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Lactoferrin regulates the release of tumour necrosis factor alpha and interleukin 6 *in vivo*

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Summary. The effects of bovine lactoferrin on the serum cytokine levels, induced by lipopolysaccharide (LPS) in mice, are described. Bovine lactoferrin (BLF) introduced intravenously, 24 hours before i.v. injection of 50 μg of LPS, significantly lowered the serum concentration of TNF-α. Doses of BLF lower than 100 μg as well as pretreatment of mice with BLF on days 6–2 or 12–2 hours before LPS challenge, were not effective. Moreover, BLF induces by itself a relatively high level of IL-6, peaking at 1 hour following injection.

Pretreatment of LPS-injected mice with BLF causes, in addition, a small but statistically significant drop in IL-6 level. Human albumin, used as a control protein, did not cause any changes in the cytokine levels. The data reported herein provide a satisfactory explanation with regard to preventive activity of LF in infection.

Keywords: lactoferrin, tumour necrosis factor alpha; interleukin 6, regulation, *in vivo*

Intravenous injection of high doses of bacteria or lipopolysaccharide (LPS) leads to septic shock in which TNF-α plays a major role (Beutler *et al.* 1985; Tracey *et al.* 1987; Waage *et al.* 1989; Kunkel *et al.* 1989). Beside TNF-α, other cytokines such as IL-6 and IL-1 (Everaerdt *et al.* 1989; Jirik *et al.* 1989; Ohlsson *et al.* 1990; Dinarello & Thompson 1991), and acute phase proteins (Robey *et al.* 1984) appear in the early stage of septic shock, causing, at high concentrations, tissue injury and even death. Release of IL-6, on the other hand, represents a feedback regulatory/protective mechanism in inflammation and infection (Aderka *et al.* 1989; Revel 1989; Schindler *et al.* 1990) by the inhibition of TNF-α and IL-1 release.

Since the discovery of lactoferrin (LF) in bovine (BLF) (Groves 1960) and human (hLF) (Montreuil *et al.* 1960) milk much attention has been devoted to its antibacterial properties (Arnold *et al.* 1977; Bullen & Armstrong 1979; Guttenberg *et al.* 1989; Harmon *et al.* 1978; Ellison *et al.* 1990). The antibacterial and anti-inflammatory properties of LF have been explained by its ability to chelate iron, essential for bacterial growth (Arnold *et al.* 1977; Bullen & Armstrong 1979), and to damage bacterial cell wall (Ellison *et al.* 1990); its effect on haematopoiesis (Zucali *et al.* 1989) and solubilization of immune complexes (Kijlstra & Jeurissen 1982; Kulics & Kijlistra 1987).

Recently, it has become clear that LF may also participate in the course of inflammation (Lash *et al.* 1983; Mansson *et al.* 1990; Van Snick *et al.* 1974) and can be regarded as an indicator of rheumatoid disease (Lash

Abbreviations: LF: lactoferrin, B: bovine, h: human, HA: human albumin, IL: interleukin, TNF-α: tumour necrosis factor alpha, IL-1ra: interleukin 1 receptor antagonist, i.v.: intravenous, r: recombinant, DMF: dimethylformamide, PMN: polymorphonuclear leucocytes, PGE: prostaglandin E, SDS: sodium dodecyl sulphate, LAL: Limulus Amoebocyte Lysate, IMDM: Iscove's Modified Dulbecco's Medium.

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et al. 1983). Patients with LF deficiency are prone to repeated infections (Trumpler *et al.* 1989); however, duration and severity of infection in neutropaenic patients can be reduced by LF treatment. Other interesting features of LF action include inhibition of prostaglandin E (PGE) release (Pelus *et al.* 1979), formation of classic C3 convertase (Kijlstra & Jeurissen 1982) and generation of hydroxyl radicals (Britigan *et al.* 1989).

One of us (Zagulski *et al.* 1989) has previously described a preventive function of BLF during lethal bacteraemia in mice treated with a high dose of LF 24 hours before infection. The time of treatment and the fact that the LF was fully saturated with iron excluded the possibility of prevention simply by iron chelating. Therefore, other mechanisms could be involved, enabling the animals to survive the shock. The results presented in this paper clearly show that pretreatment of mice with BLF, injected 24 hours later with a sublethal dose of LPS, causes a block in TNF-α production. In addition, BLF, given alone to mice, induces release of IL-6 into circulation. These findings represent, to our knowledge, the first report on the effect of LF on the production of these cytokines during infection and satisfactorily explain the preventive properties of LF.

Materials and methods

Animals

Balb/c female mice, bred in our Institute and fed a standard laboratory food and tap water *ad libitum*, were used at 7–9 weeks of age.

Treatment of mice and preparation of sera for the determination of cytokines

Mice were treated with LF, human albumin (HA) and LPS intravenously in a volume of 0.2 ml. All the reagents were dissolved in 0.9% NaCl. Animals were bled at 2 hours after injection of LPS. The blood from each group of mice was pooled and sera were isolated and sterilized by passing through 0.22 μm membranes. Sera were then diluted in 0.9% NaCl and used on the same day for determination of TNF-α and IL-6. TNF-α and IL-6 were determined in sera diluted in culture media at initial concentrations of 5% for the assay for TNF-α and 0.5% for the IL-6 assay.

Reagents

Bacterial LPS (from *E. coli* 026:B6), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue), were from Sigma Chemicals, St Louis, MO; DMF from Fluka; FCS, IMDM were from Gibco, human albumin (5% solution for i.v. infusion) was from Behringwerke AG, Marburg, Germany.

Bovine lactoferrin

This was prepared and saturated with iron by Dr T. Zagulski (Institute of Genetics and Animal Breeding, Jastrzębiec/Warsaw). This BLF preparation (Zagulski *et al.* 1989) was essentially endotoxin-free as assessed by the limulus lysate test (Sigma).

Cytokines

Recombinant human IL-6 (specific activity 10^6 U/μg;B9 assay) was a generous gift from Dr L. Aarden (Brakenhoff *et al.* 1987), recombinant human TNF-α (specific activity 10^7 U/mg; L929 assay, essentially LPS free as assessed by LAL test) was donated by Dr W.J. Stec (Centre of Molecular and Biomolecular Studies, Łodź, supported by grant No PONT 73/43/92).

Cell lines

IL-6-dependent cell line 7TD1, and highly TNF-sensitive clone WEHI 164. 13, were the generous gifts of Dr J. van Snick (Van Snick *et al.* 1986) and Dr T. Espevik (Espevik & Nissen-Meyer 1986), respectively. These cell lines were maintained as described.

IL-6 bioassay

Interleukin-6 activity was measured as previously described (Van Snick & Nissen-Meyer 1986). Briefly, IL-6-dependent murine B cell hybridoma 7TD1 cells were incubated (2000 cells/well) in 96-well plates (Nunc) with serial dilutions of rhIL-6 as a standard, or serum (first dilution was 0.5%). Three days later the surviving cells were estimated by the MTT colorimetric assay. Absorbances were measured at 550 nm with the reference wavelength 630 nm (Dynatech MR5000). The detection limit of the assay was about 1.5 pg/ml. IL-6-dependent cell line 7TD1 does not respond to other growth factors such as IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF, G-CSF and M-CSF (Van Snick 1989).

TNF-alpha bioassay

TNF-α level was determined by the WEHI 164.13 bioassay (Espevik & Nissen-Meyer 1986). Briefly, WEHI 164.13 cells were seeded at a concentration of 2×10^4 cells/well. Decreasing dilutions of the assayed sera (first concen-

tration of the serum was 5%) or rhTNF-α were mixed with the target cells in the presence of actinomycin D (1 μg/ml). After 20 hours of incubation, MTT was placed into the wells and the cultures were incubated for an additional 4 hours. Next, a lysing buffer was added and the optical density was measured at 550 nm. The detection limit of the WEHI 164.13 assay was about 2.5 pg/ml. The activity was identified by the use of neutralizing concentrations of anti-mouse TNF-α (Genentech).

MTT colorimetric assay for cell growth and kill

The method was developed according to Mosmann (1983) in a modification of Hansen *et al.* (1989). Briefly, MTT (5 mg/ml stock solution) was added to volumes of 25 μl per well at the end of the assay and after 3 hours of incubation at 37°C in 100% humidity, 100 μl of the extraction buffer (20% sodium dodecyl sulphate with 50% dimethylformamide pH 4.7) was added. After an additional overnight incubation, the optical density was measured at 550 nm (Dynatech 5000).

Statistics

Groups of mice consisted of five animals as indicated in the figure legends. The sera were pooled for determination of IL-6 and TNF-α. The experiments were repeated at least three times and representative results were shown in the text. The differences were statistically significant (at least $P<0.01$ by Student's *t*-test); the s.e. never exceeded 10% between three individual wells (within one serum dilution). Some determinations were performed on sera from individual mice (five mice per group) and the mean values as well as standard deviations between the experimental groups were shown (Table 2).

Results

Inhibitory effect of bovine lactoferrin on TNF-alpha release in mice

Zagulski *et al.* (1989) have shown that BLF can prevent the lethal infection of mice injected with *E. coli* when given 10 mg of BLF i.v. 24 hours before infection. Therefore, we used that dose of LF and various times of administration to check whether such a preventive effect could be due to an inhibition of LPS-induced TNF-α release. According to our expectations, the most profound inhibitory effect of BLF on TNF-α production was observed when the protein was given into mice 24 hours before LPS (Table 1). Some inhibition was noted when LPS was administered 48 hours before LPS; however, an earlier pretreatment with BLF was ineffective (data not shown). Human albumin, which has been selected as a control protein, did not cause any significant inhibitory effect on TNF-α release when administered 24 hours before LPS challenge. BLF or HA alone (10 mg/mouse), introduced i.v., induced minimal TNF-α concentrations in the circulation ranging from 38 to 68 U/ml (mean 52 U/ml) for BLF, comparing to 10–38 U/ml (mean 21 U/ml) for HA.

Although WEHI-164.13 has been selected as the most

Table 1. Time dependent inhibition of LPS-induced TNF-α release by bovine lactoferrin*

Time (hours)	TNF-α (U/ml) Pretreatment BLF	HA	LPS‡
24	335.9 (88.3)§	2465.3 (13.8)	
22	666.1 (76.7)		
20	619.7 (78.3)		
18	666.1 (76.7)		
2	2606.3 (8.8)	2710.6 (5.3)	
2			2861.2

* Mice were pretreated with 10 mg of bovine lactoferrin or human albumin and after the times indicated in the table mice were challenged with LPS. After an additional 2 hours blood was collected and sera subjected to determination of TNF-α activity.

† Time before LPS injection.

‡ 50 μg per mouse.

§ Percentages of inhibition LPS induced TNF-α are given in parentheses.

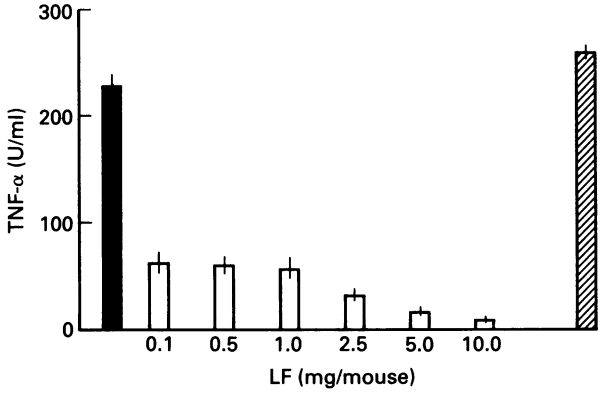

Figure 1. Dose dependent suppression of TNF-α activity by bovine lactoferrin in mice injected with LPS (50 μg/mouse). Mice were pretreated with increasing doses of BLF (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 mg/mouse) or HA (10 mg/mouse). Sera were pooled and tested for the presence of TNF-α. Five mice group were used. ■, LPS; □, LF/LPS; ▨, HA/LPS.

Table 2. Lactoferrin inhibits LPS-induced TNF-α release in mouse sera*

	1	2	3	4	5	Average	s.d.
BLF†	72.2	89.0	81.0	68.9	38.5	69.9 (97.3)§	19.3
LPS‡	2749.6	2689.8	2432.0	2410.3	2521.6	2560.7	152.5

* In the first group, mice were pretreated with 10 mg of BLF. After 24 hours they were injected with LPS. In the second group animals were treated only with LPS. Two hours after LPS injection blood was collected and sera from individual mice were assayed for the presence of TNF-α.

† 10 mg per mouse.

‡ 50 μg per mouse.

§ Percentage of average inhibition of LPS induced TNF-α.

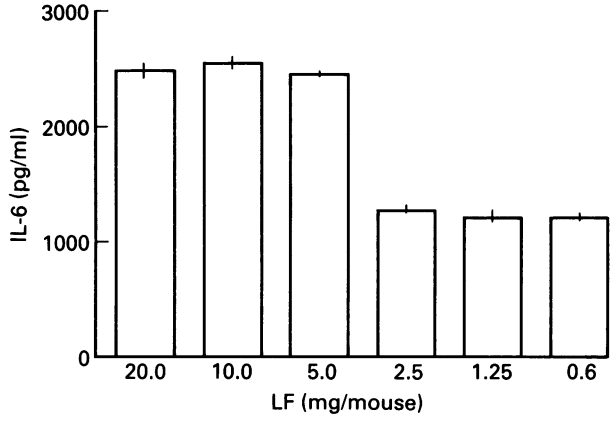

Figure 2. Dose dependent induction of IL-6 by bovine lactoferrin. Mice were treated with decreasing doses of lactoferrin and blood was collected 2 hours later for IL-6 determination. Six separate groups of mice were treated as described in Figure 1. Five mice per group were used.

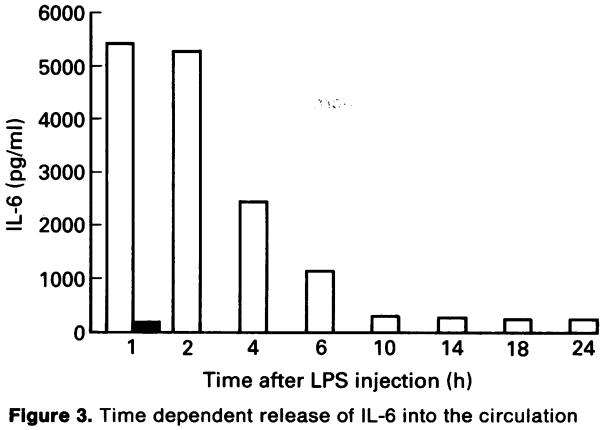

Figure 3. Time dependent release of IL-6 into the circulation induced by □, bovine lactoferrin. Mice were treated with 10 mg of lactoferrin and blood was collected at times indicated in the figure for IL-6 determination. Five mice per group were used. ■, 0.9% NaCl.

sensitive target cell for demonstration of the cytolytic activity of TNF-α (Espevik & Nissen-Meyer 1986) we used polyclonal rabbit anti mouse TNF-α antibodies (Genentech) to show that sera of mice treated with LPS contain TNF-α. The sera were preincubated into an ice bath with diminishing concentrations of the anti TNF-α antibodies for 1 hour and tested for TNF-α activity. It appeared that the α-TNF antibodies, at a high concentration, prevented completely the lytic activity of the serum and that action of the antibodies could be titrated down (not shown).

The inhibitory action of BLF was dose dependent and could be still observed at a dose of 100 μg/mouse; the dose of 10 mg/mouse, however, was most efficient causing more than 95% inhibition of TNF-α release (Figure 1). This very profound inhibitory effect of BLF on LPS-induced TNF-α production was confirmed in experiments where the levels of TNF-α were determined in sera from individual mice (Table 2).

Doses of BLF lower than 100 μg/mouse were not effective and doses higher than 10 mg (20 mg) did not cause further inhibition of TNF-α production (data not shown).

Effect of lactoferrin on IL-6 production

Because of its regulatory function in inflammation, it was important to establish the concentration of serum IL-6 in mice treated with BLF, and BLF plus LPS. It appeared that BLF induced production of IL-6 when mice were treated with 20–0.6 mg of the protein (Figure 2) in a dose dependent manner.

In doses lower than 0.5 mg, the level of induced IL-6 is not detectable (not shown). The control protein (human albumin) did not elicit a significant concentration of IL-6 compared with BLF. Timing experiments showed that IL-6 concentration in serum peaked at about 1 hour after BLF administration (Figure 3).

We also examined how BLF, given 24 hours earlier, affected LPS induced IL-6 release. A small but significant decrease in IL-6 production was found (not shown).

Discussion

The aim of our studies was to reveal the mechanism of the preventive action of LF in infection. Our investigations extended the results of Zagulski *et al.* (1989) who found that pretreatment of mice with high doses of BLF protected the animals from lethal effects of *E. coli* infection. The data presented herein indicate that such animals, pretreated with BLF, could be saved as the result of significantly reduced levels of serum TNF-α. Our finding may also explain the therapeutic value of lactoferrin in inflammation (Kijlstra & Jeurissen 1982; Van Snick *et al.* 1974) and infection (Harmon *et al.* 1978; Zucali *et al.* 1989), since it is clear that TNF-α is responsible for tissue damage in patients and experimental animals with septic shock (Libert *et al.* 1991; Waage & Espevic 1988) and use of anti-TNF-α antibodies reduces the lethal effect of bacteraemia (Beutler *et al.* 1985; Tracey & Cerami 1989). In addition, our results may explain why the serum levels of lactoferrin are drastically elevated in infection (Guttenberg *et al.* 1989) indicating that LF could also play a role as an anti-inflammatory factor. In fact, LF has already been shown to inhibit IL-1 production induced by LPS (Zucali *et al.* 1989).

In our experiments, BLF also affected serum levels of IL-6. IL-6 was found to play a regulatory role in inflammation by inhibition of TNF-α production when administered 4 hours before LPS injection into mice (Aderka *et al.* 1989; Revel 1989; Schindler *et al.* 1990). IL-6 suppresses IL-1 and TNF-α production, elicited by LPS *in vitro* and *in vivo*, and the acute phase plasma proteins which are postulated to prevent tissue injury during inflammation (Revel 1989) but cannot protect mice against a lethal dose of TNF-α (Libert *et al.* 1991). Our results showed that BLF injected alone induces significant IL-6 levels when measured at 2 hours following BLF injection. On the other hand, BLF suppresses IL-6 serum concentration in mice treated with LPS.

It is difficult to evaluate the significance of the altered IL-6 levels in our experiments. IL-6 could play an anti-inflammatory role by suppressing TNF-α production; however, the concentration of serum IL-6 at 24 hours following BLF injection was almost physiological (not shown) and the protective activity of exogenously introduced IL-6, shown by others (Aderka *et al.* 1989; Schindler *et al.* 1990), was seen only when IL-6 was injected 4 hours before LPS. In our model, treatment of mice with BLF at 6, 4 or 2 hours before LPS injection, thus indicating high levels of endogenous IL-6 4 hours before administration of LPS, had no influence on TNF-α levels (not shown). This discrepancy may be due to a possible involvement of other cytokines in the protective phenomenon. It seems likely that the mechanism of the protective action of the exogenously administered IL-6 is different from that of BLF (this paper) and of IL-1 or TNF-α (Schindler *et al.* 1990; Libert *et al.* 1991). The possibility that the protective effect of BLF does not involve desensitization mediated by TNF-α or IL-1 is further supported by the inability of LF to induce significant concentrations of TNF-α (this paper) and IL-1 (Zucali *et al.* 1989). The inhibitory activity of lactoferrin was not changed in adrenalectomized mice indicating that corticosteroids are also not involved in this phenomenon (our unpublished data). Actions of BLF, IL-1 or TNF-α, when administered 24 hours before LPS, may depend on a transient inactivation of the reticuloendothelial system cell function, rendering the cells unresponsive to a second challenge with LPS or TNF-α. Recent findings, however, argue strongly against such a possibility (T. Zagulski, personal communication). Alternatively, the desensitization phenomenon, induced by LF, may involve appearance of receptor antagonists and/or soluble receptors for IL-1 and TNF-α which will effectively block TNF-α and IL-1 activity. The decreased level of IL-6 in mice, pretreated at 24 hours with BLF and injected with LPS, could be explained as a result of an unknown mechanism leading to a reduced reactivity of cells with LPS, possibly by the mechanism described by Flohé *et al.* (1991).

Physiological serum concentration of lactoferrin is normally very low (0.25 μg/ml) but is drastically elevated (five to six-fold) after injection of LPS (Sawatzki & Rich 1989). A 20-fold increase in lactoferrin level is observed after challenge of PMN with bacteria (Harmon *et al.* 1978; Guttenberg *et al.* 1990). It seems unlikely that LF changes the activity of LPS by binding of LPS molecule (Miyazawa *et al.* 1991) since LF disappears quickly from circulation (Harmon *et al.* 1978) and, moreover, it is most active when given 24 hours before LPS. The BLF preparation used for the experiments was free of detectable amounts of endotoxin. In addition, very low levels of TNF-α, induced by BLF, were comparable to those elicited by the control protein, human albumin. Lastly, human albumin did not cause release of IL-6 and did not lower the levels of LPS induced TNF-α.

It is clear that the protective effect of lactoferrin can not be explained solely by the inhibition of TNF-α production (this paper) or IL-1 (Zucali *et al.* 1989). Recent reports indicate that blocking the release of other relevant, proinflammatory cytokines such as IL-1 (Ohlsson *et al.* 1990; Alexander *et al.* 1991; McIntyre *et al.* 1991), IFN-γ (Silva & Cohen 1992), and TNF-α (Beutler *et al.* 1985), will prevent death of lethally infected animals. Even the use of antibodies to IL-6 prevents the death of lethally

infected animals (Starnes *et al.* 1990), though IL-6 by itself inhibits the production/release of IL-1 and TNF-α (Revel 1989). These findings reveal a strict interdependence between the cytokines and suggest ways of potential therapeutic interventions in the course of sepsis. Recent data obtained in our laboratory indicate that lactoferrin not only inhibits the release of TNF-α, but also prevents rh TNF-α induced death of experimental animals (manuscript in preparation). These results suggest the value of LF application in all states where high levels of TNF-α occur.

Taken together, we can explain in part the preventive effect of lactoferrin in infection. The role of IL-6 in this phenomenon, as well as the nature of other intermediate events, leading to the significant drop of serum TNF-α levels, need to be clarified.

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