



Lactoferrin Stimulates Killing and Clearance of Bacteria but Does Not Prevent Mortality of Diabetic Mice

TADEUSZ ZAGULSKI^{1*}, ZOFIA JARZĄBEK¹, ALINA ZAGULSKA¹, MAGDA JASZCZAK²,
IWONA E. KOCHANOWSKA³ and MICHAŁ ZIMECKI³

¹ Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzębiec, 05-552 Wólka Kosowska, Poland, ² Dental Institute, Medical University of Warsaw, Miodowa 18, 00-246 Warsaw, Poland, ³ Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Weigla 12, 53-114 Wrocław, Poland

Abstract. We have previously shown that bovine lactoferrin (BLF) given intravenously (i.v.) protected mice against a lethal dose of *Escherichia coli* and strongly stimulated both the clearing and killing activities in liver, lungs, spleen and kidney. Since some studies indicated a reduction of the manifestation of experimental pancreatitis with lactoferrin (LF), we decided to examine the protective activity of BLF against lethal *E. coli* infection in animals with alloxan (Alx)-induced diabetes. It appeared that 48 h diabetes substantially lowered the killing activity in all four organs as well as the clearing rate of *E. coli* from the circulation. BLF given i.v. reduced this undesirable effect of diabetes. However, in 10- and 20-day diabetic animals, the diabetes alone stimulated the killing activity in the organs investigated, and upregulated the clearing rate of *E. coli* from the circulation. Lactoferrin significantly increased both the killing and the clearing activity in these long-term diabetic animals. In some cases the stimulating effect of BLF was very high, suggesting a concerted action of BLF and diabetes in that category of mice. Despite these beneficial effects of BLF and diabetes on the killing process in the investigated organs, the survival time of animals from all the diabetic groups (48 h, 10 and 20 days) was not prolonged by BLF. The protective properties of BLF did not depend on the blood glucose levels in the diabetic animals. BLF partly delayed the development of experimental Alx-induced diabetes, measured by the glucose level, but only if administered shortly after Alx injection. In conclusion, we demonstrated that the state of diabetes alone could increase killing of bacteria in the investigated organs and LF enhanced this process. However, LF had no protective effect against the mortality of diabetic mice infected with a lethal dose of *E. coli*.

Key words: lactoferrin; protective activity; lethal infection; diabetes; *E. coli*; mice.

Introduction

A substantial part of the biological role of lactoferrin (LF), present in secretory fluids and neutrophils, appears to be connected with the natural defense of mammals^{38, 43}. Lactoferrin alone or together with an-

other iron-binding protein, serum transferrin, is able to generate a microbiostatic defense mechanism by depriving microorganisms of iron in their physiological environment^{5, 12}. However, some microorganisms are able to acquire iron ions during growth in iron-limited conditions³ by scavenging the iron ion from iron-bind-

* Correspondence to: Dr. Tadeusz Zagulski, Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzębiec, 05-552 Wólka Kosowska, Poland, tel.: +48 22 756 14 17, fax: +48 22 756 16 99, e-mail: zagulski@yahoo.com or panighz@atos.warman.com.pl

ing proteins of the host, thus overcoming the defense system. Previously, we found that bovine lactoferrin (BLF) given intravenously (i.v.) to mice, before *Escherichia coli* lethal experimental infection, protected a substantial percentage of the animals from death⁴⁷. Recently, we showed that this protective activity, generated *in vivo* by LF, was primarily a killing system⁴⁹. We also demonstrated that BLF, given i.v. to mice prior to administration of endotoxin, significantly reduced serum tumor necrosis factor α (TNF- α)²⁸.

Insulin-dependent diabetes mellitus (IDDM) is a serious chronic disorder caused by a immune-mediated, selective destruction of β cells. Although insulin-replacement therapy increases life expectancy, the disease is characterized by severe macro- and microvascular complications that include blindness and kidney failure, and a considerably increased mortality². It is well understood that persistent hyperglycemia contributes to tissue and organ damage by promoting the formation of advanced glycosylation end-products (AGEs)⁴. Diabetes is also associated with the down-regulation or dysregulation of some natural defense parameters of mammals^{18, 31, 40}, and acceleration of the ageing process^{31, 33, 39}. Apart from other sources LF was also identified in pancreatic homogenate^{7, 13} and its concentration was found elevated in patients with chronic pancreatitis^{6, 16, 17}; nevertheless, its biological function during the disease remains a matter of hypothesis¹⁵. Recently, it was suggested that LF had a protective effect against the induction of acute pancreatitis in rats by caerulein²³. Taking into consideration the above-cited associations between LF and pancreatitis, we decided to find out whether experimentally induced diabetes had any effect on the protective activity of LF in mice given a lethal dose of *E. coli*.

Materials and Methods

Animals. Experimental protocols were in accordance with our Institute's Ethics Committee. Male and female (50/50%) CFW mice were bred at the Institute of Genetics and Animal Breeding (Jastrzębiec, Poland). The animals had free access to standard laboratory food and water, and were kept at 24–25°C, with a light-darkness cycle of 12 h. The mice were used at the age of 8–10 weeks, weighing 25–30 g.

Preparation and use of BLF. BLF was isolated from milk as described earlier⁴⁵. The purity of the BLF preparations, as checked by SDS-PAGE and immunoelectrophoresis was not less than 99%, with iron saturation of about 100%. Lipopolysaccharide (LPS) contamina-

tion was removed according to KARPULUS et al.²². Briefly, BLF was passed through a Polymyxin B-Sepharose 4B (Sigma Chem. Co., St. Louis) preparative affinity chromatography column. After eluting from the column, the BLF solution was dialysed against LPS-free water and recovered by lyophilization. After passing through the column, the BLF contained 3–9 pg LPS/mg protein, depending on the preparation used. These levels were below any biological activity of the LPS in our experimental conditions. To determine LPS-contamination of our BLF preparations we used the Quantitative Chromogenic Limulus Amebocyte Lysate assay (QCL-000, BioWhittaker, Walkersville, MD). For all experiments, BLF was diluted with pyrogen-free phosphate-buffered saline (PBS), pH 7.2, and was given to mice i.v. in a 5 mg dose in a total volume of 0.1 ml.

Other chemicals used were obtained from commercial companies (mainly Serva, Heidelberg and Sigma Chem. Co., St. Louis) and were of the highest commercially available grade.

Preparation and use of bacteria. The enterotoxigenic strain of *E. coli* 844 0-78 K 80/B was obtained from the Veterinary Museum of Microorganisms, National Veterinary Research Institute, Puławy, Poland. For all experiments the bacteria were prepared as described earlier⁴⁶. Mice were injected i.v. (into the lateral tail vein) with a single dose of living *E. coli* containing 2×10^8 cells suspended in 0.1 ml PBS. The dose given i.v. was lethal for nondiabetic control mice.

Development of diabetes. Diabetes was induced by a single injection of alloxan (Alx; Sigma Chem. Co., St. Louis, USA) into the tail vein; 60 mg/kg body weight with 0.1 ml PBS. Mice were considered diabetic when their blood glucose level was > 300 mg/dl (approx.). The control group was injected with the same volume of PBS. Control mice had glucose levels between 90 and 110 mg/dl.

Determination of blood glucose. The glucose levels was determined in the blood of all the animals using "Glucocard" (Kyoto Daiichi Kagaku Co., Japan). Glucose was measured before the start of the experiment (i.e. before Alx or PBS injection), and then on days 1, 5, 10 and 20 of diabetes.

Determination of the number of living of *E. coli* cells in blood and tissues. In these experiments, diabetes was induced at three different time points before i.v. injection of *E. coli* LD₁₀₀ 48 h, 10 days and 20 days. These mice were further divided into untreated animals and mice given BLF (5 mg in 0.1 ml PBS, i.v.) 24 h before *E. coli* LD₁₀₀. Nondiabetic mice were treated with PBS instead of Alx or BLF. All the animals from this series of experiments were analyzed 5 h after

E. coli LD₁₀₀ injection. The living *E. coli* cells in blood and tissues were counted as described earlier⁴⁹. Blood samples from individual mice were collected aseptically from the portal vein. Serial 10-fold dilutions of blood in sterile PBS were plated in a volume of 0.1 ml on McConkey agar (Difco Lab., Detroit, Michigan). The plates were incubated at +37°C for 18 h. Colonies were counted and expressed as the log of the colony-forming units (CFU) per 1 ml of blood. After blood collection, the lungs, liver, spleen and kidney were removed from the mice, weighed and homogenized in sterile PBS (1 g of wet tissue per 25 ml PBS) with a Glass-Coll TRI-R K41 homogenizer, 3 × 10 s at 6000 cycles/min using a glass tube and teflon pestle. At the same time, the culture of *E. coli* from the breeding bottle, used for the infection of the mice, was homogenized under the same conditions. The homogenates of organs and *E. coli* suspension were serially 10-fold diluted with sterile PBS and 0.1 ml of the dilutions were plated on McConkey agar plates, incubated and counted as described above for the blood samples. The number of colonies was expressed as the log CFU per 1 g wet tissue or for 1 ml of *E. coli* culture. The preparation of the homogenates and their plating on the McConkey agar plates were performed in sterile or semi-sterile conditions.

Survival experiments. Diabetes was induced as described above. Half of the diabetic mice was treated with BLF (5 mg in 0.1 ml PBS, i. v.) 24 h before *E. coli* LD₁₀₀. One control group was given BLF 24 h before A1x, and *E. coli* LD₁₀₀ was injected 48 h after A1x. Other control mice were treated with BLF and 24 h later injected with *E. coli* LD₁₀₀, and the third control group was injected with PBS and 24 h later with *E. coli* LD₁₀₀. The survival experiment was performed on mice given A1x 20 days, 10 days or 48 h before BLF administration, followed by *E. coli* injection after 24 h. The survival rate of the animals was determined on days 1, 3, 7, 14, 21 and 30 after *E. coli* LD₁₀₀ injection.

Studies on insulin treatment. The animals were injected with A1x as described above. Starting 48 h later, the diabetic animals (blood glucose levels >300 mg/dl) received subcutaneously (s.c.) one unit of insulin (Humulin M4 (40/60), Lilly, France S.A., F-67640 Fegersheim, France) once daily for 20 days. On day 19 after administration of A1x, the animals were given BLF as described above and 24 h later injected with *E. coli* LD₁₀₀. The survival rate was determined on days 1, 3, 7 and 10 after *E. coli* LD₁₀₀ injection. The blood glucose levels were determined as described above.

Determination of the effects of BLF administration on the development of diabetes. The animals were

divided into two groups: A1x- or PBS-treated. Next, each category of animals was injected with BLF as follows: the first dose 12 h after A1x or PBS (5 mg BLF in 0.1 ml PBS i.v. permice) and then, continuously, 2 mg BLF in 0.1 ml PBS per mouse every two days. The glucose level was determined as previously described, the first time just before A1x or PBS, and thereafter once a day.

Statistical analysis. Analysis of variance using the SAS General Linear Model was made for the clearing and killing rates to establish significant differences (*p<0.01) between means for different experimental groups. Results are presented as means from at least 3 parallel experiments. Each experimental group consisted of 10 mice.

Results

Killing and clearing rate of E. coli in investigated organs and in blood of diabetic and nondiabetic mice

The live *E. coli* cells in suspension from the breeding bottle, homogenized under the same conditions as for tissue samples, were not killed during the homogenization procedure (control, data not shown). The killing rate of *E. coli* among the investigated organs (Fig. 1) was differential and also depended on the duration of diabetes. In the nondiabetic control groups, the best effects of BLF pretreatment were seen in the lung (an about 400 times increase of the killing capacity); the effects of BLF in other organs were, however, also very significant. It appeared that 48 h diabetes lowered the killing rate of *E. coli* and BLF was, in all cases, able to significantly enhance the killing rate, but not to the levels observed in nondiabetic BLF-treated mice. Further analysis of the figure revealed that long-term, in particular 10-day diabetes significantly increased the killing rates of *E. coli* in all organs, with the best effects observed in the lungs and in the livers. Pretreatment with BLF gave the best results in the case of 10- and 20-day diabetes in the lung and 20-day diabetes in the kidney. These effects of BLF were, however, proportional to those reported in nondiabetic the control animals. BLF also stimulated the clearing rate of *E. coli* in all groups (Fig. 2). In 10-day diabetic animals, the stimulatory effect of BLF was unusually high (1.2×10^4 CFU), as compared with nondiabetic mice the control, where 5×10^8 in unprotected, and 3×10^6 CFU in BLF-protected mice were found. This result strongly suggests a concerted stimulatory action of diabetes and BLF on the clearing of *E. coli* from the circulation.

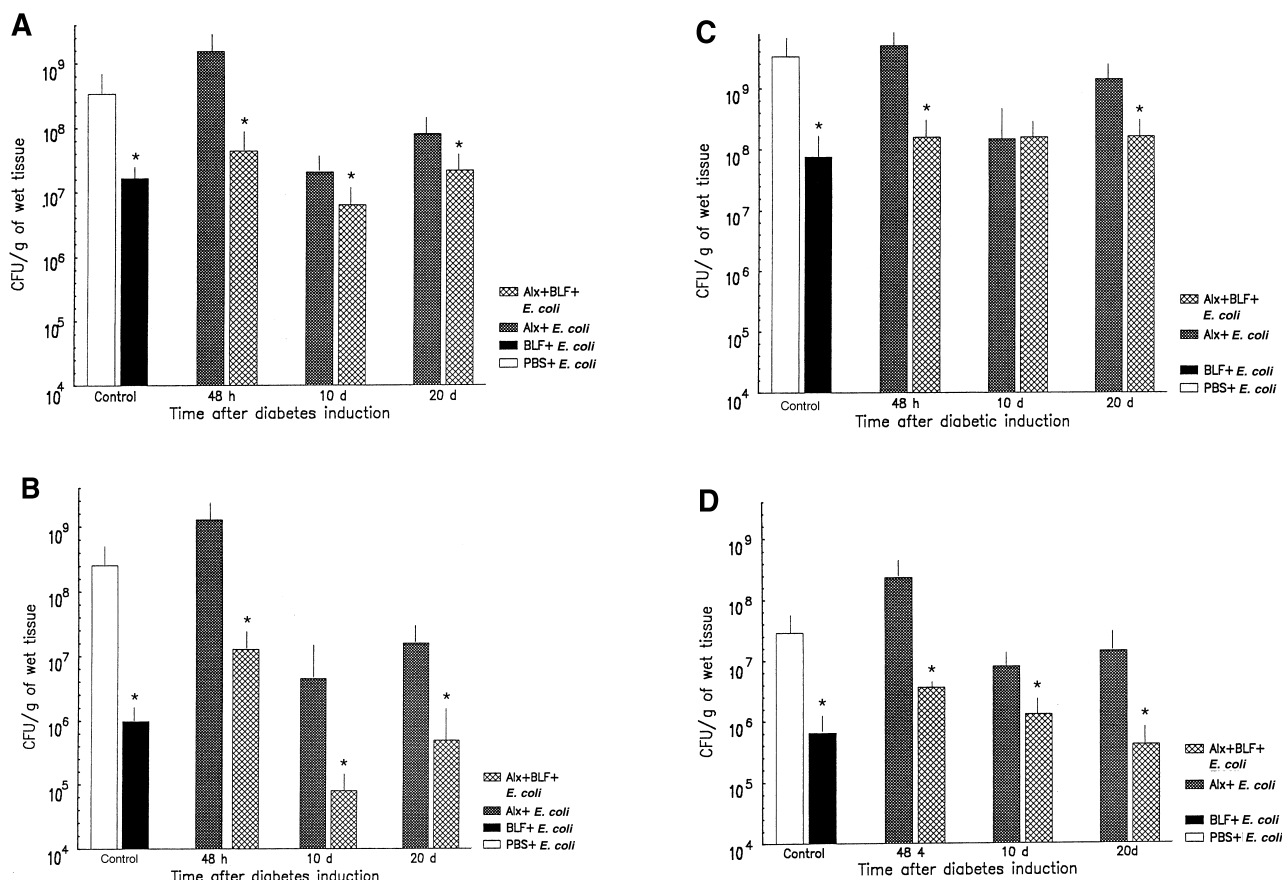


Fig. 1. The killing rate of live *E. coli* in the liver (A), lung (B), spleen (C), and kidney (D) of normal and diabetic mice treated with BLF and infected with *E. coli*. Results are expressed on a logarithmic scale as means $\pm$ SE. Means differ significantly from the control group (Alx+*E. coli*) at * $p < 0.01$

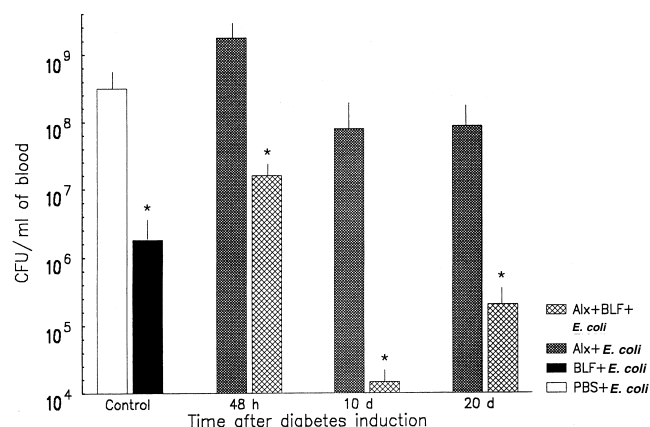


Fig. 2. The clearing rate of live *E. coli* from the blood of normal and diabetic mice treated with BLF and infected with *E. coli*. Results are expressed on a logarithmic scale as means $\pm$ SE. Means differ significantly from the control group (Alx+*E. coli*) at * $p < 0.01$

Effect of BLF on survival of diabetic mice

In the survival experiments we demonstrated (Table 1) that BLF given i.v. protected only a small fraction of the diabetic animals (survival rate 18–22% on day 30

after *E. coli* LD₁₀₀), compared with nondiabetic control groups (survival rate 0 and 82% for untreated and BLF-treated, respectively). This result was not significantly dependent on the duration of diabetes (2 to 20 days).

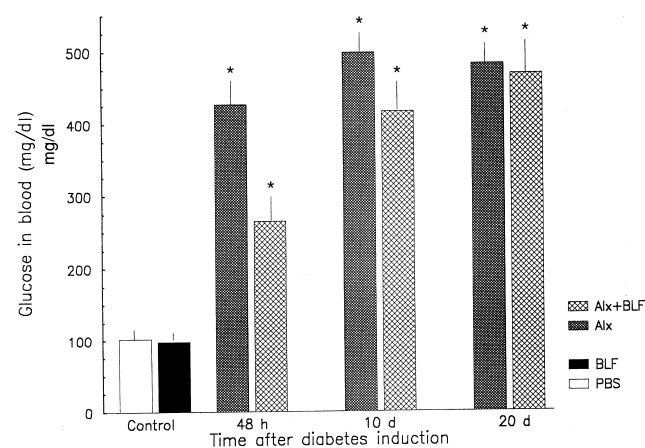
Effect of BLF on glucose levels in diabetic mice

The glucose levels in the blood were very high at all times during these experiments. In Fig. 3, blood glucose levels are shown in all 3 groups of diabetic control animals, as measured just before *E. coli* LD₁₀₀ administration. They varied 270–500 mg glucose/dl of blood in diabetic animals, compared with nondiabetic controls (90–110 mg/dl). A single injection of LF markedly downregulated the glucose levels in the blood of 48 h diabetic animals (Fig. 3). In 10- and 20-day diabetic mice, the effect of BLF was not substantial. Similar results were observed in the experiment where BLF was administrated repeatedly after A1x (data not shown). The persistent lowering of blood glucose levels, elevated after A1x injection, to physiological levels using insulin did not restore the BLF-generated

Table 1. The effects of BLF on the survival of normal and diabetic mice infected with *E. Coli*: dependence on duration of diabetes

Experimental group	Survival rate in groups (%) day of experiment						
	0	1	3	7	14	21	30
PBS + <i>E. coli</i>	100	2 ± 1	0	0	0	0	0
PBS + BLF + <i>E. coli</i>	100*	100*	96 ± 2*	90 ± 4*	90 ± 4*	84 ± 5*	84 ± 5*
BLF + Alx 48 h + <i>E. coli</i>	100	36 ± 6	18 ± 3	18 ± 3	18 ± 3	18 ± 3	18 ± 3
Alx 20 d + <i>E. coli</i>	100	0.5 ± 1	0.5 ± 1	0.5 ± 1	0.5 ± 1	0.5 ± 1	0.5 ± 1
Alx 10 d + <i>E. coli</i>	100	1.5 ± 2	1.5 ± 2	1.5 ± 2	1.5 ± 2	1.5 ± 2	1.5 ± 2
Alx 48 h + <i>E. coli</i>	100	11 ± 3	11 ± 3	11 ± 3	11 ± 3	11 ± 3	11 ± 3
Alx 20 d + BLF + <i>E. coli</i>	100	54 ± 2	33 ± 3	33 ± 3	29 ± 2	20 ± 8	20 ± 8
Alx 10 d + BLF + <i>E. coli</i>	100	50 ± 1	27 ± 2	21 ± 3	21 ± 3	21 ± 3	21 ± 3
Alx 48 h + BLF + <i>E. coli</i>	100	60 ± 4*	40 ± 6	20 ± 3	20 ± 3	20 ± 3	20 ± 3

For details of treatment of mice see Materials and Methods. Results are expressed as means SE. Means differ significantly from the control group (PBS + *E. coli*) at * $p < 0.01$.

**Fig 3.** Blood glucose levels in normal and diabetic mice treated with BLF. Results are expressed as means $\pm$ SE. Means differ significantly from the control group (PBS) at * $p < 0.01$

protecting mechanism(s), since BLF could not protect 10-day diabetic mice against *E. coli* LD₁₀₀ (survival 9% compared with 0% in Alx + *E. coli*, 0% in PBS + *E. coli* and 100% in BLF + *E. coli* group).

Discussion

In this report we demonstrated several interesting phenomena associated with the antibacterial activity of diabetic mice and the effects of BLF administration to diabetic mice infected with a lethal dose of *E. coli*. First, long-term, but not early (48 h), diabetes enhanced the killing of bacteria in the studied organs and the clearing rate from the circulation. Secondly, BLF retained its antibacterial activity in diabetic mice and, in the case of clearing bacteria from circulation, it even exhibited a synergistic stimulatory effect with the con-

dition generated by diabetes. Thirdly, BLF delayed the development of diabetes as measured by glucose levels in the circulation. Lastly, BLF could not prevent the mortality of diabetic mice infected with *E. coli*, although it exhibited such a protective property in non-diabetic mice.

There are at least three phenomena which require special attention and discussion: 1) the advantageous effect of diabetes alone on the killing and clearing rate of bacteria, 2) the costimulatory effect of BLF and 3) the lack of protection against mortality by BLF in diabetic mice. The first issue may be difficult to explain. In fact, there is only one report available describing the activation of reticuloendothelial macrophages by diabetes⁴⁴. This study supports that observation since the state of diabetes preferentially stimulated the killing of bacteria in reticuloendothelial system (RES)-rich organs, such as lungs and liver, and affected to a much lesser degree spleen and kidney (Fig. 1). There are, however, two other reports indirectly explaining the phenomenon of the increased capacity of RES to phagocytize and kill bacteria. First, it was shown that macrophage mannose/N-acetylglucosamine receptors are downregulated by elevated glucose concentrations⁴². Second, the macrophage candidacidal activity, enhanced by interferon γ (IFN- γ), was correlated with a decreased number of mannose receptors²⁹. The mechanism of the second phenomenon, i.e. the stimulatory action of BLF and diabetes in clearing bacteria from the circulation, may be complex and result from a variety of direct and indirect actions of LF on the immune system, bacteria and pancreas. The protective effect of LF on experimental acute pancreatitis, induced by caerulein, was studied by KOIKE and MAKINO²³. They found that LF significantly reduced the serum amylase level, pancreatic wet weight and histological alterations in that organ. In our study,

BLF delayed development of diabetes as judged by glucose levels, which supported the observations mentioned above. Although 48 h diabetes lowered the killing and clearing rates of *E. coli*, BLF significantly reduced that undesirable effect of early diabetes. The reduction of the clearing rate of bacteria by 48 h diabetes itself cannot be reasonably explained at present, however. The protective activity of BLF in diabetes may also result from the ability of this immunoregulatory protein to induce or modulate activities of several cytokines. We have previously found that BLF was also able to preferentially inhibit the activity of TH1 but not TH2 antigen specific ones⁵². Data on the role of cytokines in diabetes clearly indicate that this autoimmune disorder, mediated by TH1 cells²⁷, may be inhibited by blocking IFN- γ ⁸, but can be prevented by application of TH2-type cytokines such as IL-4³⁶ or IL-10^{25, 34}. We have recently shown that BLF was able to induce substantial amounts of IL-10 *in vivo*⁵⁰. Diabetes in mice could also be prevented by TNF- α ¹⁹, adjuvant therapy³⁰ and treatment with 65 kDa heat shock protein (HSP65)¹¹. It appears that all this information may also indirectly explain the protective effect of LF in diabetes. Lactoferrin was shown to induce TNF- α *in vivo*⁵³, exhibit adjuvant properties⁵¹ and to crossreact with HSP65¹⁴. Other properties of LF may also account for its antibacterial actions, including activation of RES^{48, 49}, ability to chelate free iron ions¹², reduction of hypothermia in endotoxemia²⁴, activation of lysozyme¹, binding of LPS¹⁰ and preservation of the gut structure²⁴.

Although BLF appeared to be effective in increasing the killing and clearing rates of *E. coli* in diabetes, it was somewhat disappointing that it did not improve survival of infected mice in comparison to nondiabetic control mice. Several factors may account for that phenomenon. *In vitro* studies showed, for example, that the antibacterial activity of LF was inhibited by the binding of advanced glycation end products to a specific domain in lactoferrin²⁶. Such a process is not very likely in our model, since BLF had a significant ability to enhance the killing and clearing processes of *E. coli*. It was, however, demonstrated that diabetes induced substantial endothelial dysfunction^{9, 20}. A possibility also exists that diabetic mice are more susceptible to septic shock and, consequently, to organ failure, since the disease leads to vascular complications and kidney dysfunction². Lastly, the high glucose levels could simply promote bacteria growth. We suggest that a combination of several pathological changes caused by diabetes may impair defense mechanisms, causing high mortality in diabetic mice despite advantageous effects of BLF in the killing and clearing processes. We recently

demonstrated⁵⁰ that BLF may mediate two kinds of effects on LPS-induced serum cytokine levels: 1) a strong decrease of both proinflammatory TNF- α and antiinflammatory IL-6 and IL-10 or 2) a strong decrease of TNF- α with a prevalence of IL-6 and IL-10. It is known that a dominance of IL-10 may cause a state of hyporeactivity which is detrimental in models of experimental infection^{32, 41}. It is possible that the state of diabetes favors such a condition upon infection. We have also found (unpublished) that treatment of normal mice i.v. or *per os* before or after performing a cecal ligation and puncture procedure (polymicrobial sepsis) did not influence the survival of the mice despite a significant alteration in peritoneal exudate cytokine levels. BLF may be also indirectly involved in shedding soluble TNF- α receptors by induction of IL-10^{21, 50}. Mice lacking TNF- α receptors may be resistant to endotoxic shock^{35, 37}, but are not protected against bacterial infection. Thus, in some experimental models BLF may not be effective in preventing mortality.

It is clear that at this stage of investigation no definite explanation regarding the described phenomena in diabetic mice may be provided. To achieve that, a more complex methodological approach would be required, including measurement of cytokine activities and other indicators of inflammation and assessment of the immune status of the cells involved.

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