

Research Paper

Expression of the B subunit of the heat-labile enterotoxin of *Escherichia coli* in tobacco mosaic virus-infected *Nicotiana benthamiana* plants and its characterization as mucosal immunogen and adjuvant

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Abstract

We have produced biologically active recombinant (r) LTB, the nontoxic B subunit of heat-labile toxin (LT) of *Escherichia coli* (LT) in tobacco mosaic virus (TMV)-infected *Nicotiana benthamiana* plants. We amplified the LTB encoding sequence with its leader and introduced a hexahistidyl tag and an endoplasmic reticulum retention signal. The resulting product was ligated into a TMV-based plant viral expression vector that was used for the generation of recombinant viral RNA. Eighty-nine percent of *N. benthamiana* plants inoculated with the recombinant viral RNA were systemically infected as determined by anti-TMV enzyme-linked immunosorbent assay (ELISA) experiments. The rLTB monomer was identified by LT-specific as well as by histidyl-tag-specific immunoblots. rLTB from plant extracts of TMV-infected *N. benthamiana* leaves was purified to give 75 µg rLTB pentamers per gram fresh plant material and was capable of binding G_M1 ganglioside. The immunogenicity of the plant-produced rLTB was tested in mice and showed that intranasal application of rLTB (15 µg per mouse) induced LTB-specific IgG1 antibodies. To prove its adjuvanticity, rLTB was intranasally co-administered with the *Hevea* latex allergen Hev b 3, leading to allergen-specific IgG1 and IgG2a antibody production. The fact that intranasal application of rLTB and Hev b 3 prior to systemic challenge with the allergen enhanced the Th2 responses at the humoral and cellular level indicated that rLTB promoted immune responses that were naturally induced by the antigen/allergen. In conclusion, these results indicate that the plant viral expression system is suitable for the rapid large-scale production of biologically active LTB with strong mucosal adjuvant capacity.

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Keywords: Recombinant LTB; Tobacco mosaic virus expression vector; *Nicotiana benthamiana*; Mouse model; Mucosal adjuvant; Latex allergen Hev b 3

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolylphosphate; BSA, bovine serum albumin; Con A, concanavalin A; CT, cholera toxin; ELISA, enzyme-linked immunosorbent assay; i.n., intranasal; i.p., intraperitoneal; IFN-γ, interferon-γ; IL-5, interleukin-5; kDa, kilodalton; LTB, B subunit of heat-labile enterotoxin of *Escherichia coli*; NBTC, 4-nitroblue-tetrazolium-chloride; *P*, probability; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; r, recombinant; RT, reverse transcriptase; SDS, sodium dodecylsulfate; SI, stimulation index; T, Tween; TBS, Tris-buffered saline; Th, T helper cell; TMV, tobacco mosaic virus; TSP, total soluble protein.

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1. Introduction

To date, most vaccines have been given parenterally. The development of vaccines delivered at mucosal surfaces could provide a safe method for inducing modulated systemic immune responses without injection-related hazards (Pizza et al., 2001). As the mucosal application of antigen alone mostly shows low efficacy, co-administration of certain mucosal adjuvants is necessary (Pizza et al., 2001).

Two well-characterized bacterial proteins with an outstanding potential to function as immunogens and as mucosal adjuvants are the heat-labile enterotoxin (LT) of *Escherichia coli* and the cholera toxin (CT) of *Vibrio cholerae* (Elson and Ealding, 1984; Clements et al., 1988). These structurally and functionally highly homologous toxins are the causal agents of the watery secretions typical of traveller's diarrhoea and cholera, respectively (Douce et al., 1997). For application in humans, several non-toxic derivatives of LT/CT and their non-toxic mucosal binding B subunits (LTB/CTB) have already been tested for their immunogenicity and their ability to act as mucosal adjuvants (Douce et al., 1997, 1998; Williams, 2000; Pizza et al., 2001). The non-toxic LTB has been found to be a more potent adjuvant than CTB (Douce et al., 1997; Millar et al., 2001). Therefore, we focused on the large-scale production of LTB in plants and an investigation of its immunological properties.

An LTB oligomer consists of five identical B subunits, each of 11 kDa, which are held together by non-covalent interactions. Nevertheless, this pentameric ring is extremely stable, a result of the strong hydrophobic interactions at adjacent surfaces. Heat treatment at temperatures over 70 °C in the presence of sodium dodecylsulfate (SDS) leads to the destruction of the pentameric ring and the release of the monomers. The high affinity interaction of LTB with the G_M1 ganglioside and other glycosphingolipids promotes the uptake of the toxic A subunit into eukaryotic cells (Williams, 2000). An intact receptor binding site of the LTB pentamer is necessary for the potent immunogenicity and adjuvant activity of LTB (Nashar et al., 1996; De Haan et al., 1998b). Whereas LTB is able to bind several receptors in addition to G_M1, CTB only has high affinity for this ganglioside. These differences in the ability of LTB to bind to

receptors not accessible to CTB could explain the differences in the nature of the immune responses elicited by LT or CT, respectively (De Haan et al., 1998a).

Recombinant (r) LTB has been successfully produced in *E. coli* (Clements et al., 1983) as well as in transgenic tobacco and potato plants (Haq et al., 1995; Mason et al., 1998; Lauterslager et al., 2001). For clinical trials in humans, contamination of a recombinant protein with pathogenic microbes, prions, or bacterial endotoxins must be avoided. Using bacterial or animal expression systems, the recombinant proteins need to be purified from such contaminants. In contrast, the production of a recombinant protein in plants may even allow the oral application of vaccine antigens without purification (Walmsley and Arntzen, 2000). Furthermore, the expression of immunotherapeutic reagents in plants offers the possibility of production on a large agricultural scale, resulting in reduced costs overall (Walmsley and Arntzen, 2000).

Compared to the cultivation of transgenic plants, virus based expression in plants offers rapid production of diverse types of antigens, using the plants' machinery for glycosylation, formation of disulfide bridges, and postranslational modifications (Pogue et al., 1998; Yusibov et al., 1999). Plant virus based expression systems exploit the ability of small RNA viruses to infect higher plants via infectious RNA, to rapidly amplify viral RNA and to produce large amounts of the recombinant protein in the plant (Pogue et al., 1998; Yusibov et al., 1999). Transcription of a heterologous sequence of interest is driven by a second subgenomic viral coat protein promotor (Shivprasad et al., 1999). Recently, plant virus based expression systems have been successfully used for the inexpensive production of large quantities of correctly processed and functional therapeutic reagents (Turpen et al., 1995; Krebitz et al., 2000, 2003; Dirnberger et al., 2001; Lim et al., 2002; Yusibov et al., 2002).

In this study, we produced purified, biologically active rLTB in tobacco mosaic virus (TMV)-infected *Nicotiana benthamiana* plants. Furthermore, we investigated its ability to act as both immunogen and adjuvant in a mouse model. As a model antigen for co-application, we used rHev b 3, an allergen of the latex of *Hevea brasiliensis*, which is recognized by

the IgE of more than 80% of latex allergic patients with spina bifida (Wagner et al., 1999).

2. Materials and methods

2.1. Construction of the pBSG-LTB expression plasmid and inoculation of *N. benthamiana* plants with recombinant viral RNA

For polymerase chain reaction (PCR) amplification of the coding sequence of LTB, a genomic DNA preparation of *E. coli* (ATCC 43886), kindly provided by Alexander M. Hirschl (Division of Clinical Microbiology, General Hospital of Vienna, Austria), served as template. At the 5'-end of the construct, a *PacI* restriction site and the start codon followed by GGT to facilitate initiation of translation in eukaryotes according to Kozak (Kozak, 1981) were added (primer LTB1: 5'-TTAATTAAG ATG GGT AAT AAA GTA AAA TGT TAT G-3'). At the 3'-end of the coding sequence of LTB, the primer LTB2 (5'-CCTAGG TCA TAG CTC ATC TTT CTC AGA GTG ATG GTG ATG GTG ATG GTT TTC CAT ACT GAT TG-3') introduced an *AvrII* restriction site, the stop codon, the coding sequence for the SEKDEL retention signal (Munro and Pelham, 1987; Haq et al., 1995), as well as a hexahistidyl tag (Fig. 1).

The resulting PCR product was excised from agarose gels, cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced. The insert was released with *PacI* and *AvrII* and ligated into pBSG735A66 (Large Scale Biology, Vacaville, CA, USA), resulting in the plasmid pBSG-LTB. Plasmid pBSG-LTB carries the insert within the genomic sequence of a TMV-based expression vector that had been optimized according to criteria identified by Shivprasad et al. (1999) in a study of a series of TMV expression vectors (Fig. 1). Transformed X11 blue *E. coli* cells were screened for the presence of recombinant plasmids with vector- or LTB-specific primers. Plasmid-DNA of positive clones was prepared.

Template plasmids were linearized with *Sse8387I* and digested with Klenow fragment (Promega) in preparations to be used for the subsequent in vitro transcription. Capped infectious viral RNA was generated in vitro from the linearized plasmid with

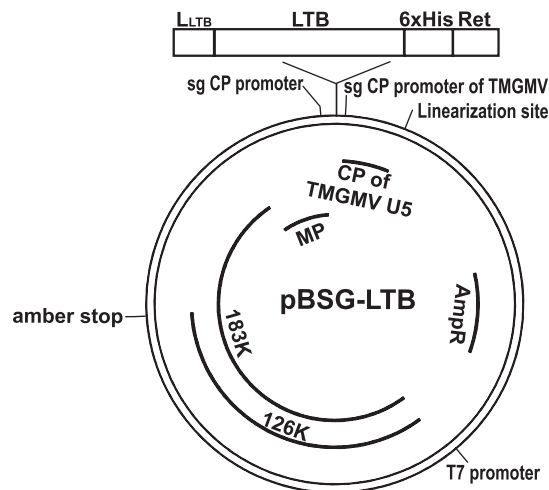


Fig. 1. Schematic of pBSG-LTB harbouring the genome of a tobacco mosaic virus (TMV)-derived dual subgenomic vector containing a hybrid fusion of TMV U1 and tobacco mild green mosaic virus variant U5 (TMGMV U5) (Shivprasad et al., 1999; Yusibov et al., 1999). 126K/183K: RNA-dependent RNA polymerase; MP: movement protein; CP: coat protein; sg: subgenomic. The linearization site and the T7 promoter are shown—both are necessary for the successful production of infectious viral RNA by in vitro transcription. The inserted coding sequence for rLTB produced in TMV-infected *N. benthamiana* plants is boxed: L_{LTB}: bacterial leader sequence of LTB; 6xHis: hexahistidyl affinity tag; Ret: endoplasmic reticulum retention signal.

Promega's Ribomax Large Scale RNA Production System-T7 kit and Cap-Analog (Ambion, Austin, TX, USA). The integrity of the transcribed RNA was assessed by electrophoresis on a 1% agarose gel. After dusting *N. benthamiana* leaflets (two per plant) lightly with carborundum powder, 2 µg RNA was gently rubbed on their surface to initiate infection.

2.2. Detection of TMV infection by enzyme-linked immunosorbent assay (ELISA)

One week after inoculation, plant tissue of newly formed upper leaves was ground with coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6; 1 ml buffer per 100 mg tissue) and centrifuged for 6 min at 7000 rpm at room temperature. From the resulting supernatant, 100 µl plant extract, together with positive and negative controls, was used to coat in duplicates the wells of microtiter plates overnight at 4 °C. After washing three times

with 200 μ l phosphate-buffered saline containing 0.05% Tween 20 (PBST), 200 μ l blocking buffer (PBST containing 0.5% non-fat dried milk powder) was added to each well. The plate was incubated at 37 °C for 1 h, washed as described above and incubated with polyclonal anti-TMV antibodies (No. 1065-05, Adgen, Ayr, UK; 100 μ l/well, diluted 1/2000 in PBST containing 0.2% bovine serum albumin (BSA)) at 37 °C for 2 h. After washing three times, alkaline-phosphatase-conjugated goat anti-rabbit IgG (No. 04007, Adgen; 100 μ l per well, diluted 1/4000 in PBST/0.2% BSA) was added and the plate was incubated at 37 °C for 1 h. After washing four times with PBST, color development was performed using the Fast *p*-Nitrophenyl Phosphate Tablet Set (Sigma, St. Louis, MO, USA). Absorbance values were measured at 405 nm (550 nm as reference wavelength) using an ELISA reader (Dynatech MR 7000).

2.3. RNA isolation of TMV-infected plants

Ten days after inoculation, the occurrence of a systemic infection in the plants and the presence of RNA coding for LTB was determined by reverse transcriptase (RT)-PCR with TMV- and LTB-specific primers. Total RNA isolated from newly formed upper leaves of plants that had been positive in TMV ELISA experiments was used. For RNA isolation, TRI Reagent (Molecular Research Center (MRC)) was employed followed by chloroform extraction and iso-propanol precipitation.

2.4. Protein extraction of TMV-infected *N. benthamiana* plants

Between 2 and 3 weeks after inoculation, leaflets of infected plants were frozen in liquid nitrogen and pulverized. The powder was stirred in extraction buffer, a modified version of the extraction buffer described by Arakawa et al. (200 mM Tris pH 8.0, 100 mM NaCl, 400 mM sucrose, 0.05% Tween 20, and Complete™ EDTA free protease inhibitor tablets from Roche, Mannheim, Germany) (Arakawa et al., 1997), and centrifuged for 6 min at 7000 rpm. The supernatant was loaded on 15% SDS–polyacrylamide gels. SDS–polyacrylamide gels were Coomassie stained or transferred to nitrocellulose membranes for immunoblotting.

2.5. Characterization of rLTB by immunoblots, microsequencing, and ELISA

LTB-specific immunoblots were carried out as previously described (Arakawa et al., 1997). Briefly, nitrocellulose membranes were blocked with Tris-buffered saline (TBS) containing 5% fat-free milk powder. After washing, the membranes were incubated with rabbit anti-CTx antibody (Sigma C3062, 1/5000 in TBS/0.05% Tween/1% milk) specific for CTB and the homologous LTB, as well as for LT, CT, and the corresponding A subunits (Gill et al., 1976). After incubation with alkaline-phosphatase-conjugated swine anti-rabbit IgG (Dako, Carpinteria, CA, USA; 1/2000 in TBST/1% milk), the membranes were treated with 4-nitroblue-tetrazolium-chloride (NBTC) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) for color development.

For detection of rLTB via its histidyl tag, nitrocellulose membranes were blocked with TBST/1% fat-free milk powder followed by incubation with Penta His Antibody (Qiagen, Hilden, Germany; diluted 1/2000 in TBST/milk). After incubation with alkaline-phosphatase-conjugated rabbit anti-mouse IgG + IgM (Jackson Immuno Research, West Grove, PA, USA; 1/5000 in TBST/milk), the membranes were treated with NBTC and BCIP for color development.

Recombinant LTB monomer was electroblotted onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA), Coomassie stained, excised, and sequenced using a Procise 491 sequencer (Applied Biosystems, Foster City, CA).

The G_M1-ELISA was performed on duplicate samples. Microtiter plates (Nunc, Polysorp Immuno Plate) were coated with 100 μ l/well G_M1-monosialoganglioside (Sigma G7641, 3 μ g/ml in 50 mM NaHCO₃ pH 9.6) overnight at 4 °C. As controls for non-specific binding, equivalent amounts of BSA or sucrose (3 μ g/ml) were coated (Arakawa et al., 1997). The plates were blocked with PBS/1% BSA (300 μ l/well) and coated G_M1 was incubated with 30 ng purified plant-expressed rLTB or bacterial CT as positive control (Sigma C3012). After incubation with rabbit anti-CTx (Sigma C3063, 1/8000 in PBS), alkaline-phosphatase-conjugated swine anti-rabbit IgG (Dako, 1/3000 in PBS, 100 μ l/well) was applied to the microtiter plates and incubated for 2 h at 37 °C. Color development was performed using

Fast *p*-Nitrophenyl Phosphate Tablet Sets (Sigma). Absorbance values were measured at 405 nm (550 nm as reference wavelength).

2.6. Purification of rLTB using Ni-chelate affinity chromatography

Fifteen days after inoculation, the plants with positive results from anti-CTx immunoblots were harvested including leaf, stem, and root material, frozen in liquid nitrogen, pulverized, and extracted for 1 h at 4 °C in a threefold excess of the extraction buffer described above. After the extract had been centrifuged for 10 min at 3000 rpm at 4 °C, the supernatant was filtered, the pH adjusted to 8.0 (necessary for Ni-chelate affinity chromatography), and incubated with equilibrated Ni-NTA agarose beads (Qiagen, 1 ml beads per 20 g plant material) in a batch procedure for 1 h at 4 °C. The beads were packed into a column and washed with extraction buffer, followed by washing with extraction buffer containing 20 mM imidazole or 100 mM imidazole, respectively. LTB was eluted with extraction buffer containing 250 mM imidazole. Purified LTB was dialyzed against PBS and checked in anti-CTx immunoblots and G_M1-ELISA experiments for biological activity.

2.7. Animals

Female 7-week-old BALB/c mice were obtained from Charles River (Sulzfeld, Germany). All experiments were approved by the Animal Experimentation Committee of the University of Vienna and the Federal Ministry of Education, Science, and Culture of Austria.

2.8. Immunization protocol

To evaluate the immunogenicity of rLTB, 15 µg plant-produced rLTB in 30 µl 0.9% NaCl was intranasally (i.n.) applied three times with 7-day intervals. To test the adjuvanticity of rLTB, rLTB (15 µg per mouse) was co-applied i.n. with 10 µg of the latex allergen rHev b 3 (Biomay, Vienna, Austria) (Wagner et al., 1999). Ten days after the i.n. treatment, the mice were systemically challenged by three intraperitoneal (i.p.) injections of 5 µg rHev b 3 adsorbed to alumin-

ium hydroxide with 14-day intervals between injections. Control mice were treated i.n. with 10 µg rHev b 3 in 30 µl of 0.9% NaCl or sham-treated with 30 µl of 0.9% NaCl and then all mice were challenged as described above.

2.9. Sampling

Blood samples were taken before and seven days after i.n. treatment as well as seven days after i.p. challenge by tail bleeding. Serum was prepared and stored at –20 °C until analyzed. Spleens were removed under sterile conditions. The organs were homogenized and filtered through sterile filters. After lysis of erythrocytes, cells were washed and resuspended in complete medium (Wiedermann et al., 1999).

2.10. Detection of antigen-specific antibody levels in mouse sera

Microtiter plates (Nunc-Nalge, Roskilde, Denmark) were coated with rHev b 3 or plant-produced rLTB (5 µg/ml 50 mM sodium carbonate puffer, pH 9.6) overnight at 4 °C. After washing and blocking with 1% BSA–PBS/Tween, serum samples were serially diluted and plates were incubated with sera overnight at 4 °C, followed by incubation with rat anti-mouse IgG1, IgG2a, and IgE antibodies (1/500, Pharmingen, San Diego, CA, USA). Thereafter, peroxidase-conjugated mouse anti-rat IgG antibodies (diluted 1/2000, Jackson Immuno Lab, West Grove, PA, USA) were applied. Color development was performed with the tetramethylbenzidine substrate (Chemicon, Temecula, CA, USA). Antibody titers were calculated as the reciprocal dilution of the serum samples corresponding to an absorbance of 0.2 above the background of pre-immune sera.

2.11. Lymphocyte proliferation assay

Spleen cell suspensions were plated into 96-well round-bottom plates (Nunc) at a concentration of 2×10^5 cells/well and incubated with or without rHev b 3 (3 µg/well) for 4 days. The cultures were then pulsed with 0.5 µCi/well tritiated thymidine (Amersham, Little Chalfont, UK) for 16 h and harvested. The proliferative responses were measured by scintil-

lation counting. The experiments were performed in triplicate. The ratio of the mean proliferation after stimulation with antigen (cpm) and the medium control values (cpm), i.e., stimulation index (SI), was calculated.

2.12. Measurement of cytokine production

For determination of interleukin-5 (IL-5) and interferon- γ (IFN- γ) production, spleen cell suspensions were cultured with or without rHev b 3 (15 μ g/well) in 48-well plates (Costar, Cambridge, MA, USA) at a concentration of 5×10^6 cells/well. Supernatants were taken after 40 h of antigen stimulation and stored at -20°C until analyzed. IL-5 levels were measured using a mouse ELISA kit (Endogen, Cambridge, MA, USA). IFN- γ levels were measured by ELISA as previously described (Winkler et al., 2002). The results reflect the measured cytokine levels in pg/ml after subtraction of baseline levels of non-stimulated cultures.

2.13. Statistics

The Mann–Whitney U test was used for statistical analysis. Probability (P) values <0.05 were considered significant. If possible, given values are the means of determinations with individual mice or single samples. The standard error of the mean is also given.

3. Results

3.1. Cloning of the expression construct pBSG-LTB for the production of rLTB in TMV-infected *N. benthamiana* plants

The sequence coding for LTB including its bacterial leader was obtained by PCR of a genomic DNA preparation of an enterotoxigenic *E. coli* strain. The LTB sequence was modified by PCR to include the sequence for the histidyl tag and the endoplasmic retention signal. Fragments with the correct sequence were subcloned from the pGEM-T Easy vector to pBSG735A66, a dual subgenomic TMV-based expression vector, resulting in the plasmid pBSG-LTB (Fig. 1).

3.2. Infectivity of recombinant pBSG-LTB and analysis of the production of rLTB in TMV-infected *N. benthamiana* plants

Infectious viral RNA was produced by in vitro transcription of the TMV part of the linearized pBSG-LTB with T7 RNA polymerase. The transcript length was checked on 1% agarose gels and estimated to be larger than 7 kb. This is in good agreement with the expected size of an intact, full size viral TMV-RNA (8 kb) (data not shown).

Ten days after mechanical inoculation of *N. benthamiana* plants with the recombinant viral RNA, TMV infection was visible as mild leaf deformations, some variable leaf mottling, and growth retardation, and resulted in systemic infection of 17/19 (89%) inoculated plants as determined by anti-TMV ELISA experiments. Furthermore, RNA isolation of infected plants followed by RT-PCR resulted in a 700-bp PCR product corresponding to the sequence of LTB including 200 additional nucleotides corresponding to the sequence flanking the coding region.

To screen for the production of rLTB, leaflets of each of the infected plants were harvested and proteins extracted fifteen days after inoculation. An 11-kDa protein band corresponding to the LTB monomer including an intact histidyl affinity tag was visible in anti-CTx as well as in anti-Penta His immunoblots of all of these 17 extracts.

3.3. Production and molecular characterization of purified rLTB

Approximately 100 g plant material, corresponding to five *N. benthamiana* plants, was extracted for 1 h. rLTB was purified under native conditions by Nickelate chromatography. After heat treatment of the protein fractions, the progress of purification of rLTB was visualized by Coomassie stained SDS–polyacrylamide gels (Fig. 2A). The major portion of purified rLTB was eluted with 250 mM imidazole in extraction buffer (Fig. 2A, lane 5). In an immunoblot, the anti-CTx antibody recognized a weak band corresponding to the monomeric rLTB in heat-treated total plant extract as well as in the fraction eluted with 100 mM imidazole in addition to a major band corresponding to monomeric rLTB in the fraction eluted with 250 mM imidazole (data not shown). The purified recombinant

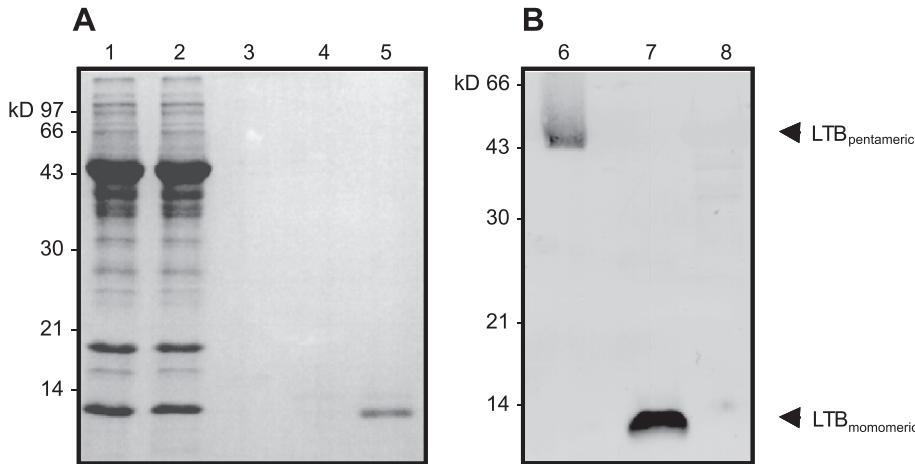


Fig. 2. Purification of rLTB produced in TMV-infected *N. benthamiana* plants using Ni-chelate chromatography. (A) Protein fractions at different stages of purification were boiled for 5 min in the presence of SDS, loaded onto a 15% SDS–polyacrylamide gel and stained with Coomassie brilliant blue R-250. Total protein extracted from LTB-TMV-infected *N. benthamiana* plants (lane 1), flow through from the column (lane 2), washing step at 20 mM imidazole (lane 3), washing step at 100 mM imidazole (lane 4), aliquot of the fraction eluted at 250 mM imidazole (lane 5). (B) Purified rLTB corresponding to lane 5 was transferred to nitrocellulose membranes after SDS–PAGE and detected with anti-CTx antibody. rLTB loaded onto SDS–polyacrylamide gel without prior heating (lane 6), after heating for 5 min at 95 °C (lane 7), and extract of non-TMV-infected *N. benthamiana* plants as negative control (lane 8).

protein was dialyzed against PBS to provide a physiological buffer system.

The purity of rLTB was tested by loading 5 µg rLTB on a 15% SDS–polyacrylamide gel after boiling the sample in the presence of SDS. No other plant components except the 11-kDa band corresponding to monomeric rLTB were visible on the Coomassie stained gel demonstrating a purity of about 98% (data not shown). The final yield of the purified recombinant protein was calculated to be 75 µg purified rLTB per gram fresh plant material. Total soluble protein (TSP) extracted from leaves was determined by the Bradford method to be 10 mg per gram fresh leaf. Therefore, rLTB was at least accumulated to 0.75% of the TSP in leaves as measured after purification.

To confirm pentamerization of rLTB monomers in plants, rLTB was loaded on a 15% SDS–polyacrylamide gel with and without prior heat treatment. Detection via anti-CTx antibody showed a band of approximately 50 kDa on the immunoblot corresponding to the rLTB pentamer (Fig. 2B, lane 6), instead of the 11-kDa band corresponding to the monomer that was observed when rLTB was loaded on the gel after heat treatment (Fig. 2B, lane 7).

Purified rLTB produced in plants was also tested for its biological activity by studies on its ability to bind to G_{M1} ganglioside in ELISA experiments. rLTB revealed G_{M1} ganglioside binding capacity comparable to that of commercially available cholera toxin (Fig. 3). Coated BSA or sucrose, instead of G_{M1} ganglioside, served as negative controls. Minimal

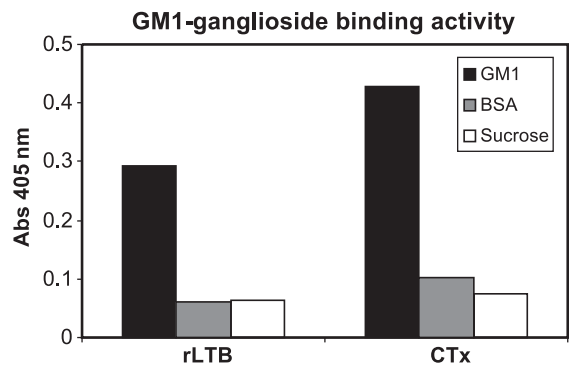


Fig. 3. G_{M1} ganglioside binding capacity of plant-derived rLTB. The ELISA experiment was performed by incubation of coated G_{M1} ganglioside with purified rLTB produced in TMV-infected *N. benthamiana* plants or with commercially available CTx (including subunit A and B, Sigma) as positive control. Coated BSA or sucrose served as negative controls. Bars illustrate means of single determinations performed in duplicate.

binding to BSA and sucrose was observed confirming the specificity of the LTB-G_{M1} ganglioside binding (Fig. 3).

To test whether rLTB was correctly processed in *N. benthamiana* plants, the N-terminal amino acid residues of the purified rLTB monomer were determined by protein sequencing revealing the peptide sequence APQSI. These experimental data are in accordance with the predicted N-terminus of the mature protein corroborating the correct cleavage of the bacterial 21 amino acid residue leader sequence in the plant.

3.4. Efficacy of plant-expressed rLTB as immunogen/mucosal adjuvant

In order to test its immunogenicity as well as its adjuvanticity, rLTB was mucosally applied alone or in combination with the latex allergen Hev b 3. After three i.n. applications of rLTB, LTB-specific IgG1 antibodies were detected in the pooled mouse sera, demonstrating the immunogenicity of the rLTB-molecule (Fig. 4A). Applying rLTB together with rHev b

3, LTB-specific IgG1 antibody levels, but also LTB-specific IgG2a antibody titers were measured, suggesting that co-application of LTB with Hev b 3 resulted in an additive effect in the humoral immune response (Fig. 4A). Moreover, in the group immunized i.n. with rLTB and Hev b 3, Hev b 3-specific IgG1 and IgG2a antibody titers were determined in contrast to mice immunized i.n. with Hev b 3 alone ($P \leq 0.05$) (Fig. 4B).

3.5. Efficacy of rLTB as a mucosal immunomodulator

To test whether i.n. application of rLTB not only enhanced antigen specific immune responses, but also influenced the quality of the response, mice were systemically challenged with the Hev b 3 antigen subsequent to i.n. immunization. Mice sensitized i.p. with rHev b 3/Al(OH)₃ displayed substantial anti-Hev b 3 IgG1, IgG2a, and IgE antibody levels (Table 1). Intranasal pretreatment with rLTB alone did not influence the antigen specific response upon systemic challenge with the allergen. However,

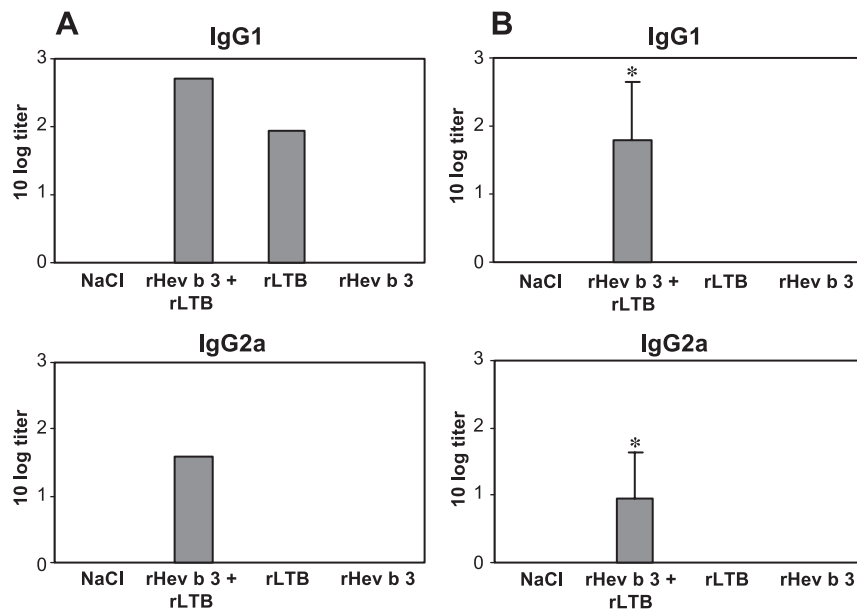


Fig. 4. Serum antibody responses in mice after intranasal treatment with plant-produced rLTB or with LTB and Hev b 3 as model antigen. LTB-specific (A) and Hev b 3-specific (B) immune responses after intranasal administration of NaCl, co-administration of rHev b 3 and rLTB, rLTB, or rHev b 3, respectively. Blood samples were taken seven days after the third i.n. treatment. IgG1 and IgG2a ELISA experiments were performed with pooled sera in the case of LTB-specific Igs (A) or with individual sera for determination of Hev b 3-specific antibodies (B). Bars indicate mean antibody titers $\pm$ the standard error of the mean. Asterisks denote statistically significant differences between titers of mice given NaCl, rLTB or rHev b 3 and co-administration of rHev b 3 and rLTB ($P \leq 0.05$).

Table 1

Hev b 3-specific immunomodulation after mucosal priming (intranasal) followed by systemic challenge (intraperitoneal) with rHev b 3

Mucosal priming with	T cell proliferation	Humoral immune response ^a	
	SI	IgE (A_{450})	IgG2a (A_{450})
NaCl	3.50 ± 1.09	0.81 ± 0.64	0.61 ± 0.40
rHev b 3	4.44 ± 0.61 ^{*,b}	1.06 ± 0.76	0.18 ± 0.00 ^{*,c}
+ rLTB			
rLTB	2.19 ± 1.02	0.59 ± 0.36	0.50 ± 0.38
rHev b 3	1.96 ± 0.32	0.75 ± 0.62	0.06 ± 0.04

^a Absorbance values of the humoral immune responses are given as means ± standard error of the mean obtained by ELISA experiments performed with mouse sera diluted 1/10 for Hev b 3-specific IgE measurement or 1/500 for Hev b 3-specific IgG2a determination.

^b Significant differences between the group of mice treated i.n. with rHev b 3 and rLTB and the groups that received rLTB or rHev b 3 alone prior to i.p. challenge.

^c Significant differences between the group of mice treated i.n. with rHev b 3 and rLTB and the groups that received NaCl or rHev b 3 prior to i.p. challenge.

* Probability values < 0.05 using the Mann–Whitney *U* test are defined as significant.

i.n. immunization with Hev b 3 and LTB prior to i.p. challenge with Hev b 3 resulted in a significant decrease of Hev b 3-specific IgG2a versus an increase of Hev b 3-specific IgE, indicating that LTB application might favour the induction of Th2-biased responses (Table 1). In contrast to the immunostimulatory effects of rLTB, i.n. pretreatment with Hev b 3 alone prior to sensitization rather resulted in suppression of antigen-specific antibody responses (Table 1).

In vitro stimulation of murine spleen cells with rHev b 3 resulted in a strong T cell proliferative

response in the group of mice pretreated i.n. with Hev b 3 and LTB. This response was slightly higher compared to that of non-pretreated mice, but was significantly stronger than that of the groups pretreated with rLTB or Hev b 3 alone prior to systemic challenge (Table 1).

IL-5 and IFN- γ levels were measured in spleen cell cultures upon in vitro stimulation with rHev b 3. Along with the humoral responses, the pretreatment with rLTB mixed with Hev b 3 strongly favored the induction of Th2 biased T cell responses, as the IL-5/IFN- γ ratio was higher in this group (mean of IL-5: 95.77 pg/ml, mean of IFN- γ : 7.47 pg/ml) compared to that of LTB-pretreated mice (mean of IL-5: 51.95 pg/ml, mean of IFN- γ : 19.87 pg/ml) or Hev b 3-pretreated animals (mean of IL-5: 26.33 pg/ml, mean of IFN- γ : 42.88 pg/ml), and even significantly ($P < 0.05$) higher compared to the ratio in the group of non-pretreated mice (mean of IL-5: 85.50 pg/ml, mean of IFN- γ : 147.05 pg/ml) (Fig. 5).

4. Discussion

In the present study, we report for the first time the production of the B subunit of the *E. coli* heat-labile enterotoxin in a transient virus based plant expression system. The infectivity of the recombinant TMV was tested, rLTB was isolated from infected *N. benthamiana* plants and purified by nickel chelate chromatography. The rLTB was tested for its biological activity in vitro and in vivo. In a mouse model, we investigated the efficacy of rLTB to act as a mucosal adjuvant, immunogen, and immunomodulator.

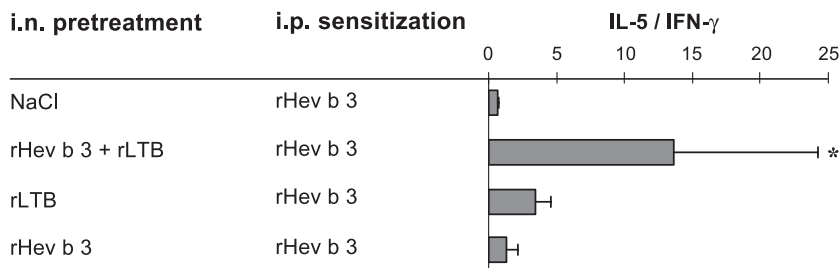


Fig. 5. In vitro cytokine production of spleen cell cultures after stimulation with rHev b 3. Bars illustrate the mean ratio of IL-5/IFN- γ ± standard error of the mean. The asterisk denotes a statistically significant difference ($P < 0.05$) between the group of mice pretreated with a combination of rHev b 3 and rLTB and the group of mice that was not pretreated prior to systemic challenge with rHev b 3.

The application of soluble antigens via the mucosa usually does not—or only weakly—induce an effective immune response but rather leads to tolerance induction (Weiner, 1994). Therefore, the use of mucosal adjuvants is necessary for vaccination approaches that utilize the mucosal route for vaccine delivery (McGhee et al., 1992). Mucosa-binding molecules, such as CT from *V. cholerae* or LT from *E. coli*, have been used as mucosal adjuvants and carriers for many types of antigens, including keyhole limpet hemocyanin, the influenza virus surface antigen, a birch pollen allergen, or virus-like particles of rotavirus (Elson and Ealding, 1984; Verweij et al., 1998; Wiedermann et al., 1998; Fromantin et al., 2001). However, since CT and LT are toxins and cannot be used as part of human vaccines, several studies—including the present one—have focused on non-toxic derivatives of CT or LT for the induction of antigen-specific immune responses (Douce et al., 1997, 1998; Williams, 2000; Pizza et al., 2001).

Different strategies for the large-scale production of adjuvants and immunomodulators exist. The non-toxic B subunit of *E. coli* heat-labile enterotoxin was successfully produced in *E. coli* (Clements et al., 1983), as well as in transgenic tobacco and potato plants (Haq et al., 1995; Mason et al., 1998; Lauterslager et al., 2001). Recombinant LTB delivered in a transgenic potato has even been tested in human studies (Tacket et al., 1998). Bacterial expression systems require additional purification procedures to produce recombinant proteins free of endotoxins and fit for clinical applications. Transgenic potato plants expressing high levels of rLTB from a constitutive promoter showed stunting of shoot growth and poor potato tuber yield, indicating that rLTB may exert a toxic effect in plant cells (Mason et al., 1998). Therefore, we selected a transient plant virus based expression system to bypass possible phytotoxicity of rLTB by inoculation of mature plants (Shivprasad et al., 1999). This TMV-based expression system combines the rapid amplification of the viral genome including the LTB sequence and high expression levels due to the strong viral CP promoter with the advantages of plant expression systems in general (Pogue et al., 1998).

Haq et al. (1995) showed that transgenic tobacco and potato plants that produced a fusion of rLTB and the endoplasmic reticulum retention signal SEKDEL

(Munro and Pelham, 1987) yielded threefold higher amounts of biologically active rLTB than plants producing rLTB without the SEKDEL extension. This observation was explained by the increased concentration of LTB monomers due to retention in the endoplasmic reticulum (Haq et al., 1995). As observed in the bacterial periplasm (Hirst and Holmgren, 1987), this cellular compartmentation of rLTB facilitated the pentameric oligomerization (Haq et al., 1995) that is essential for its biological activity (Nashar et al., 1996; De Haan et al., 1998b). We therefore introduced the endoplasmic retention signal in our recombinant pBSG-LTB construct downstream of the sequence encoding the bacterial leader as well as the mature LTB (Fig. 1).

In addition, the pBSG-LTB construct was equipped with a hexahistidyl tag for easy purification via Ni-chelate chromatography. One native Ni-affinity chromatography step yielded the exclusive recovery of rLTB with a purity of about 98% after elution with 250 mM imidazole. Furthermore, the 65-kDa rLTB pentamer seemed to be highly enriched in the purified rLTB sample as shown in Fig. 2B lane 6, as the monomeric 11-kDa rLTB was hardly detectable on the anti-CTx immunoblot when rLTB was loaded onto an SDS–polyacrylamide gel without prior heat treatment. It is likely that pentameric rLTB containing five hexahistidyl tags shows a higher affinity for binding to the Ni-NTA agarose matrix than the rLTB monomer with only one hexahistidyl tag or the non-specifically bound plant proteins. This resulted in the production of highly enriched rLTB pentamer visualized in Fig. 2B lane 6, indicating that *N. benthamiana* favors the correct assembly of rLTB and that our purification procedure via Ni-NTA agarose does not destabilize the pentameric structure of plant-produced rLTB.

As observed by Arakawa et al. (1997) for a commercially available CTB pentamer, our purified plant-produced rLTB pentamer migrated on SDS–polyacrylamide gels at a lower molecular weight (about 50 kDa) than expected from its calculated molecular weight of 65 kDa—including the histidyl tag and the endoplasmic reticulum retention signal (Fig. 2B, lane 6). Correct processing of rLTB produced in TMV-infected plants was determined by N-terminal sequencing. We could prove that the natural bacterial leader of LTB was correctly cleaved off in *N. benthamiana* resulting in the

amino acid sequence APQSI at the N terminus of mature rLTB.

N. benthamiana plants infected with the viral RNA transcribed from the pBSG-LTB construct yielded 75 µg rLTB per gram fresh leaf, corresponding to 7.5 µg per milligram of TSP as measured after the purification by nickel chelate chromatography. In contrast, Haq et al. (1995) obtained up to 0.014 µg rLTB per milligram TSP produced in transgenic *Nicotiana tabacum* leaves and 0.11 µg/mg TSP in transgenic potato tubers. Transgenic potato tubers expressing a plant optimized synthetic LTB gene under the control of a constitutive promoter yielded up to 10 µg rLTB per gram tuber (Mason et al., 1998). Lauterslager et al. (2001) obtained up to 17 µg rLTB per gram potato tuber using a plant optimized synthetic LTB gene including nucleotides coding for the SEKDEL retention signal and by driving the expression of rLTB under the control of a tuber specific promoter in transgenic potato plants.

Recombinant LTB was tested for its biological activity in a G_{M1} binding ELISA (Fig. 3). Recombinant LTB produced in TMV-infected *N. benthamiana* bound specifically to G_{M1} ganglioside (Fig. 3). This proves that the plant-produced rLTB was present as the pentamer that is essential for its immunogenicity and adjuvant activity as only correctly folded LTB pentamers are biologically active and capable of binding to the G_{M1} ganglioside receptor (Nashar et al., 1996; De Haan et al., 1998b).

In a mouse model, we tested rLTB, which bound to the G_{M1} ganglioside in vitro (Fig. 3), for its immunogenicity and adjuvant activity in vivo. LTB-specific IgG1 antibodies were induced upon i.n. immunization with rLTB (Fig. 4A). Interestingly, the immunogenicity of LTB was further enhanced after co-application with our model antigen Hev b 3, since not only LTB-specific IgG1 but also LTB-specific anti-toxic IgG2a antibodies were detected after i.n. application of both antigens (Fig. 4A).

Some reports demonstrated that recombinant LTB or CTB lacked the capacity to stimulate systemic and mucosal antibody responses towards the co-administered antigen, and that the adjuvant effects of LT and CT are therefore directly linked to the enzymatic (ADP-ribosylation) activity of these molecules (Lycke et al., 1992; Tamura et al., 1994a). In contrast, more recently, it has been shown that rLTB and rCTB also

had potent adjuvant activities towards i.n. administered antigens (Tochikubo et al., 1998; Verweij et al., 1998), which—similar to their immunogenicity—are directly related to G_{M1}-binding activities (De Haan et al., 1998b). In the present study, we demonstrated that LTB, recombinantly produced via a plant viral expression system, also acted as an efficient mucosal adjuvant, as anti-Hev b 3 IgG1 and IgG2a antibody titers were significantly higher in the group of mice immunized with LTB together with Hev b 3 than in the group of mice i.n. immunized with Hev b 3 alone (Fig. 4B).

Besides their immunoenhancing effects, mucosal adjuvants can also influence the quality of the immune response. It is assumed that the phenotype of an immune response depends on the type of immunocompetent cells that are present in the effector region. With respect to the mucosal immune system the effector sites are suggested to preferentially mount Th2-biased immune responses (Taguchi et al., 1990). Indeed, using CT as an adjuvant, it has been demonstrated that nasal and/or oral immunization with a protein antigen and CT induces local IgA and high levels of serum IgG and IgE, achieved through a selective induction of Th2-type cytokines (Snider et al., 1994; Tamura et al., 1994b; Marinaro et al., 1995). However, using LT as a mucosal adjuvant, it has also been reported that the antibody profiles induced by LT are regulated by both CD4⁺Th2 and Th1 cell types (Takahashi et al., 1996).

Apart from the influence of the cellular environment of the effector regions, the immunostimulating effects of a mucosal adjuvant are also influenced by the nature of the co-administered antigen. In this regard, it has been shown that mucosal adjuvants enhance the immune responses that are naturally induced by the antigen, i.e., viral or bacterial antigens preferentially induce Th1-like immune responses (Reuman et al., 1991; Bourguin et al., 1993), whereas soluble protein antigens promote Th2-like immune responses (Xu-Amano et al., 1993; Marinaro et al., 1995). In this regard, we demonstrated in the present study that i.n. co-application of rLTB and the allergen Hev b 3 enhanced the production of allergen-specific Th2 responses at the humoral and cellular level, i.e., allergen-specific IgG1 and IgE as well as IL-5 production in vitro. In view of future vaccine development, it will be

important to determine whether LTB generally favors the induction of Th2 responses—including IgE—or rather enhances immune responses in an antigen dependent manner.

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