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Intraperitoneal injection of lactoferrin ameliorates severe albumin extravasation and neutrophilia in LPS-induced inflammation in neonatal rats

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ABSTRACT

Lactoferrin (LF) plays various anti-inflammatory roles in inflammation experimentally induced by lipopolysaccharides (LPS). But the effects of LF on albumin extravasation and neutrophilia have not been elucidated. We aimed to study the effects of LF on albumin extravasation, neutrophilia and/or on other symptoms in inflammation caused by LPS in rats. Human lactoferrin (hLF) was injected (10 mg/100 mL in PBS) 18 h, or 15 min prior to, or 60 min after intraperitoneal injection of LPS in 13 days old Sprague Dawley rats. Prophylactic injection of hLF significantly ameliorated albumin extravasation in ascitic fluid at 5 h and neutrophilia in the blood at 24 h after LPS injection, but the after-injection of hLF did not. Interestingly, an injection of rat anti-TNFα IgG 15 min prior to LPS injection did not ameliorate albumin extravasation. Prophylactic injection of hLF significantly ameliorated other symptoms like mortality, and the decrease of phagocytotic activity of peritoneal polymorpho-nuclear leukocytes (PMNL), but did not ameliorate the decrease of platelets in the plasma. These findings suggest that hLF may be available as a medical treatment prior to surgery for prophylaxis of side effects like albumin extravasation or neutrophilia.

Lactoferrin (LF) has diverse homeostatic activities in relation to anti-bacterial, anti-viral and anti-inflammatory immunological activities (12, 13). It is reported that prophylactic treatment by injection of human lactoferrin (hLF) reduced lipopolysaccharide (LPS)-induced TNFα production and the death rate in mice (15). In general, intraperitoneal injection of LPS (LPS-ip-injection), a cell wall component of gram-negative bacteria, induces severe septic like inflammation with production of TNFα and neutrophilia (6) in the plasma of experimental animals. In clinical patients suffering from spontaneous bacterial peritonitis, ascites are frequently found. More than 92% of these patients have monomicrobial infections with aerobic gram-negative bacilli that contain LPS in their cell walls (7). However, it is not known whether a prophylactic injection of hLF has homeostatic effects on albumin extravasation or neutrophilia after LPS-ip-injection. In the present study, we showed gross albumin accumulation in ascitic fluid 5 h after LPS injection in neonatal rats. Using this neonatal model, we examined whether LF had the ability to ameliorate gross albumin extravasations in the peritoneal cavity that followed LPS injection. We also examined changes in blood components like platelets, and what does relate to rat anti-TNFα IgG injected at the same site just prior to LPS injection. We also examined whether hLF had prophylactic effects on the decrease of the phagocytotic activity after LPS injection.

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MATERIALS AND METHODS

Animals. Rats (Sprague Dawley) were purchased from Japan SLC (Shizuoka, Japan) at 5 days after birth and were kept under 12 h light-dark cycles at

25°C. At 13 days after birth, rat pups were weighed and assigned randomly to eliminate the influence of inter-litter variation. Meiji Dairies Corporation's Committee for Research on Experimental Animals approved the experimental protocol. Experiments were conducted in accordance with the *NRC Guide for the Care and Use of Laboratory Animals* (NRC, 1985).

Reagents. Casein was purchased from Fontera LTD (Auckland, New Zealand). hLF was purchased from Sigma-Aldrich Japan (Tokyo, Japan), and bovine lactoferrin (bLF) from both Sigma-Aldrich Japan and Wako Pure Chemical Industries Ltd. (Osaka, Japan). LPS from *Escherichia coli* (B E.Coli 055:B5) was purchased from Difco Laboratories (Detroit, Michigan, USA). Rat anti-TNFα IgG was purchased from Dainippon Pharm. Co. Ltd. (Osaka, Japan).

Experiments. One hundred mL of casein-Na solution (5 mg in 100 μL of PBS) was injected intraperitoneally (ip) to stimulate polymorpho-nuclear leukocyte (PMNL), and 100 μL of hLF(10 mg in 100 mL of PBS) was injected ip 18 h prior to (hLF-LPS(−18 h)), 15 min prior to (hLF-LPS(−15 m)), or 1 h after LPS (hLF-LPS(+60 m)) injection (0.2 μg/g of body weight). The same volume of PBS was injected in a control group (LPS-cont). At 5 h after LPS injection, ascitic lavage fluid was taken after washing by injecting 1 mL of ice-cold PBS to the peritoneal cavity and systemic blood was withdrawn for plasma from the abdominal aorta after laparotomy under anesthetization by ethyl ether. All samples of ascitic lavage were centrifuged, and the supernatants kept at −80°C until analysis. The cells precipitated were examined by assay for phagocytotic activity. In an inhibitory experiment, rat anti-TNFα IgG was injected 15 min prior to LPS injection.

Analytical methods. Albumin in ascitic lavage and plasma was measured using A/GB-test (WAKO Pure Chemical Industries, Osaka, Japan). Total protein in ascitic lavage was measured using Coomassie Protein Assay Reagent (Pierce, Rockford, Illinois, USA). The concentration of TNFα in plasma was measured by an ELISA kit (Endogen, Woburn, Massachusetts, USA). Total volume of accumulated ascitic fluid was calculated by its dilution rate from the optical density of phenol red solution (20 μg/mL) of 559 nm, which was injected at 1 mL volume into the peritoneal cavity to dilute the ascitic lavage fluid.

Phagocytotic activity of PMNL was measured as follows. Pups were injected with 100 μL of 5% casein 18 h prior to LPS injection. Peritoneal cells including PMNL were taken as above into ascitic lavage fluid at 5 h after LPS-ip-injection, and washed 2 times with cold PBS. Cells were finally suspended in 0.2% fetal calf serum (FCS)-RPMI medium (to 5×10^6 /mL). Forty μL of the cell mixture was layered on collagen-coated type 1 cover glasses (Cover glass coat C, IWAKI Glass Ltd. Tokyo, Japan). The cover glasses were then incubated in a CO_2 incubator for 5 min at 37°C. Thereafter, cells adhered on the cover glass were washed three times by 0.2% FCS-RPMI medium. Phagocytotic assay was started by addition of 200 μL of 0.4% FITC-latex beads and 1% FCS-RPMI at the top of the cells on each cover glass. The fluorescent beads (Fluoresbrite YG0.75) were purchased from Polyscience (Warrington, England). After incubation, unphagocytosed beads were washed out by the beads-free medium and cells that adhered on the cover glass were fixed in 4% formaldehyde-PBS. Phagocytosed PMNL was counted under a fluorescent microscope after the May Giemsa staining method.

Blood was taken from the abdominal aorta in EDTA-containing tubes. The analysis of the blood cells was externalized to BML Inc. (Tokyo, Japan).

Statistic analysis. Data were compared between groups using a one-way analysis of variance (ANOVA), and then by applying Fisher's post hoc test ($P < 0.05$). Mortality rate was examined for significance using the chi-square test ($P < 0.05$). All statistical analysis was performed using STATVIEW 4.1 (Abacus Concepts, Inc., Berkeley, CA, USA).

RESULTS

Effect of human lactoferrin on mortality

In the LPS group, 63% (5/8) of the pups had died by 24 h after LPS-ip-injection. However, all pups (7/7) in hLF-LPS(−18 h) group were alive at 24 h after LPS injection, as shown in Table 1.

Effect of human lactoferrin on phagocytotic activity

The phagocytotic activities of the PMLN in the LPS-cont group were significantly lower than in pups of the control without treatment. The prophylactic injection of hLF 18 h prior to LPS-ip-injection (hLF-LPS(−18 h)) ameliorated the decrease of phagocytotic activity of PMNL that was induced by LPS as shown in Fig. 1.

Table 1 *Effects of lactoferrin on mortality*

Groups	Dead/tested	Mortality (%)
none	0/5	0
LPS	5/8	63*
hLF-LPS	0/7	0
bLF-LPS	0/7	0

Mortality of rat pups was observed 24 h after LPS intraperitoneal injection (0.2 μg/g of body weight in 100 μL PBS). Both human lactoferrin (hLF 10 mg/100 μL of PBS) and bovine lactoferrin (bLF 10 mg/100 μL of PBS) were injected 18 h prior to LPS injection. (*P < 0.05)

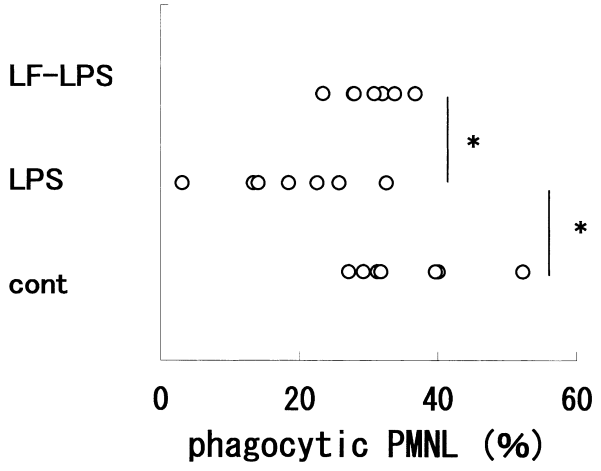

Fig. 1 Prophylactic effects of lactoferrin on the decrease of phagocytotic activity of polymorpho-nuclear leukocytes (PMNL) 5 h after LPS injection. Human lactoferrin (hLF 10 mg/100 μL of PBS) was injected 18 h prior to intraperitoneal injection of LPS. Bars indicate standard deviation. (*P < 0.05).

Effect of human and bovine lactoferrin on accumulation of ascitic fluid

The volume of ascites, the concentration of albumin, and the concentration of total protein in ascitic lavage fluid at 5 h after the LPS injection were significantly higher than that observed in the no treatment control group (Fig. 2, 3a). The volume of ascitic fluid at 5 h after LPS injection in the hLF-LPS(−18 h) group was two times smaller than that in the LPS-cont group, as shown in Fig. 2. The protein accumulation in the hLF-LPS(−18 h) and the hLF-LPS(−15 m) groups significantly decreased, but the protein content in the hLF-LPS(+60 m) group did not differ from that in the LPS group, although the mean value was lowered (Fig. 3a). In the same experiment, concentration of albumin in the lavage fluid was also lower in the hLF-LPS(−18 h) group than in the hLF-LPS(−15 m) and the hLF-LPS(+60 m) groups (Fig. 3b). While, the concentration of albumin in plasma was not significantly lower in the hLF-LPS(+60 m) group comparing with the no treatment group (Fig. 3c). On the other hand, the concentration of TNFα in plasma was lower in all of hLF-LPS groups than that in the LPS-cont group (Fig. 3d).

Prophylactic injection of bLF (bLF-LPS(−18 h)) did not decrease total protein (Fig. 4a) and albumin in ascitic lavage fluid (Fig. 4b) after LPS injection. In contrast, the concentration of TNFα in plasma was extremely lowered by ip injection of bLF 18 h prior to LPS injection (Fig. 4c).

Effects of TNFα antibody

The concentration of TNFα in the plasma increased 5 h after LPS injection. But, an intraperitoneal injection of rat anti-TNFα IgG 15 min prior to LPS injection did not ameliorate total protein accumulation in ascitic lavage fluid, as shown in Fig. 5.

Effects of human lactoferrin on neutrophilia and platelet number

The number of neutrophils were counted 5 h and 24 h after LPS injection. Neutrophilia was not found in blood 5 h after LPS injection but was found 24 h after LPS injection in the LPS-control group. In contrast, the neutrophilia was ameliorated by the hLF injection at 18 h prior to the LPS injection (Fig. 6).

The number of platelets in plasma decreased at 5 h after LPS-ip-injection in LPS-control group, and that in the hLF-LPS (−18 h) group decreased also to the same level. The number of platelets did not recover at 24 h after the LPS-injection, indicating that hLF had no prophylactic effect on the decrease of platelets after LPS injection, as shown in Fig. 7.

DISCUSSION

TNFα is known as a potent inflammatory cytokine for LPS inflammation. It is reported that production of TNFα by LPS-ip-injection is inhibited by prophylactic administration of LF (10, 15). We also showed here the prophylactic effect of hLF on albumin extravasation, and an increase in ascitic fluid after LPS injection, although the rat anti-TNFα IgG that was injected ip 15 min prior to the LPS injection did not ameliorate total protein accumulation in ascitic lavage fluid, as shown in Fig. 5. This finding indicates that hLF may ameliorate the accumulation of albumin in ascitic fluid with an indirect relationship in respect to blocking the potent inflammatory activity of TNFα.

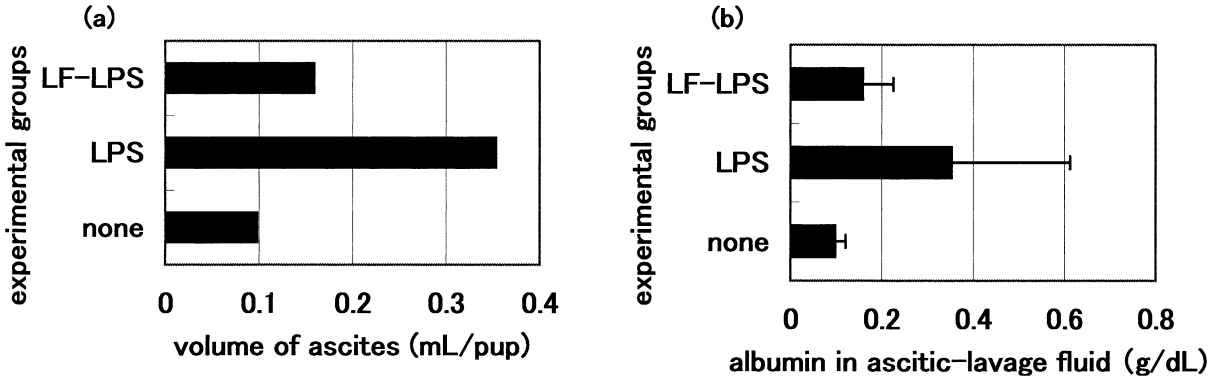

Fig. 2 Prophylactic effects of lactoferrin on LPS-induced ascites. The volume of the ascitic fluid was calculated from the dilution rate and the optical density of phenol-red solution at 559 nm injected into the peritoneal cavity. (n = 2)

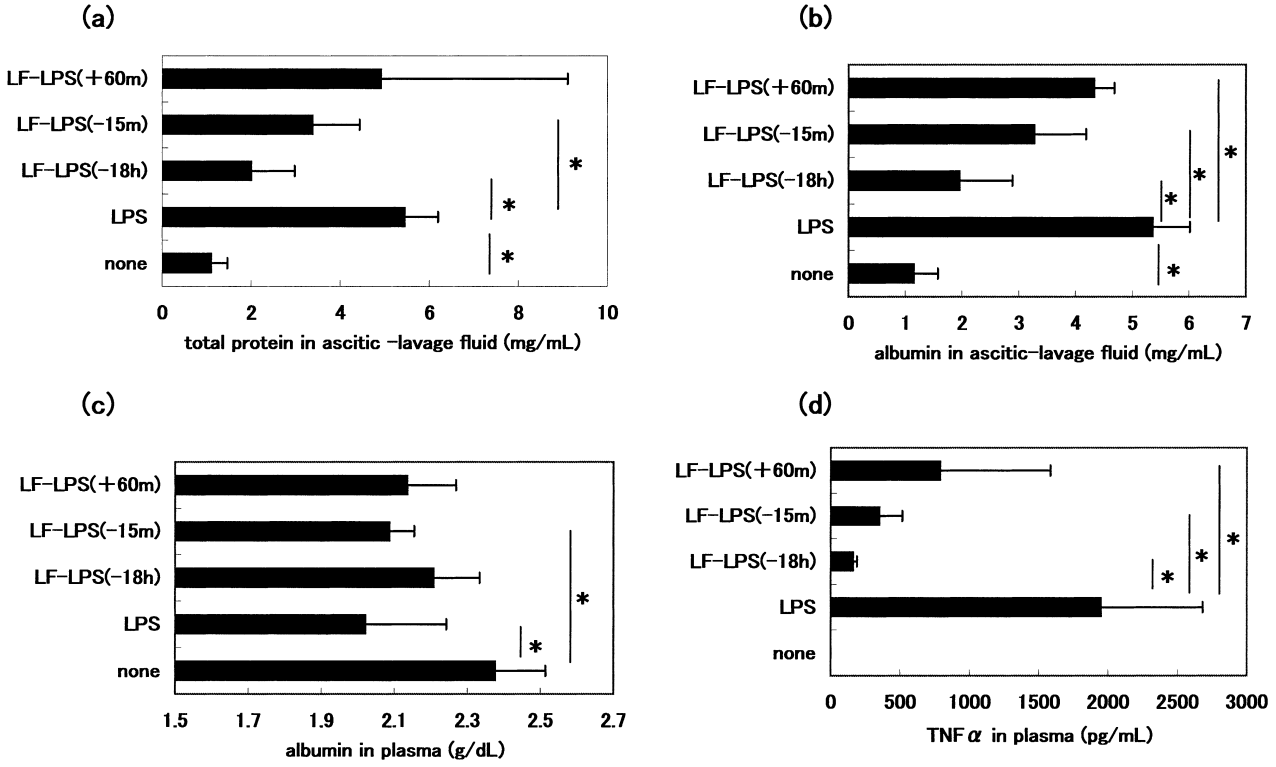

Fig. 3 Prophylactic effects of human lactoferrin (hLF) on LPS-induced inflammation. The time effect by the injection of hLF was examined. hLF was intraperitoneally injected 18 h (hLF-LPS(−18 h)) or 15 min (hLF-LPS(−15 m)) prior to intraperitoneal injection of LPS (LPS-ip-injection). One hundred μL of PBS was injected 18 h prior to LPS-ip-injection (LPS) or PBS injection (none). (a) total protein in ascitic fluid (mg/mL), (b) albumin in ascitic fluid (mg/mL), (c) albumin in plasma (g/dL), and (d) TNF α in plasma (pg/mL). Bars indicate standard deviation. (*P < 0.05)

Kijlstra and Jeurissen (8) suggested that human tear LF might play an anti-inflammatory role by modulating activation of the complement system. Meanwhile, Shibasaki *et al.* (11) showed that complement-dependent accumulation and degradation of platelets in the lung and liver were induced by injection of LPS. In respect to the platelet response to LPS, they also proposed that the lectin pathway to form C3 convertase from C4 and C2 is involved in the rapid accumulation of platlets in the liver and lungs, and that the pathway from C5 to C9 is involved in the destruction of platelets and the conse-

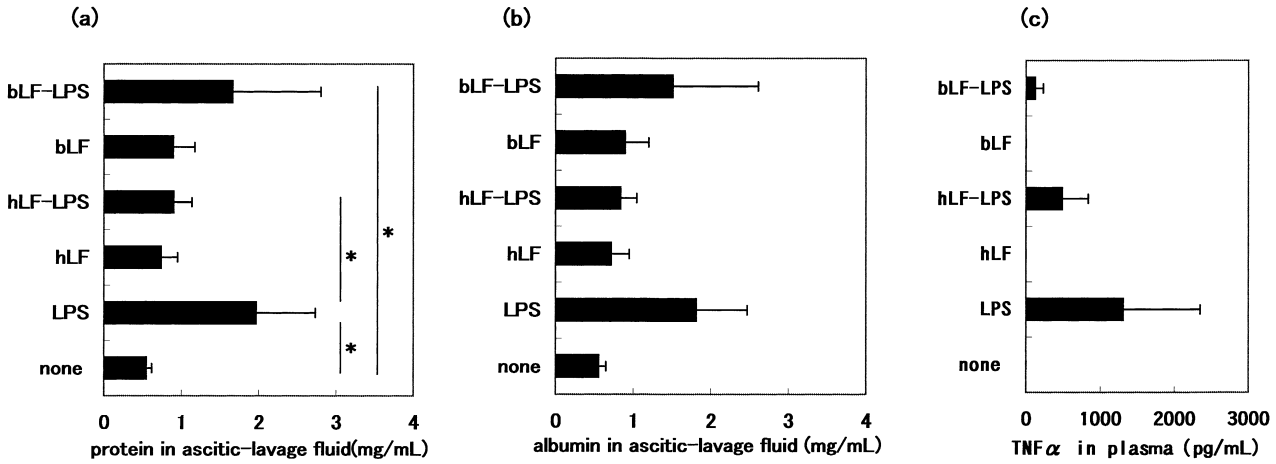

Fig. 4 Prophylactic effects of human lactoferrin (hLF) or bovine lactoferrin (bLF) on LPS-induced inflammation. Both lactoferrins (10 mg/100 µL PBS) were injected 18 h prior to intraperitoneal injection of LPS. Ascitic fluid was washing out with 1 mL of cold PBS, and blood taken from the abdominal aorta 5 h after LPS injection. Figure shows the concentration of total protein (a) and albumin (b) in ascitic fluid, and TNF α concentration (c) in plasma. Bars indicate standard deviation. (*P < 0.05)

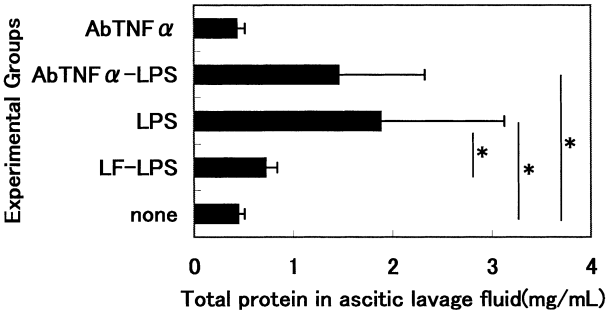

Fig. 5 Effects of rat anti-TNF α IgG on LPS-induced ascites. AbTNF α: rat anti-TNF α IgG (200 ng/pup in 100 µL of PBS) but not LPS was injected. AbTNF α-LPS: rat anti-TNF α was injected 15 min prior to inraperitoneal injection of LPS (LPS-ip-injection). LPS: 100 µL of PBS was injected 15 min prior to LPS-ip-injection. Bars indicate standard deviation. (*P < 0.05)

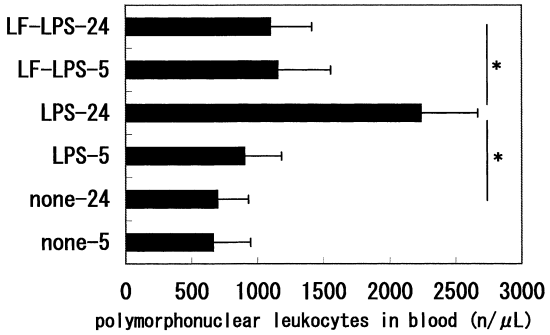

Fig. 6 Prophylactic effects of human lactoferrin on neutrophilia induced by intraperitoneal injection of LPS (LPS-ip-injection). Polymorphonuclear leukocytes were counted in blood at 5 and 24 h after LPS-ip-injection. Bars indicate standard deviation. (*P < 0.05)

quent anaphylactic shock. In our experiment, extreme degradation of platelets occurred in plasma after the LPS injection, but this effect was not ameliorated by a prophylactic injection of hLF (Fig. 7). In the LPS-cont group, the total amount of PMLN in lavage fluid was lower than that in hLF-LPS (−18 h) (data not shown). It was observed that there were many destroyed or apoptotic leukocytes (data not shown) in the LPS-cont group. These findings suggest that hLF may modulate activation of complement just after injection of LPS, or that hLF may make hosts tolerant to inflammatory stresses.

The effect of bLF on albumin extravasation and ascites were different from those of hLF after LPS injection (Fig. 4). The injured cells were observed more in peritoneal cells on the glass-slide after LPS injection in bLF-LPS(−18 h) than that in hLF-LPS(−18 h). More experiments need to explain those differences between hLF and bLF.

The activity of ameliorating the severe accumulation of protein and albumin in lavage fluid after LPS injection was higher in the hLF-LPS(−18 h) group than in the hLF-LPS(−15 m) as shown in Fig. 3. These findings suggest that the mechanism of amelioration by hLF may not directly depend on antagonistic competition of hLF with LPS-binding proteins, which inhibit the endotoxin interaction with CD14 (2, 5).

We showed in a previous paper that breast milk

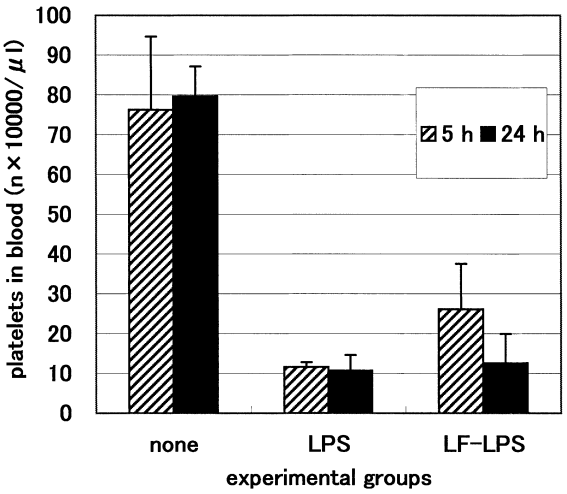

Fig. 7 Prophylactic effects of human lactoferrin on the number of platelets in blood at 5 h and 24 h after intraperitoneal injection of LPS. Bars indicate standard deviation. (*P < 0.05)

decreased bacterial translocation more than an artificial formula for rats free from LF and that bacterial translocation by enterobacteriaceae occurred more frequently in artificially reared rat pups than in breast-fed (14). The decrease of the phagocytotic activity of the PMNL by LPS in the neonatal rats and the amelioration of the phagocytotic activity by hLF injection (Fig. 2) may explain the bacterial translocation increased in the artificially reared pups.

In patients, surgery or traumatic abdominal injury may cause bacterial translocation (3, 9) and ascites, resulting in adverse effects, thereafter pneumonitis and/or bronchitis occasionally occur (1) causing bronchocavernous plasma exudation. This is a serious problem and such patients may die by suffocation with their own sputum. It is well known that LF is secreted by neutrophils that migrate at inflammation sites, like tracheo-bronchia in bronchitis or synovial fluid in arthritis (4). The injection of hLF may create a condition that mimics inflammation, and may attract healing functions by some unknown mechanism. Recombinant hLF (Agenix, Houston, Tex., USA) significantly ameliorated albumin accumulation in ascitic fluids (data not shown) after LPS injection, the same as hLF derived from human milk (Fig. 3). These findings suggest that a prophylactic injection of hLF may help avoid adverse effects resulting from surgical stress. However, more works remain to be done for human application of this proposition.

In conclusion, the present study demonstrates that a prophylactic intraperitoneal injection of hLF has the ability to ameliorate LPS-induced septic-like inflammatory responses such as neutrophilia and albumin extravasations, and may not involve the mediation of the inactivation of the plasma TNFα. The present results also show that hLF might be available as a prophylactic treatment to be administered prior to surgery.

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