

Effect of Maternal Dietary Cow's Milk on the Immune Response to β -Lactoglobulin in the Offspring: A Four-Generation Study in Mice

Susanne Brix Hanne Risager Christensen Vibeke Barkholt Hanne Frøkiær

BioCentrum-DTU, Technical University of Denmark, Lyngby, Denmark

Key Words

Food protein · β -Lactoglobulin · Maternal transfer · Offspring · Oral tolerance

Abstract

Background: Evaluation of immune responses to food proteins in animal models requires that the animals are not already sensitized or orally tolerized against the proteins in question. Since maternal transfer of specific immune responses has been observed, breeding of animals on an antigen-free diet for several generations may be necessary to obtain immunologically naïve animals. **Methods:** To determine the most appropriate breeding conditions of mice to be used in immunological studies on food proteins, we examined immune responses towards β -lactoglobulin (BLG) in mice bred on a milk-containing diet (F0) and then for three generations (F1–F3) on a commercially available milk-free diet. The specific antibody and cell-proliferative response to BLG was compared in non-immunized and immunized BALB/c mice, and in mice orally tolerized to BLG prior to immunization. **Results:** The immune response to BLG in the F1 generation deviated from the response observed in the F0 and F2/F3 generations. Importantly, trace amounts of

BLG detected in the commercial milk-free diet did not induce oral tolerance. **Conclusions:** The study showed that breeding mice on an antigen-free diet for at least two generations is required to attain animals appropriate for immunological studies of food proteins. Although the small quantity of BLG in the milk-free diet did not induce detectable oral tolerance in the present study, it is strongly recommended that the potential effect of contaminating dietary antigen is considered in future studies on food proteins.

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Introduction

Induction of IgE-mediated allergic reactions to food proteins is currently believed to result from a breakdown of normal oral tolerance [1]. Although extensively studied, the factors controlling these adverse reactions are still not fully understood. Development of appropriate animal models may help to delineate the mechanisms involved in the initiation or prevention of allergic responses. To this end, several different approaches have been tried during the last decade [2–8]. However, common food proteins such as milk, soy and other plant proteins are used exten-

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Correspondence to: Dr. Hanne Frøkiær
BioCentrum-DTU, Biochemistry and Nutrition
Technical University of Denmark
DK-2800 Kongens Lyngby (Denmark)
Tel. +45 45252753, Fax +45 45886307, E-Mail hf@biocentrum.dtu.dk

sively as inexpensive protein sources in animal diets. As such, these dietary components are confounding factors in animal models of food allergy [9–12]. It is imperative, therefore, that diets free of the antigen in question are used in the breeding of animals intended for such studies. Consequently, diets claiming to be free of specific antigens are now commercially available and are being used in many studies examining responses to food proteins. However, the widespread use of common food proteins such as cow's milk and soy in animal diets may lead to cross-contamination during the manufacture of different diets. As a result, trace amounts of common antigens may nevertheless be present in the diet. More attention must therefore be paid to the content of specific antigens in animal diets and the effect of these antigens on immune responses in animals.

Another equally important factor to consider is the number of generations animals must be bred on an antigen-free diet. Maternal transfer of specific immune responses (i.e. suppression/priming) towards food proteins has previously been observed in experimental animals [9, 12–15]. However, only two studies have addressed how many generations of animals are influenced by the maternal antigen exposure [9, 12]. These studies, employing rats (soy protein) and guinea pigs (cow's milk), revealed that immune responses of the F1 generation on an antigen-free diet deviated from those of later generations.

Since mice have often been used to study immunological responses to food antigens [3, 5–8, 16], and since milk proteins are present in many rodent diets and have been extensively studied in many animal models [2, 3, 5, 6, 16, 17], the present study examined how many generations of mice must be bred on a milk-free diet to negate the maternal influence on the immune response to the bovine milk protein β -lactoglobulin (BLG). Furthermore, since trace amounts of BLG were detected in the commercial milk-free diet, we also studied the effect of this low amount of contaminant on the induction of oral tolerance to BLG.

Materials and Methods

Animals

BALB/c mice, 6–8 weeks old (M&B, Ry, Denmark), were bred and maintained on a standard rodent diet (Altromin 1314/1324, Altromin, Lage, Germany) containing skimmed cow's milk powder (4 g/100 g). All animal studies were approved by the Danish Animal Experiments Inspectorate and were carried out according to the guidelines of the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

Breeding Protocol

Starting with mice bred on a milk-containing diet (F0), three generations (F1–F3) of mice were bred concurrently on a diet claimed to be free of cow's milk proteins (TD 9608, Harlan Teklad, Zeist, The Netherlands). The F0 animals were maintained on the milk-free diet from 14 days before mating. The subsequent generations were obtained from brother-sister mating of F1 and F2 parents. The male offspring used for experiments were age-matched within the same week and were 9 weeks old at the beginning of the experiments. The two diets used were found to support growth equally well, in that the mice in all four generations gained weight equally, and no significant differences were observed in the mean body weights at either 12 or 23 weeks of age (data not shown).

Measurement of the BLG Content in Rodent Chow by ELISA

Extracts of finely grinded mouse chow were made in phosphate-buffered saline (PBS, 0.01 M, pH 7.4, 20% w/v) at 4°C for 16 h, and the supernatant collected after centrifugation (1,000 g, 10 min). The BLG content was quantified using a sandwich ELISA employing two anti-BLG monoclonal antibodies produced in our laboratory. Briefly, microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with anti-BLG monoclonal antibody in carbonate buffer (0.05 M, pH 9.6) and incubated for 1 h at room temperature (RT). After washing, the chow extracts or BLG standard (>90%, variant A+B, 3 $\times$ crystallized; Sigma, St. Louis, Mo., USA) were serially two-fold diluted in PBS containing 0.1% Triton X-100 (PBST). The plates were incubated for 2 h at RT, then washed and incubated for another hour at RT with biotinylated anti-BLG monoclonal antibody. After washing, the plates were incubated for 1 h at RT with peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark), and then washed. Enzyme-catalyzed color development was accomplished by adding TMB (Merck, Darmstadt, Germany, diluted in peroxide solution) for 10 min and then stopped with phosphoric acid (2 M). Plates were read at 450 nm using 630 nm as reference. Extract and standard curves were parallel, and the detection limit was <1 ng/ml (corresponding to approximately 5 ng BLG/g feed).

Immunization Protocol

Mice were immunized intraperitoneally on days 1 and 14 with a cow's-milk-based formula (Nan-1, Nestlé, Vevey, Switzerland, whey/casein ratio, w/w: 60/40) diluted in distilled water and mixed 1:1 in Freund's incomplete adjuvant (Difco, Detroit, Mich., USA). Each mouse received 22 μ g casein and 33 μ g whey proteins (approximately 20 μ g BLG). The protein content was determined by amino acid analysis as previously described [18]. Blood was collected from the retro-orbital plexus into heparin-coated capillaries on days 1 and 21, immediately mixed with PBST (1:16) and stored at –20°C.

Protocol for the Induction of Oral Tolerance

Oral tolerance was induced in F0 and F3 mice by adding BLG (4 mg/ml, Sigma) to the drinking water for 3 consecutive days (days 1–3). Control groups were given normal drinking water. Mice were immunized intraperitoneally on days 12 and 26 with 20 μ g BLG (Sigma) diluted in sterile saline and mixed 1:1 with Freund's incomplete adjuvant (Difco). Blood samples were collected on days 0, 12, 26 and 33 as described above.

Determination of Specific Antibody by ELISA

BLG (10 μ g/ml, Sigma) or α -casein (1 μ g/ml, Sigma) was dissolved in carbonate buffer (0.05 M, pH 9.6) and coated onto microti-

ter plates (Maxisorp, Nunc) for 1 h at RT. After washing, blood samples were added to the plates and then serially twofold diluted in PBST. The plates were incubated for 1 h at RT, washed and incubated with peroxidase-conjugated anti-mouse immunoglobulin (Dako) for 1 h at RT. Plates were developed with TMB as described above. Pooled blood obtained from multiple Nan-1-boosted mice was included as internal standard on each plate. Samples were run in duplicate on different days. Results were expressed as log₂ titers defined as the sample dilution giving an absorbance of 0.2 above background. Measurement of ovomucoid-specific antibody was performed similarly except that the ELISA plates were coated with 0.3 µg/ml ovomucoid (type II-0, Sigma).

Cell Proliferation Assay

Single-cell suspensions of the spleen and mesenteric lymph nodes were prepared and resuspended in culture medium (X-vivo 10, serum-free medium, BioWhittaker, Verviers, Belgium) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all Life Technologies, Paisley, UK). The cells were cultured at 5×10^5 viable cells/200 µl/well in 96-well flat-bottom tissue culture plates (Nunc) with 25 µl of BLG (1, 5 or 25 µg/ml, >90%, variant A+B, Sigma), α-casein (50 µg/ml, >85%, Sigma), ovomucoid (70 µg/ml, type II-0, Sigma) or in unstimulated cultures with PBS (0.01 M, pH 7.2). Within each group, spleen cells were cultured individually from each mouse while equal cell numbers were pooled from the mesenteric lymph nodes. After incubation for 48 h at 37°C in 5% CO₂, cells were pulsed with [³H]-thymidine (TdR, 1 µCi/ml, Amersham, UK) for the final 18 h of incubation. The cells were harvested onto fiberglass filters (Whatman, Maidstone, UK) using a 12-well cell harvester (Automash 2000, Dynex Technologies, Denckendorf, Germany), and the amount of TdR incorporated was determined by liquid scintillation counting (Tri-Carb®, Packard, Meriden, Conn., USA). Mean [³H]-TdR incorporation in antigen-stimulated cultures was determined by subtracting the mean for unstimulated cultures.

Statistical Analysis

The data were analyzed for statistical significance (GraphPad Prism, version 3.0, GraphPad software, San Diego, Calif., USA) using one-way ANOVA followed by Tukey's multiple comparison test. A p value <0.05 was considered significant.

Results

Immune Responses to BLG in Four Generations of Mice

To compare specific immune responses between different generations of mice bred first on a milk-containing diet (F0) and for up to three generations thereafter on a commercial milk-free diet (F1–F3), we examined the antibody and cellular responses towards BLG in mice immunized with a bovine milk formula and in non-immunized mice. All generations were bred concurrently in order to perform all manipulations, including the cell proliferation, at the same time. Bovine BLG was used as the

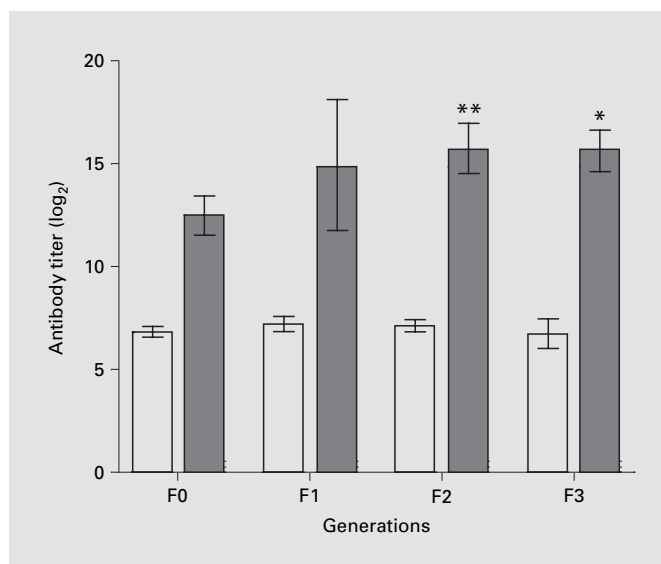


Fig. 1. Anti-BLG-specific antibody titers in non-immunized (white bars) and immunized (hatched bars) BALB/c mice bred concurrently on a standard milk-containing diet (F0) and for up to three generations (F1–F3) on a commercial milk-free diet. The F0 mice were switched to the milk-free diet 14 days before mating. In immunized mice, intraperitoneal immunizations with a milk formula were performed at weeks 9 and 11. For all mice, blood samples were taken at weeks 9 (control) and 12. Antibody titers at week 12 are presented as the log₂ of the reciprocal of the sample dilution giving an absorbance of 0.2. Data are means ± SD (n = 8). * p < 0.05, ** p < 0.01, compared to the relevant F0 generation group.

main model milk protein, since BLG is not expressed in the rodent mammary gland [19].

In immunized mice, the immunoglobulin response to BLG was significantly lower in the F0 generation compared to that in either the F2 or the F3 generation (fig. 1), strongly suggesting that the F0 mice have been rendered orally tolerant to the BLG in the milk-containing diet. In the F1 mice, a greater variability in the BLG-specific immunoglobulin response was observed, ranging from values observed in the F0 generation to those in the F2/F3 generations. The F2 and F3 generations did not significantly differ in their immunoglobulin response to BLG, suggesting that from the F2 generation onwards, the humoral response is no longer affected by the F0 exposure to BLG. The same patterns were observed for both BLG-specific IgG1 and IgG2a (data not shown).

In non-immunized mice, no BLG-specific immunoglobulin response above background was revealed in any of the four generations (fig. 1), showing that no circulating

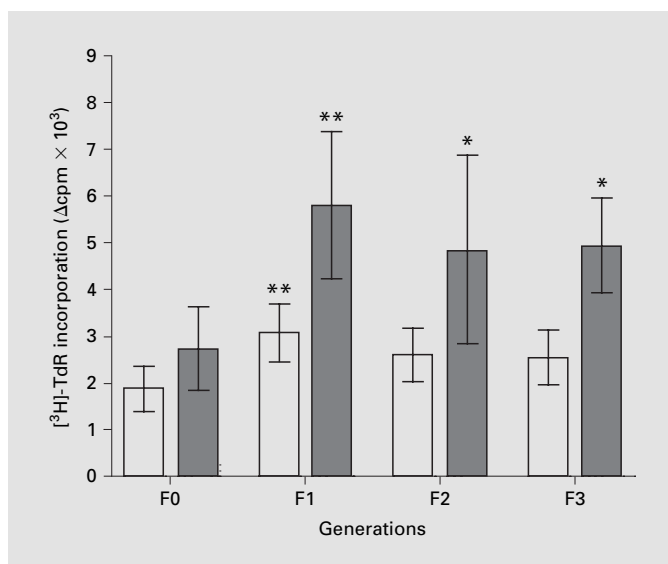


Fig. 2. Proliferation of spleen cells from four generations of non-immunized (white bars) and immunized (hatched bars) BALB/c mice stimulated in vitro with 5 µg/ml BLG. The four generations of mice were bred and maintained as described in figure 1. Cell-proliferative responses were measured at week 12. The results are expressed as the mean [3H]-TdR incorporation in antigen-stimulated cultures after subtracting the mean value of the unstimulated cultures. Means $\pm$ SD (n = 8). * p < 0.05, ** p < 0.01, compared to the relevant F0 generation group.

BLG-specific antibodies were generated per se in the 12-week-old mice fed either of the two diets.

The cellular response achieved after in vitro BLG stimulation of spleen cells from immunized mice (fig. 2) was significantly lower in F0 generation mice when compared to the F1, and the F2 and F3 generations. Furthermore, no difference in the BLG-specific cellular response was observed between the F2 and F3 mice. Once again, the response of F1 mice differed from those of the other generations. More specifically, the cellular response of the F1 mice was significantly higher than that of the F0 mice, and slightly, though not significantly, elevated in comparison to the F2 and F3 mice.

Interestingly, the non-immunized F1 mice also displayed significantly increased cell proliferation when compared to the non-immunized F0 mice, and a weak but non-significantly elevated response in comparison to F2/F3 mice (fig. 2).

To confirm that the differences observed were not due to non-diet-related differences between the mice, the egg white protein ovomucoid (absent in both diets) was used

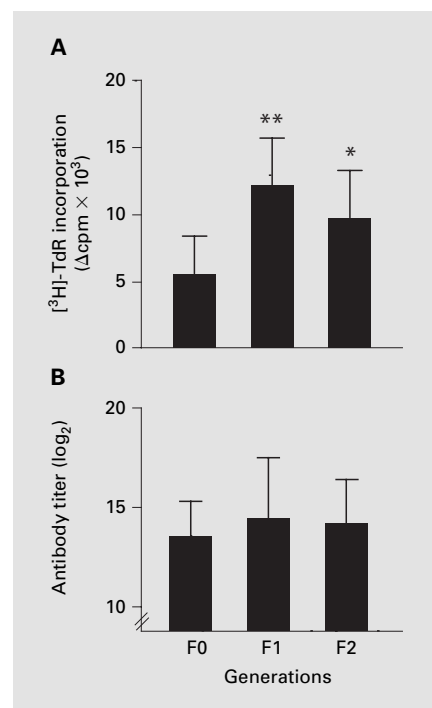


Fig. 3. The cellular and humoral responses to α -casein in F0–F2 immunized mice. The three generations of mice were bred and maintained as described in figure 1. After 9 and 11 weeks, the mice were immunized intraperitoneally with a milk formula. Blood samples were taken at weeks 9 (control) and 12. Cell proliferation was performed at week 12. **A** Cell proliferation of spleen cells cultured in vitro with 50 µg/ml α -casein. The proliferation is shown as the mean [3H]-TdR incorporation in antigen-stimulated cultures after subtracting the mean value of the unstimulated cultures. **B** Antibody titers at week 12 are presented as the log₂ of the reciprocal of the sample dilution giving an absorbance of 0.2. Means $\pm$ SD (n = 8). * p < 0.05, ** p < 0.01, compared to the F0 generation group.

as a control antigen in the experimental assays. No differences in specific immunoglobulins or in cell-proliferative responses towards ovomucoid were observed among the four generations of immunized or non-immunized mice (data not shown), which confirmed that the difference in the immune response of the F1 mice compared to the other generations was specifically related to BLG in the diet.

A second study using F0, F1 and F2 generations confirmed the above-described effects observed on humoral and cellular responses to BLG (data not shown). Furthermore, due to immunization with the milk formula containing a mixture of bovine milk proteins, we were also capable of evaluating the antigen-specific immune re-

sponse towards other milk proteins. Examination of the α -casein-specific response of spleen cells also revealed a deviating response in the F1 generation (fig. 3A), as was observed for BLG. However, no differences between the three generations were seen on the humoral response to α -casein (fig. 3B).

In summary, the results showed the expected tolerance to BLG in F0 generation mice. The response of the F1 generation is less easy to explain. However, it is evident that F1 mice, unlike later generations, are neither immunologically naïve towards BLG nor (presumably) towards other bovine milk proteins, too.

Detection of BLG in the Commercial Milk-Free Diet

Another important consideration when breeding experimental animals to study immunological responses to food proteins is the presence of minute amounts of antigen in diets claiming to be free of antigen. The BLG content in the commercial milk-free diet used in our studies was therefore analyzed by ELISA. Unfortunately, the diet was found to contain trace amounts of BLG of approximately 1 μ g/g feed. In comparison, the BLG content of the milk-containing diet was approximately 400 μ g/g feed. Thus, the daily intake of BLG in an adult mouse consuming approximately 5 g feed/day is about 5 μ g and 2 mg for the milk-free and the milk-containing diets, respectively. Since small amounts of contaminating antigen are often inevitable in manufactured diets claimed to be antigen free, the present study was carried out using the commercial 'milk-free' diet described above.

Comparison of Oral Tolerance to BLG in F0 versus F3

To examine the consequences of the different levels of BLG content in the two diets, we compared the capacity to induce oral tolerance to BLG in the F0 and F3 generations. The F0 and F3 were each divided into two groups. One group in each generation was administered BLG (20 mg/day for 3 days) in the drinking water (fed) while the second group received water alone (non-fed). All groups received subsequently twice intraperitoneal immunization with purified BLG on days 12 and 26.

As expected from the previous results, the humoral response to BLG in the non-fed F0 generation was significantly lower than that of the non-fed F3 generation due to the pre-experimentally induced oral tolerance in the former (fig. 4A). In the F0 generation, there was no difference between the fed and non-fed group in their humoral response to BLG. However, in the F3 generation, the immunoglobulin response of the fed animals was significantly lower than that of the non-fed animals. This differ-

ence was observed for both BLG-specific IgG1 and IgG2a (data not shown).

Likewise, the specific proliferation of spleen cells to BLG was reduced in both the fed and non-fed F0 groups as well as in the fed F3 group when compared to the non-fed F3 group (fig. 4B). Interestingly, BLG-stimulated proliferation of mesenteric lymph node cells from F0 mice, whether fed or non-fed, was statistically significantly different from that of the fed F3 group (fig. 4C), suggesting that the continuous intake of approximately 2 mg BLG/day in the milk-containing diet induced a more pronounced oral tolerance than feeding for only 3 days with 20 mg BLG/day in the F3 mice.

The present data suggest that oral tolerance to BLG is induced in the F0 generation due to exposure to BLG in the milk-containing diet. Furthermore, inducing tolerance to BLG in F3 animals results in a tolerance which closely resembles the response usually achieved using other model proteins like ovalbumin, which are not present in the diet [20]. Thus, trace amounts of BLG in the milk-free diet do not seem to be sufficient to induce oral tolerance defined by antibody and in vitro cellular responses.

Discussion

The present study demonstrates that maternal dietary cow's milk, in conjunction to the suppressed immune response to BLG in the mother, also influences the immune response to BLG in the first-generation offspring.

Upon immunization, the immunoglobulin and cell-proliferative responses to BLG in the F0 mice were significantly lower than that of the F2/F3 mice, clearly indicating that BLG in the standard diet has induced oral tolerance to the antigen in the F0 mice. This is in agreement with earlier studies in mice, which have reported oral tolerance induction towards proteins present in the diet [10, 11, 21, 22]. Whether this presence of oral tolerance in mice fed a BLG-containing diet is linked with the absence of hypersensitivity reactions remains to be tested. However, in recent studies employing mice fed a soy-protein-containing diet, we found that the IgE response induced to soy protein by use of the Th2-driving mucosal adjuvant cholera toxin was considerably suppressed in the soy-protein-fed mice [22].

In the F1 generation, the immune response to BLG was different from the response of both the F0 mice and the F2/F3 mice. For the antibody response, the greater variability in F1 mice resulted in an immunoglobulin re-

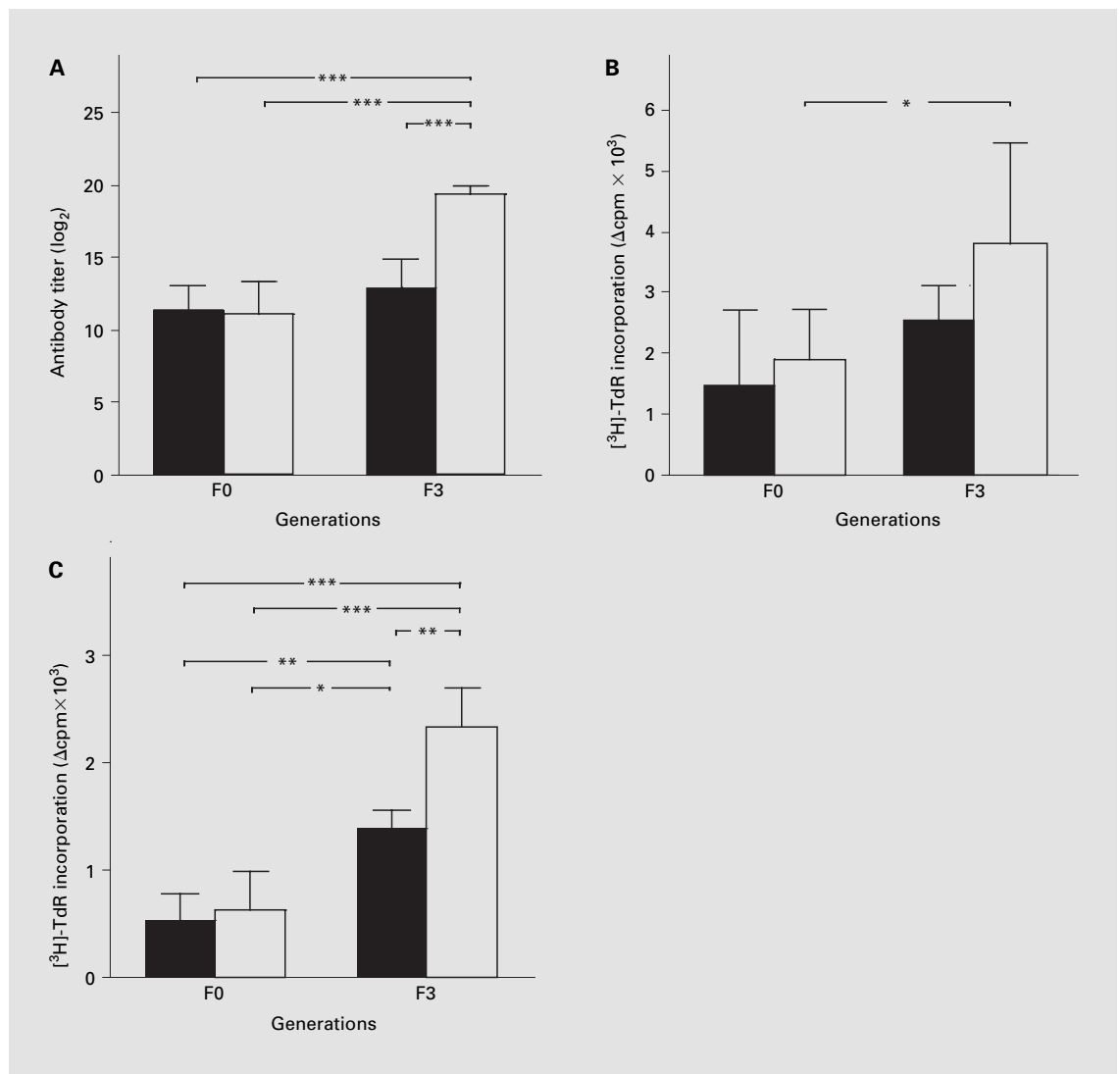


Fig. 4. Effect of feeding BLG to the F0 and the F3 generations on the specific antibody titers and cellular responses to BLG. Mice from the F0 (milk-containing diet) and the F3 (commercial milk-free diet) generations were fed soluble BLG (approximately 20 mg/day) via the drinking water (fed groups, black bars) or given pure drinking water (non-fed groups, white bars) for 3 days (days 1–3) and then immunized intraperitoneally on days 12 and 26 with 20 µg BLG. Blood samples and cells from spleen and mesenteric lymph nodes were collected on day 33. Antibody titers are shown as log₂ of the reciprocal of the sample dilution giving an absorbance of 0.2. The cell-prolifera-

tive responses are expressed as the mean [³H]-TdR incorporation in BLG-stimulated cultures after subtraction of the mean value of unstimulated cultures. **A** BLG-specific antibody titers. Means ± SD (n = 4–6). **B** Proliferative response of spleen cells stimulated with 1 µg/ml BLG. Means ± SD (n = 4–6). **C** Proliferative response of mesenteric lymph node cells stimulated with 25 µg/ml BLG. Means ± SD of quadruplicates from pooled cell cultures within each group. * p < 0.05, ** p < 0.01, *** p < 0.001; Tukey's multiple comparison test.

response intermediate between that of the F0 and the F2/F3 mice. A similar BLG-specific immunoglobulin response pattern was observed in an additional study involving mice immunized at 4 weeks of age and to a lesser extent in mice immunized at 20 weeks of age [unpubl. observations]. In vitro cellular proliferation in the presence of

BLG was slightly increased in the cells of the F1 mice compared to those of the F2/F3 mice. This increased response was observed in both immunized and non-immunized mice and could be due to priming of fetal or neonatal immune cells by maternally derived factors transferred via the placenta or the milk. Such a phenome-

non has been reported previously for the offspring of mice fed a gluten-containing diet [14]. Likewise, in humans, fetal priming of cord blood mononuclear cells to ovalbumin and to the milk proteins BLG, α -lactalbumin and α -casein has also been described [23, 24]. Correspondingly, we observed here systemic cellular priming towards α -casein in the F1 generation of milk-formula-immunized mice. However, the humoral response towards α -casein did not reveal any differences between the three generations. Analyses of specific immunoglobulin in the blood samples towards other bovine caseins (β -casein and κ -casein) showed no differences either [unpubl. observations]. This discrepancy between the anti-BLG and the anti-casein antibodies detected in the four generations of mice could be due to analytical conditions as bovine caseins may denature during plastic coating to microtiter plates resulting in changes in conformational epitopes. Another explanation could be that the amount of caseins in the standard chow does not suffice to suppress the humoral response, which is known to be less susceptible to suppression than the cellular response [1].

In non-immunized mice, no circulating anti-BLG-specific antibodies were detected in any of the four generations of mice at either 9 or 12 weeks of age. Since a transient antibody response to BLG has been reported by Kato et al. [25] in the offspring of dams fed a milk-containing diet during pregnancy, we performed an additional study collecting blood samples in 4-week-old mice. However, we could not detect anti-BLG-specific antibodies in any of the generations of mice examined at this age either [unpubl. observations]. Different BLG contents in the diets could perhaps explain the disparities between our results and those of Kato et al. [25]. However, the lack of information concerning the BLG content of the diet used in the latter study makes it difficult to make any firm conclusions.

When interpreting the results of the present study, the small amount of BLG detected in the commercial milk-free diet needs to be considered. However, it seems unlikely that the unique immune response of the F1 mice is due to contaminating levels of BLG in the milk-free diet, as a lower response was detected in the F2/F3, which were fed the same commercial milk-free diet. On the other hand, it could be that this low-level BLG contamination has triggered an otherwise latent response to maternally transferred factors. Irrespective of the underlying mechanisms, our data support earlier reports of a deviation in the response of F1 generation animals bred on an antigen-free diet [9, 12]. Thus, it appears that breeding of mice for at least two generations on an antigen-free diet is required

for studies examining the immune responses towards food proteins.

The presence of small amounts of antigen in commercial diets claiming to be antigen-free highlights the need to consider the impact of such contamination on the immune response in experimental animals. In this study, it is difficult to draw firm conclusions concerning the immunological effect of small amounts of BLG (approximately 1 μ g/g) in such diets as we could not compare the responses to those of mice bred on a strictly antigen-free diet. Only a few studies have focused on the effect of feeding low doses of antigens to experimental animals bred on a strictly antigen-free diet [26–28]. Applying the same experimental protocol for the detection of oral tolerance, following the administration of ovomucoid to BALB/c mice in the drinking water, we found in a previous study the threshold for tolerance induction to be in the range of 0.1 mg of ovomucoid/day for 50 days [28]. Neither tolerance nor priming were observed at lower antigen doses, supporting the present findings that ingestion of an amount of BLG of approximately 5 μ g/day does not affect the immune response to BLG in mice. In contrast, Gonnella et al. [27] found that administration of as low as 250 ng ovalbumin five times by gavage induced oral tolerance in C57BL/6 mice, whereas 500 pg ovalbumin caused priming. In addition to the different mouse strains, antigens and immunization strategies used, the discrepancy between the two studies could be due to the different routes of administration. Indeed, gavage administration has been reported to stimulate immune responses (IgE, IgG1 and histamine release) to ovalbumin (0.1 mg/mice) in mice whilst administration of the same amount of antigen in drinking water had no effect [8].

Our results comparing oral tolerance induction in F0 and F3 mice suggest that the small amount of BLG in the milk-free diet did not affect either the antibody or cell-proliferative response to BLG in F3 mice after high-dose feeding of BLG. Thus, exposure to approximately 1 μ g BLG/g feed does not appear to have a suppressive effect on mice. However, in experiments on sensitization, a potential effect of these small amounts of BLG cannot be excluded. To evaluate this further, the effect of small amounts of dietary antigen on sensitization in a murine model is currently under investigation in our laboratory.

In conclusion, our results suggest that in addition to breeding mice on an antigen-free diet for at least two generations, it is also imperative to control the specific antigen content in the diets used for breeding.

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