Chapter 7

General discussion

Mucosal immune responses upon oral vaccination

The efficacy of vaccination via the oral route was evaluated first. This was determined by measuring the antigen-specific titres in faeces, intestinal scraping and blood samples. Oral vaccines are known to induce both mucosal and system immune responses [1]. Mucosal immune responses are dominated by immunoglobulins of the IgA isotype and are best monitored in mucosal secretions. Surprisingly, we found the highest antigen-specific antibody titres in blood and these were of the IgG1 isotype. IgA antibodies were hardly measurable, especially not in faeces or intestinal scraping (Chapters 2, 3, 4, 5 and 6). It was not expected that the systemic immune response was predominant, but we experienced difficulties in determining the mucosal immune response. In our hands, low mucosal responses were measurable in faeces after oral immunisation with OVA with or without CT (Chapters 2, 3) or transgenic potatoes expressing LTB (Chapter 4) and in intestinal scrapings after oral immunisation with transgenic potatoes expressing LTB-fusion proteins (Chapter 5).

Besides immunological active components, mucosal secretions contain other functional substances. Secretions in the gastrointestinal (GI) tract play a major role in the digestion of food and for this purpose, proteolytic enzymes are abundantly present. Although immunoglobulins, especially sIgA, are relatively insensitive to proteolytic degradation, the enzymes may still interfere with the test system. Therefore protease inhibitors were added to the secretion samples, which slightly diluted the antibodies present in these samples. Another reason why blood titres are higher may be that blood is a closed compartment where antibodies are circulating. Mucosal antibodies, on the other hand, are constantly secreted.

Although mucosal secretion samples were collected on the same days as blood samples, Van der Heijden *et al.* has observed significant day to day fluctuations of the mucosal response [2], thus repeated sampling would be more appropriate for a better evaluation of the mucosal immune response. Animal samples were collected every week in the here described experiments, but for future studies daily collection is recommended to obtain more insight in the mucosal immune response. Measurement of antibodies in intestinal scrapings has restrictions because a single animal can be sampled only once.

High titres in blood were always found together with low but detectable responses in secretions and low titres were accompanied by absence of a mucosal response. Faeces IgA titres were lower than serum IgA titres (various Chapters). This is in agreement with findings of Jertborn *et al.* [3], who found that serum IgG and IgA are indicative for the sIgA response. Others, however stated that blood reflects only systemic and not mucosal immunity [2,4].

Analysis of the antibody-secreting cells (ASCs) in the mucosal associated lymphoid tissue (MALT) is another, probably more sensitive method to quantify the number of activated and mature plasma B-cells [5]. Most antigen-specific ASCs in the MALT isolated

after oral immunisation produced antibodies of the IgA isotype. An easier and less laborious method might be measurement of ASCs in blood. Blood ASCs elicited by oral vaccination are proven to home to mucosal tissues and are independent from serum antibody responses. But since sensitivity of measurement on blood ASCs is low, these are not appropriate alternatives for MALT-derived ASCs to measure the mucosal immune response [6].

Interpretation of the antibody titre into protective levels is rather complicated. The protective value of the antibody responses elicited by the transgenic potatoes was not validated. In a study on oral immunisation with virus-like-particles (VLPs) of rabbit hemarraghic disease virus (RHDV) in rabbits, significant protection against a subsequent challenge with virulent virus were observed, while the IgA titre in serum was 64 at maximum (manuscript in preparation). Such protection against an infection with virus or bacteria despite low IgA titres have been confirmed by others [7]. So, relatively low antibody titres can still be successfully protective.

An applicable immunisation protocol for edible vaccines

Oral immunisation has proven to be more successful when the vaccine was administered frequently, preferably on several consecutive days, followed by boost immunisations three to four weeks later [8-11]. This immunisation protocol had to be optimised in the light of potato tuber as edible vaccines. The frequency of feeding of raw potatoes to mice was limited by the maximal oral intake, the lack of nutritional value and the presence of toxic ingredients. Immunisation on consecutive days was therefore undesirable, because daily repeated immunisations with potatoes appeared to be too aggravating for the animals. A protocol was designed in which the animals were immunised on alternating days. First, the protocol was optimised using a soluble protein: ovalbumin (OVA). IG priming and boosting with OVA on three alternating days ('triple dose') resulted in higher responses in serum than single immunisations. Local responses were only obtained after 'triple dose' immunisation and CT was necessary as adjuvant. Immune responses against this toxin were also elicited (Chapter 2). Then it was investigated whether oral immunisations were more effective in a primed immune system. Chapter 3 describes that subcutaneous (SC) or intraperitoneal (IP) immunisations with OVA could prime the immune system for a subsequent oral booster. Interestingly, use of an adjuvant in the priming significantly increased serum IgA and, to a lesser extend, IgG1 upon the booster. As we expected, oral boosts significantly enhanced the serum IgA titres in systemically primed mice compared to naive mice. Although this was independent of the use of CT, the use CT was continued in order to maximise the mucosal immune response. However, the maximal IgA titre in serum was on average 128 and the maximal IgA titre in faeces or intestinal scrapings was 8 when CT was used.

Oral boosting of primed mice with 'triple doses' and CT as adjuvant induced antibody responses in faeces whereas local responses were not significant with 'single dose' boosts. This systemic priming/oral boost strategy was first described by Pierce *et al.* who found that SC/oral immunisation of dogs with cholera toxoid enhanced the anti-toxoid response in serum and duration of protection against a challenge with live bacteria [7]. Local responses were not determined in the study of Pierce, but antigen-specific ASCs were found in the small intestine and other lymphoid organs by Van der Heijden *et al.* [5]. Chapter 3 clearly demonstrated that antibodies were secreted and detectable in faeces when the parenterally primed animals were boosted with 'triple doses' of the antigen.

One explanation of how a parenteral immunisation can prime the mucosal immune system (MIS) for a subsequent booster is that systemic immunisation generates a population of primed lymphocytes. This results in increased responsiveness to relatively small amounts of antigen that pass the physical barrier of the GI tract. Another explanation is that systemic priming activates the expression of mucosal homing receptors on the surface of antigen-specific lymphocytes. How and where these lymphocytes are induced by systemic immunisation remains obscure [12]. But homing of primed lymphocytes to the MALT results in a population of memory cells and a state of increased responsiveness of the MALT. Upon an oral antigen booster, the primed MALT will develop a secondary response manifested by IgG and IgA antibodies (Chapter 3)

Since it was already known that expression levels of recombinant proteins in plants could be very low, we determined the minimal effective oral dose of OVA. Oral boosting of SC primed mice required doses of at least 300 μg OVA provided that CT was added as adjuvant. Without this adjuvant, this minimal effective dose was significantly higher. Van der Heijden *et al.* demonstrated that without CT, systemically primed mice must be boosted orally with at least 10 mg OVA [5]. These results again demonstrated that large amounts of antigen are needed.

Taken together these findings, an immunisation protocol for mice was proposed which involved SC priming on day 0 with 100 μg antigen plus 50 μg of the adjuvant Butyl-16-p(AA) [13] and an oral booster with three doses of at least 300 μg antigen plus 5 μg CT three to four weeks later. In retrospect, evaluation of this protocol with LTB or CTB instead of CT as adjuvant would have been more appropriate since the protocol was intended to be used with edible vaccines expressing LTB with or without co-expressed antigens.

Chapter 4 describes the application of this immunisation protocol with edible vaccines expressing LTB as immunogen. SC priming with tuber extract followed by 'triple dose' oral boost immunisations appeared to be efficient in inducing serum and faecal IgA. A decrease in body weight of the animals during the immunisation period was observed (data not shown), which was considered to be caused by the intense immunisation schedule, including three oral immunisations on alternating days which were preceded by an overnight fasting period. This immunisation protocol affected the general condition

of the animals resulting in clear morbidity for one or a few days and even mortality in a few cases in one experiment (Chapter 5).

The studies described in Chapter 2 revealed that a second series of 'triple dose' boost immunisations further enhanced the serum IgA response while the other responses were maintained at the same level. Cebra reported that cells in the germinal centres of Peyers Patches (PPs) were transient and that successful secretory IgA responses attenuated the stimulation by secondary mucosal challenge. This may explain why traditional boost responses are often not induced after oral immunisation [14].

Furthermore, the duration of the response must be assessed in order to establish the optimal time intervals for subsequent oral boost immunisations. We have only followed the immune response until three to four weeks after the last boost. Lycke *et al.* however, demonstrated that up to two years after an initial series of oral immunisation with CT, a single oral boost with 10 μg CT evoked a clear anti-CT IgA response in the lamina propria [15]. This indicated that long-term memory in the gut could be established.

Edible vaccines based on LTB

Edible vaccines were made in potato plants under control of the tuber specific patatin promotor. The first transgenic plant generated contained expressed recombinant LTB (pL421). Then, other transgenic plants were constructed for production a LTB-fusion protein. For this purpose, the potato cultivar Desiree was transformed to express E2-LTB (pL1317). LTB was also co-expressed in one plant together with E2 (pL4+14#109). The majority of the transgenic plants produced tubers within 2 – 4 months after transfer to the greenhouse (personal communication). The expression of GM1-binding antigens was determined in extracts from freshly harvested tubers. Potato tubers contained approximately 7 mg of water-soluble protein per gram of fresh weight tuber. Most of the tubers analysed contained GM1-binding LTB or LTB-fusion proteins. LTB as well as the LTB-fusion proteins were intact (including pentamer formation), as confirmed by ELISA (Florack *et al.*, manuscript in preparation) and Western blot analysis (Fig. 7.1).

Chapter 4 describes the use of plant LTB as immunogen. The expression level of GM1-binding LTB was on average 0.25% LTB per total soluble protein (TSP), which was comparable to findings by others (Chapter 1, Table 1.1). The IG administered dose was of about 2 μg of LTB and the dose by oral feeding about 65 μg of LTB. The plant-produced LTB was immunogenic and oral administration elicited both systemic and local IgA responses in parenterally primed but not in naive animals. Our results corresponded partially with those of others. Mason *et al.* demonstrated that feeding of naive mice with LTB tubers with similar antigen doses induced local IgA and the toxic effects of LT could be neutralised *in vivo* [16]. Why oral immunisation of naive mice was not effective in our hands was not elucidated. Despite lower dose, IG administration

induced higher antibody titres than feeding of intact tuber. This demonstrated that other factors than antigen dose influence the outcome of oral vaccination.

The E2-LTB-fusion protein could be produced correctly in potatoes. The expression of E2-LTB was more than 10 times lower (Chapter 5) than LTB alone as reported by us (Chapter 4) and Mason *et al.* [17], most likely because of the enormous size of the fusion protein. With E2-LTB, a weak response against LTB but no response against E2 was detected. Possible explanations are too low dose of E2 or relatively too rapid

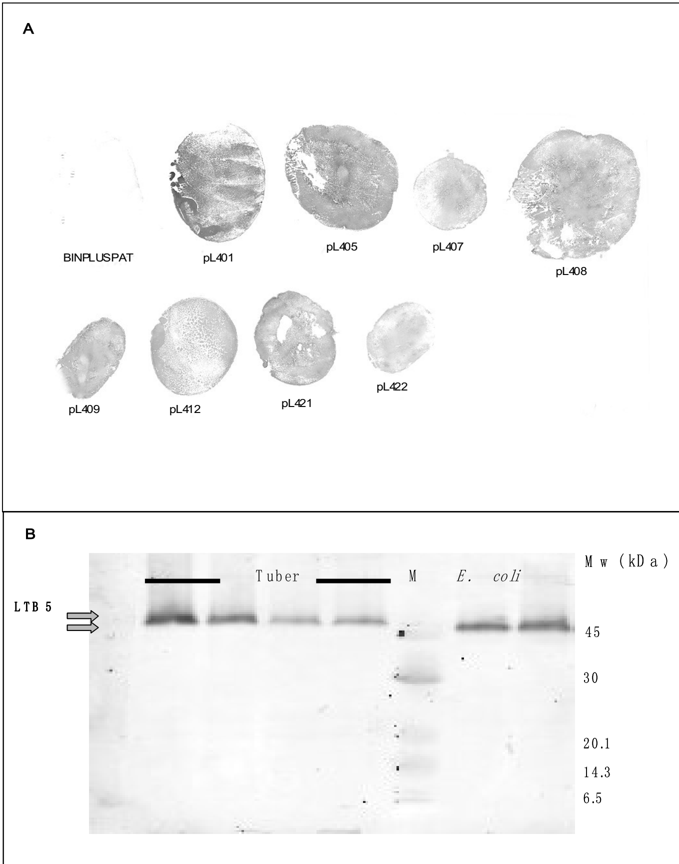

Figure 7.1
Immunoblots of LTB produced in transgenic potato tubers. Accumulation of LTB in the tuber was demonstrated using a LTB-specific monoclonal antibody on a nitrocellulose tissueblot. BINPLUSPAT contains the empty vector. pL401 until pL422 contain the LTB-coding vector (A). Presence of pentameric LTB in tuber extracts is demonstrated by westernblot analysis under semi-native conditions (B).

degradation of the antigen. Apparently, LTB did not function as an adjuvant for E2 (Chapter 5). Co-expression of LTB with E2 (E2 + LTB) again induced antibody responses against LT only and not against E2.

Degradation of the edible vaccine in the GI tract could also have been responsible for the poor efficacy of LTB as adjuvant. Transport studies in the human intestinal epithelial cell line Caco-2 with radioactively labelled LTB revealed that after two hours of incubation, the radioactivity was transported from the apical to basolateral and visa versa. Less than 1% of the transported radioactivity could be immunoprecipitated with anti-LTB antiserum indicating that LTB was extensively degraded during the transport [22]. With rapid degradation and low expression levels in potatoes, it can be expected that only a small quantity of the vaccine finally reach the MIS. Chapter 3 demonstrated that 300 μg of OVA was the minimal effective dose for oral immunisation. Increase of expression levels in plants might improve the probability of success. Furthermore, the use of another, more potent adjuvant is desirable. In general, LTB and CTB are weaker adjuvants than the complete holotoxin [23] but toxicity of the latter has hindered its practical use. Non-toxic mutants of LT and CT that have been developed recently [20,24-26] are interesting adjuvant candidates for future edible vaccine studies.

Oral immunisation versus oral tolerance

The antibody responses we measured in serum and faeces were low or, in some cases, totally absent. There is a major paradox in oral vaccination: feeding of vaccines must result in protective immune responses instead of oral tolerance (Fig. 7.2). Normally, the GI tract does not develop an immune response against food components but is tolerant towards them [27]. With edible vaccines, the MIS must be told that the transgenic potato is not a common potato but contains a vaccine, and that the MIS must respond to this vaccine but not to the potato itself.

The biological function of the immune system is first and foremost to protect against dangerous pathogens. Live microorganisms are able to infect and invade the host, cause damage and generate danger signals that stimulate and activate immune responses [28]. Non-living antigens without adjuvant lack danger signals and are therefore less effective than live vaccines. Obviously, food products do not contain these danger signals. Perturbation of the physiological balance in the GI tract might lead to the unwanted situation in which food induces mucosal immune responses and diseases like food allergy or coeliac disease can be induced. An edible vaccine must therefore provide the necessary signals for an immune response, because food normally does not. The response must, however, be controlled to prevent unwanted diseases.

Remarkably, the systemic prime/oral boost protocol was successful for oral boosts immunisations, whereas oral boosting of naive mice was often unsuccessful. We

suggested that this immunisation protocol could prevent oral tolerance, because an established immune response is difficult to tolerise (Chapter 3).

Although systemically primed mice could be tolerised by feeding OVA, the degree of tolerance and its effects on the systemic immune response were more limited than that found in equivalent naïve animals [29]. This indicates that induction of oral tolerance is relatively ineffective in a situation with an established immune response [30]. Antigen-experienced T cells may be inherently resistant to tolerogenic signals, perhaps because their increased expression of adhesion molecules and altered signalling pathways make them less dependent on co-stimulation for their activation than naïve T cells [29].

Adoptive transfer studies with lymphocytes of orally treated animals are needed to proof oral tolerance. Recipients should not respond to a systemic immunisation. These type of experiments were not performed. Therefore, the role of oral tolerance in our experiments could not be determined. Nevertheless, study of this phenomenon is an important consideration in further studies on edible vaccines. Especially since induction of oral tolerance using edible vaccines might broaden the application area of such

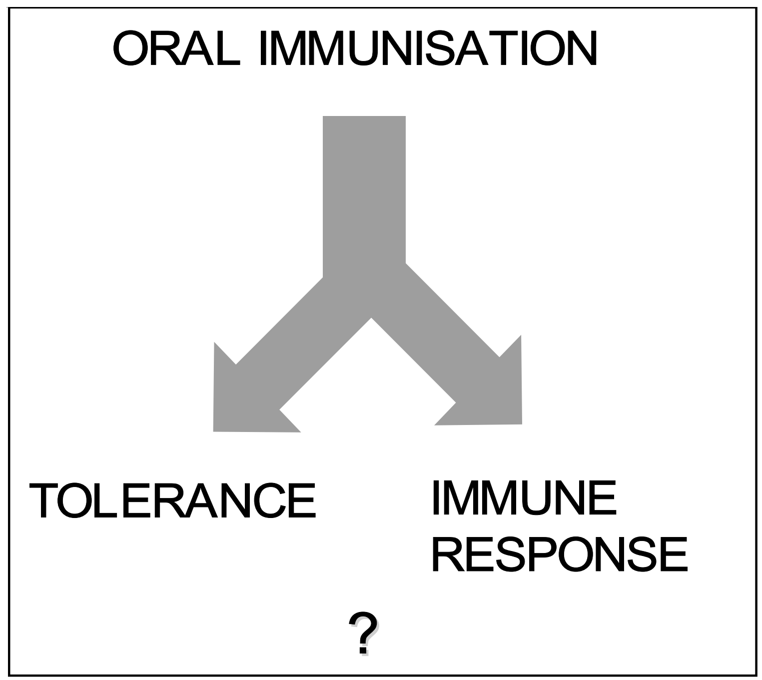

Figure 7.2
The oral vaccination paradox: oral vaccination is intended to active, protective immunity against pathogens invading mucosal tissues, but the GI-tract is programmed to respond with a state of immunotolerance against oral antigens

vaccines. Oral tolerance induction can be exploited as immunotherapy for diseases like allergy or some autoimmune diseases [31]. Williams *et al.* summarised the use of LTB and CTB conjugates for the induction of tolerance as immunotherapy for experimental allergic encephalomyelitis (EAE) in the rat and diabetes in the nonobese diabetic (NOD) mouse.

Edible vaccines as genetically modified organisms; the public opinion

The development of transgenic plants with improved production or resistance has initiated public debate and awareness on the subject. Although edible vaccines have obvious advantages, the use of transgenic plants distress critics. The main concern is the introduction of new genetically modified variants in the environment via pollen, seeds or pieces of root or tubers since these are capable to grow out into full transgenic plants. In 1999, Losey *et al.* published a 'Scientific Correspondence' in Nature that pollen from corn engineered to express proteins from *Baccilus thuringiensis* (Bt) pose a potential risk to monarch butterfly populations growing on milkweed. However, this controversial publication lacked solid scientific data (e.g. missing proper controls and details such as the dose used, the unspecified endotoxin concentration in the pollen themselves, and the lack on information on the potential for temporal and spatial overlap of pollen shed, milkweed plants and monarchs under natural field conditions). The scientific community rejected the works validity. Sears *et al.* reported recently that Bt expression in pollen is low and no acute toxic effects were observed at any pollen density that would be encountered in the field. In addition, only a portion of the monarch populations utilises milkweed in and near cornfields. These researchers concluded that the impact of Bt corn pollen from current commercial hybrids on the monarch butterfly populations is negligible [32]. Nevertheless, the Losey *et al.* report was immediately embraced by the media and the public [33]. Shelton and Sears reviewed the history of the monarch controversy in a special GM issue of The Plant Journal [33]. Adequate data is not yet available to provide an appropriate risk assessment by the scientific community. At this moment, risk communication has been left largely in the hands of non-scientists [34]. It is clear that the impact of transgenic food on its environment must be assessed with absolute care to ensure proper, well-thought research. The results must then be evaluated in a more reserved manner without letting emotions prevail.

Perspectives of edible vaccines

In the early 1990s, Charles Arntzen mused about genetically engineered plants producing vaccines in their edible parts. Ten years later, many publications and patents have been published on this subject. The research described in this thesis, clearly demonstrated that potato tubers could be used to produce complex (fusion) proteins

of which LTB, E2 + LTB, and CVP-LTB producing tubers were immunogenic upon subcutaneous immunisation. The ideal edible vaccine has high protein content and high expression levels of the antigen, grows rapidly under a wide range of conditions and is easy to propagate. Finally, the edible vaccine is not toxic when given in large amounts, because oral vaccination may require high and repeated doses. In this respect, the use of potatoes has several drawbacks. Consumption of raw potatoes is not preferable and cooking might denature the antigen. Being a member of the family of solanaceae, potatoes contain several toxic glycoalkaloids ('solanins') with the highest levels found in the foliage, blossoms and sprouts, followed by the peel and the tuber flesh [37,38]. These solanins can cause hemolytic and hemorragic damage to the gastrointestinal (GI)-tract if ingested in excess of a few mg per kg body weight [39]. Solanins 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 [37,40,41]. Potatoes were initially not intended to be used as vaccine vehicles, but merely as model system to prove the concept. However, potatoes may be practical as certain kinds of potatoes are actually eaten raw in South America and cooking of potatoes does not destroy the antigen per se [42]. Table 1.1 to 1.3 (Chapter 1) summarise the plants which have been transformed

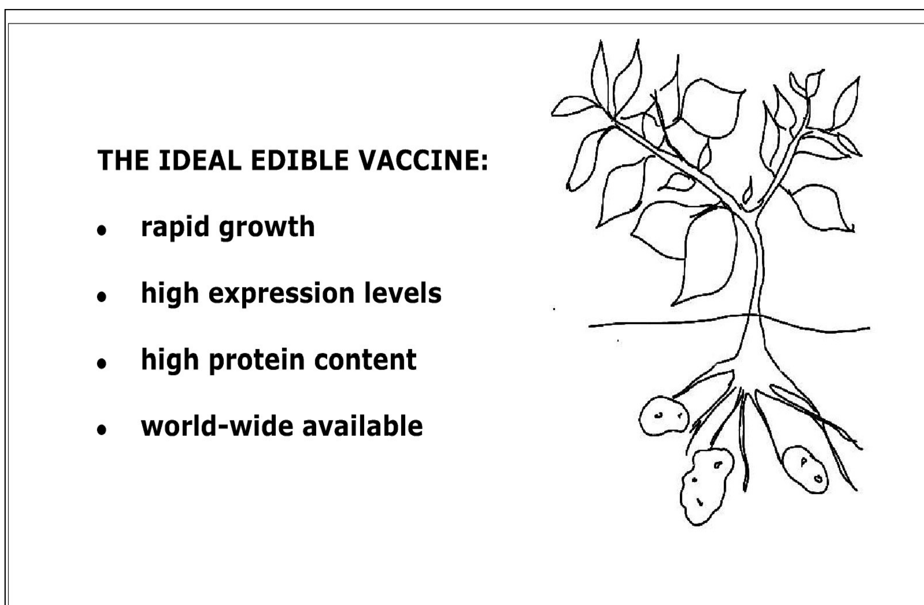

Figure 7.3
Characteristics of the ideal plant for edible vaccine production

yet. For future edible vaccine studies, a more suitable plant like tomato with relatively high expression levels should be chosen (Fig. 7.3).

Despite limited progress, researchers still believe in the use of plants for medical purposes. Besides protection against infectious pathogens, oral administration of antigen may be of interest in suppressing autoimmunity. Furthermore, plants can be used to produce therapeutical antibodies for example a chimeric IgG-IgA antibody against a surface antigen of *Streptococcus mutans* to prevent tooth decay, or to produce pharmaceutical proteins like human serum albumin, epidermal growth factor or interferon- [Streatfield, 2001]. The prediction that an applicable edible vaccine will be ready in the near future is overly optimistic, however, the still increasing knowledge in molecular biology and immunology makes it less fiction.

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