Liposomalization of Lactoferrin Enhanced It's Anti-inflammatory Effects *via* Oral Administration

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It is known that lactoferrin is one of the functional proteins contained in mammalian milk and that it plays an important role in the immune system. In this study, we prepared multi-lamellar liposomal bovine lactoferrin composed of egg yolk phosphatidylcholine and phytosterol for oral delivery, and examined any resulting anti-inflammatory effects. Oral pretreatment of liposomal lactoferrin exhibited more suppressive effects than did nonliposomal lactoferrin on CCl_4 -induced hepatic injury in rats as well as on lipopolysaccharide-induced TNF-alpha production from mouse peripheral blood mononuclear leukocytes. Further investigation revealed that the liposomalization did not exert influence on the absorbability of lactoferrin to the venous blood or lymph following an intraduodenal administration in rats. Furthermore, there was no significant difference exhibited between the antigenicity of liposomal and non-liposomal lactoferrin, which was measured using the passive cutaneous anaphylaxis reaction following oral sensitization to them in guinea pigs. These results suggest that liposomal lactoferrin might act more effectively than conventional lactoferrin in the intestinal site, which is regarded as an active site of orally administered lactoferrin, although the biological mechanism is not fully understood yet. Consequently we propose that liposomal lactoferrin could be a novel active constituent useful for preventive and therapeutic treatment of inflammatory diseases.

Key words lactoferrin; liposome; oral delivery; anti-inflammatory

Lactoferrin is an 80 kD iron-binding protein contained mainly in mother's milk and also in tears, ¹) saliva, nasal secretions, semen²) and neutrophilic leukocytes^{3,4}) of mammals. It is considered to be a bioactive milk protein that plays versatile roles in the immune system responses and helps to protect the body from various infections.^{5,6} Many studies have already demonstrated that bovine lactoferrin (bLF) features a variety of bioactivities such as anti-bacterial,^{6–9} antiviral,^{10–12} anti-oxidative,¹³ anti-tumor,^{14–16} anti-inflammatory,^{17–20} immunomodulatory,²¹ analgesic²² and antistress²³ effects. Recently, it has also been reported that oral administration of bLF to patients suffering chronic hepatitis C virus.²⁴

Liposomes, on the other hand, are spherical vesicles whose membranes are composed of one or more bilayers of phosphatidylcholine. They can be utilized as drug carriers for a variety of substances such as small molecular drugs, proteins, nucleotides and plasmids. Some studies have already demonstrated that liposomalization of therapeutic and cosmetic agents can enhance their activity by improving their stability and permeability, and also giving a targeting ability and time release. Some protein agents such as insulin,^{25,26)} calcitonin,^{27,28)} parathyroid hormone²⁸⁾ and erythropoietin,²⁹⁾ which are susceptible to being digested and also have low permeability for the intestinal membrane, have been shown to improve their pharmacological effects through oral administration by various liposomal applications.

In this study, bLF is encapsulated into liposome consisting of egg yolk phosphatidylcholine (EPC) and phytosterol in order to orally administer bLF more effectively. We prepare two types of liposomal lactoferrin (L-bLF1 and L-bLF2) using different methods and confirm their potential for oral delivery with a few anti-inflammatory animal models. Furthermore, we examine their absorbability to the external jugular vein or lymph and their antigenicity to consider the mechanisms of their anti-inflammatory effects.

MATERIALS AND METHODS

Preparation of L-bLF1 1.05% of EPC (Q.P. Corporation, Japan) and 0.243% of phytosterol (Tama Biochemical Co., Ltd., Japan) at a molar ratio of 7:3 were dissolved in ethanol, and the solvent was evaporated. Citric buffer (pH 6.7) containing 3.0% of bLF (Morinaga Milk Industry Co., Ltd., Japan) was added to the lipid film. Sonication of the solution produced multi-lamellar vehicles (L-bLF1) of 580 nm in mean diameter. The multi-lamellar formation of liposome was confirmed by the negative staining method using electron microscopy (JEM-100SX, JEOL Ltd., Japan), and the diameter of the liposome was determined by NICOMP 370 (Particle Sizing Systems, U.S.A.). The encapsulating ratio of bLF into liposomes (42%) was also calculated by determining each level of bLF and EPC in the liposomal solution dialyzed using Spectra / Por CE membrane (U.S.A., MWCO: 300,000).

Preparation of L-bLF2 Multi-lamellar vehicles were prepared by hydrating 1.0% of food grade EPC (Q.P. Corporation, Japan) and 0.12% of phytosterol (Tama Biochemical Co., Ltd., Japan) with the aqueous solution containing 3.0% of bLF (Morinaga Milk Industry Co., Ltd., Japan) and 10% of maltitol (Towa Chemical Industry Co., Ltd., Japan). The solution was preliminarily emulsified and subsequently liposomalized by a high-pressure homogenizer. The average vehicle diameter was 70 nm. The multi-lamellar formation of L-bLF2 and the diameter were confirmed in a similar manner to L-bLF1.

Hepatic Injury Assay of L-bLF1 on Rats The experimental design followed the method of Abe *et al.*,³⁰⁾ where

bLF (300 mg/kg), L-bLF1 equivalent with 300 mg/kg of bLF and vehicle (Citric buffer) were orally administered to male Wistar rats (n=5), weighing 200±20 g at 80, 56, 32 and 8 h before a single dose of CCl₄ (1 ml/kg in olive oil / 1 : 1, *p.o.*). Meanwhile, silymarin (Aldrich, U.S.A., 300 mg/kg, *p.o.*) was used as a positive control agent and administered orally to test animals at 30 min before and at 4 and 8 h after CCl₄ injection. The rats were sacrificed 24 h after administration of CCl₄, when serum glutamic pyruvate transaminase (GPT) and glutamic oxaloacetate transaminase (GOT) levels were determined by the optimized UV method (GPT or GOT assay kit, Wako, Japan).

TNF-Alpha Release Assay from Mice Peripheral Blood Mononuclear Leukocyte (PBML) The experimental design followed the method of Bundschuh et al.³¹⁾ The experiments with L-bLF1 and L-bLF2 were carried out separately (Test 1 and Test 2 in Table 3). Groups of six BALB/c male mice, weighing 22 ± 2 g were used. We orally administered bLF (300 mg/kg), L-bLF1 or L-bLF2 equivalent to 300 mg/kg of bLF and the vehicle (citric buffer or maltitol solution) once daily for seven consecutive days, whereas we intraperitoneally administered cyclophosphamide (Sigma, U.S.A., 30 mg/kg), which served as the positive control, to test animals with a single dose on day 6. Blood samples were obtained by cardiac puncture on day 8 and PBML was then isolated by density-gradient separation. The cells (10^6) from each group of three animals were incubated in AIM-V growth medium (pH 7.4) at 37 °C for 24 h with and without lipopolysaccharide (LPS from E. coli, $1 \mu g/ml$). Levels of TNF-alpha were then determined from the culture supernatant using a specific ELISA kit (R&D Systems, U.S.A.); individual values from the two groups of three mice were averaged.

bLF Absorbability to the External Jugular Vein The experimental design was conducted according to the method of Harada *et al.*,³²⁾ whereby bLF (300 mg/kg, i.d.) or L-bLF2 (300 mg/kg, i.d.) was infused into the duodenum of Wistar rats (n=8, 8 weeks old) under urethane anesthesia. Venous blood of each animal was collected from the cannulated external jugular vein (before, and 30 min, 1 h, 2 h, 4 h, 6 h and 8 h after their infusions). These samples were centrifuged and the bLF concentration in plasma was quantitatively assayed by ELISA.

bLF Uptake to Thoracic Duct Lymph The experimental design was conducted according to the method of Harada *et al.*³²⁾ Under urethane anesthesia, bLF (200 mg/kg, i.d.) or L-bLF2 (200 mg/kg, i.d.) was infused into the duodenum of Wistar rats (n=5-7, 8 weeks old). Thoracic lymph fluid was harvested and collected in heparin-coated tubes throughout the sampling period (before and hourly after their infusions, for 4 h). These samples were centrifuged and the concentration of bLF was measured by ELISA. The amounts of absorbed bLF in the thoracic duct lymph were calculated from the flow rate and the concentration of bLF in lymph.

Antigenicity Study in Guinea Pigs Hartley female guinea pigs (n=3, 6 weeks old) were each orally sensitized with a concentration (4.5 mg or 45 mg/body) of bLF and L-bLF1 for three weeks (5d per week). Furthermore, 45 mg/body of bLF and L-bLF1 with FCA at the first sensitization, and with FIA at the second and third times as an adjuvant, were also injected into other animals (n=3) subcuta-

neously once a week for 3 weeks, as well as OVA (Ovalbumin, 1.0 mg/body) as a positive control. Five weeks after the initial administrations, all animals were sacrificed in order to collect blood samples and antiserum from them. The serum obtained from all animals in the bLF and L-bLF1 sensitization groups were diluted in saline from 10 to 10000 times, and each diluted serum was injected (0.1 ml) intracutaneously into the backs of two recipient female Hartley guinea pigs (8 weeks old). Each elicitor solution (bLF and L-bLF1) with equal saline in quantity containing 1% Evans Blue was also injected (1.0 ml) intravenously to recipient animals 4 h after the injections of diluted antiserum. When the pigment spots, which appeared in the injection sites within 30 to 60 min, exceeded 5 mm in diameter, the test substances were determined to be antigenicity-positive.

RESULTS AND DISCUSSION

Multi-lamellar liposomal lactoferrin (L-bLF1 and L-bLF2) composed of EPC and phytosterol were prepared in two different methods. Electron microscopy observation by the negative staining method clarified each multi-lamellar liposomal formation (Fig. 1). The liposomes' mean particle diameter turned out to be 580 nm and 70 nm, respectively, as Table 1 shows.

In the first test to confirm the potential of the liposomalization, we examined the suppressive effect of bLF and L-bLF1 on CCl₄-induced hepatic injury in rats after pre-administration of 300 mg/kg to them at 80, 56, 32 and 8 h before CCl₄ treatment. The results indicate that bLF pretreatment slightly suppressed any GPT or GOT increase in serum induced by CCl₄, while L-bLF1 pretreatment caused a more significantly intense effect (GOT; p < 0.05), as did the positive control silymarin, in comparison with the control group (Table 2). The potentials of L-bLF1 (Test 1) as well as L-bLF2 (Test 2) were also examined respectively using the



Fig. 1. Morphology of Multi-Lamellar Liposomes (L-bLF1 and L-bLF2) Used in the Present Study

L-bLF1 and L-bLF2 were prepared as described in Materials and Methods. Negatively stained electron microphotographs of the liposomal samples are shown.

Table 1. Physical Properties of Liposomal Lactoferrin (L-bLF1 and L-bLF2)

	Manufacturing process/additive	EPC : phytosterol (mol : mol)	bLF conc. (mg/ml)	Mean diameter (nm±S.D.)
L-bLF1	Sonication	70:30	30	580 ± 560
L-bLF2	High-pressure homogenizer/maltitol	84:16	30	70 ± 40

Table 2. Suppressive Effect of L-bLF1 on CCl₄-Induced Hepatic Injury in Rats

Test	Serum GPT		Serum GOT		
substances	U/1	Dec. %	U/l	Dec. %	
Control bLF L-bLF1 Silymarin	421.6±91.8 386.8±79.1 306.4±105.4 292.8±85.2*	8 27 31	1146.4±345.1 966.4±262.3 723.6±221.6* 740.4±243.2*	16 37 35	

All data are expressed as means \pm S.D. and were compared with the Student's *t*-test. Differences with p < 0.05 are considered statistically significant. *p < 0.05 (vs. control group).

suppressive tests on LPS-induced TNF-alpha production from PBML after 300 mg/kg of them were pre-administered to BALB/C mice (n=6 per group) for seven consecutive days in both tests. Both L-bLF1 and L-bLF2, as well as cyclophosphamide as a positive control, showed markedly suppressive effects of TNF-alpha in culture supernatant produced from PBML in contrast to the much less weaker effects of non-liposomal b-LF in each test (Table 3). It has been already reported that recombinant human lactoferrin intravenously administered 24 h before LPS injection has a protective effect against the development of hepatitis, as assessed by the level of serum ALT in the mouse hepatitis model induced by zymosan and LPS, and it was suggested that this was due to the suppression of proinflammatory cytokine TNF-alpha from hepatic macrophages (Kupffer cells).¹⁸⁾ Oral administration of bLF was also shown to suppress TNF-alpha production and increase IL-10 production in the LPS-stimulated adjuvant arthritis rats.²⁰⁾ Our study indicates that liposomal bLF taken orally has a more potent anti-inflammatory activity than that of normal bLF taken likewise.

These enhanced anti-inflammatory effects of L-bLF1 and L-bLF2 pre-administration via the oral route suggest that liposomalization of bLF contributed somewhat to bLF absorption into the circulatory system via the intestinal tract, which led to some tests of bLF transfer to blood or lymph circulation from the duodenum. It is already reported that bLF administered orally to neonatal pigs appeared in blood circulation and was excreted into the bile through entero-hepatic circulation,33) and in the experiment using adult rats, bLF infused intraduodenally was transported into blood circulation via the lymph pathway.32) In this study, bLF and L-bLF2 were administered intraduodenally to Wistar rats in order to confirm the bLF absorption to the external jugular vein (300 mg/kg each) and also its transportation to thoracic duct lymph (200 mg/kg each). From these studies, bLF was reaffirmed to be transported into the blood circulation from the lymphatic pathway as reported in the previous study,³²⁾ but the result of the former test revealed that there was no difference in bLF concentration in plasma collected from the external jugular vein in both bLF and L-bLF2 groups (Fig. 2). Moreover, there was no significant difference in the bLF output to the thoracic duct lymph following duodenal administration (Fig. 3). These results showed that the liposomalization did not enhance the absorbability of bLF from the intestinal lumen.

Atkinson and Meredith (1998) reported that bLF given through i.p. exhibited antigenicity in the brown Norway rat model using carrageenan as an adjuvant, and the $ED_{50}s$ for lactoferrin was 40-50 ng.34) In a more recent study (Meredith and Atkinson, 2000), bLF was dosed orally using the same animal model and resulted in the possibility of antigenicity.³⁵⁾ In this work, we measured the passive cutaneous anaphylaxis (PCA) reaction following bLF and L-bLF1 oral dosing 5 times a week for 3 weeks to confirm their antigenicity, since liposomalization might be also considered to enhance the adverse affect of bLF. The data is shown in Table 4. The serum obtained from all animals in the bLF and L-bLF1 sensitization groups showed a positive reaction. The PCA antibody titers from the oral administration group and the subcutaneous administration group ranged from less than 10 to 100 and from 1000 to 10000, respectively. There was no difference in the antibody titer in the oral administration group for bLF and L-bLF1; however, the antibody titer for bLF seemed to be little higher than that of L-bLF1 in the subcutaneous administration group. At the same time, the serum obtained from all animals in the OVA sensitization group exhibited a severe positive reaction, and the PCA antibody titer was 10000. Judging from these results, we concluded that L-bLF1 induced antigenicity in the guinea pigs under the conditions of the present study, but the potential of antigenicity with it seemed to be lower than that with bLF.

CONCLUSION

To date, the mechanisms behind the various functions featured by orally dosed bLF remain partially unrevealed, but particular studies have suggested engagement of the cell immunity mediated by IL-18 production on the intestinal epithelial tissue.^{36,37)} It has also been reported that oral administration of bLF to mice increased the secretion of anti-bLF IgA and IgG in the intestinal fluid but made no difference in the serum, and it is suggested that bLF could act as an immunostimulating factor of the mucosal immune system.³⁸⁾ On the other hand, it has been demonstrated that a portion of bLF is transported into blood circulation through the intestine^{32,33)} as previously noted. In addition, bLF is known to be comparatively robust to digestive enzymes such as pepsin or trypsin.^{39,40)} One recent study on determining the extent of gastric degradation of bLF in humans reports that the total amount of intact bLF entering the small intestine is 60-80% after oral ingestion.⁴¹⁾ In fact, there is a chance that bLF's robustness to digestive hydrolysis improved by liposomal en-

Table 3.	Suppressive Effects of L-bLF	1 and L-bLF2 on LPS-Induced	TNF-Alpha Release from Mouse PBML
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	Track and store and	TNF-alpha (pg/ml)			
	lest substances	LPS(+)	LPS(-)	Net	% Inh.
Test 1	Control	169.4	0.0	169.4±95.1	_
	bLF	125.8	0.0	125.8 ± 86.3	26
	L-bLF1	50.1	0.0	50.1 ± 34.2	70
	Cyclophosphamide	69.2	0.0	69.2 ± 37.5	59
Test 2	Control	209.3	20.1	189.2 ± 8.2	
	bLF	193.7	43.0	150.8 ± 6.7	20
	L-bLF2	38.2	0.0	38.2 ± 1.8	80
	Cyclophosphamide	159.2	69.9	89.4 ± 0.4	53

LPS(+): The cells treated with LPS for incubation. LPS(-): The cells without treatment with LPS for incubation. Net: subtracted TNF-alpha level of LPS(-) from that of LPS(+), and shown as means \pm S.D.



Fig. 2. Changes in Time Course of bLF Concentration in Plasma Triangle and circle show data from bLF and L-bLF2, respectively. Data represent means±S.E. from 8 rats.

 Table 4.
 Homologous Passive Cutaneous Anaphylaxis in Guinea Pigs



Fig. 3. Changes in Time Course of bLF Total Output in Thoracic Duct Lymph

Triangle and circle show data from bLF and L-bLF2, respectively. Data represent means \pm S.E. from 5—7 rats.

Immunization		Elicitation		PCA titer ^a)		
Immunogen (Route)	Dose (Dosing frequency)	Elicitor	Dose (i.v.)	1 ^{<i>b</i>)}	2	3
L-bLF1 (p.o.)	4.5 mg/body (5 times/week)	L-bLF1	4.5 mg/body	10	10	100
L-bLF1 (p.o.)	45 mg/body (5 times/week)	L-bLF1	4.5 mg/body	10	10—100	100
bLF (p.o.)	4.5 mg/body (5 times/week)	bLF	4.5 mg/body	100	<10	100
bLF (p.o.)	45 mg/body (5 times/week)	bLF	4.5 mg/body	10—100	10	10
L-bLF1+FCA (s.c.)	45 mg/body (1 time/week)	L-bLF1	4.5 mg/body	1000	1000	1000
bLF+FCA (s.c.)	45 mg/body (1 time/week)	bLF	4.5 mg/body	1000-10000	1000-10000	1000-10000
OVA+FCA (s.c.)	1 mg/body (1 time/week)	OVA	1 mg/body	10000	10000	10000

a) The highest dilution rate of serum showing a blue spot with a diameter of more than 5 mm as the mean value. b) Antiserum number. OVA; Ovalbumin, FCA; Freund's complete adjuvant.

capsulation could enhance it's anti-inflammatory effects. To determine whether bLF is more robust to gastric digestion due to its liposomalization, we performed digestive tests on bLF and liposomal bLF using artificial gastric fluid. Liposomalization improved bLF's robustness to the artificial gastric digestion, but a certain level of intact bLF still remained after digestion, even when not liposomalized. Therefore, bLF taken orally can be considered to enter the intestinal lumen where an active site of bLF is assumed to exist, regardless of whether the bLF is liposomalized or not.

In this study, we also examined whether liposomalization of bLF could affect its transportation from the intestine and its antigenicity in order to consider the mechanisms of their action. These results revealed that liposomalization of bLF did not improve its absorbability from the duodenum into the bloodstream or into lymph circulation. The lack of significant difference of the antigenicity between liposomal bLF and non-liposomal bLF also supports the hypothesis that liposomalization has no influence on the absorption of bLF from the intestinal region.

Yamamoto *et al.* (2002) have already reported that vacant MLV liposomes composed of EPC stimulate the release of proinflammatory cytokines such as TNF-alpha, IL-1 beta and IL-6 from human PBML *in vitro*,⁴²⁾ but no other studies of vacant liposome with phosphatidylcholine on inflammatory response are known to exist. Furthermore, it has not yet been demonstrated that only vacant liposome administered orally exhibits other biological activities in addition to an anti-inflammatory effect.

According to the animal models used in this study, liposomes might assist the function of bLF in the intestine, which is considered to be an active site for orally administered bLF. Meanwhile it can not be denied that the possible anti-inflammatory effects of vacant liposome contributed synergistically to the more intensive effects of liposomal bLF.

Liposomal bLF remains a matter for further investigation as a novel component useful for the preventive and therapeutic treatment for various inflammatory diseases, although from the results given in this study the biological mechanism is not yet fully understood. We would like to carry out further studies on the potential of liposomal lactoferrin.

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