RESEARCH COMMUNICATION

Lack of Chronic Oral Toxicity of Chemopreventive Bovine Lactoferrin in F344/DuCrj Rats

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Abstract

Studies were undertaken to determine whether bovine lactoferrin (bLF) and related compounds, shown to prevent carcinogenesis in the colon and other organs in rats, have any toxic effects in long-term feeding studies. In experiment I, male F344/DuCrj rats received a basal diet containing 0.2% bLF for 40 weeks. No adverse findings were noted, furthermore, serum triglyceride level was significantly decreased to 72% of the control level, suggesting preventive effects against the metabolic syndrome. In experiment II, male and female F344/DuCrj rats were fed a basal diet containing 0.02, 0.2, 2.0 and 5.0% bLF, 2.0% bLF hydrolysate (bLF-H) or 0.1% lactoferricin (LFcin), a peptide derived from bLF, for 60 weeks in males and 65 weeks in females. No toxicological effects, including carcinogenicity, were evident in either sex. The results of the studies provide subjective support for safety of clinical studies of bLF for supplement use.

Key Words: Chemopreventive agent - bovine lactoferrin - *in vivo* toxicity - F344/DuCrj rat

Asian Pacific J Cancer Prev, **9**, 313-316

Introduction

Lactoferrin is a multi-functional iron-binding glycoprotein, which is present at high concentration in mammalian colostrums, as well as neutrophilic leukocytes (Masson et al., 1969), which release the compound in response to inflammatory stimuli (Lash et al., 1983). The primary biological function of lactoferrin is related to its anti-bacterial, anti-viral, anti-fungal and immune-modulating effects (Bullen, 1975; Arnold et al., 1980; Brock, 1995; Lönnerdal and Iyer, 1995). Furthermore, lactoferricin (LFcin), a pepsin digested peptide of lactoferrin, has been shown to possess potent and wide-spectrum anti-microbial effects (Tomita et al., 1991; Bellamy et al., 1992), as confirmed by extensive analyses of the mechanisms (Wakabayashi et al., 2003). Recently, bovine lactoferrin (bLF) was shown to exert cancer preventive effects in various organs of rodents including the colon, lung and esophagus (Sekine et al., 1997; Tsuda, et al., 2000b, 2004). Furthermore, bLF was also demonstrated to have anti-metastatic effects in mice (Iigo et al., 1999; Tsuda et al., 2000a).

However, for application of bLF in human trials of its ability to prevent carcinogenesis and tumor metastasis with long-term ingestion, it is obviously necessary to conduct chronic feeding studies to detect any adverse effects. Prior to the current studies, acute and subchronic exposure indicated no obvious toxicological effects after 4-weeks and 13-weeks feeding with doses up to 2,000 mg/kg/day (Yamauchi et al., 2000a). Lack of mutagenicity was also reported (Yamauchi et al., 2000b). However, chronic toxicology studies have hitherto not been performed, promoting the present studies of long-term toxic effects of dietary bLF in F344 rats of both sexes.

Materials and Methods

Test Chemical

Bovine lactoferrin (bLF), lactofferin hydrolysate (bLF-H), generated by acid-pepsin hydrolysis (Tomita et al., 1991), and lactoferricin (LFcin), identified as an antimicrobial peptide derived by pepsin digestion of lactoferrin (Bellamy et al., 1992)(Morinaga Milk Industry Co., Ltd., Zama, Japan) were used.

Diet preparation and analysis

The compounds were mixed at the designated levels into powdered basal diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan) after being previously confirmed to be stable in diet for 3 months when stored in a cold room controlled at the Food Science and Technology Institute, Morinaga Milk Industry Co., Ltd.. Therefore, the diets were prepared at intervals of 3 months and stored in a cold room. Amounts of bLF in the diet preparations were

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within ± 5% of the target concentrations. Homogeneity was confirmed as satisfactory.

Animals and their maintenance

Male and female F344/DuCrj (F344) rats, 5 weeks of age, were purchased from Charles River Japan (Atsugi, Japan) and allowed a 7 days quarantine and acclimation period. After confirmation of normal health status, they were used for the studies.

Animals of the same sex were housed two or three to a polycarbonate cage, with wood chip (Oriental Yeast Co., Ltd., Tokyo, Japan) for bedding. They were placed on powdered MF basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) with or without test compounds and tap water ad libitum. The room temperature and relative humidity were controlled at 21~25˚C and 50~60%, respectively, and the room air was changed 15 or more times per hour. Fluorescent tube lighting was employed to provide a 12-hr light/dark cycle.

Experimental procedures

Experiment I: Starting at 6 weeks of age, groups of 15 male rats were given diet containing 0% (control) or 0.2% of bLF for 40 weeks. Diet and drinking water were available ad libitum. The animals were observed for general conditions every day and weighed once weekly for the initial 4 weeks and once every 4 weeks thereafter. Determination of food consumption by cage was performed at the same time as body weight measurement. Test material intake (mg/kg body weight/day) was calculated for each group from mean food consumption and body weight data and the nominal dietary levels.

At the end of the treatment, all animals were fasted overnight and then killed in the next morning under deep ether anesthesia. Whole blood samples were collected from the all rats via the inferior vena cava and blood biochemistry determinations were performed with an Automatic Analyzer Model 7070 (Hitachi Co., Ltd., Tokyo, Japan). Parameters were aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (É¡-GTP), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), total cholesterol (T-CHO), trigryceride (TG), total protein (TP), albumin (ALB), serum iron. Gross inspections for any lesions were made at autopsy and the findings recorded. The liver, kidneys and spleen were weighed and the organ-to body weight ratios were determined.

Experiment II: A total of 100 F344 rats each sexes were used. Starting at 17 (in males) or 11 (in females) weeks of age, groups of 25 rats (groups 1 and 5) and 10 rats (groups from 2 to 4, and 6 and 7) of each sex were given powdered diet as in Experiment Öü containing 0% (control), 0.02, 0.2, 2.0 and 5.0% bLF, 2.0% bLF-H or 0.1% bLFcin for 60 weeks in males and 65 weeks in females. The animals were observed for general conditions every day and weighed 8 times during the experiment. Measurement of food consumption and water intake by cage were performed once every 2 weeks for the first 16 weeks and once every 4 weeks thereafter. Test material intake (mg/kg body weight/day) was calculated for each group from mean food consumption and the nominal dietary levels. Careful gross examinations were made at autopsy. The following organs, the liver, kidney, spleen, adrenal and pituitary were weighed for each animal. Samples of these organs, thymus, lungs (including trachea), salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, pancreas, urinary bladder testes, prostate, seminal vesicle, ovaries (including oviducts), uterus, vagina, spinal cord and grossly visible lesions were fixed in 10% buffered formalin solution. Tissues were routinely processed for histopathological examination.

Statistical analyses

For body weight, blood biochemistry and organ weight data, the significance of inter group differences was assessed using the Bartlett's test (Bartlett, 1937). If homogeneous, the data were analyzed with the Dunnett's multiple comparison test (Dunnett, 1955) and if not then with the Steel's test (Steel, 1959). For the incidences of histopathological lesions, the significance of differences observed between the control and treated groups was evaluated with the Fisher's exact probability test (Fisher, 1955). The Mann-Whitney U test was employed for comparison of degrees of change (Gad and Weil, 1989). The levels of significance were set at $P<0.05$ and $P<0.01$.

Results

Experiment I

Neither clinical signs related to bLF treatment nor deaths were observed throughout the 40 weeks of the study. No alteration in body weights related to bLF treatment was noted (data not shown). However slight, but significantly decreased relative liver weights (but not absolute weights) were noted in the 0.2% bLF treated animals (data not shown). No treatment-related macroscopic changes were observed in the bLF fed animals (data not shown). Selected blood biochemistry data are given in Table 1. AST, ALT, ALP, BUN and TG were significantly lowered in the 0.2% bLF group. No treatment-related histopathological lesions were observed.

Experiment II

Neither clinical signs related to bLF treatment nor deaths were observed throughout the study period. No significant alteration in body weights related to bLF treatment was noted. Average food consumption values were comparable to the targeted doses of test compound in both sexes, exhibiting dose-dependent increase in total bLF intake. Average water intake values in bLF-treated groups were not different from controls in either sex. There were no significant differences in final body, liver, kidneys, spleen, adrenal and pituitary weights between treated groups and control groups in either sex. No treatment-related macroscopic changes were found (data not shown).

No treatment-related histopathological changes were observed in either sex of the treated and control groups. All incidences of histopathological alterations observed in the present study were within the ranges for spontaneously occurring lesions in F344 rats (Goodman et al., 1979; Haseman et al., 1990).

Table 1. Blood Biochemistry Data for F344 Rats Fed a Diet Containing 0.2% bLF for 40 weeks

	Control	0.2% bLF
GOT (IU/L)	135.1 ± 30.1	100.0 ± 24.0[c]
GPT (IU/L)	85.3 ± 11.7	68.3 ± 19.0[b]
γ-GTP (IU/L)	N.D.	N.D.
ALP (IU/L)	714.6 ± 108.5	585.3 ± 93.7c
Creatinine (mg/dL)	0.3 ± 0.028	0.28 ± 0.025
BUN (mg/dL)	18.5 ± 2.00	16.0 ± 1.33c
Total Protein (g/dL)	6.7 ± 0.38	6.6 ± 0.22
Albumin (g/dL)	4.3 ± 0.26	4.4 ± 0.13
Cholesterol (mg/dL)	77.1 ± 10.9	78.3 ± 8.26
Triglyceride (mg/dL)	117.3 ± 44.9	83.9 ± 21.8[a]
Blood Glucose (g/dL)	174.4 ± 24.1	168.5 ± 21.3
Serum Iron (μg/dL)	152.7 ± 19.5	155.9 ± 25.6

Values are means±S.D. [a,b,c] P<0.05, P<0.01, P<0.005 compared to the control group. N.D., Not detected

Discussion

The present 40 week-chronic oral administration of dietary 0.2% bLF (Experiment I) demonstrated no cause of any toxicological lesions in male F344 rats. Decrease in serum GOT, GPT and ALT may be related to improvement of the impaired liver function possibly due to aging. Similarly, the lower level of BUN may be at least partly associated with protection of kidney function.

The findings, however, could not be confirmed in Experiment II in which animals were fed longer duration because serum was not taken. However, the serum triglyceride level was clearly decreased to 72 % of the control level. Although serum glucose level did not decrease, the result may be beneficial for protecting against the metabolic syndrome associated with hyperlipidemi. Administration of bLF has been reported to induce the production of cytokine IL-18 in the mouse small intestine (Kuhara et al., 2000). Recently, Netea et al. reported that deficiency of interleukin-18 leads to hyperphagia, obesity and hyperglycemia resulting from insulin resistance in Il-18 knockout mice (Netea et al., 2006). Furthermore, the molecular mechanisms responsible for the hepatic insulin resistance in Il-18 knockout mice involve an enhanced expression of genes associated with gluconeogenesis in the liver, resulting from defective phosphorylation of STAT3 (Netea et al., 2006). Decreased triglyceride level might therefore imply induction of cytokine IL-18 by lactoferrin in experiment I. Practically, serum IL-18 levels are significantly increased in patients with HVC associated chronic hepatitis C (Ishii, 2004). In this context it should be noted that blood biochemistry revealed significant lowering of AST and ALT in the 0.2% bLF treatment group, which might be related to gluconeogenesis in the liver.

In experiment II, there was no evidence of long term toxicity or carcinogenicity in either sex of rat fed 5.0% bLF for 60 or 65 weeks. Total and daily intake in the 5.0% bLF group were 46,201 g or 54,175 g, and 109 g or 119 g, when extrapolated to male or female human beings, respectively. Thus, we conclude that these ranges of dosage levels can be administered to humans safely in clinical trials. Our results are in line with the lack of any adverse effects reported in lactoferrin-treated patients with chronic hepatitis C, healthy subjects positive for helicobacter pylori infection and high risk subjects of colon and lung cancers (Tanaka et al., 1999; Iwasa et al., 2002; Okuda et al., 2005).

Accordingly, the results indicated that the NOAEL for bLF with 60 or 65 weeks dietary treatment is at least 5.0% for both sexes. Therefore, the results of the current study provide strong support for safety in accepted dose ranges of lactoferrin and related compounds for further clinical and intervention studies.

Acknowledgements

This work was supported in part by a Grant-in-Aid for the Second 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, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

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