Stimulating effect of bovine lactoferrin (bLF) on the level of lipopolisaccharide-binding protein (LBP) during experimental diabetes in mice

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Reduced inflammatory responses are frequently associated with *diabetes mellitus*. In order to investigate the influence of *diabetes mellitus* on the activation of lipopolysaccharide-binding protein (LBP), diabetic mice with high alloxan hyperglycaemia (higher than, or equal to 300 mg/100 ml of blood glucose) have been produced. It was shown that association of diabetes with abnormally high susceptibility to infections is partially caused by decreased level of LBP and that this negative phenomenon is linked to disturbances in lactoferrin action. Intravenous injection of bovine lactoferrin (bLF) may prevent against negative results of diabetes on immune system.

Insulin-dependent *diabetes mellitus* is a serious chronic disorder caused by an immune-mediated, selective destruction of β cells. Although insulin-replacement therapy increases life expectancy, the disease is characterized by severe complications that include down-regulation or deregulation of some natural defence parametres in mammals [Geerlings and Hoepelman 1999].

Most studies concerning cellular innate immunity show decreased functions (chemotaxis, phagocytosis) and killing of diabetic polymorphonuclear cells and

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diabetic monocytes/macrophages compared to control cells. Furthermore, some microorganisms become more virulent in a high-glucose environment. Another mechanism which can lead to the increased prevalence of infections in diabetic patients is an increased adherence of microorganisms to diabetic than to non-diabetic cells [Geerlings and Hoepelman 1999].

Protective activities of bovine lactoferrin (bLF) were reported against infections during *diabetes mellitus* [Zagulski *et al*. 2001]. The mechanisms of protective actions of bLF against microbial, for example oral candidiasis, were particularly elucidated to include augmentation of T-cell activities of lesional lymphoid tissues [Abe 2004]. However, antimicrobial and anti-inflammatory activity of bLF seems to be more complicated.

During diabetes antibacterial action of lactoferrin (LF) is decreased, mainly by the binding to glucose-modified proteins bearing advanced glycation endproducts (AGEs), which formation and deposition are much enhanced in diabetes, mainly as a consequence of hyperglycaemia. Exposure to AGE-modified proteins blocks the bacterial agglutination and bacterial killing activities of LF. Modified LF mediates the interaction of AGEs with endothelial cells [Li 1998].

Since earlier studies indicated a stimulation of killing and clearance of bacteria in diabetic animals [Zagulski *et al*. 2001], we decided to examine whether these phenomena are based on LF-LBP interaction. With the enzyme linked immunosorbent assay (ELISA), we subsequently tested: (1) the induction of LBP by bLF and (2) the co-reaction of bLF with alloxan on the level of LBP.

Material and methods

Animals

The Swiss Webster (CFW®) male mice bred at the Polish Academy of Sciences Institute of Genetics and Animal Breeding, Jastrzębiec, were used at the age of 3 months, weighing 25-30 g. The animals were housed in the same-family groups, 4-5 per cage (290 × 210 × 100 mm), at 23°C, on 12:12 light-dark cycle, and had unlimited access to murine chow (LABOFEED H, Poland), containing 1340 kcal ME, and 22 g total protein (with 1.5% of lysine), 5 g crude fibre, 4 g ether extract and 6,5 g crude ash per 100 g fresh matter.

The protocol of the experiments on live mice was approved by the State Ethics Commission for Experimentation on Animals. All procedures adopted for this research are commonly used and considered ethically acceptable in all countries of the European Union and North America [Stafleu *et al*. 1999].

Preparation and use of chemicals

Lactoferrin. Bovine lactoferrin (bLF) was isolated from milk according to Zagulski *et al*. [1979]. The purity of bLF preparations, as checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoelectrophoresis, was not less than

99%, and iron saturation was 100%. To remove endotoxin, bLF was further purified by preparative Detoxi-Gel Endotoxin Removing Gel chromatography following the "Instruction for use" by PIERCE CHEMICAL Co. (Rockford, IL, USA). The final preparation of bLF was dialyzed extensively against LPS-free water, and recovered by freeze drying. The final bLF contained less than 4 ng endotoxin/g protein, as determined by Quantitative Chromogenic *Limulus Amebocyte* Lysate assay (QCL-1000, BioWhitaker, Qualkersville, MD) – Gardi and Arpagaus [1980].

For all experiments bLF was diluted (5 mg/ml) in pyrogen-free phosphate buffered saline (PBS), pH 7.2 and administered to mice in a total volume of 0.1 ml. In all experiments the control animals were injected with 0.1 ml pyrogen-free PBS.

Bovine serum albumin (BSA) – 98% purity – was obtained from SIGMA and administered to mice as control protein in the same dose and by the same route(s) as bLF.

Alloxan (Alx) – was obtained from SIGMA and used at a dose 60 mg/kg body weight with 0.1 ml PBS (Alx-2,4,5,6-tetraoxypyrimidine; SIGMA Chem. Co., St. Louis, USA).

Experimental

Development of diabetes. Diabetes was induced by a single intravenous injection (tail) of Alx (60 mg/kg body weight in 0.1 ml PBS). Mice were considered diabetic when their blood glucose level was >300 mg/dL (approx.). The control animals were injected with the same volume of PBS and had glucose levels between 90 and 110 mg/dL. The glucose level was determined in the whole blood of all the animals using "Glucocard" of KYOTO DAIICHI KAGAKU Co., Japan)

LBP assay. In all experiments the plasma LBP was quantified by means of an ELISA using monoclonal coating antibody (generated in LBP knocked out mice) against mouse LBP (BIOMETEC, bIG). Fifty microliters of affinity purified rabbit anti-rLBP antibody (2 μg/mL in PBS) were incubated overnight at 2-8°C (or alternatively, 1 hour at 37°C) in the wells of Immulon 2 (DYNATECH Laboratories Inc., Chantilly, VA) microliter plates. The antibody solution was removed and 200 μL of 1% non-fat milk in PBS (blocking agent) was added to all wells. After blocking the plates for 1 hour at room temperature, the wells were washed 3 times with 300 μL of wash buffer (PBS/0.05% Tween-20).

Standards, samples and controls were diluted in triplicate with PBS containing 1% BSA, 0.05% Tween 20 (PBS-BSA/Tween) and 10 units/mL of sodium heparin (SIGMA Chemical Co., St. Louis, MO) in separate 96-well plates. rLBP or rLBP25 standard solutions were prepared as serial two-fold dilutions from 100 to 0.012 ng/mL. Each replicate and dilution of the standards, samples and controls (50 μL) was transferred to the blocked microtiter plates and incubated for 1 hour at 37°C. After the primary incubation, the wells were washed 3 times with wash buffer. Biotin-labelled rabbit anti-LBP antibody was diluted 1/2000 in PBS-BSA/Tween and 50 μL was added to all wells. The plates were then incubated for 1 hour at 37°C. Subsequently, all wells

were washed 3 times with wash buffer. Alkaline phosphatase-labeled streptavidin (ZYMED Laboratories Inc., San Francisco, CA) was diluted 1/2000 in PBS-BSA/ Tween and 50 µL was added to all wells. After incubation for 15 minutes at 37°C., all wells were washed 3 times with wash buffer and 3 times with deionized water and the chromogenic substrate p-nitrophenylphosphate (1 mg/mL in 10% diethanolamine buffer) was added in a volume of 50 µL to all wells. Colour development was allowed to proceed for 1 hour at room temperature, after which 50 µL of 1 N NaOH was added to stop the reaction. The absorbance at 405 nm was determined for all wells using a Vmax Plate Reader (MOLECULAR DEVICES Corp., Menlo Park, CA).

The mean absorbance at 405 nm (A_{405}) for all samples and standards (in triplicate) were corrected for background by subtracting the mean A_{405} of wells receiving only sample dilution buffer (no LBP) in the primary incubation step. A standard curve was then plotted as A_{405} against 1 ng/mL of LBP. The linear range was selected, a linear regression analysis was performed and concentrations were determined for samples and controls by interpolation from the standard curve.

Evaluation of bLF effect. To investigate whether bLF can affect the LBP level *in vivo*, mice were injected intraperitoneally with 10 mg/mouse of bLF in a total volume of 0.1 ml. The control animals were injected with the same volume of BSA. About 2 ml of fresh peripheral blood was collected after decapitation to a sterile S-Monovette 2,7 ml EDTA (as a anticoagulant) coated syringe (SARSTEDT AG, Germany). Blood was collected at 0, 2, 6, 12, 24 and 48 hours (different groups of mice) after bLF (or BSA) injection and next plasma was obtained by centrifugation (600 rpm, 20 minutes). Plasma was kept frozen at -80°C. LBP concentration was measured with ELISA, as described above.

Statistical

In all experiments the groups of mice consisted of 9-10 animals. The experiments were performed on plasma from individual mice. LBP levels were measured using five concentrations of plasma (1:50, 1:100, 1:125, 1:200, 1:250) and repeated at least three times for each concentration. The mean values as well as standard deviations are given in the text. Statistical analyses were done using ANOVA test to compare intergroup differences. When appriopriate, post hoc analysis of significant differences revealed by ANOVA was performed using an all pairwise Tukey HSD test. The differences were aimed as significant at P≤0.05*; P≤0.01** and P≤0.001***.

Results and discussion

Effect of lactoferrin on LBP level

A two-way ANOVA, calculated from the changes from LBP baseline levels, showed significant main effects for treatment (BSA *vs*. bLF); [$F(1,52) = 32.43$; P<0.001]. The increase of LBP lasted 24 h with a peak after 6 h, and subsided 48 h after bLF injection. BSA, which has been used as a control protein, did not cause any

significant effect on LBP level. In addition, significant interaction treatment × time [$F(5,48) = 42.06$; P<0.001] was identified.

Effect of bLF on alloxan-diabetic mice

The mean LBP basal concentration of plasma was 58.55 µg/ml (SD = 5.05, n = 11). BLF intraperitoneal injection (10.0 mg/kg/mouse) induced significant increase in LBP content [$F(1,38) = 82.04$; P<0.001]; 187.69% of preinjection baseline level – Figure 2 – confirming the results of previous experiment – Figure 1. After intravenous Alx injections of 60 mg/kg, significant decrease appeared [$F(1,38) = 41.75$; P<0.001] in plasma LBP concentration at 48 h (63.84% of preinjection baseline level).

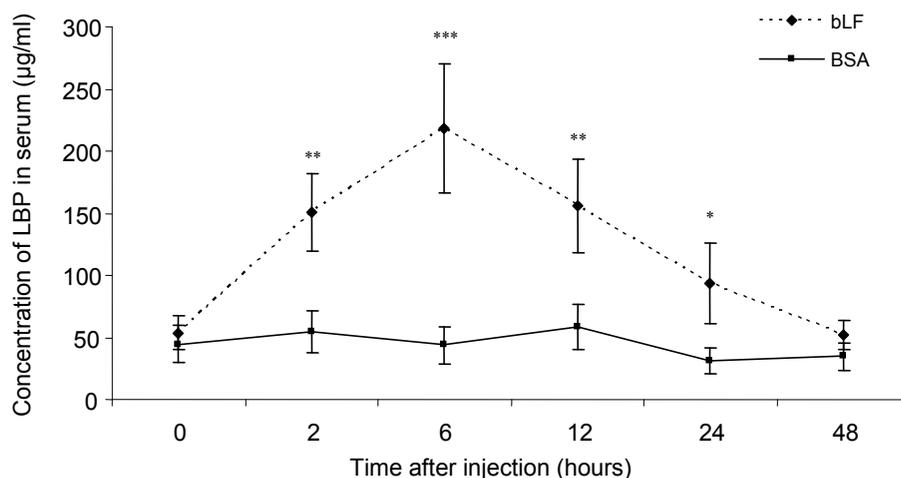Fig. 1. Kinetics of LBP concentrations after bLF injection. Each time point represents 9 mice. *P<0.05, **P<0.01, *** <0.001, (post hoc test: bLF-treated *versus* BSA control group).

Injection of bLF (24 h after injection of Alx) almost completely prevented the Alx effect on LBP concentration (interaction Alx × LF) at 48 h after Alx administration (by significant elevation of LBP level; 273.07% of only Alx treated mice) – [$F(1,38) = 31.58$; P<0.001]. With administration of bLF after Alx injection, there were no significant differences in LBP contents when compared to the mice treated only with bLF [$F(1,17) = 0.36$; P=0.56]. Finally the differences between control and diabetic mice (induced by Alx) in LBP level were signficantly higher than between mice treated with bLF and diabetic mice injected with bLF interaction Alx × LF [$F(1,39) = 4.12$; P<0.05].

The results presented here show that LF plays an important regulatory role in LBP concentration of blood. When administered intravenously this protein increased LBP concentration measured of plasma. The stimulatory action of bLF seems to be
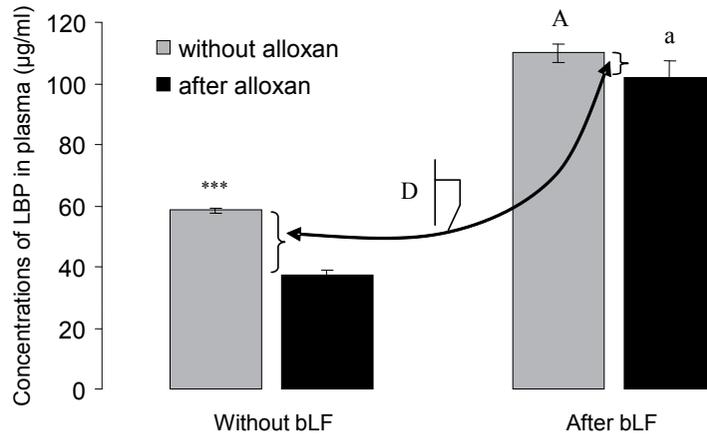
Fig. 2. The effect of bLF on the concentrations of LBP in control and alloxan-treated mice.
***P<0.001, alloxan-treated, n = 10 *vs*. control group, n = 11.
A – P<0.001, bLF-treated mice, n = 9 *vs*. control group, n = 11.
a – P<0.001, alloxan and bLF-treated mice, n = 10 *vs.* alloxan-only treated mice, n = 10.
D – Significant effect of interaction between Alx and BLF.

dose-dependent. Preliminary studies showed that the dose of 10 mg/mouse used in experiment was most efficient. Doses of bLF lower than 100 μg/mouse are not effective and doses higher than 10 mg/mouse did not cause further increase of LBP level (figures not tabulated).

The kinetics of LBP induced by bLF seems to be highly associated with the antibacterial activity of diabetic mice. As shown, LBP concentration in diabetic animals was reduced significantly. Surprisingly, injection of bLF reversed this effect, and 24h after injection of bLF the LBP level was almost the same as in control animals (Fig. 2). It indicates that bLF may be used as an effective immunostimulating agent for diabetic patients. It is in accordance with authors' earlier study [Zagulski *et al*. 2001] showing that bLF administered intravenously abolished unfavourable effect of diabetes which substantially downregulated killing and clearing rate of *E. coli* cells in many organs and from the blood of infected animals. As it was shown earlier bLF injection prevented the mortality of non-diabetic mice infected with *E. coli*. However, this effect was not observed in diabetic mice. Moreover, bLF delayed the development of diabetes as measured by glucose levels in the circulation, but the role of LBP in this phenomenon is unknown [Zagulski *et al*. 2001]. Finally, it is concluded that elevated level of AGEs in tissues and plasma of diabetic patients may inhibit endogenous activity of LF on LBP transcription, thereby increasing susceptibility to bacterial infections.

On the other hand, bLF in the pancreatic acinus may aim as candidate for the target antigen and may participate in the development of pancreatitis leading to the insulin-dependent *diabetes mellitus* [Okazaki *et al*. 2000, Taniguchi *et al*. 2003]. It should be explained definitely to prevent against negative results of lactoferrin-based therapy.

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Stymulujący wpływ bydlęcej laktoferyny (bLF) na poziom białka wiążącego lipopolisacharyd (LBP) podczas doświadczalnej cukrzycy myszy

S t r e s z c z e n i e

Jednym z najpoważniejszych powikłań w przebiegu cukrzycy są zaburzenia związane z odpornością organizmu. Ich mechanizm jest dotychczas w małym stopniu poznany. Przypuszcza się, że odgrywa w tym dużą rolę osłabienie odporności nieswoistej, w której decydującą rolę pełni białko wiążące się z lipopolisacharydem bakterii gram(-), tj. LBP. W celu zbadania wpływu cukrzycy na aktywację LBP, przebadano myszy ze sztucznie indukowaną hiperglikemią poprzez iniekcję aloksan. W badaniach wykazano, że spowodowane cukrzycą predyspozycje do infekcji są spowodowane przez obniżony poziom LBP, co wynika z zaburzeń aktywności laktoferyny. Suplementacja laktoferyny podczas cukrzycy może te niekorzystne objawy hamować.