Jpn. J. Cancer Res. **90**, 262–267, March 1999

Possible Chemopreventive Effects of Bovine Lactoferrin on Esophagus and Lung Carcinogenesis in the Rat

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A milk component, bovine lactoferrin (bLF), previously shown by us to be a strong chemopreventive of colon carcinoma development, was examined for its influence on other organs using a rat multi-organ carcinogenesis model. Male F344 rats, aged 6 weeks, were treated sequentially with diethylnitrosamine (DEN, i.p.), dihydroxy-di-*N*-propylnitrosamine (DHPN, in drinking water) and *N*-nitrosomethylbenzylamine (NMBA, s.c.) during the first 8 weeks (DDN treatment), and then bLF was administered in the basal diet, at a dose of 2, 0.2, 0.02 or 0.002%. Other groups were given DDN treatment or bLF alone as controls. All surviving animals were killed at week 41, and major organs were examined histopathologically for neoplastic lesions. In the esophagus, a tendency for reduction in development of papillomas was evident in the bLF-treated animals, along with a significant suppression of relatively large-sized papillomas (more than 50 mm^3 volume) at the 0.2% dose ($P<0.05$, 11% of the control). The multiplicity of tumors (adenomas and carcinomas) in the lung was also decreased in animals fed 0.02% bLF (1.98±0.41 per cm^2 lung tissue section, $P<0.05$) compared to the control group (3.48±0.33). No enhancing or inhibitory effects of bLF on tumor development in other organs were noted. The present results indicate that bLF exerts chemopreventive effects in the esophagus and lung in addition to the colon.

Key words: Bovine lactoferrin — Chemoprevention — Esophagus — Lung — Rat

Lactoferrin (LF) is a multifunctional iron-binding glycoprotein which is particularly abundant in colostrum (approximately 10 mg/ml) and is also present in mammalian epithelial cell secretions such as tears, saliva and seminal fluid in various amounts (0.01–2 mg/ml).[1)] It is reported to have bacteriostatic properties,[2)] antiviral activity[3, 4)] and immunomodulatory functions, such as natural killer (NK) cell activation,[5)] stimulation of lymphokine-activated killer (LAK) cell activity[6)] and potentiation of macrophage cytotoxicity.[7)] Recently it was reported that lactoferrin can directly activate NK cells and arrest cell proliferation of epithelial tumor cell lines.[8)] Thus, its physiological importance may be related to host primary defense mechanisms.

We have recently shown that dietary supplementation with bovine lactoferrin (bLF), derived from bovine milk,[2)] can inhibit the development of azoxymethane (AOM)-induced aberrant crypt foci (ACF) as precursor lesions of tumor development,[9)] as well as carcinomas[10)] in the rat colon, without any toxic effects in major organs. It was also found to reduce growth and metastasis of solid tumors.[11)] Therefore, bLF is considered to be a good candidate for a chemopreventive agent of human cancer development.

However, several studies of chemopreventive agents have demonstrated that preventive and occasionally promoting or carcinogenic effects of individual exogenous agents may markedly differ from organ to organ.[12–16)] Therefore, research into chemoprevention should be based on a wide-spectrum organ analysis. To assess whether bLF might influence tumorigenesis in other organs, we investigated the effects of bLF supplementation using a rat multi-organ carcinogenesis model,[17, 18)] in which complex modifying effects of chemopreventive agents on major organs can be detected in a single experiment.

MATERIALS AND METHODS

Materials Diethylnitrosamine (DEN) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo) and dihydroxy-di-*N*-propylnitrosamine (DHPN) from Nacalai Tesque Inc. (Kyoto). *N*-Nitrosomethylbenzylamine (NMBA) was synthesized in our laboratory from *N*-methylbenzylamine (Tokyo Chemical Industry Co., Ltd.). bLF, obtained from bovine skim milk by the method of Law

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and Reiter, as described previously,[19] was kindly provided by Drs. Tomita and Shimamura of Morinaga Milk Industry Co., Ltd. (Zama).

Animals Male F344 rats, aged 5 weeks, were obtained from Charles River Japan Inc. (Kanagawa) and randomly divided into 4 groups of 20 animals each (groups 1–4). Additional groups of 35 animals (group 5), 5 animals (groups 6–9) and 6 animals (group 10), were also housed in plastic cages with wood chips for bedding in an air-conditioned room with a 12 h light/dark cycle. They were maintained on Oriental MF powdered basal diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*.

Treatment The experimental protocol is shown in Fig. 1. Animals in groups 1–5 received a single i.p. injection of 100 mg/kg body weight DEN in saline, 0.1% DHPN for 4 weeks in the drinking water and 5 s.c. injections of 0.5 mg/kg body weight NMBA in 20% dimethyl sulfoxide (DMSO) solution in saline over 2 weeks, during the initial 8-week period, for multiple initiation (DDN treatment). Animals in groups 1–4 were then administered 2, 0.2, 0.02 and 0.002% bLF, respectively, in basal diet, starting at 2 weeks after the completion of DDN treatment until the end of the experiment. Those in group 5 were treated with DDN treatment alone as the carcinogen control. Those in groups 6–9 were given 2, 0.2, 0.02 or 0.002% bLF, respectively, in basal diet, without DDN treatment (bLF controls), from week 10 to the end of the experiment. Animals in group 10 were maintained on basal diet without any treatment throughout the experimental period. All the rats were weighed once every 2 weeks during the DDN treatment, and then once every 4 weeks until the end of the experimental period. Animals that died during the experiment or became moribund were autopsied. Those surviving longer than 38 weeks were included in the effective numbers. The final numbers of rats analyzed were as follows, group 1 (2% bLF), 14; group 2 (0.2% bLF), 19; group 3 (0.02% bLF), 16; group 4 (0.002% bLF), 19; and group 5 (basal diet), 29. All survivors were killed under deep ether anesthesia at the end of week 41, and subjected to complete autopsy.

Histopathological examination At autopsy, the liver and kidney were weighed. The major organs were excised, fixed in buffered formalin, and routinely processed for embedding in paraffin. Sections were stained with hematoxylin and eosin for histopathological examination. Esophageal tumor volumes were calculated from measurements of 3 dimensions. Neoplastic lesions (adenomas and carcinomas) in the lungs were analyzed with the aid of an image processor (IPAP, Sumika Technos Co., Ltd., Osaka), and the data were expressed as numbers and areas per lung tissue.

Statistical analysis Statistical analysis of differences between the treated and control groups was performed with Dunnett's *t*-test for body weights, organ weights and the multiplicities of neoplastic lesions. The χ^2 test was used for the incidences of neoplastic lesions. The criterion of significance was set at $P<0.05$ and all analyses were carried out with a JMP software package (Version 3.1, SAS Institute Japan, Tokyo) on a Macintosh computer.

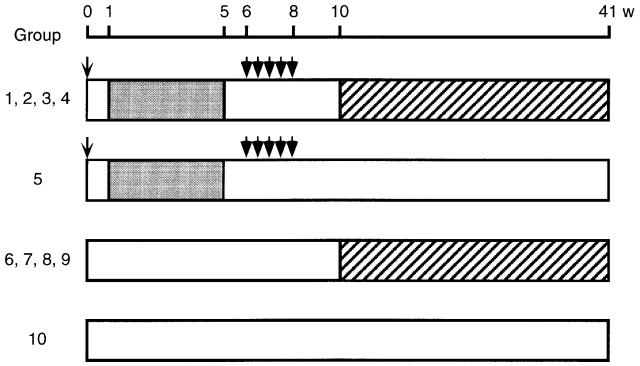

Fig. 1. Experimental protocol. ▼, DEN 100 mg/kg body weight i.p. injection; ▩, DHPN 0.1% in drinking water; ▼, NMBA 0.5 mg/kg body weight s.c. injection; ▨, bLF: 2, 0.2, 0.02 and 0.002%; □, basal diet.

RESULTS

No clinical signs or effects on body weight gain, liver or kidney weights related to bLF administration were apparent in any of the rats with or without DDN treatment during the experiment (Table I). In addition, no neoplastic changes were observed in animals without DDN treatment (in groups 6–10). A total of 16 rats were found dead or were killed on becoming moribund before week 38: 4 in group 1, 1 in group 2, 4 in group 3, 1 in group 4 and 6 in group 5. The deaths were considered to have been caused by the DDN treatment in all cases. The mean body weights of rats injected with DDN were reduced approximately 20% compared with animals without DDN. The administration of 0.002–2% bLF did not influence the food intake, so that the intake of bLF was directly proportional to the dose.

Incidences and multiplicity of neoplastic lesions are presented in Tables II–VII. Although without obvious dose dependence, a tendency for reduction of papilloma development in the esophagus was observed in the bLF-treated animals (groups 1–4) compared with the controls, along with significant suppression of relatively large papillomas (more than 50 mm^3) at the 0.2% dose (incidence, $P<0.005$, 11% of the control; multiplicity, $P<0.05$, 10%) (Table II). Lung tumor (adenoma and carcinoma combined) multiplicities (No./cm^2) also showed tendencies for decrease in the bLF-treated animals (groups 1–4) and a

Table I. Effects of bLF on Body and Organ Weights at Week 41

Group	Treatment	No. of rats	Body (g)[a)]	Liver (g)[a)]	Kidney (g)[a)]
1	DDN→bLF 2%	14	324.9± 7.5	7.73±0.38	8.35±4.70
2	DDN→bLF 0.2%	19	311.7±10.3	7.31±0.26	2.75±0.65
3	DDN→bLF 0.02%	14	309.3±12.7	7.05±0.48	2.21±0.15
4	DDN→bLF 0.002%	17	340.4± 7.1	8.20±0.27	2.51±0.36
5	DDN→control	29	318.6± 6.8	7.71±0.29	6.57±1.73
6	bLF 2%	5	395.9± 9.4	9.20±0.19	2.05±0.05
7	bLF 0.2%	5	404.0± 5.1	9.67±0.19	2.11±0.09
8	bLF 0.02%	5	406.4± 7.6	9.69±0.22	2.08±0.05
9	bLF 0.002%	5	392.5± 5.1	9.63±0.31	2.02±0.04
10	Control	6	394.9± 7.3	9.51±0.31	2.07±0.02

a) Values given are mean±SE.

Table II. Effects of bLF on Incidence and Multiplicity of Tumors[a)] in the Esophagus

Group	Treatment	No. of rats	Total tumors		Tumor volume (mm^3)					
			Incidence (%)	No./rat[b)]	1–<20		20–<50		50≤	
					Incidence (%)	No./rat[b)]	Incidence (%)	No./rat[b)]	Incidence (%)	No./rat[b)]
1	DDN→bLF 2%	14	14 (100)	4.36±0.31	14 (100)	3.14±0.40	9 (64)	0.93±0.22	4 (29)	0.29±0.13
2	DDN→bLF 0.2%	19	18 (95)	4.47±0.45	18 (95)	3.63±0.41	10 (53)	0.79±0.21	1 (5)[c)]	0.05±0.05[d)]
3	DDN→bLF 0.02%	14	14 (100)	4.57±0.69	13 (93)	3.64±0.73	5 (36)	0.50±0.20	5 (36)	0.43±0.17
4	DDN→bLF 0.002%	17	17 (100)	4.65±0.62	15 (88)	3.18±0.41	10 (59)	0.88±0.21	7 (41)	0.59±0.21
5	DDN→control	29	29 (100)	5.93±0.58	28 (97)	4.10±0.45	21 (72)	1.34±0.24	13 (45)	0.48±0.11

a) Papillomas and carcinomas.
b) Values given are mean±SE.
c) $P<0.005$ compared with group 5.
d) $P<0.05$ compared with group 5.

Table III. Effects of bLF on Incidence and Multiplicity of Tumors[a)] in the Lung

Group	Treatment	No. of rats	Incidence (%)	Multiplicity[b)]	
				Numbers (No./cm^2)	Areas (mm^2/cm^2)
1	DDN→bLF 2%	14	14 (100)	2.75±0.34	11.48±1.50
2	DDN→bLF 0.2%	19	19 (100)	2.74±0.37	11.21±1.43
3	DDN→bLF 0.02%	14	14 (100)	1.98±0.41[c)]	11.13±3.21
4	DDN→bLF 0.002%	17	17 (100)	2.36±0.27	9.49±1.08
5	DDN→control	29	29 (100)	3.48±0.33	12.78±2.15

a) Adenomas and carcinomas.
b) Values given are mean±SE.
c) $P<0.05$ compared with group 5.

significant reduction was observed at the 0.02% dose ($P<0.05$, 60% of the control) (Table III). No enhancing or inhibitory effects of bLF were noted for tumor development in the liver (including GST-P-positive foci, data not shown), kidney and thyroid (Tables IV–VII).

DISCUSSION

Recently, we have shown that bLF inhibits the development of AOM-induced aberrant crypt foci (precursor lesions)[9)] and carcinomas[10)] in the rat colon when given as

Table IV. Effects of bLF on Incidence and Multiplicity of Tumors in the Hepatocellular Lesions

Group	Treatment	No. of rats	Adenoma		Carcinoma		Total tumors	
			Incidence (%)	No./rat[a)]	Incidence (%)	No./rat[a)]	Incidence (%)	No./rat[a)]
1	DDN→bLF 2%	14	4 (29)	0.36±0.17	2 (14)	0.14±0.10	5 (36)	0.50±0.20
2	DDN→bLF 0.2%	19	3 (16)	0.16±0.09	2 (11)	0.11±0.07	5 (26)	0.26±0.10
3	DDN→bLF 0.02%	14	4 (29)	0.36±0.17	1 (7)	0.07±0.07	5 (36)	0.43±0.17
4	DDN→bLF 0.002%	17	3 (18)	0.24±0.14	2 (12)	0.12±0.08	5 (29)	0.35±0.15
5	DDN→control	29	5 (17)	0.21±0.09	1 (3)	0.03±0.03	6 (21)	0.24±0.09

a) Values given are mean±SE.

Table V. Effects of bLF on Kidney Tumor Incidences

Group	Treatment	No. of rats	Renal cell (%)			Transitional cell (%)			Nephro-blastoma (%)
			Adenoma	Carcinoma	Total tumors	Papilloma	Carcinoma	Total tumors	
1	DDN→bLF 2%	14	5 (36)	1 (7)	5 (36)	1 (7)	3 (21)	4 (29)	6 (43)
2	DDN→bLF 0.2%	19	6 (32)	2 (11)	8 (42)	3 (16)	5 (26)	8 (42)	8 (42)
3	DDN→bLF 0.02%	14	5 (36)	2 (14)	6 (43)	0 (0)	2 (14)	2 (14)	10 (71)
4	DDN→bLF 0.002%	17	9 (53)	1 (6)	10 (59)	1 (6)	2 (12)	3 (18)	5 (29)
5	DDN→control	29	13 (45)	1 (3)	13 (45)	1 (3)	7 (24)	7 (24)	15 (52)

Table VI. Effects of bLF on Kidney Tumor Multiplicity

Group	Treatment	No. of rats	Renal cell[a)]			Transitional cell[a)]			Nephro-blastoma[a)]
			Adenoma	Carcinoma	Total tumors	Papilloma	Carcinoma	Total tumors	
1	DDN→bLF 2%	14	0.50±0.20	0.07±0.07	0.57±0.25	0.07±0.07	0.29±0.16	0.36±0.17	0.64±0.23
2	DDN→bLF 0.2%	19	0.53±0.21	0.16±0.12	0.68±0.22	0.16±0.09	0.26±0.10	0.42±0.12	0.42±0.12
3	DDN→bLF 0.02%	14	0.50±0.20	0.14±0.10	0.64±0.23	0.00±0.00	0.21±0.15	0.21±0.15	1.14±0.29
4	DDN→bLF 0.002%	17	0.76±0.24	0.06±0.06	0.82±0.23	0.06±0.06	0.12±0.08	0.18±0.10	0.35±0.15
5	DDN→control	29	0.55±0.14	0.03±0.03	0.59±0.15	0.03±0.03	0.28±0.10	0.31±0.11	0.66±0.14

a) Values given are mean±SE.

Table VII. Effects of bLF on Thyroid Tumor Incidences and Multiplicity

Group	Treatment	No. of rats	Adenoma		Carcinoma		Total tumors	
			Incidence (%)	No./rat[a)]	Incidence (%)	No./rat[a)]	Incidence (%)	No./rat[a)]
1	DDN→bLF 2%	14	6 (43)	0.50±0.17	6 (43)	0.50±0.17	8 (57)	1.00±0.28
2	DDN→bLF 0.2%	19	9 (47)	0.68±0.22	9 (47)	0.79±0.22	14 (74)	1.47±0.28
3	DDN→bLF 0.02%	14	4 (29)	0.36±0.17	7 (50)	0.71±0.24	10 (71)	1.07±0.25
4	DDN→bLF 0.002%	17	6 (35)	0.47±0.19	10 (59)	0.76±0.18	12 (71)	1.24±0.28
5	DDN→control	29	15 (52)	0.66±0.13	11 (38)	0.41±0.11	19 (66)	1.07±0.17

a) Values given are mean±SE.

a dietary supplement, and also suppresses spontaneous intestinal polyposis in Apc^{Min} mice as a model of familial adenomatous polyposis.[20)] Furthermore, we have observed activation of NK activity in AOM-treated rats fed bLF.[8)] Findings have accumulated pointing to a wide array of functions of bLF in the immune system; thus NK cell activation,[5)] stimulation of LAK cell activity[6)] and potentiation of macrophage cytotoxicity[7)] have been documented,

among other effects. The chemopreventive potential of bLF on carcinogenesis in animal models might therefore result from activation of immune responses.

Many compounds have been demonstrated to exert chemopreventive effects. However, their action is usually tissue- or organ-specific and some of them also enhance carcinogenesis in other animal models or target sites. For instance, it is well established that aspirin, a nonsteroidal anti-inflammatory drug (NSAID), has chemopreventive effects on colon carcinogenesis,[12] whereas it enhances forestomach carcinogenesis in rats.[13] Catechol, an antioxidant, reduces carcinogenesis in rat colon, lung, kidney and mammary gland, but causes tumors in the rat esophagus.[13] In relation to this point, a Working Group from the National Cancer Institute and the Food and Drug Administration in the United States concluded that efficacy studies should include 2 or more tumor model systems in different organs, in preclinical efficacy studies for initiation of Phase I–II clinical trials for chemopreventive investigational drugs.[21]

Although no obvious dose-dependence was observed in the present multi-organ carcinogenesis model, bLF tended to inhibit esophagus and lung carcinogenesis at the 0.002–2% doses, along with significant inhibition at the 0.2% dose in the esophagus and at the 0.02% dose in the lung (Tables I and II). However, no significant modification was apparent in the liver, kidney or thyroid (Tables III–VII). There was no evidence of histopathological toxicity in animals administered bLF, with or without DDN treatment and the results indicated that bLF does not exert promoting effects in any major organs.

In conclusion, bLF exerts a beneficial effect which is limited in terms of the target organ spectrum. However, no adverse effects were noted. The question of whether bLF stimulation of immune responses is responsible for its influence on carcinogenesis in animal models remains to be determined. Further investigations of its absorption, distribution and biological effects are warranted.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare in Japan, by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan and by a grant from the Foundation for Promotion of Cancer Research in Japan.

(Received November 13, 1998/Revised January 4, 1999/ Accepted January 8, 1999)

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