

Effect of Orally Administered Bovine Lactoferrin on the Growth of Adenomatous Colorectal Polyps in a Randomized, Placebo-Controlled Clinical Trial

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Abstract

Lactoferrin (LF), a secreted, iron binding glycoprotein originally discovered as a component of milk, is found in a variety of exocrine secretions and in the secondary granules of polymorphonuclear leukocytes. Animal experiments have shown that oral administration of bovine lactoferrin (bLF) exerts anticarcinogenesis effects in the colon and other organs of the rat. The aim of this study was to determine whether oral bLF could inhibit the growth of adenomatous colorectal polyps in human patients. A randomized, double-blind, controlled trial was conducted in 104 participants, ages 40 to 75 years, with polyps ≤ 5 mm in diameter and likely to be adenomas. Participants were assigned to receive placebo, 1.5-g bLF, or 3.0-g bLF daily for 12 months. Target adenomatous polyps were monitored by colonoscopy. Ingestion of 3.0-g bLF significantly retarded adenomatous polyp growth in participants 63 years old or younger. Removal of adenomatous colorectal polyps is done as a preventative measure against colorectal cancer; however, polyps can be overlooked, and when detected, polypectomy is not always 100% effective in eradicating a polyp. Our study suggests that daily intake of 3.0 g of bLF could be a clinically beneficial adjunct to colorectal polyp extraction.

Colorectal cancer is one of the most frequent causes of death from cancer (1–5). Most colorectal cancers arise from benign adenomas (6). Adenoma formation and colorectal cancer incidence have been reported to be influenced by food elements and nutrition (7–10), making diet and dietary supplements factors in colorectal cancer. Bovine lactoferrin (bLF) isolated from cow milk has been studied for inhibition of colorectal and other cancers (11–15). Specifically, supplementation of bLF to the diet of azoxymethane-treated rats decreased the

incidence of both colorectal cancer and aberrant crypt foci (12, 14, 15). Importantly, bLF has been reported to be well tolerated in clinical research (16, 17).

In the present study, we conducted a randomized controlled trial to evaluate whether a 1-year oral intake of bLF-containing tablets (Morinaga Milk Industry Co. Ltd.) inhibits the growth of colorectal polyps < 5 mm in diameter with pit pattern III. Because polyps with pit pattern III have been reported to be histologically adenomatous in $\sim 90\%$ of the cases (18–20) and polyps < 5 mm in diameter show a tendency to grow in size (21), our target lesion is suitable for the current study and measurement of polyp diameter is a promising surrogate end point. We found a statistically significant retardation of polyp growth in participants 63 years old or younger ingesting 3.0-g bLF daily over the course of 12 months.

Ingestion of bLF inhibits colorectal carcinogenesis in animal studies (12, 14, 15). In addition, ingestion of bLF enhances immune function, both in animal studies (22, 23) and in human patients (24). Finally, numerous studies have revealed the crucial role of the immune system in inhibiting the neoplastic process (25). Therefore, immunologic parameters associated with bLF ingestion were measured: interleukin-18 and IFN γ , because ingestion of bLF enhances expression of interleukin-18 and IFN γ in the mouse small intestine (22); T-cell subpopulation numbers, because ingestion of bLF reconstitutes T-cell populations in immunosuppressed mice (26); natural killer (NK) cell number and activity, because ingestion of bLF enhances NK cell activity in rats (12); and neutrophil number, because

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ingestion of bLF increases the number of neutrophil precursor cells in human patients (24, 27). In addition, because LF is an important component of the human immune system (23, 28–31), we also measured the levels of human lactoferrin (hLF) in the serum of trial participants.

At the end of the trial period, the target lesions were removed and examined. The number of neutrophils in these specimens was of particular interest: Several reports indicate that neutrophils can enhance tumor growth (32–35), but in rodents, ingestion of bLF attenuates the movement of neutrophils to the small intestine (36).

Materials and Methods

Trial profile

An outline of the trial profile is shown in Supplementary Fig. S1. The trial was initiated after approval by the Ethical Committee of the National Cancer Center Hospital, Tokyo, Japan, and continued until January 2006. This study is registered in the University Hospital Medical Information Network Clinical Trials Registry (Tokyo, Japan; no. C000000182). The Independent Data Monitoring Committee determined the trial should have approximately 105 participants. Between February 2002 and January 2005, 307 patients scheduled for colonoscopic examination at the National Cancer Center Hospital, Tokyo, Japan, were invited to join the trial; patients were approached before their examinations. Of these, 215 patients provided written informed consent.

The 215 potential trial participants underwent their scheduled colonoscopic examinations, and during the examination, potential target polyps were marked by injection of india ink close to the polyp and photographed. Patients were excluded from the trial who met any of the following criteria: no target lesion present; dairy product allergies; use of nonsteroidal anti-inflammatory drugs (NSAID) or statins for >50 d in the previous 3 mo (use of these drugs could affect polyp size refs. 37, 38); diagnosed as having cancer; a history of colectomy within the previous 3 y; inflammatory bowel disease, familial adenomatous polyposis, or hereditary nonpolyposis colorectal cancer; or active infection such as hepatitis B or C. Patients with a history of cancer were included in the trial only if they were diagnosed as being cured of cancer and unlikely to suffer a relapse for at least 1 y. Ultimately, 108 participants ages 40 to 75 y with ≤5-mm-diameter polyps showing a pit pattern III were enrolled in the trial; the classification of pit patterns was based on Kudo's classification (39).

Participants were randomized (Randomization Center; Japan Clinical Research Support Unit, Tokyo, Japan) and assigned to one of three treatment groups (the placebo group, the 1.5-g bLF group, or the 3.0-g bLF group). Trial participation commenced within 30 d after colonoscopic examination. At commencement, patients underwent their initial trial interviews.

Participants took six tablets orally everyday for 12 mo. One tablet (1.5 g) contained bLF at 0, 250, or 500 mg. In addition, the tablets contained carbohydrate (D-sorbitol, maltitol, and corn starch), but not fat or dietary fiber. The caloric value of six tablets was 36 kcal for all groups. Tablets were designed to be indistinguishable from each other in appearance, smell, and taste. Good compliance was defined as having taken two thirds or more of the tablets prescribed, and poor compliance as having taken less than two thirds of the tablets prescribed. Intake of any product containing bLF was prohibited throughout the entire study period. Participants were verbally instructed to continue with their usual food (especially fat and fiber), alcohol, and supplement intake at trial commencement and 3, 6, and 9 mo, although they were not requested to record their meals during the trial period. Treatment assignments and participant assessments were not revealed to investigators, participants, or the sponsor over the study period.

Colonoscopy

Endoscopists performed initial and final total colonoscopic examinations with zoom colonoscopes (CF-240ZI, PCF-240ZI, and CF-200Z, Olympus). During the initial examination, before the commencement of the trial, all lesions detected by colonoscopy were observed at 100-fold magnification after spraying 0.2% indigo carmine dye over the lesion to easily differentiate polyp from normal mucosa and to more clearly observe the pit patterns of the polyps. Of these, ≤5-mm-diameter polyps with pit pattern III at the most proximal sites of the right colon (cecum to transverse colon) and the left colon (descending colon to rectum) were identified as target lesions. The classification of pit patterns was based on Kudo's classification (39). This classification system assigns colonic lesions into six categories: types I and II are designated nonneoplastic; types IIIS, IIIL, IV, and V are designated neoplastic. Type IIIS shows small tubular or roundish pits; type IIIL shows large tubular or roundish pits. Macroscopically, intramucosal polypoid growths (pedunculated polypoid lesions and sessile and broad-based polypoid lesions) and nonpolypoid growths showing infiltration of tumor cells below the submucosal layer were excluded from being used as target lesions because they would most probably show malignant characteristics (40–43). These lesions and >5-mm-diameter polyps with pit pattern III were removed at the time of detection. A total of 119 lesions in the full analysis set (FAS) population (104 participants) with pit pattern III were identified as target polyps.

Target polyps were photographed and marked by injection of india ink close to the polyp. Target polyp size was estimated using both open biopsy forceps (44–46) and a 5-mm paper disc and open scale forceps (see Supplementary data for a description of the use of the 5-mm paper disc to estimate polyp size). At the end of the trial period, target polyps were identified by location and the presence of the india ink marker. The size and pit pattern of the polyps were measured by endoscopy, and the polyps were excised for examination. (All other premalignant and malignant growths were also removed at this time.) Target polyps were immediately fixed in phosphate-buffered neutral formalin, embedded in paraffin, and stained with H&E.

The Endoscopic Data Adjudication Committee (Drs. Akasu and Gotoda) evaluated the size of the polyps and pit pattern by photographs. Excised lesions were histopathologically diagnosed by a board-certified pathologist.

Assessment of safety

To assess safety, medical health checkups and evaluation of clinical findings through diary writing and patient interviews were done at trial commencement and every 3 mo. Safety analyses were done for all participants who took at least one tablet.

Immunologic parameters

Peripheral blood was collected from participants at trial commencement (before treatment), 3 mo, 6 mo, 9 mo, and 12 mo. Parameters to be measured were selected based on the results of previous experimental studies (12, 14, 15, 47). ELISA, lymphocyte subset (CD4, CD8, CD16, and CD56) measurement, and NK cell activity were measured as described below.

ELISA. Peripheral blood was collected from trial participants as noted above. ELISA for hLF was done as follows. Microtiter plates were coated with a mouse anti-hLF antibody (mouse, clone: 2B8, IgG1, Advanced Immuno Chemical) and incubated at 5°C overnight. To block the wells, 250 μL of 0.5% gelatin in PBS were added to the wells and the plate was incubated at 37°C for 1 h. One hundred microliters of sample were applied to the blocked wells and the plate was incubated at 5°C overnight. Captured hLF was detected using horseradish peroxidase-labeled polyclonal antibodies against hLF (rabbit; Cappel) and visualization was done using *o*-phenylenediamine (Sigma). The minimal detectable concentration of hLF was ~200 pg/mL; the minimal detectable concentration of bLF was >20 μg/mL. An ELISA specific for bLF using a specific monoclonal

antibody against bLF, developed by Morinaga Milk Industry, was done similarly. Briefly, microtiter plates were coated with capture antibody (anti-bLF rabbit polyclonal, Morinaga Milk Industry) and blocked, and then 100- μ L sample was applied. Captured bLF was detected using a biotin-labeled anti-bLF polyclonal antibody (rabbit; Nacalai, Japan) and visualized with horseradish peroxidase-labeled streptavidin (Zymed) and *o*-phenylenediamine. The minimal detectable concentration of bLF was \sim 500 pg/mL; the minimal detectable concentration of hLF was $>$ 20 μ g/mL. An ELISA kit (minimum detection limit, 25.0 pg/mL; Medical & Biological Laboratories Co. Ltd.) was used to measure mature human interleukin-18. Human IFN γ levels were determined by SRL, Inc.

Lymphocyte subsets (CD4, CD8, CD16, and CD56). Peripheral blood was collected from trial participants as noted above. Blood samples were diluted with saline, and lymphocytes were separated from the diluted blood samples using Ficoll-Conray solution (relative density, 1.077; IBL). Fluorescence-activated cell sorting was used to isolate the different lymphocyte subsets. The following antibodies were used for immunofluorescence staining of lymphocytes: two-color antihuman CD4-FITC (T4, Beckman Coulter), CD8-RD1 (T8, Beckman Coulter), CD16-FITC (Becton Dickinson), and CD56-RD1 (Beckman Coulter). Relative fluorescence intensities of single- or two-color staining were then measured with a FACSCalibur (Becton Dickinson).

NK cell activity. Peripheral blood was collected from trial participants and lymphocytes isolated using Ficoll-Conray solution as noted above. The entire lymphocyte population (containing effector cells) was washed twice with PBS. Cell killing activity was then measured using K-562 cells labeled with 51 Cr as target cells. Target cells (1×10^6 /mL, 10 μ L) and effector cells (1×10^6 /mL, 200 μ L) were mixed and incubated at 37°C, 5% CO $_2$ for 3.5 h. The medium was then removed and clarified by centrifugation, and soluble 51 Cr released by killed K-562 cells was measured with a gamma-counter (1470 Wizard, Perkin-Elmer Life and Analytical Sciences).

Polymorphonuclear leukocyte infiltration into target polyps

At the end of the trial period, a final colonoscopic examination was done and target polyps (and all other premalignant and malignant growths found) were removed. Target polyps were immediately fixed in buffered formalin, embedded in paraffin, and stained with H&E, followed by histopathologic examination. Polymorphonuclear leukocytes (PMN) were counted in the stroma of adenomatous polyps. Only polyp sections containing at least five atypical adenoma glands and mucosa propria to a depth equal to at least the average gland diameter (*d*) were used. PMNs in the stroma of five glands and the underlying mucosa propria to depth *d* were counted. The area was measured using an image analysis system (Image Processor for Analytical Pathology, Sumika Technos Corp.).

Statistical analyses

Fifteen participants had two target polyps; these participants were assessed according to the change in the average of the diameter of the two polyps. Polyp assessment was done based on the Response Evaluation Criteria in Solid Tumors (48). Analysis of covariance and Dunnett's multiple comparison test were used to compare the change in polyp size in the LF-treated groups with that in the placebo group, using the initial polyp size as a covariate. The assumption for analysis of covariance was checked, and there was no interaction between baseline and treatment. To check the interaction between treatment outcome and each prognostic factor (age, sex, presence or absence of previous colectomy, and site of target lesion), multiple regression analysis was done with the following four variables: treatment, sum of target-lesion diameters by colonoscopy at trial commencement, factor, and factor-by-treatment interaction. For grouping by age, trial participants were divided into two groups: participants at or below the overall median age of the trial participants (\leq 63) and participants above the overall median age of the trial participants ($>$ 63). For prog-

nostic factors showing significant interaction with treatment outcome, subgroup analyses were done separately in each subgroup population. The data for NK activity and hLF levels were analyzed using Dunnett's test. Pearson coefficients of correlation were calculated to determine degrees of association between different variables. Levels of significance were set at 0.05 (two-sided) for all statistical analyses. All calculations were conducted using SAS version 8.2 (SAS Institute).

Results

Disposition of subjects

The trial profile is shown in Supplementary Fig. S1 and detailed in Materials and Methods. Briefly, over the course of \sim 3 years (February 2002 to January 2005), patients scheduled for a colonoscopic examination were approached before their examinations and invited to join the trial. Each of the 215 patients who agreed to join the trial provided written informed consent and underwent their scheduled examination. During this examination, potential target polyps were marked and photographed for further evaluation by the Endoscopic Data Adjudication Committee. Each of these patients also underwent a pre-trial interview to determine their suitability for inclusion in the trial. For each trial participant, trial commencement began within 30 days after their initial colonoscopic examination.

Of the 215 patients who initially agreed to join the trial, 108 patients met the trial criteria and agreed to continue with the trial. These 108 patients were each enrolled and randomized into one of three treatment groups (the placebo group, the 1.5-g bLF group, or the 3.0-g bLF group). After randomization into treatment groups, two participants originally enrolled into the trial were excluded: One participant assigned to the placebo group did not have a target polyp, as judged by the Endoscopic Data Adjudication Committee after further evaluation of the photographs taken during the initial colonoscopic examination, and during the initial trial interview, one participant assigned to the 3.0-g bLF group was found to have used statins. Therefore, the initial trial population consisted of 106 participants. Table 1 shows the characteristics of this population at trial commencement. The overall age (mean \pm SD) was 62.4 ± 6.9 years. Eighty-seven subjects (82.1%) were men. A total of 121 polyps with pit pattern III were identified by colonoscopy in this population, and the estimated diameter (mean \pm SD) was 3.5 ± 0.9 mm. All those ($n = 27$) with a history of colectomy had undergone it as a result of colorectal cancer.

Two participants withdrew from the trial after commencement (both in the placebo group). The FAS population for the trial, therefore, consisted of 104 participants. The per-protocol set (PPS) population included 102 participants after exclusion of two from the FAS population who used NSAIDs (both in the 3.0-g bLF group). The FAS population was used to analyze the effects of bLF treatment on target polyp size, and the PPS population was used to analyze the effects of bLF treatment on immunologic parameters; see Supplementary data for a brief explanation of our use of the FAS and PPS population data.

Compliance

In the FAS population ($n = 104$), tablet intake rates (mean \pm SD) were $92.1 \pm 9.0\%$ in the placebo group ($n = 33$), $94.3 \pm 5.0\%$ in the 1.5-g bLF group ($n = 37$), and $92.1 \pm 9.3\%$ in the 3.0-g

Table 1. Characteristics of the initial trial population at trial commencement

	Placebo group (n = 35)	1.5-g bLF group (n = 37)	3.0-g bLF group (n = 34)
Age (y), mean ± SD	63.0 ± 6.4	61.4 ± 7.3	63.0 ± 6.8
Male, n (%)	29 (82.9)	29 (78.4)	29 (85.3)
Height (cm), mean ± SD	165.4 ± 7.4	164.5 ± 7.0	164.4 ± 7.8
Weight (kg), mean ± SD	64.2 ± 7.6	65.9 ± 11.9	63.9 ± 9.0
Previous colectomy, n (%)	10 (28.6)	8 (21.6)	9 (26.5)
Alcohol consumption, n (%)	26 (74.3)	28 (75.7)	26 (76.5)
Smoking, n (%)	5 (14.3)	13 (35.1)	8 (23.5)
Target-lesion diameters (mm) by colonoscopy, mean ± SD	4.0 ± 1.4	3.8 ± 1.4	4.1 ± 1.7
Site of target lesions by colonoscopy			
Right colon, n (%)	23 (65.7)	23 (62.2)	20 (58.8)
Left colon, n (%)	7 (20.0)	9 (24.3)	9 (26.5)
Right and left colon, n (%)	5 (14.3)	5 (13.5)	5 (14.7)

bLF group ($n = 34$). There was poor compliance (as defined in Materials and Methods) from two participants: one participant of two withdrawing consent from the placebo group (tablet intake rate, 54.6%) and one participant of two excluded from the 3.0-g bLF group due to NSAID use (tablet intake rate, 51.7%).

Pit patterns and histologic diagnosis

A total of 119 polyps in the FAS population with pit pattern III were identified by colonoscopy as target polyps. The pit pattern of two (both in the 3.0-g bLF group) changed into pit pattern I (regular round crypts, normal mucosa) at 12 months. All the others showed pit pattern III. Of these lesions, 91 were histologically diagnosed: 31 in the placebo group, 30 in the 1.5-g bLF group, and 30 in the 3.0-g bLF group. (Twenty-eight polyps were used up during RNA extraction; the analysis of the RNA is ongoing and not part of this report.) Eighty-nine (97.8%) were adenomas and two were hyperplasias (both in the 1.5-g bLF group), verifying our identification of polyps with pit pattern III as a predictor of adenoma.

Safety

One participant in each of the three groups had an adverse event for which a causal relationship with tablets could not be determined: A mild decrease in triacylglycerol levels was observed in a participant in the placebo group; a mild increase in alkaline phosphatase levels was observed in a participant in the 1.5-g bLF group; and a moderate increase in total bilirubin levels (with a mild increase observed at trial commencement) was observed in a participant in the 3.0-g bLF group. Levels of alkaline phosphatase and total bilirubin spontaneously returned to normal after the end of the study treatment. In the 3.0-g bLF group, lung metastases from colorectal cancer were observed in one participant and liver metastases from colorectal cancer were observed in a second participant (both participants had a history of colon cancer). Because both were diagnosed with metastases within 1 week before the end of the treatment and were found to have no laboratory abnormalities associated with them, the treatment was continued and completed. No other serious adverse events occurred during this study, and the safety of the treatment was confirmed.

Efficacy of bLF treatment on polyp size

A comparison of the change in polyp size among treatment groups in the FAS population is shown in Table 2. Although the differences between LF-treated groups and the placebo group were not significant ($P = 0.098$), some reduction in polyp diameter in the 3.0-g bLF group (-0.2 mm, 4.9% regression) was observed, whereas an increment in polyp size was observed in the placebo group ($+0.2$ mm, 5.0% increase). Similar results were obtained in the analysis of the PPS population (Table 2).

A comparison of the change in polyp size among groups and in subgroups (age or sex) is shown in Table 2. Multiple regression analysis revealed both age-by-treatment ($P = 0.034$) and sex-by-treatment ($P = 0.043$) interactions. Significant retardation of target polyp diameter was found in participants ≤ 63 years of age ingesting 3.0 g of bLF per day ($P = 0.006$; Table 2) and possibly in female participants ingesting 3.0 g of bLF per day ($P = 0.019$; Table 2). The number of female participants, however, was small; therefore, whereas the retardation of target polyp diameter was statistically significant, the effect of bLF in women needs to be confirmed in trials with a larger number of female participants.

The number and site of target lesions and the presence or absence of previous colectomy showed nonsignificant interaction with treatment outcome ($P = 0.39$ and $P = 0.57$, respectively).

Efficacy of bLF treatment on immunologic parameters

The effect of bLF ingestion on polyp size was equivalent in the FAS and PPS populations (Supplementary Table S1); therefore, the PPS population was used to examine the effect of bLF treatment on immunologic parameters (see Supplementary data for a brief explanation of our use of the FAS and PPS population data).

Ingestion of 3.0-g bLF resulted in significantly elevated serum hLF levels ($P < 0.001$; Fig. 1A), suggesting that ingestion of 3.0-g bLF affected the immune system. Multiple regression analysis revealed age-by-treatment interaction on serum levels of hLF ($P < 0.001$). As with the effect of bLF on polyp size, bLF significantly affected serum levels of hLF only in participants ≤ 63 years of age. In the ≤ 63 years age subgroup ($n = 54$),

serum hLF levels changed by -1.63 ± 10.69 ng/mL in the placebo group and by 25.43 ± 19.35 ng/mL in the 3.0-g bLF group ($P < 0.001$); in the ≥ 64 years age subgroup ($n = 48$), serum hLF levels changed by -3.18 ± 5.69 ng/mL in the placebo group and by 5.25 ± 14.16 ng/mL in the 3.0-g bLF group ($P = 0.079$). Analysis of serum hLF levels in participants ingesting 3.0-g bLF revealed that in this group, induction of hLF weakened with aging ($r = -0.642$, $P < 0.001$; Fig. 1B). Serum levels of (ingested) bLF were below the limit of detection in all groups (data not shown).

Ingestion of 1.5-g bLF increased NK cell activity ($P = 0.048$; Fig. 2); however, the increase in NK cell activity in participants ingesting 3.0-g bLF did not attain statistical significance ($P = 0.058$). There was no age-by-treatment interaction on NK cell activity ($P = 0.911$).

For all other immunologic parameters measured, no differences between LF-treated groups and the placebo group were observed (data not shown).

Changes in polyp size and possible associated factors

As noted above, the effect of bLF ingestion on polyp size was equivalent in the FAS and PPS populations. Therefore, the PPS population was used to examine correlations between changes in target polyp size and NK cell activity, serum hLF levels, and PMN infiltration into target polyps.

Participants with growth-retarded polyps had significantly higher NK cell activity compared with participants with growing polyps ($P = 0.037$; Supplementary Fig. S2A). In addition, increased NK cell activity correlated with increases in the CD16⁺/CD56⁺ subset of NK cells in the blood ($r = 0.371$,

Table 2. Analysis of the growth of polyps by colonoscopy

Variable	Baseline,* mm \pm SD	Change, [†] mm \pm SD (%)	95% CI, [‡] mm	P [§]
Full analysis set ($n = 104$)				
Placebo group ($n = 33$)	4.0 \pm 1.4	0.2 \pm 0.8 (5.0)		
1.5-g bLF group ($n = 37$)	3.8 \pm 1.4	0.1 \pm 0.8 (2.6)	-0.70, 0.26	0.490
3.0-g bLF group ($n = 34$)	4.1 \pm 1.7	-0.2 \pm 1.3 (-4.9)	-0.91, 0.07	0.098
Per-protocol set ($n = 102$)				
Placebo group ($n = 33$)	4.0 \pm 1.4	0.2 \pm 0.8 (5.0)		
1.5-g bLF group ($n = 37$)	3.8 \pm 1.4	0.1 \pm 0.8 (2.6)	-0.70, 0.26	0.500
3.0-g bLF group ($n = 32$)	4.1 \pm 1.8	-0.2 \pm 1.3 (-4.9)	-0.95, 0.05	0.081
Subgroup				
Age ($n = 104$)				
≤ 63 -year-old ($n = 55$) [¶]				
Placebo group ($n = 18$)	3.9 \pm 1.5	0.5 \pm 0.8 (12.8)		
1.5-g bLF group ($n = 21$)	4.0 \pm 1.6	-0.1 \pm 0.5 (-2.5)	-1.08, 0.03	0.066
3.0-g bLF group ($n = 16$)	4.0 \pm 1.7	-0.4 \pm 1.3 (-10)	-1.41, -0.22	0.006
≥ 64 -year-old ($n = 49$)				
Placebo group ($n = 15$)	4.1 \pm 1.3	-0.1 \pm 0.8 (-2.4)		
1.5-g bLF group ($n = 16$)	3.5 \pm 1.2	0.3 \pm 1.0 (8.6)	-0.66, 1.02	0.840
3.0-g bLF group ($n = 18$)	4.1 \pm 1.8	-0.1 \pm 1.3 (-2.4)	-0.82, 0.80	>0.950
Sex ($n = 104$)				
Male ($n = 85$)				
Placebo group ($n = 27$)	3.9 \pm 1.4	0.2 \pm 0.8 (5.1)		
1.5-g bLF group ($n = 29$)	3.9 \pm 1.5	0.1 \pm 0.9 (2.6)	-0.66, 0.39	0.790
3.0-g bLF group ($n = 29$)	4.1 \pm 1.9	-0.1 \pm 1.2 (-2.4)	-0.72, 0.33	0.600
Female ($n = 19$)				
Placebo group ($n = 6$)	4.6 \pm 1.2	0.3 \pm 0.9 (6.5)		
1.5-g bLF group ($n = 8$)	3.4 \pm 0.6	0.1 \pm 0.4 (2.9)	-2.06, 0.73	0.410
3.0-g bLF group ($n = 5$)	3.7 \pm 0.5	-1.1 \pm 1.4 (-29.7)	-3.10, -0.29	0.019

Abbreviation: 95% CI, 95% confidence interval.

*Baseline refers to the polyp measurements obtained during the initial examination.

[†]Difference between initial polyp measurements (baseline) and month 12 values. Percentages show the ratio of mean change to mean baseline values.

[‡]95% CI with Dunnett's adjustment using the baseline value as a covariate.

[§]P values versus placebo, calculated by Dunnett's test using the baseline value as a covariate.

^{||}Two participants in the placebo group who withdrew from the trial after commencement were excluded from the full analysis set population. As a result, 104 participants were analyzed.

[¶]The overall median age of the trial participants is 63 y.

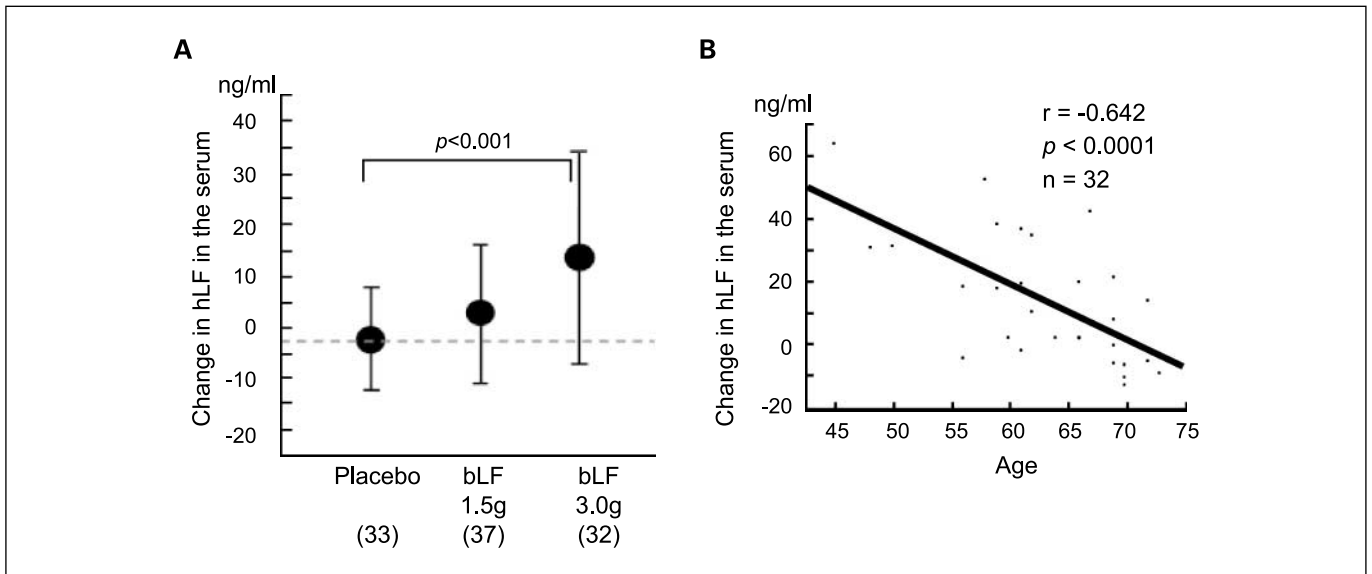


Fig. 1. Serum levels of hLF were measured every 3 mo during the trial period. Changes in serum hLF over the course of the trial period ($t = 0$ to $t = 12$ mo) in the PPS population ($n = 102$) are shown (see Supplementary data for a description of the PPS population). **A**, effect of bLF ingestion on serum levels of hLF in the PPS population. **B**, correlation between age and hLF induction in PPS members ingesting 3.0-g bLF. The numbers in parentheses indicate the number of participants in the separate groups. Bars, SD.

$P < 0.001$; Supplementary Fig. S2B). Our data suggest that higher NK cell activity may be associated with retardation of target polyp growth.

Participants with growth-retarded polyps had higher levels of hLF in their serum than participants with growing polyps ($r = -0.279$, $P = 0.005$; Fig. 3A). Our data suggest a possible negative correlation between serum hLF levels and polyp growth; however, because bLF ingestion affects both polyp growth and serum hLF in the ≤ 63 years age subgroup, the

association between hLF and polyp growth is currently unknown.

Growth-retarded polyps contained a lower density of PMNs compared with faster-growing polyps. A total of 91 target polyps were histologically diagnosed, and the density of PMNs in the stroma surrounding the target polyps could be determined in 88 of these polyps, defining a subgroup referred to as the PPS population with countable PMNs (PCP). In the PCP subgroup, the stroma surrounding growth-retarded polyps contained a lower density of PMNs than the stroma surrounding growing polyps ($r = 0.440$, $P < 0.001$; Fig. 3B). There was no statistically significant age-by-treatment interaction on PMN infiltration into target polyps ($n = 88$, $P = 0.819$).

Finally, PCP subgroup participants in whom ingestion of bLF resulted in induction of serum hLF levels by more than 5 ng/mL had a lower density of PMNs in the stroma surrounding the target polyps than PCP subgroup participants in whom ingestion of bLF resulted in induction of serum LF levels by 5 ng/mL or less ($P = 0.021$; Fig. 3C). These results suggest a possible negative correlation between serum levels of hLF and PMN infiltration into the stroma surrounding adenomatous polyps; however, because the variables being measured were not independent, the actual association between hLF and PMN infiltration into the target polyp is currently unknown.

Discussion

Diet and dietary supplements are factors in colorectal cancers (7–10). When used as a dietary supplement, bLF isolated from cow milk decreases the incidence of both colorectal cancer and aberrant crypt foci in animal models (12, 14, 15). We conducted a randomized, double-blind, controlled trial with patients ages 40 to 75 years with ≤ 5 -mm-diameter adenomatous colorectal polyps to determine whether supplementation of bLF to the human diet had an effect on these

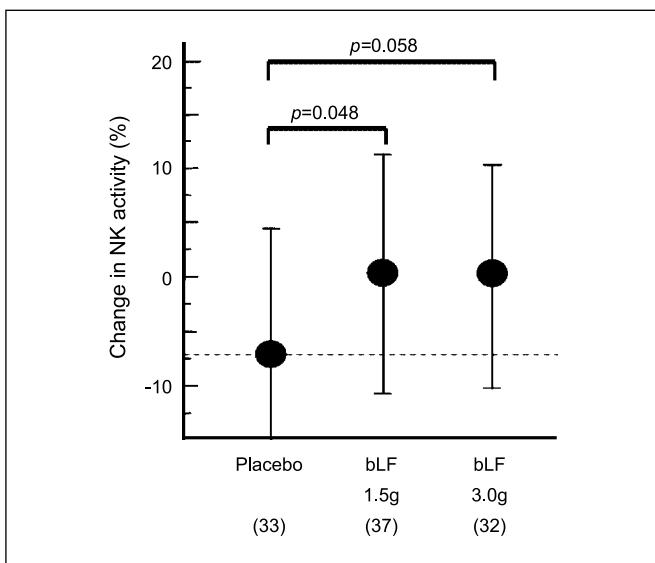


Fig. 2. NK cell activity in the serum was measured every 3 mo during the trial period. Changes in NK cell activity over the course of the trial period ($t = 0$ to $t = 12$ mo) in the PPS population ($n = 102$) are shown (see Supplementary data for a description of the PPS population). The numbers in parentheses indicate the number of participants in the separate groups. Bars, SD.

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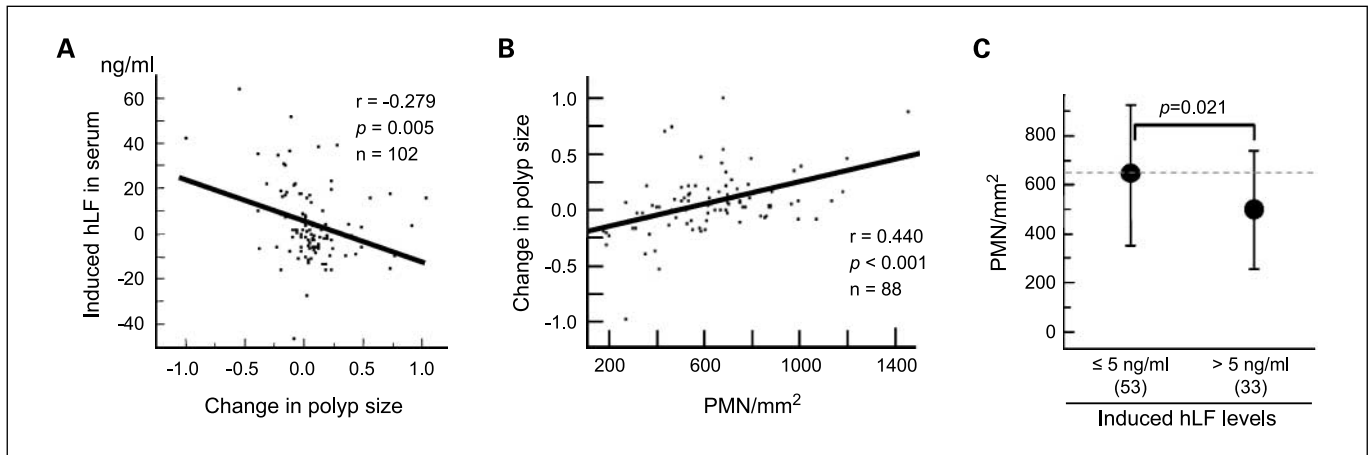


Fig. 3. Comparison of the change in target polyp size, change in serum levels of hLF, and the density of PMNs in the stroma of the target polyps. **A**, changes in serum hLF over the course of the trial period ($t = 0$ to $t = 12$ mo) in the PPS population ($n = 102$) were determined (see Supplementary data for a description of PPS) and the change in hLF levels was compared with the change in the size of the targets polyps in these participants. **B**, a total of 91 target polyps from the PPS population were histologically diagnosed. The density of PMNs in the stroma surrounding the target polyps could be determined in 88 of these polyps; this defines a PPS subgroup referred to as the PPS population with countable PMNs (PCP) subgroup. The change in polyp size was compared with the density of PMNs at the target polyp in the PCP subgroup. **C**, PMN counts at the target polyps of PCP members in whom ingestion of bLF resulted in induction of serum hLF by 5 ng/mL or less and participants in whom ingestion of bLF resulted in induction of serum hLF by more than 5 ng/mL. Relative change in polyp size (**A** and **B**) was determined as $[(\text{size at the end of the trial}/\text{initial size}) - 1]$; this method normalizes the changes in polyp sizes, with negative values representing regressing polyps and positive values representing growing polyps. In **C**, 5 ng/mL was the mean change in serum hLF levels among the participants during the course of this study

(i.e., $\frac{\sum_{i=1}^n (\text{hLF}_{\text{after}} - \text{hLF}_{\text{before}})_i}{n} = 5 \text{ ng/mL}$). The numbers in parentheses indicate the number of participants in the separate groups. Bars, SD.

polyps. Participants were assigned to receive placebo, 1.5-g bLF, or 3.0-g bLF daily for 12 months. In sum, a 1-year oral intake of 3.0 g of bLF per day induced statistically significant retardation of colorectal adenomatous polyp size in participants 63 years old or younger.

The beneficial effect of bLF may be more prominent in women than in men (see Table 2). However, because of the small number of women taking part in the present study, the retardation of polyp size in the 3.0-g bLF group versus the placebo group, although statistically significant, is not a reliable finding. Further studies with an increased number of participants are warranted.

The exact mechanisms by which bLF affects colorectal polyps in humans were not directly addressed in this clinical trial. One possibility is that oral intake of bLF affects human immunologic activities, and that this in turn retards polyp growth; immune modulation mediated by oral administration of bLF has been shown in animal models (12, 22, 47, 49, 50). Notably, at the time of writing of this article, a study sponsored by the National Cancer Institute⁸ is recruiting participants to examine the effects of talactoferrin, a recombinant form of hLF that has been successfully tested in phase II clinical trials with patients with refractory metastatic renal cell carcinoma (51), on the immune system and its effectiveness in treating non-small cell lung cancer.

Intake of 3.0 g of bLF increased serum hLF levels (Fig. 1A). Because hLF is an important component of the innate immune system (23, 28–31), the increase in hLF levels suggests that ingestion of bLF did affect the immune system. Importantly, increased serum hLF correlated well with retardation of polyp size (Fig. 3A). However, whereas ingestion of bLF induced serum hLF levels and serum hLF levels correlated with retar-

dation of polyp size, a statistically significant association between bLF ingestion and retardation of polyp size was obtained only in participants 63 years old or younger. Intriguingly, the ability of bLF ingestion to elevate serum hLF weakened with aging (Fig. 1B and C), suggesting that the effect of bLF on immune function may weaken with age, and this weakening may be the reason that bLF inhibition of adenomatous polyp growth seems to be age dependent.

The mechanisms by which ingested bLF exerts its effects on the immune system are currently being studied in several laboratories (see ref. 23). Possibly, ingested bLF peptides interact with gut-associated lymphoid tissue. Several pathways have been proposed, but the exact cell types and the receptors with which ingested bLF interacts remain undefined.

Another consideration is that LF is an iron chelator (23), and the bLF used in this study was approximately 10% to 20% iron saturated. Consequently, this bLF had a high iron chelating ability. Tumor cells, like all cells, require iron, and removal of iron from the tumor environment results in regression of tumor growth (52, 53). However, the ability of bLF peptides to chelate iron after passage of the bLF-containing tablets through the stomach and small intestine has not been determined, and due to the nature of this trial, we were unable to measure iron levels in the target polyp environment. Therefore, the effect, if any, of the iron chelating ability of bLF on target polyp growth is unknown, but it is a possible factor.

LF is found in a variety of exocrine secretions (e.g., tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, bile, cervicovaginal mucus, and seminal fluid); it is also a major component of the secondary granules of neutrophils and is released by activated neutrophils (see ref. 23). Therefore, should ingested bLF cause activation of neutrophils, this would result in elevated serum hLF levels. Due to the nature of this study, however, we could not directly measure neutrophil activity in the serum samples obtained

⁸ Available from: <http://ClinicalTrials.gov>; identifier NCT00923741.

from the participants in the trial. Consequently, bLF-mediated induction of serum hLF levels via activation of neutrophils is hypothetical, and it remains a possibility that induction of serum hLF proceeds wholly or in part by other routes.

The induction of serum hLF is most likely due to the effect of ingested bLF on the immune system. Whether serum hLF itself caused regression of polyp growth or was simply a consequence of bLF treatment is not known, and the exact relationship between tumor growth and serum hLF levels was not elucidated in this study. The major difficulty in determining whether serum hLF affects polyp growth is the lack of knowledge of possible mechanisms by which serum hLF could affect polyp growth.

NK cells are a principal effector of immunosurveillance against tumors (54). The effect of bLF on NK cell activity in this study was inconsistent. There was a tendency for NK cell activity to increase in participants ingesting bLF, and the increases were statistically significant in participants ingesting 1.5 g of bLF per day, but not in participants ingesting 3.0 g of bLF. Importantly, participants with growth-retarded polyps had higher NK cell activity than participants with growing polyps. Therefore, whereas this study did not establish a relationship between ingestion of bLF and NK cell activity, it remains a reasonable possibility that ingestion of bLF is associated with increased NK cell activity, and that this increased NK cell activity may have retarded polyp growth. A second trial with an increased number of participants extended over a longer period of time is needed to resolve these points.

Infiltration of PMNs into a tumor site can enhance tumor growth (32–35). In this study, participants with higher induction of serum hLF levels had both retarded polyp growth (Fig. 3A) and a lower density of PMNs in the stroma surrounding their target polyps (Fig. 3C). In rodents, oral administration of recombinant human LF attenuates neutrophil migration to the intestine (36). Therefore, in our study, bLF acting directly in the colon or possibly through serum hLF may have inhibited PMN infiltration into the target area.

In summary, ingestion of bLF had two significant effects: First, it resulted in regression of polyp growth in participants ≤ 63 years of age; second, it resulted in increased serum hLF levels in participants ≤ 63 years of age. In addition, induction of serum hLF was statistically associated with decreased infiltration of PMNs into the target area, and decreased infiltration of PMNs into the target area correlated with decreased polyp growth. Finally, enhanced NK cell activity was associated with decreased polyp growth, and there was a tendency (not con-

sistent statistically) for NK cell activity to be enhanced in participants ingesting bLF. Taken together, our findings suggest that ingested bLF inhibits the growth of adenomatous colon polyps and that this inhibition proceeds via bLF modulation of immune system function.

Colonoscopy with clearing of neoplasms by polypectomy significantly reduces colorectal cancer; however, colorectal cancer incidence after clearing colonoscopy is appreciable (55). Factors considered to be involved in colorectal cancer that arise after clearing colonoscopy include detection failures during colonoscopy and incomplete polyp extraction (55, 56). Agents associated with retardation of adenomas are likely to reduce colorectal cancer risk because the cumulative incidences of progression from ≥ 10 -mm-diameter polyps to cancer at 5, 10, and 20 years are reported to be 2.5%, 8%, and 24%, respectively (57), significantly higher than those for 2- to 5-mm-diameter polyps (58). Therefore, a supplement effective in the retardation of polyp growth would be a clinically useful adjunct to colorectal polyp extraction. Cyclooxygenase-2 inhibitors have been shown both to decrease the incidence of sporadic colorectal polyps (38) and to induce regression of colorectal polyps already present (44, 45); however, these drugs can have severe adverse effects (38, 44). Similarly, NSAIDs can be beneficial, but because of possible severe adverse effects, the U.S. Preventive Services Task Force recommends against their routine use to prevent colorectal cancer in individuals at average risk (59, 60). bLF is well tolerated in preclinical (61) and clinical research studies (16, 17), and no severe adverse effects related to bLF were observed in the present trial. Our study suggests that daily intake of 3.0 g of bLF could be a useful adjunct to colorectal polyp extraction.

Disclosure of Potential Conflicts of Interest

T. Kozu, G. Iinuma, Y. Saito, T. Akasu, D. Saito, and T. Kakizoe report receiving funding from the Morinaga Milk Industry Co. Ltd. in accordance with the provisions of their respective institutions; Y. Ohashi reports receiving consultancy fees from Morinaga Milk Industry. D.B. Alexander, M. Iigo, and H. Tsuda report no potential conflict of interest.

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