Chapter 4

Oral immunisation of naive and primed animals with transgenic potato tubers expressing LTB

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Chapter 4

Summary

The efficacy of edible vaccines produced in potato tubers was examined in mice. Transgenic plants were developed by *Agrobacterium tumefaciens*-mediated transformation. The antigen selected was the non-toxic B subunit of the *Escherichia coli* enterotoxin (recLTB). A synthetic gene coding for recLTB was made and optimised for expression in potato tubers and accumulation in the endoplasmic reticulum. Introduction of this gene under control of the tuber-specific patatin promoter in potato plants resulted in the production of functional, i.e. Gm1-binding, recLTB pentamers in tubers. Selected tubers containing about 13 μg recLTB per g fresh weight were used for immunisation. Subcutaneous immunisation with an extract of recLTB tubers yielded high antibody titres in serum, which were similar to those obtained with bacterial recLTB. The efficacy of oral administration of recLTB tubers was determined by measuring mucosal and systemic immune responses in naive and primed mice. Animals were primed by subcutaneous injection of an extract of recLTB tuber plus adjuvant. Naive and primed mice were fed 5 g of tubers (~ 65 μg recLTB) or were intubated intragastrically with 0.4 ml tuber extract (~ 2 μg recLTB). In naive mice, feeding recLTB tubers or intubation of tuber extract did not induce detectable anti-LT antibody titres. In primed animals, however, oral immunisation resulted in significant anti-LT IgA antibody responses in serum and faeces. Intragastric intubation of tuber extract revealed higher responses than feeding of tubers.

These results indicate clearly that functional recLTB can be produced in potato tubers, that this recombinant protein is immunogenic and that oral administration thereof elicits both systemic and local IgA responses in parentally primed but not in naive animals.

Introduction

Oral vaccination can lead to protection against infectious agents entering the body via mucosal surfaces of the host [1-3]. Oral vaccines have the advantage of being safe and easy to administer but exploitation is hampered by low efficacy, induction of immunotolerance rather than an immune response, proteolytic degradation of the antigens during passage through the gastro-intestinal tract and exposure to extremely acidic conditions in the stomach [4]. Naturally stable and encapsulated antigens may survive the harsh environment of the gastro-intestinal tract. At present, heat-labile enterotoxin of *Escherichia coli* (LT), its homologue *Vibrio cholerae* toxin (CT) and their B-subunits are among the very few effective, nonliving mucosal immunogens known. The relatively strong immunogenicity after local administration might be related to the intrinsic mucosal adjuvant activity of these components [5]. The mechanisms underlying the mucosal immunogenicity of these substances are still not precisely known [6].

The widespread use of these antigens requires large-scale fermentation and purification. In principle, plants, especially edible plants or parts thereof, are simple production systems, as they do not require complicated production facilities and purification procedures [7]. It has been shown that recombinant multimers of B-subunits of LT and CT can be produced successfully in potato and that they are immunogenic upon oral intake in animals [8-10] and humans [11]. In these studies, oral application resulted in significant levels of protection against a challenge with the respective toxin [10,11]. Recombinant CT-B in potato tuber is stable upon cooking and preserved its biologic activity including its ability to bind to Gm1 [9]. This suggested that the tuber matrix might provide some degree of protection of the antigens against rapid degradation.

Here we report the expression of functional recLTB in tubers of transgenic potato plants and the capability of tuber-derived recLTB to evoke mucosal and systemic antibody responses in mice. Effects of oral immunisation with recLTB tubers were examined in both naive and primed animals. For this purpose, mice were primed by subcutaneous injection of an extract of recLTB tubers plus adjuvant. The adjuvant chosen for parenteral injection was a synthetic polymer of polyacrylate modified chemically with butyl esters (butyl16-p(AA)). This adjuvant has been shown to be a strong mucosal adjuvant for intranasal immunisation [12].

Materials and Methods

Design of LTB plant expression cassette

For expression in potato tubers, a synthetic gene for LTB (synLTB) was prepared based on the sequence of pYA3047 [13]. At the 3'-end a nucleotide sequence was added coding for the hexapeptide Ser-Gln-Lys-Asn-Gln-Leu (SEKDEL) for retention of the protein in the endoplasmic reticulum. Unique restriction sites were introduced in the flanking and coding regions of synLTB at intervals. Putative polyadenylation stop and mRNA instability motifs were removed and codon usage was altered in favour of use in solanaceous crops. SynLTB was made by ligation of fragments each obtained upon enzymatic conversion of two synthetic complementary oligonucleotides with overlapping 3'-ends as described previously [14]. The resulting gene was placed under control of the class I patatin promoter [15] and nopaline synthase terminator (Tnos) sequence and cloned in the binary vector pBINPLUS [16] generating pLANTIGEN4 (Figure 4.1). As a control, an empty expression cassette comprising the patatin promoter and nopaline synthase terminator sequence was cloned in pBINPLUS generating pBINPLUSPAT.

Potato transformation and production

pBINPLUSPAT and pLANTIGEN4 were introduced in *Agrobacterium tumefaciens* strain Agl0 [17] by electroporation and used for transformation of *Solanum tuberosum* cultivar Désirée (De Z.P.C., Leeuwarden, The Netherlands) essentially as described by Stiekema *et al*

[18]. Shoots were rooted on MS20 supplemented with 0.05 mg.l^{-1} indole acetic acid (Duchefa), 7 g.l^{-1} purified agar, 100 mg.l^{-1} kanamycin, 250 mg.l^{-1} cefotaxim and 250 mg.l^{-1} vancomycin. For bulk tuber production, selected transgenic plants were multiplied *in vitro* by cutting. All experiments with transgenic plants were performed under the auspices of the Dutch Committee for Genetically Modified Organisms (COGEM) according to the Dutch law and European guidelines 90/219/EC and 90/220/EC.

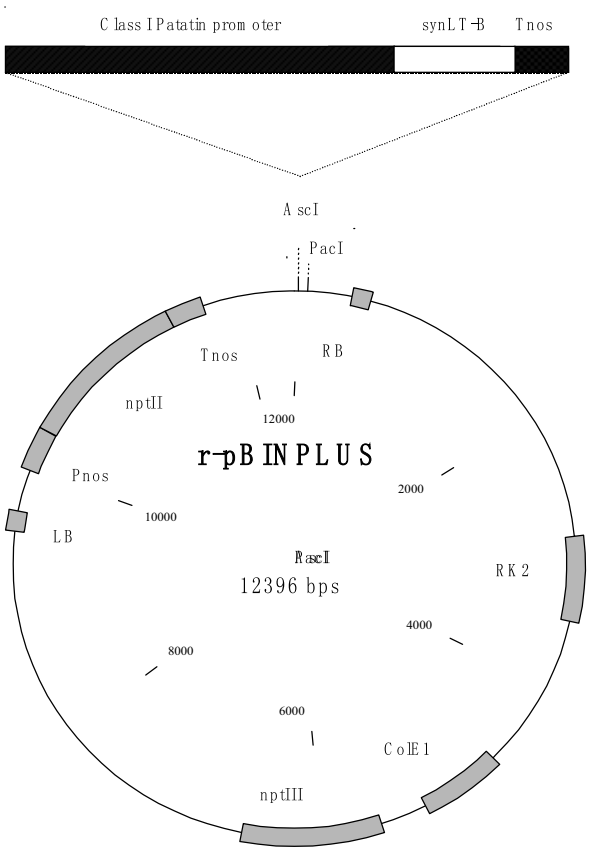

Figure 4.1
Schematic representation of the binary vector pLANTIGEN4 containing the synthetic gene for LT-B. RB, right T-DNA border sequence; Tnos, nopaline synthase terminator; SynLT-B, synthetic gene construct coding for LT-B; nptII, neomycin phosphotransferase II gene, plant selectable kanamycin resistance marker; Pnos, nopaline synthase promoter; LB, left T-DNA border sequence; nptIII, neomycin phosphotransferase III gene, bacterial selectable kanamycin resistance marker; CoIE1 and RK2, origins of replication.

Preparation of tuber extracts and protein analysis

Skinless tuber was extracted in 25 mM sodium phosphate pH 6.6, 100 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 50 mM sodium ascorbate, 1% Triton X-100 and 20 mM sodium metabisulphite. Tissue homogenate was centrifuged at 4°C, 12000 rpm for 5 min and supernatant was collected and transferred to a fresh tube. Total soluble protein was estimated by the method of Bradford. For subcutaneous and intragastric immunisation, the supernatant was dialysed extensively (MWCO 10.000 Da), and freeze-dryed. Proteins were re-suspended in small volumes of de-ionised water for immunisation purposes. Ganglioside GM1 ELISA was performed as described [19] with 5 μg ml^{-1} Gm1(Sigma, St. Louis, MO). Fixed amounts of total soluble tuber protein were loaded onto the plates. As a standard, control tuber extract was spiked with twofold serial dilutions of bacterial recLTB (kindly provided by Dr. L. de Haan, Groningen).

Immunisation of mice

Female Swiss mice (6 to 8 weeks old) were obtained from Charles River (Sulzfield, Germany) and housed under D2 conditions. Experimental groups consisted of five animals. All animal experiments were held under auspices of the ID-Lelystad B.V. Animal Experimentation Committee according to the Dutch Law on Animal Experimentation. To evaluate immunogenicity, mice were subcutaneously immunised on day 0 with 0.1 ml extract from recLTB tuber or with 0.1 ml bacterial recLTB. As adjuvant, 50 μg ml^{-1} butyl16-p(AA)[13] dissolved in PBS was used. To evaluate immunogenicity upon oral administration, some groups of naive mice were immunised orally with tuber-derived recLTB on day 0, 2, and 4, a so-called triple-dose schedule. Oral immunisations were administered by feeding 5 gram of non-peeled, sliced tuber for 24 hours, or by intragastric intubation with 0.4 ml tuber extract. Before oral immunisation, mice fasted overnight while water was provided *ad libitum*. Other groups of mice were primed subcutaneously with 0.1 ml tuber-derived recLTB mixed with 50 μg ml^{-1} butyl16-p(AA) on day 0. Booster immunisations were given orally 3 weeks later, at day 21, 23, and 25. Mice were sacrificed at day 42. Control animals were immunised orally with control tubers (pBINPLUSPAT).

Serum samples were collected at several time intervals from tail blood. Four to six fresh faeces pellets were collected and immediately frozen at –20°C. Before analysis, faeces pellets were dissolved in 750 μl protease inhibitor solution (2 mM phenylmethyl sulfonyl fluoride in isopropanol, 44 mg ml^{-1} bovine serum albumin, 0.002 mg ml^{-1} trypsine inhibitor, 1 mM ethylenediamine tetraacetic acid (all from Sigma), and 0.002 mg ml^{-1} sodium azide (Merck)).

Antibody determination in serum and faeces by ELISA

ELISA plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 0.1 $\mu g.\mu l^{-1}$ LT (Sigma) dissolved in PBS. Serum and faeces samples were twofold serial diluted and antibody subclasses were detected with biotin-labelled goat anti-mouse IgG_1 and IgA (diluted 1/2000, Zymed, San Francisco, CA) and horseradish peroxidase-labelled streptavidin (diluted 1/2000; DAKO A/S, Glostrup, Denmark) was used for detection. As the substrate, 0.1 $mg.ml^{-1}$ tetramethylbenzidine and 0.005 v/v% H_2O_2 was used. Extinctions were measured at 450 nm. Antibody titres were calculated as the dilution of the sample giving an extinction value of 1 above the background. Geometric mean titres (GMT) of individual 2-log titres, SEM and antilog (2^{GMT}) values were calculated. Statistical analysis was performed by the two-side Student's t-test. Differences between groups with P values > 0.05 were considered not to be significant.

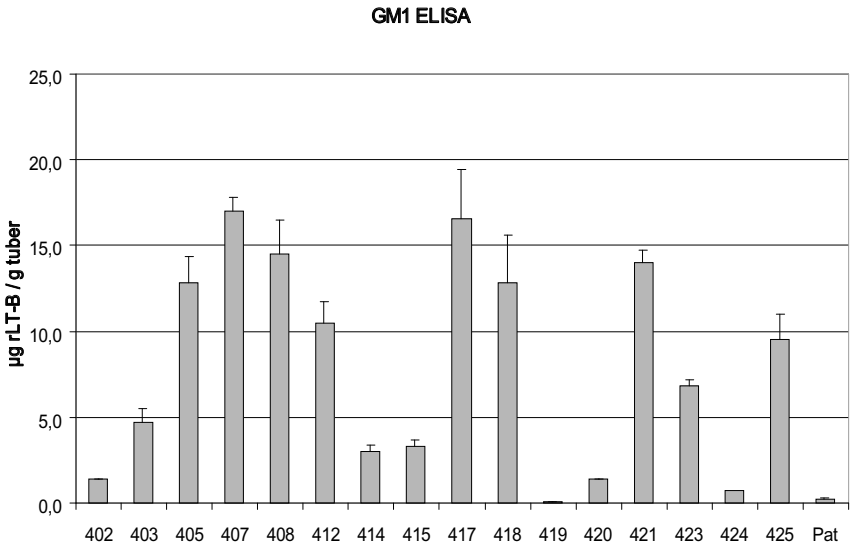

Figure 4.2
RecLT-B expression levels in transgenic potato tubes. Production of recLT-B in tubers of 16 independent transgenic recLT-B plants numbered arbitrarily and one control plant (PAT) was analysed by Gm1-ELISA and expressed in $\mu g\ g^{-1}$ FW. Mean values and standard deviations of three independent analysis are represented. Detection limit is < 13 nmole kg^{-1}.

Results

Genetic modification of potato

pLANTIGEN4 was introduced in *Agrobacterium tumefaciens* and used in transformation experiments. Transformation of internodes of cultivar Désirée finally generated 22 independent transgenic plants containing the pLANTIGEN4 gene construct (Figure 4.1). As a control, pBINPLUSPAT was used in transformation experiments, comprising the binary vector with an 'empty' patatin class I promoter cassette. Tubers were harvested after 2 to 4 months from 16 out of the 22 pLANTIGEN4 transgenic plants and from one pBINPLUSPAT plant.

rec*LTB production*

Analysis of tuber material spiked with bacterial recLTB revealed that 25 pg recLTB per μg water-soluble tuber protein could still be measured in the Gm1-ELISA. Most of the tubers contained GM1-binding recLTB and this could be detected using a LTB_5 specific monoclonal antibody (Figure 4.2). The concentration of recLTB was calculated based on an estimated protein content of 7 mg g^{-1} fresh weight (FW) tuber as was measured for the potato cultivar that was used (data not shown). Maximal concentration of recLTB was 17 μg recLTB per g tuber corresponding with 1.3 μmoles kg^{-1} monomeric recLTB and 0.26 μmoles kg^{-1} pentameric recLTB.

Table 4.1 Anti-LT IgG1 responses in serum after subcutaneous immunisation with potato recLTB or with bacterial recLTB.

	2-log anti-LT IgG1 antibody titre in serum at:					
Antigen	**day 0**			**day 14**		
	GMT	**SEM**	**antilog**	**GMT**	**SEM**	**antilog**
tuber recLTB	6.1	1.7	71	13.9	0.5	14766
bacterial recLTB	8.0	0.6	249	15.1*	0.6	35610

Groups of 5 mice were immunised subcutaneously and antibody titres against LT in serum were measured by ELISA. The mean of 2-log antibody titres (GMT), SEM and antilog values were calculated.
**Significantly different ($P < 0.05$).*

Immunogenicity of tuber-derived recLTB

To determine immunogenicity of tuber-derived recLTB, mice were immunised subcutaneously with a tuber extract containing 45 μg recLTB or with 45 μg bacterial recLTB. Serum samples were collected at days 0 and 14. Both recLTB proteins elicited significant anti-LT antibody responses in serum (Table 4.1). At day 14, the 2-log antibody titre in serum was 13.9 and 15.1 for tuber recLTB and bacterial LTB, respectively. This indicates that plant recLTB is comparably immunogenic as the bacterial LTB.

Oral immunisation of naive mice with recLTB

Groups of mice were fed 5 g of sliced, non-peeled tubers containing ~ 65 μg recLTB or 5 g control tubers. Tubers were swallowed completely within 24 hours leaving only

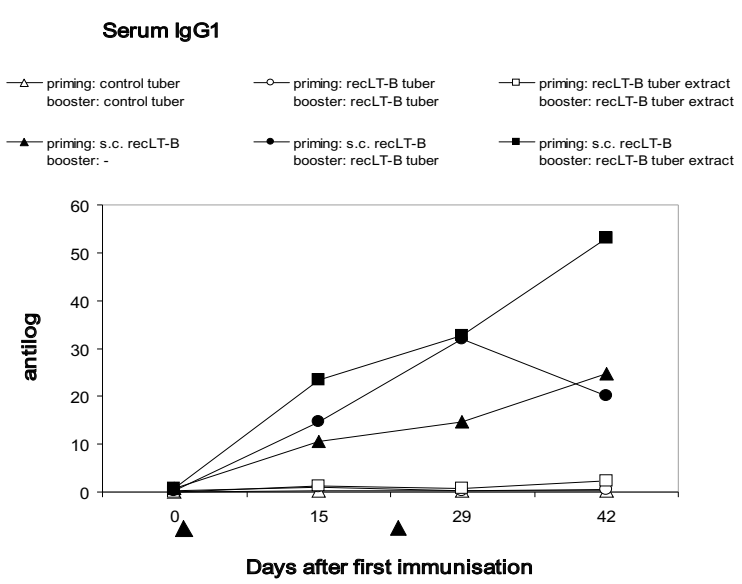

Figure 4.3
Anti-LT IgG1 responses in serum after oral immunisation of naive and primed mice. Antibody titres were determined by ELISA at different time intervals after oral immunisation of naive mice (open figures) and subcutaneously primed animals (filled figures) with recLT-B tubers (circles), extract of recLT-B tubers (squares) or control tubers (triangles). Arrowheads mark time of immunisation.

the tuber skin. Other groups of mice were intragastrically immunised with 0.4 ml tuber extract containing ~ 2 μg recLTB. Mice fed with control tuber did not develop significant antigen-specific antibody titres. Neither IgG1 nor IgA antibodies against LT were detected in serum and in faeces after administration of recLTB tubers or tuber extract (Figures 4.3 to 4.5).

Oral booster of primed mice with recLTB

Groups of mice were primed subcutaneously with ~ 0.5 μg recLTB in tuber extract mixed with the adjuvant butyl16-p(AA). Oral booster immunisations were given 3 weeks later using ~ 65 μg recLTB in tuber or ~ 2 μg recLTB in tuber extract. Two weeks after s.c. priming, IgG1 antibody titres were detectable in serum and antilog

values ranged from 10 to 23 (Figure 4.3). No IgA was found in serum or in faeces (Figure 4.4 and 4.5). When no booster immunisation was given, serum IgG1 and IgA titres at day 29 and 42 remained at the level of day 15 (filled triangle).

An oral boost after subcutaneous priming increased serum IgG1 titres but the differences with primed but non-boosted animals, were not statistically significant (Figure

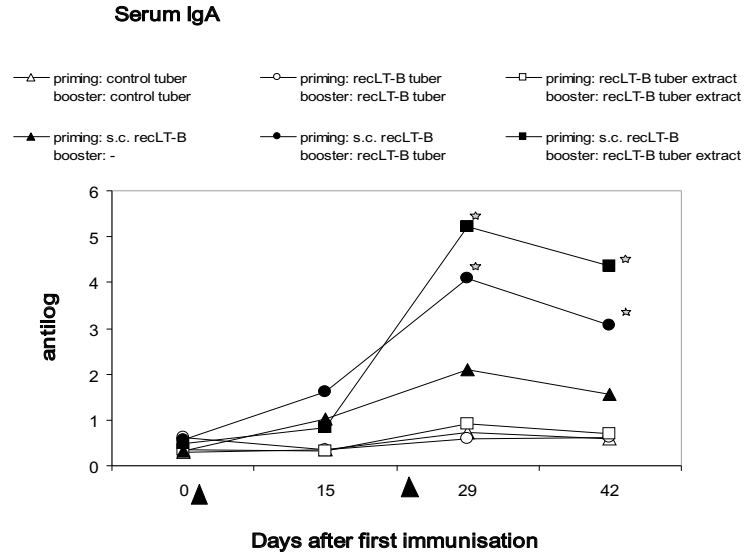

Figure 4.4
Anti-LT IgA responses in serum after oral immunisation of naive and primed mice. Antibody titres were determined by ELISA at different time intervals after oral immunisation of naive mice (open figures) and subcutaneously primed animals (filled figures) with recLT-B tubers (circles), extract of recLT-B tubers (squares) or control tubers (triangles). Arrowheads mark time of immunisation. Statistically significant values are indicated by an astrix.

4.3). After oral booster immunisations, serum IgA antibody titres were significantly enhanced at day 29 and 42 as compared to titres in primed but non-boosted animals (Figure 4.4). Intragastric immunisation with tuber extract generated higher IgA responses than feeding tubers though these differences were not statistically significant.
In faeces, a small but significant increase in IgA titre was detected after intragastric booster immunisation with tuber extract but not after feeding with tubers (Figure 4.5).

Discussion

Plants are recognised as safe and cheap production system for proteins of pharmaceutical interest including vaccines [20,21]. In the past few years, recombinant plants expressing antigens or antibodies have been developed successfully by using plant viruses or *Agrobacterium tumefaciens*-mediated transformation [22,23]. We examined the production of recLTB in potato tubers and the applicability of these tubers for oral immunisation. In order to obtain sufficient production levels of this bacterial protein in transgenic plants, a synthetic gene was made for retention in the endoplasmic reticulum. Introduction of this synthetic gene in potato under control of a tuber-specific promoter yielded several independent transgenic lines. Most of these lines produced LTB pentamers as became apparent from tissue blotting (data not shown), Gm1-ELISA and Western analysis under non-reducing conditions (data not shown) using a LTB_5 specific monoclonal antibody. As compared to bacterial recLTB, a slightly lower migration rate of tuber recLTB was observed suggesting a slightly higher molecular weight (data not shown). This difference might be the consequence of the addition of the flexible linker and the ER-retention signal at the extreme carboxyterminus. About half of the transgenic plants produced Gm1-binding recLTB at levels of 10 μg up to 17 μg per gram fresh weight (FW). Modification of the coding sequence greatly affects expression levels [10,24]. It was reported that the incorporation of ER-retention signal can augment expression levels of recLTB [25] but our data do not support this observation. However, the production levels measured in our transgenic plants underestimate the real values as recovery of recLTB from tuber material is not complete (data not shown). In addition, quantitative analysis of recLTB by the Gm1-ELISA is affected negatively by tuber material such that the sensitivity is reduced dramatically and in a dose-dependent fashion (data not shown).

Subcutaneous immunisation of extract of recLTB tuber elicited high anti-LT antibody titres in mice. This indicated that the tuber-derived recLTB is immunogenic (Table 4.1). However, oral immunisation of naive mice by feeding recLTB tubers or by intragastric intubation of recLTB tuber extracts did not evoke detectable IgG1 or IgA antibody titres in serum. In addition, these animals were not protected against a challenge with LT (data not shown). Possible explanations for the absence of anti-LT responses in our studies include an inadequate immunisation schedule, too low doses of antigen, too low immunogenicity of the tuber recLTB, too low sensitivity of the detection system and interference of tuber material with the development of an immune response. Using our triple-dose schedule, the total amount of recLTB antigen administered by intragastric intubation and feeding was 6 and 200 μg per immunisation per animal, respectively. An increase of the number of immunisations from two to four series of triple oral feedings with 5 g recLTB tubers per immunisation and a total dose of 780 μg recLTB per

mouse, also did not result in detectable IgG1 or IgA titres in serum or faeces. Other investigators reported LTB-specific antibody responses in serum and mucosal secretions after oral immunisation of mice by intubation of leaves of transgenic plants expressing *Escherichia coli* LTB at comparable doses [24]. Similar experiments with potato tubers containing recLTB or its homologue recCT-B resulted in antibody responses in mice

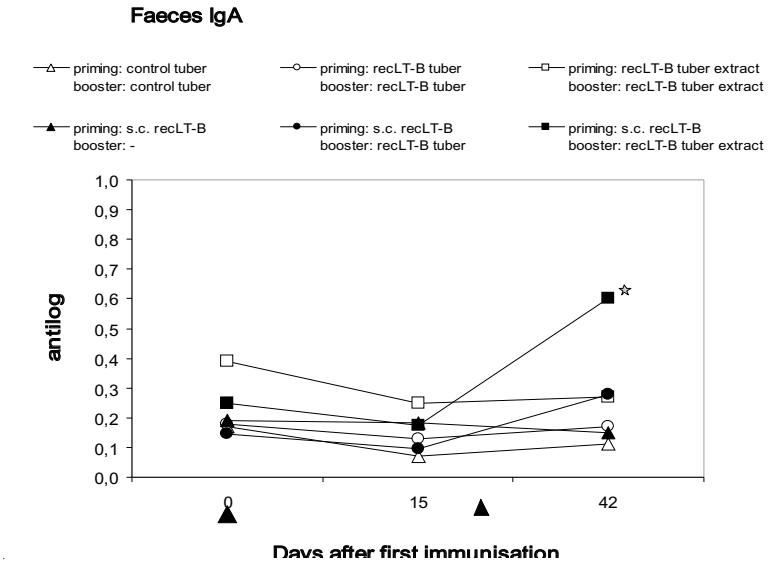

Figure 4.5
Anti-LT IgA responses in faeces after oral immunisation of naive and primed mice. Antibody titres were determined by ELISA at different time intervals after oral immunisation of naive mice (open figures) and subcutaneously primed animals (filled figures) with recLT-B tubers (circles), extract of recLT-B tubers (squares) or control tubers (triangles). Arrowheads mark time of immunisation. Statistically significant values are indicated by an astrix.

and humans and protection against a challenge with the respective toxin [9-11]. Furthermore, we measured antibody titres against the holotoxin LT and not against LTB, which was the antigen, used for immunisation. It can not be excluded that a large portion of antibodies generated by tuber recLTB are unable to bind to LT due to difference in structure [26] or to the inaccessibility of antigenic determinants.

As a consequence of the lack of responses, we examined other possibilities for oral immunisation. Previously, it was reported that intraperitoneal injection of mice with antigen primed for subsequent oral booster immunisation as shown by increased intestinal as well as systemic immune responses [27]. This phenomenon of oral boosting of

primed animals is not exclusive for the intraperitoneal route of immunisation, the antigen tested or the adjuvant used. In the study presented, we compared the effects of oral immunisation of subcutaneously primed mice with that of naive animals. The primary subcutaneous immunisation induced IgG1 responses in serum whereas IgA responses in both serum and faeces remained low. Subsequent oral immunisation augmented significantly the humoral response in primed but not in naive animals. In the primed mice, IgA antibody titres in serum and faeces were augmented significantly while the IgG1 titre in serum remained unaltered. Apparently, once the immune system is primed, oral immunisations are capable of boosting the IgA but not the IgG1 response. The lack of effect on serum IgG1 suggests that the response to the oral booster immunisation is induced and developed at the mucosal site. The mechanisms underlying the increased responsiveness of parenterally primed animals to orally delivered antigens are not clear yet. It is known that lymphocytes can migrate from spleen and circulation to mucosal tissues, where they can proliferate and produce IgA upon encountering antigens entering the body via a mucosal route [28]. Explanations for the immune response to orally administered antigens in parenterally primed animals include improved absorption or uptake of antigens by the gut, altered antigen processing favouring immunoresponsiveness rather than immunotolerance, enhanced capability of the primed immune system to react to small quantities of antigen entering the body, etc. Further research is needed to elucidate the processes involved. This concept of the 'oral boost' is of great interest to the development of oral vaccines as it may render detectable responses in situations where normally no immune response would occur.

There was an obvious difference between feeding tubers and the intubation of tuber extracts. Despite the 30-fold higher antigen dose, feeding tubers was significantly less effective than intubation in inducing IgA in primed animals. This might be due to the method of administration or the antigen preparation. Experiments performed previously by our group demonstrated that oral immunisation by feeding standard food supplemented with antigen is less effective in inducing local and systemic immune responses than intragastric intubation of the same dose of that antigen [29]. This difference indicates that protein degradation in the gastro-intestinal tract is an important factor. These observations implicate that studies on intragastric intubation are limited indicative for the efficacy of edible vaccines and overestimate the potentials of oral vaccines produced in plants.

In conclusion, we demonstrated that potato tubers might be a potential source of oral vaccines for direct application and that they can produce considerable levels of functional recLTB pentamers. Transgenic tubers induce specific immune responses depending on the route of administration and immune status of the animal. In our hands, oral immunisation solely by feeding transgenic tubers was not effective. Activation of the immune system by a primary immunisation via a parenteral route followed by

booster via the oral route resulted in significant systemic and local responses. The administration of edible vaccines in primed instead of naïve subjects reveals a more sensitive test system and higher probability of success. Further research is required to optimise this approach and to identify the underlying mechanisms.

Acknowledgements

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Chapter 4