

Lipopolysaccharide Contamination of β -Lactoglobulin Affects the Immune Response against Intraperitoneally and Orally Administered Antigen

Susanne Brix Tanja M.R. Kjær Vibeke Barkholt Hanne Frøkiær

BioCentrum-DTU, Biochemistry and Nutrition Group, Technical University of Denmark, Lyngby, Denmark

Key Words

Emulsion · Food allergy · Food protein · Intraperitoneal immunization · β -Lactoglobulin · Lipopolysaccharide · Oral tolerance

Abstract

Background: Microbial components in the environment are potent activators of the immune system with capacity to shift the active immune response towards priming of Th1 and/or Th2 cells. Lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria, is extensively present in food products like cow's milk. It is not well established, however, how this presence of LPS affects oral tolerance induction. **Methods:** We studied the effect of LPS contamination in a commercial preparation of the cow milk protein β -lactoglobulin (β -LG) on antigen-specific immune responses. IgG1/IgG2a production upon intraperitoneal immunization without adjuvant was measured, and oral tolerance induction against β -LG after administration of either an aqueous solution or water-in-oil (w/o) emulsion of β -LG was evaluated. **Results:** LPS contamination of β -LG provoked a β -LG-specific IgG2a response, as well as an enhanced β -LG-specific IgG1 response upon intraperitoneal immunization. Oral tolerance induction to β -LG was induced by aqueous solutions of β -LG with and without LPS administration. Conversely, oral administration of w/o-emulsified β -LG

prevented oral tolerance to β -LG only when the β -LG was contaminated with LPS. **Conclusions:** LPS contamination of an aqueous protein solution does not affect oral tolerance induction, whereas LPS present in emulsion prevents oral tolerance induction towards the food protein.

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Introduction

Several commercial products applied in immunological research have recently been found to contain bioactive lipopolysaccharide (LPS) [1–5], and LPS has therefore unintentionally been introduced into experimental animal models. Accordingly, LPS contamination may influence results obtained in experimental animal models used to evaluate allergenic or other immunomodulating effects of food proteins or products.

Immunization with antigens is used in various animal models to induce an antigen-specific response, and in some experiments intraperitoneal immunization of food antigens is even used to assess the allergenic potential of the food proteins [6]. It seems likely that contamination of food proteins with LPS may prime for antigen-specific Th1 responses upon immunization due to the Th1-driving adjuvant effect of LPS [7–9]. On the other hand, contamination with LPS in food products used for oral inges-

tion may have no impact due to the usual presence of numerous Gram-negative bacteria, and hence the presence of LPS in the intestine [9]. Despite this, it has previously been shown that LPS, when administered simultaneously with aqueous solutions of pure proteins, actually enhances oral tolerance induced towards that specific protein [10, 11]. Currently, however, we lack information on how the presence of LPS in different oil-based food formulations may influence the induction of oral tolerance.

In the present study, we focused on the effect of LPS contamination on immune responses against the cow milk protein β -lactoglobulin (β -LG) when administered either intraperitoneally or orally. For oral administration, both aqueous solutions and a w/o-emulsion of β -LG were tested.

Material and Methods

Antigens and Endotoxin Content

β -LG (>90%, L-0130, lot 124H7045) was purchased from Sigma-Aldrich, St. Louis, Mo, USA. Purified β -LG was prepared from β -LG (>95%, variant A+B, Arla Foods, AF, Videbæk, Denmark, PSDI-2400) by size exclusion chromatography using a Superdex 75 column (Hiload 26/60; AP Biotech, Uppsala, Sweden) and 150 mM ammonium acetate, pH 6.0. Antigen preparations were tested for endotoxin contamination by the chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, Md., USA). For the Sigma β -LG batch, the LPS content was 2,952 EU/mg, whereas for purified β -LG it was 1.5 EU/mg.

Intraperitoneal Immunization with Antigen Solutions

Antigen solutions of 2% (w/v) in sterile phosphate-buffered saline (PBS, pH 7.4) were intraperitoneally injected into BALB/c mice in a volume of 0.25 ml on days 1 and 7, as described by Dearmann et al. [6]. On day 14, blood was collected by cardiac puncture, and serum was prepared. The BALB/c mice (12–13 weeks old) were bred and maintained on a commercially available milk- and egg-free diet (TRM 9608, Harlan Teklad, The Netherlands) for at least two generations. Antigen-specific immunoglobulin isotypes (IgG1 and IgG2a) in serum samples were determined by ELISA as described by Kjær and Frøkiær [12]. All animal experiments were approved by the Danish Animal Experiments Controlling Body.

Protocol for Induction of Oral Tolerance to β -LG

For 3 consecutive days (days 1–3), BALB/c mice were fed 0.2 ml by gavage with one of the following protein solutions: 50 mg/ml β -LG (Sigma, L-0130) or 50 mg/ml purified β -LG, both diluted in drinking water. Other groups received by gavage 0.2 ml of a sonicated emulsion of either 100 mg/ml β -LG (Sigma) or 100 mg/ml purified β -LG mixed 1:1 with four parts of triolein (Sigma, T-7752) and one part of a solution containing 20 mmol/l taurocholate (Sigma) and 10 mg/ml choline (Sigma) in distilled water. Control groups received drinking water similarly. On days 10 and 24, all mice were immunized intraperitoneally with 20 μ g purified β -LG diluted in sterile PBS and mixed 1:1 with Freund's incom-

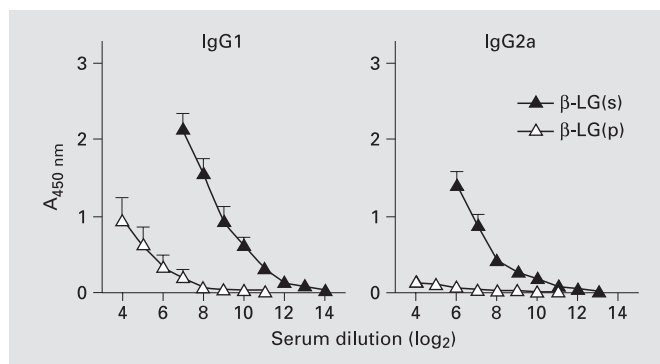


Fig. 1. Antibody response towards β -LG after intraperitoneal immunization in PBS. BALB/c mice were immunized intraperitoneally with 2% β -LG solutions, and serum-derived antigen-specific IgG1 and IgG2a were measured individually for each mouse by ELISA. Responses towards LPS-containing β -LG and purified β -LG are shown. Data are means + SEM (n = 9). The immunoglobulin titer represents the $\log_2$ of the serum dilution. S = Sigma-Aldrich; P = Purified.

plete adjuvant (Difco, Detroit, Mich., USA). Blood samples were collected from the retro-orbital plexus on days 0, 10, 24 and 31. After blood collection on day 31, spleens were sampled to assess cell proliferation using purified β -LG. Both cell proliferation and β -LG-specific immunoglobulin levels were measured as described by Kjær and Frøkiær [12].

Statistical Analysis

The data were analyzed for statistical significance (GraphPad Prism, version 3.02, GraphPad Software, San Diego, Calif., USA) using one-way ANOVA followed by Tukey's multiple comparison test. In one set of experiments, the Student t test was employed. A p value <0.05 was considered significant.

Results

To examine the influence of LPS contamination in antigen preparations used for intraperitoneal immunization, the humoral response upon intraperitoneal immunization without an adjuvant towards purified and LPS-contaminated β -LG was evaluated. Measurement of antigen-specific IgG1 and IgG2a was used to reflect the ability of the antigen preparations to induce a Th2 or Th1 response, respectively [13]. Comparisons of the IgG1 and IgG2a responses generated towards the purified and LPS-contaminated β -LG (fig. 1) showed that only LPS-containing β -LG induced an IgG2a response, whereas other tested proteins (ovalbumin, ovomucoid and bovine serum albumin) only provoked IgG1 responses (data not

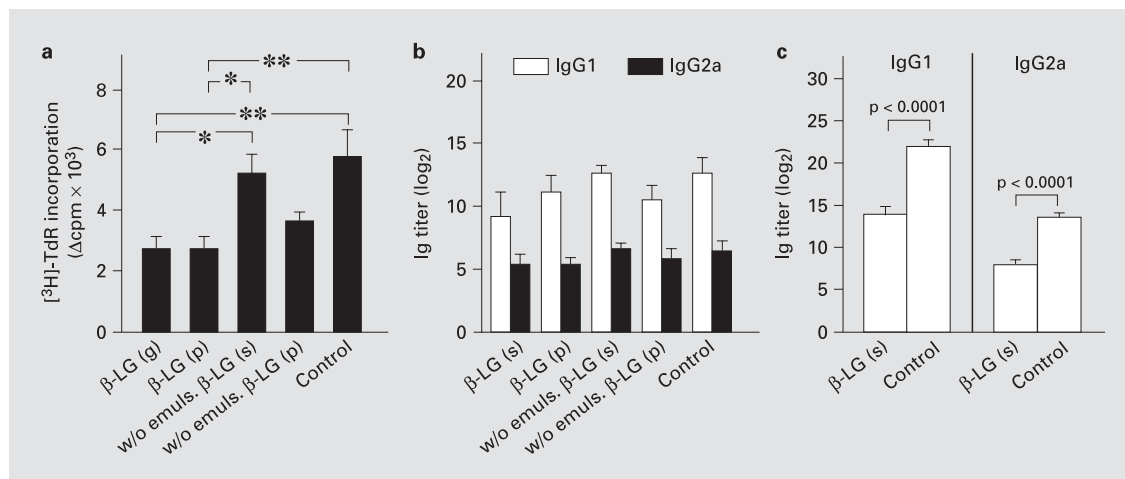


Fig. 2. Effect of co-administration of LPS on oral tolerance induction to β -LG. BALB/c mice were fed by gavage with 10 mg β -LG/day for 3 days and control groups with water. All groups were immunized either with purified β -LG (**a**, **b**) or LPS-containing β -LG (**c**) in Freund's incomplete adjuvant 7 and 21 days after the last feedings. Blood samples and spleen cells were collected on day 31. **a** Proliferative response of spleen cells cultured at 5×10^5 cells/well and stimulated with 200 μ g/ml purified β -LG for 4 days. **b** β -LG-

specific IgG1 and IgG2a antibody levels shown as log₂ of the reciprocal of the sample dilution giving an absorbance of 0.2. Data are means + SEM (n = 7). * p < 0.05, ** p < 0.01 (Tukey's multiple comparisons test). **c** β -LG-specific IgG1 and IgG2a antibody responses of mice immunized with LPS-containing β -LG. Data are means + SEM (n = 6) and analyzed by Student's t test. S = Sigma-Aldrich; P = Purified; emuls. = emulsion.

shown). The β -LG per se did not provoke the Th1-mediated response (IgG2a) observed for the LPS-contaminated β -LG, as no IgG2a was induced after injection of purified β -LG (fig. 1). This indicated that the presence of LPS induced the IgG2a response. Furthermore, the Th2-mediated response (IgG1) towards LPS-contaminated β -LG was found to be enhanced approximately 20 times as compared with that of purified β -LG (fig. 1).

To examine the effect of LPS-contaminated β -LG on oral tolerance induction, a classical oral tolerance protocol was employed. Purified β -LG was used for intraperitoneal immunization, and the effect of oral co-administration of LPS in aqueous solution or in a w/o emulsion of β -LG was considered (fig. 2a, b). The proliferation of spleen cells was significantly different depending on the feeding regimen (fig. 2a). Administration of β -LG in aqueous solution with and without LPS contamination induced a significantly reduced cellular response (p < 0.01) as compared to the non-fed β -LG-immunized control group. In contrast, co-administration of LPS in the w/o emulsion of β -LG significantly enhanced the cellular response as compared with that of mice fed the aqueous solution of LPS-contaminated β -LG (p < 0.05) and was at the same level as that of the control group. Feeding with w/o emulsion of purified β -LG resulted in a cellular

response ranking between that of mice fed LPS-contaminated β -LG oil emulsion and that of mice fed the aqueous solutions of β -LG. For the β -LG-specific IgG1 and IgG2a responses, the same tendencies as those seen for the cellular responses were observed (fig. 2b), although no significant differences were revealed between groups. However, in another experiment (fig. 2c) where LPS-containing β -LG was used for immunization instead of purified β -LG, higher specific antibody titers were observed for immunized controls who were not fed β -LG, and oral administration of β -LG induced significantly suppressed immunoglobulin levels compared to the control levels (fig. 2c).

Discussion

In the present study, we found that LPS present in a commercial preparation of β -LG significantly influenced the immune response to β -LG. We have shown that LPS contamination of a bovine β -LG preparation provoked a β -LG-specific IgG2a response and enhanced the IgG1 response after intraperitoneal immunization without an adjuvant. This result suggests that unintended presence of LPS in an antigen solution induces an antigen-specific

Th1 (IgG2a) response and enhances the antigen-specific Th2 (IgG1) response, and hence works as a Th1-polarizing adjuvant. Previously, Dearmann et al. [6] proposed that induction of a specific IgG2a response towards a protein after intraperitoneal immunization may reduce the potential allergenicity of that protein. However, our findings demonstrate that unintended presence of Th1-driving contaminants such as LPS in protein preparations may prevent that proteins possessing allergenic potential are discovered using the intraperitoneal immunization approach. In agreement with our findings, Watanabe et al. [14] have shown that LPS contamination of ovalbumin results in suppression of IgE in an airway hyperactivity mouse model, indicating a shift away from a Th2 response.

Focusing on the influence of LPS contamination on oral tolerance induction to β -LG, we found that administration of LPS and β -LG in a w/o emulsion prevented induction of oral tolerance to β -LG. In previous studies by Kaneko et al. [15, 16], a soy-oil emulsion of β -LG has been shown to prevent oral tolerance induction as well as to induce a systemic antibody response. Whether LPS was involved in these responses was not studied. In the present study, when LPS was present in an aqueous β -LG solution, we did not detect an enhanced oral tolerance induction to β -LG, as has earlier been described for non-food-related antigens [10, 11].

It is well known that it is more difficult to visualize oral tolerance measured as an antibody response as compared to a cellular response. Here we show that when immunizing with an antigen contaminated with LPS, then it is possible to show oral tolerance induction on the antibody level as well as on the cellular level. On the contrary, using the purified β -LG for immunization, we were not able

to detect significant differences between feeding groups on IgG1 and IgG2a responses. Differences in the results in investigations on oral tolerance might therefore be ascribed to contaminations with LPS, emphasizing the significance of the adjuvant dependency on oral tolerance induction.

The observed duality in effect of LPS on β -LG-specific responses when co-administered with β -LG in emulsion versus solution may be due to increased absorption of oil-emulsified protein as opposed to soluble protein [17, our own unpubl. observations]. Accordingly, absorption of greater amounts and/or different absorption pathways may cause LPS to be co-transferred with oil-emulsified protein. A subsequent systemic response against β -LG may then arise as a cause of dissemination of absorbed β -LG together with LPS to draining lymph nodes and spleen.

In conclusion, in protocols aiming at clarifying the immunoregulatory mechanisms induced by the antigen per se, it is compulsory to take into account the possible contamination of the antigen source with microbial components such as LPS. In addition, more information is required regarding the immunological effects of LPS contamination of foods used in different food formulations, as oral tolerance induction was found to be hindered after oral administration of a LPS-contaminated w/o emulsion of β -LG.

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