

Immunostimulatory potential of β -lactoglobulin preparations: Effects caused by endotoxin contamination

Susanne Brix, MS,^a Lionel Bovetto, PhD,^b Rodolphe Fritsché, PhD,^b
Vibeke Barkholt, PhD,^a and Hanne Frøkiaer, PhD^a Lyngby, Denmark, and Lausanne,
Switzerland

Background: The immunomodulating potential residing in cow's milk proteins is currently receiving increasing attention because of growing interest in functional foods and the complex problem of cow's milk allergy. One of the major cow's milk allergens, whey protein β -lactoglobulin, has previously been shown to mediate cellular activation in both human and murine immune cells.

Objective: We examined the response to different β -lactoglobulin preparations in naive immune cells.

Methods: Splenocytes and cells from mesenteric lymph nodes derived from BALB/c mice bred and maintained on a milk-free diet were cultured in vitro with different β -lactoglobulin preparations. Cell proliferation, cytokine production, and increases in intracellular glutathione were used as cellular activation markers. Moreover, the effect of β -lactoglobulin on cytokine production in murine bone-marrow-derived dendritic cells was examined.

Results: We observed that some commercial β -lactoglobulin preparations induced pronounced proliferation of both spleen cells and cells from mesenteric lymph nodes; production of TNF- α , IL-6, IL-1 β , and IL-10; and an increased level of intracellular glutathione in spleen cell cultures. Furthermore, TNF- α , IL-6, IL-1 β , and IL-10 production was induced in murine bone-marrow-derived dendritic cells. Purification of β -lactoglobulin from raw milk using nondenaturing conditions, however, revealed that the β -lactoglobulin per se did not possess the immunomodulatory activity. Eventually, the immunostimulatory effect was found to be caused by endotoxin contamination.

Conclusion: These results identify endotoxin as the main immunostimulatory component present in some commercial β -lactoglobulin preparations. Moreover, the present study makes it evident that immunomodulatory effects attributed to β -lactoglobulin need to be reassessed. (*J Allergy Clin Immunol* 2003;112:1216-22.)

Key words: β -Lactoglobulin, bioactive, immunomodulatory, endotoxin, lipopolysaccharide, milk proteins

Abbreviations used:

CBMC: (Human) cord blood mononuclear cell
DC: Dendritic cell
GSH: Glutathione (reduced)
 α -LA: α -Lactalbumin
 β -LG: β -Lactoglobulin
MBM: Murine bone marrow (cells)
MLN: Mesenteric lymph node (cell)
OVA: Ovalbumin
PMB: Polymyxin B

Cow's milk contains proteins that are reported to possess immunomodulatory activity¹⁻⁵ and, at the same time, are recognized as food allergens in susceptible individuals.^{6,7} Among the milk proteins, whey protein β -lactoglobulin (β -LG), 1 of the major allergens of cow's milk,⁸ has been observed specifically to stimulate the proliferation of ex vivo cultured human cord blood mononuclear cells (CBMCs),⁹⁻¹⁵ PBMCs,¹⁵⁻²⁰ lamina propria lymphocytes,¹⁹ and Peyer's patches mononuclear cells,²¹ as well as to induce specific proliferation and IgM production in murine immune cells.⁵ In addition, cytokine production was observed in human CBMCs (IFN- γ ¹⁰ and IL-5, IL-10, IL-13, and IFN- γ ²⁰), PBMCs (TNF- α ; IL-10¹⁹; and IL-5, IL-10, and IL-13²⁰; as well as IFN- γ ^{20,22}), lamina propria T cells (IL-2, TNF- α , IL-10, and IFN- γ ¹⁹), and Peyer's patches (IFN- γ ²¹) after ex vivo exposure to commercial preparations of purified β -LG. In an attempt to explain these observations, the results observed with CBMCs were taken as evidence for in vivo priming of naive immune cells toward β -LG because of maternal intake of cow's milk products.^{11,13} Nevertheless, conflicting data emerge when focusing on the relevance of the ex vivo immunostimulatory activity of β -LG in relation to cow's milk allergy. In some studies, the β -LG proliferative response of CBMCs was found to be higher in atopy-prone newborns compared with non-atopy-prone control newborns,^{9,10} whereas in other studies, no differences were observed.^{12-14,23} Thus, the significance of the ex vivo immunostimulatory activity of β -LG on the risk of developing cow's milk allergy remains to be clarified.

As a consequence, the aim of the present study was to contribute to the current understanding of the mechanisms involved in the ex vivo immunostimulatory effect exerted by β -LG. To examine whether the effect of β -LG was caused by antigen-specific cell proliferation or was dependent on a mitogenic activity residing in some com-

From ^athe BioCentrum-DTU, Technical University of Denmark, and ^bNestlé SA, Nestlé Research Center.

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Reprint requests: Dr Hanne Frøkiaer, BioCentrum-DTU, Biochemistry and Nutrition, Technical University of Denmark, DK-2800 Kgs Lyngby, Denmark.

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mercial β -LG preparations, we used cow's-milk-naïve immune cells. Because of the ubiquitous presence of cow's milk products in the human diet, the availability of truly cow's-milk-naïve human immune cells is very limited. Accordingly, the naïve immune cells were obtained from BALB/c mice bred and maintained on a strictly cow's-milk-free diet for at least 2 generations. Using such cells, we compared the immunostimulatory capacity of the most commonly used commercial β -LG preparations (Sigma-Aldrich) with that of other purified β -LG preparations.

MATERIALS AND METHODS

Animals

Female BALB/c mice (IFFA-Crédo, L'Abresle, France), 10 to 12 weeks old, were bred and raised on a strictly cow's-milk-free diet for several generations. Female C3H/HeJ and C3H/HeOJ mice were obtained from The Jackson Laboratory (Bar Harbor, Me) and were used at 10 weeks of age.

In Switzerland, the animal studies were performed according to the regulations of the Veterinary Service of the Canton de Vaud, Switzerland, and in Denmark, all animal studies were approved by the Danish Animal Experiments Inspectorate.

Antigens

β -LG (>90%, variant A+B, Sigma-Aldrich, St Louis, Mo; L-3908 and L-0130), casein (Sigma, C-5890), α -lactalbumin (α -LA, ~85%, Sigma, L-6010), bovine serum albumin ($\geq$ 99%, Sigma, A-7638), ovalbumin (OVA, >98%, grade V, Sigma, A-5503), and β -LG (>95%, variant A+B, Arla Foods, Videbæk, Denmark, PSDI-2400) were used.

Dialysis of β -LG

β -LG (Sigma), dissolved in water (MilliQ, Millipore) at 50 mg/mL, was dialyzed 3 times at 4°C using dialysis membranes (Spectra/Por, Gardena, Calif) with a 6- to 8-kDa or a 12- to 14-kDa cutoff.

Purification of β -LG from raw milk

Milk from a β -LG-heterozygous cow was received cold in the morning before pasteurization. After fat removal by centrifugation (5000g, 1 hour, 10°C), skimmed milk was acidified at pH 4.6 by acetic acid addition to eliminate casein. The whey supernatant was collected, and pH was adjusted to 7.0 with sodium hydroxide before freezing. The separation of whey proteins was accomplished using a 7-mL anionic ion-exchange column (D-ZEPHYR/10, IBF Biotechnics, Villeneuve la Garenne, France) and a nonlinear pH gradient from 7.0 to 4.0 generated by stepwise gradients of the A and B ammonium acetate buffers (A, 50 mmol/L, pH 7.0; B, 100 mmol/L, pH 4.0) performed as follows: 0% to 2.8% buffer B by use of 10 mL, 2.8% to 4.1% buffer B by use of 65 mL, and then 4.1% to 100% buffer B by use of 25 mL. The flow rate was 10 mL/min. The content of whey proteins in each fraction was analyzed using SDS-PAGE and HPLC, and fractions containing both variants (A and B) of β -LG were pooled. The pH was adjusted to 6.5 and the protein lyophilized. For a further purification step, the β -LG was dissolved in 50 mmol/L ammonium acetate, pH 6.0, at 10 mg/mL and subjected to size-exclusion chromatography on a Superdex 75 column (Hiload 26/60, AP Biotech, Uppsala, Sweden) using 150 mmol/L ammonium acetate, pH 6.0. The β -LG was collected in 2 peaks. The fractions were pooled, dialyzed (Spectra/Por, with a cutoff of 12 to 14 kDa) in water (MilliQ, Millipore) at 4°C, and lyophilized.

SDS-PAGE

Gel electrophoresis was performed using NuPAGE, Bis-Tris 4% to 12% gels (Invitrogen, Paisley, UK) on reduced (0.5 mol/L DTT) samples. Staining was performed with SilverXpress (Invitrogen, Paisley, UK) in accordance with the manufacturer's instructions.

Isolation and culture of spleen and MLN cells

Single-cell suspensions of spleen and mesenteric lymph nodes (MLNs) were prepared and suspended in culture medium (X-vivo 10, serum-free medium; BioWhittaker, Verviers, Belgium, supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L L-glutamine [all Life Technologies]). For proliferation, the spleen and MLN cells were cultured at 3×10^5 /well and 5×10^5 /well, respectively, in flat-bottomed, 96-well tissue-culture plates (polystyrene, Costar) and stimulated for 66 hours at 37°C, 5% CO₂, with various antigen concentrations or culture medium (background). In some experiments, LPS (*Escherichia coli* O55:B5, Sigma) was used at 10 μ g/mL. All stimulants were sterile filtered (0.22 μ m, Millex, Millipore). Cultures were pulsed with 1 μ Ci/well of [³H]thymidine (Amersham, Amersham, United Kingdom) after 48 hours and harvested 18 hours later. Stimulation index was calculated as the ratio of counts per minute for antigen-stimulated and background cultures.

To measure cellular glutathione (GSH) content and cytokine production, spleen cells were cultured at 2×10^6 /well in 24-well tissue-culture plates (polystyrene, Costar) with 1 mg/mL antigen or culture medium. Cells and supernatants were collected after 48 hours of stimulation, which was shown in preliminary experiments to be optimal for both GSH and the cytokine measurements. For GSH measurements, the cells were labeled immediately after collection, and for cytokine analysis, supernatants were stored at -80°C.

GSH measurements

Intracellular GSH content was measured by flow cytometry of cells labeled with the fluorescent GSH-reactive probe 5-chloromethylfluorescein diacetate (Molecular Probes, Eugene, Ore).

The following optimized staining procedure was used. Cells (1.5×10^6) gently suspended in 200 μ L of 37°C warm 5-chloromethylfluorescein-diacetate-containing culture medium (0.5 μ mol/L) were incubated for 25 minutes at 37°C, 5% CO₂. After washing, cells were incubated for a further 15 minutes in culture medium. After 2 additional washes in cold PBS (0.01 mol/L, pH 7.2), analysis was performed using a FACSScan flow cytometer with CellQuest software (BD Biosciences, San Jose, Calif). A total of 10,000 cells were counted for each analysis, and the results are presented as the percent increase in cellular thiol, calculated as the percentage of thiol-containing cells in antigen-stimulated cultures subtracted by that of background cultures.

Isolation and culture of MBM cells

Dendritic cells (DCs) were isolated and cultured from murine bone marrow (MBM) cells derived from C57BL/6 mice (M&B, Lille Skensved, Denmark) as previously described.²⁴ To induce cytokine production, 8-day-old, DC-enriched cultures were seeded in 48-well tissue-culture plates at 1.4×10^6 /well with 1 mg/mL of selected fractions from size-exclusion chromatography of β -LG (Sigma) or unfractionated β -LG (Sigma or AF) in duplicate. As a positive control, 1 μ g/mL LPS (*E coli* O26:B6, Sigma) was added. After stimulation of DCs for 19 hours at 37°C, 5% CO₂, the supernatants were collected and stored at -80°C until cytokine measurements.

Cytokine ELISA

The culture supernatants were assayed for TNF- α , IL-1 β , IL-6, and IL-10 content by ELISA (R&D Systems, Minneapolis, Minn) in accordance with the manufacturer's instructions.

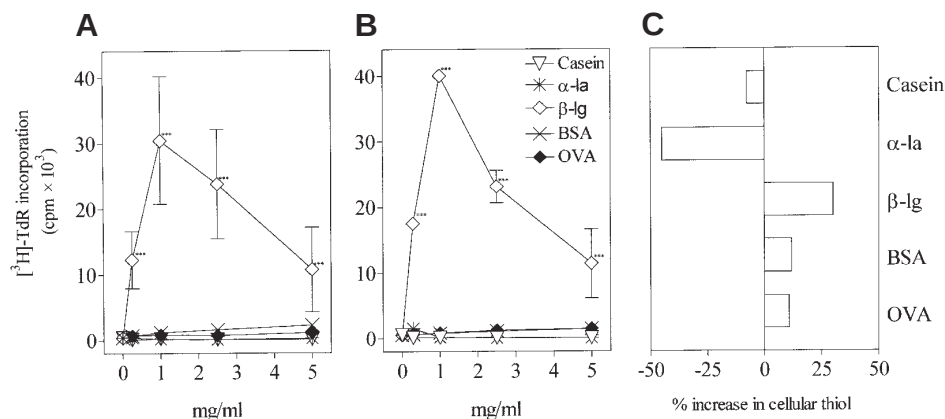


FIG 1. Induction of proliferation and intracellular thiol increase in cow's-milk-naïve immune cells after in vitro exposure to β -LG. Splenocytes and MLNs from BALB/c mice were cultured with various concentrations of the different milk proteins and OVA. **A**, Proliferation of spleen cells. **B**, Proliferation of MLNs. **C**, Intracellular thiol level in splenocytes cultured with 1 mg/mL protein. Data are mean $\pm$ SD for proliferation, $n = 6$. Differences in cell proliferation toward the proteins were analyzed for significance using 1-way ANOVA. *** $P < .001$ by Tukey's multiple-comparisons test.

Determination of endotoxin content

Protein preparations dissolved in endotoxin-free PBS (BioWhittaker) were tested for the presence of endotoxin using a *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, Md). LPS-spoke recovery experiments were carried out for all samples. The analyses were performed at the microbiologic test laboratory (AKA-MIK, Statens Serum Institut, Copenhagen, Denmark). The endotoxin content (endotoxin units per milligram protein) was calculated based on amino acid analysis of each protein sample, performed as previously described.²⁵

Statistical analysis

The data were tested for statistical significance using 1-way ANOVA (GraphPad Prism, version 3.02, GraphPad Software). If significant, data were further analyzed by multiple-comparison tests as described. $P \leq .05$ was considered significant.

RESULTS

Commercial β -LG induces stimulation in cow's-milk-naïve immune cells

To assess and compare the effect of β -LG with that of other cow's milk proteins on proliferation of cow's-milk-naïve immune cells, spleen cells and cells from MLNs derived from BALB/c mice bred and maintained on a cow's-milk-free diet were cultured in vitro with the commercially available milk proteins casein, α -LA, BSA, and β -LG (Fig 1, A and B). The egg white protein ovalbumin (OVA) was included as a control nonmilk food protein.

The results showed a significantly higher ($P < .001$) dose-dependent proliferation induced by β -LG in both spleen and MLN cells when compared with the other milk proteins and OVA (Fig 1, A and B). None of the other proteins tested gave rise to a significantly enhanced proliferation in spleen and MLN cells in comparison with unstimulated cells.

Furthermore, as another indicator of immunostimulation,²⁶ we evaluated the effect of the selected milk proteins

and OVA on the cellular GSH content (Fig 1, C). The results showed an elevated thiol level in cells stimulated with commercial β -LG when compared with the other antigen preparations tested. Interestingly, the commercial α -LA exhibited the capacity to reduce the level of intracellular thiol substantially when compared with unstimulated cells.

Taken together, these data confirmed the presence of an immunostimulating component in the commercial β -LG preparation capable of activating cow's-milk-naïve immune cells in a mitogenic manner. The effect of the commercial bovine α -LA preparation on murine immune cells was not further examined in the present study.

Purified β -LG from raw milk exerts no immunostimulatory effect

To elucidate whether the effect of the commercial β -LG preparation was caused by β -LG per se or alternatively caused by mitogenic contaminants present in the commercial preparation, we used various strategies to remove possible contaminants from β -LG. One approach was to purify β -LG from raw milk, whereas another was to dialyze the commercial β -LG preparation to eliminate any lower-molecular-weight immunostimulatory components, such as polyamines.²⁷

The purification of β -LG from whey proteins was performed in 2 chromatographic steps using nondenaturing conditions (Fig 2, A and B). The purity of the β -LG was analyzed by SDS-PAGE (Fig 2, C), and the presence of both the A and B variants of β -LG was confirmed by HPLC (data not shown). Both the commercial β -LG and the β -LG purified from raw milk were shown by size-exclusion chromatography to be primarily of the physiologically predominant dimeric form at pH 6.0 (Fig 2, B).

Immunomodulatory effect of the 3 β -LG preparations

Commercial β -LG (Sigma), dialyzed commercial β -LG (Sigma), and purified β -LG from raw milk were eval-

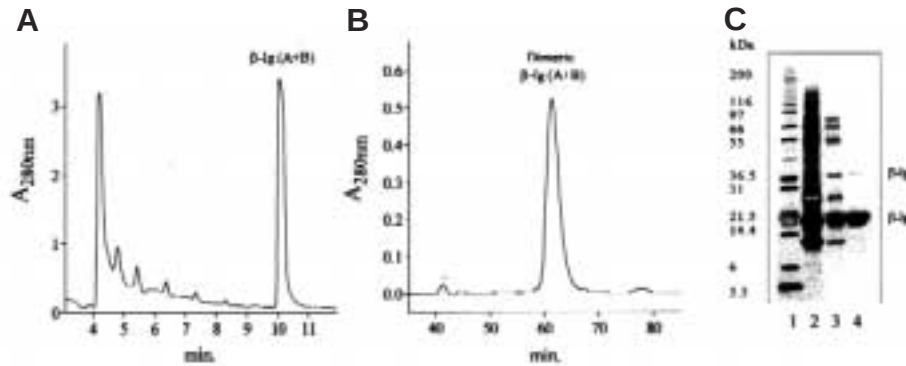


FIG 2. Purification of β -LG from raw milk using non-denaturing conditions. **A**, Anionic ion-exchange chromatography of whey proteins. **B**, Size-exclusion chromatography (Superdex 75 pg column) of the β -LG variant A+B fraction from **A**. **C**, SDS-PAGE (4%-12% NuPAGE) after silver staining: molecular weight marker (lane 1), unfractionated whey proteins (lane 2), the purity of the β -LG preparation before (lane 3), and after size-exclusion chromatography (lane 4). The protein amount applied to the SDS-PAGE was $\sim 2.5 \mu\text{g}$.

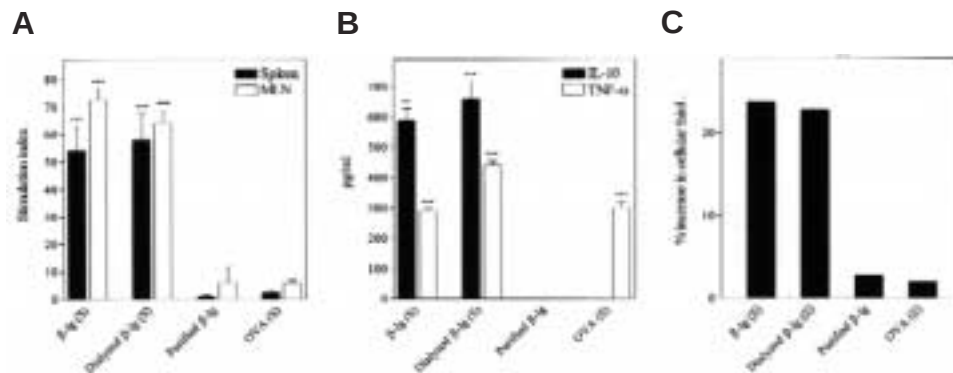


FIG 3. No immunomodulatory effects of β -LG from raw milk. Splenocytes and MLNs from cow's-milk-naïve BALB/c mice were cultured with an optimal dose of 1 mg/mL protein. **A**, Proliferation of splenocytes and MLNs. Data (mean $\pm$ SD, $n = 6$) are stimulation index calculated as counts per minute (cpm) of antigen-stimulated cultures divided by that of unstimulated cultures. The cpm of unstimulated cultures was 332 ± 88 for spleen cells and 342 ± 38 for MLNs. **B**, Cytokine production by spleen cells (mean $\pm$ SD, $n = 6$). **C**, Increase in cellular thiol in spleen cells. All data are representative of 2 independent experiments. Differences in cell proliferation and cytokine production between proteins were tested using 1-way ANOVA. $**P < .01$. $***P < .001$ by Tukey's multiple-comparisons test. S, Sigma-Aldrich.

uated using cell cultures of spleen and MLN from cow's-milk-naïve mice (Fig 3). The purification of β -LG resulted in a significantly reduced cell proliferation of spleen and MLN cells, as well as a significantly lower increase in intracellular thiol in spleen cells when compared with the response induced by the commercial β -LG (Fig 3, A and C). Likewise, the TNF- α and IL-10 production by spleen cells was eliminated after purification of β -LG from raw milk (Fig 3, B), as was the IL-6 and IL-1 β production (data not shown).

Dialysis of the commercial β -LG (cutoff, 6 to 8 kDa or 12 to 14 kDa) did not remove any lower-molecular-weight immunostimulatory contaminants (Fig 3). For all of the activation parameters evaluated, the same level of stimulation was observed towards OVA and the purified β -LG from raw milk, except for the TNF- α production induced by OVA (Fig 3, B). Thus, purification of β -LG from raw milk resulted in a substantially reduced cellular

response compared with the response induced by the commercial β -LG.

Gel filtration of commercial β -LG separates the immunomodulatory component from the β -LG

In an attempt to separate the components present in the commercial β -LG preparation, we performed size-exclusion chromatography of Sigma β -LG and collected 29 fractions of 5.2 mL each. The effect of each fraction (F1 to F29) on proliferation of cow's-milk-naïve MLNs was then assessed (Fig 4). This fractionation of Sigma β -LG showed that 1 fraction (F6) especially contained the immunostimulatory material and that no substantial activity was present in the fractions corresponding to the dimeric β -LG (F13 to F18).

Moreover, to assess the effect of F6 and 1 of the frac-

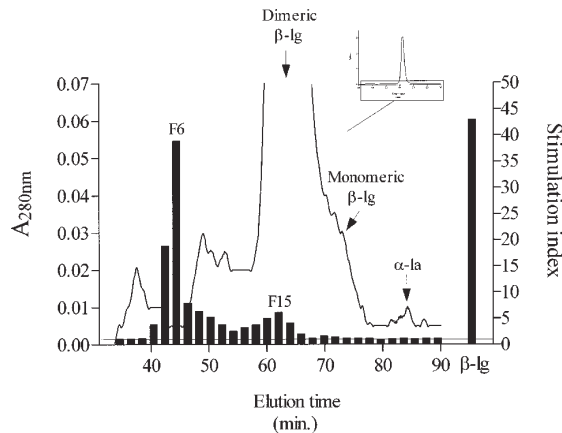


FIG 4. Size-exclusion chromatography of Sigma β -LG separates the immunostimulatory component from β -LG. The Sigma β -LG preparation (20 mg/mL) was fractionated on a Superdex 75 pg column using 150 mmol/L ammonium acetate, pH 6.0. The absorbance was measured at 280 nm (solid black line). The insert presents the whole chromatogram. Proliferation in response to each collected fraction and the unfractionated β -LG was tested on MLNs from cow's-milk-naïve BALB/c mice. The proliferation data (mean, $n = 6$) are shown as stimulation index (black bars). Results are representative of 2 independent experiments.

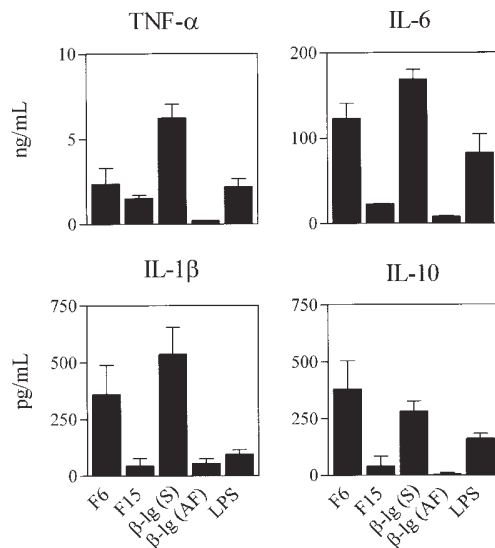


FIG 5. The immunostimulatory component of the Sigma β -LG preparation induces cytokine production in murine DC cultures. MBM cells were in vitro expanded by GM-CSF for 8 days and then cultured with 1 mg/mL antigen or 1 μ g/mL LPS (*E coli* O26:B6). After 19 hours, the supernatants were harvested, and the cytokines TNF- α , IL-6, IL-1 β , and IL-10 were measured by ELISA. Data (mean $\pm$ SD) are derived from 1 experiment using cells from 2 mice and tested in duplicate wells. The F6 and F15 were obtained from size-exclusion chromatography of the Sigma β -LG preparation (Fig 4). S: Sigma-Aldrich; AF: Arla Foods, Denmark.

tions containing dimeric β -LG (F15) on cytokine production in antigen-presenting cells, we expanded DCs from MBM cells and measured TNF- α , IL-1 β , IL-6, and IL-10 in the supernatant after culturing with F6 and F15 as well as the unfractionated Sigma β -LG and another commercially available β -LG (AF) (Fig 5). LPS from *E*

coli O26:B6 was included as a positive control. The F6 component and the unfractionated β -LG from Sigma were capable of inducing TNF- α , IL-6, IL-1 β , and IL-10 production in MBM-derived DCs. The other commercially available β -LG (AF) did not induce substantial amounts of any of the measured cytokines.

Consequently, separation of a bioactive component from the Sigma β -LG (designated F6), which is capable of inducing proliferation in MLNs as well as the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 in DCs, provides further evidence that the activity of the Sigma β -LG is caused by contamination with a mitogenic component and not by β -LG per se.

Reduced effect of commercial β -LG on cells from an LPS-hyporesponsive mouse strain

Because LPS is a recognized mitogenic component and a very likely contaminant of cow's milk,²⁸ we first examined whether the cell proliferation induced by Sigma β -LG could be a cause of LPS contamination. Our first approach was to use the LPS-specific inhibitor polymyxin B (PMB) because of its universal LPS-inhibitory activity.²⁹ The PMB were capable of inhibiting the cell proliferation induced by Sigma β -LG and LPS when used in concentrations from 10 to 50 μ g/mL (data not shown). However, in a further control experiment, we observed that PMB inhibited cell proliferation induced by other mitogenic components (concanavalin A and phytohemagglutinin) as well. Thus, the inhibiting action of PMB on Sigma β -LG and LPS did not prove that LPS was the contaminating immunostimulatory component in the Sigma β -LG.

Therefore, we tested the ability of Sigma β -LG to induce proliferation in cells from the LPS-hyporesponsive mouse strain C3H/HeJ and compared the response with that of the genetically very similar strain C3H/HeOuJ, recognized to be a normal responder to LPS.³⁰ The Sigma β -LG, LPS, and F6 induced proliferation in MLNs from both C3H/HeJ and C3H/HeOuJ mice, with a significantly reduced cell proliferation in C3H/HeJ compared with C3H/HeOuJ (Fig 6). No proliferation was observed toward the other commercial β -LG (AF) and the dimeric β -LG obtained from fractionation of Sigma β -LG (F15) in either of the 2 mouse substrains (Fig 6). Similar results were also achieved with spleen cells (data not shown).

Although cells from the C3H/HeJ mice responded toward the Sigma β -LG, F6, and LPS, this response was substantially lower than that of C3H/HeOuJ, strongly suggesting that the cell proliferation induced in murine immune cells by the Sigma β -LG preparation could be attributed to the presence of LPS.

Some commercial preparations of β -LG contain endotoxin. Eventually, the endotoxin content in the different protein preparations examined in the present study was tested using the *Limulus* amoebocyte lysate assay (Table I). All of the preparations shown to possess immunostimulatory capacity did contain LPS, whereas only low levels of LPS were detected in F15 obtained after gel filtration of Sigma β -LG (Fig 4), as well as in the β -LG (AF). The OVA preparation was found to contain some LPS.

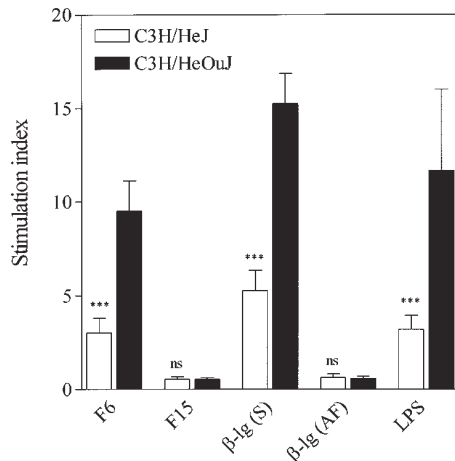


FIG 6. The cellular response to Sigma β-LG is reduced in the LPS low-responder strain C3H/HeJ. Proliferation of MLNs from C3H/HeJ and C3H/HeOuJ mice ($n = 6$) cultured with 1 mg/mL antigen or 10 μ g/mL LPS (*E coli* O55:B5). The F6 and F15 were obtained from size-exclusion chromatography of the Sigma β-LG preparation (Fig 4). *** $P < .001$ by 2-way ANOVA followed by the Bonferroni multiple-comparisons test. NS: Not significant; S: Sigma-Aldrich; AF: Arla Foods, Denmark.

In addition, other batches of Sigma β-LG (L-3908, purified by chromatography) and the Sigma β-LG preparation (L-0130, purified by crystallization) were found to contain significant amounts of LPS.

DISCUSSION

It has long been speculated that cow's milk proteins might differentially exert immunomodulatory effects on various cell populations.³¹ However, caution must be taken before drawing conclusions from in vitro studies regarding the immunostimulatory potency of common cow's milk proteins, especially when only limited attention has been paid to excluding the effect possibly caused by bioactive substances, such as bacterially derived LPS,³²⁻³⁵ likely to be present in milk preparations.

The present study provides data that document the presence of endotoxin in some commercially available β-LG preparations in amounts giving rise to significantly immunostimulatory activities on various murine cell populations.

Most important, we found that the β-LG per se did not possess the potential to induce either proliferation or cytokine production (TNF- α , IL-1 β , IL-6, IL-10) or an increase in intracellular GSH in cow's-milk-naïve immune cells. This finding is significant for the conclusions of previously reported ex vivo studies using the Sigma β-LG,^{5,10,11,13,17-23,36,37} as the substantial immunostimulatory activity toward β-LG observed in those studies primarily might be a cause of nonspecific endotoxin-induced stimulation and therefore, not exclusively a cause of β-LG-specific activity. Especially the earlier findings of production of the T_H1-cytokine IFN- γ in CBMCs,^{10,20} PBMCs,^{20,22} lamina propria T cells,¹⁹ and Peyer's patches²¹ point to the presence of a T_H1-dri-

TABLE I. Contamination of commercial β-LG preparations by endotoxin

Preparation	Endotoxin (EU/mg)
F6	1331
F15	<2
β-LG (S, L-3908, no. 1*)	6486
β-LG (AF)	3.4
OVA (S)	169
Other batches of β-LG (S) tested	
β-LG (S, L-3908, no. 2)	1136
β-LG (S, L-0130, no. 1)	2480
β-LG (S, L-0130, no. 2)	3341

AF, Arla Foods; EU, Endotoxin units; S, Sigma-Aldrich.

*Preparation used in the present study.

ving immunostimulatory component, like endotoxin, in the commercial β-LG preparations used for these studies. The current finding of a contaminating immunostimulatory component to be present in some β-LG preparations might also explain the inconsistencies among previously reported results in relation to ex vivo stimulation of CBMCs toward β-LG and the risk of developing cow's milk allergy,^{9,10,12-14,23} because a general, nonspecific proliferation clearly will blur subtle specific response differences. Therefore, from a clinical standpoint, the finding of a general nonimmunomodulatory capacity of β-LG per se on cow's-milk-naïve immune cells has potential importance, as future studies using purified β-LG on CBMCs might disclose whether CBMCs are truly stimulated on ex vivo exposure to β-LG. If so, this will imply the existence of an antigen-specific response and hence, demonstrate in vivo priming toward β-LG.

Regarding the GSH measurements, our finding that LPS was the main component in the Sigma β-LG preparation to induce an increase in the intracellular thiol level significantly reduces the probability of β-LG's playing a central role in the GSH-enhancing effect observed after feeding ~1 g/d of whey protein concentrate to adult mice,¹ as was proposed by Wong et al.⁵ Inhibition of the activity of GSH peroxidase might well be the mechanism by which LPS enhances the intracellular GSH in spleen cells. Such regulation was recently reported to take place in hepatocytes after in vitro culturing with LPS.³⁸ Inhibition of GSH peroxidase by LPS would impair the normal utilization of GSH.³⁹

Recently, other commercially available protein preparations, like hen's egg lysozyme,⁴⁰ collagen,⁴¹ and Gram-positive bacterially derived lipoteichoic acid,⁴² have equally been shown to contain bioactive endotoxin. In relation to this, the OVA preparation used in the present study did contain LPS, which presumably accounts for the TNF- α production observed in the OVA-cultured spleen cells. Hence, increased awareness of the potential presence of bioactive contaminants in commercial products is needed to achieve enhanced research products along with consistent results.

In conclusion, reassessments of previously reported effects of β-LG are needed, as endotoxin contamination of some of the most commonly used commercial β-LG preparations induces various immunostimulatory activities that cannot be ascribed to the β-LG itself.

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