilization stress as well as on rats with experimentally induced autoimmune encephalomyelitis (EAE). We demonstrated that LF accelerated reconstitution of the immune system function after administration of a sublethal dose cyclophosphamide (CP) and normalized the ratio of major blood cell types in that model. Also, after application of methotrexate (MTX) LF was effective to speed up reconstitution of the cellular and humoral immune response. Mice treated with lethal dose of busulfan (Bu) and CP and reconstituted with bone marrow cells (BMC) were able to quicker develop optimal immune responses when administered LF. In addition LF was shown to accelerate engraftment of bone marrow cells from syngeneic donors in that model. Using immobilization stress model was shown that LF accelerates reconstitution of the cellular and humoral immune response. In rats with EAE lactoferrin lowered the clinical score of the disease and diminished pathohistological changes in the spinal cord. In summary, in a series of studies we demonstrated a benefit of orally administered LF in immunocompromised animals.

Key words: **lactoferrin • cyclophosphamide • methotrexate • busulfan • bone marrow • mice • rats • psychic stress • EAE • immune response • reconstitution • engraftment**

Streszczenie

W artykule dokonano przeglądu prac zespołu Laboratorium Immunobiologii, Instytutu Immunologii i Terapii Doswiadczalnej, Polskiej Akademii Nauk we Wrocławiu, dotyczących wpływu laktoferryny (LF), podanej doustnie w wodzie pitnej, na status immunologiczny zwierząt poddanych działaniu chemioterapeutyków, stresowi psychicznemu oraz z doświadczalnie wywołanym zapaleniem mózgu i rdzenia kręgowego. Okazało się, że LF w istotny sposób przyspieszała odnowę zarówno komórkowej, jak i humoralnej odpowiedzi immunologicznej myszy po podaniu subletalnej dawki cyklofosfamidu (CP). Obserwowano też odnowę puli krążących leukocytów

oraz normalizację zmienionego obrazu krwi obwodowej. Podobne właściwości ochronne LF zanotowano u myszy traktowanych metotreksatem (MTX). LF podawana myszom po letalnej dawce busulfanu (Bu) i CP (odpowiednik stosowanej w klinice procedury kondycjonowania biorcy przeszczepu szpiku) oraz poddanych przeszczepowi szpiku kostnego znacznie przyspieszała odnowę komórkowej i humoralnej odpowiedzi immunologicznej. Na tym samym modelu wykazano, że LF przyspieszała także zasiedlanie szpiku kostnego przez przeszczepione komórki. Na modelu doświadczalnym, w którym myszy poddawano długotrwałemu stresowi immobilizacji, LF znacznie przyspieszała odnowę odpowiedzi komórkowej. U szczurów z doświadczalnym zapaleniem mózgu i rdzenia LF łagodziła objawy choroby, co korelowało ze złagodzeniem zmian histologicznych w obrębie rdzenia kręgowego. Reasumując, na kilku odmiennych modelach doświadczalnych udało się wykazać, że LF przyspiesza odnowę funkcji układu immunologicznego. Takie działanie zaobserwowano u zwierząt po zastosowaniu chemioterapeutyków, długotrwałego stresu psychicznego oraz z wywołaną chorobą autoimmunologiczną.

Słowa kluczowe: **laktoferryna • cyklofosfamid • metotreksat • busulfan • szpik kostny • myszy • szczury • stres psychiczny • EAE • odpowiedź immunologiczna • rekonstytucja • zasiedlanie**

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Abbreviations: **LF** – lactoferrin; **BLF** – bovine lactoferrin; **HLF** – human lactoferrin; **holo-LF** – iron-saturated lactoferrin; **apo-LF** – iron-free lactoferrin; **i.v.** – intravenously; **i.p.** – intraperitoneally; **Bu** – busulphan; **CP** – cyclophosphamide; **MTX** – methotrexate; **BMT** – Bone Marrow Transplantation.

Lactoferrin (LF), among well established actions directed against pathogens, exhibits differential immunoregulatory properties in health and disease [9]. Although its effects on maturation of T and B cells [18,19] are clearly expressed, the role of LF in differentiation of the myeloid cell lineage is controversial [14]. In addition, until recently, no data have been available on possible effects of LF on the immune system of immunocompromised or animals with experimentally-induced autoimmune disorder. The aim of this minireview was to present our recent data in several models including mice treated with sublethal doses of immunosuppressive drugs and/or reconstituted with bone marrow cell transfer, subjected to psychic stress and rats with induced experimental encephalomyelitis. In all these studies the animals were given LF in drinking water (0.25–0.5% solution) in order to examine possible effects of LF on reconstitution of the immune system function in immunosuppression or/and counteraction of the autoimmune disease development.

In the first model we examined a potential capability of LF to accelerate reconstitution of the innate and acquired, antigen-specific cellular and humoral immune response after application of a sublethal (350 mg/kg/b.w.) dose of cyclophosphamide (CP), an alkylating agent, used to treat both malignant and non-malignant immune-mediated inflammatory disorders in humans [8]. We showed that LF almost completely reconstituted the delayed type hypersensitivity (DTH) to ovalbumin (OVA) in CBA mice on 14 day after CP administration [3] (Figure 1). Oral LF treatment also led to a partial recovery of Concanavalin-A-induced splenocyte proliferation in these mice. These effects of LF were correlated with an increase of the $CD3^+$ and $CD4^+$ T cell content. In the same model the humoral immune response to sheep red blood cells was also partially reconstituted, although not so effectively since after 37 days following CP administration LF elevated almost nonexisting antibody production (4.7% of the control) to 47% of the control values [7]. That phenomenon could be explained by a longer life span of B, in contrast to T cells. On the other hand, the proliferative response of splenocytes to a B-cell mitogen – pokeweed mitogen, was reconstituted more quickly. Flow cytometry also showed that the content of Ig^+ splenocytes rose following LF treatment. Administration of LF in drinking water in CP-treated mice had also a profound effect on leukocytosis and blood picture i.e. proportions of major blood cell types [1]. Mice treated with CP demonstrated severe leukopenia, strong eosinophilia (day 4) and an altered lymphocyte/neutrophil ratio (days 8–22). LF, given to mice for 21 days, partially normalized the cell composition in CP-treated mice (increase of lymphocytes and decreased eosinophil content). The content of leukocytes increased upon LF administration on days 4, 8, 15 and 22 by 36.8, 39.5, 72.0 and 70.7%, respectively, in comparison to CP-treated group. Interestingly, LF partly normalized the neutrophil and lymphocyte composition on day 22 (neutrophils: 29.2% in control mice, 50.6% in CP-treated and 39.16% in CP/LF-treated; lymphocytes: 66.18%

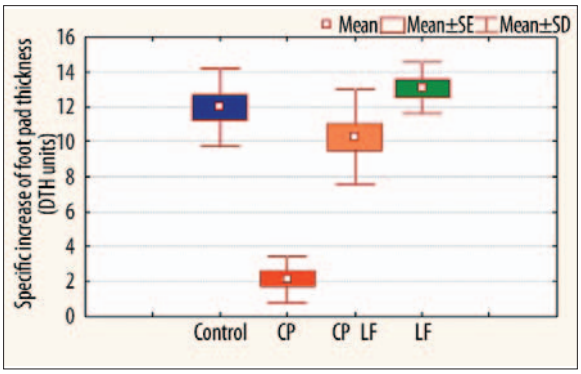

Figure 1. Reconstitution of the delayed type hypersensitivity reaction to OVA by LF in CP-treated mice. Mice were given CP (350mg/kg b.w, *i.p.*) and seven oral doses LF (1 mg each) on alternate days.

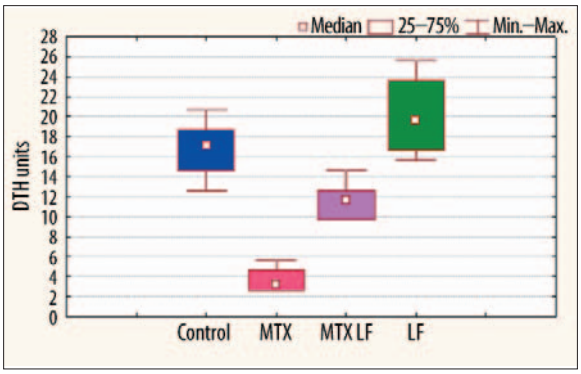

Figure 2. Effect of LF on delayed type hypersensitivity suppressed by MTX. Mice were sensitized with OVA and treated with MTX (200 mg/kg b.w, i.p.) 48h following sensitization. Mice were given LF in drinking water (0.5% solution) for the whole duration of the experiment.

in control mice, 35% in CP-treated and 48.8% in CP/LF-treated). Other investigations in that experimental model revealed that administration of LF elevated the total number of functional phagocytes (neutrophils and eosinophils) in circulation [2] and enhanced the ability of splenocytes and macrophages to produce interleukin 6 [5].

Next, we used methotrexate (MTX), an antagonist of folic acid synthesis, which causes apoptosis in activated cells primarily in the G1 and S-phases of the cell cycle [10]. We found that MTX, given intraperitoneally (i.p.) at a dose of 200mg/kg b.w, 48h following sensitization of mice with OVA, reduced by 80% the DTH response [4]. Co-administration of LF for the duration of the experiment (4 days) restored the DTH response almost to the control level (Figure 2). Nevertheless, LF was not able to restore the primary humoral immune response to SRBC when MTX (1 mg/kg b.w.) was administered to mice i.p. 48 h post immunization. On the other hand, mice treated with LF after second challenge with antigen showed significant restoration of the MTX-suppressed secondary immune response. In addition, LF (1 µg/ml) restored the secondary humoral immune response to SRBC *in vitro* when MTX (0.05–1 mM) was added to cell cultures 24 h following cell culture initiation (Figure 3). These data demonstrated that LF preferentially restores the cellular immune response impaired by MTX treatment. It seems also that LF may prevent the block of the activity of T memory cells in the secondary, humoral immune response.

The above described results indicated that LF may find application in cases where the immune system requires prompt reconstitution after chemotherapy. Therefore in a next study, we adopted in mice so called "conditioning" procedure for patients scheduled for allogeneic bone marrow transplant [6]. CBA mice were treated orally with busulfan (Bu) and CP, followed by intravenous (i.v.) injection of 10^8 syngeneic bone marrow cells. Bu, a myeloablative, but not immunosuppressive agent, is cytotoxic for early hematopoietic cells, providing similar benefit as whole-body irradiation [11]. Mice were given LF in drinking water immediately following the bone marrow transfer (BMT) and for the continuation of experiments (7–31 days). As expected, both humoral and cellular immune responses of mice that were treated with the chemotherapeutic agents, were markedly impaired. Cellular immunity was inhibited by chemotherapy treatment to a lesser degree than the humoral response (50% vs. 88% inhibition on day 14 after BMT) (Figures 4 and 5). LF appeared to effectively reconstitute both types of the immune response. Furthermore, in mice treated with the drugs and reconstituted with a small number of BM cells (10^5) LF was shown to accelerate lympho-, erythro-, and myelopoiesis in the bone marrow and appearance of transforming lymphocytes in the spleen, similarly as a control cytokine – human granulocyte colony-stimulating factor. In summary, the study suggests that LF may be a useful agent to accelerate restoration of immune system function induced by chemotherapy in BMT recipients.

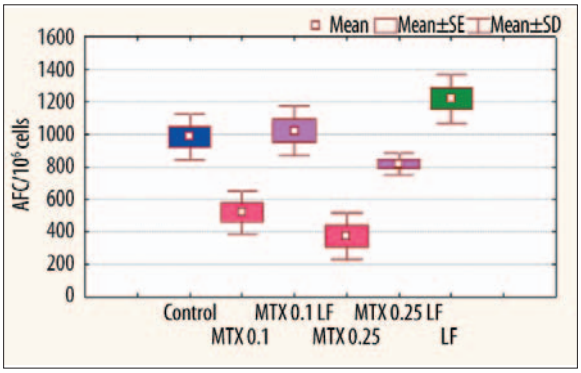

Figure 3. Effects of LF on the secondary humoral immune response *in vitro*. Splenocytes from mice sensitized with SRBCs were restimulated with SRBCs *in vitro*. MTX was added to the cultures at concentrations of 0.1 and 0.25 mM 48h following immunization. LF was added to the cultures at a concentration of 1 µg/ml at the beginning of 4-day incubation.

Interdependence between the function of the central nervous system and the immune system is well established [15]. Stressful conditions may differentially affect the immune response in experimental animals and in humans. Acute immobilization stress (IS) was shown to inhibit cellular [12] and humoral [13] immune responses. We recently demonstrated [16] that long-term IS induced significant suppression of cellular and humoral immune response in CBA mice but the

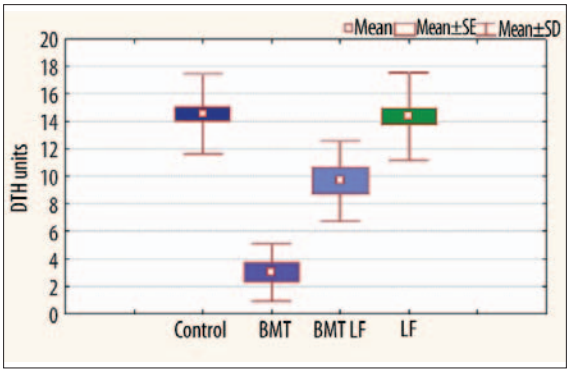

Figure 4. Effect of LF on reconstitution of DTH in Bu/CP-treated and BMC-reconstituted mice. On day 7 the ability of mice to develop cellular immune response was determined.

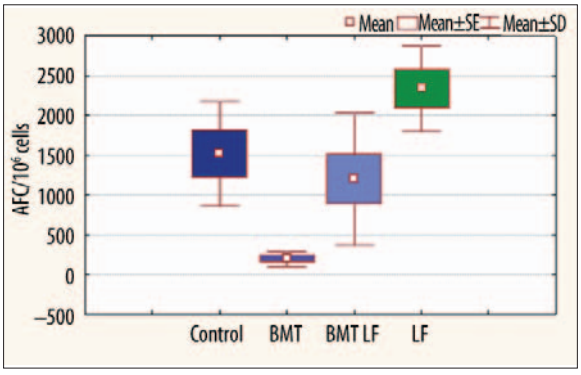

Figure 5. Effect of LF on reconstitution of humoral immune response in Bu/CP-treated and BMC-reconstituted mice. On day 21 the ability of mice to develop humoral immune response was determined.

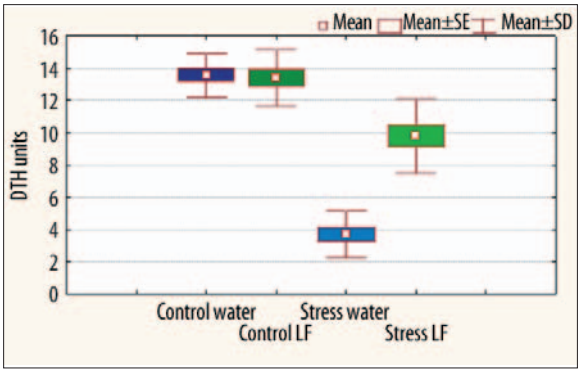

Figure 6. Up-regulation of the immobilization stress (IS)-suppressed DTH response by LF. Mice were subjected to IS (5 h daily for 5 days) before immunization with OVA. LF was applied to mice as 5% solution in drinking water throughout the experiment.

Table 1. Effects of oral LF administration to rats with elicited encephalomyelitis. LF_0 – LF given rats in the drinking water as a 0.25% solution from the day of EAE elicitation; LF_7 – LF given to rats 7 days following EAE elicitation.

	Severity of Disease			
Group	**No. Rats**	**Incidence**	**Day of onset**	**Max Score (mean ±*SE*)**
Control	8	8/8	12	3.5±0.5
LF_0	8	8/8	12	3.1±0.7
LF_7	8	8/8	12	2.6±0.6

suppression was attenuated by LF given to mice in drinking water (Figure 6). That was the first report on the regulatory effect of LF on the immune response modified by the psychic stress, consistent with other reports on antinociceptive and analgesic actions of LF in experimental animals.

LF was already shown to inhibit development of naturally occurring hemolytic anemia in New Zealand Black mice [20]. In our latest study we investigated the effect of LF, administered in drinking water to Lewis rats with experimentally induced encephalomyelitis (EAE) [17]. LF was provided to rats as 0.25% solution in drinking water from the day of immunization or beginning day 7 after immunization. The clinical score of the disease and histology of the dorsal region of the spinal cord were evaluated. The experiments showed that LF significantly lowered the clinical score (Table 1) of the disease as well as the cell infiltration in the white and gray matter of the spinal cord and around the abdominal artery. In addition, in LF-treated rats the cell numbers in the peripheral, draining lymph nodes, greatly elevated in the untreated rats, were similar as in naive animals. Also, the serum levels of both pro- and anti-inflammatory cytokines were reduced in LF-treated rats to control values. Both schedules of LF administration were effective indicating that LF may also inhibit already existing autoimmune response.

In summary, the above described results demonstrated therapeutic benefit of LF in immunocompromised animals, thus opening perspectives for therapeutic application of LF in clinic.

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