Pharmacological Reports
2005, 57, 811–817
ISSN 1734-1140

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Effects of lactoferrin on the immune response modified by the immobilization stress

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Abstract:
Effects of orally administered lactoferrin (LF) on the cellular and humoral immune responses in mice subjected to immobilization stress (IS) were investigated. Here, we demonstrate that long-term IS induced significant suppression of cellular and humoral immune responses in CBA mice. The suppression was attenuated by LF given to mice in drinking water as determined by the number of antibody-forming cells (AFC) in the spleen and the magnitude of delayed type of hypersensitivity (DTH). On the other hand, LF lowered the elevated DTH response in mice exposed to short-term IS (5 h only) on the day of elicitation of the DTH reaction. We also showed that LF up-regulated spontaneous transforming growth factor beta (TGF-β) production in the cultures of mesenteric lymph node cells derived from short-term stressed mice. This is the first report on the regulatory effect of LF on the immune response modified by the psychic stress and is consistent with other reports on antinociceptive and analgesic actions of LF in experimental animals.

Key words:
lactoferrin, immobilization stress, immune response

Introduction

Interdependence between the function of the central nervous system and the immune system is well established [35]. The signaling between these two systems is accomplished by hormones and cytokines, so-called hypothalamic-pituitary-adrenal axis (HPA) and occurs during various types of immune response including trauma and stress [5]. Depending on duration, type and intensity, stressful conditions may differentially affect the immune response in experimental animal models and in humans.

Restraint stress in the mouse model induces severe lymphopenia absent in adrenalectomized mice indicating a role of endogenous steroid hormones [26]. Mature T cells are more resistant to stress than B cells [3]. Stress is also associated with the increased percentage of neutrophils in circulation [4]. Stress affects both humoral [12] and cellular immune response [11] but not in submissive animals [6]. Stress was also found to modify experimental autoimmune encephalomyelitis (EAE) in rats [2]. In humans, mild stress causes a shift towards T-helper type 2 (Th2) cytokine production [18]. A short reexposure of stressed macaques to a moderate stressor caused higher release of

transforming growth factor beta (TGF-β) compared with control animals [28], indicating a role for that cytokine in counteracting adverse consequences of stress.

Lactoferrin (LF) belongs to the family of proteins involved in iron metabolism and is a very important constituent of the innate immune system [15]. The protein content is high in secretory fluids of mammals [15]. It is also present in secondary granules of circulating neutrophils [32]. The serum level of LF upon infection [23] and clinical procedures [32] is significantly elevated. LF presents a wide array of antibacterial actions, both direct [34] and indirect [37] as well as protective properties in experimental endotoxemia [13, 16]. Inhibition of proinflammatory cytokines [16] and free radicals [1] may account for those actions. Several LF cell surface receptors have been described including those on intestinal cells [10]. Although LF is partially degraded by proteolytic enzymes, a part of the protein can cross the intestinal barrier intact [31] and the LF-derived peptides are also strongly immunotropic. LF was shown to inhibit effector phase of delayed type hypersensitivity (DTH) [40] and to selectively suppress function of Th1 cells [41]. In addition, LF was shown to induce interleukin (IL)-10 when given *per os* or intravenously [39], which links LF to the cytokine involved in down-regulation of Th1 type response [22].

The aim of this investigation was to assess potential role of LF in stress-related immune responses in mice.

Materials and Methods

Animals

CBA mice, males and females 12 weeks old, were delivered by Animal Facility of the Institute of Immunology and Experimental Therapy (IIET), Wroclaw, Poland. Mice were fed a pelleted commercial food and water *ad libitum*. The IIET Ethics Committee approved the study.

Reagents

Low-endotoxin bovine milk lactoferrin (LF) (0.16 E.U./mg, < 25% iron saturated) was purchased from Morinaga Milk Industry Co, Japan. Sheep red blood cells (SRBC) were delivered by Wroclaw Agriculture Academy. SRBC were kept in Alsever's solution at 4°C until use. Ovalbumin (OVA) and RPMI-1640 medium were purchased from Sigma (USA); complete (cFa) and incomplete (iFa) Freund's adjuvants were delivered by Difco (USA). Fetal calf serum (FCS) was supplied by Gibco. The level of TGF-β was determined by ELISA kit from R&D Systems.

Immobilization stress (IS) and treatment with LF

Prior to immunization for induction of the humoral and cellular immune response, mice were kept in specially designed restraining device each day for 5 h, for 5 consecutive days (long-term stress). Alternatively, mice were exposed to IS for 5 h before elicitation of DTH response (short-term stress). Mice, including control animals, were not given access to food and water during the restraint period. LF was applied to mice as a 0.5% addition to drinking water for the entire duration of the experiments (5 days before immunization with OVA or SRBC until determination of DTH reaction or AFC numbers) (long-term stress). That concentration of LF has been chosen in the course of our previous studies regarding immunotropic action of LF. In the model of short-term stress, LF was given to mice from the day of immunization to the day of DTH elicitation. For determination of TGF-β production by mesenteric lymph node cells, mice were given LF (0.5% solution in drinking water) for 3 days before short-term stress and cell isolation.

Humoral immune response

Mice (5 per group) were immunized intraperitoneally (*ip*) with 5% suspension of SRBC (0.2 ml). Four days later spleens were isolated and the number of antibody-forming cells (AFC) was determined by a test of local hemolysis in agar according to Mishell and Dutton [19]. The results are presented as the mean AFC values per 10^6 splenocytes ± standard error (SE).

Cellular immune response

Mice were sensitized with 5 μg of OVA emulsified in cFa, injected subcutaneously (*sc*) into the tail base. Four days later mice were given *sc* an eliciting dose of OVA (50 μg) in iFa in the hind foot pads and 24 h later the foot pad edema was measured using a cali-

per. Nonspecific foot pad edema in naive mice, given an eliciting dose of antigen, was subtracted. The results are presented as the mean antigen-specific increase in the foot pad thickness expressed in DTH units (1U = 0.1 mm) ± SE. The experimental groups consisted of 5 mice and the results are given as the mean values from 10 measurements (foot pads) ± SE.

Isolation of mesenteric lymph nodes lymphocytes and determination of TGF-β

Mesenteric lymph nodes were pressed through plastic screens into cold RPMI-1640 medium. The cells were washed two times, resuspended in a cell culture medium (RPMI-1640, 10% FCS, glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics) placed onto 96-well plates (Nunc) at a density of 2×10^5 cells/well, and cultured at 37°C. The level of TGF-β in 30 h cell culture supernatants was determined by ELISA. The results are expressed in pg/ml.

Statistics

The results are presented as the mean values ± SE. The Levene's test was used to determine the homogeneity of variance between groups. When the variance was homogeneous, analysis of variance (ANOVA) was applied, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of the difference between groups. Significance was determined at $p \leq 0.05$.

Results

Effects of LF on stress-elicited changes in the delayed type hypersensitivity

Mice were exposed to an IS regimen for 5 days before sensitization with antigen (OVA) (long-term stress). The results indicate that DTH response inhibited by IS was significantly restored by administration of LF in drinking water (Fig. 1). On the other hand, the DTH response, elevated after short exposure to IS (5 h before administration of the eliciting dose of antigen), was normalized in mice administered LF (Fig. 2).

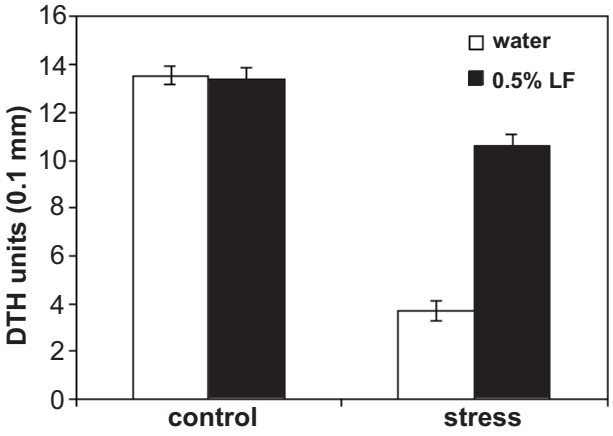

Fig. 1. Up-regulation of the IS-suppressed DTH response by LF. Mice were subjected to IS (5 h daily for 5 days) before sensitization with OVA. LF was applied to mice as 0.5% solution in drinking water throughout the experiment. The results are presented as mean values from 10 determinations (DTH units) ± SE. Control water *vs.* control LF 0.5% NS, control water *vs.* stress + water < 0.001, stress + water *vs.* stress + LF 0.5% < 0.001, control LF 0.5% *vs.* stress + LF 0.5% < 0.01

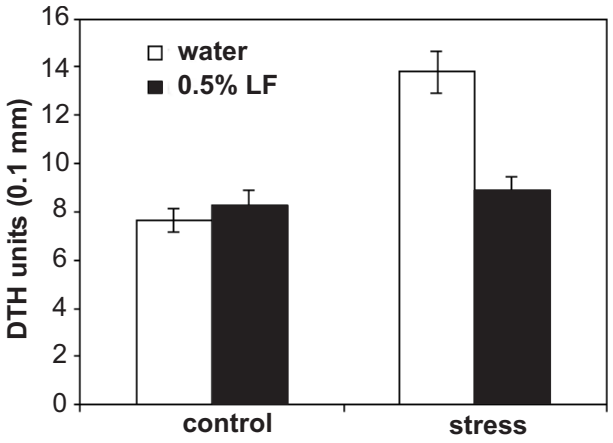

Fig. 2. Down-regulation of the IS-stimulated DTH response by LF. Mice were subjected to 5 h IS just before elicitation of the DTH reaction. LF was applied beginning on the day of immunization. The results are presented as mean values from 10 determinations (DTH units) ± SE. Control water *vs.* control LF 0.5% NS, control water *vs.* stress + water < 0.001, stress + water *vs.* stress + LF 0.5% < 0.001, control LF 0.5% *vs.* stress + LF 0.5% NS

Effects of LF on stress-elicited inhibition of the humoral immune response

Mice exposed to IS regimen for 5 days (long-term stress) exhibited suppressed humoral immune response as determined by the number of antibody-producing cells. As presented in Figure 3 the suppression was reversed in mice administered LF in drinking water in parallel to IS.

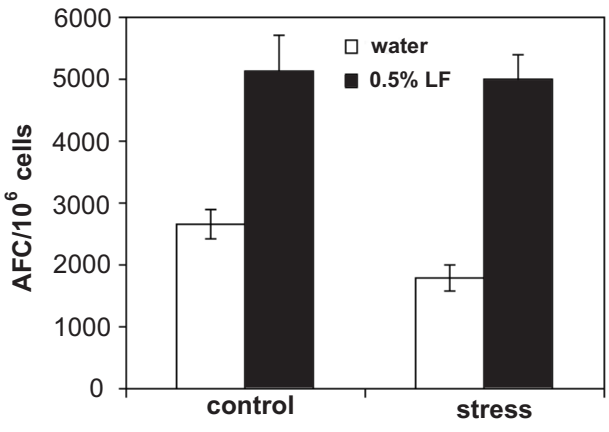

Fig. 3. Reversal of the IS-induced suppression of the humoral immune response by LF. Mice were subjected to IS (5 h for 5 consecutive days before immunization with SRBC). LF was applied in parallel with IS until determination of AFC numbers. The results are presented as mean values from 5 determinations of the number of AFC per 10^6 spleen cells ± SE. Control water *vs.* control LF 0.5% < 0.01, control water vs. stress + water < 0.05, stress + water *vs.* stress + LF 0.5% < 0.001, control LF 0.5% *vs.* stress + LF 0.5% NS

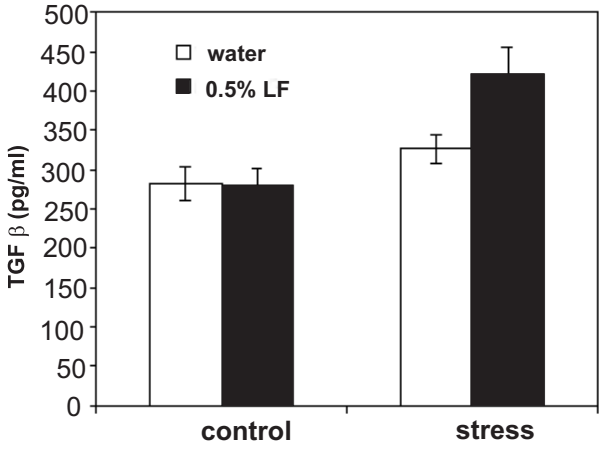

Fig. 4. LF up-regulates spontaneous TGF-β production in mesenteric lymph node cultures from stressed mice. Mice were given 0.5% LF solution in drinking water for 3 days and subjected to IS (5 h) before isolation of mesenteric lymph nodes. TGF-β level (pg/ml) was determined in 30 h culture supernatants. The data are presented as mean values from 5 mice/cell cultures ± SE. Control water *vs.* control LF 0.5% NS, control water *vs.* stress + water NS, stress + water *vs.* stress LF 0.5% < 0.05, control LF 0.5% *vs.* stress + LF 0.5% < 0.01

Up-regulation of spontaneous TGF-β production in mesenteric lymph node cells derived from stressed mice treated orally with LF

The effects of LF on the spontaneous TGF-β production in stressed and unstressed mice are presented in Figure 4. Mice were drinking 0.5% LF solution for 3 days, then were stressed for 5 h (short-term stress) and mesenteric lymph nodes were isolated on the next day. The levels of TGF-β in 30 h cell cultures were comparable in control unstressed mice and unstressed control mice drinking LF solution. The concentration of TGF-β in cell cultures from control stressed mice was slightly elevated but lymph node cells from stressed mice additionally given LF produced significantly more TGF-β.

Discussion

In this report we demonstrated that oral administration of LF might reverse stress-elicited immune responses in mice. We relate these effects of LF in part to its ability to "sense" an abnormal immune status to act accordingly [36, 43]. It is, however, difficult to provide satisfactory explanation for LF actions without a complex study of the stress-induced immune responses over long periods of time. One could also envisage that LF can counteract changes in stress-elicited release of endogenous steroids by several mechanisms. We recently found (unpublished) that LF may transiently increase cortisol serum level when given intravenously to mice. The ability of LF to reverse the consequences of the IS could be also related to induction of IL-6 [39] or nitric oxide [38], that are signaling molecules in the nervous and immunological systems. Others have found that blockage of nitric oxide synthase prevented LF protective action in a rat stress model [29]. The authors also demonstrated that LF suppressed distress in that model *via* an opioid-mediated mechanism. LF applied spinally may also produce analgesia, which could be reversed by its co-administration with nitric oxide synthase inhibitor [9]. A possibility exists that LF, by inhibiting free radical formation [1], may attenuate lipid peroxidation in piriform cortex as reported for topiramate in kainate-induced status epilepticus in rats [14]. In addition, LF may cause a decrease in the blood pressure [8] possibly counteracting the effects of IS on blood pressure. Antinociceptive effect in the rat adjuvant-induced arthritis model was recently found upon oral LF administration [7]. The regulatory action of LF in the IS model may also share a similar mechanism as in the case of LPS-induced desensitization of the HPA axis [30], since in several *in vitro* [36, 42] and *in vivo* [39] models LF demonstrated comparable effects as LPS.

The regulatory effect of LF in the mouse model may also be associated with the ability of LF to induce a potent, immunosuppressive cytokine TGF-β.

That phenomenon could be particularly relevant to the model of short-term stress where LF exerted the down-regulatory activity. In fact, we demonstrated that oral treatment of stressed mice with LF increased TGF-β production by mesenteric lymphocytes (Fig. 4). Since others reported that TGF-β might inhibit liberation of corticotropin releasing factor (CRF) [21], it is possible that stress-generated changes in the functioning of the immune system may be, in this way, reversed. Furthermore, the level of IL-10, a down-regulatory cytokine [25], was also increased (2-fold) in the cultures of mesenteric lymph node cells, derived from stressed mice drinking LF solution (not shown). Therefore, the protective action of LF in diminution of stress-related disorders may be due to its regulatory action on TGF-β and IL-10. Still another possible mechanism of counteracting effect of LF in the short-term stress model is its ability to inhibit migration inhibitory factor (MIF) [40]. That is a unique cytokine which is activated by a stress mediator – CRF [33].

LF is not the only protein, present in milk, exhibiting analgesic and relaxing properties. Similar activities are displayed also by casein peptides [24], α-lactalbumin [17] and peptides derived from α-lactalbumin and β-lactoglobulin – α- and β-lactorphin, respectively [20, 27]. Casein peptides decreased blood pressure, elevated during stress, by inhibiting the activity of the enzyme converting angiotensin I to active angiotensin II (a strong hypertensive compound acting on blood vessels). Hypotensive action of the lactorphins was attributed to its effect on endothelial function [27] and was abolished by naloxone, an antagonist of the opioid receptors [20]. One of those casein peptides demonstrated also opioid activity [24]. Nevertheless, there have been no reports on the counteracting actions of these proteins and peptides on stress-modified immune response.

In conclusion, this study demonstrated that LF could normalize the IS-induced immune response in mice. The above-described antinociceptive and anti-inflammatory actions of LF and its regulatory effect on cytokine production may account for these effects. These results suggest a possible application of LF for treatment of patients suffering from stress-related disorders.

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Received:
November 16, 2004; in revised form: September 19, 2005.