Oral bovine lactoferrin improves bone status of ovariectomized mice

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Running head: Lactoferrin reduces in vivo bone resorption

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Abstract

The aim of the present study was to evaluate the effect of dietary lactoferrin on bone metabolism in vivo using a postmenopausal animal model. We investigated whether bovine lactoferrin (bLF) ingestion could prevent bone loss in ovariectomized mice. Twelve-week-old female C3H mice either ovariectomized or sham-operated were fed for 27 weeks with the control diet (AIN-93M with 140 g of total milk protein as a protein source per kg of diet). Four groups of ovariectomized mice received diets including different concentrations of bLF (1, 5, 10 or 20 g of total milk protein were replaced by bLF). Ovariectomy induced a decreased uterine weight and a smaller gain of bone mineral density. Immunoreactive bLF was detected in the peripheral blood and its concentration was related to the amount of bLF ingestion. bLF supplementation to the diet improved bone mineral density (BMD) and femoral failure load in a dose-dependent manner. We confirmed the direct effects of bLF in vitro using established and primary cultures of murine bone cells. Addition of bLF to the culture medium at a concentration of between 1 and 1000 µg/ml stimulated both cell growth and differentiation of osteoblastic MC3T3 cells while inhibiting the growth of pre-osteoclastic RAW 267.4 cells. In primary culture of mixed bone cells, an enhanced osteoblast differentiation was associated with an inhibition of osteoclasts differentiation at lower bLF concentrations (1-10 µg/ml). In conclusion, these findings suggest that dietary lactoferrin supplementation can have a beneficial effect on postmenopausal bone loss by modulating bone formation and resorption.

Keywords: Lactoferrin; Osteoblast; Osteoclast; bone
Introduction

Osteoporosis is one of the most critical disorders occurring in postmenopausal women. It is characterized by a reduced bone mineral density and an increased risk of fracture. The postmenopausal bone loss is a consequence of estrogen deficiency which increases osteoclast activity (37) leading to an imbalance between bone formation and bone resorption. Estrogens play a fundamental role in skeletal growth and bone metabolism. It has been recognized that in response to estrogen deficiency, osteoclastogenesis occurs (39). TNF is one of the cytokines responsible for the augmented osteoclastogenesis (47). Ovariectomy increased T cell TNF production which increased macrophage colony-stimulating factor induced and RANKL-induced osteoclastogenesis (8). Moreover the presence of increased levels of TNF was reported in the bone marrow of Ovx animals and in blood cells of postmenopausal women (38, 44). Postmenopausal osteoporosis should be also regarded as the result of an inflammatory process (52). Recent animal studies demonstrated that estrogen deficiency causes bone loss by mechanisms associated with inflammatory and oxidative processes (34, 23, 13). Recent studies suggest a strong relation between bones and the immune system; this interface should play a role not only in the regulation of inflammatory bone turnover, but also in animal models of postmenopausal osteoporosis, and in basal regulation of bone homeostasis (52).

Interestingly Lactoferrin (LF), an 80 kDa iron-binding glycoprotein of the transferrin family, has been demonstrated to inhibit in vitro osteoclast-mediated bone resorption (31). LF was also demonstrated to have in vitro anabolic, differentiating and anti-apoptotic effects on osteoblasts, and to inhibit osteoclastogenesis (8). Moreover in vivo local injection of LF above the hemicalvaria increases bone formation and bone area in adult mice (8). LF is found in milk secreted by the mammary gland but also in tears, synovial fluids, saliva, seminal fluid
and to a lesser extent in the specific granules of neutrophils (27, 29). LF has a role in host non-specific defense (9, 12, 14, 25, 42). This property is related to its ability to either sequester iron in biological fluids or to destabilize the membranes of microbes, this plays a direct anti-microbial role in secretion and at the surface of epithelia by limiting the proliferation and adhesion of microorganisms. In addition to its direct antimicrobial effects LF is believed to modulate the inflammatory process mainly by preventing the release of inflammatory cytokines which induce recruitment and activation of immune cells at inflammatory sites (26). Moreover LF’s biological functions are dependent on its target cells, and might be related to its capacity to bind to various molecules in the cells.

The present study addresses the bone action of bLF in vitro and in vivo. We used ovariectomized mice as an animal model of postmenopausal osteoporosis to study the effect of dietary bLF supplementation on bone status. In vitro studies were performed using established cell lines and an original primary cell culture system allowing the growth of both differentiated osteoblasts and osteoclasts. The results support the potential of oral LF supplementation to improve postmenopausal bone loss.

**Materials and methods**

*Lactoferrin preparation*

Bovine lactoferrin was isolated from fresh skimmed milk by dual cation exchange chromatography. Briefly, fresh skimmed bovine milk was passed through a sulphopropyl-type ion exchanger SPEC 70 (Pall Biosepra) at 4 °C and the bound proteins were eluted with 1.7 M NaCl. Demineralized eluted protein fraction was reapplied to the cation exchanger S Sepharose Fast Flow (GE Healthcare) at 4 °C and the bound proteins were eluted in steps with 0.5 M NaCl at pH 6.5 and 0.9 M NaCl at pH 8.5. The latter fraction containing
Lactoferrin was demineralized, microfiltrated and spray-dried. bLF purity of the final product was greater than 98% as assessed by reversed-phase HPLC using VYDAC 214TP54 (Grace).

**Ovariectomized mice model**

Female 6-week-old C3H/HeN strain mice (n=48) were purchased from Harlan (France). They were housed in a room controlled for temperature (22 ± 1°C) under a 12:12 light-dark cycle and were given free access to a standard pellet diet and water. All experimental procedures used during these experiments complied with institutional guidelines and policies to prevent pain and distress. Forty-two 12-week-old female C3H mice were ovariectomized (Ovx) and eight mice were sham-operated (Sham). Mice were anesthetized with ketamine (100 mg/kg) + xylazine (10 mg/kg) and morphine was given to avoid pain. One week after surgery, Ovx mice were divided into 5 groups of 8 animals and fed for 27 weeks with either the control diet, AIM-93M including 140 g of total milk protein per kg of diet (Ovx C) or with a diet in which the total milk protein content had been adjusted to allow the incorporation of 1 (Ovx 1), 5 (Ovx 5), 10 (Ovx 10) or 20 (Ovx 20) g/kg of bLF to the diet. Diet composition is shown in Table 1. At weeks 5, 9, 13, 17 and 27 after surgery, at the beginning of the light cycle, the blood of each mouse was collected from the orbital sinus to evaluate immunoreactive bLF concentrations. To avoid contamination by food, each mouse was cleaned before sampling. At 27 weeks after surgery mice were anesthetized, whole blood was collected by cardiac puncture, and they were sacrificed. The body composition was determined by dissection. Four white adipose tissue (WAT) pads, i.e. periovarian, retroperitoneal, mesenteric and total subcutaneous WAT, were removed and weighed; the liver, intestine, uterus, brown adipose tissue and carcass (muscles and skeleton) were also weighed.
Detection of bovine lactoferrin by ELISA

The rabbit polyclonal antibodies against bLF were prepared in our laboratory as previously described (10). The affinity-purified antibodies from rabbit were biotinylated using biotinamidocaproate N-hydroxysuccinimide ester in a biotin/antibody ratio of 0.08 (w/w). Immunoreactive bLF concentration in mouse serum was measured by ELISA as previously described (24). Maxisorp™ 96-well microplates (Nunc) were coated overnight at 4 °C with 100 µl of affinity-purified rabbit anti-bLF antibodies, 2 µg/ml in 0.1 M NaHCO₃, pH 9. After blocking with phosphate buffered saline (PBS)-3% gelatine, the standards and sample diluted in PBS-1% gelatine were incubated for 1.5 h at 37 °C. After washing with PBS-0.05% Tween 20, the microplates were incubated at 37 °C for 1.5 h with biotin-conjugated anti-bLF antibodies which were diluted in PBS-1% gelatine (100 ng/ml). After washing, Extravidin® peroxidase (Sigma) 1:5000-diluted in PBS-1% gelatine was added for 30 minutes incubation at 37 °C. After washing, 0.025% H₂O₂ (Sigma) and 0.5 mg/ml o-phenylenediamine (Sigma) were added as substrates and the reaction was stopped by addition of 2 M H₂SO₄. The optical density of each well was measured at 490 nm using a microplate reader (Molecular Devices). The standard curve indicated that the detection limit in this assay was approximately 1 ng/ml bLF. No significant cross-reaction was observed with murine lactoferrin (less than 0.1%).

Bone mineral density, biomechanical strength of femurs, calcium contents of femora

Radiographic dual-energy X-ray absorptiometry, analysis using a Lunar Piximus densitometer (GE Medical Systems, software version 1.4x lunar) was performed to determine the entire body, lumbar and femoral bone mineral density (BMD) and bone mineral content (BMC). The BMD of the entire body, lumbar spines and the right femoral bone of each mouse were measured, under anesthesia, 1, 5, 9, 13, 17, 22 and 27 weeks after the ovariectomy. Through
whole-body scanning, the BMD and BMC of the entire body, lumbar spine and right femur were analyzed.

The right femur of each mouse was cleaned from muscle, preserved in a tube and kept on ice until the three-point bending test was performed few hours later. Three-point bending evaluates the elastic and plastic properties contributing to femoral strength. Yield load which is a measure of the femoral elastic limit was determined as the point where the slope of the load–deformation curve deviates from being a straight line. The peak load is a measure of the maximum force that the femur withstands before fracture. This test was carried out at the femoral midpoint where cortical bone is predominant. The biomechanical strength testing was determined using digital callipers (Texturometer TA XT2i using expert exceed version 07.13, Cedarlane Laboratories, Hornby, Canada). Femurs were positioned such that the posterior side was placed on two base supports with the midpoint directly under the crosshead. The crosshead was lowered at a constant speed of 0.1 mm/second until fracture occurred. The peak load and yield load were calculated from the load-displacement curve.

The left femur of each mouse was cleaned from muscle and dried overnight at 100 °C, weighed and then ashed at 550 °C for 48 h. The ashes were extracted with 1 ml of 1 M HCl. The amount of calcium in femurs was determined by atomic absorption spectrophotometry using a Zeeman polarized spectrophotometer (Hitachi Z-5000).

Cell line cultures

The osteoblastic cell line MC3T3-E1 obtained from the European collection of cell cultures (ECACC) was cultured at 37 °C under a 5% CO2-95% air atmosphere in Petri dishes with α-MEM (Invitrogen France) supplemented with 10% fetal bovine serum (FBS) (HyClone, Perbio). Cells were subcultured every 5 days using 0.05% trypsin and 0.54 mM EDTA in Ca²⁺/Mg²⁺-free PBS. For the experiment, cells were seeded onto 48-well plates (IWAKI, ATCG) at a density of 5 x 10⁴ cells/cm². LF was added to the culture medium, 48 hours after
seeding, at final concentrations between 1 and 1000 µg/ml. Cell growth after 72 hours of incubation in the presence of LF, was evaluated using a FluoReporter® Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes). The fluorometric method we used has been developed to count adherent cells in the range of 1000 to 100000 cells per well for proliferation studies. In a first experiment, cells were counted and compared to DNA quantification. The standard curve obtained was not significantly different at 5, 10 or 14 days of culture. For the DNA quantification cells were rinsed with PBS and frozen at – 80 °C until DNA quantification. On the day of the determination, cells were unfrozen in water and homogenized, half of the cell homogenate was used for DNA quantification and the other half to evaluate alkaline phosphatase (ALP) activity as a marker of bone formation (43). To evaluate ALP activity we used a fluorescent substrate, 4-methylumbelliferyl phosphate (Sigma) and the activity was expressed as function of DNA content.

The pre-osteoclastic RAW 264.7 monocyte/macrophage cell line, obtained from ECACC, was cultured at 37 °C under a 5% CO₂-95% air atmosphere in Petri dishes with DMEM (Invitrogen) supplemented with 10% FBS (HyClone, Perbio). For the experiments, cells were seeded onto 48-well plates (IWAKI, ATCG) at a density of 5 x 10⁴ cells/cm². Forty eight hours after seeding, bLF was added to the culture medium at final concentrations between 1 and 1000 µg/ml. Cell growth after 72 hours of incubation in the presence of LF, was evaluated using a FluoReporter® Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes).

*Primary culture of murine bone cells*

BALB/c mice were sacrificed by decapitation and tibias were removed aseptically. Bones were placed in a Petri dish containing α-MEM including 10% FBS. Bones were broken with a scalpel and bone marrow was scraped out. Bone pieces and the medium with the cells were collected in a tube and vigorously shaken. The solution was filtrated through a 70 µm cell
strainer. Cells were collected by centrifugation for 5 min at 800 g and resuspended in α-MEM with 10% FBS and 10⁻⁸ M of 1α, 25-dihydroxyvitamin D₃. Cells obtained from 4 to 5 tibias were plated in one 75 cm² flask. After 6 days of culture, the cells were scraped, collected, counted and resuspended in α-MEM with 10% FBS and 10⁻⁸ M of 1α, 25-dihydroxyvitamine D₃ and seeded in Petri dishes at density of 2.5 x 10⁴ cells/cm². BSA or bLF was added to the culture medium 24 hours after seeding. Cells were incubated for 14 days in presence of BSA or bLF, then washed with PBS, fixed and stained with Giemsa (Sigma). Osteoclast-like multinucleated cells which express specifically tartrate resistant acid phosphatase (TRAP) were evaluated using the method described by Sakai et al. (41). DNA quantification and ALP activity were determined as described in the previous section. Morphological observation was done by light microscopy with 100× magnification to look at the effect of LF on cell growth. The BD BioCoat™ Osteologic™ bone cell culture system (BD Bioscience) was used to characterize and measure osteoclast-mediated bone resorption. The mixed bone cell suspension prepared as described above was seeded at a density of 4 x 10⁴ cells/cm². BSA (final concentration 1000 µg/ml) or bLF (final concentration 10, 100 or 1000 µg/ml) was added to the culture medium 24 hours after seeding. Cells were incubated for 14 days in the presence of BSA or bLF. The digestion of sub-micron synthetic calcium phosphate thin films was observed with a light microscope with 10× magnification to evaluate cell resorption activity.

Statistical analysis

Results are expressed as means ± standard deviation. Statistical analyses were performed using SAS software. Results were compared using a one-way analysis of variance (ANOVA) to assess the effect of diets. Significance was established at P< 0.05.

Results
Body composition and blood LF concentration of ovariectomized mice fed LF-supplemented diet

Effectiveness of the Ovx procedure was confirmed by the reduction in uterine weight of Ovx mice as compared to Sham mice (Sham 114 ± 41 mg vs Ovx 67 ± 29 mg, P<0.001). However LF supplementation did not significantly modify uterine weight.

At the end of the experiment, after 27 weeks, there were no significant differences in body weight between the different mice groups (Figure 1), although Ovx mice tended to gain more weight than the Sham mice. Indeed, carcass, WAT and subcutaneous fat weight of Ovx mice were significantly heavier as compared to Sham mice. Both WAT and carcass increased but the body composition (WAT/carcass) of Ovx mice was significantly increased as compared to Sham mice (Sham 0.70 ± 0.12 versus Ovx 0.84 ± 0.012 P<0.05). Addition of bLF to the diet did not significantly modify any parameter.

Immunoreactive bLF (iLF) concentration in individual mouse peripheral blood was evaluated after bLF ingestion for 4, 8, 12, 16 and 26 weeks. The iLF blood concentrations of mice receiving the bLF-supplemented diets, for 8 weeks, were significantly increased as compared to those receiving the control diet (Figure 2) and were correlated to the bLF concentration in the diet (r=0.998). Similar results were obtained after 4, 12, 16 and 26 weeks (data not shown).

LF-supplemented diet improved BMD and bone mechanical properties of ovariectomized mice

Total, femoral and lumbar BMD were evaluated every month during the 6 months of the experiment. The total BMD of Sham mice was significantly higher (P<0.05) than that of Ovx mice fed with the control diet (Ovx C) from week 5 to week 27 post-surgery (Figure 3A). Moreover, this figure shows that the ovariectomy delayed the progress of BMD since the Ovx
C mice reached the maximal BMD at about week 17 post-surgery versus week 9 post-surgery for the Sham mice.

bLF supplementation restored a reduced gain of total BMD in Ovx mice as compared to the Sham mice (Figure 3B). A significant increase in the total BMD as compared to Ovx C was reported 9 weeks after surgery for Ovx 10 and Ovx 20, at week 17 and week 27 for Ovx 5, Ovx 10 and Ovx 20. The Ovx 20 total BMD at week 27 was even higher than that of Sham mice.

After 26 weeks of bLF supplementation, BMD was also evaluated at the femoral (Figure 4A) and lumbar levels (Figure 4B). Femoral and lumbar BMD values measured in Ovx mice were smaller as compared to Sham mice values. bLF supplementation restored BMD at both femoral and lumbar level. At the femoral level, a significant increase of the BMD as compared to Ovx C was observed for Ovx 5, Ovx 10 and Ovx 20. Moreover bLF supplementation at 20 mg/kg of diet also significantly increased femoral BMD as compared to the Sham mice. At the lumbar level, a significant increase of the BMD as compared to Ovx C was observed only for Ovx 10 and Ovx 20. bLF supplementation appeared to be more efficient at the femoral level. Figure 4D also shows femoral calcium content at week 27 post-surgery. bLF supplementation increased femoral calcium content of Ovx mice in a dose-dependent manner, and a significant increase was observed for Ovx 5, Ovx 10 and Ovx 20.

Biomechanical properties of femur were also evaluated at 26 weeks after bLF supplementation (Figure 4C). As the primary effects of estrogen deficiency on bone biomechanical properties were on stiffness (yield load) and failure load (peak load) (20), both were measured. Yield load and peak load of Ovx C mice were significantly reduced as compared to those of Sham mice. bLF supplementation to the diet increased both parameters as compared to Ovx C. In contrast with what was observed for BMD and calcium content, the lowest concentration of bLF (1 g/kg of diet) was effective at protecting both yield load and
peak load. Furthermore, in all Ovx groups receiving bLF, both parameters were even higher than those of Sham mice. These results suggest that dietary bLF supplementation should be effective at reducing post-menopausal bone lost and fracture at the femoral level.

*LF stimulated osteoblastic and inhibited osteoclastic cell activity in vitro*

We verified that bLF can have direct effects on bone cells using established cell lines and primary cultures of murine bone cells. The effect of bLF on osteoblast growth and differentiation was evaluated using the established osteoblastic MC3T3 cell line. Addition of bLF to the culture media dose-dependently increased MC3T3 cell growth (Figure 5A). The results were expressed as a stimulation index (SI), calculated as the ratio of DNA content in presence of bLF as compared to BSA as control. A significant increase in cell growth was observed at a concentration as low as 5 µg/ml (SI = 1.25) which is a physiological range of lactoferrin concentration. The highest growth stimulation (SI = 1.82) was observed at a concentration of 100 µg/ml LF.

To evaluate if bLF addition to the cell culture media can also stimulate osteoblast activity of MC3T3 cells, we measured ALP activity which is a well-known marker for osteogenic activity in osteoblasts. Figure 5B shows that, after 3 days of incubation, a significant increase in ALP activity was observed only at the higher bLF concentrations (50 to 1000 µg/ml), and unlike the effect on cell growth, the highest bLF concentration (1000 µg/ml) was the most efficient to stimulate cell differentiation.

The effects of bLF on osteoclast development were assessed in pre-osteoclastic established RAW cells. Addition of bLF to the culture media bathing RAW cells produced a decrease in cell growth (Figure 5A), in contrast with the effect observed in osteoblastic MC3T3 cells. At a bLF concentration of 1000 µg/ml, no cell growth was observed. These opposite effects of bLF on osteoclast development and on osteoblast growth and differentiation support the
importance of studying the effect of bLF on a cell culture model including both osteoblasts and osteoclasts.

We then developed a primary culture of murine bone cells. After 14 days of culture, the presence of two cell types was observed in the culture, i.e. round shape osteoclast-like multinuclear giant cells that express TRAP and smaller osteoblast-like mononuclear cells (Figure 6A). The proportion of TRAP positive cells was never more than 5%. Figure 6B shows that when mixed primary culture of murine bone cells was grown in presence of a low concentration of bLF (10 µg/ml) the number of cells increased. However, Figure 6C and 6D show that higher concentration of bLF (100 and 1000 µg/ml) decreased the number of cells. To evaluate the effect of bLF on growth of mixed primary cells, DNA was quantified. We observed that as bLF concentration in the culture medium increased, the number of osteoclast decreased. Indeed, no multi-nucleated cells were observed at 1000 µg/ml, indicating a complete inhibition of osteoclast differentiation (Figure 6D). Moreover at this concentration, the cells seeded in the plates attached but never developed, the number of cells remaining the same during the next 14 days. DNA quantification confirmed that low concentration of bLF significantly increased cell growth but a concentration of 100 or 1000 µg/ml inhibited cell growth (Figure 7). We report a strong dose dependent effect of bLF on primary culture of murine bone cells. The cell growth increase was coupled to a significant increase in ALP activity at the lower bLF concentrations (1 and 10 µg/ml). Osteoclast numeration showed that bLF at concentrations ranging from 1 to 1000 µg/ml inhibited osteoclast differentiation (Figure 7). The higher bLF concentrations (100 and 1000 µg/ml) significantly inhibited growth of both osteoblasts and osteoclasts.

To better characterize the effect of bLF on osteoclast activity, primary culture of murine bone cells was performed for 14 days on the BD BioCoat™ Osteologic™ bone cell culture system. This allowed the measurement of osteoclast-mediated bone resorption. Osteoclast activity was
shown by digestion of sub-micron synthetic calcium phosphate thin films: digestion of a wide surface of synthetic films was observed when cells are grown without bLF, in the presence of BSA (Figure 8). The digestion was drastically diminished in the presence of bLF at a concentration of 10 and 100 µg/ml (Figure 8B, 8C) and completely abolished at 1000 µg/ml (Figure 8D). These results indicate that bLF inhibits osteoclast differentiation and its resorption activity at a physiologically occurring concentration.

Discussion

Osteoporosis is the consequence of an inadequate bone formation to compensate for the increased bone resorption associated with estrogen deficiency. In this study we used *in vivo* and *in vitro* models to evaluate the effect of oral LF on bone status. The results showed that oral bLF supplementation dose-dependently improves bone mineral density and femoral failure load of ovariectomized mice. Both direct action, through increased blood concentration and indirect action of LF by modulation of the immune system are probably involved in both enhanced osteoblastic activity and inhibition of osteoclastic activity.

In the present study, C3H ovariectomized mice or sham-operated mice were used as an *in vivo* animal model of postmenopausal osteoporosis (11, 50). In this C3H mouse model the ovariectomy was performed at 12 weeks when BMD was still increasing. Accordingly we observed a reduced rate of bone mass gain. These results are in accordance with recent studies showing that in mice bone cell sensitivity to estrogen deficiency is different, leading to various bone loss patterns, according to the developmental stages. Moreover bone loss after ovariectomy has been shown to depend on both the genetic background and on the skeletal site (1, 4, 19, 21, 30, 36, 53, 54, 59). In the mouse model bLF-supplemented diet improved BMD and bone mechanical properties after ovariectomy. The lowest bLF concentration tested was effective at maintaining femur mechanical properties but did not have any significant
effect on the calcium content or BMD. Biomechanical principles dictate the anatomic location of bone growth which is a factor that determines the magnitude of the loss in strength after ovariectomy. Cortical bone mechanical properties are typically determined by whole bone mechanicals test. In this study, whole femur was subjected to three-point bending and stiffness and failure load measurements, which have been shown to be the most sensitive indicator of changes in bone morphology (20). Although bone mechanical tests do not exactly mimic \textit{in vivo} loading, these tests provide quantitative assessment of how mechanical behaviour is affected by the change in morphological architecture of the bone after ovariectomy. Considering that anatomic location of bone loss is a factor that determines bone strength, further micro-architecture analyses should help us to better identify where and how LF modulates bone metabolism and to understand LF mechanism of action on bones.

The lowest LF concentration used (1g/kg of diet) did not significantly modify any of the parameters tested except femoral failure load. Indeed, maximum break and yield loads in Oxv1 were significantly improved for femurs as compared to those of Ovx C. The morphology of long bone is different from that of short bone, such as vertebrae, and this may explain why different effects on femur and vertebrae were reported in the present study.

Both \textit{in vivo} and \textit{in vitro} experiments suggested that the improvement of bone metabolism by LF given orally could partly result from a direct local action of LF on bone. Evaluation of blood immunoreactive bLF showed that dietary bLF absorption into peripheral blood was directly related to bLF content in the diet and that iLF blood concentration remained at the same level from 4 to 26 weeks of bLF ingestion. Moreover, we observed that the peripheral iLF blood concentrations went back rapidly to control level when the LF supplementation was stopped. This strongly suggests that the immunoreactive bLF measured in peripheral blood is derived from the diet. In a previous study (10), we have shown that intact immunoreactive
bLF is absorbed from mouse intestine into the blood and subsequently localized within various tissues. LF resistance to proteolytic digestion (22, 51) and the presence of LF-receptor in the mouse intestinal brush border (17) may explain the rapid bLF uptake from the lumen to the blood (15, 18). Moreover, our in vitro experiments demonstrated that LF could directly act on bone cells. bLF at low physiological concentrations (5 µg/ml) stimulated osteoblastic MC3T3-E1 cells growth and can stimulate growth of osteoblast and inhibit osteoclastogenesis in primary culture of murine bone cells. A more important stimulation of cell growth was obtained in the presence of 100 µg/ml of LF. In addition osteoblast differentiation increased gradually up to a LF concentration equal to 1000 µg/ml. bLF at a concentration ranging from 10 to 1000 µg/ml was found to inhibit RAW cell growth. As previously reported, LF action on osteoclasts is strikingly different since it produces an important arrest of osteoclastogenesis (8, 31). These results were confirmed in mixed primary culture of murine bone cells. At high concentration, growth and differentiation of both osteoclasts and osteoblasts were completely arrested, a phenomenon which has been correlated to a decreased expression of nuclear factor-κB (8). However, in agreement with Cornish et al. (8), at low physiological concentrations LF exerted a dual effect characterized by an important inhibition of osteoclast differentiation with a stimulating effect on osteoblast proliferation. The transcription factor NF-kB is known to play a central role on the regulation of inflammatory and immune responses and on the control of cell mitosis and apoptosis (45). In opposition to Conish’s studies, Oh et al. (35) reported in neutrophils an activation of NF-κB by LF concentration ranging from 20 to 100 µg/ml. This suggests that LF can trigger different pathways depending of the target cells.

LF is also presumed to have an indirect action on bone metabolism. An increasing number of studies indicate that LF modulates inflammatory processes and antioxidant activity mainly by
preventing the release of cytokines which induce recruitment and activation of immune cells at inflammatory sites (26). It is also possible that LF regulates bone homeostasis through the modulation of cytokine production. One of the mechanisms responsible for ovariectomy-induced bone loss is a cytokine-driven increase in osteoclast formation (7, 51). Osteoclast differentiation takes place when bone marrow macrophages are co-stimulated by the two osteoclastogenic factors, i.e. the receptor activator of nuclear factor-κB ligand (RANKL) and the macrophage colony-stimulating factor (M-CSF) (7). In an estrogen deficient situation (such as in postmenopausal women and Ovx animals), TNF-α up-regulates octeoclast formation (39, 51) by stimulating the production of RANKL and M-CSF and causes bone loss in rodents and human (6, 39). Thus, we propose that dietary intake of bLF can have an indirect effect on bone through its capacity to regulate the immune system by decreasing TNF production. In agreement with this hypothesis, bLF oral administration has been shown to suppress TNF-α production and to increase IL-10 production in adjuvant-stimulated arthritic rats (16) and to lower the expression of TNF-α in intestinal lymphocytes of healthy mice (46). Thus, dietary supplementation of bLF to OVX mice would decrease TNF production which subsequently normalized the elevated osteoclastogenesis observed in oestrogen-deficient situation.

Interestingly, other milk whey protein fractions have been demonstrated to have an effect on bone resorption. Several human studies have confirmed the beneficial effects of the basic protein fraction from bovine milk on bone metabolism (2, 3, 48, 57). Furthermore, in vitro studies have demonstrated that high mobility group-like protein (47) and kininogen fragment 1.2 (56, 58) which are found in the basic protein fraction of bovine milk, promote osteoblast proliferation. Cystatin C (32) and angiogenin (33), two proteins which are also found in milk
basic protein fraction, are known to act as inhibitory factors on osteoclastic bone resorption. Whether Lf is an active component in these basic protein fractions remains to be demonstrated.

In conclusion, the results obtained in the present study with Ovx mice show the effectiveness of dietary bLF supplementation on postmenopausal bone loss by modulating bone formation and resorption. The demonstration of the dietary bLF transfer into peripheral blood in an immunoreactive form and the dual effects of bLF on osteoblasts and osteoclasts support a direct action of bLF on bone cells. Moreover the involvement of indirect actions of ingested bLF via the modulation of cytokine production remains to be proven.

Altogether, our data suggest that dietary bLF supplementation may represent a preventive strategy for bone disorders in our experimental model. The question of the relevance of such a strategy for postmenopausal bone disorder treatment in humans requires clinical investigations.
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Figure legends

Figure 1 Final body weight, white adipose tissue (WAT), carcass and subcutaneous weight of sham-operated mice (Sham) and ovariectomized mice (Ovx) fed the control or bovine lactoferrin-containing diets. The ovariectomized (white) or sham-operated (black) mice were fed for 6 months either with the control diet for Sham and Ovx control, or with a diet including 1 (diagonal marking), 5 (grey), 10 (vertical marking) or 20 g/kg (doted) of bovine lactoferrin (see Table 1 for the diet compositions). Values are expressed as the mean ± SD, n = 8. Values with different letters are significantly different (P < 0.05).

Figure 2. Immunoreactive bovine lactoferrin in the serum of mice fed with diet including different concentration of bovine lactoferrin. The ovariectomized (Ovx) or sham-operated (Sham) mice were fed for 2 months either with the control diet for Sham and Ovx control (Ovx C), or with a diet including 1 (Ovx1), 5 (Ovx5), 10 (Ovx10) or 20 g/kg (Ovx20) of bovine lactoferrin (see Table 1 for the diet compositions). Values are expressed as the mean ± SD, n = 8. * indicates significant difference from Ovx C, (P < 0.05).

Figure 3. Evolution of bone mineral density (BMD) of whole body throughout the experiment period for the sham-operated (Sham) and the ovariectomized (Ovx C) mice fed the control diet (A). Data are means ± SD, n=8. Statistical difference between Sham and Ovx C are indicated by *, P < 0.05. Effect of bovine lactoferrin ingestion for 2, 4 and 6 months on whole body BMD (B). The ovariectomized (white) or sham-operated (black) mice were fed for 6 months either with the control diet for Sham and Ovx C, or with a diet including 1 (diagonal marking), 5 (grey), 10 (vertical marking) or 20 g/kg (doted) of bovine lactoferrin. Values are expressed as the mean ± SD, n = 8. Values with different letters are significantly different (P < 0.05).
Figure 4: Femur mineral density (A), lumbar mineral density (B), mechanical properties of the right femur, maximum break load (grey) and yield load (black) (C) and calcium content of the left femur (D) after 26-week ingestion of the experimental diets. Ovariectomized mice were fed for 26 weeks with the control diet (Ovx C), or the diet supplemented with 1 (Ovx1), 5 (Ovx5), 10 (Ovx10) or 20 g/kg (Ovx20) of bovine lactoferrin. The sham-operated mice were fed for 26 weeks with the control diet. Values are expressed as the mean ± SD, n = 8. Values with different letters are significantly different (P < 0.05).

Figure 5: Effect of bovine lactoferrin on proliferation of MC3T3 cells (open circle) and RAW 267.4 cells (black square) (A), and alkaline phosphatase activity of MC3T3 cells (B). DNA content quantification was used to evaluate cell growth in presence of bLF for three days. ALP activity was evaluated as function of DNA content in the presence of bLF for three days. The values are expressed as a stimulation index. Data are means ± SD of three determinations done on three different cultures. * indicates significant difference from control (P < 0.05).

Figure 6: Effect of bovine lactoferrin on primary culture of murine bone cells. Cells obtained from tibias were grown on standard Petri dishes for 14 days in presence of BSA (A) or bovine lactoferrin at concentrations of 10 (B), 100 (C) or 1000 µg/ml (D). Cells were fixed and stained with Giemsa. Photographs were taken at the same magnification (100×).

Figure 7: Effect of bovine lactoferrin on proliferation of primary culture of murine bone cells (open circle). DNA content quantification was used to evaluate murine bone cell growth in presence of bovine lactoferrin for 14 days. The values are expressed as a stimulation index. Data are means ± SD of three determinations done on four different cultures. Effect of bovine lactoferrin on alkaline phosphatase activity of primary culture of murine bone cells (black
Alkaline phosphatase activity was evaluated as function of DNA content for cells grown in the presence of bovine lactoferrin. The values are expressed as stimulation index. Data are means ± SD of three determinations done on four different cultures ($P < 0.05$). Effect of bovine lactoferrin on osteoclast differentiation of primary culture of murine bone cells (black triangle). The number of differentiated osteoclasts, designated as cells with 3 or more nucleus, was counted after 14 days of culture in the presence of bovine lactoferrin. The values are expressed as inhibition index as compares to control. Values are means ± SD of three determinations done on four different cultures. * indicates significant difference from control ($P < 0.05$)

**Figure 8.** Primary culture of murine bone cells were grown for 14 days on BD BioCoat™ Osteologic™ bone cell culture system to characterize and measure osteoclast-mediated bone resorption shown by digestion of sub-micron synthetic calcium phosphate thin films. Cells were grown in the presence of BSA (A) or bovine lactoferrin at concentrations of 10 (B), 100 (C) and 1000 µg/ml (D). Photographs were taken at the same magnification (10×).
<table>
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<tr>
<th>Ingredient</th>
<th>Control</th>
<th>Ovx1</th>
<th>Ovx5</th>
<th>Ovx10</th>
<th>Ovx20</th>
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<tr>
<td>Total cow’s milk protein (^a)</td>
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<td>139</td>
<td>135</td>
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<tr>
<td>Bovine lactoferrin (^a)</td>
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<td>Corn starch (^b)</td>
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<tr>
<td>Sucrose (^c)</td>
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<tr>
<td>Soybean oil (^d)</td>
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<td>Alpha-cellulose (^e)</td>
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<td>AIN 93 M mineral mixture (^f)</td>
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<td>AIN 93 M vitamin mixture (^f)</td>
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<td>Choline (^f)</td>
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<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
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</tr>
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</table>

\(^a\) Armor Protéines, Saint-Brice-en-Coglès, France

\(^b\) Ceresstar, Haubourdin, France

\(^c\) Eurosucré, Paris, France.

\(^d\) Bailly SA, Aulnay-sous-Bois, France.

\(^e\) Medias Filtrants Durieux, Marne-la-Vallée, France (Alphacel, ICN Pharmaceuticals, Orsay, France).

\(^f\) ICN Pharmaceuticals, Orsay, France.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A DNA quantification

B Phosphatase alkaline activity

[Diagram showing DNA quantification and Phosphatase alkaline activity data with significant differences marked by asterisks.]
Figure 6
Figure 7