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Inflammation Research

Protective effects of lactoferrin in *Escherichia coli*-induced bacteremia in mice: Relationship to reduced serum TNF alpha level and increased turnover of neutrophils

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Abstract. *Objective and Design:* Previous studies demonstrated that lactoferrin (LF), given intravenously (i.v.), 24 h before lethal *Escherichia coli* (*E. coli*) infection, protects mice against mortality. The aim of this investigation was to determine whether downregulation of serum TNF alpha activity and increase of neutrophil number in the circulation and bone marrow by LF could contribute to the protective action of LF against *E. coli*-induced sepsis.
Material and subjects: CBA female mice, 10–12 week old, weight 20–22 g, were used.
Treatment: Mice were given 10 mg LF i.v. either 2 h or 24 h before i.v. administration of lethal dose of *E. coli* (5×10^8).
Methods: Serum activities of TNF alpha and IL-1 were determined by bioassays 2 h following *E. coli* or LF injection. The blood and bone marrow smears were stained with Giemsa and May-Grünwald reagents and reviewed histologically.
Results: LF given 24 h before *E. coli* caused a 60% reduction of TNF alpha released into circulation. However, pretreatment of mice with LF 2 h before bacterial challenge resulted in strong (15 fold) increase of TNF alpha serum level. Analysis of bone marrow cell composition revealed a significant increase in neutrophil lineage cell content (myelocytes, bands and mature neutrophils) following 24 h pretreatment with LF (51.8% of the total cell count), versus PBS control (32.7%) and 2 h LF pretreatment (35.8%). The percentage of neutrophils (bands and mature forms) in the peripheral blood rose to 47.4% versus 32% and 32%, respectively. Intravenous administration of LF increased also interleukin 1 (IL-1) concentration in the circulation of noninfected mice.
Conclusions: This investigation has added more information regarding the mechanism of the protective action of LF in *E. coli*-induced bacteremia by revealing the phenomenon of accelerated neutrophil recruitment and down-regulation of *E. coli*-induced TNF alpha serum level.

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Key words: Bacteremia – Lactoferrin – TNF alpha – Neutrophils

Introduction

Mortality rate resulting from Gram-negative sepsis still remains high in intensive care units despite advances of modern medicine and associated disciplines [1]. A number of new therapeutic approaches, including application of anti-endotoxin antibodies [2] or anti-cytokine therapy [3] have proved to be disappointing. Not only have these therapies been ineffective, but, they may increase mortality. It also became clear that use of the immune therapies for prevention or treatment of septic shock may lead to a better outcome only, if we can assess the patient's immune status prior to such therapy [4, 5]. Under some conditions it may be dangerous to treat a patient with a putative risk of developing sepsis with an immunomodulatory approach [6].

A biphasic immunological pattern during the initial and later phase of sepsis is characterized by the early hyperinflammatory phase, which is counterbalanced by the later hypo-inflammatory responses. Although a massive release of both pro- and anti-inflammatory cytokines upon infection is regarded as the major cause of organ dysfunction and death [4], a significant decrease of the cytokine activity may be beneficial in avoiding lethal effects of endotoxemia [7–11] but not bacteremia, where the pro-inflammatory cytokines play an important role in the stimulation of the immune system [11–14]. The prevalence of anti-inflammatory cytokines, such as interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) may also be detrimental in various models of sepsis [9, 15].

More recently increasing attention has been paid to the innate immunity in combating pathogen infection [16]. Lactoferrin (LF) represents one of the elements of the innate

immunity system [17] and is abundant in secretory fluids of mammals and secondary granules of neutrophils [18]. LF is considered an important mediator in host defence against various environmental insults. It is a pleiotropic immunoregulator with receptors on major immune cell types and gut epithelium [19–21]. Among many properties, LF exhibits a wide array of actions against pathogens [22–25]. Interestingly, the antimicrobial activities of LF are selectively directed against pathogenic intestinal flora [26]. Originally, the antibacterial properties of LF were explained by its ability to chelate free iron ions needed for bacterial growth, a bacteriostatic effect [27]. However, beside the direct activities against bacteria [23, 28, 29] LF may have an indirect effect by alleviating consequences of interactions of bacterial products with host's cells. For example, LF can bind to lipopolysaccharide (LPS) [30] and compete with LPS-binding protein for CD14 receptors [31]. In addition, LF was found to strongly downregulate the release of both pro- and anti-inflammatory cytokines upon LPS challenge [7].

The protective role of LF in prevention of the lethal consequences of *Escherichia coli (E. coli)*-induced sepsis was first demonstrated by Zagulski et al. [32]. The authors revealed that an intravenous injection of LF significantly increased survival in mice challenged with a lethal dose of *E. coli*. Also, it was demonstrated that the protective effect was both time- and dose-dependent. The septic conditions were clearly diminished in mice pre-treated with LF 24 h but not 2 h before bacterial challenge. In that experimental model LF was also found to rapidly increase clearance of bacteria from circulation and RES organs and to enhance the killing activity of the RES cells [33, 34]. The time-dependent protection by LF was also shown in *Staphylococcus aureus* infection model [25].

The aim of this investigation was to further explain the mechanisms of the time-dependent protective action of LF in experimental bacteremia. The study provides evidence that the protection conferred by LF given 24 h before bacterial infection is associated with increased output of neutrophils into circulation and reduced TNF alpha levels.

Materials and methods

Mice

CBA mice (10–12 weeks old, 20–22 g) were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy (IIET) – Wroclaw, Poland. Mice were fed a commercial, pelleted food and water ad libitum. The animal ethics committee at the Institute of Immunology and Experimental Therapy approved the study.

Reagents

Low endotoxin bovine milk lactoferrin (0.16 E.U./mg, < 25% iron saturated) was obtained from Morinaga Milk Industry Co, Japan. Lactoferrin was dissolved in PBS and filtered through 0.2 µm Milipore filters before injection to mice. *E. coli* strain O55 was obtained from the IIET collection. LPS (E. coli serotype O111:B4; 3×10^6 E.U./mg) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All tissue culture plates and flasks were purchased from Nunc (Nunc, Denmark).

Treatment of mice with LF and E. coli

LF was given intravenously (i.v.) – 10 mg in 0.2 ml PBS – into the lateral tail vein 2 h or 24 h before the *E. coli* strain O55 (5×10^8 cells suspended in 0.1 ml PBS) or saline was injected. Two hours later the blood and bone marrow were taken for biological and histological evaluations.

Determination of TNF alpha activity

TNF alpha level was determined by the WEHI 164.13 bioassay [35]. Briefly, WEHI 164.13 cells (ATCC CRL 1751) were seeded at a concentration of 2×10^4 cells/well in quadriplicate. Increasing dilutions of the assayed serum were mixed with the target cells in the presence of actinomycin D (1 µg/ml). After 20 h of incubation, Thiazolyl blue (MTT) was added into the wells and the cultures were incubated for an additional 4 h. Next, a lysing buffer was added and the optical density at 500 nm (Dynatech 5000) was measured. The detection limit of the WEHI 164.13 assay was about 2.5 pg/ml. One unit of TNF alpha activity was defined as an inverse of serum dilution where 50% cell death took place.

Determination of IL-1 activity

IL-1 level was determined in bioassay by using the LRBM-33-1A5 (ATCC CLR 8079) [36]. Briefly, 100 µl of LRBM-33-1A5 cells (5×10^4 cells/ml) were cultured in 96-well flat-bottom plates in quadriplicate in the presence of equal volumes of supernatant with phytohemaglutinin (5 µg/ml) for 24 h in a cell culture incubator. Samples of pooled serum from LBRM cultures (100 µl) were added to 100 µl of CTLL-2 (ATCC TIB 21) cells (10^4 cells/ml) in quadriplicate and cultured in an incubator for 24 h. CTLL-2 growth was estimated by MTT colorimetric assay as described above. One unit of IL-1 equals one unit of IL-2 (50% of maximal CTLL-2 growth) as defined in the method [36].

Analysis of the cell type profile in the bone marrow and circulating blood

Mice were subjected to halothane anaesthesia and bled from the retro-orbital plexus. Femurs were isolated and bone marrow cells obtained by flushing with 0.45 mm needles with PBS. The blood and bone marrow smears were prepared on microscopic glasses, dried and stained with Giemsa and May-Grünwald reagents. The smears were subsequently reviewed histologically; the pathologist viewing and interpreting the slides was blinded to type of experiment and treatment.

Statistics

All data are expressed as mean ± SD. Differences between groups were analyzed by the Mann-Whitney U test. P value of 0.05 or less was considered significant.

Results

Effects of LF pre-treatment on TNF alpha following E. coli injection

The results shown in Table 1 revealed that pretreatment of mice with LF 24 h before *E. coli* administration was associated with a significant ($P < 0.05$) 60% decrease of TNF alpha level, measured at 2 h after *E. coli* injection. In contrast, 2 h pretreatment with LF caused a very strong (15 fold) elevation of TNF alpha activity in the circulation. That group of mice was also distinctly hypothermic and lethargic.

Table 1. Effects of LF pretreatment on *Escherichia coli* elicited TNF alpha serum levels. 10 mg of LF was given i.v. 24 h or 2 h before i.v. *Escherichia coli* (5×10^8) challenge or 0.2 ml PBS as a control. TNF alpha was determined by a bioassay 2 h after *Escherichia coli* injection. The data are presented as mean values from 6 mice/group ± SD. *Escherichia coli* control vs LF – 24 h ($P < 0.05$), *Escherichia coli* control vs LF – 2 h ($P < 0.01$).

	Experimental group					
	Control PBS	Control LF – 2 h	Control LF – 24 h	Control *Escherichia coli*	LF – 2 h + *Escherichia coli*	LF – 24 h + *Escherichia coli*
TNF alpha, pg/ml	224 ± 61.3	286 ± 24.4	281.4 ± 51.2	12913.7 ± 5101.1	196615.2 ± 180848.4	5238.4 ± 2732.6

NS, not significant; SD, standard deviation.

Induction of IL-1 by lactoferrin

To check whether LF given i.v. could elicit IL-1 release into circulation mice were injected with increased LF doses. The ability of LF to induce IL-1 release was compared to a reference factor – LPS (50 µg/mouse; 1.5×10^5 E.U.). Figure 1 demonstrates that LF induced IL-1 release in a dose-dependent manner, measured at 2 h after injection. The increase of IL-1 level, elicited by administration of 10 mg LF was about one-third of that induced by LPS.

Profile of neutrophils in the bone marrow and circulating blood following LF administration

Effects of LF injection on neutrophil recruitment and turnover were established by the content of cells of the neutrophil lineage in the bone marrow and circulating blood following 24 h and 2 h i.v. pretreatment of mice with LF. The results shown in Table 2 indicate that administration of LF 24 h before analysis of bone marrow cell composition, caused increases in the content of all cell types of the neutrophil lineage (myelocytes from 10.7% to 14.6%, bands from 6.3% to 9.2% and neutrophils from 15.7% to 28.0%). The increase of the all cell types of the neutrophil lineage was 58% (from 32.7% to 51.8%). On the other hand, pretreatment of mice with LF 2 h before cell analysis resulted only in a negligible increase of the neutrophil lineage cell content (from 32.7% to 35.8%). 24 h pretreatment of mice with LF also caused a significant (53%) increase of the total neutrophil cell content in the peripheral blood (bands and mature neutrophils) – from 32.0% to 47.4% (Table 3). Again, the rise of neutrophils in mice pretreated 2 h before cell determination was absent. Of particular interest was a very strong elevation in the percentage of band cells in the circulation (from 1.0% in the PBS control to 6.7% in mice pretreated 24 h with LF) representing over 6 fold increase.

Table 2. LF increases the neutrophil lineage compartment in the bone marrow. Mice were treated i.v. with 10 mg LF. Bone marrow smears were made and stained (Materials and Methods) 2 h and 24 h after LF injection. Data are presented as mean values (percentage) of a given cell type from 5 mice ± SD. Changes in myelocytes – NS among groups, bands: NS among groups, neutrophils: PBS control vs LF – 24 h ($P < 0.05$), PBS control vs LF – 2 h (NS).

Experimental group	Bone marrow content		
	Myelocytes	Bands	Neutrophils
Control	10.7 ± 1.15	6.3 ± 3.21	15.7 ± 4.04
LF – 2 h	12.0 ± 2.35	5.4 ± 1.34	18.4 ± 2.70
LF – 24 h	14.6 ± 3.97	9.2 ± 1.92	28.0 ± 3.87

NS, not significant; SD, standard deviation.

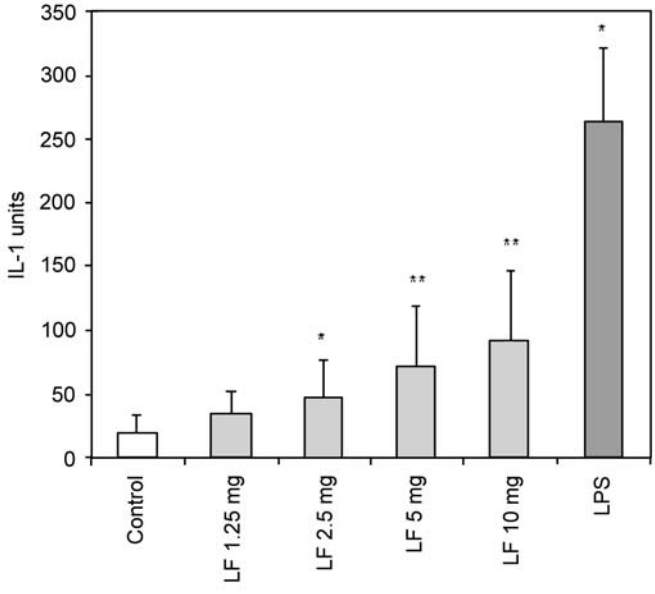

Fig. 1. Induction of serum IL-1 activity by LF. Mice were injected i.v. with increasing doses of LF (1.25 – 10 mg) or LPS (50 µg). IL-1 activity was determined by the bioassay in sera isolated from mice 2 h after injection. Data are presented as mean values (units/ml) from 5 mice/group ± SD. PBS control vs LF 1.25 mg (NS), PBS control vs LF 2.5 mg ($P < 0.01$), PBS control vs LF 5.0 mg ($P < 0.001$), PBS control vs LF 10.0 mg ($P < 0.001$), PBS control vs LPS ($P < 0.01$). *$P < 0.01$; **$P < 0.001$.

Discussion

In this investigation we provide evidence that the enhancement of the protective activity of LF against *E. coli* infection may result from a significant mobilization of the neutrophil pool in the bone marrow and circulating blood. In addition, LF given 24 h before *E. coli* infection caused significant reduction of TNF alpha released into circulation. Moreover, as we demonstrated, i.v. administration of 10 mg of LF induced a marked increase in the serum IL-1 level. These find-

Table 3. LF increases the percentage of immature and mature neutrophils in the circulating blood. Mice were treated i. v. with 10 mg LF. Blood smears were prepared and stained (Material and Methods) 2 h and 24 h after LF injection. Data are presented as mean values (percentage) of all major blood cell types from 5 mice ± SD. Bands: PBS control vs LF – 24 h ($P < 0.05$), PBS control vs LF – 2 h (NS), neutrophils: PBS control vs LF – 24 h ($P < 0.05$), PBS control vs LF – 2 h (NS). Other relation not significant.

Experimental group	Peripheral blood content					
	Bands	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Control	1.0 ± 0.0	31.0 ± 4.58	3.7 ± 1.15	0.0	64.3 ± 3.51	0
LF – 2 h	1.6 ± 1.14	30.4 ± 6.84	4.0 ± 1.22	0.0	64.0 ± 6.04	0
LF – 24 h	6.2 ± 1.48	41.2 ± 2.49	5.4 ± 2.41	0.2 ± 0.45	47.6 ± 4.34	0

NS, not significant; SD, standard deviation.

ings provide additional and complementary explanations for the phenomenon of the protection exhibited by LF in experimental bacteremia [17]. Zagulski et al. postulated that the predominant antibacterial role in the described model was played by the cells of the reticuloendothelial system [32–34]. This was demonstrated by a significant decrease in the protective activity of LF against *E. coli* infection when blocking the RES function with dextran sulphate prior to LF administration [33]. The authors found that the protective effect of LF was time-dependent and 24 h pretreatment gave maximum (76% survival) protection. They concluded that a certain period of time was required for the immune system to generate the antibacterial RES activity. From our study it became clear that 24 h period was sufficient to strongly enhance recruitment of neutrophil cell type at various stages of differentiation in the bone marrow and circulating blood. The increase of neutrophil precursors (bands) in blood was particularly striking (6 fold). A mobilization of neutrophil precursors, although not to such a degree, was also observed upon oral treatment of volunteers with LF [37]. Other studies confirmed that neutrophils play a crucial role in the protection against *E. coli* infection in mice [38] and interleukin 6 (IL-6) was also required for recruitment of neutrophils [38, 39]. The induction of IL-6 and TNF alpha by i. v. LF injection was demonstrated by us previously [10, 40] and suggests that it could contribute to the increased mobilization of neutrophils in the bone marrow. During infection the number of neutrophils infiltrating RES organs increases several fold and these cells are predominantly responsible for the clearance of bacteria in organs of infected mice [41, 42]. Other studies demonstrated that bovine lactoferrin can stimulate the phagocytic activity of human neutrophils [43]. That finding further supports the importance of neutrophils as targets for LF action.

In our study LF elicited relatively high IL-1 serum levels, corresponding to 30% of the value induced by a reference inducer LPS. This could represent an additional protective action induced by LF since IL-1, given 24 h before bacterial challenge, enhanced resistance to infection, probably by elicitation of acute phase protein synthesis [44]. The protective effect of LF, given 24 h before bacteria, may in part be explained by a reduction of serum TNF alpha levels. Although, high levels of TNF alpha are frequently considered lethal [1], it has to be stressed, however, that the suppressive effect of LF on TNF alpha release was only partial and could be still associated with the need for that cytokine to perform its antibacterial activity [12]. In our study the detrimental effect of the exceptionally high TNF alpha level was evident in mice pretreated with LF (2 h prior to infection) already after 2 h following *E. coli* infection (a deep hypothermia and lethargy). In other experiments, we demonstrated that 24 h pretreatment of mice with LF significantly reduced (by 90%) TNF alpha release elicited by a sub-lethal dose of LPS [40]. The phenomenon of the remarkable increase of TNF alpha production, when mice were pretreated 2 h before bacteria challenge, is difficult to explain. It is possible that at the time of *E. coli* injection, LF may directly disrupt the cell wall causing increased LPS spill due to interaction with *E. coli* cell wall porins [29, 45]. In addition, LF could transiently increase the sensitivity of RES cells to bacterial products [28].

In conclusion, the time-dependent protective effect of LF against lethal *E. coli* bacteremia may have a differential basis. Beside the postulated activating effect on the killing and clearing function of RES, LF clearly induces rapid mobilization of neutrophils in the circulation, down-regulates TNF-alpha release and by eliciting IL-1 production may lead to increased resistance to bacterial infection, probably by synthesis of acute phase proteins. The present study provides supplementary evidence for the pleiotropic mechanisms of antibacterial actions of LF, a protein of potential clinical application.

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