

Original article

Modulation of allergic immune responses by mucosal application of recombinant lactic acid bacteria producing the major birch pollen allergen Bet v 1

Background: Probiotic lactic acid bacteria (LAB) are able to modulate the host immune system and clinical trials have demonstrated that specific strains have the capacity to reduce allergic symptoms. Therefore, we aimed to evaluate the potential of recombinant LAB producing the major birch pollen allergen Bet v 1 for mucosal vaccination against birch pollen allergy.

Methods: Recombinant Bet v 1-producing *Lactobacillus plantarum* and *Lactococcus lactis* strains were constructed. Their immunogenicity was compared with purified Bet v 1 by subcutaneous immunization of mice. Intranasal application of the live recombinant strains was performed to test their immunomodulatory potency in a mouse model of birch pollen allergy.

Results: Bet v 1 produced by the LAB was recognized by monoclonal anti-Bet v 1 and IgE antibodies from birch pollen-allergic patients. Systemic immunization with the recombinant strains induced significantly lower IgG1/IgG2a ratios compared with purified Bet v 1. Intranasal pretreatment led to reduced allergen-specific IgE vs enhanced IgG2a levels and reduced interleukin (IL)-5 production of splenocytes *in vitro*, indicating a shift towards non-allergic T-helper-1 (Th1) responses. Airway inflammation, i.e. eosinophils and IL-5 in lung lavages, was reduced using either Bet v 1-producing or control strains. Allergen-specific secretory IgA responses were enhanced in lungs and intestines after pretreatment with only the Bet v 1-producing strains.

Conclusions: Mucosal vaccination with live recombinant LAB, leading to a shift towards non-allergic immune responses along with enhanced allergen-specific mucosal IgA levels offers a promising approach to prevent systemic and local allergic immune responses.

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Key words: mucosal vaccination; recombinant lactic acid bacteria; respiratory allergy; secretory IgA; Th1/Th2.

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Type I allergy is characterized by an imbalance of T helper (Th)-1- and Th-2-like immune responses with exaggerated production of interleukin (IL)-4, IL-5 and IL-13 leading to production of immunoglobulin (Ig)-E towards otherwise innocuous molecules. In the last decade, the prevalence of type I allergy has increased markedly with approximately 20% of the population suffering from allergic rhinitis, conjunctivitis, or bronchial asthma mostly caused by airborne inhalant aller-

gens. Apart from a genetic predisposition and specific properties of allergenic molecules, this constant increase in the prevalence of allergic disorders has been linked to the high hygienic standards in industrialized countries associated with a reduced microbial exposure and to a concomitant lack of counter-regulatory immune responses, referred to as 'hygiene hypothesis' (1, 2).

Until now, the only established curative approach against type I allergy is specific immunotherapy (3). However, frequent injections, anaphylactic side reactions during the treatment, or the use of aluminium salts, known as adjuvants with Th-2-inducing capacity (4), might limit the efficacy of this treatment. In this context, the design of Th-1-promoting vaccines (5) and less invasive treatment strategies such as mucosal application (6, 7) might ameliorate the management of allergic manifestations.

Abbreviations: Th, T helper; Ig, immunoglobulin; LAB, lactic acid bacteria; i.n., intranasal; i.p., intraperitoneal; s.c., subcutaneous; CFUs, colony forming units; BAL, bronchoalveolar lavage; BP, birch pollen; Ll, *Lactococcus lactis*; Lp, *Lactobacillus plantarum*; LPS, lipopolysaccharide; con, control strains; rec, Bet v 1-producing strains (recombinant).

Dietary lactic acid bacteria (LAB) are non-invasive and non-pathogenic Gram-positive bacteria with GRAS (generally regarded as safe) status that have been used for food processing and preservation for centuries. In addition, specific strains were reported to exert health beneficial or probiotic effects (8). A possible role of LAB in the prevention of allergic diseases has been suggested. In particular, a relationship between the composition of the intestinal flora and the prevalence of allergic diseases has been epidemiologically documented (9). Furthermore, clinical trials have shown a reduced incidence of allergic symptoms after ingestion of particular LAB strains (10–12). In this context, we recently demonstrated in a mouse model of type I allergy, that selected LAB strains (*Lactococcus lactis* and *Lactobacillus plantarum*) have Th-1-promoting capacities *in vitro* and *in vivo* (13).

Nowadays, LAB are increasingly being recognized as effective carriers capable of delivering antigens via different mucosal routes, i.e. intranasal, intragastric and vaginal. Hence, live bacterial vectors seem to be superior to inactivated ones (14, 15). Therefore, the present study was carried out to develop a mucosal vaccine against birch pollen allergy based on live recombinant LAB strains producing the major birch pollen allergen Bet v 1. We describe the construction and characterization of these strains and the successful prevention of allergic immune responses in mice after mucosal vaccination with the recombinant LAB.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* MC1061 was cultured at 37°C in Luria broth. *L. plantarum* was grown at 37°C in MRS medium (Difco, Becton Dickinson, Franklin Lakes, NJ, USA). *L. lactis* was grown at 30°C in M17 medium (Difco) supplemented with 0.5% of glucose. *L. plantarum* and *L. lactis* strains harbouring the empty-expression plasmid pTG2247 (16) and pTREX1 (17), respectively, were used as negative controls. Antibiotics (Sigma-Aldrich, St Quentin Fallavier, France) were used at the following final concentrations: for *E. coli*, chloramphenicol (20 µg/ml) and ampicillin

(100 µg/ml); for *L. plantarum* and *L. lactis*, chloramphenicol (10 µg/ml) and erythromycin (5 µg/ml).

Transformation, DNA manipulations and construction of plasmids

Escherichia coli, *L. plantarum*, and *L. lactis* were electrotransformed as previously described (16, 17). *E. coli* MC1061 was used as an intermediate host for cloning. Molecular biology techniques were performed as described by Sambrook *et al.* (18). A polymerase chain reaction (PCR) reaction was performed on the *E. coli* expression vector pMW175 (19) carrying the full Bet v 1a-encoding cDNA fragment using the specific sense OMEC190 and antisense OMEC191 primers (OMEC190, 5'-CAGGCCACCATGGGTGT TTTC-3' and OMEC191, 5'-CAAGTTTCTAGATTAGTTGTA GG-3') which hybridize with the 5'- and 3'- ends of the protein-encoding region, respectively. The sense and antisense primers contain an *Nco*I or *Xba*I restriction site, respectively (underlined). The amplified Bet v 1-encoding fragment was subcloned into the pZero-2 plasmid (Invitrogen, Groningen, The Netherlands) and the construction was verified by DNA sequencing (Applied Biosystems, Warrington, UK). Subsequently, the insert was digested with the restriction enzymes *Nco*I–*Xba*I and subcloned into the *Nco*I–*Xba*I-digested plasmid pNZ8037 (20). The resulting construct was designated pMEC184 and carries the Bet v 1-coding sequence under the control of the lactococcal nisin-inducible promoter P_{nisA} . The appropriate host strains *L. plantarum* NCIMB8826 Int-1 (21) and *L. lactis* NZ9800 (22) were electrotransformed with plasmid pMEC184 giving rise to the respective recombinant strains (Table 1). Strain stability was tested by standard methodology in our laboratory (21).

Nisin induction and protein extractions

Induction of Bet v 1 production in recombinant *L. lactis* and *L. plantarum* was performed using nisin as previously described (20, 21). Cell extracts were prepared with a French® Press and mixed with a protease inhibitor (Complete®, Boehringer Mannheim, Germany). Total protein concentration was measured using a protein assay kit (Bio-Rad, Munich, Germany) after removing cell debris.

Immunological characterization of recombinant LAB *in vitro*

Cell extracts of recombinant or control strains were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose by electroblotting according to standard protocols (23). A mouse monoclonal

Table 1. Characteristics of bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
<i>E. coli</i> MC1061	<i>araD139 Δ(ara-leu)7696 lacX74 galV galK hsr-hsm rpsL</i>	Sambrook <i>et al.</i> (18)
<i>L. plantarum</i> (Lp) NCIMB8826	Originally isolated from human saliva	NCIMB
<i>L. plantarum</i> (Lp) NCIMB8826 Int-1	NCIMB8826 containing <i>nisRK</i> genes stably integrated to the <i>tRNA^{Ser}</i> locus	Pavan <i>et al.</i> (21)
<i>L. lactis</i> (Ll) MG1363	Subsp. <i>cremoris</i> , plasmid-free	Wells <i>et al.</i> (17)
<i>L. lactis</i> (Ll) NZ9800	Strain derived from <i>L. lactis</i> MG1363; <i>ΔnisA</i> , non-nisin producer, <i>pepN: nisRnisK</i>	Kuipers <i>et al.</i> (22)
pNZ8037	Chloramphenicol resistance, <i>L. lactis</i> pSH71 replicon, pNZ 8008 derivative carrying a multiple-cloning site; allows translational fusion to the <i>nisA</i> promoter	de Ruyter <i>et al.</i> (20)
pMEC184	Chloramphenicol resistance, pNZ8037 derivative carrying the <i>Bet v 1</i> gene translationally fused to the <i>nisA</i> promoter	This study

NCIMB, National Collection of Industrial and Marine Bacteria, Terry Research Station, Aberdeen, Scotland.

anti-Bet v 1 IgG [1:10, BIP1 (23)], or sera from birch pollen-allergic, grass pollen-allergic or non-atopic subjects (1:4, $n = 4$), followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:5000; Promega, Madison, WI, USA) or goat anti-human IgE (1:700; Biosource International, Camarillo, CA, USA) were used for detection. Recombinant Bet v 1 (Biomay, Vienna, Austria) produced in *E. coli* served as control. Measurement of bacterial endotoxin contamination in recombinant Bet v 1 was performed using the LAL ENDOCHROME test (Charles River Endosafe®, Wilmington, NC, USA) revealing lipopolysaccharide (LPS) levels ranging from 0.015 to 0.05 EU/ μ g protein (which are the common baseline levels for commercially obtained recombinant allergens).

Quantification of Bet v 1 production by ELISA

Microtitre plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated with BIP1 (5 μ g/ml, 0.1 M carbonate-bicarbonate buffer) and thereafter incubated with increasing concentrations of Bet v 1 or serial dilutions of cell extracts of recombinant or control LAB strains. Rabbit anti-Bet v 1 polyclonal serum (1:10 000) followed by peroxidase-conjugated mouse anti-rabbit IgG (1:2000; Jackson Immuno Lab. Inc., West Grove, PA, USA) was used for detection. Colour development and measurement were performed as previously described (24). Production of Bet v 1 by strains was calculated from three independent experiments.

Immunological characterization of recombinant LAB *in vivo*

Animals. Seven-week-old female BALB/c mice were purchased from Charles River (Sulzfeld, Germany). The Animal Experimentation Ethics Committee of the University of Vienna approved all experiments. The LAB strains were grown as described above, pelleted, washed twice and re-suspended in saline at the required concentration for *in vivo* application.

Subcutaneous immunization of BALB/c mice. Groups of nine mice were subcutaneously (s.c.) immunized on days 0, 21 and 42 with 1 μ g of purified Bet v 1 or recombinant LAB strains containing 1 μ g Bet v 1 [recombinant *L. lactis*: 3.3×10^9 colony-forming units (CFUs), recombinant *L. plantarum*: 6.2×10^8 CFUs], or the same quantity of cells from the control strains adsorbed to 2 mg of Al(OH)₃ (Serva, Heidelberg, Germany) in 150 μ l phosphate-buffered saline. At killing, 7 days after the last immunization, blood samples were taken and spleens removed for preparation of spleen cell cultures as previously described (24).

Mucosal pretreatment with recombinant LAB in a mouse model of allergic asthma

Experiment I. For evaluation of systemic immune responses, mice ($n = 8$) were intranasally (i.n.) pretreated on days 0–3 and 6–9 with 10^9 CFUs of either recombinant or control strains, or saline. Seven days later, allergic sensitization was performed by three intraperitoneal (i.p.) injections in 10-day intervals using 1 μ g Bet v 1/Al(OH)₃. At killing, 8 days after the last i.p. injection, serum and spleen were collected (Fig. 1A).

Experiment II. For evaluation of effects on airway inflammation and IgA production in lungs and gut, mice ($n = 8$) were i.n. pretreated (days 0–3 and 6–9) with recombinant or control strains (10^9 CFUs) and thereafter sensitized via an aerosol of 0.1% birch pollen extract solution after i.p. priming with Bet v 1/Al(OH)₃ as previously described (24). Allergic airway inflammation was pro-

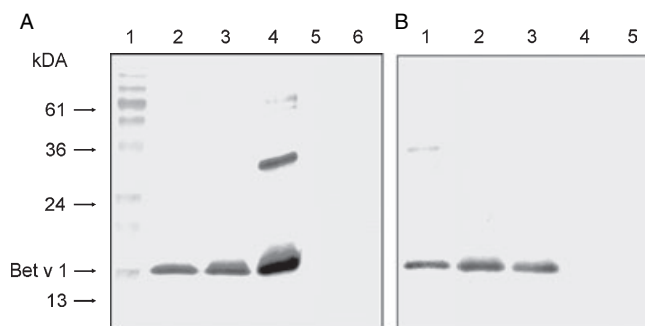


Figure 1. Immunoblot analyses of Bet v 1-producing LAB. (A) Bet v 1 production from total cell extracts detected with a monoclonal antibody against Bet v 1 (BIP1). Lane 1: Molecular weights, lane 2: Bet v 1-producing (recombinant) *Ll*, lane 3: Bet v 1-producing (recombinant) *Lp*, lane 4: recombinant Bet v 1, lane 5: control *Ll*, lane 6: control *Lp*. (B) Recognition of Bet v 1 by a representative serum of a birch pollen-allergic patient. Lane 1: recombinant Bet v 1, lane 2: recombinant *Ll*, lane 3: recombinant *Lp*, lane 4: control *Ll*, lane 5: control *Lp*. *Ll* = *L. lactis*, *Lp* = *L. plantarum* (Bet v 1 monomer at 17 kDa, Bet v 1 dimer at 34 kDa).

voked by two aerosol challenges with a 1% birch pollen solution. Bronchoalveolar lavages (BAL) and small intestine lavages were collected 3 days after challenge (Fig. 2A).

Translocation of recombinant bacteria to the blood stream was studied after i.n. treatment of mice ($n = 6$) with 10^9 CFUs of recombinant LAB. Blood samples were taken (15, 30, 60, 90, 180 min; 4 and 18 h after application) and plated on selective agar. Bacteria were enumerated and authentication of strains performed by detection of the Bet v 1 plasmid by PCR as described. A maximum of 10^3 bacteria (equalling 0.4–1.6 μ g of Bet v 1) in total blood was found 15 min after one i.n. administration of the bacteria but was totally cleared after 180 min. Similar results were obtained after four consecutive i.n. administrations.

Sampling and read outs

Bronchoalveolar and small intestine lavages. Bronchoalveolar lavages were collected and used for assessment of IL-5 and IgA levels by enzyme-linked immunosorbent assay (ELISA), as well as eosinophilic airway inflammation by cytocentrifugation and staining as previously described (25). Small intestine lavages for assessment of IgA were performed as previously described (14).

Determination of allergen-specific antibody levels. Allergen-specific antibody levels in mouse sera (IgE, IgG1, IgG2a) or in bronchial and intestinal lavages (IgA) were determined by ELISA as previously described (24). Briefly, microtitre plates (Nunc Immuno Plate) coated with Bet v 1 were incubated with mouse sera in serial dilutions, or in dilutions of 1/1000 for IgG1, 1/500 for IgG2a and 1/10 for IgE detection. Lavage fluids were applied undiluted for detection of IgA. Isotype detection was performed using rat anti-mouse IgE, IgG1, IgG2a or IgA (1:500; Pharmingen, San Diego, CA, USA) followed by peroxidase-conjugated mouse anti-rat IgG (1:1000; Jackson Immuno Lab. Inc.). For determination of total IgA, microtitre plates were coated with rat anti-mouse IgA (2 μ g/ml, Pharmingen). Detection was performed using a biotin-labelled rat anti-mouse IgA antibody (2 μ g/ml, Pharmingen) followed by streptavidin-peroxidase (1/10 000; Endogen, Woburn, MA, USA).

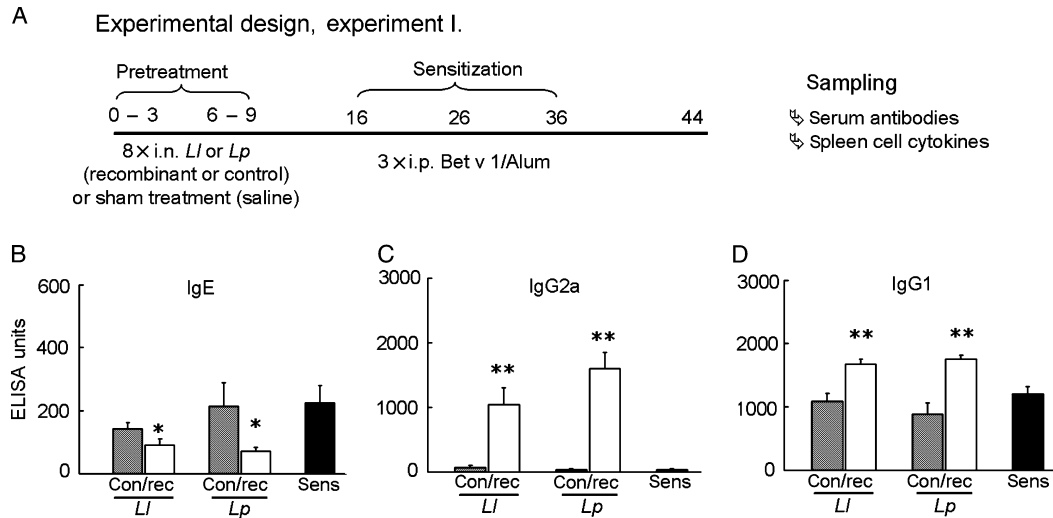


Figure 2. Experiment I: allergen-specific antibody levels after mucosal pretreatment with recombinant LAB. (A) Experimental design: after intranasal pretreatment with Bet v 1-producing (rec) LAB, control (con) LAB or saline, mice were sensitized three times intraperitoneally with Bet v 1/Al(OH)₃. Bet v 1-specific IgE (B), IgG2a (C) and IgG1 (D) antibody levels of mice pretreated with control (grey bars, con) or recombinant (white bars, rec) *L. lactis* (*Ll*) or *L. plantarum* (*Lp*), or saline (black bars, sens). Serum antibody levels were expressed as arbitrary ELISA units. Significantly different from saline pretreated controls (* $P \leq 0.05$, ** $P \leq 0.01$).

Colour development and measurement were performed as previously described (24).

Measurement of cytokine production. Spleen cells were cultured with or without Bet v 1 as previously described (24). Interferon (IFN)- γ and IL-5 production was measured by ELISA and expressed in pg/ml after subtraction of baseline levels of non-stimulated cultures as previously described (25).

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical differences were evaluated by Mann–Whitney *U*-test and considered significant at $P < 0.05$.

Results

Construction and *in vitro* characterization of recombinant LAB producing Bet v 1

Recombinant *L. plantarum* and *L. lactis* strains producing high levels of Bet v 1 were constructed (see Materials and Methods; Table 1). Intracellular production of the allergen was examined in immunoblots of total cell extracts from the recombinant strains (10 μ g per lane) using the monoclonal antibody BIP1. Bet v 1 (Fig. 3A, lane 4; approximately 17 kDa) was detected in cell extracts of recombinant strains (lanes 2, 3), but not in those of control strains (lane 5, 6). Bet v 1 production was quantified by ELISA, showing that 10^9 CFUs of recombinant *L. lactis* or *L. plantarum* strains were equal to 0.4 ± 0.15 and 1.6 ± 0.3 μ g Bet v 1, respectively, which correspond to 0.7–1% and 1–2% of total soluble cellular proteins.

Immunoblot analyses with sera from birch pollen-allergic patients displayed comparable binding of IgE to recombinant Bet v 1 and to Bet v 1 produced by the recombinant LAB strains. No IgE binding was detected using the control strains (Fig. 3B). Sera of four non-atopic or grass-pollen allergic control donors showed no IgE binding (data not shown).

The 34-kDa band (lane 4 Fig. 3A, lane 1 Fig. 3B) present in the recombinant Bet v 1 control, represents a dimer of the Bet v 1 molecule produced by *E. coli*.

Immunogenicity of recombinant LAB strains *in vivo*

Antibody levels and cytokine production profiles were compared after s.c. immunization of mice with recombinant LAB or with purified Bet v 1 (Table 2). Allergen-specific IgG1 and IgE responses were highest in mice immunized with Bet v 1, while IgG2a antibody responses were most pronounced in mice immunized with recombinant *L. plantarum*. Immunization with recombinant *L. lactis* elicited significantly lower IgG1 and IgG2a antibody levels compared with Bet v 1-immunized mice. Nevertheless, the IgG1/IgG2a ratio was significantly lower in both recombinant LAB immunized groups compared with the Bet v 1 group (Table 2). No significant allergen-specific antibody responses were detectable in control animals. Production of IL-5 was significantly lower in spleen cell cultures of mice immunized with the recombinant LAB strains when compared with the Bet v 1-sensitized mice (Table 2). Although IFN- γ levels were lower using recombinant LAB, the IL-5/IFN- γ production ratio was still significantly lower compared with Bet v 1-immunized mice (Table 2).

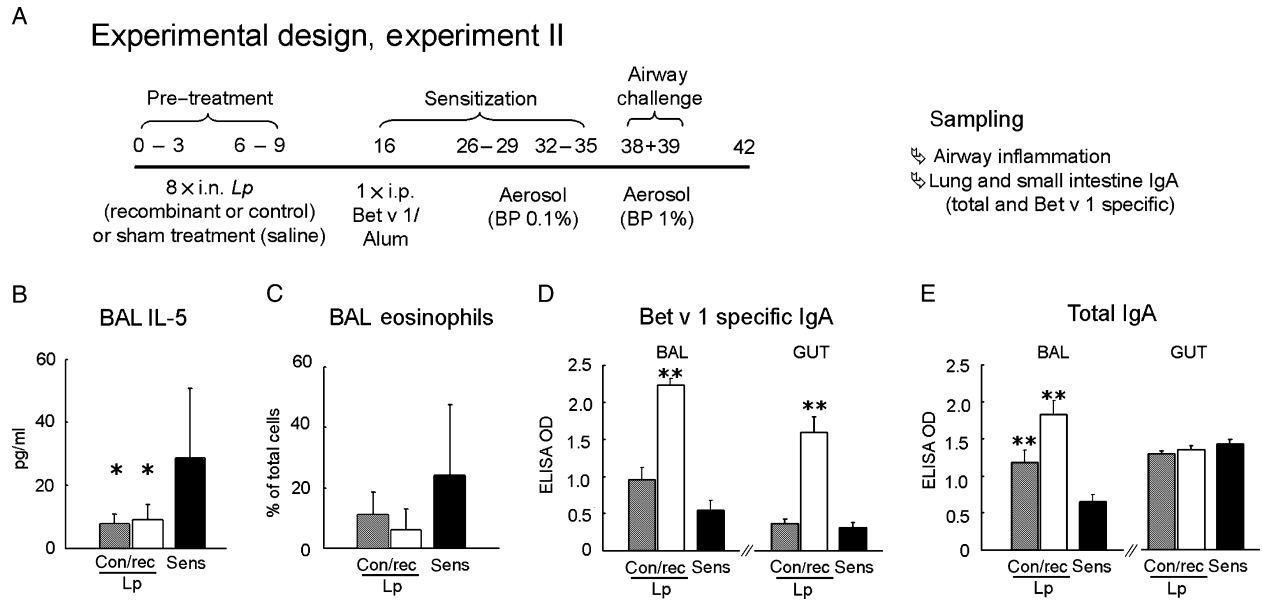


Figure 3. Experiment II: Allergic airway inflammation and mucosal IgA after mucosal pretreatment with recombinant LAB. (A) Experimental design: after pretreatment with Bet v 1-producing (rec) LAB, control (con) LAB or saline, mice were aerosol-sensitized using 0.1% birch pollen extract subsequent to intraperitoneal priming with Bet v 1/Al(OH)₃. Allergic airway inflammation was induced by aerosol challenge with 1% birch pollen solution. Allergic airway inflammation in BAL: IL-5 (B) and eosinophils expressed as % of BAL cells (C). Mucosal IgA: Bet v 1-specific IgA in BAL and small intestine lavages (D) and total IgA (E) of mice pretreated with control (grey bars, con) or recombinant (white bars, rec) *L. plantarum* (Lp), or saline (black bars, sens). IgA levels in lavage fluids are expressed in optical density (OD) units. Significantly different from saline-pretreated controls (***P* ≤ 0.01).

Table 2. Allergen-specific serum antibody levels and cytokine production of spleen cell cultures in subcutaneously immunized mice (*n* = 8)

Sensitization	IgG1	IgG2a	IgE	IgG1/IgG2a ratio	IFN- γ † (pg/ml)	IL-5 (pg/ml)	IL-5/IFN- γ ratio
Bet v 1	1.1 ± 0.3 × 10 ⁶	9.7 ± 3.2 × 10 ²	5.5 ± 2.1 × 10 ¹	17.1 ± 3.7	348.1	789.3 ± 203.5	2.27 ± 0.58
Rec L1	3.8 ± 2.2 × 10 ³ *	2.2 ± 0.6 × 10 ¹ *	0.1 ± 0.0 × 10 ¹ *	3.3 ± 1.6**	22.4	2.7 ± 1.8**	0.14 ± 0.07*
Rec Lp	4.4 ± 0.8 × 10 ⁵ _{n.s.}	3.5 ± 2.3 × 10 ³ _{n.s.}	1.7 ± 1.2 × 10 ¹ _{n.s.}	8.7 ± 4.5*	78.2	53.3 ± 45.1**	0.68 ± 0.58*
Con L1	1.1 ± 0.2 × 10 ² **	0.1 ± 0.0 × 10 ¹ *	0.1 ± 0.0 × 10 ¹ *	—	12.6	0.1 ± 0.0	—
Con Lp	0.3 ± 0.2 × 10 ² **	0.1 ± 0.0 × 10 ¹ *	0.1 ± 0.0 × 10 ¹ *	—	15.6	0.1 ± 0.0	—

Bet v 1-specific antibodies are reported as mean end point titre ± SEM.

Cytokines were measured in supernatants of splenocytes stimulated with Bet v 1. Data are mean values ± SEM.

Significantly different from Bet v 1-immunized animals (**P* < 0.05, ***P* < 0.01). n.s. = not significant.

†Measured from pooled samples.

s.c. = subcutaneous. L1 = *Lactococcus lactis*, Lp = *Lactobacillus plantarum*; rec = recombinant Bet v 1-producing strain, con = control strains.

Mucosal pretreatment with recombinant LAB in a model of type I allergy

Allergen-specific antibody levels and cytokine production – experiment I. Intranasal pretreatment of mice with recombinant *L. lactis* or *L. plantarum* prior to i.p. sensitization with Bet v 1 led to a significant reduction of allergen-specific IgE compared with sham pretreated controls (*P* < 0.05, Fig. 1B). High levels of allergen-specific IgG2a antibodies were found in sera of mice pretreated with either of the recombinant LAB strains (*P* < 0.01, Fig. 1C). Mice pretreated with control strains or saline (sens) displayed only marginal levels of this isotype. A concomitant increase of allergen-specific IgG1

was noted for both groups receiving the recombinant strains (*P* < 0.01, Fig. 1D). IL-5 production of splenocytes was significantly reduced by pretreatment with recombinant *L. plantarum* compared with sensitized controls while in the groups pretreated with recombinant *L. lactis* or the control strains, IL-5 levels did not significantly differ from sham pretreated controls. No significant differences in IFN- γ levels were noted between the groups (Table 3). The strongest reduction in IL-5/IFN- γ ratio (reduced by 66%, data not shown) was achieved with the recombinant *L. plantarum* strain.

Allergic airway inflammation and mucosal IgA – experiment II. Interleukin-5 levels in BAL (Fig. 2B) were

Table 3. Allergen-induced cytokine production of spleen cell cultures (intranasal pretreatment, experiment I)

Intranasal pretreatment	IFN- γ (pg/ml)	IL-5 (pg/ml)
rec <i>L</i>	830.6 $\pm$ 122.8	22.7 $\pm$ 7.6
rec <i>Lp</i>	999.6 $\pm$ 340.8	12.1 $\pm$ 1.8*
con <i>L</i>	970.3 $\pm$ 194.6	24.8 $\pm$ 5.8
con <i>Lp</i>	1614 $\pm$ 423.8	34.2 $\pm$ 12.2
saline (sens)	1047.5 $\pm$ 219.5	37.7 $\pm$ 8.4

*Cytokines measured in supernatants of splenocytes stimulated with Bet v 1. Mice ($n = 8$) were pretreated eight times i.n. with 10^9 CFUs of the indicated strain or NaCl, prior to intraperitoneal sensitization with $3 \times 1 \mu\text{g}$ Bet v 1/Al(OH) $_3$.

Data shown are mean $\pm$ SEM.

rec = recombinant strain producing Bet v 1; con = control strain.

significantly lower in mice i.n. pre-immunized with the recombinant *L. plantarum* compared with saline pretreated controls (sens). A similar reduction of IL-5 was also achieved using the control *L. plantarum*. In accordance, airway eosinophilic infiltration was less pronounced (n.s.) in mice pretreated with either recombinant or control *L. plantarum* (Fig. 2C). Bet v 1-specific IgA responses in BAL and small intestine lavages were significantly increased after pretreatment with the recombinant LAB (Fig. 2D). Total IgA levels in BAL were significantly elevated in mice i.n. pretreated with either recombinant or control *L. plantarum*, whereas total gut IgA levels were not influenced by the intranasal pretreatment (Fig. 2E).

Discussion

Based on the assumption that a lack of counter-regulatory immune responses may favour the development of type I allergy, the induction of allergen-specific Th1 responses has been proposed as a promising concept for treatment of Th2-biased hyper-responsiveness (26). From existing literature, adequate microbial intervention seems to constitute a promising approach to reach this goal (9). Combining the probiotic concept (10) with the fact that LAB have increasingly been used to deliver bioactive compounds by different mucosal routes (15), we engineered two LAB strains, *L. plantarum* and *L. lactis*, to intracellularly produce the airborne allergen Bet v 1 with the aim of using them as live vectors for specific prophylaxis of birch pollen allergy. A few papers reported a similar approach (27–30), but only one study described in depth the protective capacity of recombinant LAB strains producing a food allergen in a mouse model (30). In our study, the recombinant Bet v 1 produced in LAB exhibited correct conformation and equivalence to natural Bet v 1.

For immunological characterization of our constructs, we first compared the allergen-specific immune responses induced after s.c. immunization of BALB/c mice with purified Bet v 1 and with Bet v 1 delivered by live LAB strains. In contrast to immunization with purified allergen alone, recombinant *L. plantarum* and to a lower degree recombinant *L. lactis* also led to a shift towards Th1-like

immune responses. The fact that *L. lactis* poorly elicits immune responses after s.c. application is in accordance with a previous study using recombinant *L. lactis* for production of a food allergen (28). In view of these results and our previous data on immune modulation by non-recombinant LAB (13), we used the Bet v 1-producing LAB strains for mucosal prophylaxis in a mouse model of birch pollen allergy. As it has been shown that the strongest immune regulations take place at the mucosa directly exposed to the vaccine (31), the i.n. route of administration was chosen to influence both systemic and respiratory responses.

In contrast to the different immunogenicity of *L. plantarum* and *L. lactis* after s.c. application, both strains elicited a comparable immunomodulatory capacity when applied via the nasal mucosa: i.n. pretreatment with both live recombinant LAB strains led to significantly reduced allergen-specific IgE antibody responses, associated with the induction of allergen-specific IgG2a antibody responses. Modulation of humoral systemic immune responses by mucosal vaccination has been well described occurring through antigen transportation to systemic lymphoid organs preferentially via the lymphatics (32, 33). Besides indicating a shift towards Th1-like immune responses, IgG2a antibodies themselves might exert beneficial 'blocking' activities as it has been described for enhanced IgG responses after specific immunotherapy (34).

At the cellular level, pretreatment with recombinant *L. plantarum* seemed to be superior to that with *L. lactis*, inducing a significant suppression of the Th2 cytokine IL-5 in spleen cell cultures. Therefore, the effects on airway inflammation were studied after i.n. pretreatment with only the stronger immunomodulating *L. plantarum* strain. In bronchoalveolar lavages, IL-5 levels were significantly reduced after i.n. pretreatment with either the recombinant or control *L. plantarum* strain (Fig. 2B). As this cytokine is crucial for induction of eosinophilic airway inflammation, an overall reduction of eosinophils in BAL was achieved. This indicates that the bacterial vectors or components thereof have the capacity to locally suppress allergic inflammation. In contrast to pretreatment with recombinant *L. plantarum*, non-specific suppressive effects of the wild-type bacteria might however be of shorter duration, as previously described in studies using empty bacterial DNA vectors and CpG motifs for allergy treatment (35, 36). Finally, a major advantage of mucosal application of recombinant LAB is the potential to induce local immune responses at the antigen-exposed mucosae (15). 'Immune exclusion' mediated by secretory IgA antibodies is known to have a protective effect against infectious agents (37). With respect to allergy, the protective capacity of secretory IgA is still under debate. In favour of a protective role, it has been shown that allergen-specific secretory IgA can act as 'blocking antibody' in nasal secretions and inhibit IgE-mediated histamine release (38). In other studies, a lack of allergen-

specific IgA antibodies in tears (39) and low IgA levels in saliva (40) of allergic patients have been shown to contribute to the severity of the allergic symptoms. Thus, our finding that prophylactic vaccination using recombinant *L. plantarum* induced allergen-specific IgA, both in the airways and the gut, indicates the potential to induce protective immune responses at the site of allergen encounter. Moreover, concerning a Bet v 1-associated oral allergy syndrome because of cross-reacting food allergens, local Bet v 1-specific IgA responses might be of further advantage (41).

Taken together, the present data demonstrate that LAB producing a particular aeroallergen are promising vaccine candidates against type I allergy, as i.n. pretreatment with these recombinant LAB led to reduced allergen-specific IgE concomitantly with increased allergen-specific IgA at the mucosae in mice. Compared with conventional immunotherapy, the advantages of such a mucosal allergy vaccine lie in the change to a less invasive route, the use of defined recombinant allergens instead of extract-based vaccines, and the ease of production and

effective delivery to mucosal sites by live carriers with intrinsic immunomodulatory capacities. We conclude that the induction of protective immune responses at the sites of direct allergen exposure linked to counter-regulatory systemic immune responses, as it was achieved by mucosal delivery of innocuous recombinant LAB, might become an effective strategy in primary prevention of type I allergy. Ongoing studies aim at evaluating this vaccination system also in a therapeutic setting.

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References

- Bresciani M, Parisi C, Manghi G, Bonini S. The hygiene hypothesis: does it function worldwide? *Curr Opin Allergy Clin Immunol* 2005;**5**:147–151.
- Eder W, von Mutius E. Hygiene hypothesis and endotoxin: what is the evidence? *Curr Opin Allergy Clin Immunol* 2004;**4**:113–117.
- Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998;**102**:558–562.
- Grun JL, Maurer PH. Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. *Cell Immunol* 1989;**121**:134–145.
- Wohlleben G, Erb KJ. Atopic disorders: a vaccine around the corner? *Trends Immunol* 2001;**22**:618–626.
- Morris DL. WHO position paper on oral (sublingual) immunotherapy. *Ann Allergy Asthma Immunol* 1999;**83**:423–424.
- Wiedermann U. Mucosal immunity–mucosal tolerance. A strategy for treatment of allergic diseases. *Chem Immunol Allergy* 2003;**82**:11–24.
- Mercenier A, Pavan S, Pot B. Probiotics as biotherapeutic agents: present knowledge and future prospects. *Curr Pharm Des* 2003;**9**:175–191.
- Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001;**108**:516–520.
- Rautava S, Kalliomaki M, Isolauri E. New therapeutic strategy for combating the increasing burden of allergic disease: Probiotics – A Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota (NAMI) Research Group report. *J Allergy Clin Immunol* 2005;**116**:31–37.
- Kalliomaki M, Salminen S, Poussa T, Arvilommi H, Isolauri E. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. *Lancet* 2003;**361**:1869–1871.
- Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH et al. Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *J Allergy Clin Immunol* 2003;**111**:389–395.
- Repa A, Grangette C, Daniel C, Hochreiter R, Hoffmann-Sommergruber K, Thalhamer J et al. Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy. *Vaccine* 2003;**22**:87–95.
- Grangette C, Muller-Alouf H, Goudercourt D, Geoffroy MC, Turneer M, Mercenier A. Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant *Lactobacillus plantarum*. *Infect Immun* 2001;**69**:1547–1553.
- Hanniffy S, Wiedermann U, Repa A, Mercenier A, Daniel C, Fioramonti J et al. Potential and opportunities for use of recombinant lactic acid bacteria in human health. *Adv Appl Microbiol* 2004;**56**:1–64.
- Hols P, Slos P, Dutot P, Reymund J, Chabot P, Delplace B et al. Efficient secretion of the model antigen M6-gp41E in *Lactobacillus plantarum* NCIMB 8826. *Microbiology* 1997;**143**:2733–2741.
- Wells JM, Wilson PW, Le Page RW. Improved cloning vectors and transformation procedure for *Lactococcus lactis*. *J Appl Bacteriol* 1993;**74**:629–636.
- Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd edn. New York: Cold Spring Harbor Laboratory, 1989.
- Weiss C, Kramer B, Ebner C, Susani M, Briza P, Hoffmann-Sommergruber K et al. High-level expression of tree pollen isoallergens in *Escherichia coli*. *Int Arch Allergy Immunol* 1996;**110**:282–287.

20. de Ruyter PG, Kuipers OP, de Vos WM. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl Environ Microbiol 1996;**62**:3662–3667.
21. Pavan S, Hols P, Delcours J, Geoffroy MC, Grangette C, Kleerebezem M et al. Adaptation of the nisin-controlled expression system in *Lactobacillus plantarum*: a tool to study in vivo biological effects. Appl Environ Microbiol 2000;**66**:4427–4432.
22. Kuipers OP, Beerthuyzen MM, Siezen RJ, De Vos WM. Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. Requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur J Biochem 1993;**216**:281–291.
23. Jarolim E, Tejkl M, Rohac M, Schlerka G, Scheiner O, Kraft D et al. Monoclonal antibodies against birch pollen allergens: characterization by immunoblotting and use for single-step affinity purification of the major allergen Bet v 1. Int Arch Allergy Appl Immunol 1989;**90**:54–60.
24. Wiedermann U, Jahn-Schmid B, Fritsch R, Bauer L, Renz H, Kraft D et al. Effects of adjuvants on the immune response to allergens in a murine model of allergen inhalation: cholera toxin induces a Th1-like response to Bet v 1, the major birch pollen allergen. Clin Exp Immunol 1998;**111**:144–151.
25. Repa A, Wild C, Hufnagl K, Winkler B, Bohle B, Pollak A et al. Influence of the route of sensitization on local and systemic immune responses in a murine model of type I allergy. Clin Exp Immunol 2004;**137**:12–18.
26. Romagnani S. Immunologic influences on allergy and the TH1/TH2 balance. J Allergy Clin Immunol 2004;**113**:395–400.
27. Kruisselbrink A, Heijne Den Bak-Glashouwer MJ, Havenith CE, Thole JE, Janssen R. Recombinant *Lactobacillus plantarum* inhibits house dust mite-specific T-cell responses. Clin Exp Immunol 2001;**126**:2–8.
28. Chatel JM, Nouaille S, Adel-Patient K, Le Loir Y, Boe H, Gruss A et al. Characterization of a *Lactococcus lactis* strain that secretes a major epitope of bovine beta-lactoglobulin and evaluation of its immunogenicity in mice. Appl Environ Microbiol 2003;**69**:6620–6627.
29. Scheppeler L, Vogel M, Marti P, Muller L, Miescher SM, Stadler BM. Intranasal immunisation using recombinant *Lactobacillus johnsonii* as a new strategy to prevent allergic disease. Vaccine 2005;**23**:1126–1134.
30. Adel-Patient K, Ah-Leung S, Creminon C, Nouaille S, Chatel JM, Langella P et al. Oral administration of recombinant *Lactococcus lactis* expressing bovine beta-lactoglobulin partially prevents mice from sensitization. Clin Exp Allergy 2005;**35**:539–546.
31. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005;**11**:S45–53.
32. Almeida AJ, Alpar HO. Nasal delivery of vaccines. J Drug Target 1996;**3**:455–467.
33. Eyles JE, Bramwell VW, Williamson ED, Alpar HO. Microsphere translocation and immunopotential in systemic tissues following intranasal administration. Vaccine 2001;**19**:4732–42.
34. Valenta R. The future of antigen-specific immunotherapy of allergy. Nat Rev Immunol 2002;**2**:446–453.
35. Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. J Immunol 1999;**162**:6284–6293.
36. Jilek S, Barbey C, Spertini F, Cortes B. Antigen-independent suppression of the allergic immune response to bee venom phospholipase A(2) by DNA vaccination in CBA/J mice. J Immunol 2001;**166**:3612–3621.
37. Brandtzaeg P, Baekkevold ES, Farstad IN, Jahnsen FL, Johansen FE, Nilsen EM et al. Regional specialization in the mucosal immune system: what happens in the microcompartments? Immunol Today 1999;**20**:141–151.
38. Platts-Mills TA, von Maur RK, Ishizaka K, Norman PS, Lichtenstein LM. IgA and IgG anti-ragweed antibodies in nasal secretions. Quantitative measurements of antibodies and correlation with inhibition of histamine release. J Clin Invest 1976;**57**:1041–1050.
39. Aghayan-Ugurluoglu R, Ball T, Vrtala S, Schweiger C, Kraft D, Valenta R. Dissociation of allergen-specific IgE and IgA responses in sera and tears of pollen-allergic patients: a study performed with purified recombinant pollen allergens. J Allergy Clin Immunol 2000;**105**:803–813.
40. Bottcher MF, Haggstrom P, Bjorksten B, Jenmalm MC. Total and allergen-specific immunoglobulin A levels in saliva in relation to the development of allergy in infants up to 2 years of age. Clin Exp Allergy 2002;**32**:1293–1298.
41. Bolhaar ST, Tiemessen MM, Zuidmeer L, van Leeuwen A, Hoffmann-Sommergruber K, Bruijnzeel-Koomen CA et al. Efficacy of birch-pollen immunotherapy on cross-reactive food allergy confirmed by skin tests and double-blind food challenges. Clin Exp Allergy 2004;**34**:761–769.