Chapter 5

Oral immunisation with LTB co-expressed or fused to E2 expressed in transgenic potato tubers

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Chapter 5

Summary

Two types of transgenic potato plants were developed expressing the B-subunit of heat-labile enterotoxin of Escherichia coli (LTB) together with a glycoprotein (E2) of Classical Swine Fever virus: 1) E2 co-expressed with LTB (E2 + LTB) and 2) E2 genetically conjugated to LTB (E2-LTB. Subcutaneous (SC) immunisation of extracts of these E2 + tubers induced significant serum antibody titres against LT indicating that these constructs were immunogenic. Oral immunisation of naive mice with these tubers or tuber extracts did not result in detectable serum responses. In addition, serum antibody responses in SC primed mice were not enhanced by oral booster immunisations. At mucosal level, feeding of E2 + LTB tubers elicited significant IgA responses in intestinal scrapings against LT but not against E2.

We concluded that LTB-fusion proteins expressed in potato plants are immunogenic and that oral administration mostly evoked low IgA responses at local level but not in serum and only in SC primed animals. LTB did not increase the response against fused or co-administered E2. Our results are discussed in the light of feasibility of edible vaccines.

Introduction

Oral vaccination is regarded to be an attractive alternative for injected vaccines as it is easy to apply, cheap and safe. Furthermore, it can induce protection at mucosal level, at the site of entrance of many pathogens and it enables mass vaccination via food or drinking water. However, oral vaccination is often not very effective. The immune response is short lasting and large doses of antigen are needed, even when alive microorganisms are used [1-3]. Strong mucosal adjuvants and antigen-presentation systems are needed, especially for non-living antigens. Until today, the only known strong mucosal immunogens are the heat-labile toxin of *Escherichia coli* (LT) and cholera toxin of *Vibrio cholerae* (CT) and their A and B subunits which might be related to their intrinsic adjuvant activity. These toxins are interesting candidates for edible vaccines as they can be expressed in plants such as tobacco [4], maize [5] and potato [4,6-10] and retained their biological activity (i.e. GM1-binding) even after boiling [9]. Feeding of transgenic potato tubers to mice [7] and humans [8] resulted in the induction of specific antibodies in serum and faeces. The antibody titres were often low but still high enough to confer protection against a challenge with the toxin [7]. Despite considerable variation in expression levels between individual plants and plant tissue parts and the low immune responses upon oral intake, edible vaccines are believed to be promising. Oral vaccination of primed but not naive mice with LTB tubers induced serum and local IgA [6].

Here we examined whether LTB produced in plants is capable of enhancing the immune response against co-expressed of genetically fused antigens. LTB is the non-

toxic part of LT and the adjuvant function depends on the pentameric conformation and is most pronounced when conjugated chemically to the antigen. We describe the production and testing of a fusion protein in *Solanum tuberosum* Desiree tubers namely the fusion protein of LTB and a glycoprotein of classical swine fever (E2) [11]. Furthermore, LTB and E2 co-expressed as separate molecules in one single potato plant is examined. The immune responses against these antigens after feeding with tubers and oral administration of tuber extracts are compared.

Materials and methods

Potato tuber-derived vaccines

For immunisation experiments tubers from four different selected transgenic potato lines were used: PAT, pL421, pL1317 and pL4+14#109. Control plants (PAT) and potato plants accumulating LTB pentamers in tubers (pL421) have been described before [6]. The expression level of LTB in pL421 tubers approximates 15 μg pentamers per gram fresh weight (FW). pL1317 harbours a gene construct coding for a fusion protein consisting of LTB and the classical swine fever virus (CSFV) E2 glycoprotein as present in the pRb2 vector described before [6]. The expression level of LTB-E2 fusion protein in tubers of pL1317 approximates 0.1 μg pentamers per gram FW. pL4+14#109 harbours the expression cassette giving rise to accumulation of LTB pentamers in tubers similar to that of pL421 in conjunction with an expression cassette giving rise to accumulation of the CSFV E2 glycoprotein. pL4+14#109 tubers hence accumulate both LTB and CSFV E2 in one cell. The expression level of LTB in these tubers approximates 10 μg per gram FW and that of CSFV E2 approximates 1 μg per gram FW.

Immunisation of mice

For subcutaneous and intragastric immunisation, large scale extracts were made as described before [6] from 300 g of tubers of pL1317 (LTB-E2), PAT (control tubers) and 600 g of tubers from pL4+14#109 (E2 + LTB). Supernatants harbouring the vaccines were dialysed once against excessive extraction buffer and six times against excessive de-ionised water for 48 h using SnakeSkin pleated dialysis tubing (Pierce Chemical Company, Rockford, USA; MWCO 10 kDa) and concentrated by freeze-drying and subsequently re-suspended in small volumes of de-ionised water for immunisation purposes. The final concentration of the vaccines was determined by Gm1-ELISA or Western blotting as described. For oral immunisations using tubers, these were peeled and sliced prior to oral immunisations.

Female Swiss mice (6-8 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 the auspices of the ID-Lelystad B.V.

Animal Experimentation Committee according to the Dutch Law on Animal Experimentation. Oral immunisations were administered on three alternating day, a so-called triple dose schedule [12] by feeding 5 g peeled and sliced tuber for 24 h, or by intragastric (IG) administration with 0.4 ml of tuber extract. Before oral immunisation, mice were fasted overnight while water was provided *ad libitum*. Naive mice were orally immunised on day 0, 2, and 4 and were given boost immunisations on day 21, 23, and 25. Other groups of mice were primed subcutaneously (SC) with 0.1 ml tuber extract mixed with 50 μg ml^{-1} of butyl16-p(AA) on day 0 and received oral boost immunisations on day 21, 23, and 25. Mice were immunised with tuber-derived E2 + LTB (pL4+14#109), LTB-E2 (pL1317), and control tuber extract respectively. Mice were sacrificed on day 35 or on day 42. Control animals were immunised with control tubers (pBINPLUSPAT).

Sample collection

Serum samples were collected at several time intervals from tail blood. Four to six fresh faeces pellets were collected and pre-treated as described previously [6]. From some groups, intestinal scrapings were collected on the day of sacrifice as described before [13].

Antibody determination by ELISA

High binding ELISA plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 0.1 μg ml^{-1} of LT (Sigma) dissolved in PBS or with 2.5 μg ml^{-1} of recombinant CSFV E2 produced in insect cells using a baculo expression vector, in coating buffer (ID-Lelystad, pH 9.6). ELISA plates (Polysorb, Nunc) were coated overnight at 4°C with 2.5 μg ml^{-1} CPV dissolved in coating buffer (ID-Lelystad, pH 9.6) [14]. Serum and faeces samples were twofold serially diluted in PBS, 0.05% Tween 20, and 1% bovine serum albumin. ELISA was performed as described earlier [6].

Extinctions were measured at 450 nm and antibody titres were expressed as the dilution factor of the sample giving an extinction value of 1 above the background. Geometric mean titres (GMT) of individual 2-log titres, standard deviation (SD), standard error of the mean (SEM) and antilog (2^{GMT}) values were calculated. Statistical analysis was performed by Student's two-tailed *t*-test. Differences between groups with P value < 0.05 were considered to be significant.

Results

Tuber-derived vaccines

The tubers used in this study were selected on basis of accumulation of significant amounts of the vaccine in fresh tubers. pL4, pL1317 and pL4+14#109 tubers were chosen on basis of Gm1 receptor binding of LTB subunit. The amount of CSFV E2

glycoprotein was estimated by Western blotting using known amounts of CSFV E2 produced in a baculo system. Expression levels of LTB-E2 in tubers of pL1317 were very low compared to LTB only (pL421), most likely because of the enormous size of the fusion protein (mol. wt. of LTB-E2 is 50 kDa compared to 11 kDa for LTB)

Subcutaneous immunisation with tuber extracts

Expression levels of the concentrated antigens isolated from tuber by extraction, dialysis and freeze-drying were determined by Gm1 ELISA and Western blotting. To determine the immunogenicity of the various LTB vaccines, antibody responses were measured after subcutaneous (SC) immunisation of 0.1 ml tuber extract with butyl16-p(AA) as adjuvant. E2 + LTB was immunogenic upon SC immunisation revealing anti-LT IgG1 but not IgA in serum (Table 1). No responses against the co-expressed CSFV E2 or to the fused E2 present in pL4+14#109 and pL1317 respectively, were detected. The antigen dose for SC immunisation was very low for all tuber constructs (Table 1). Experiments were performed with one dose for each vaccine and hence a correlation

Table 5.1 Anti-LT IgG1 responses in serum in subcutaneously primed mice.

antigen	prime immunisation		boost immunisation		anti-LT IgG1	
	route of immunisation	LTB per dose (µg)	route of immunisation	LTB per dose (µg)	day 14	day 35
control tuber	SC	-	--	--	< 1	< 1
E2 + LTB	SC	2.9	--	--	2.8 ± 1.0	5.7 ± 1.5
E2 + LTB	SC	2.9	Tuber	50	3.0 ± 0.3	4.6 ± 1.1
E2 + LTB	SC	2.9	IG	11.7	4.3 ± 1.7	6.6 ± 1.1
E2-LTB	SC	0.05	--	--	<1	<1
E2-LTB	SC	0.05	tuber	0.75	<1	<1
E2-LTB	SC	0.05	IG	0.2	<1	<1

Antibody titres are given as GMT ± SEM. Mice were primed subcutaneously on day 0 with 0.1 ml tuber extract together with adjuvant. Oral boosts were given on day 21, 23, and 25 as 5 g of intact tuber or as 0.4 ml tuber extract. Antibody titres of boosted and non-boosted mice were not significantly different. For E2 + LTB, plant (4+14)-109 harbouring 280 nM LTB was used. This plant also contained 8 µg of E2 per gram fresh weight tuber. For E2-LTB, plant 1317 harbouring 3.95 nM LTB was used. As a control, transgenic plants harbouring an empty tuber expression cassette was used.

between dose and immune response could not be determined. Antibody responses in faeces could not be detected (data not shown).

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Oral immunisation of naive mice

Mice were immunised orally either by feeding intact tuber or by intragastric (IG) administration of tuber extract according to a triple dose schedule [12]. Most animals ate more than 2.5 gram tuber within 24 h. Oral intake of E2 + LTB tubers but not IG administration of tuber extract revealed low but significant anti-LT IgG1 titres in serum on day 14 (Table 2) in contrast to previous experiments where intragastric immunisation

Table 5.2 Anti-LT IgG1 responses in serum in naive mice.

antigen	prime immunisation		boost immunisation		anti-LT IgG1	
	route of immunisation	LTB per dose (µg)	route of immunisation	LTB per dose (µg)	day 14	day 35
E2 + LTB	tuber	50	tuber	50	2.7 ± 1.3	2.3 ± 1.0
E2 + LTB	IG	11.7	IG	11.7	< 1	< 1
E2-LTB	tuber	0.75	tuber	0.75	< 1	< 1
E2-LTB	IG	0.2	IG	0.2	< 1	< 1

Antibody titres are given as GMT ± SEM. Mice were primed orally on day 0, 2, and 4, and boosted on day 21, 23, and 25.

experiments performed best [6,15]. IgA was not induced. Oral boosting (either with tuber or tuber extract) did not enhance these responses. No immune responses against the co-expressed or fused E2 and CPV were observed. In addition, antibody responses against LTB, E2 or CPV in faeces were not detected (data not shown).

Oral immunisation of primed mice

To enhance the immune response against orally administered antigens, we applied the systemic priming/oral boost strategy [16]. Subcutaneously primed mice were orally boosted either by feeding with of tuber or IG administration of tuber extract. To determine the effects of the oral booster, one group was primed but not boosted. Oral boosting with E2 + LTB did not significantly increase the anti-LT IgG1 titre compared to non-boosted animals. Neither did it boost the antibody response against E2. Oral boosting with E2 + LTB only slightly increased the IgA response (data not shown). Neither IgG1 nor IgA were detected in faeces (data not shown). In addition, intestinal scrapings of mice immunised with E2 + LTB were collected on day 35 and tested on presence of antigen-specific antibodies. Compared to groups immunised with control tubers, we found significantly higher anti LT IgA titres in groups fed with E2 + LTB tubers but not in the groups administered IG with tuber extract (Fig. 1). Intestinal IgG1 was not enhanced by oral immunisation and anti-E2 antibodies were not detected.

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Clinical observation of mice after oral boost immunisation

During the experiments, we monitored daily the conditions of the animals. We observed side effects after oral intake of raw potatoes. The mice displayed signs of nausea and

Table 5.3 Number of animals that were ill/died/tested after the oral boost.

Priming	booster	E2 + LTB	E2-LTB	Total
tuber	tuber	0/0/5	2/1/5	4/2/20
IG	IG	0/0/5	0/0/5	1/1/20
SC	-	0/0/5	0/0/5	0/0/20
SC	tuber	1/1/5	1/0/5	7/2/20
SC	IG	0/0/5	0/0/5	3/0/20
	Total	1/1/25	3/1/25	15/5/100

During the experiments, all mice were clinically observed. Ill mice suffered from nausea and, when fed tuber, refused to eat. In that case they were fed oat to recover or they died within 3 days.

reduced appetite and refused to eat the whole 5 gram of potato. Table 3 summarises the number of affected and dead mice as well as the total number of mice. To recover, the mice were fed oat instead of the vaccine. Complications only occurred after the booster and within three days after immunisation. No correlation could be found between route of priming or boosting.

Discussion

In the present study, we explored the efficacy of edible vaccines based on the B subunit of the heat-labile enterotoxin of *E. coli* (LTB). The CSFV E2 glycoprotein was expressed as fusion protein with LTB and produced in potato tubers and compared with CSFV E2 and LTB co-expressed in tubers order to establish the role of conjugation. It has been proven by many others that LTB and CTB (B-subunit of the *Vibrio cholerae* toxin) are not only effective mucosal immunogens but act also as adjuvant towards antigens co-administered [17-19]. The strongest response was obtained when antigens were conjugated chemically to LTB or CTB [20]. Others and we demonstrated that LTB and CTB can be produced in their pentameric forms and with their ability to bind to GM1 gangliosides in plants, e.g. in tobacco [4], maize [5] and potato [4,6-10]. Oral administration of these plants in mice [4,6,7,10] or in humans [6,21] induced systemic and local antibody responses and conferred protection against a subsequent challenge with the natural toxin [7].

The immunogenicity of the plant produced proteins, was determined by a single subcutaneous (SC) injection of tuber extracts plus adjuvant in mice. E2 + LTB and CPV-LTB appeared to be immunogenic and elicited IgG antibodies against LT in serum. Responses were low or absent which was explained by the low antigen dose varying between less than 1 µg and a few µg. In our previous study with LTB alone, the dose

was about 50 μg. [6]. Immunisation with extracts with E2-LTB did not result in detectable responses most probably due to extremely low doses. These constructs were not further examined.

Mice with a body weight of between 20 and 40 g have a maximal daily intake of about 5 g potatoes. Feeding this quantity of pL4+14#109 (E2 + LTB) tubers to naive mice corresponded with a dose of 50 μg LTB. It induced significant anti-LT IgG1 (Table 2, day 14) but not IgA in serum. Intragastric (IG) administration of E2 + LTB tuber extract corresponding with about 11.7 μg LTB did not induce an immune response. This was in contrast with our previous findings that feeding with 65 μg LTB was less effective than IG administration of 2 μg LTB. We then suggested that besides the antigen dose, the route of administration could be crucial for the outcome of oral immunisation [6] because the gastro-intestinal tract is originally not designed to respond immunologically towards orally administered food antigens [22]. Instead, oral tolerance is induced [1]. In contrast, we now observed that E2 + LTB tubers are capable to trigger the immune system whereas tuber extract is not.

Oral boosting of SC primed mice did not enhance the IgG1 or IgA response in serum (Table 1) or in faeces (data not shown). This did not corresponded to our previous study where we demonstrated that oral immunisation with plant produced material augmented the IgA titre [6]. However, in the present study significant IgA responses were observed in intestinal scrapings after boosting with E2 + LTB tubers.
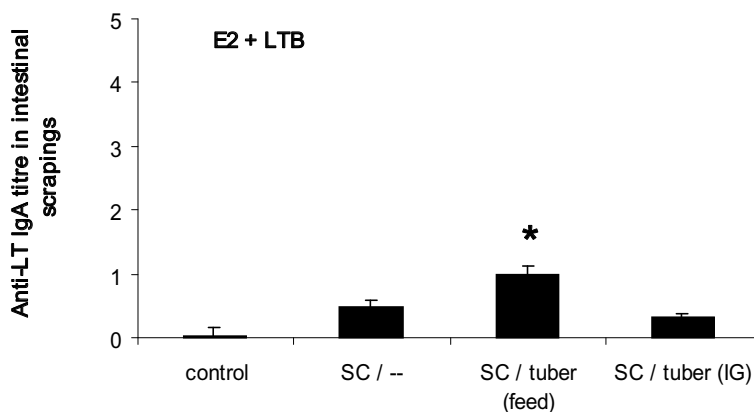

Figure 5.1
Anti-LT IgA antibody titres in intestinal scrapings on day 35, two weeks after oral boost immunisations with E2 + LTB tuber material. Control mice were immunised with tuber material transformed with an empty vector. An asterisk indicates significant responses induced by the oral boost.

LTB did not enhance the immune response against co-administered or fused proteins although correct assembly of the fusion proteins in potato plants and intact GM1-binding in ELISA. Most probably, the E2 and CPV doses were too low to induce an immune response, despite the use of LTB. LTB is a weaker adjuvant than both the toxic holotoxin and LTA [17] but toxicity of the latter two hampers their clinical use. Recently, non-toxic forms retaining mucosal adjuvanticity have been created by site-directed mutagenesis [23-28]. These mutant toxins are interesting adjuvant candidates for future transgenic edible vaccines.

All oral immunisations were preceded by overnight fasting to enable quick consumption of the tuber material by mice. Animals were monitored daily during the experiments and no abnormalities were seen after parenteral and oral priming. After the oral boosters, some of the animals were clearly affected for one or a few days. Weight loss of more than 5 g was noted and even mortality was observed. These systemic side effects were noted from day the first day of booster on and disappeared within the subsequent 24 h. These adverse reactions were registered only after the boost and not after priming. But we do not have proof that these reactions were the result of priming using a total protein extract of tuber, giving rise to immune responses to many tuber proteins resulting in loss of oral tolerance and/or induction of an allergic reaction. It can also be the consequence of changes in the material administered. Being a member of the family of solanaceae, potatoes contain several toxic glycoalkaloids, socalled solanins, with the highest levels found in the foliage, blossoms and sprouts, followed by the peel and the tuber flesh [29,30]. These solanins can cause haemolytic and hemorragic damage to the gastrointestinal-tract if ingested in excess of a few mg per kg body weight [31]. They are not destroyed by boiling and cooking of potatoes and its concentration can increase substantially on exposure to light, environmental changes during growing seasons and harvest, and as a result of mechanical injury, including peeling and slicing [29,32,33]. However, the dosage of these compounds is least in peeled tubers which was the material used for immunisation and is expected to be absent in the extracts that were dialysed for several days. Another, less aggravating immunisation protocol must be developed to overcome these adverse effects of edible vaccines or another, non-toxic plant species (e.g. banana or corn) should be used.

In summary, we demonstrated that E2 co-expressed with LTB but not E2 fused to LTB was immunogenic and E2 + LTB evoked serum responses towards LT after SC priming and oral feeding of mice. Feeding boosted the local but not the serum response. LTB, however, could not act as an adjuvant towards E2. CPV-LTB was immunogenic upon SC but not upon oral immunisation.

Oral immunisation using edible vaccines remains an attractive concept, but several problems must be solved before an effective edible vaccine is available. First, expression levels of recombinant proteins in plants must be increased. Second, appropriate plants

or plant parts should be selected. And third, appropriate mucosal adjuvants should be incorporated.

Acknowledgements

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