Available online at www.sciencedirect.com
ScienceDirect
Nutrition Research 28 (2008) 583–589
Nutrition Research
www.nrjournal.com

Bovine lactoferrin supplementation supports immune and antioxidant status in healthy human males

Ann M. Mulder[a], Paul A. Connellan[a], Christopher J. Oliver[b], Carol A. Morris[a,*], Lesley M. Stevenson[a]

[a]*Center for Phytochemistry and Pharmacology, Southern Cross University, Lismore, NSW 2480, Australia*
[b]*Blackmores Research Office, Southern Cross University, Lismore, NSW 2480, Australia*

Received 27 March 2008; revised 16 May 2008; accepted 16 May 2008

Abstract

Dietary supplements of bovine lactoferrin are purported in consumer literature to enhance and support the immune system response through their antioxidant, antibacterial, and antiviral properties. Our aim was to investigate more fully the potential immune modulating properties and antioxidant activity of an oral supplementation of bovine lactoferrin in humans. Using an intraindividual repeated measure design, 8 healthy males aged 30 to 55 years, self-administered daily for 21 days, one capsule of placebo for 7 days, followed by 100 mg of lactoferrin for 7 days, followed by 200 mg of lactoferrin for 7 days. Peripheral blood lymphocyte subset counts, T-cell activation, natural killer (NK) cell cytotoxicity, serum cytokine levels (tumor necrosis factor [TNF]-α, interferon [IFN]-γ, interleukin [IL]-2, IL-4, IL-6, and IL-10), and serum hydrophilic, lipophilic, and total antioxidant capacity were repeatedly measured before and after each progressive supplementation. Statistically significant increases were found between presupplementation levels and levels after 200 mg of supplementation in total T-cell activation (as measure by $CD3^+$) ($P < .001$), helper T-cell activation (as measure by $CD4^+$) ($P < .001$), cytotoxic T-cell activation (as measured by $CD8^+$) ($P < .001$), and hydrophilic antioxidant capacity ($P < .05$). No significant changes were seen in the other parameters measured. These results support the proposal that oral supplements of bovine lactoferrin may be a useful adjunct toward modulation of immune activity, in particular T-cell activation and antioxidant status.

Keywords: Lactoferrin; T-lymphocytes; Immunology; Antioxidants; Dietary supplements; Humans

Abbreviations: AAPH, 2,2′,Azobis(2-amidino-propane) dihydrochloride; AC, antioxidant capacity; APC, allophycocyanin; BMI, body mass index; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; IFN, interferon; NK, natural killer; $ORAC_{FL}$, oxygen radical absorbance capacity (fluorescent); PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; PCA, perchloric acid; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PHA, phytohemagglutinin; TE, trolox equivalents; TNF-α, tumor necrosis factor α.

1. Introduction

Lactoferrin is a protein found in many body fluids including colostrum, milk [1], tears, nasal secretions, saliva, vaginal secretions, and seminal fluid [2]. These fluids interface with the body's external environment and play an important role in the body's innate immune system; they are the first-line of host defense. In addition, lactoferrin is produced in high levels in neutrophils [3]. A number of physiologic bioactive functions have been ascribed to lactoferrin including antibacterial, antifungal, antiviral [4], antiparasitic [5], antitumor activity [6], immunomodulatory effects [3,7], and regulation of iron absorption during

* Corresponding author.
E-mail address: carol.morris@scu.edu.au (C.A. Morris).

0271-5317/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.nutres.2008.05.007

inflammation and infection [8]. It is the ability to sequester free ferric ions that gives lactoferrin its potential antioxidant properties [1]. Numerous in vitro studies demonstrate lactoferrin's activation and support of the immune process. In vivo studies on mice reinforce bovine lactoferrin's immune-modulating effects against a number of diseases involving bacterial and fungal infections, including *Escherichia coli* [9], *Helicobacter pylori* [10], *Candida albicans* [11], and in inflammation [12] and cancer [13]. In addition, a small number of clinical trials have supported bovine lactoferrin as an immune modulator through the demonstration of increased phagocytic activity of polymorphonuclear leukocytes [14], decreased production of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in cell cultures [15] and supporting eradication of *Helicobacter pylori* [16], *Trichophyton rubrum* [17], and treatment of hepatitis C [18].

On this evidence, bovine lactoferrin supplements are reported to have the ability to support the immune system and influence immune cell activity potentially via these antioxidant, antibacterial, and antiviral properties.

The aim of this study was, therefore, to investigate changes in immune and antioxidant status, in particular lymphocyte subset counts, natural killer (NK) cell cytotoxicity, T-cell activation, serum cytokines IL-2, TNF-α, interferon (IFN)-γ, IL-4, IL-6, IL-10, and both hydrophilic and lipophilic antioxidant status. The hypothesis of this study is that either or both, 100 mg and 200 mg of bovine lactoferrin supplements will enhance immune and antioxidant status in human males.

2. Methods and materials

2.1. Study participants

Eight healthy male volunteers, mean age of 40 years (range, 31-52 years), were recruited from Southern Cross University (SCU, Lismore, NSW, Australia). Participants were healthy nonsmokers and ceased all medications including vitamin, mineral, and herbal supplements for 14 days before and during the study period. The study excluded those participants with immune or autoimmune disorders, diabetes, or those on any medication. Potential participants then underwent a clinical health assessment that included medical history collection, blood pressure, heart rate, and body mass index (BMI) measurements, and blood safety parameter determination; full blood count, liver function test, urea electrolytes and creatinine levels, and C-reactive protein. All procedures and processes were approved by the SCU Human Research Ethics Committee and the University of Queensland Medical Research Ethics Committee (Brisbane, QLD, Australia). Participants were fully informed, and written consent was obtained from each subject.

2.2. Study design

The trial design was an intraindividual, repeated measure, dose-response study. It involved one group of 8 participants who all simultaneously underwent 3 successive treatments—placebo, 100 mg of lactoferrin, and 200 mg of lactoferrin—for a period of 21 days. All participants commenced with 1 capsule daily of a placebo for 7 days (days 0-6), followed by 1 capsule daily of 100 mg of lactoferrin for 7 days (days 7-13), followed by 1 capsule daily of 200 mg of lactoferrin for 7 days (days 14-20). Measurement of immune and antioxidant parameters were performed at each visit (ie, on days 0, 7, 9, 14, 16, and 21).

2.3. Study intervention

The dosage of both placebo and lactoferrin was 1 capsule per day, self-administered with breakfast each morning. Placebo capsules contained 200 mg of calcium phosphate; 100 mg lactoferrin capsules contained 100 mg of bovine lactoferrin and 100 mg of calcium phosphate; and 200 mg lactoferrin capsules contained 200 mg bovine lactoferrin.

2.4. Study outcomes

Primary outcome measures included in vivo changes in lymphocyte subset counts including total ($CD3^+$) T cells, helper ($CD4^+$) T cells, cytotoxic ($CD8^+$) T cells, total NK cells ($CD3^-$, $CD16^+$, and/or $CD56^+$), and total B cells ($CD19^+$); and ex vivo changes in nonspecific immune parameters including NK cell cytotoxicity. Secondary outcome measures included ex vivo changes in specific immune parameters including T-cell activation; in vivo changes in cytokine IL-2, TNF-α, and IFN-γ levels that predominantly regulate a cell-mediated immune response and IL-4, IL-6, and IL-10 levels that predominantly regulate antibody-mediated responses; and in vivo changes in hydrophilic and lipophilic antioxidant status.

2.5. Blood collection

Blood collection for both blood safety parameters, full blood count, liver function test, urea electrolyte and creatinine levels, and C-reactive protein and blood study outcome parameters were performed on days 0, 7, 9, 14, 16, and 21 after a 12-hour overnight fast. Samples for safety parameters were analyzed according to standard diagnostic laboratory procedures at the Northern Rivers Pathology Service, Lismore Base Hospital, Lismore, NSW, Australia. Samples for study outcome parameters were analyzed at the Center for Phytochemistry and Pharmacology, SCU, Lismore, NSW, Australia.

2.6. Immune parameter measurements

2.6.1. Lymphocyte subsets

Five milliliters of blood was collected in an EDTA tube, stored at room temperature (RT), and analyzed within 24 hours of collection. Flow cytometric analysis was used for monitoring the expression of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $CD16^+$, and $CD56^+$ antigens on peripheral blood lymphocytes (PBL). Staining of PBL was performed by

the Lyse/No Wash method using MultiTest IMK kit reagents (catalogue no. 340503, Becton Dickinson, San Jose, Calif). Briefly, 50 μL of whole blood (EDTA) was added to 20 μL of both fluorochrome-conjugated monoclonal antibodies (anti-CD3fluorescein isothiocyanate (FITC)/CD8phycoerythrin (PE)/CD45peridinin chlorophyll protein (PerCP)/CD4allophycocyanin (APC) and anti-CD3FITC/CD(16+56)PE/CD45PerCP/CD19APC). Tubes were vortexed and incubated for 15 minutes in the dark at RT. Four hundred fifty microliters of 1 × MultiTest lysing solution was added to each tube, and tubes were vortexed and incubated for 15 minutes in the dark at RT. Analysis was carried out on a fluorescence activated cell sorter (FACS) Calibur 4-color flow cytometer and MultiSet software (Becton Dickinson), using excitation wavelengths of 488 nm and 635 nm.

2.6.2. T-cell activation

Four milliliters of blood was collected in a lithium heparin vacutainer tube, stored at RT, and analyzed within 24 hours of collection. Lymphocyte activation was measured on whole blood according to the expression of an early activation marker $CD69^+$ in T cells stimulated with phytohemagglutinin (PHA-M). This was achieved by using FastImmune T-cell Value Bundle II (catalogue no. 340408, Becton Dickinson) and flow cytometry to determine the population of activated T cells. Briefly, 5 μL of 10 μg/mL PHA-M *Phaseolus vulgaris* (Sigma L2646, St Louis, MO) was added to 500 μL of whole blood and incubated for 4 hours at 37°C (5% CO_2). Unstimulated controls were run in parallel for each subject. Aliquots of 50 μL of preincubated blood were then added to 10 μL of fluorochrome-labeled monoclonal antibodies (anti-γ1FITC/γ1PE/CD3PerCP, anti-CD4FITC/CD69PE/CD3PerCP, and anti-CD8FITC/CD69PE/CD3PerCP) in 3 separate tubes. Tubes were mixed and incubated for 15 minutes at RT in the dark. Four hundred fifty microliters of 1 × FACS lysing solution was added to each tube; tubes were vortexed and incubated for 15 minutes in the dark at RT. The percentage of T cells (total T cells, $CD4^+$ T cells, and $CD8^+$ T cells) expressing $CD69^+$ was then analyzed using a 4-color FACS Calibur flow cytometer and CellQuest Pro software (Becton Dickinson).

2.6.3. Natural killer cell cytotoxicity

Four milliliters of blood was collected in a lithium heparin vacutainer tube, stored at RT, and analyzed within 12 hours of collection. Peripheral blood mononuclear cells (PBMCs) were prepared from the whole blood using Isopaque-Ficoll (Amersham Biosciences, Uppsala, Sweden). The PBMCs (effectors) were incubated at 37°C for 2 hours with K562 target cells (ATCC [American Type Culture Collection], Manassas, Va) at a ratio of 25:1 (effector-target). The target cells were prelabeled with a green fluorescent dye, DiO (V-22886, Molecular Probes, Eugene, Ore), to allow precise differentiation from the effector cells. After incubation, a red fluorescent DNA dye, propidium iodide (P-3566; Molecular Probes, Eugene), was added to label the target cells permeabilized by NK cell activity. A target cell control was also run to monitor spontaneous target cell death. The percentage of dead target cells was determined by flow cytometry using a 4-color FACS Calibur flow cytometer and CellQuest Pro software. Percentage of specific cytotoxicity was determined by subtracting the percentage of dead cells in the target control tube from the percentage of dead target cells in each test sample.

2.6.4. Cytokines

Five milliliters of blood was collected in a serum separator tube. The blood was allowed to stand at RT for at least 30 minutes then centrifuged at 1500 relative centrifugal force (rcf) for 10 minutes. The serum was removed and placed in a sealed 1.5 mL polypropylene tube and stored at −80°C. The samples were thawed and mixed thoroughly immediately before testing. Serum cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α were measured using Th1/Th2 cytometric bead array kit (catalogue no. 551809; Becton Dickinson). Cytokine concentrations for each sample were determined using a 4-color FACS Calibur flow cytometer and software. The sensitivities for IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ were 2.6, 2.6, 3.0, 2.8, 2.8, and 7.1 pg/mL, respectively. The assay was conducted according to manufacturer's instructions. The assay sensitivities were defined as the corresponding concentration of 2 SD above the mean fluorescence of 20 replicates of negative (0 pg/mL) control.

2.7. Oxygen radical absorbance capacity measurements

Five milliliters of blood was collected in a serum separator tube. The blood was allowed to stand at RT for at least 30 minutes then centrifuged at 1500 rcf for 10 minutes and serum stored at −80°C. An assay, developed and optimized based on a previously published method [19], was used to measure serum oxygen radical absorbance capacity ($ORAC_{FL}$) as follows.

2.7.1. Serum extraction before $ORAC_{FL}$ assay

One hundred microliters of serum, 100 μL of water, 200 μL of ethanol, and 400 μL of hexane were added to a clear glass vial, mixed on a mechanical shaker for 5 minutes, let stand for 2 minutes then centrifuged for 5 minutes at 5000 rcf. The hexane layer was removed and placed into an amber glass vial. Another 400 μL of hexane was added to the original clear glass vial, and the process repeated. The hexane extracts were combined and dried under nitrogen flow, ready for lipophilic $ORAC_{FL}$ analysis.

To the original clear glass vial was added 400 μL of 0.5 mol/L perchloric acid (PCA) (catalogue no. 24425-2; Sigma) and 200 μL of 75 mmol/l of phosphate buffer, then vortexed and centrifuged for 10 minutes at 5000 rcf. The supernatant was removed into a second clear glass vial ready for hydrophilic $ORAC_{FL}$ analysis.

2.7.2. Hydrophilic $ORAC_{FL}$ assay

Twenty microliters of Trolox (Fluka, Buchs, Switzerland) standards (12.5, 25, 50, and 100 μmol/L), controls (40 μmol/L of Trolox), and samples were added in duplicate to a 96-well microplate (Greiner U bottomed). A row of 20 μL of 1.48 μmol/L of fluorescein solution (catalogue no. 166308; Sigma) was also added, to test for background noise. One hundred seventy microliters of 30 mmol/L of 2,2′,azobis(2-amidino-propane) dihydrochloride (AAPH) (catalogue no. 017-11062; Wako, Osaka, Japan) was then added; readings were started immediately. All dilutions were made using a mix of MilliQ water, ethanol, PCA, and phosphate buffer in a ratio 1:2:4:2.

2.7.3. Lipophilic $ORAC_{FL}$ assay

Two hundred microliters of acetone was added to the lipophilic extract, let stand for 10 minutes, then 700 μL of 7% randomly methylated-β-cyclodextrin (RMCD) (Wacker-Chemie, Burghausen, Germany) was added. The microplate (JRH flat bottomed) procedure is the same as for the hydrophilic assay. Dilutions of 100 μmol/L of Trolox were used as standards and 500 μmol/L of butylated hydroxytoluene (Sigma) was the control. All dilutions were made using 7% RMCD.

All samples were analyzed at 4 different dilutions in duplicate, and all assay plates were read in a prewarmed 37°C Wallac Victor 2 multiplate analyzer (Perkin Elmer, Turku, Finland). The area under the curve was calculated by point-to-point integration using slow kinetics.

2.8. Statistical analysis

Changes in immune and antioxidant markers over time were compared using repeated measures analysis of variance (1-way ANOVA) (ie, 6 visits—day 0, 7, 9, 14, 16, and 21) or paired *t* tests (ie, 2 visits—day 0 vs day 7), using a statistical software package (SPSS for Windows, version 14.0, Chicago, Ill) [20]. Results were checked for normal distribution using a Kolmogorov-Smirnov and Shapiro-Wilk tests before further analysis and adjustment were made accordingly. Results were corrected by the Greenhouse-Geisser procedure where appropriate (violation of sphericity assumption). All values were expressed as mean ± SEM. *P* values of less than .05 were considered significant.

3. Results

3.1. Immune parameters

One major finding of the present study was that after 2 weeks of lactoferrin supplementation there was a statistically significant increase in total ($CD3^+$), helper ($CD4^+$), and cytotoxic ($CD8^+$) T-cell activation as measured by the percentage of T cells expressing the early activation marker $CD69^+$ (Table 1). Normally less than 2% of unstimulated T cells express $CD69^+$; however, stimulation of T cells by the mitogen *Phaseolus vulgaris* has been shown to result in an increase in CD69 expression. The percentage of $CD69^+$ expression after stimulation reflects the degree of T-cell activation. The ANOVAs for repeated measures of changes over time from day 0 to day 21 revealed significant increases for T-cell activation; total ($CD3^+$) T-cell activation increased by 29% over baseline ($F_{(4,28)} = 36.1$; $P < .001$), helper ($CD4^+$) T-cell activation increased by 22% over baseline ($F_{(4,28)} = 23.1$; $P < .001$), and cytotoxic ($CD8^+$) T-cell activation increased by 25% over baseline ($F_{(4,28)} = 22.5$; $P < .001$). Similarly, repeated measures ANOVAs from day 0 to day 16, day 7 to day 21, and day 7 to day 16 also revealed significant increases ($P < .001$) for total T-cell activation, helper T-cell activation, and cytotoxic T-cell activation. Furthermore, there were no

Table 1
Serum immune markers and antioxidant capacity concentrations for all participants at each visit

	Day 0	Day 7	Day 9	Day 14	Day 16	Day 21
T-cell activation (% $CD69^+$)						
Total ($CD3^+$)	42.9 ± 4.6	44.4 ± 3.8	40.4 ± 3.8	NA	53.3 ± 5.3**	55.2 ± 4.6**
Helper ($CD4^+$)	43.4 ± 4.9	44.6 ± 3.9	39.7 ± 4.4	NA	52.8 ± 5.3**	52.8 ± 5.1**
Cytotoxic ($CD8^+$)	43.4 ± 5.3	42.6 ± 4.4	40.2 ± 4.3	NA	52.0 ± 5.6**	54.4 ± 5.2**
Lymphocyte subset ($\times 10^9$/L)						
Total ($CD3^+$) T cells	1.53 ± .13	1.65 ± .14	1.71 ± .18	1.47 ± .19	1.66 ± .23	1.67 ± .21
Helper ($CD4^+$) T cells	.99 ± .08	1.03 ± .09	1.11 ± .12	.91 ± .12	1.07 ± .15	1.07 ± .12
Cytotoxic ($CD8^+$) T cells	.48 ± .07	.54 ± .08	.54 ± .08	.48 ± .08	.53 ± .09	.54 ± .09
Total B cells	.24 ± .04	.25 ± .03	.26 ± .05	.23 ± .04	.25 ± .05	.26 ± .05
Total NK cells	.26 ± .05	.27 ± .05	.28 ± .06	.27 ± .05	.24 ± .05	.27 ± .05
NK cell cytotoxicity (% K562 cells killed)	30.8 ± 4.9	28.7 ± 5.1	28.0 ± 4.9	25.9 ± 4.4	23.6 ± 4.0	24.0 ± 5.0
Antioxidant capacity (μmol/L Trolox equivalents/L)						
Total	2536 ± 208	2709 ± 174	2796 ± 254	2654 ± 249	2813 ± 274	2745 ± 268
Hydrophilic	979 ± 69	1050 ± 94	1050 ± 85	1081 ± 81	1208 ± 113*	1154 ± 65*
Lipophilic	1556 ± 173	1658 ± 164	1745 ± 225	1573 ± 185	1604 ± 193	1591 ± 217

All participants self-administered for 21 days, 1 capsule of placebo (calcium carbonate) for 7 days, followed by 100 mg of lactoferrin capsule for 7 days, followed by 200 mg of lactoferrin capsule for 7 days. Blood samples were collected after overnight fasting on days 0, 7, 9, 14, 16, and 21 of the study. All values are expressed as mean ± SEM (n = 8).

* $P < .05$ vs day 0; $P > .05$ vs day 7 (significance assessed by 1-way ANOVA). NA indicates results not available because of technical failure.

** $P < .001$ vs day 0 and day 7.

significant changes in all T-cell activation markers because of the placebo as determined by paired t test analysis between day 0 and day 7 (P = .287, P = .451, and P = .664, respectively). Unfortunately, through lack of data because of technical difficulties, we were not able to look for increases at day 14 after 1 week of 100 mg of lactoferrin supplementation.

Changes in lymphocyte subset counts and NK cell cytotoxicity were also evaluated, neither of which showed significant change between baseline and either 1 or 2 weeks of supplementation (Table 1).

The values for most cytokines measured, TNF-α, IFN-γ, IL-2, IL-4, IL-6, and IL-10, were relatively low with results in most cases (63%) falling below the minimum level of detection. This inability to detect changes was therefore because of either the low serum levels, as reflective of the healthy status of participants and/or the sensitivity limits of the assay.

3.2. Antioxidant capacity

The other major finding was a statistically significant increase in hydrophilic antioxidant capacity (AC) after 2 weeks of lactoferrin supplementation (Table 1). Repeated measures of ANOVAs over time from baseline (day 0) to the completion of lactoferrin supplementation (day 21) revealed hydrophilic AC increased from 979 ± 69 μmol/L trolox equivalents (TE)/L to 1154 ± 65 μmol/L TE/L ($F_{(2.3,16.3)}$ = 3.2; $P < .05$). Paired t test analysis between day 0 and day 7 revealed no significant changes because of placebo ($t(7)$ = −1.107; P = .305); however, repeated measures ANOVA from day 7 (commencement of 100 mg of lactoferrin) to day 21 revealed no significant increases ($F_{(4,28)}$ = 2.0; P = .127). In addition, there were no significant increases between baseline and day 14 after 1 week of 100 mg of lactoferrin ($F_{(3,21)}$ = 1.4; P = .280). Changes in lipophilic and total AC were also evaluated; however, results showed no significant change in serum levels between baseline and either 1 or 2 weeks of supplementation (Table 1).

3.3. Safety

Overall, participants tolerated the placebo and lactoferrin supplements well. A small number of adverse events were reported including headache, toothache, and hay fever; all were transient, minor, and unrelated to the study medication. Blood pressure, heart rate, body weight, and the blood safety parameters, full blood count, liver function tests, C-reactive protein, and urea electrolytes and creatinine levels, were assessed at each visit (ie, on days 0, 7, 9, 14, 16, and 21). Results remained stable, and no adverse changes were reported for any participant.

4. Discussion

In this study, a bovine lactoferrin supplement given for 2 weeks to healthy males modulated immune function and antioxidant status of these participants. This change was reflected through a significant increase in total ($CD3^+$) T-cell activation ($P < .001$), helper ($CD4^+$) T-cell activation ($P < .001$), and cytotoxic ($CD8^+$) T-cell activation ($P < .001$) that occurred between days 0 and 16, days 0 and 21, days 7 and 16, and days 7 and 21. There was no demonstrated significant increase, however, after only 1 week of a 100 mg daily lactoferrin supplement (day 14) because of insufficient data. Furthermore, there was a significant increase in hydrophilic antioxidant levels between both days 0 and 16 and days 0 and 21 ($P < .05$) however not between days 0 and 14, days 7 and 14, days 7 and 16, or days 7 and 21 (P = not significant) (Fig. 1). Therefore, this increase in T-cell activation and hydrophilic AC was observed only subsequent to 1 week of 100 mg of supplementation followed by 1 week of 200 mg of supplementation.

Activation of helper $CD4^+$ T cells stimulates production of plasma B cells, memory B cells, and antibodies, resulting in increased surveillance and tagging of bacteria and fungi. Previous in vivo studies on mice using bovine lactoferrin have demonstrated its efficacy against challenge with *Escherichia coli* [9,21], *Helicobacter pylori* [10], *Helicobacter felis* [22], *Candida albicans* [11], and *Trichophyton rubrum* [17], which may be explained via the above mechanism.

In addition, $CD4^+$ T-cell activation enhances the function of macrophages, stimulating release of cytokines, both antiinflammatory (IL-4, IL-10) and proinflammatory (IL-6, TNF-α, IFN-γ). It would appear that lactoferrin may therefore enhance antiinflammatory activity and/or decrease proinflammatory mechanisms. This has been demonstrated in studies with rodents, which showed decreased production of IL-6 and TNF-α after thymectomy and splenectomy after supplementation with lactoferrin [23] occurred. Increased production of IL-4 and IL-10 and reduction of IL-6 and TNF-α in lactoferrin-supplemented rats was reported, after induced intestinal colitis [12]. In addition, a small clinical trial on healthy individuals showed that, after supplementation with either 10 mg or 50 mg of lactoferrin per day, the ability of peripheral blood cells to spontaneously produce proinflammatory IL-6 and TNF-α was also significantly reduced [15]. Our results, however, are based on a method that assesses serum cytokine levels and demonstrated no changes in cytokine levels over time; probably because of initial low levels in healthy participants that ranged below the level of sensitivity. Production of cytokines could have been measured ex vivo from stimulated peripheral blood mononuclear cells, but this was not undertaken in this study.

Activation of helper ($CD4^+$) T cells also stimulates production of cytotoxic ($CD8^+$) T cells that attack and destroy virus-invaded cells, cancer cells, intracellular bacteria, intracellular parasites, and foreign cells as supported by in vitro studies on herpes simplex [24], rotavirus [25], hepatitis C virus [18], and experimentally induced cancers [6,26]. Consistent with results from a small clinical trial by Ishikado et al [27], our results also support no change in numbers of lymphocyte subset counts, including T cells, B cells, and NK cells.

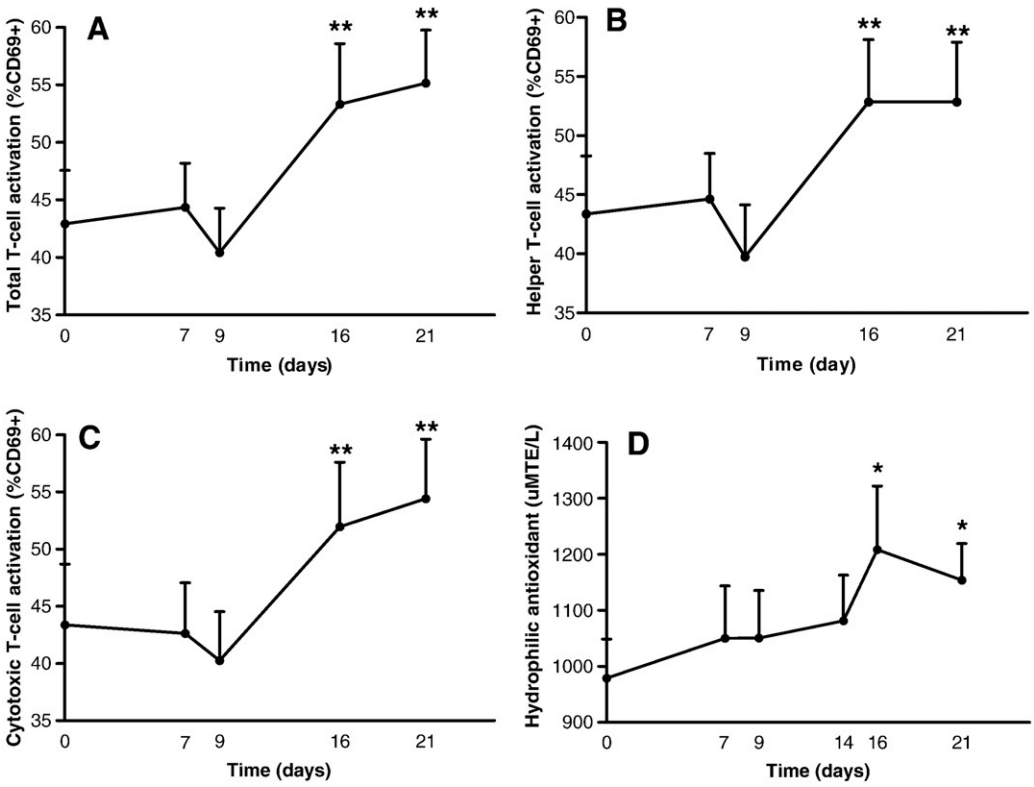

Fig. 1. Total ($CD3^+$) T-cell activation (A), helper ($CD4^+$) T-cell activation (B), cytotoxic ($CD8^+$) T-cell activation (C), and hydrophilic antioxidant capacity (D) responses to lactoferrin supplementation for all participants (n = 8). Values represent the mean ± SEM. Data were analyzed using a repeated measures ANOVA. ** indicates significant difference from day 0 to day 7 ($P < .001$). * indicates significant difference from day 0 ($P < .05$) but not day 7 ($P > .05$).

Endogenous lactoferrin is generally thought to exhibit hydrophilic antioxidant properties because of its iron-sequestering ability. However, it is unknown whether oral supplementation of lactoferrin will translate into an increased systemic antioxidant capacity. Our results demonstrate a significant increase in serum hydrophilic antioxidant capacity ($P < .05$). If the antioxidant properties of lactoferrin exist by virtue of its iron-sequestering ability, it functions through a preventative mechanism such as decreasing hydroxyl radical formation via the Fenton reaction. The $ORAC_{FL}$ assay, however, is considered to be specific for antioxidants using the chain breaking mechanism [28]. Therefore, our results suggest that the antioxidant capacity determined in serum after lactoferrin intervention may not be totally because of its iron-sequestering ability. One explanation, given that lactoferrin per se is thought not to be absorbed through the gut wall [29], is that digested fragments of lactoferrin such as lactoferricin, bind to gastrointestinal epithelial cells modulating antioxidant production. Therefore, possibly only in the gastrointestinal tract does lactoferrin act as an iron-sequestering antioxidant.

Results from this study reinforce the safety profile associated with bovine lactoferrin as safe for oral administration at dosages up to 200 mg/d, that is, no significant changes in either physiologic or blood parameter safety measurements were observed.

This study has several limitations including the small, male only, sample size. The trial design also contained no placebo group. Instead, each participant ingested a placebo (calcium carbonate), before lactoferrin supplementation and, therefore, effectively acted as their own control. The lack of placebo limits interpretation of data as it is possible, though not likely, that changes were because of ingestion of calcium carbonate for the 21 days rather than lactoferrin. In addition, the trial was not blinded, that is, participants knew when the active medication commenced. Bidirectional communication between the brain and immune system could indicate the potential necessity for blinding [30]. Future studies could use a randomized crossover trial design to alleviate this problem.

Finally, the absence of T-cell activation data on day 14 raises speculation regarding the efficacy of 100 mg of lactoferrin supplementation for 7 days, and it may be that a lower dose than 200 mg would also provide the same results for 14 days.

These results, nevertheless, add to an increasing body of knowledge on the use of bovine lactoferrin as a dietary supplement. Therefore, the results from this study provide a foundation for further trials that may include larger sample size with both males and females, addition of a placebo group, and a randomized crossover trial design. In addition, it would be interesting to determine if the helper ($CD4^+$) T-cell activation observed in this study, applied to

$CD4^+$ Th1 lymphocytes (promoting the classical cell-mediated immunity), $CD4^+$ Th2 lymphocytes (involved in allergy type reactions), or all helper ($CD4^+$) T lymphocytes.

This report demonstrates that oral supplementation of bovine lactoferrin, in particular 100 mg for 7 days followed by 200 mg for 7 days, enhances total, helper, and cytotoxic T-cell activation and hydrophilic antioxidant status. This finding suggests that bovine lactoferrin supplements have the potential to be a useful nutritional adjunct supporting immune stimulation and antioxidant capacity status in otherwise healthy individuals.

Acknowledgment

This study was funded by Blackmores Pty Ltd (Sydney, NSW, Australia). We thank Probiotec (Melbourne, VIC, Australia) for donating the lactoferrin (Glycomax Lactoferrin) and the volunteers for their participation in the study. We acknowledge that the author Christopher Oliver is an employee of Blackmores Pty Ltd, and Ann Mulder was the recipient of a scholarship paid by Blackmores Pty Ltd.

References

[1] Larkins N. Potential implications of lactoferrin as a therapeutic agent. Am J Vet Res 2005;66(4):739-42.
[2] Farnaud S, Evans R. Lactoferrin—a multifunctional protein with antimicrobial properties. Mol Immunol 2003;40(7):395-405.
[3] Legrand D, Elass E, Carpentier M, Mazurier J. Lactoferrin: a modulator of immune and inflammatory responses. Cell Mol Life Sci 2005;62(22):2549-59.
[4] Valenti P, Berlutti F, Conte M, Longhi C, Seganti L. Lactoferrin functions: current status and perspectives. J Clin Gastroenterol 2004;38 (6 Suppl):S127-9.
[5] Chierici R. Antimicrobial actions of lactoferrin. Adv Nutr Res 2001; 10:247-69.
[6] Tsuda H, Ohshima Y, Nomoto H, Fujita K, Matsuda E, Iigo M, et al. Cancer prevention by natural compounds. Drug Metab Pharmacokinet 2004;19(4):245-63.
[7] Wakabayashi H, Takakura N, Yamauchi K, Tamura Y. Modulation of immunity related gene expression in small intestines of mice by oral administration of lactoferrin. Clin Vaccine Immunol 2006;13(2): 239-45.
[8] Brock J. The physiology of lactoferrin. Biochem Cell Biol 2002;80(1): 1-6.
[9] Zagulski T, Lipinski P, Zagulska A, Broniek S, Jarzabek Z. Lactoferrin can protect mice against a lethal dose of *Escherichia coli* in experimental infection in vivo. Br J Exp Pathol 1989;70(6):697-704.
[10] Wada T, Aiba Y, Shimizu K, Takagi A, Miwa T, Koga Y. The therapeutic effect of bovine lactoferrin in the host infected with *Helicobacter pylori*. Scand J Gastroenterol 1999;34(3):238-43.
[11] Takakura N, Wakabayashi H, Ishibashi H, Yamauchi K, Teraguchi S, Tamura Y, et al. Effect of orally administered bovine lactoferrin on the immune response in the oral candidiasis murine model. J Med Microbiol 2004;53(Pt 6):495-500.
[12] Togawa J, Nagase H, Tanaka K, Inamori M, Nakajima A, Ueno N, et al. Oral administration of lactoferrin reduces colitis in rats via modulation of the immune system and correction of cytokine imbalance. J Gastroenterol Hepatol 2002;17(12):1291-8.
[13] Kuhara T, Iigo M, Itoh T, Ushida Y, Sekine K, Terada N, et al. Orally administered lactoferrin exerts an antimetastatic effect and enhances production of IL-18 in the intestinal epithelium. Nutr Cancer 2000;38(2):192-9.
[14] Yamauchi K, Wakabayashi H, Hashimoto S, Teraguchi S, Hayasawa H, Tomita M. Effects of orally administered bovine lactoferrin on the immune system of healthy volunteers. Adv Exp Med Biol 1998;443: 261-5.
[15] Zimecki M, Spiegel K, Wlaszczyk A, Kubler A, Kruzel M. Lactoferrin increases the output of neutrophil precursors and attenuates the spontaneous production of TNF-alpha and IL-6 by peripheral blood cells. Arch Immunol Ther Exp (Warsz) 1999;47(2):113-8.
[16] de Bortoli N, Leonardi G, Ciancia E, Merlo A, Bellini M, Costa F, et al. *Helicobacter pylori* eradication: a randomised prospective study of triple therapy versus triple therapy plus lactoferrin and probiotics. Am J Gastroenterol 2007;102(5):951-6.
[17] Yamauchi K, Hiruma M, Yamazaki N, Wakabayashi H, Kuwata H, Teraguchi S, et al. Oral administration of bovine lactoferrin for treatment of tinea pedis. A placebo-controlled, double-blind study. Mycoses 2000;43(5):197-202.
[18] Ishii K, Takamura N, Shinohara M, Wakui N, Shin H, Sumino Y, et al. Long term follow-up of chronic hepatitis C patients treated with oral lactoferrin for 12 months. Hepatol Res 2003;25(3):226-33.
[19] Prior R, Hoang H, Gu L, Wu X, Bacchiocca M, Howard L, et al. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. J Agric Food Chem 2003;51(11): 3273-9.
[20] Field A. Discovering statistics using SPSS. 2nd ed. London: Sage Publications; 2006.
[21] Lee WJ, Farmer JL, Hilty M, Kim YB. The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets. Infect Immunol 1998;66(4):1421-6.
[22] Dial EJ, Lichtenberger LM. Effect of lactoferrin on *Helicobacter felis* induced gastritis. Biochem Cell Biol 2002;80(1):113-7.
[23] Zimecki M, Wlaszczyk A, Zagulski T, Kubler A. Lactoferrin lowers serum interleukin 6 and tumor necrosis factor alpha levels in mice subjected to surgery. Arch Immunol Ther Exp (Warsz) 1998;46(2): 97-104.
[24] Marchetti M, Trybala E, Superti F, Johansson M, Bergström T. Inhibition of herpes simplex virus infection by lactoferrin is dependent on interference with the virus binding to glycosaminoglycans. Virology 2004;318(1):405-13.
[25] Superti F, Ammendolia MG, Valenti P, Seganti L. Antirotaviral activity of milk proteins: lactoferrin prevents rotavirus infection in the enterocyte-like cell line HT-29. Med Microbiol Immunol (Berl) 1997;186:83-91.
[26] Yoo YC, Watanabe S, Watanabe R, Hata K, Shimazaki K, Azuma I. Bovine lactoferrin and Lactoferricin inhibit tumor metastasis in mice. Adv Exp Med Biol 1998;443:285-91.
[27] Ishikado A, Imanaka H, Kotani M, Fujita A, Mitsuishi Y, Kanemitsu T, et al. Liposomal lactoferrin induced significant increase of the interferon-alpha (IFN-alpha) producibility in healthy volunteers. Biofactors 2004;21:69-72.
[28] Ou B, Hampsch-Woodill M, Prior R. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem 2001;49(10):4619-26.
[29] Teraguchi S, Wakabayashi H, Kuwata H, Yamauchi K, Tamura Y. Protection against infections by oral lactoferrin: evaluation in animal models. Biometals 2004;17(3):231-4.
[30] Maier S, Watkins L. Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behaviour, mood, and cognition. Psychological Review 1998;105(1): 83-107.