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Liposomal lactoferrin induced significant increase of the interferon-alpha (IFN-α) producibility in healthy volunteers

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Abstract. Interferon-alpha (IFN-α) producibility has been widely accepted as one of the important markers to evaluate the immune status [1,2]. In this study, preliminary clinical tests were carried out to confirm the immunomodulatory activity of liposomal lactoferrin including IFN-α producibility and NK activity. In a primary open trial, the liposomal lactoferrin was administered to five healthy males for one week and various immunological indices were evaluated. Furthermore, ten healthy males were administered 319 mg per day of liposomal or non-liposomal lactoferrin for four weeks, and immune status was monitored at 0, 1 and 4 weeks after the intake as well as three weeks after stopping it. In this double-blinded comparative study, the IFN-α producibility was significantly increased only in the liposomal lactoferrin group during administration and decreased 3 weeks after stopping it, while the IFN-α producibility was unchanged in the non-liposomal lactoferrin group. Although the biological mechanism of IFN-α producibility enforced by liposomal lactoferrin has not been wholly understood, it is suggested to be a novel active constituent having preventive and therapeutic effects on inflammatory diseases, cancer and infectious diseases such as chronic hepatitis C.

Keywords: Lactoferrin, liposome, multi-lamellar vehicle, immunological indices, interferon-alpha producibility, NK activity, hepatitis C

1. Introduction

Lactoferrin is a bioactive milk protein that plays versatile roles in the immune system responses and it helps to protect the body against infections. Recently, it has been reported that oral administration of bovine lactoferrin to the patients of chronic hepatitis C reduced the amount of hepatitis C virus [3]. On the other hand, liposomes are spherical vesicles whose membranes are composed of one or more bilayers of phosphatidylcholine. They can be used 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 insulin gives more intensive hypoglycemic effect than naked insulin via oral route [4]. In this study, the immunomodulatory activity of liposomal lactoferrin as a novel functional food was preliminarily examined on healthy volunteers in comparison with non-liposomal lactoferrin.

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Table 1
Formulation of the tablets for the clinical tests

Ingredients	Open trial	Double-blinded comparative study	
	LLF1	LLF2	NLLF
Liposomal lactoferrin*	23.5%	55.7%	–
Lactoferrin	–	–	11.8%
Maltitol	26.2%	24.0%	67.9%
Other ingredients**	50.3%	20.3%	20.3%
Weight (g)/tablet	0.5	0.3	0.3
Lactoferrin content (mg)/tablet	25.0	35.4	35.4

*Liposomal lactoferrin contains 70.8% of maltitol, and therefore each maltitol content of LLF1 and LLF2 is 42.8% and 63.4%, respectively.
**Lactulose, lactose, sucrose esters of fatty acids and silicon dioxide.

2. Material and method

2.1. Preparation of liposomal lactoferrin

Multi-lamellar vehicles were prepared by hydrating the food grade lecithin (Q.P. Corporation, Japan) and other lipids with the aqueous solution containing lactoferrin (Morinaga Milk Industry Co., Ltd., Japan) and maltitol (Towa Chemical Industry Co., Ltd., Japan). The solution was preliminarily emulsified and then liposomalized by a high-pressure homogenizer. An average of the diameter was 60 nm. 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 liposome was determined by NICOMP 370 (Particle Sizing Systems, USA).

2.2. Preparation of tablets with liposomal lactoferrin

Powdered liposomal lactoferrin to be compressed directly into the tablets for the clinical tests was obtained by freeze-drying liposomal lactoferrin solution and then milling the dried product. Liposomal lactoferrin tablet 1 (LLF1) for the open trial test, and liposomal lactoferrin tablet 2 (LLF2) and non-liposomal lactoferrin tablet (NLLF) for the double-blinded comparative study were prepared as shown in Table 1. One tablet of LLF1 was designed as 0.5 g in total weight, while LLF2 and NLLF as 0.3 g. To confirm that the liposome could be properly formed after oral intake, the crushed LLF2 was re-dispersed in water and centrifugated. The evaluation result of the supernatant by electron microscopy as well as NICOMP 370 showed that dried liposome in the tablet might be possibly reconstructed after oral intake.

2.3. Design of the open trial test

In-house five healthy males aged 26–37 years old (32.4 ± 4.2 y) with no food allergy, who did not take any foods containing lactoferrin during the two weeks before the test start were selected. Written informed consent was obtained from all subjects. Four tablets of LLF1 were given to each subject three times a day for a week (300 mg of lactoferrin per day). At the end of each period, blood samples were collected, and general, biological and immunological blood examinations as indicated below were conducted.

2.4. Design of the double-blinded comparative study

In-house healthy males aged 30–39 years old (34.5 ± 3.0 y) with no experiences of hepatitis and blood transfusion were recruited for the study. The subjects were allocated into two groups in randomized, double-blinded, crossover study (five subjects per each group). The subjects from each group were administered nine tablets of LLF2 or NLLF for four weeks. The same clinical examinations were conducted as described in the "design of the open trial test" at baseline, one and four weeks after the first administration of LLF2 or NLLF as well as three weeks after the end of the administration.

2.5. General blood examinations

Erythrocyte count, hemoglobin, hematocrit value, WBC count, differential count of leukocytes and platelet count were measured by Japan Medical Laboratory Co., Ltd.

2.6. Blood biological examinations

GOT, GPT, ALP, γ-GTP, total cholesterol, HDL-cholesterol, arteriosclerosis index, triglyceride, blood glucose, HbA1c, amylase, urea nitrogen, albumin and A/G ratio were measured by Japan Medical Laboratory Co., Ltd.

2.7. Immunological examinations

Virus-induced IFN-α or -γ producibilities, PHA-induced IFN-γ, IL-4, IL-12 or TNF-α, NK activity, GSH score, CD19, CD3-56+, CD3+56+, CD4, CD8, CD4/CD8 and CD3/GD were measured by Louis Pasteur Center for Medical Research.

2.8. Statistical analysis

Statistical analysis was carried out with a Windows computer using the VisualStat Ver. 4.5 package. Data are shown as the mean ± SD. Where appropriate, the data were analyzed by one-way, repeated-measures analysis of variance followed by the LSD test. Significance was set at $p < 0.05$.

3. Result and discussion

No adverse effect has been observed during both the open trial test and the double-blinded comparative study, which showed the safety of liposomal lactoferrin. In the open trial test, IFN-α producibility in all of healthy volunteers increased after the intake of LLF1 (from 8,408 ± 3,108 IU/ml to 14,966 ± 3,442 IU/ml). The increase showed a statistical significance ($p = 0.04$), and NK activity was unchanged. In the double-blinded comparative study as well as the open trial test, IFN-α producibility of liposomal lactoferrin (LLF2) group was also significantly increased during the intake period and returned to the preintake level at 3 weeks after the end of the intake, although it was unchanged in the non-liposomal lactoferrin (NLLF) group (Table 2). During the test periods, NK activities in both groups were increased after one week and declined to the lower level than that of initial after four weeks, and the wash-out for three weeks also restored the activities to the same level as that at the baseline (Table 3). Appreciable changes of other immunological indices were not observed in both groups.

Table 2
The changes of IFN-α producibility in the open trial test and the double-blinded comparative study

		IFN-α producibility (IU/ml)			
		0 W	1 W	4 W	3 W after intake
Open trial	LLF1	$8{,}408 \pm 3{,}108^{a}$	$14{,}966 \pm 3{,}442^{a}$	–	–
Double-blinded	NLLF	$6{,}423 \pm 3{,}701$	$7{,}585 \pm 3{,}006$	$7{,}063 \pm 3{,}638$	$5{,}699 \pm 3{,}536$
comparative study	LLF2	$7{,}123 \pm 2{,}982^{b,c}$	$14{,}418 \pm 6{,}016^{b}$	$16{,}661 \pm 3{,}931^{c}$	$8{,}985 \pm 3{,}249$

All data are presented as means $\pm$ SDs.
[a]Significantly different, $p < 0.05$, [b,c]Significantly different, $p < 0.01$.

Table 3
The changes of NK activity in the open trial test and the double-blinded comparative study

		NK activity (%)				
		E/T ratio	0 W	1 W	4 W	3 W after intake
Open trial	LLF1	40:1	27 ± 13	26 ± 19	–	–
		20:1	20 ± 10	22 ± 14	–	–
Double-blinded comparative study	NLLF	40:1	34 ± 15^{a}	$50 \pm 13^{a,d}$	23 ± 13^{d}	31 ± 13
		20:1	23 ± 14	31 ± 12	22 ± 15	22 ± 8
	LLF2	40:1	30 ± 8^{b}	40 ± 13^{e}	$13 \pm 5^{b,e}$	28 ± 7
		20:1	19 ± 8^{c}	23 ± 9^{f}	$9 \pm 3^{c,f}$	21 ± 9

All data are presented as means $\pm$ SDs.
[a,c,f]Significantly different, $p < 0.05$, [b,d,e]Significantly different, $p < 0.01$.

IFN-α producing effect of liposomal lactoferrin in the double-blinded comparative study corresponds with that of the open trial test for a week in contrast to ineffectiveness of non-liposomal lactoferrin. These results showed that liposomalization of lactoferrin increased IFN-α producibility, even though the biological mechanism has not been wholly understood yet. IFN-α is widely used for a treatment of hepatitis C due to its antiviral activity, and therefore liposomal lactoferrin which increases IFN-α producing capacity can be considered useful as a novel supplementary food for the treatment of hepatitis C.

References

[1] K. Uno, K. Nakano, N. Maruo et al., Determination of interferon-α-producing capacity in whole blood cultures from patients with various diseases and from healthy persons, *J. Interferon Cytokine Res.* **16** (1996), 911–918.
[2] K. Uno and T. Kishida, Clinical utility of the measurement of IFN producing capacity, *PasKen Journal* **9–10** (1997), 1–6.
[3] K. Tanaka, M. Ikeda, A. Nozaki et al., Lactoferrin inhibits hepatitis C virus viremia in patients with chronic hepatitis C: a pilot study, *Jpn. J. Cancer Res.* **90** (1999), 367–371.
[4] J.A. Rogers and K.E. Anderson, The potential of liposomes in oral drug delivery, *Crit. Rev. Ther. Drug Carrier Syst.* **15** (1998), 421–480.