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ORIGINAL ARTICLE

In vivo *transfer of plasmid from food-grade transiting lactococci to murine epithelial cells*

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We recently demonstrated that noninvasive food-grade Lactococcus lactis (L. lactis) can deliver eukaryotic expression plasmid in mammalian cells in vitro. Here, we evaluated, in vivo, whether a eukaryotic expression plasmid carried by lactococci can translocate to the epithelial cells of the intestinal membrane. The strain LL(pLIG:BLG1) carrying one plasmid containing a eukaryotic expression cassette encoding β-lactoglobulin (BLG), a major allergen of cow's milk, was orally administered by gavage to mice. BLG cDNA was detected in the epithelial membrane of the small intestine of 40% of the mice and BLG was produced in 53% of the mice. Oral administration of LL(pLIG:BLG1) induced a low and transitory Th1-type immune response counteracting a Th2 response in case of further sensitization. We demonstrated for the first time the transfer of a functional plasmid to the epithelial membrane of the small intestine in mice by noninvasive food-grade lactococci.

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Introduction

Lactococcus lactis is a food-grade Gram-positive lactic acid bacterium considered as noninvasive and noncolonizing. Its use to deliver several antigens and cytokines has been intensively studied (for reviews see Hanniffy *et al.*[1] and Nouaille *et al.*[2]). Recently, we explored the potential use of noninvasive *L. lactis* strains as a DNA delivery vehicle.[3] We constructed one *Escherichia coli L. lactis* shuttle plasmid, pLIG:BLG1, which contains a eukaryotic expression cassette with the cDNA of bovine β-lactoglobulin (BLG). We first showed that the resulting *L. lactis* strain MG1363(pLIG:BLG1) was not able to express BLG. Its potential as a BLG cDNA delivery vehicle was confirmed by detection of BLG in Caco-2 human colon carcinoma cells after 3 h of coincubation. Both BLG cDNA and BLG expression were detected only in Caco-2 cells coincubated with MG1363(pLIG:BLG1). We conclude that lactococci can deliver BLG cDNA into mammalian epithelial cells, demonstrating their potential to deliver *in vivo* a DNA vaccine. Here, the same strain was orally administered to mice to validate, *in vivo*, this plasmid delivery strategy. In addition, the potential translocation of such cDNA carried by transitory lactococci raises several questions on the fate of foreign DNA in the digestive tract and the modulation of host response by transiting food-grade bacteria.

In contrast to bacteria-mediated delivery of protein antigens, bacteria-mediated delivery of DNA vaccines could lead to host expression of post-translationally modified antigens and therefore to the presentation of conformationally restricted epitopes. Most of the bacteria used as DNA vaccine carrier are enteroinvasive species such as *Shigella flexneri*, *Yersinia enterocolitica* or *Listeria monocytogenesis* (for review, see Schoen *et al.*[4]). Despite the use of attenuated strains, the risk associated with the potential reversion to a pathogenic phenotype is a major concern.[5] The capacity of invasivity is encoded by different surface proteins as either internalin A (inlA) for *L. monocytogenes*[6] or invasin (inv) for *Y. enterocolitica*.[7] Noninvasive bacteria as *E. coli*, *L. lactis* or *Enterococcus faecalis* were rendered invasive after expression of either *inlA* or *inv* gene.[8–10] Nevertheless, it has been demonstrated *in vitro* that noninvasive bacteria as *L. lactis* or *E. coli* are able to transfer genetic material.[3,9,11] *In vivo*, *E. coli* has been used as vector for DNA vaccines by intramuscular, intraperitoneal or subcutaneous administrations.[12,13]

Here, we explored the ability of not genetically modified *L. lactis* to deliver plasmid *in vivo* to mice. We showed that oral administration of lactococci carrying a BLG eukaryotic expression plasmid induces a local production of BLG in the small intestine. The *in situ* production elicits a specific immune response protecting the mice from further sensitization with cow's milk proteins. To our knowledge, this is the first evidence of functional genetic material transfer from food-grade transiting bacteria to host.

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Results

BLG cDNA is detected in the small intestine after oral administration of LL(pLIG:BLG1) strain

Three days after the last gavage with either *L. lactis* LL(pLIG:BLG1) or *L. lactis* LL(pLIG1), mice were killed and the small intestine was withheld from each mouse and washed. To detect the presence of the BLG cDNA, DNA was extracted, purified and amplified by PCR using specific oligonucleotides of the BLG. Specific amplification was found in 40% of the mice treated with LL(pLIG:BLG1) ($n=5$) (Figure 1). No specific amplification was ever detected in either (pLIG1) group or naive mice.

BLG protein is detected in the small intestine after oral administration of LL(pLIG:BLG1) strain

Mice were force fed 1, 3 or 5 days. Three days after the last gavage, BLG was assayed using two enzyme immuno assays (EIA) specific of either BLG under native conformation (BLGn) or BLG under denatured conformation (BLGd) on soluble and insoluble proteins extracted from the small intestine. Three daily gavages are necessary to detect BLG only in the soluble fraction and exclusively under native conformation in eight of the mice treated with LL(pLIG:BLG1) (Figure 2b). Increasing the number of gavages did not change significantly the quantity of BLG detected or the number of mice expressing BLG (Figure 2c). Neither BLGn, nor BLGd was found in mice treated with either LL(pLIG1) or naive mice. BLG was never found in spleen or liver ($n=5$).

BLG protein is produced transitorily in the small intestine after oral administration of LL(pLIG:BLG1) strain

Mice were force fed daily three times. One, three or six days after the last gavages, BLG was assayed using two EIA specific of either BLGn or BLGd on soluble and insoluble proteins extracted from the small intestine. In mice killed 1 day after the last gavage, ~2.5 ng of BLG per ml were found exclusively under native conformation in the soluble fraction in two of the mice treated with LL(pLIG:BLG1) ($n=10$) (Figure 3). In mice killed 3 days after the last gavage, ~1.5 ng of BLG per ml was found exclusively under native conformation in the soluble fraction in four of the mice treated with LL(pLIG:BLG1) ($n=10$) (Figure 3). No BLG was found in mice killed 6 days after the last gavages ($n=10$) (Figure 3).

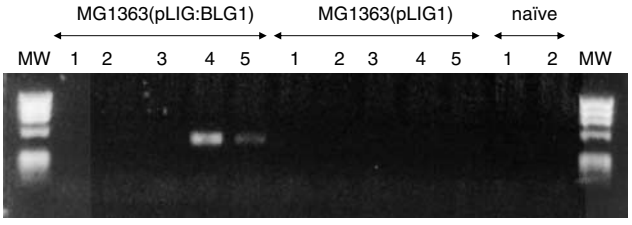

Figure 1 BLG cDNA detection by PCR amplification in the small intestine wall. Mice were force fed 3 days consecutively with LL(pLIG:BLG1) and LL(pLIG). Three days after the last gavage, mice were killed and DNA was extracted and purified. PCR amplification was performed using oligonucleotides specific from BLG. BLG, β-lactoglobulin.

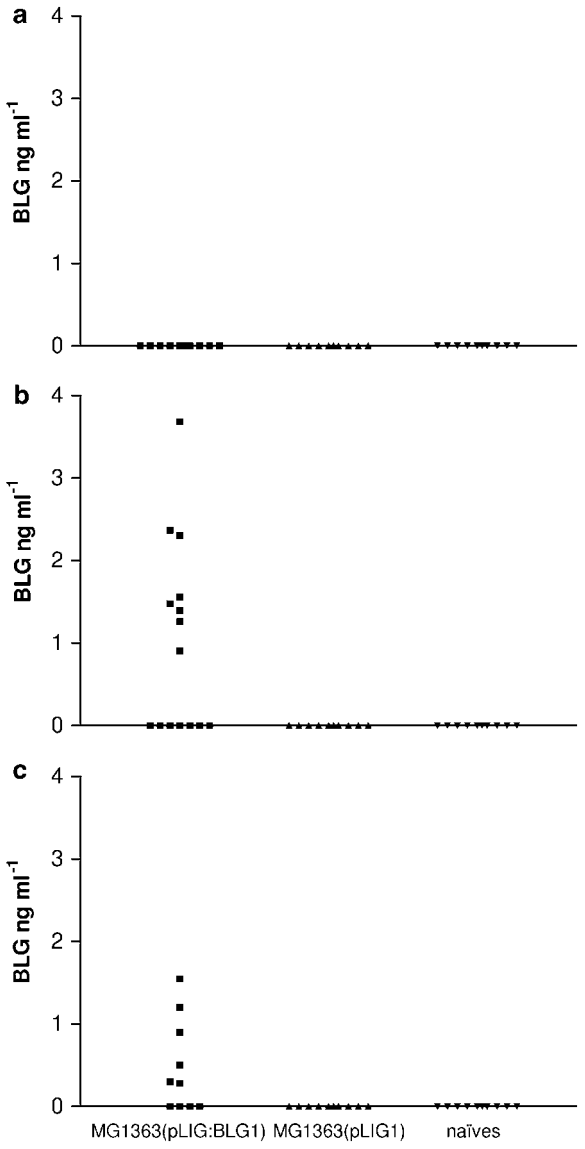

Figure 2 BLG detection in the small intestine wall. Mice were force fed 1 (**a**), 3 (**b**) or 5 days (**c**) consecutively with LL(pLIG:BLG1) and LL(pLIG). Three days after the last gavage, mice were killed and proteins were extracted. BLG was detected by enzyme immuno assay in protein extracts. BLG, β-lactoglobulin.

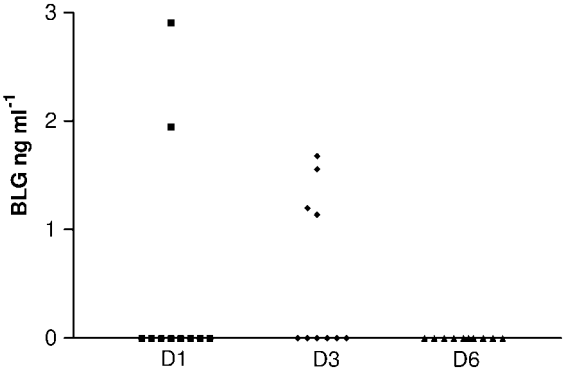

Figure 3 Kinetics of BLG expression. Mice were force fed 3 days consecutively with LL(pLIG:BLG1) and LL(pLIG). After 1, 3 and 6 days of the last gavage, mice were killed and proteins were extracted. BLG was detected by enzyme immuno assay in protein extracts. BLG, β-lactoglobulin.

Oral administration of LL(pLIG:BLG1) strain elicits antibody response against BLG

Three weeks after the last gavage with either LL(pLIG:BLG1) or LL(pLIG1) strains, mice were again force fed 5 days consecutively with the same strains to boost the immune response. BLG-specific immunoglobulin (Ig) G and IgA were assayed in fecal pellets, whereas BLG-specific IgG1, IgG2a and IgE were assayed in sera. Four days after the boost, a low IgG2a response was found in six of the mice treated with LL(pLIG:BLG1) strain ($n = 13$) (Figure 4a). Twelve days after the boost, no immune response was detected anymore (Figure 4b).

Mice were killed and spleens were harvested for cytokines secretion assays. After reactivation with BLG, no cytokines (interleukin (IL)-5 or interferon (IFN)-γ) was detected in any group. No IgA or IgG could be assayed in fecal pellets.

Mice pretreated with LL(pLIG:BLG) strain are protected from further oral sensitization

Mice were treated as previously described and 3 weeks after the boost, mice were sensitized orally with cholera toxin and milk proteins. BLG-specific IgG1, IgG2a and IgE were assayed in sera. Four days after the last sensitization, either untreated mice or mice treated with LL(pLIG1) showed a strong Th2 immune response with high level of IgE (Figure 5c) and IgG1 (Figure 5a). The IgE concentration was decreased in average by 70% between mice pretreated with LL(pLIG:BLG1) and untreated sensitized mice (Figure 5c). No differences were observed in IgG2a (Figure 5b) or IgG1 (Figure 5a) concentrations between pretreated or untreated mice.

Mice were killed and spleens were harvested for cytokines secretion assays. IL5 and IFN-γ were assayed after reactivation with BLG. Mice pretreated with LL(pLIG:BLG1) strain showed a statistically significant decrease of 40% in IL5 secretion (Figure 6a) and are the only one to secrete IFN-γ (Figure 6b).

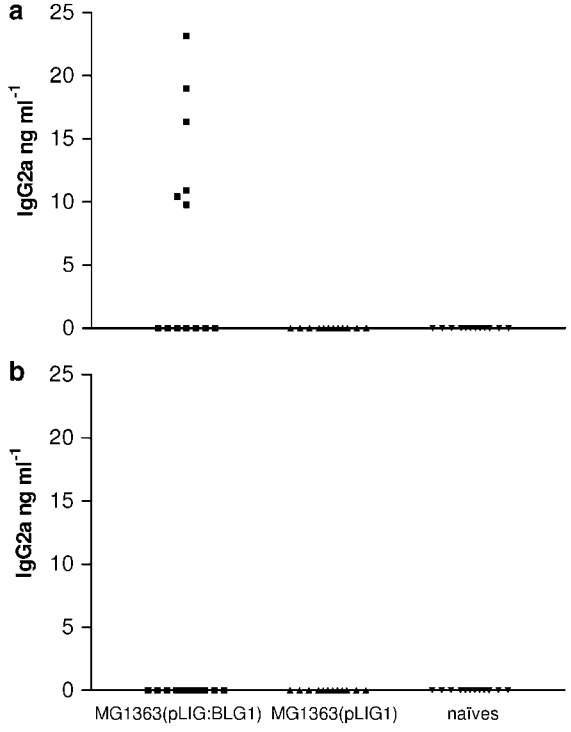

Figure 4 BLG-specific IgG2a detection in sera of mice treated with LL(pLIG:BLG1) or LL(pLIG1). Mice were force fed 5 days consecutively with LL(pLIG:BLG1) and LL(pLIG) and again 3 weeks after the last gavage. Mice were bled 4 (**a**) or 12 days (**b**) after the last gavage. BLG-specific IgG2a was assayed in different sera. BLG, β-lactoglobulin; Ig, immunoglobulin.

Discussion

Recent data confirmed the crucial role of bacterial genomic DNA in host–bacteria interactions.[14–16] Bacterial genomic DNA is thus suspected to play a key role in

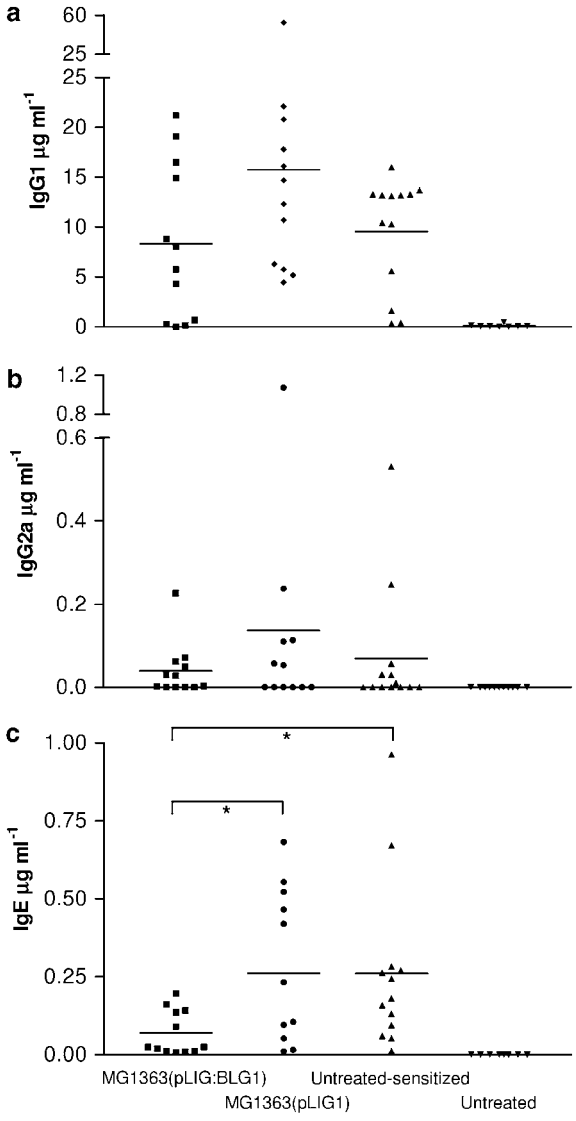

Figure 5 BLG-specific IgE detection in sera of mice pretreated or not with LL(pLIG:BLG1) or LL(pLIG1) after sensitization. Mice were treated or not with LL(pLIG:BLG1) or LL(pLIG1) and then sensitized by intraperitoneal injection of cow's milk mixed with cholera toxin. Mice were bled 4 days after the last sensitization and BLG-specific IgG1 (**a**), IgG2a (**b**) and IgE (**c**) were assayed in sera. Statistical analyses were performed by analysis of variance and Tukey's multiple comparison tests. * indicates $P < 0.05$. BLG, β-lactoglobulin; Ig, immunoglobulin.

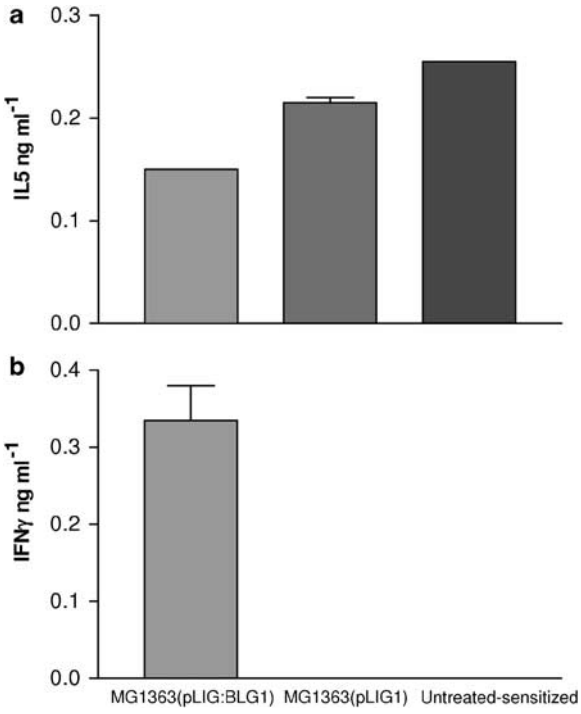

Figure 6 Cytokines secretion after BLG reactivation of splenocytes from mice pretreated or not with LL(pLIG:BLG1) or LL(pLIG1) and BLG sensitized. Mice were treated and sensitized as described in Figure 5 and killed at the end of experiment. Spleens were removed and splenocytes reactivated as explained in the Materials and methods section. Interleukin-5 (**a**) and interferon-γ (**b**) are represented as cytokine concentrations in supernatants of BLG-reactivated splenocytes after subtraction of cytokines assayed in supernatants of nonreactivated splenocytes. BLG, β-lactoglobulin.

mucosal immunology via the dendritic cells and the TLR9 pathway. Until now, the capture of bacterial DNA by the epithelial membrane cells had never been demonstrated *in vivo*. Here, we designed a system to evaluate the fate of a eukaryotic expression cassette delivered by a food-grade bacterium in digestive tract of mice. We showed that such DNA fragment could translocate from its bacterial carrier to host and thus modulate host immune response. This is the first demonstration of such phenomenon after oral administration of food-grade transiting bacteria.

Similar experiments were already performed using purified naked DNA molecules. Twenty-four hours after a gavage with one green fluorescent protein (GFP) plasmid DNA, GFP DNA was detected in the nucleus of epithelial cells from cecum wall by fluorescent *in situ* hybridization technique.[17] Nevertheless, even after 21 days of gavage, no mRNA was detected.[18] In other experiments, they were not able to observe any GFP activity[19] in contrast to our experiments, which led to BLG production by host. We have recently shown *in vitro* that transformation of Caco-2 cells is more efficient when the plasmid is inside the bacterium than just associated with bacteria.[3] This could partly explain the lack of GFP expression after intragastric gavage of naked DNA.

This absence of detection could also be due to the nature of the used reporter and its sensitivity threshold. As previously mentioned, sensitivity of the reporter system is a crucial key for detection of gene transfer.[8] In most of these experiments, the used reporter is either GFP or variant of GFP, which can be detected at a minimum of 1 μM expressed in cytoplasm of HeLa cells.[20] Our EIA, specific of native BLG, is 1000-fold more sensitive with a detection limit of 1 nM.[21] After coculture of Caco-2 cells and *L. lactis* carrying a functional eukaryotic *gfp* gene, no fluorescent cells could be detected.[9] In similar experiments, the use of *L. lactis* LL(pLIG:BLG) to deliver a functional eukaryotic *blg* gene led to both expression and secretion of BLG.[3]

In our experiments, the BLG cDNA was detected in DNA extracted from the small intestinal wall that was washed of gut contents 72 h after the last gavage. This result can be considered as an indirect evidence of plasmid transfer from lactococci to epithelial mouse intestinal cells. Lactococci are not commensal bacteria and they are considered as transiting bacteria. Drouault *et al*.[22] showed a dramatic lactococcal lysis in each compartment of the digestive tract 1 h after uptake of pure culture of *L. lactis* by force-feeding. We also previously showed that population levels dropped to $\sim 10^3$ CFU per g of fecal sample at 48 h postinoculation of conventional mice with 10^8 CFU per ml of bacterial cells.[23] Thus, we considered that 72 h after the last gavage, most of lactococci administered have been lysed and eliminated suggesting that the BLG cDNA detected at this time is probably located in either the intestinal lumen or inside the epithelial membrane rather than in lactococci. Considering the short half-life of naked DNA in the small intestine,[24] most of BLG cDNA detected is probably inside the murine epithelial cells. The question of how DNA plasmid entered epithelial membrane cells is raised and no clear answers are available at this time. For instance, two nonexclusive options can be considered: either as naked DNA after bacterial lysis or as DNA contained by bacteria passively internalized in intestinal membrane cells.

We previously expressed BLG in different bacteria such as *E. coli*, *L. lactis* and more recently in *Lactobacillus casei* using various expression systems.[2,25,26] In all the bacteria tested, significant amounts of BLGn were never produced in contrast to BLGn in eukaryotic cells.[26] For example, a maximum of ~1% of BLG was produced in its native conformation in *E. coli* and *L. lactis*.[2,25] In this study, we used the strain LL(pIL:BLG1), which is not able to produce BLG[3] and BLG was only detected under its native conformation. This is an evidence of both BLG synthesis in cells of the murine intestinal membrane and of plasmid translocation from *L. lactis* to the murine intestinal membrane. BLG was detected in treated mice between 1 and 3 days and considering that enterocytes are renewed each 2–5 days,[27] this observation suggests that BLG could be mainly expressed by enterocytes.

Another criterion should be taken into account: we previously showed that more than 30 μg of BLG per mice should be orally released by lactococci to decrease significantly IgE response in mice.[28] In this study, no BLG, mRNA or protein was detected in LL(pLIG:BLG1), suggesting that even if this strain produces BLG, this production will be below detection limit that is 30 pg ml^{-1}.[21] Considering that a maximum of 3 ng of non-detectable BLG (contained in 100 ml of lactococcal culture used for the gavage) can be possibly administered to mice, this quantity is 10 000-fold lower than 30 μg of BLG necessary to modulate IgE level.

The oral administration of the strain LL(pIL:BLG1) induced a low and transitory specific Th1 immune response. Neither IgA nor IgG could be detected in feces and no cytokines (IFN-γ, IL-5, IL-4) secretion was observed after splenocytes reactivation with BLG *in vitro*. In contrast, oral administration of an *L. lactis* strain producing BLG led to the detection of IgA in feces.[29] Despite these results, mice were effectively protected against further sensitization by a specific Th1 response as also shown by the increase of Th1-cytokines levels. Recently, it was shown that mice can be protected from foot-and-mouth disease virus infection by DNA vaccines without specific humoral responses.[30] Injection of low doses of cDNA BLG in mice tibialis muscles induced protection of mice against sensitization without detectable specific humoral responses (JM Chatel, unpublished data). We can conclude that DNA delivered by transiting bacteria can specifically modulate host immune response.

One major hypothesis, which could explain the expression of the cDNA of BLG is the translocation of the lactococcal vehicle. Such phenomenon in epithelial membrane has never been demonstrated for *L. lactis* but it cannot be excluded. Several species of lactic acid bacteria[31,32] and commensal bacteria as *Enterobacter cloacae* are able to penetrate the dendritic cells and even to reside in these cells for several days.[33]

To conclude, we demonstrated the ability of *L. lactis* to deliver *in vivo* a fully functional plasmid to the small intestine wall of mice. This observation will contribute to better understand the interactions between host and both transiting and commensal bacteria and more precisely the potential role of their DNA in modulation of host response. In future, the understanding of this mechanism will involve the use of other bacteria and other cDNA reporters.

Materials and methods

Plasmids, bacterial strains, media and growth conditions

Plasmids pLIG:BLG1, pLIG1 and strains LL(pLIG:BLG1) and LL(pLIG1) have been described previously.[3] Briefly, pLIG:BLG1 is a cointegrate obtained by fusing pIL253,[34] an erythromycin-resistant plasmid replicating in *L. lactis*, with pcDNA3BLG5,[35] an ampicillin-resistant plasmid replicating in *E. coli* and carrying an eukaryotic BLG expression cassette. pLIG1 is a cointegrate obtained by fusing pIL253 and pcDNA3. pLIG:BLG1 and pLIG1 were introduced by electrotransformation in *L. lactis* subsp. *cremoris* MG1363,[36] as described elsewhere[37] resulting to LL(pLIG:BLG1) and LL(pLIG1), respectively. These strains were grown ON at 30 °C in M17 medium containing 0.5% glucose (GM17) and erythromycin 5 μg ml^{-1}. Bacteria were then centrifuged (5000 *g*, 10 min, 4 °C), washed two times in phosphate buffer saline (PBS) and then concentrated to get 100 ml of culture in 300 μl of concentrate.

Mice handling

Female Balb/c mice were from CERJ (Centre d'Elevage René Janvier, Le Genest Saint-Isle, France), and were housed under normal husbandry conditions. Mice were acclimated for 2 weeks before experiments, and were used when they were 6 weeks old. All experiments were performed according to the European Community rules of animal care, and with authorization 91–122 of the French Veterinary Services.

Apparatus and reagents

All EIA were performed in 96-well microtiterplates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using specialized Titertek microtitration equipment from Labsystems (Helsinki, Finland). Unless otherwise stated, all reagents were of analytical grade from Sigma (St Louis, MO, USA).

IgA and IgG assays in fecal extracts and sera

Specific IgA was assayed in sera and fecal samples as previously described.[29] Sera (1/50) or feces (1/30) diluted in EIA buffer (0.1 M phosphate buffer, 0.1% bovine serum albumin, 0.15 M NaCl, 0.01% sodium azide) plus 0.1% Tween was incubated in plates coated with BLG. Specific IgA was detected using a goat polyclonal serum anti-mouse IgA (Southern Biotechnology Associated, Birmingham, AL, USA) labeled with acetylcholinesterase (AChE).[29] Fecal BLG-specific IgG were detected using an AChE-labeled goat anti-mouse IgG (Jackson ImmunoResearch laboratories, West Grove, PA, USA).

Samples collection

Blood samples were obtained from the retro-orbital venous plexus, centrifuged, 0.1% sodium azide was added as preservative, and the sera were stored at −20 °C until further assay. Naive mice were bled on the same days. Fecal pellets (0.1 g) were immediately dropped in 1 ml phosphate buffer containing 1% bovine serum albumin, 50 mg ml^{-1} of bacitracin, 300 mg ml^{-1} of benzamidine, 80 mg ml^{-1} of leupeptin, 20 mg ml^{-1} of chymostatin and 25 mg ml^{-1} of pepstatin and incubated on rotary shaker overnight at 4 °C. The tubes were vortexed to disrupt all solid material and then centrifuged at 16 000 *g* for 5 min at 4 °C. The supernatant was removed and stored at −20 °C until further assays.

Intragastric sensitization of mice with cow's milk

Mice received 5 mg of cow's milk protein on days 41, 47, 53, 59 and 65, by oral administration of homogenized commercial whole milk (Lait entier Lactel, France), mixed with 0.6 μg g^{-1} of cholera toxin as described previously.[38]

Cytokine productions

Spleens were harvested under sterile conditions. After lysis of red blood cells (180 mM NH_4Cl, 17 mM Na_2EDTA) and several washes, splenocytes were resuspended in RPMI-10 (RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin, 100 mg ml^{-1} streptomycin). Cells were incubated for 60 h at 37 °C (5% CO_2) in 96-well culture plates (10^6 cells per well) in the presence of BLG (20 mg ml^{-1}) or concanavalin A (1 mg ml^{-1}, positive control). Incubations with PBS or ovalbumin (20 mg ml^{-1}) were made as negative controls. Supernatants were then removed and stored at −80 °C until further assay. IFN-γ was assayed using the CytoSetsTM kits (BioSource International Europe, Nivelles, Belgium). IL-5 was assayed using an immunometric assay using monoclonal antibodies TRFK4 for

capture and AChE-labeled TRFK5 monoclonal antibodies for development.[39]

Quantification of bovine BLG-specific IgE, IgG1 and IgG2a

β-lactoglobulin-specific IgE, IgG1 and IgG2a were measured using immunoassays, as previously described. Antibodies directed either against the native or the denatured forms of BLG were determined.[40]

Detection of BLG under native and denatured conformation by two-site EIA

Two-site EIA for native (BLGn) and denatured BLG (BLGd) were performed as previously described.[21] Briefly, assays were performed in 96-well microtiter plates coated with a monoclonal antibody (capture antibody) specific for either BLGn or BLGd. A 50-µl volume of standard (BLGn or BLGd) or 50 µl of the samples was added; then 50 µl of tracer was added, consisting of a second monoclonal antibody labeled with AChE, a conjugate recognizing either BLGn or BLGd. The capture and tracer antibodies were directed against different complementary epitopes. After 18-h reaction at 4 °C, the plates were washed and solid-phase-bound AChE activity was measured using Ellman's method.[41] Detection limits of 30 and 200 pg ml^{-1} were obtained for BLGn or BLGd, respectively.

PCR detection of BLG cDNA

Mice were fed orally 5 days with 100 ml of culture grown ON. Three days after the last gavage, mice were killed and the small intestine was withdrawn. Gut contents were removed by washing with 15 ml of PBS containing an antiprotease cocktail (50 µg ml^{-1} of bacitracin, 300 µg ml^{-1} of benzamidine, 80 µg ml^{-1} of leupeptin, 20 µg ml^{-1} of chymostatin, 25 µg ml^{-1} of pepstatin and 200 µM of phenylmethylsulfonyl fluoride). Membranes were homogenized by Ultra-Turax in ATL buffer from DNeasy tissue kit (Qiagen, Courtabeuf, France), 140 mg of tissue per ml ATL buffer. Then DNA was extracted and purified as described by supplier. PCR amplification was performed using oligonucleotides specific from BLG. Purified plasmid pLIG:BLG1 was used as control template.

Protein extraction from the small intestine

Typically, mice were fed orally 5 days with 100 ml of culture grown ON. Three days after the last gavage, mice were killed and the small intestine was withdrawn. Gut contents were removed by washing with 15 ml of PBS. Membranes were homogenized by Ultra-Turax in PBS added with antiprotease cocktail (0.2 mg of intestine per ml of PBS). After centrifugation for 45 min, 20000 *g*, the supernatant (soluble fraction) is removed and kept until further investigations at −20 °C. Pellet is resuspended in PBS, urea 8 M, dithiothreitol 1 mM to extract insoluble proteins. After centrifugation for 45 min, 20 000 *g*, pellet is discarded and supernatant (insoluble fraction) is dialyzed against PBS before use.

Statistical analyses

Data were analyzed using analysis of variance and Tukey's multiple comparison test. A *P*-value less than 0.05 was considered significant.

Abbreviations

LL, *Lactococcus lactis* MG1363; PBS, phosphate buffer saline; EIA, enzyme immuno assay

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