Analysis of the intestinal IgA response in mice exposed to chronic stress and treated with bovine lactoferrin

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Abstract. Immunoglobulin (Ig) A, an antibody with a pivotal role in gut homeostasis, can be modulated by stress and bovine lactoferrin (bLf). The aim of the present study was to analyze the impact of chronic stress on the IgA response in the small intestine during bLf treatment. Male BALB/c mice (n=6 mice/group) underwent 1 h of chronic stress by immobilization for 7 consecutive days or were left unstressed, and were untreated or treated with bLf (50, 500 or 5,000 μg). Plasma corticosterone expression levels were determined by ELISA. The distal small intestine was dissected to analyze: i) total IgA, secretory IgA and IgG, as well as and specific IgA and IgG antibody levels in the intestinal liquid by ELISA; ii) α-chain and polymeric immunoglobulin receptor (pIgR) protein expression in epithelial cell extracts analyzed by western blotting; iii) the mRNA expression levels of α-/J-chains, pIgR, IL-2, IL-4, IL-5 and IL-6 in whole mucosal samples by reverse transcription-quantitative PCR. Data were analyzed by one-way ANOVA, and the differences were analyzed by the Holm-Šidák post hoc test and were considered significant if $P<0.05$. Results from the present study revealed the upregulatory effects of chronic stress on the total antibody levels, protein (α-chain; 78-kDa pIgR) and mRNA (α- and J-chains; pIgR; IL-6) expression levels were restricted by bLf under stress. The effects of chronic stress on the downregulation of IL-2 and IL-4 mRNA expression were not changed by bLf under stress. The corticosterone response in unstressed mice treated with 5,000 μg bLf and the specific-IgG levels in the unstressed and stressed groups treated with bLf at all doses were increased. The findings suggested an effect of bLf in maintaining homeostasis under stress.

Introduction

Repeated stress events have a substantial role in the modulation of immunoglobulin (Ig)A, a component of mucosal immunity with a key anti-inflammatory role that is regarded as a biomarker of animal wellness (1,2). Experimental assays in animal models have shown, in most cases, the downregulatory effects of chronic stress on parameters associated with the IgA response, which include total and secretory IgA production and the number of IgA^+ plasma cells (3-9). In other studies, the IgA response was found to be either significantly or non-significantly increased under chronic stress conditions (8,10,11). The modulatory role of chronic stress on the intestinal IgA response seems to entail crosstalk between the humoral and cellular components of intestinal immunity and stress hormones, such as corticosterone release by the adrenal glands (5,8,9). The elevation of corticosterone levels is the endpoint of the hypothalamic pituitary adrenal (HPA) axis activation via the corticotropin-releasing hormone (CRH) pathway (12). The underlying molecular mechanisms that control intestinal immunity are not fully known; however, in the brain, corticosterone positively or negatively modulates genomic and nongenomic expression through cytoplasmic and membrane glucocorticoid receptors, respectively (12).

Bovine lactoferrin (bLf) is a multifunctional iron-binding glycoprotein that enhances the IgA response and even IgG expression levels, as previously shown in the distal or full-length small intestine of healthy mice (13-15). Similar to IgA, locally synthetized IgG contributes to the protection of the intestinal mucosa (16). The effects of lactoferrin on the IgA response result from the role of lactoferrin as a modulatory factor in antigen presenting cells (17) and $CD3^+$ T cells (18) as well as its action as a class-switch factor for IgA via the transforming growth factor (TGF)-β receptor III signaling pathway (19). In experimental models of chronic immobilization stress, bLf treatment increases the number of splenic antibody-secreting

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Abbreviations: bLf, bovine lactoferrin; SIgA, secretory IgA; pIgR, polymeric immunoglobulin receptor

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cells (20). The underlying mechanism seems to entail both the upregulatory and downregulatory effects of bLf on stress hormones such as corticosterone (21,22). Chronic stress has an earlier proinflammatory effect in the ileum compared with that in the duodenum, as previously documented in mice that underwent crowding stress, a model that is used to address the role of psychosocial stress in human disorders such as irritable bowel syndrome (IBS) (23). The harmful effect of chronic stress is associated in part with perturbations in the luminal microbiota that, in turn, exhibits a gradient that increases from the proximal to the distal small intestine (24,25).

Although previous studies have focused on separately examining the effects of stress or bLf on intestinal immunity, the present study assessed the effects of chronic stress on parameters associated with the IgA response in the distal small intestine during bLf treatment. As the modulatory properties of chronic stress on the IgA response-associated parameters in the distal intestine under conditions of bLf treatment have not yet been documented, this approach may provide insights into the potential use of bLf in pharmacological and neuroimmunological interventions to maintain intestinal homeostasis.

Materials and methods

Animal handling. A total of 48 male BALB/c mice (age, 6 weeks; body weight, 21-23 g) were purchased from Unidad de Producción y Experimentación Animal, Universidad Autónoma Metropolitana Unidad Xochimilco (Mexico City, Mexico). Mice individually housed, were provided with water and fed Laboratory Rodent Diet 5001 (LabDiet) *ad libitum*, and kept on a 12-h light/dark cycle in a noiseless room, at 20˚C and a relative humidity of 55%. The mice were left for 2 weeks to adapt to the environmental conditions, and the experimental interventions were started when the mice were 8-weeks-old. The animals were handled in accordance with Mexican Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico City, Mexico) in concordance with ARRIVE guidelines for reporting animal research (26). The protocol was approved by the Institutional Animal Care and Use Committee of the Instituto Politécnico Nacional. All handling and assays were carried out from 8:00-11:00 a.m. to normalize the influence of the circadian cycle on adrenocorticotropic hormone and corticosterone fluctuations.

For immobilization, each mouse was placed in a prone position, and the four limbs were gently stretched and attached with adhesive tape to an expanded polystyrene board enveloped with plastic film for easy cleaning. Limb immobilization occurred in the following order: Forepaws, hindpaws and tail. This process was accomplished by first placing very low-adhesive tape and then covering that with high-adhesive tape to minimize pain after tape removal. The adhesive tape was placed at the dorsum of the forepaws, on the footpads of the hindpaws and in the middle of the tail. Curve strips produced from paperboard-adhesive tape reels were placed upon the adhesive tape as chewers for mice to prevent self-inflicted injuries on the foreleg skin. In this experiment, the head of each mouse was allowed to move freely, and the twisting of limbs and tearing of whiskers were avoided by gentle and careful handling of mice. After 1 h of immobilization, the adhesive tape was removed carefully to reduce pain in the following order: Tail, followed by the hindpaws and then the forepaws. This protocol was repeated daily for 7 days. Food and water were removed from the control group during the period of immobilization applied to the stressed mice group.

Bovine lactoferrin. Iron-saturated (3%) bLf was purchased from NutriScience Innovations, LLC, and the purity was confirmed. Briefly, total protein of bLf was quantified using Bradford reagent (cat. no. 500 0006; Bio-Rad Laboratories, Inc.) and 2 μg protein mixed with NuPAGE™ LDS Sample Buffer (4X; cat. no. NP0007; Invitrogen; Thermo Fisher Scientific, Inc.) was boiled at 85˚C for 5 min and loaded onto a 10% SDS-PAGE gel alongside a molecular weight marker (cat. no. MPSTD3; MilliporeSigma). Electrophoresis was performed at 88 V for 2 h. After, the gels were stained with Coomassie blue R solution (cat. no. 1610436; Bio-Rad Laboratories, Inc.) for 1 h at room temperature with gentle agitation and the bLf purity was confirmed by the presence of a single 80 kDa band (Fig. S1). A volume of 100 μl saline solution 0.9% w/v NaCl (Solución CS-PiSA®; PiSA Pharmaceutical) containing 50, 500 or 5,000 μg bLf for the bLf-treated groups or vehicle only (saline solution) for the bLf-untreated groups were delivered by oral deposition with a 200-μl pipette tip.

Experimental protocol. A total of eight groups of six mice were used in this study. Three groups were treated with either 50, 500 or 5,000 μg of bLf and underwent chronic stress for 1 h a day for 7 days by board immobilization. bLf administration occurred 1 h before the start of the stress session. Three groups of unstressed mice were used as the controls, which were treated with corresponding bLf doses. Two groups were untreated basal groups, one of which underwent stress and one that was not stressed. On day 7, the mice were sacrificed by exposing them with an isoflurane overdose (Sofloran™; PiSA Pharmaceutical). Euthanasia procedure was conducted by placing each mouse within a 500 ml clean chamber glass jar (wide mouth) with a tight-fitting lid containing 300 μl of 100% isoflurane. Following 3 min of isoflurane exposure, cardiac arrest (no palpable heartbeat) and sphincter distention (urine and feces release) were detected to confirm death, and the mice were immediately exsanguinated by cardiac puncture with a heparinized syringe. Plasma samples were collected and stored at -70˚C to analyze the corticosterone level by ELISA. A small 5-cm distal segment proximal to the ileocecal valve was collected and dissected for intestinal washings to evaluate antibody levels by ELISA. Epithelial cells purified from the distal intestinal segment were used to analyze the protein expression levels of α-chain and polymeric immunoglobulin receptor (pIgR) by a chemiluminescent western blot assay. The intestinal strip was scraped with a coverslip to collect whole mucosa samples to analyze the mRNA expression levels of α-chain, J-chain, pIgR, IL-2, IL-4, IL-5 and IL-6 by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

ELISAs for antibody levels. The collection of intestinal washings was accomplished by flushing the intestinal segment with 3 ml of sterile PBS (pH 7.2) containing 0.02% sodium azide and the Complete Mini protease inhibitor cocktail

(cat. no. 11 836 153 001; Roche Diagnostics GmbH). The intestinal washings were centrifuged at 10,000 x g for 20 min at 4˚C, and the supernatants were removed and stored at -70˚C until used. The levels of antibodies in the intestinal washings were tested in 96-well microtiter ELISA plates using 100 μl of all reactants in each well in successive steps. For this study, rabbit anti-mouse IgA myeloma serum to detect total IgA was prepared as follows: Two male New Zealand rabbits (age, 12 weeks; body weight, 2.5 kg) were purchased from Unidad de Producción y Experimentación Animal, Universidad Autónoma Metropolitana Unidad Xochimilco (Mexico City, Mexico). Rabbits were individually housed, provided with water and fed Laboratory Rabbit Diet 5321 (LabDiet) *ad libitum* and kept on a 12-h light/dark cycle in a noiseless room, at 20˚C and a relative humidity of 55%. Rabbits were immunized intramuscularly with IgA κ-chains from murine myeloma (cat. no. M1421; Sigma-Aldrich; Merck KGaA). An immunogen volume of 500 μl containing 0.3 μg was mixed with 500 μl of either complete (cat. no. F5881; Sigma-Aldrich; Merck KGaA) or incomplete Freund's adjuvant (cat. no. F5506; Sigma-Aldrich; Merck KGaA). Three immunizations were applied for 7 days each, the first with the immunogen plus complete Freund's adjuvant and the last two with the immunogen plus with incomplete Freund's adjuvant. A total of 1 ml blood was collected once from the marginal vein for monitoring the immune response of rabbit. One week after the last immunization, 10 ml blood was collected from the central ear artery using a sterile syringe and applying 70% ethanol as a disinfectant and 1 ml (1% w/v) lidocaine (Pisacaina®1%; PiSA Pharmaceutical) as a local anesthetic subcutaneously. Blood was centrifuged at 515 x g for 10 min at room temperature and serum collected was aliquoted and stored at -70˚C. Thereafter, euthanasia was conducted by an intraperitoneal injection of a sodium pentobarbital overdose (100 mg/kg body weight), death was confirmed by detecting lack of pulse, breathing, corneal reflect, cardiac arrest and sphincter distention.

For sandwich-type ELISA, coating was performed by overnight incubation at 4˚C with 0.1 μg/ml rabbit polyclonal anti-myeloma mouse IgA antibody (prepared as described above) for total IgA, 0.4 μg/ml goat polyclonal anti-SC (N-16) antibody (cat. no. sc-20485; Santa Cruz Biotechnology, Inc.) for secretory IgA or 0.1 μg/ml rabbit polyclonal anti-mouse IgG secondary antibody (cat. no. 61-6000; Invitrogen; Thermo Fisher Scientific, Inc.) for total IgG in 0.1 M carbonate-bicarbonate buffer (pH 9.6). A total of five rounds of washes with PBS containing 0.05% Tween-20 (PBST) were performed before and after each incubation step at 37˚C. Blocking was performed with 3% skimmed milk in 0.1 M carbonate-bicarbonate buffer (pH 9.6), and incubation for 2 h at 37˚C. For the samples, two-fold sample dilutions in a saline solution were tested in duplicate and incubated for 1 h at 37˚C. The conjugation step was performed by 1 h incubation at 37˚C with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA (1:5,000; cat. no. 626720; Invitrogen; Thermo Fisher Scientific, Inc.) for total and secretory IgA or HRP-conjugated rabbit anti-mouse IgG (1:5,000; cat. no. 816720; Invitrogen; Thermo Fisher Scientific, Inc.) for total IgG antibodies. A volume of 10 μl 30% hydrogen peroxide (cat. no. H1009; Sigma-Aldrich; Merck KGaA) was mixed with 10 mg of O-phenylenediamine (cat. no. P9029; Sigma-Aldrich; Merck KGaA) in 50 ml phosphate-citric acid citrate buffer (0.05 M, pH 5.0) was prepared as the substrate and incubated at room temperature in the dark for 20 min. The enzymatic reaction was stopped with 2.5 M sulfuric acid (cat. no. 320501; Sigma-Aldrich; Merck KGaA). For normalization purposes, the absorbance value (λ=490 nm) of each sample was divided by the absorbance of an internal control (pool of intestinal liquid samples from mice treated with bLf) and multiplied by the corresponding dilution factor. The data from six mice per group are reported in relative units as the mean ± SD.

For the evaluation of specific IgA and IgG levels, an anti-bLf coating was performed with 0.2 μg/ml of bLf using carbonate-bicarbonate buffer (pH 9.6) and the same incubation conditions. Thereafter, the successive steps followed those described for the total antibody ELISAs, but using HRP-conjugated anti-mouse IgA (1:5,000; cat. no. 626720; Invitrogen; Thermo Fisher Scientific, Inc.) or HRP-conjugated anti-mouse IgG (1:5,000; cat. no. 816720; Invitrogen; Thermo Fisher Scientific, Inc.) antibodies. The absorbance of the samples was divided by the absorbance of the internal standard and multiplied by the corresponding dilution factor. Data are expressed in relative units as the mean and SD.

Western blot analysis. Epithelial cell protein extracts were examined by western blot analysis to determine the expression of the α-chain component of IgA and pIgR, which is involved in IgA-mediated transcytosis (27). The expression of the 78-kDa and 120-kDa forms of the pIgR protein were measured (28). Epithelial cells were purified as following: Intestinal segments were everted to expose the luminal surface and mixed with 1.5 mM EDTA (pH 7.3) plus 1% antibiotic-antimycotic (cat. no. 15240062; Gibco; Thermo Fisher Scientific, Inc.) and 1% FBS (cat. no. 10437; Gibco; Thermo Fisher Scientific, Inc.) in RPMI-1640 medium (cat. no. 11875085; Gibco; Thermo Fisher Scientific, Inc.). Thereafter, the samples were incubated for 30 min at 37˚C in a water bath at 50 x g, and then a gentle disaggregation was applied using a syringe rubber plunger before passing the cell suspension through a 100-μm cell strainer (cat. no. Z742101; Sigma-Aldrich; Merck KGaA) to remove the larger debris. The cell suspension was centrifuged at 515 x g for 15 min at 4˚C, the supernatant was removed, and the cell pellet was resuspended in RPMI-1640 medium, passed through a gauze and then centrifuged as before. The cell pellet was subsequently suspended in 20% Percoll (HyClone; Cytiva) and this mixture was overlaid upon 40% Percoll and then centrifuged at 515 x g for 30 min at 4˚C. The epithelial cells were recovered at the interphase between 20 and 40% and then washed with PBS and then centrifuged as indicated above. From each group (n=6), epithelial samples of two mice were mixed and three pools were obtained and analyzed in duplicate. The epithelial cells were treated with protease inhibitor cocktail (cat. no. 11836153001; Roche Diagnostics GmbH) and disrupted by sonication with one 10 sec pulse at 100 W amplitude (Fisher Sonic Dismembrator Model 3000). The samples were then centrifuged at 10,000 x g for 10 min at 4˚C and the supernatants were collected and stored at -70˚C.

Total protein concentration was quantified using Bradford reagent and 20 μg protein/lane was separated via 10% SDS-PAGE at 88 V for 2 h. The separated proteins

Table I. Nucleotide sequences of primers used for reverse transcription-quantitative PCR.

Primer name	Sequence (5'→3')	Sequence (3'→5')
α-chain	acctcagtcaccgtctcctc	cggaagggaagtaatcgtga
J-chain	gaactttgtataccatttgtcagacg	ctgggtggcagtaacaacct
pIgR	ctgtgcccgaaactggat	tcaggttggcttcttgtatgag
IL-2	gctgttgatggacctacagga	ttcaattctgtggcctgctt
IL-4	catcggcattttgaacgag	cgagctcactctctgtggtg
IL-5	acattgaccgccaaaaagag	atccaggaactgcctcgtc
IL-6	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa
18S ribosomal RNA	gcaattattccccatgaacg	gggacttaatcaacgcaagc

were transferred onto PVDF membranes and blocked with 5% milk/1X TBST at room temperature for 1 h. The membranes were then incubated at room temperature for 1 h with primary antibodies [rabbit HRP-conjugated anti-mouse IgA (1:10,000; cat. no. 626720; Invitrogen; Thermo Fisher Scientific, Inc.), goat polyclonal anti-human SC (N-16) antibody (1:400; cat. no. sc-20485; Santa Cruz Biotechnology, Inc.) to detect pIgR or goat polyclonal anti-actin C-11 antibody (1:500; cat. no. SC-1615; Santa Cruz Biotechnology, Inc.)]. Following the primary antibody incubation, the membranes were washed with TBST (thrice) and incubated with a secondary antibody just for SC and actin [1:5,000; HRP-conjugated rabbit anti-goat polyclonal IgG (H+L; cat. no. 61-1620; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The membranes were washed with TBST (thrice) and protein bands were detected by chemiluminescence, which was carried out using a streptavidin-HRP conjugate (cat. no. 18-152; Millipore; Merck KGaA) and the luminol-based SuperSignal West Femto substrate (cat. no. 3409; Thermo Fisher Scientific, Inc.). The protein bands were detected using an ImageQuant LAS 4000 instrument (GE Healthcare), and the bands were semi-quantified using the ImageQuant TL v8.1 software (Amersham; Cytiva). The data are reported in relative units and were computed by dividing the signal of each band (α-chain, 60 kDa; pIgR, 78 and 120 kDa) by the signal of the actin band (42 kDa). The data were normalized with the control basal group and reported as the mean ± SD.

RT-qPCR. RT-qPCR was performed to assess the expression of regulatory ILs involved in the generation of IgA-associated proteins (α- and J-chains) and pIgR (27,29). RNA extraction, cDNA synthesis and qPCR for α-chain, J-chain, pIgR and IL-2, -4 and -6 (30) and IL-5 were performed. Briefly, total RNA from whole mucosa samples was extracted using TRIzol® reagent (50 mg/ml; Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's protocol. Reverse transcription was performed using M-MLV reverse transcriptase (cat. no. 28025-021; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol at 37˚C for 50 min. qPCR was subsequently performed using a LightCycler TaqMan Master reaction mix (cat. no. 04 535 286 001; Roche Diagnostics GmbH) in a LightCycler instrument (Roche Diagnostics GmbH). The following thermocycling conditions were used: Initial denaturation at 95˚C for 10 min; followed by 45 cycles for amplification at 95˚C for 10 sec, 60˚C for 35 sec and 72˚C for 1 sec; and 1 cycle for cooling at 40˚C for 30 sec.

Original specific oligonucleotide primers were generated by using the online assay design software ProbeFinder (https://lifescience.roche.com/en_mx/brands/universal-probe-library.html) as previously reported (30). The nucleotide sequence of primers used for RT-qPCR are shown in Table I. Universal ProbeLibrary probes are all from Roche Diagnostics GmbH (α-chain, probe #27, cat. no. 04687582001; J-chain, probe #11, cat. no. 04685105001; pIgR, probe #80, cat. no. 04689038001; IL-2, probe #15, cat. no. 04685148001; IL-4, probe #2, cat. no. 04684982001; IL-5, probe #91, cat. no. 04692080001; IL-6, probe #6, cat. no. 04685032001; 18S ribosomal RNA, probe #48, cat. no. 04688082001. The samples were analyzed in duplicate and the mRNA expression levels were calculated by using the comparative parameter quantification cycle method and normalized to the level of the 18S ribosomal RNA subunit (31).

Corticosterone assay. Plasma corticosterone expression levels were determined using a commercially available Corticosterone ELISA kit (cat. no. 901-097; Enzo Life Sciences, Inc.). Two-fold sample dilutions were analyzed in duplicate, and corticosterone concentrations were calculated from a standard curve and are reported in ng/ml as the mean ± SD of 6 mice per group.

Statistical analysis. Three independent assays were performed, and the results are expressed as the mean ± SD from one representative assay of 8 groups with 6 mice each. Data comparisons were analyzed by one-way ANOVA followed by a Holm-Šidák pos hoc test. All analyses were performed using the statistical program SigmaPlot version 11 (Systat Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

bLf counteracts the effect of stress on the increase of total IgA and IgG responses. Analysis between the basal groups indicated that the levels of total IgA ($P<0.01$) and SIgA ($P<0.001$) were significantly greater in the stressed group compared with levels in the control group. Compared with the corresponding basal group, the expression levels of total IgA, SIgA (at all bLf dosages) and specific IgA (50 μg bLf) were significantly lower in the bLf and stress treated groups ($P<0.001$, except total IgA

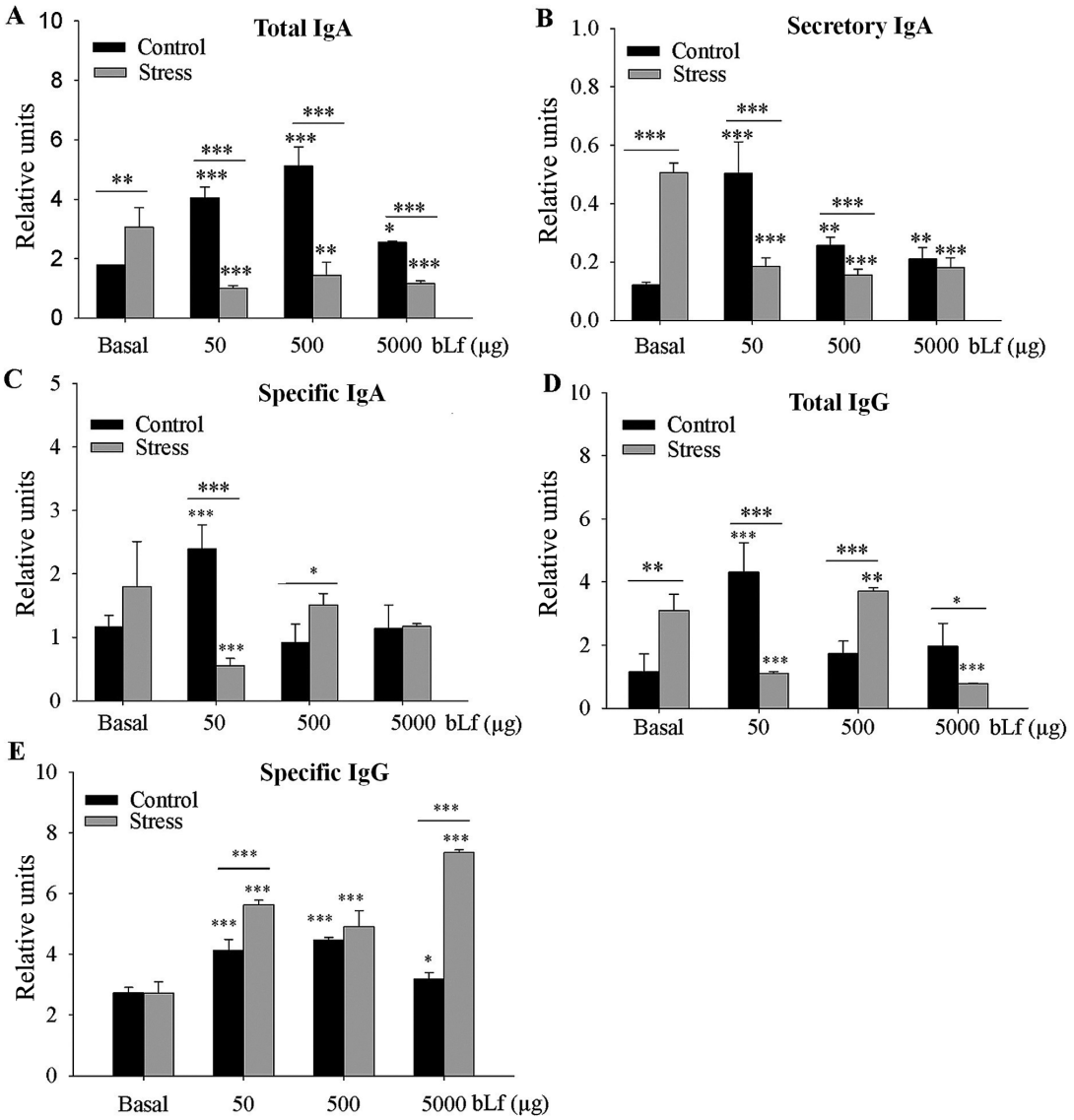

Figure 1. Concentrations of (A) total IgA, (B) secretory IgA, (C) specific IgA, (D) total IgG and (E) specific IgG antibodies in the distal small intestine of mice that underwent chronic immobilization stress and treatment with 50, 500 or 5,000 μg bLf. Unstressed control and stressed mice not given bLf were included in the basal groups. Data are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 vs. the respective basal group or comparisons indicate by the lines. bLf, bovine lactoferrin; IgA, immunoglobin A; IgG, immunoglobin G.

500 μg bLf, P<0.01). In comparison with their corresponding basal group, expression levels of all bLf dosages of total IgA (Fig. 1A), SIgA (Fig. 1B) and specific IgA at 50 μg bLf (Fig. 1C) were significantly higher in control bLf-treated mice (P<0.001); total IgA treated with 5,000 μg bLf (P<0.05) and SIgA treated with 500 or 5,000 μg bLf (P<0.01) were also significantly higher. Within bLf-treated groups expression levels of total IgA at all bLf treatment doses and SIgA treated with 50 or 500 μg bLf were significantly lower (P<0.001) in stressed mice compared with control mice; the specific-IgA expression levels were significantly lower (P<0.001) when treated with 50 μg bLf, or significantly higher (P<0.05) when treated with 500 μg bLf, in the stressed compared with the unstressed mice.

Between the basal groups, the total-IgG expression levels were significantly higher (P<0.05) in the stressed mice compared with the control mice (Fig. 1D). In comparison with their corresponding basal group, total IgG expression levels were significantly greater in the control mice treated with 50 μg bLf (P<0.001) and stressed mice treated with 500 μg bLf (P<0.05), whereas total IgG expression was significantly lower in stressed mice treated with 50 or 5,000 μg bLf (P<0.001). All bLf-treated mice had significantly higher expression levels of specific-IgG compared with their corresponding basal groups (all P<0.001, except P<0.05 for control mice treated with 5,000 μg of bLf; Fig. 1E). Within bLf-treated groups anti-bLf IgG expression levels were significantly higher in stressed mice treated with 50 or 5,000 μg bLf compared with the control mice with the same bLf treatment (P<0.001).

bLf counteracts the effects of stress on increase protein expression levels of α-chain and 78-kDa pIgR. Within the basal groups, α-chain (P<0.01; Fig. 2A) and 78-kDa pIgR (P<0.001; Fig. 2B) protein expression levels were significantly higher in the stressed compared with the control mice. In comparison with the basal group of the stressed mice, expression levels of the α-chain treated at any dose (Fig. 2A) and 78-kDa pIgR

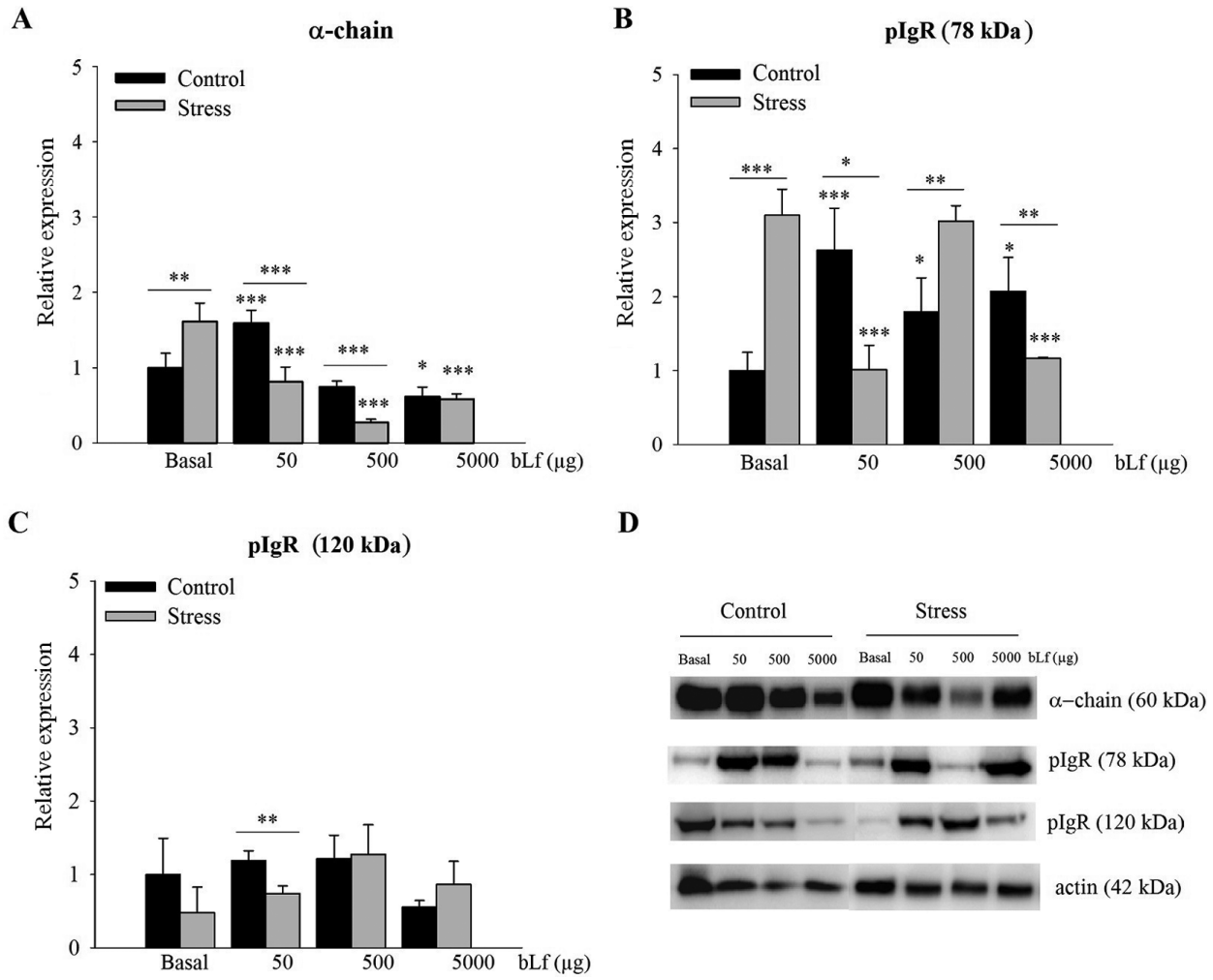

Figure 2. Relative protein expression levels of (A) α-chain, (B) 78-kDa pIgR and (C) 120-kDa pIgR in the distal small intestine of mice that underwent chronic immobilization stress and treatment with bLf at different doses. (D) Representative scans of blots for the proteins and actin control from the control and experimental groups were included. Unstressed control and stressed mice not given bLf were included in the basal groups. Data are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 vs. the respective basal group or comparisons indicate by the lines. bLf, bovine lactoferrin; pIgR, polymeric immunoglobulin receptor.

treated with 50 or 5,000 μg of bLf (Fig. 2B) were significantly lower in bLf-treated mice (P<0.001); no significant difference was detected in the expression of 78-kDa pIgR of mice treated with 500 μg bLf (Fig. 2B). In the control group, α-chain protein expression in mice treated with 50 μg bLf (Fig. 2A) and 78-kDa pIgR expression in mice treated with any bLf dose (Fig. 2B) were significantly greater in the treatment groups compared with the basal group (P<0.001 for both at 50 μg bLf; P<0.05 for 78-kDa pIgR at 500 or 5,000 μg bLf). Notably, the expression of the α-chain was significantly lower when treated with 5,000 μg bLf, compared with the basal control (P<0.05; Fig. 2A) in bLf-treated control mice.

Comparisons within the bLf-treated groups indicated that in stressed mice, the relative protein expression levels of α-chain following treatment with 50 μg bLf (P<0.01) or 500 μg bLf (P<0.001) (Fig. 2A), 78-kDa pIgR following treatment with 50 μg bLf (P<0.05) or 5,000 μg bLf, (P<0.01) (Fig. 2B), and 120-kDa pIgR in mice treated with 50 μg bLf (P<0.01) (Fig. 2C) were significantly lower, whereas 78-kDa pIgR protein expression was significantly higher following treatment with 500 μg bLf (P<0.01; Fig. 2B). Representative western blotting images are presented in Fig. 2D.

bLf counteracts the effects of stress on increased α-chain and 78-kDa pIgR mRNA expression levels. Within the basal groups, α-chain (P<0.05), J-chain (P<0.05) and pIgR (P<0.001) mRNA expression levels were significantly higher in the stressed compared with the control mice (Fig. 3). In comparison with their corresponding basal group, α-chain mRNA expression levels were significantly elevated following treatment with 5,000 μg bLf in both the control (P<0.001) and stressed (P<0.01) groups (Fig. 3). Notably, following treatment with 50 or 500 μg bLf, stressed mice experienced a significant reduction in α-chain mRNA expression (P<0.001; Fig. 3A). The mRNA expression levels of J-chain following treatment at all bLf doses (P<0.001; Fig. 3B) and pIgR treated with 500 μg bLf (P<0.01; Fig. 3C) in the bLf-treated control mice were significantly elevated. By contrast, J-chain mRNA expression following treatment with 5,000 μg bLf (P<0.05; Fig. 3B) and pIgR mRNA expression levels treated with 50 μg bLf (P<0.05), 500 μg bLf (P<0.001) or 5,000 μg bLf (P<0.01) were significantly lower in the stressed bLf-treated mice compared with the basal control (Fig. 3C). Analysis within the bLf-treated groups showed that mRNA expression levels of α-chain following treatment with 50 or 5,000 μg bLf (P<0.01)

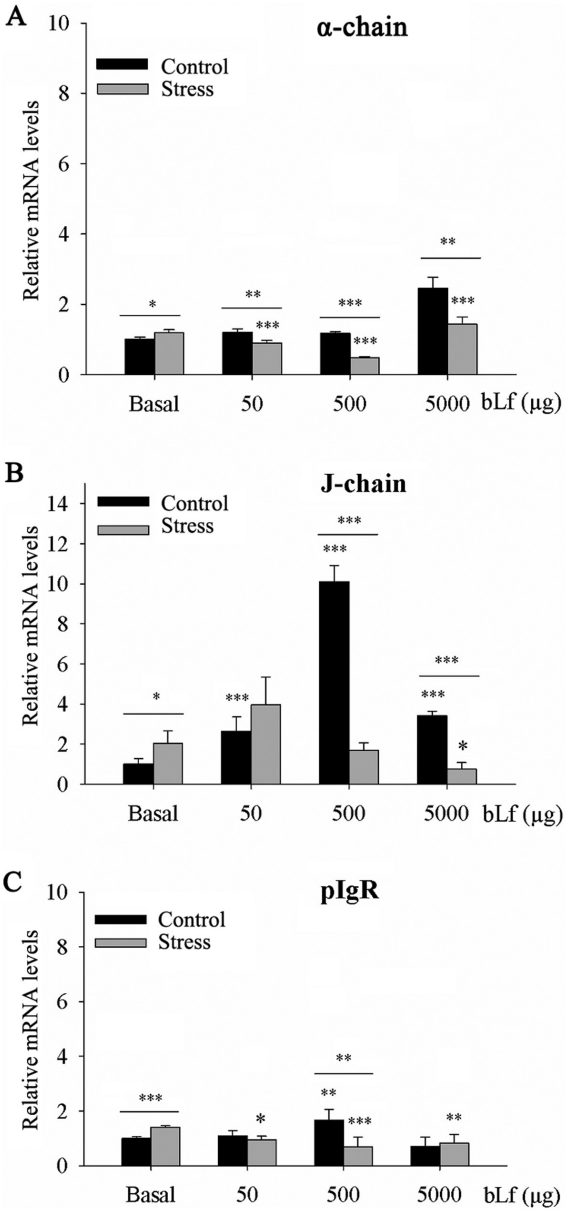

Figure 3. Relative mRNA expression levels of (A) α-chain, (B) J-chain and (C) pIgR in the distal small intestine of mice that underwent chronic immobilization stress and treatment with bLf. Unstressed control and stressed mice not given bLf were included in the basal groups. Data and are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 vs. the respective basal group or comparisons indicate by the lines. bLf, bovine lactoferrin; pIgR, polymeric immunoglobulin receptor.

or 500 μg bLf (P<0.001), the J-chain expression following treatment with 500 or 5,000 μg bLf (P<0.001), and pIgR in mice treated with 500 μg bLf (P<0.01) were lower in the stressed mice compared with the unstressed mice (Fig. 3A-C).

bLf does not counteract the effects of stress on the mRNA expression of IgA-associated ILs. Within the basal groups, stressed mice showed significantly elevated IL-6 mRNA expression levels (P<0.01; Fig. 4D) and lower IL-2 (P<0.01; Fig. 4A) and IL-4 (P<0.001; Fig. 4B) mRNA expression levels. In comparison with the corresponding basal group, stressed mice had elevated IL-2 mRNA when treated with 5,000 μg bLf (P<0.01; Fig. 4A) and significantly lower IL-5 and IL-6 mRNA expression at all bLf dosages (P<0.001; Fig. 4C and D). In comparison with the basal group, the control mice had significantly elevated IL-2 mRNA expression levels at 50 μg bLf (P<0.001) and 5,000 μg bLf (P<0.01) (Fig. 4A) and significantly lower IL-4 mRNA expression levels when treated with 5,000 μg bLf (P<0.001; Fig. 4B). There were no statistically significant differences in the mRNA expression levels of IL-5 or IL-6 of the control mice at any concentration of bLf treatment (Fig. 4C and D). Within the bLf-treated mice, stressed mice had lower expression levels of IL-2, IL-4 and IL-5 when treated with 50 or 500 μg bLf (all P<0.001, except P<0.01 IL-2 500 μg bLf; Fig. 4A-C) and significantly lower expression levels of IL-6 mRNA when treated with 500 or 5,000 μg bLf (P<0.001; Fig. 4D). Moreover, stressed mice had greater IL-4 mRNA expression levels when treated with 5,000 μg bLf (P<0.001; Fig. 4B).

bLf does not counteract the effect of stress on the elicitation of corticosterone levels. Analyses of the expression levels of corticosterone, which is used as a stress biomarker (5), indicated that within the basal groups the concentration of corticosterone were found to be significantly greater in the stressed mice compared with the control mice (P<0.001). Furthermore, in comparison with the corresponding basal groups, the unstressed control mice treated with 5,000 μg bLf had a significantly higher corticosterone expression level (P<0.001). Within the bLf-treated groups, the stressed mice treated with 50 or 500 μg bLf had a significantly elevated corticosterone concentration compared with the control mice receiving the same treatment (P<0.001) (Fig. 5).

Discussion

The effects of chronic stress on parameters associated with the IgA antibody response have mostly been analyzed in the colon (3,7,9,10,32) and, to a lesser extent, in the full-length small intestine (5), jejunum (6), duodenum or ileum (11). In addition, the modulatory properties of bLf on the antibody response have been documented in the distal and full-length small intestine of healthy mice (13-15). Moreover, the effects of chronic stress in mice treated with bLf have been demonstrated by an increased response of splenic antibody-secreting cells (20). The present study aimed to address the impact of chronic stress on some parameters associated with the IgA response in the distal small intestine under conditions of bLf treatment.

Chronic stress has a substantial upregulatory effect on the generation of IgA, as previously found in the distal small intestine and mesenteric lymph nodes (8,11). In the present study, overall data analysis within the bLf treated groups indicated that, in general terms, the upregulatory effects of chronic stress on the concentration of total antibodies (IgA, SIgA and IgG), α-chain protein and the mRNA expression of α-chain and J-chain were reduced by bLf. These findings may reflect a protective role of bLf against increased gut permeability, causing an increase in luminal IgA, as previously found in the ileal content of mice that underwent massive bowel resection and as supported by *in vitro* assays (33,34). No significant effects of chronic stress on specific IgA and IgG antibody expression levels were observed; however, specific

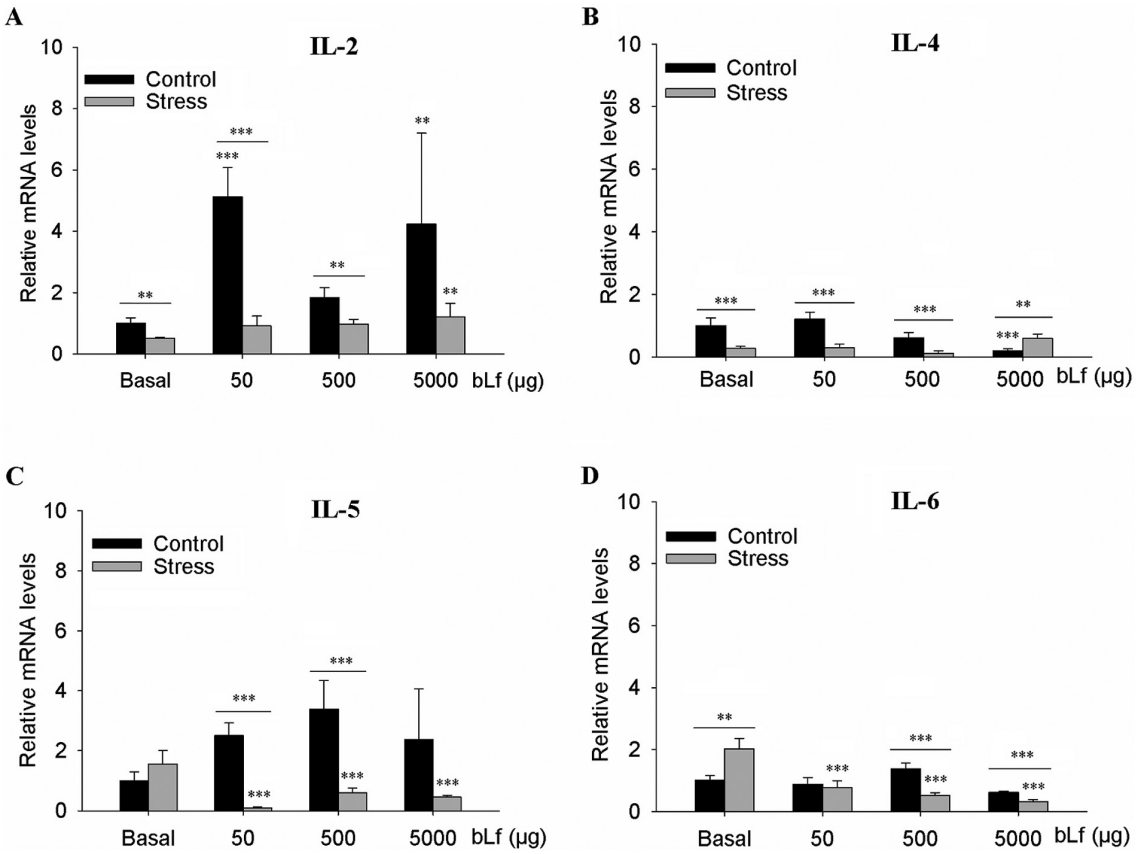

Figure 4. Relative mRNA expression levels of (A) IL-2, (B) IL-4, (C) IL-5 and (D) IL-6 in the distal small intestine of mice that underwent chronic immobilization stress and treatment with bLf. Unstressed control and stressed mice not given bLf were included in the basal groups. Data are expressed as the mean ± SD. **P<0.01 and ***P<0.001 vs. the respective basal group or comparisons indicate by the lines. bLf, bovine lactoferrin.

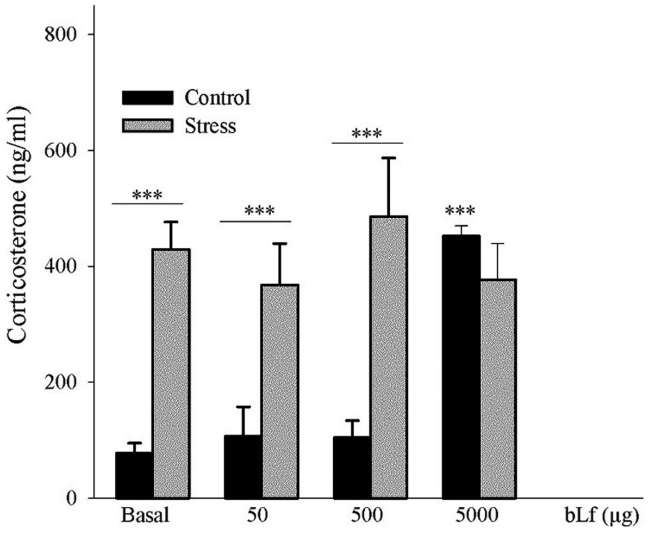

Figure 5. Plasma concentrations of corticosterone (ng/ml) in mice that underwent chronic stress and treatment with different bLf doses and in the unstressed and stressed groups that did not receive bLf treatment. Data are expressed as the mean ± SD. ***P<0.001 vs. the respective basal group or comparisons indicate by the lines. bLf, bovine lactoferrin.

IgG expression levels were triggered by bLf with and without chronic stress. The biological meaning is currently unknown, but the luminal increase of serum-derived IgG may provide a strategy of protection like that described in aged mice (35).

In the present study, the effect of chronic stress on the induction of 78-kDa pIgR protein and pIgR mRNA expression was also decreased by bLf. Similar to the IgA response, the downregulation of pIgR expression may result from the control mechanism of bLf when gut permeability is compromised under conditions of chronic stress. bLf induces an increased number of positive IL-4-TCD3^{+}CD4^{+} cells localized at the distal region of the small intestine (13) and is also known to induce the IL-4 response in the full-length small intestine of healthy mice (15). Chronic stress, by contrast, is known to downregulate the expression level of IL-4 mRNA, as previously described in the jejunum of heat-stressed rats (6). IL-4 activates signaling pathways that upregulate pIgR (28). Moreover, stress hormones such as corticosterone elicit an increase in pIgR mRNA expression levels, as previously found in the proximal intestine of suckling rats (36). In the present study, bLf did not counteract the impact of chronic stress on the decrease of IL-4 mRNA expression levels and on corticosterone triggering; it appears that the downregulation of pIgR mRNA expression levels by bLf under conditions of chronic stress involves pathways independent of IL-4 and corticosterone.

IL-4, IL-2 and IL-5 are known to be involved in signaling pathways for the generation of IgA (31). IL-2 has a key role in the transcription of the J-chain of dimeric IgA (28). Both IL-4 and IL-5 enhance the IgA class-switching of B cell precursors, elicit autocrine TGF-β release by B cells primed by CD40 ligand and promote the proliferation and plasma cell

differentiation of IgA^+ B cells (31). bLf induces the generation of not only IL-4 but also IL-2, as previously found in the full-length small intestine of healthy mice (15), whereas chronic stress has been shown to decrease IL-2 transcription in the jejunum of heat-stressed rats (6). In the present study, bLf did not counteract the downregulatory impact of chronic stress on IL-2 mRNA as was found for IL-4 mRNA. Under stress conditions, the downregulatory properties of bLf on the transcription of IL-2 and IL-4 mRNA may underlie the decreased antibody response.

Chronic stress is known to increase circulatory levels of IL-6 (37). IL-6 is a pleiotropic factor for IgA generation but is also a potent stimulator of corticosterone through a CRH-independent pathway (38). The elicitation of corticosterone by chronic stress has previously been associated with a decrease in the IgA response (3,5,8,9). In the present study, IL-6 mRNA expression levels elicited by chronic stress were reduced by treatment with bLf, and the elevation of corticosterone levels induced by chronic stress was not significantly affected by any dose of bLf. These findings may reflect a regulatory role of bLf on IL-6 and corticosterone responses to decrease the luminal IgA response and thus maintain gut homeostasis under stress conditions. In the present study, unstressed control mice treated with 5,000 μg of bLf was found to elevate serum corticosterone levels. These findings are in line with an upregulatory effect of bLf on endogenous corticosterone that relies on, in part, the propensity of bLf to elicit a Th2-associated IL profile (22). As suggested, bLf acts as a HPA axis stimulator to cause corticosterone release in healthy mice, potentially by interacting with receptors on cells in the central nervous system (22).

In the present assay, dose-dependent effects of bLf were not detected, resulting in part from the extent of the luminal elimination of bLf complexes that accumulate through the uptake of bLf and/or the removal of bLf-complexes generated by the chronic ingestion of bLf; the clearance of bLf complexes by immune exclusion may prevent their entry into systemic compartments with potentially harmful outcomes (39). Apparent inconsistencies were found between the production and mRNA expression of parameters of the IgA response evoked by chronic stress in the presence of bLf. These findings may result from the potential ability of bLf to elicit the local response of corticosterone. Indeed, corticosterone is known to be released by the epithelial monolayer of the full-length small intestine in mice (40). Moreover, bLf interacts with toll-like receptor (TLR)4, which is expressed in immune competent cells (41). In the intestinal epithelium, TLR4 expression is higher in the distal than in the proximal small intestine (42). Thus, the inconsistent data found in the present study may reflect the impact of chronic stress on the modulation of IgA and pIgR by bLf and their interplay with microbiota and TLR4 (43,44).

Although an obvious limitation involves the assessment of ILs only following transcription, the current study may be an experimental representation of human disorders such IBS that are associated with disturbances in the luminal microbiota, which exhibits a gradient that increases from the proximal to the distal small intestine (24,25). In conclusion, results from the present study indicated that bLf may counteract the upregulatory impact of chronic stress on the elevation of most parameters associated with the IgA response but at a dose of 5,000 μg, elevates the expression of corticosterone and the specific IgG antibody response at 50, 500 and 5,000 μg.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

TRCH performed the ELISAs. DCGJ performed the reverse transcription-quantitative PCR assays. RCR made substantial contributions to the conception and design of the study. MGV conducted the western blot assays, and the acquisition, analysis and interpretation of data. MEDS participated in the design of the experiments, and performed the analysis and interpretation of the data, was involved in drafting the manuscript and revised it critically for important intellectual content. Each author participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animals were handled in accordance with Mexican federal regulations for animal experimentation and care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico City, Mexico). The protocol was approved by the Institutional Animal Care and Use Committee of the Instituto Politécnico Nacional (Mexico City, Mexico).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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