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Chemopreventive Effects of Bovine Lactoferrin on *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine-induced Rat Bladder Carcinogenesis

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Chemopreventive effects of bovine lactoferrin (bLF), which is found at high concentrations in colostrum, on rat bladder carcinogenesis were investigated using a rat bladder medium-term bioassay. In experiment 1, a total of 80 F344 male rats, 6 weeks old, were divided into 5 groups. Groups 1 and 2 were treated with 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in the drinking water for 8 weeks and after a 1-week interval, received dietary supplementation with 2% and 0.2% bLF, respectively. Group 3 received 0.05% BBN for 8 weeks and then no treatment. Group 4 was administered 2% bLF alone from week 9, without prior carcinogen exposure. Group 5 was maintained without any treatment throughout the experiment. All rats were killed at the end of week 36. Group 1 demonstrated a significantly decreased multiplicity of the bladder tumors (carcinomas and papillomas) as compared with group 3. Maximum cut surface areas of bladder tumors were also significantly decreased in groups 1 and 2 compared with group 3. No bladder tumors were observed in groups 4 or 5. In experiment 2, a total of 60 rats were divided into two groups (30 rats each); both were treated with 0.05% BBN for 4 weeks and after a 1-week interval, one received 2% bLF (group 1) and the other, basal diet (group 2) for 4 weeks. Group 1 demonstrated a tendency for decrease of the 5-bromo-2′-deoxyuridine (BrdU) labeling index. bLF was detected in the urine of rats fed bLF by ELISA as well as western blot analysis. The findings indicate that 2% bLF can inhibit BBN-induced rat bladder carcinogenesis, and that this may be due to bLF in the urine.

Key words: Bovine lactoferrin — Chemoprevention — Bladder — BBN — Urine

Lactoferrin (LF), a multifunctional iron-binding glycoprotein with a molecular weight range of 76 000 to 81 000,[1–3] is found at high concentrations in colostrum (approximately 7 g/liter), some secretions of glandular epithelium (tears, saliva, seminal fluid) and the specific granules of neutrophils.[4, 5] It activates the cytotoxic effects of natural killer (NK) cells, lymphokine-activated killer (LAK) cells, polymorphonuclear (PMN) leukocytes, and macrophages.[6–9] The main biological functions of LF are considered to be in iron transportation and bacteriostasis due to sequestration of iron required for microbial growth.[10] Concerning effects on neoplasms, LF inhibits growth of solid tumors and development of experimental metastasis in mice.[11] It has also been shown to inhibit the proliferation of transformed and tumor epithelial cell lines by a block in the cell cycle progression at the G1 to S transition.[12]

It has been reported that dietary supplementation of bovine lactoferrin (bLF), derived from bovine milk, can inhibit the development of aberrant crypt foci (ACF), surrogate markers for tumors, as well as adenocarcinomas in the azoxymethane (AOM)-initiated rat colon and also intestinal polyposis in the APC^{MIN} mouse.[13–16] Moreover, it was recently found that bLF may suppress esophagus and lung carcinogeneses in rats.[17] Since the effects of bLF on tumorigenesis in the urothelial epithelium have remained unclear, in the present study, it was administered in the diet after *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) treatment in a rat bladder medium-term bioassay.

MATERIALS AND METHODS

Carcinogens and test compounds The carcinogen, BBN, was obtained from Tokyo Kasei Co., Ltd., Osaka, and bLF, isolated from bovine skim milk by the method of Law and Reiter,[18] as described previously, was kindly provided by Morinaga Milk Industry Co., Ltd. (Zama).

Animals and diets Male F344 rats were purchased from Charles River Japan (Atsugi) at 5 weeks of age and maintained in plastic cages with wood chips for bedding in an animal room with a 12 h light/dark cycle at 22±2°C and 44±5% relative humidity. The animals were allowed free access to basal diet (Oriental MF, Oriental Yeast Co., Tokyo) and tap water and used in the experiments after a 1-week acclimation period.

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Experimental protocol

Experiment 1: A total of 80 F344 male rats, 6 weeks old, were divided into five groups. Groups 1 and 2 (20 rats each) were treated with 0.05% BBN in drinking water for 8 weeks and after a 1-week interval, respectively given dietary supplementation with 2% and 0.2% bLF. These doses were based on earlier findings for rat colon carcinogenesis.[14, 15] Group 3 (20 rats) received 0.05% BBN for 8 weeks and then no treatment. Group 4 (10 rats) was administered 2% bLF alone from the beginning of week 9, without prior carcinogen exposure. Group 5 (10 rats) was maintained without any treatment. The total observation period was 36 weeks.

Body weights of all of the rats were measured every week. Food consumption and water intake in each cage were measured every 2 weeks. One hour before sacrifice, 10 rats in each group were injected i.p. with 100 mg/kg body weight of 5-bromo-2′-deoxyuridine (BrdU). All rats were killed under ether anesthesia for histological examination of the bladder. The body, liver and kidney weights of all the rats were measured. Bladders were inflated and fixed with 10% phosphate-buffered formalin (pH 7.4).

Experiment 2: A total of 60 rats were divided into two groups. Group 1 (30 rats) was treated with 0.05% BBN in the drinking water for 4 weeks and after a 1-week interval, received dietary supplementation with 2% bLF for 4 weeks. Group 2 (30 rats) received 0.05% BBN for 4 weeks and then no treatment. Body weights of all rats were measured every week. Food consumption and water intake in each cage were measured every 2 weeks. One hour before sacrifice, 10 rats in each group were injected i.p. with 100 mg/kg body weight of BrdU. All rats were killed under ether anesthesia at the end of week 9. The 10 bladders from the BrdU-injected animals were inflated and fixed with 10% phosphate-buffered formalin (pH 7.4) for histological examination. For the other 40 rats, the bladder mucosa was removed using the edge of a slide glass and frozen in liquid nitrogen for subsequent measurement of ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase (SAT) activities.

Analysis of urine Fresh urine samples were collected from 10 animals in each group in experiment 1 by forced urination in the morning 1 day before the termination. The pH was measured (Horiba model F-15 pH meter, Horiba, Ltd., Kyoto) and the urine samples were analyzed for sodium, potassium and chloride (Hitachi 710 electrolyte analyzer, Hitachi Instruments, Ltd., Tokyo). In experiment 2, samples were collected from 23 animals in group 1 and 21 animals in group 2, respectively by the same method in the morning 1 day before the termination. The urine samples in experiment 2 were used for western blot analysis and ELISA.

Histopathological and immunohistochemical analyses The bladders were cut into two equal parts with a razor blade to examine their macroscopic appearance. In experiment 1, they were then weighed, divided into strips, fixed in 10% phosphate-buffered formalin (pH 7.4) again, embedded in paraffin and sliced at 3 μm for histological examination. Sections cut at 5 μm were routinely stained with H-E. Epithelial cells incorporating BrdU were immunohistochemically demonstrated in the sections by the avidin-biotin-peroxidase complex procedure (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) using anti-BrdU mouse monoclonal antibody (DAKO, Tokyo), as described previously.[19, 20] The numbers of labeled cells were counted under a light microscope and labeling indices were expressed as percentages of the total cells (with a minimum of 4000 cells counted per slide).

Quantitative assessment of cut surface areas of bladder tumors In experiment 1, cut surface areas of bladder tumors in H-E slides were assessed using a two-dimensional approach with the aid of a color image processor (IPAP; Sumica Technos, Osaka).[21]

Measurement of ODC and SAT activities In experiment 2, enzyme activities of ODC and SAT in the mucosa of the urinary bladder were measured as biomarkers of cell proliferation by the methods of Matsui *et al*. and Otani *et al*., respectively.[22, 23] One sample comprised mucosa taken from 2 rats. Ten samples in each group were used for the analyses. Frozen specimens were homogenized with 4 vol. of 50 m*M* Tris buffer (pH 7.5) containing 0.25 *M* sucrose, the homogenates were centrifuged at 100 000*g* for 30 min and the supernatants were assayed for ODC and SAT activities. The amount of $^{14}CO_2$ produced from L-[1-^{14}C]-ornithine (2.96 mCi/mmol) and the amount of acetyl moiety transferred from [1-^{14}C]acetyl coenzyme A to spermidine were measured with a Beckman liquid scintillation counter (LC5801) for ODC and SAT activities, respectively.

Western blot analysis A total of 20 urine samples from experiment 2 were analyzed (group 1, 10 samples; group 2, 10 samples). SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out as described by Laemmli[24] using 10–20% gradient gels. Ten microliters of each urine sample was mixed with 5 μl of 5-fold standard nonreducing SDS-sample buffer and 10 μl of water and boiled for 3 min at 95°C. The mixture (25 μl) was applied into gel wells and electrophoresed for 1 h at 30 mA. The proteins in the gels were transferred with commercially available blocking buffer, Block Ace (Dainippon Pharmaceuticals, Osaka), with 50 m*M* Tris-HCl pH 7.6 containing 0.9% NaCl and 0.05% Tween 20 (T-TBS) to polyvinylydene difluoride (PVDF) sheets at 4°C overnight. After washing, the sheets were treated with mouse anti-bLF monoclonal antibody diluted 1:1000 for 30 min at room temperature. After further washing, the sheets was treated with biotin-labeled anti-mouse IgG antibody diluted 1:2000 in the same buffer as the primary anti-

body, then with alkaline phosphatase-labeled streptoavidin (1:1000) (each for 30 min at room temperature). Immunoreactive proteins were visualized using 5-bromo-4-chloro-3′-indolylphosphate and the nitroblue tetrazolium reaction. Staining intensity was determined using a laser densitometer.

ELISA Urinary levels of bLF were quantified by ELISA methods in a total of 44 samples in experiment 2 (group 1, 23 samples; group 2, 21 samples). Anti-bLF polyclonal antibody was obtained from a female rabbit immunized with bLF in Freund's complete adjuvant. Another rabbit polyclonal antibody to bLF (Yagai, Yamagata) was biotinylated by means of a biotinylation kit (American Qualex, San Clemente, CA). Urine samples and antibodies were diluted with PBS (phosphate-buffered saline) containing 10% Super Block (Pierce, Rockford, IL) and 0.05% Tween 20. The rabbit anti-bLF antibody (10 μg/ml) was immobilized on the surface of 96-well microplates for 1 h at 37°C followed by blocking with Super Block for 30 min at room temperature. After washing with PBS containing 0.05% Tween 20, 100 ml of standard (bLF 0, 1.25, 2.5, 5 ng/ml) or sample (6 samples, 1:300 diluted; 38 samples, 1:51 diluted) was added to each well in duplicate for 60 min at 37°C. After washing with the same buffer, biotinylated anti-bLF antibodies (3 μg of ml, Yagai) were added to each well and incubation was continued for a further 60 min at 37°C. The wells were washed, then alkaline phosphatase-avidin complex (Bio-Rad, Hercules, CA) diluted 1:1000 was added and allowed to react for 1 h at 37°C. After the final washing, 10 μl of *p*-nitrophenyl phosphate solution was introduced. After 30 min at room temperature, the reaction was terminated by adding 100 μl of 5% EDTA and the absorbance at 405 nm was measured with a microplate reader.

Statistical evaluation of data Data are shown as mean±SD. The results of experiment 1 were analyzed by two-way factorial ANOVA using StatView software (Abacus Concepts, Berkeley, CA), and for experiment 2, Student's *t* test using the JMP software package (Version 3.1, SAS Institute, Japan) was applied on a Macintosh. *P* values less than 0.05 were considered to be significant.

RESULTS

Experiment 1 All rats survived until the end of the experiment. No clinical symptoms due to bLF were seen in any of the rats. Data for body and liver weights of rats killed at week 36 are summarized in Table I. No significant intergroup variation was observed. Values for kidney weights, and food and water consumptions were also similar among the five groups (data not shown). However, the weights of the bladders in groups 1 and 2, receiving BBN followed by 2% and 0.2% bLF, were significantly lower than in group 3, BBN followed by the basal diet ($P<0.01$).

Macroscopically, bladder tumors were only observed in groups 1 to 3 with BBN initiation. Sizes of bladder tumors in groups 1 and 2 were smaller than that in group 3.

The incidence and multiplicity of bladder tumors, essentially papillomas and transitional cell carcinomas (TCCs), are shown in Table II. Bladder tumors were histologically apparent in all rats treated with BBN (groups 1 to 3). With regard to the multiplicity of bladder tumors, a significant reduction was observed in group 1 as compared to group 3, and a tendency for decrease was also observed in group 2. Similar results were obtained for TCCs alone. No difference in inflammatory cell infiltration in the submucosa of the bladder was apparent between bLF-treated groups and non-treated groups. Data for maximum cut surface areas of bladder tumors in groups 1 to 3 are shown in Fig. 1. Values for groups 1 and 2 were significantly lower than that for group 3.

BrdU labeling indices for histologically normal or simple hyperplastic mucosa of the bladder are shown in Table III. In groups 1 to 3, BrdU labeling indices showed a tendency to decrease dose-dependently, but without statistical significance. Without BBN treatment, BrdU labeling indices were low.

Values for pH, and sodium, potassium and chloride concentrations are shown in Table IV. Some data for sodium,

Table I. Body, Liver and Bladder Weights for Rats Killed at Week 36 (Experiment 1)

Group	No. of rats	Body wt. (g)	Liver wt. (g)	Bladder wt. (g)
G1	20	362±20[a)]	7.6±0.6	0.27±0.20[b)]
G2	20	363±15	7.6±0.5	0.25±0.14[b)]
G3	20	354±18	7.5±0.5	0.50±0.49
G4	10	363±14	7.3±0.3	0.10±0.02
G5	10	361±15	7.5±0.6	0.10±0.02

a) Mean±SD.
b) $P<0.01$ vs. G3.

Table II. Effects of bLF on the Incidence and Multiplicity of Bladder Tumors (Groups 1 to 3 with BBN Initiation) in Experiment 1

Groups	Incidence (%)	Multiplicity (number/rat)		
		Bladder tumor (Papilloma+TCC)	Papilloma	TCC
G1	100	4.3±2.4[a, b)]	0.8±1.3	3.5±2.4[c)]
G2	100	5.1±2.1	0.4±0.6	4.8±2.1
G3	100	5.7±2.0	0.4±0.5	5.3±2.0

a) Mean±SD.
b) $P<0.05$ vs. G3.
c) $P<0.01$ vs. G3.

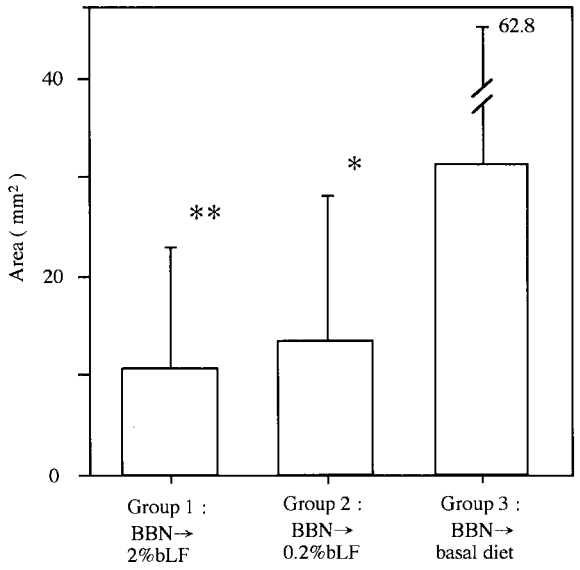

Fig. 1. Effects of bLF on cut surface areas of bladder tumors (groups 1 to 3 with BBN initiation) in experiment 1. Significant differences were observed for groups 1 and 2 compared to group 3 (* $P<0.05$, ** $P<0.01$).

Table III. BrdU Labeling Indices (%) for Normal or Simple Hyperplastic Mucosa of the Bladder

Groups	Treatment	BrdU labeling index (%)
Experiment 1		
G1	BBN →2.0% bLF	0.23±0.11[a, b]
G2	BBN →0.2% bLF	0.29±0.09[b]
G3	BBN →basal diet	0.33±0.19[c]
G4	— →2.0% bLF	0.08±0.10
G5	— →basal diet	0.08±0.10
Experiment 2		
G1	BBN →2.0% bLF	0.12±0.09
G2	BBN →basal diet	0.16±0.04

a) Mean±SD.
b) $P<0.05$ vs. G5.
c) $P<0.001$ vs. G5.

potassium and chloride in groups 1 to 3 (BBN-treated groups) were significantly lower than in group 4 or 5 (without BBN), but no significant intergroup variation related to bLF administration was observed in the BBN-treated groups.

Experiment 2 No significant intergroup variation was observed in body or liver weights of rats killed at week 9 (data not shown). Values for food consumption and water intake were also similar (data not shown).

Table IV. Urinary pH and Electrolyte Levels (Experiment 1)

Groups	pH	Electrolyte concentration (mEq/liter)		
		Na	K	Cl
G1	7.0±0.5[a]	115±22[c]	193±75[b, e]	138±41[b, c]
G2	7.0±0.7	122±58[c]	220±60[d]	146±28
G3	7.0±0.8	114±50[c]	207±61[e]	147±50
G4	6.8±0.6	167±26	306±36	174±29
G5	6.8±0.7	150±52	255±63	172±37

a) Mean±SD.
b) $P<0.05$ vs. G5.
c) $P<0.05$ vs. G4.
d) $P<0.01$ vs. G4.
e) $P<0.001$ vs. G4.

Table V. ODC and SAT Activities in the Mucosa of the Urinary Bladder in Experiment 2

Groups	ODC activity (pmol/mg protein/h)	SAT activity (pmol/mg protein/10 min)
G1	118.4±107.6[a]	32.9±42.7
G2	112.0±121.2	50.0±75.2

a) Mean±SD.

No bladder tumors were observed. However, putative preneoplastic lesions, papillary and/or nodular (PN) hyperplasia,[25] were apparent at one or two per rat, with no significant difference between the two groups. Epithelial BrdU labeling indices for bladder mucosa, excluding PN hyperplasias, are given in Table III. A non-significant tendency for decrease was apparent in group 1.

Data for ODC and SAT activities in the bladder mucosa of groups 1 and 2 are given in Table V. There are no differences in ODC activities between groups 1 and 2, while SAT activity in group 1 was lower than in group 2, but without statistical significance. Representative western blots for bLF in the urine of BBN-treated rats are shown in Fig. 2. Bands of intact bLF of about 80 000 molecular weight, were apparent for group 1 (BBN followed by 2% bLF), with densities clearly and significantly higher than in group 2. Urinary levels of bLF as assessed by ELISA are shown in Fig. 3. Values were significantly higher in group 1 (0.67±0.16 μg/ml) than in group 2 (0.02±0.02 μg/ml).

DISCUSSION

In the present study, bLF, particularly at a dose of 2%, significantly reduced the multiplicity of BBN-initiated bladder tumors and their individual sizes. bLF also reduced cell proliferation in normal or simple hyperplastic

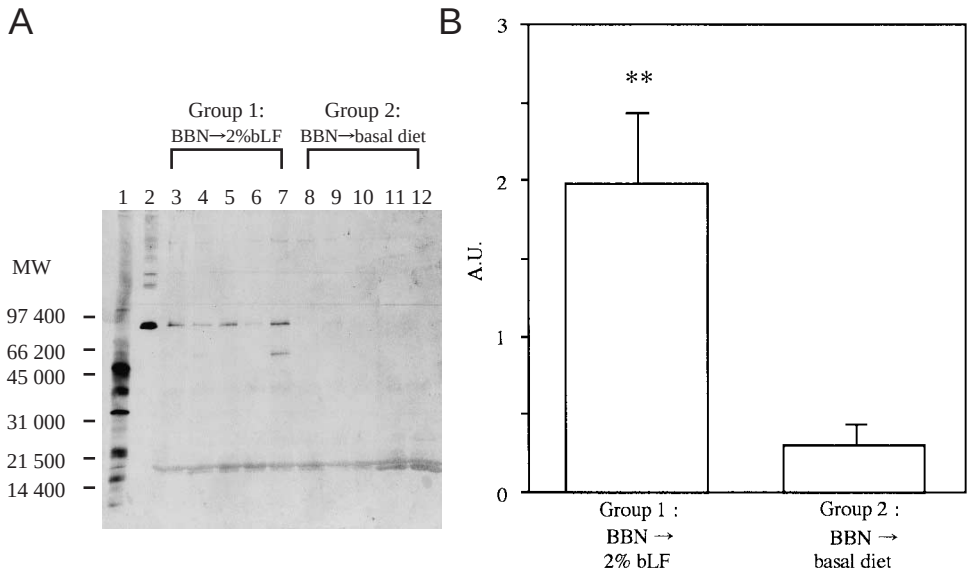

Fig. 2. Protein levels of bLF in the urine assessed by western blot analysis in experiment 2. A, Lane 1, marker; lane 2, intact bLF; lanes 3–7, urine samples of rats of group 1 (BBN followed by 2% bLF); lanes 8–12, urine samples of rats of group 2 (BBN followed by basal diet). In lanes 2–7, bands for intact bLF with a molecular weight of about 80 000 are observed. B, Density of each band. Those for urine after BBN followed by 2% bLF are significantly stronger than those after BBN followed by basal diet (** $P<0.01$).

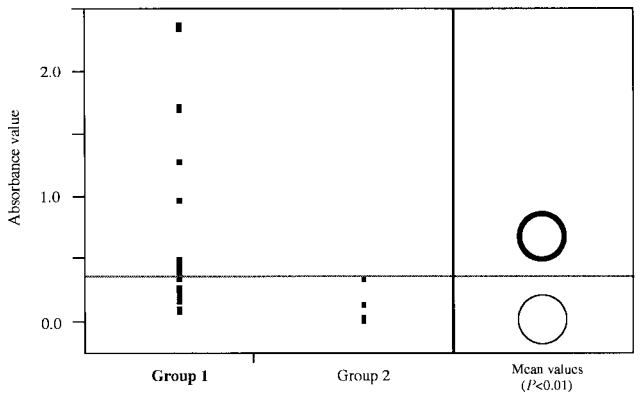

Fig. 3. Absorbance values for intact bLF in urine samples assessed by ELISA in experiment 2. Each dot is a separate value. The mean concentration±SD in groups 1 and 2 were 0.67±0.16, 0.02±0.02 mg/ml, respectively ($P<0.01$). The bold circle shows the mean of group 1, and the normal circle shows the mean of group 2.

mucosa of the bladder. These results indicated that bLF exerts chemopreventive effects in the post-initiation stage of urinary bladder carcinogenesis with a possible involvement of suppressed cell proliferation.

The presence of bLF in the fecal and urinary extracts of new infants has been previously reported,[26, 27] and elevated levels in the urine could be detected by western blot and ELISA in the present study. LF is a macromolecule, with a molecular weight of about 80 000. It is well known that such macromolecules are markedly restricted in their passage across the capillary wall. The permeability of the glomerular capillary wall to macromolecules is quantitatively expressed in terms of fractional clearance and is affected by the size, charge and shape of the molecule.[28] In the kidney of rats, the slightly cationic form, LDH5 (molecular weight 140 000) was found to have five times higher clearance than the anionic LDH1 and $2^1/_2$ times higher than albumin (molecular weight 69 000), despite being larger in size.[29] bLF would be expected to be restricted by the glomerular capillary wall due to its molecular weight, but our data clearly show that it can be excreted in the urine, possibly due to factors such as net surface charge.

In general, the modifying effects of chemicals on rat bladder carcinogenesis are mainly exerted through the urine. Since in experiment 2, bLF was significantly detected in the urine of rats fed bLF, this might also have been the case in the present study. LF itself inhibits the proliferation of the transformed and tumor epithelial cell lines by blocking cell cycle progression at the G1 to S transition.[12] Moreover, in the present study, SAT activity was slightly lower, but ODC activity was not decreased. Generally, ODC and SAT activities are markers of cell proliferation caused by either genotoxic or nongenotoxic

agents.[30] Further studies are required to discover why the present studies showed no reduction of ODC activity. Our data on reduction of BrdU incorporation suggest that bLF in the urine directly influenced the epithelium of the bladder, and reduced cell proliferation and tumor development.

Cyclooxygenase (COX) and production of eicosanoids such as prostaglandins (PGs) impact on tumor growth either by participating in signal cascades for cell proliferation or by disturbing immunological surveillance.[31–33] COX-2 is said to be related to inflammation and cell proliferation,[34] and a specific inhibitor suppressed BBN-induced rat bladder carcinogenesis.[35] Since bLF has bacteriostatic and anti-inflammatory effects,[5] it also has the potential to reduce COX-2, and suppress cell proliferation and tumor growth.

Several hypotheses have been proposed concerning the mechanism of inhibitory effects of bLF. It is well established that LF can activate NK cells and LAK cells *in vivo* and *in vitro*.[6, 36, 37] When contained in cheeses it enhances the absorption of ferric iron and this activates immunocompetent peripheral lymphocytes to inhibit tumor growth.[38] Sekine *et al*. also found a good relationship between inhibitory effects on ACF and activation of NK cells in rat colon carcinogenesis.[15] However, the lack of any obvious difference of inflammatory cell infiltration in the submucosa of the bladder between bLF-treated and non-treated groups indicates that immunological responses might not have contributed to the inhibition of bladder carcinogenesis, although further investigations are required to resolve this point.

In the present study, 2% or 0.2% bLF did not cause any signs of toxicity, including loss of body, liver, or kidney weight, in line with earlier findings. Furthermore, the lack of any promoting effect means that bLF has the potential to be a practical chemopreventive agent. In experiment 2, there were no significant differences in preneoplastic lesions between the groups with or without bLF administration but the experimental period was short. Since the multiplicity of PN hyperplasia in group 2 was only one or two per rat, comparison of preneoplastic lesions with or without bLF was difficult.

In conclusion, 2% bLF decreased tumor multiplicity and tended to reduce cell proliferation in BBN-induced rat bladder carcinogenesis, possibly by direct effects through its presence in the urine.

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