

BCG vaccine modulates intestinal and systemic response to β -lactoglobulin

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β -Lactoglobulin (BLG) is a clinically important antigen in cow's milk and one of the major allergens causing cow's milk allergy. Bacillus Calmette-Guérin (BCG) vaccination has been suggested to modify immune response possibly decreasing the risk of allergy to some antigens in both human and experimental animals. In the present study, we have analyzed whether the early BCG vaccination has any effect on the markers of systemic and gastrointestinal (GI) sensitization to BLG. We immunized two groups of Hooded-Lister rat puppets with intraperitoneal injections of native BLG at 43 and 62 days with pertussis vaccine as adjuvant, one group receiving additionally BCG. The animals were then fed native and denatured milk products twice weekly from 73 to 131 days of age, when they were killed. Control group was not vaccinated and received normal rat forage. Total immunoglobulin E (IgE) levels and BLG-specific IgG₁ and IgG_{2a} concentrations were determined in serum samples. Spontaneous interleukin (IL)-4 and interferon (IFN)- γ production from duodenal specimens were measured, and the inflammatory cells were quantitated in specimens from different sections of the GI tract. Administration of BCG simultaneously with BLG resulted in reduced IgE concentration in serum, while the specific IgG₁ and IgG_{2a} antibody responses and the spontaneous secretion of IL-4 and IFN- γ were not affected. Furthermore, BCG-induced eosinophilic infiltration and increase of intraepithelial lymphocytes (IEL) in the GI mucosa, and a trend toward increased number of lamina propria mononuclear inflammatory cells in the colon (BCG compared with BLG, $p = 0.09$; BCG compared with controls, $p = 0.02$). Controls showed increment of IgG₁ response in comparison with the BLG group ($p = 0.04$) and increase of mucosal eosinophilic infiltration. The BCG modified the response to BLG both at the systemic level as shown by decrease of total IgE and at GI mucosa where increase of eosinophilic infiltration and increased number of IEL were seen. Increment of IgG₁ level and eosinophils in the controls might be related with the lack of modulatory effect of pertussis vaccination. A shift of response toward the lower GI tract after BCG immunization as shown by a trend for increase of mononuclear inflammatory cells in colon lamina propria mimics disease development in some cases of clinical food allergy, and emphasizes the need for evaluation of the changes in the whole GI tract in food allergy models.

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Cow's milk allergy (CMA) is usually the first allergic manifestation of the child (1) and β -lactoglobulin (BLG) is one of the major food allergens (2). Clinical features include immediate reactions with skin rash, gastrointestinal (GI) and/or systemic reactions, and delayed reactions, such as

cow's milk-sensitive enteropathy (CMSE) with cell-mediated immune response locally in various segments of the GI tract and vague or severe GI complaints (3, 4). Thus, CMA is not a clear-cut clinical entity, and the mechanisms by which the different patterns develop are not known (3).

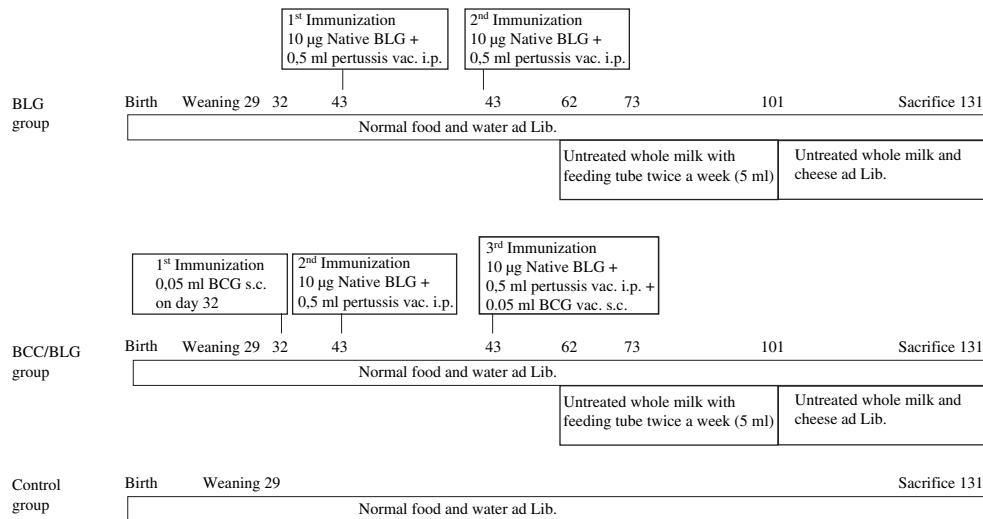


Fig. 1. Summary of the immunization procedures and the diets of the experimental animals.

At birth, both the murine and human immune system is skewed toward Th₂ immunity (5, 6). There is experimental evidence from murine models suggesting that Th₁/Th₂ balance and development of respiratory allergy may be modified by BCG vaccination or infection (7, 8). In humans, the effect of BCG vaccination on the risk of allergy and immune response is a matter of controversy (9–11).

We have recently shown that heat denaturation of BLG modifies the local and systemic response to this antigen. Reaction to native BLG having features of Th₂ balanced response while the reaction induced by heat-denatured BLG has more of Th₁ features (12). Here, we have analyzed the modulating effect of BCG vaccine on the response to low dose parenteral sensitization and GI exposure to native BLG by using the same animal model. Our hypothesis was that BCG vaccination would promote a shift of balance toward Th₁, compared with Th₂ response induced by native BLG only.

Materials and methods

Animals and immunization

Conventionally bred outbred Hooded-Lister rats (Laboratory Animal Centre, University of Oulu, Oulu, Finland) were nursed in the Laboratory Animal Centre, University of Oulu, Oulu, Finland. The stock of rats was HsdOla:LH.

The schedule of the immunization and feeding of the three groups of animals numbering 8–10 in each group is summarized in Fig. 1. Two of the groups (BLG group and BCG/BLG group) were immunized with native BLG containing the variants A and B (Sigma Chemical Co., St Louis, MO,

USA), which were purified by anion-exchange chromatography (13). The animals were immunized twice with 10 mg intraperitoneal injections of BLG and 0.5 ml of pertussis vaccine, containing whole inactivated bacteria (Public Health Institute, Helsinki, Finland) on days 43 and 62. BCG/BLG-group was additionally immunized subcutaneously with BCG vaccine (0.05 ml; BCG Vaccine SSI, Statens Serum Institut, Denmark) on days 32 and 62. Rats were allowed water and normal animal food *ad libitum* [Rat and mouse No. 3, RM3(E) Breeding expanded, SDS Special Diet Services; Witham, Essex, England, UK]. During the days 73–101, BLG and BLG/BCG groups were fed 5 ml of unprocessed milk with a feeding tube twice a week. In addition, during the days 102–130, they got cheese and unprocessed milk *ad libitum*. The control animals (n = 10) were not immunized and were fed normal animal food and water *ad libitum*. At day 131, the rats were killed using ketamine anesthesia. The study was approved by the Ethical Committee for Animal Studies, University of Oulu.

Quantitation of mucosal inflammatory cells

On day 131, tissue samples were taken from the antrum of the stomach, duodenum, proximal jejunum (about 5 cm from the pylorus) and colon. The specimens were fixed in 10% neutral buffered formaline and embedded in paraffin. Sections were cut at 5 µm and stained with hematoxylin/eosin. All cell counts were made by one of us (JR) blinded to the treatment data and other results as described earlier (12). The following cell types were counted: intraepithelial lymphocytes (IEL), intraepithelial eosinophils, lamina propria eosinophils and mononuclear

inflammatory cells from duodenum and jejunum, mononuclear inflammatory cells and eosinophils from glandular zone of antroduodenal mucosa, and lamina propria mononuclear inflammatory cells and eosinophils from colon.

Quantitation of serum total IgE levels and specific IgG₁ and IgG_{2a} antibodies to BLG

Blood samples were obtained through cardiac puncture at day 131. Serum was separated and frozen at -20°C . Total serum immunoglobulin E (IgE) level and specific IgG₁ and IgG_{2a} antibodies to BLG were measured as described earlier (12).

Measurement of IFN- γ and IL-4 secretion

For the evaluation of spontaneous secretion of interferon (IFN)- γ and interleukin (IL)-4, fresh specimens taken at day 131 from the duodenum were weighed and placed immediately in cell culture medium (RPMI, 0.5 ml) and incubated at 37°C for 5 h. The supernatants were frozen at -70°C . Secreted IFN- γ and IL-4 were measured with enzyme-linked immunosorbent assay (ELISA) (Quantikine M murine IFN- γ , RIF00; IL-4, R4000; R&D systems, Abingdon, Oxon, UK) as described earlier (12).

Statistics

Statistical analysis was performed with SPSS (9.0; SPSS Inc., Chicago, IL, USA). Groups were compared with the non-parametric Kruskal–

Wallis test or chi-square test for dichotomous variables and correlation between the variables was analyzed by the non-parametric Spearman rank correlation.

Results

Quantitation of mucosal inflammatory cells

For quantitative analysis of inflammatory cell response in different parts of GI mucosa tissue specimens from rats immunized with BLG or BCG/BLG and treated with milk products, and non-immunized control group were studied (Table 1). In the gastric lamina propria eosinophilic counts were significantly higher in the BCG/BLG ($p = 0.002$) and control ($p = 0.006$) group when compared with BLG group, while in the duodenum the controls showed more eosinophils than the BCG/BLG group ($p = 0.002$; Fig. 2). Rats immunized with BLG had significantly less IELs in the duodenum than the rats immunized with BCG/BLG ($p = 0.015$) or controls ($p = 0.01$) and a similar difference between BLG and BCG/BLG groups were seen in the jejunum as well ($p = 0.027$; Table 1). Jejunal intraepithelial eosinophil counts were similarly lower in the BLG group when compared with BCG/BLG group ($p = 0.045$; Table 1). Lamina propria mononuclear inflammatory cell densities were mostly similar, but in the colon, the BCG/BLG group showed highest density differing significantly from the control group ($p = 0.02$) and showing a similar trend in comparison with the BLG group

Table 1. Quantitation of mucosal inflammatory cells in animals immunized with native BLG with or without BCG injection and in non-immunized controls

	BCG/BLG	BLG	Controls
Gastric antrum			
Mononuclear inflammatory cells	427 (277–853)	341 (149–939)	331 (171–597)
Eosinophils	277 (197–341)*	128 (43–213)*	320 (85–576)*
Duodenum			
Mononuclear inflammatory cells	8152 (7347–9561)	7830 (6039–9964)	7750 (5535–11071)
Eosinophils	318 (173–462)†	578 (116–636)	578 (462–809)†
Intraepithelial lymphocytes	14.6 (12.4–27.2)‡	7.9 (4.9–16.7)‡	16.2 (8.8–26.4)‡
Intraepithelial eosinophils	0.4 (0.0–1.5)	0 (0–1.5)	1.0 (0–1.9)
Jejunum			
Mononuclear inflammatory cells	8354 (7649–9360)	7950 (6340–9863)	7699 (6843–8655)
Eosinophils	925 (694–2312)	895 (635–1271)	867 (462–1502)
Intraepithelial lymphocytes	23.3 (14.5–30.4)§	15.5 (9.6–21.3)§	17.9 (8.9–31.1)
Intraepithelial eosinophils	1.7 (0–5.8)¶	1.0 (0–1.6)¶	1.2 (0.4–2.2)
Colon			
Mononuclear inflammatory cells	658 (569–711)**	462 (356–676)	373 (178–818)**
Eosinophils	178 (107–391)	71 (0–462)	124 (36–284)

Cells were counted in different parts of the gastrointestinal tract. Median and range are indicated. Cell density was counted separately in the lamina propria (cells/0.001 mm²) and the surface epithelium (intraepithelial cells/100 epithelial cells).

*Bacillus Calmette-Guérin/ β -lactoglobulin (BCG/BLG) vs. BLG, $p = 0.002$; BLG vs. controls, $p = 0.006$; †BCG/BLG vs. controls, $p = 0.002$; ‡BCG/BLG vs. BLG, $p = 0.015$; BLG vs. controls, $p = 0.01$; §BCG/BLG vs. BLG, $p = 0.027$; ¶BCG/BLG vs. BLG, $p = 0.045$; **BCG/BLG vs. controls, $p = 0.02$.

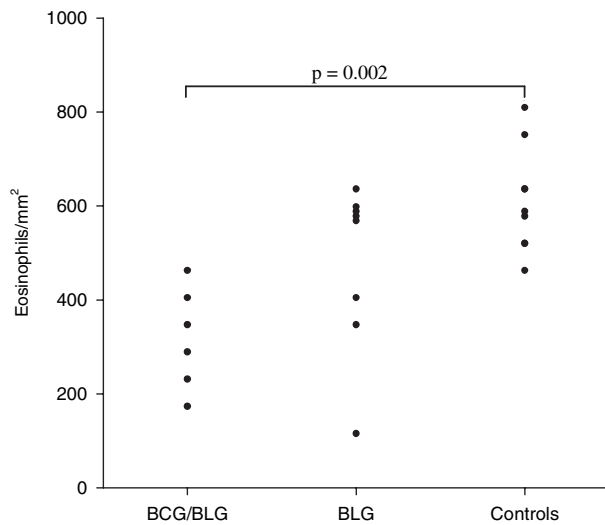


Fig. 2. Density of eosinophilic granulocytes in duodenal mucosa in rats treated with native β -lactoglobulin (BLG) with and without bacillus Calmette-Guérin (BCG) and in untreated controls.

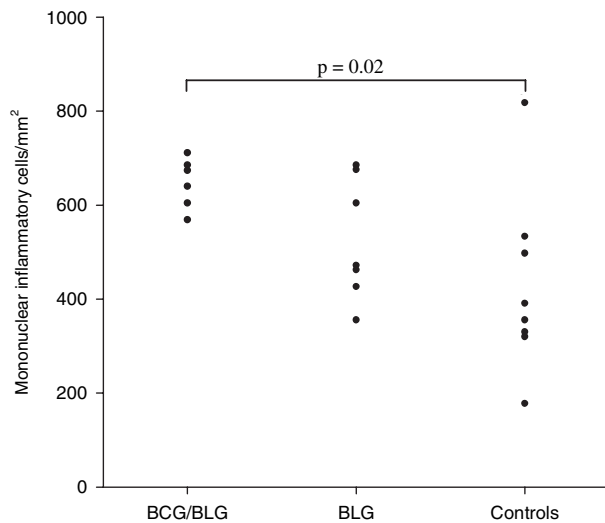


Fig. 3. Density of mononuclear inflammatory cells in colonic mucosa in rats treated with native β -lactoglobulin (BLG) with and without bacillus Calmette-Guérin (BCG) and in untreated controls.

($p = 0.09$), thus showing evidence for a chronic inflammatory reaction (Table 1; Figs. 3 and 4).

In the whole material, there was a significant negative correlation between the density of lamina propria eosinophils of the duodenum and mononuclear inflammatory cells in the colon ($c = -0.454$; $p = 0.044$, Spearman rank correlation).

Serum immunoglobulin levels

The serum IgE concentrations were lower in the BCG/BLG than in the BLG group ($p = 0.046$;

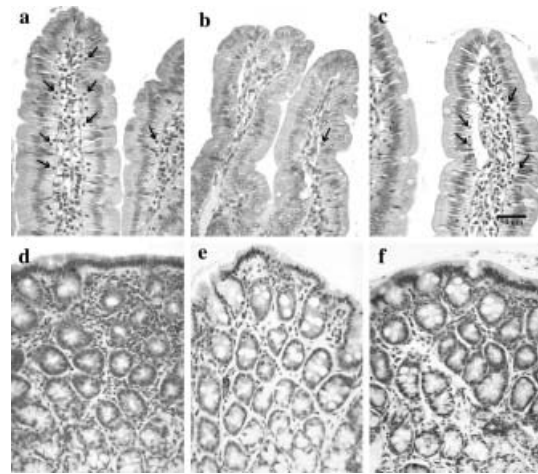


Fig. 4. Photomicrographs of duodenal (a–c) and colon (d–f) mucosa in rats immunized with bacillus Calmette-Guérin (BCG)/ β -lactoglobulin (BLG) (a,d), BLG (b,e) and untreated controls (c,f). In the duodenum, there is more intraepithelial lymphocytes (arrows) after BCG/BLG immunization (a) when compared with BLG only (b). The controls (c) show a similarly high number of intraepithelial lymphocytes. In the colon, the density of lamina propria mononuclear inflammatory cells is higher after BCG/BLG immunization (d) than in controls (f) or after BLG immunization (e). In the latter comparison, the difference between groups did not reach statistical significance (see Table 2; $p = 0.09$).

Table 2. Measurement of serum total IgE (ng/ml), BLG-specific IgG₁ and IgG_{2a} (OD = optical density values), and measurement of spontaneous secretion of IFN- γ or IL-4 (pg/ml) from duodenal mucosa in animals immunized with native BLG with and without BCG and in controls

	BCG/BLG	BLG	Controls
IgE concentration (ng/ml)	150 (130–720)*	500 (80–1370)*	200 (40–1320)
IgG ₁ (OD)	0.04 (0.01–0.07)	0.021 (0.01–0.06)**	0.03 (0.02–0.09)**
IgG _{2a} (OD)	0.07 (0.04–0.14)	0.06 (0.04–0.11)	0.09 (0.06–0.17)
IL-4 (pos./tot.)	4/8 (50%)	3/8 (36%)	7/10 (70%)
IFN- γ (pos./tot.)	2/8 (25%)	3/8 (36%)	1/10 (10%)

Median and range, and for IFN- γ or IL-4 the proportion of cases with detectable secretion (positive cases/total) are indicated.

* $p = 0.046$; ** $p = 0.037$.

IgE, immunoglobulin E; BLG, β -lactoglobulin; IFN, interferon; IL, interleukin; BCG, bacillus Calmette-Guérin.

Table 2). Rats in the BLG group had significantly ($p = 0.037$) lower levels of BLG-specific IgG₁ when compared with controls. IgG_{2a} concentrations showed no significant differences, but there was clearly a tendency for BLG group to show lower values than controls ($p = 0.055$; Table 2). The IgG₁/IgG_{2a} ratios were similar in the groups and were inversely correlated with the IL-4 concentrations ($r = -0.4$; $p = 0.042$), and the number of jejunal intraepithelial eosinophils ($r = -0.4$; $p = 0.047$).

Cytokine production

Spontaneous cytokine secretion of duodenal specimens taken after immunizations and exposure was measured to get insight of Th₁/Th₂ balance in different groups. Spontaneous secretion of IFN- γ and IL-4 from duodenal samples was low during the 5 h of incubation period and no significant differences between the groups were seen either in proportion of cases with measurable secretion or actual concentrations (Table 2). The IFN- γ concentrations showed a significant negative correlation with the number of duodenal intraepithelial eosinophils ($r = -0.532$; $p = 0.006$; Spearman), while the IL-4 concentrations did not correlate with the inflammatory cell counts. The IL-4 and IFN- γ concentrations showed a positive correlation in the individual animals ($r = 0.442$, $p = 0.008$; Spearman).

Discussion

The results show that BCG vaccination modulates immune responses to native BLG. Decreased serum IgE concentration might suggest a change toward Th₁ dominant balance at the systemic level after BCG vaccination. However, we did not find undisputable evidence to support this as no significant difference in serum IgG₁ and IgG_{2a} concentrations or their ratio were found. At the mucosal level, the effect of BCG was dependent on the location, and varied from eosinophilic cell infiltration in the most proximal part to increase of IELs in the small intestine and a tendency for a chronic inflammatory response in the colon. However, we found no significant differences in the mucosal cytokine pattern to prove any change in Th₁/Th₂ balance.

In agreement with the present finding, BCG has been reported to induce a decrease of the serum levels of IgE in experimental animals (7, 8). However, this has not been observed in all animal experiments, possibly depending on the animal strain (14, 15). Previous studies were performed in young animals and therefore are not fully comparable with our observations based on measurements on adult animals. In humans, BCG vaccination seems not to affect on total IgE concentration (9). IgG_{2a} secretion has been reported to be associated with Th₁ activation while IgG₁ mainly with Th₂ activation (16), but in our study BCG vaccination did not have any significant effect on the levels of BLG-specific IgG₁ and IgG_{2a} antibodies. We did not see any significant effect on the spontaneous secretion of IL-4 or IFN- γ in the duodenal mucosa. However, since no IFN- γ or IL-4 secretion was seen in most of the

samples, it is likely that the sensitivity of the assay was not optimal to detect a minor modification of expression.

Mucosal cellular response showed some unexpected differences in different parts of the GI tract when comparing BCG/BLG and BLG groups. In the gastric and jejunal mucosa eosinophilic infiltration was higher after BCG/BLG immunization than after BLG. In duodenal mucosa no differences in the eosinophil counts were seen, but a significant increase of IEL was apparent in the BCG/BLG group extending to jejunal mucosa. In the density of lamina propria mononuclear inflammatory cells there were no major differences in the upper GI tract, but some evidence for an increase of the density of these cells was seen in the colon mucosa ($p = 0.09$ in the BCG/BLG group in comparison with BLG-group and $p = 0.02$ in comparison with controls). These findings suggest that there is a shift of mononuclear inflammatory cell response to the distal GI tract, and even that this modification of the response by BCG vaccination may be associated with the risk of colonic inflammation.

The reasons for the different BCG-induced immune response and modification of the reaction pattern in the different parts of the GI tract are not clear and remain speculative. In the previous animal studies no morphologic or immunologic analysis of colon samples was performed and therefore no comparative data is available. The observed changes could either be a direct effect of BCG vaccination on the mucosal inflammation and immunity or induced indirectly by a modification of the antigen handling, or both. It is known that the route and dose of immunization with BCG modulate the responses in terms of Th₁/Th₂ balance. Lagrandie et al. (17) found out that a subcutaneous vaccination-induced more Th₂ biased response than mucosal (rectal) immunization, while Power et al. (18) showed that a high dose induced a Th₂ skewed response. In our previous study, we showed that the response to BLG was dependent on the denaturation of the antigen, heat-treated BLG inducing more intensive inflammation in gastric mucosa and less increment of serum IgE than native BLG (12). Although the mechanisms for this difference remained unclear, we hypothesized that the difference in the degradation might be important, denatured BLG being more prone to proteolytic degradation and therefore disclosing less conformational epitopes and more T-cell stimulating epitopes (12). Based on these observations, we speculate that BCG vaccination modifies mucosal response to BLG in two different ways depending on the state of degradation of the antigen. In the

proximal GI tract, particularly in the gastric mucosa, the antigen is intact expressing only conformational epitopes. According to our hypothesis, BCG vaccination would induce a more intensive reaction to these epitopes, which would lead to an increment of local eosinophils in the gastric mucosa. Along with the proteolytic degradation of the antigens in duodenum and more distally, peptides with sequential antigenic epitopes recognized by T-lymphocytes are released, and the response in a setting of BCG-induced modification of reaction leads to a strong lymphocyte accumulation, which becomes visible as an increase of intestinal IEL in the duodenal and jejunal mucosa. We observed a tendency for increased mononuclear cells in lamina propria in BCG-vaccinated animals.

Considering clinical relevance of the finding suggesting induction intestinal inflammatory changes with BCG, it is of interest that we and the others have shown that chronic colonic inflammation of children may be related to sensitivity to food proteins (19, 20). Moreover, we have found a rise of intraepithelial T cells including γ/δ -positive cells in the mucosa of both duodenum (21) and the ileum in subjects with colitis (19). These features bear a resemblance to those observed in our current murine model. Similarly, previous experimental colitis models have shown importance of lymphocyte subsets in the inflammatory response (22–24).

Interestingly, the non-vaccinated and non-sensitized controls showed highest density of eosinophils in the gastric antrum. This indicates that either immunization with pertussis vaccine, which was used as an adjuvant in the BLG groups, or immunization with BLG or milk proteins from the feeding ingredients of the BLG and BCG/BLG groups induce a decrease of gastric eosinophilic infiltration. In humans, it has been shown that clinical *Bordetella pertussis* infection (25) and vaccination with whole cell vaccine (26) would induce preferentially Th₁ activation. Similarly, in animal experiments, pertussis has been used to induce Th₁ activation, although in some animal experiments pertussis vaccination has been used as an adjuvant to induce immediate allergic reaction not inducible without such an adjuvant (27). In our study, consistent with uninhibited Th₂-activation in this non-vaccinated control group, IgG₁ level was increased in comparison with the BLG-group. In addition, there was some non-significant tendency to Th₂ type cytokine pattern with detectable spontaneous IL-4 secretion in majority of animals, and absence of IFN- γ in all but one animal. These findings suggest that the pertussis

vaccination might have had some modificatory effect on the immune response in BLG immunized animals. Our experimental arrangement was not planned to evaluate possible cumulative or counteracting effects of BCG and pertussis vaccinations on the response to BLG, and no constant and statistically significant evidence for either direction could be demonstrated. However, we saw some although non-significant gradual increment of the mononuclear inflammatory cell counts in the colon to lend support some cumulative effect of the two vaccinations.

In the last few years, it has been considered that a balance between the Th₂ and Th₁ developing during the infancy determines largely the development of atopy. Accordingly, the so-called 'hygiene hypothesis' favors idea that lack of microbial challenge leads to Th₂ dominant balance and a high risk of allergy, while encountering infections would lead to Th₁ dominant balance and a low risk of allergy. Observations of a protective effect of parasitic infections and allergy and also the epidemiologic findings of the emergence of Th₁-dependent autoimmune diseases along with atopic conditions argue against the hygiene hypothesis. Indeed, recently another model has been described, which better explains these observations. 'Strong regulatory network' hypothesis (28) suggests that extensive exposure to infections and antigens induces the development of regulatory network consisting of immunomodulating cells, mainly T cells, which is able to produce anti-inflammatory cytokines [IL-10 and transforming growth factor (TGF)- β], thereby suppressing both the excessive Th₂ and Th₁ activities. Although our study did not assess the regulatory network, our results indirectly suggest that the regulatory network may have different strength and effects at the systemic and local level, indicating need to extensive assaying of the response in different organ systems.

In summary, BCG induced a modification of the immune response to BLG from immediate to delayed at the systemic level, but induced a stronger local reaction in the GI tract. The inflammation was observed in all parts of the GI tract from the stomach until colon, with a pattern varying in the different parts, however. This finding emphasizes the importance of systematic morphologic analysis of the different parts of GI mucosa to show possible divergence of the reaction patterns depending of the anatomical location. By this analysis, we could find some evidence that BCG vaccination as an adjuvant to sensitization to BLG induces a chronic inflammation in the colon, but more studies are needed to confirm this finding and evaluate the mechanisms.

Acknowledgments

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References

1. SAARINEN KM, JUNTUNEN-BACKMAN K, JÄRVENPÄÄ AL, et al. Supplementary feeding in maternity hospitals and the risk of cow's milk allergy: a prospective study of 6209 infants. *J Allergy Clin Immunol* 1999; 104: 457–61.
2. WAL JM. Cow's milk allergens. *Allergy* 1998; 53: 1013–22.
3. SAMPSON HA, ANDERSON JA. Summary and recommendations: classification of gastrointestinal manifestations due to immunologic reactions to foods in infants and young children. *J Pediatr Gastroenterol Nutr* 2000; 30 (Suppl.): 87–94.
4. KOKKONEN J, RUUSKA T, KARTTUNEN TJ, NIINIMÄKI A. Mucosal pathology of the foregut associated with food allergy and recurrent abdominal pains in children. *Acta Paediatr* 2001; 90: 16–21.
5. ADKINS B. Development of neonatal Th1/Th2 function. *Int Rev Immunol* 2000; 19: 157–71.
6. MARCHANT A, GOETGHEBUER T, OTA MO, et al. Newborns develop a Th1-type immune response to *Mycobacterium bovis* bacillus Calmette-Guérin vaccination. *J Immunol* 1999; 163: 2249–55.
7. KOH YI, CHOI IS, KIM WY. BCG infection in allergen-prensensitized rats suppresses Th2 immune response and prevents the development of allergic asthmatic reaction. *J Clin Immunol* 2001; 21: 51–9.
8. YANG X, WANG S, FAN Y, ZHU L. Systemic mycobacterial infection inhibits antigen-specific immunoglobulin E production, bronchial mucus production and eosinophilic inflammation induced by allergen. *Immunology* 1999; 98: 329–37.
9. MARKS GB, NG K, ZHOU J, et al. The effect of neonatal BCG vaccination on atopy and asthma at age 7 to 14 years: an historical cohort study in a community with a very low prevalence of tuberculosis infection and a high prevalence of atopic disease. *J Allergy Clin Immunol* 2003; 111: 541–9.
10. GRÜBER C, MEINLSCHMIDT G, BERGMANN R, WAHN U, STARK K. Is early BCG vaccination associated with less atopic disease? An epidemiological study in German preschool children with different ethnic backgrounds. *Pediatr Allergy Immunol* 2002; 13: 177–81.
11. KRISHNA MT, SALVI SS. Could administration of bacille Calmette-Guérin vaccination at birth protect from the development of asthma and allergic diseases in the western world? Has this question been adequately investigated? *Pediatr Allergy Immunol* 2002; 13: 172–6.
12. RYTKÖNEN J, KARTTUNEN TJ, KARTTUNEN R, et al. Effect of heat denaturation on beta-lactoglobulin-induced gastrointestinal sensitization in rats: denatured β LG induces a more intensive local immunologic response than native β LG. *Pediatr Allergy Immunol* 2002; 13: 269–77.
13. VALKONEN KH, MARTTINEN N, ALATOSSAVA T. Preparative separation and characterization of beta-lactoglobulin genetic variants A and B from bovine milk. *Milchwissenschaft* 2000; 55: 126–9.
14. ERB KJ, HOLLOWAY JW, SOBECK A, MOLL H, LE GROS G. Infection of mice with *Mycobacterium bovis*-bacillus Calmette-Guérin (BCG) suppresses allergen-induced airway eosinophilia. *J Exp Med* 1998; 187: 561–9.
15. HYLKEMA MN, TIMENS W, LUNGE M, VAN DER WERF N, HOEKSTRA MO. The effect of bacillus Calmette-Guérin immunization depends on the genetic predisposition to Th2-type responsiveness. *Am J Respir Cell Mol Biol* 2002; 27: 244–9.
16. STEVENS TL, BOSSIE A, SANDERS VM, et al. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 1988; 334: 255–8.
17. LAGRANDERIE M, BALAZUC AM, ABOLHASSANI M, et al. Development of mixed Th1/Th2 type immune response and protection against *Mycobacterium tuberculosis* after rectal or subcutaneous immunization of newborn and adult mice with *Mycobacterium bovis* BCG. *Scand J Immunol* 2002; 55: 293–303.
18. POWER CA, WEI G, BRETSCHER PA. Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. *Infect Immun* 1998; 66: 5743–50.
19. KOKKONEN J, RUUSKA T, KARTTUNEN TJ, MAKI M. Lymphonodular hyperplasia of the terminal ileum associated with colitis shows an increase gamma delta + t-cell density in children. *Am J Gastroenterol* 2002; 97: 667–72.
20. NOWAK-WEGRZYN A, SAMPSON HA, WOOD RA, SICHERER SH. Food protein-induced enterocolitis syndrome caused by solid food proteins. *Pediatrics* 2003; 111: 829–35.
21. AUGUSTIN M, KARTTUNEN TJ, KOKKONEN J. TIA1 and mast cell tryptase in food allergy of children: increase of intraepithelial lymphocytes expressing TIA1 associates with allergy. *J Pediatr Gastroenterol Nutr* 2001; 32: 11–8.
22. DOHI T, FUJIIHASHI K, KOGA T, et al. T helper type-2 cells induce ileal villus atrophy, goblet cell metaplasia, and wasting disease in T cell-deficient mice. *Gastroenterology* 2003; 124: 672–82.
23. TOZAWA K, HANAI H, SUGIMOTO K, et al. Evidence for the critical role of interleukin-12 but not interferon-gamma in the pathogenesis of experimental colitis in mice. *J Gastroenterol Hepatol* 2003; 18: 578–87.
24. SCHMIDT C, MARTH T, WITTIG BM, HOMBACH A, ABKEN H, STALLMACH A. Interleukin-12 antagonists as new therapeutic agents in inflammatory bowel disease. *Pathobiology* 2002; 70: 177–83.
25. RYAN M, MURPHY G, GOTHEFORS L, NILSSON J, STORSAETER J, MILLS KH. *Bordetella pertussis* respiratory infection in children is associated with preferential activation of type 1 T helper cells. *J Infect Dis* 1997; 175: 1246–50.
26. AUSIELLO CM, URBANI F, LA SALA A, LANDE R, CASSONE A. Vaccine- and antigen-dependent type 1 and type 2 cytokine induction after primary vaccination of infants with whole-cell or acellular pertussis vaccines. *Infect Immun* 1997; 65: 2168–74.
27. FREIER S, ERAN M, GOLDSTEIN R. The effect of immediate-type gastrointestinal allergic reactions on brush border enzymes and gut morphology in the rat. *Pediatr Res* 1985; 19: 456–9.
28. YAZDANBAKHSH M, KREMSNER PG, van REE R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002; 296: 490–4.