FEASIBILITY OF ORAL IMMUNISATION WITH LTB-BASED EDIBLE VACCINES

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MOGELIJKHEDEN VAN ORALE IMMUNISATIE MET OP LTB GEBASSEERDE EETBARE VACCINS

(met een samenvatting in het Nederlands)

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A student once asked: 'what is the difference between a man of Tao and a little man?'. The sensei replied: 'it is simple: when the little man receives his first dan, he can hardly wait to run home and tell everyone he made his first dan. Upon receiving his second dan, he will climb to the roof and shout to the people. Upon receiving his third dan, he will jump in his automobile and parade through town with its horn blowing, telling everyone about it.'

The sensei continued: 'when the man of Tao receives his first dan, he will bow his head in gratitude. Upon receiving his second dan, he will bow his head and his shoulders. Upon receiving his third dan, he will bow at the waist and quietly walk alongside the wall so people will not see him or notice him.'

First know yourself, then know others

Gichin Funakoshi (1868-1957)

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General introduction

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Context

History has taught us that vaccines are suitable for controlling many infectious diseases. The worldwide eradication of smallpox is an example of a successful vaccination campaign. The level of other diseases like polio, diphtheria, tetanus, pertussis, and rabies is significantly reduced by vaccination. But infectious diseases still pose major health problems, especially in the more impoverished parts of the world and the veterinary world in which vaccines are too costly or not available [1]. Furthermore, there is much concern about new and re-emerging infectious diseases in the developed world. The increased (widespread) use of antibiotics has significantly increased the emergence of antibiotic-resistant organisms (e.g. *Streptococcus pneumoniae*, enterococci and gram-negative enteric pathogens) worldwide [2]. In addition, immunodeficient people and travellers can be a carrier of old or foreign infectious diseases. Therefore, development of vaccines and vaccination strategies remains important.

Two types of vaccines can be discerned: live vaccines (composed of live, attenuated micro-organisms) and non-living vaccines (composed of inactivated micro-organisms, subunits thereof, recombinant DNA products or chemically synthesised peptides or oligosaccharides). Live vaccines possess many of the biological characteristics of the virulent microorganism like receptor binding, passage through physical barriers and production of active components. They are presented via the major histocompatibility (MHC) class-I-pathway. Although they are highly immunogenic, the microorganisms in live vaccines have the risk of return to virulence [1,3]. Obviously, non-living vaccines do not hold this risk. Since they lack certain characteristics of the alive counterpart, they are in general less immunogenic and are presented via the MHC class-II-pathway. When antigens are reduced into peptides, the biological complexity of the original microorganism is also reduced and this is accompanied by a further loss in immunogenicity.

In the last two decades, considerable scientific progress has been made, which has revolutionised the way both live and non-living vaccines can be designed, formulated and produced. Moreover, new immunisation strategies are being explored in order to simplify the administration of vaccines.

Mucosal vaccination

Most living pathogens enter the body via the mucosal tissues of the gastrointestinal (GI), urigenital or respiratory tract. Specific and non-specific defence mechanisms must limit the consequences. Specific immunity at mucosal tissues is brought about by both the local (mucosal) and systemic immune system and the first is the most important one

(box 1.1). Parenteral vaccination induces the systemic immune system, but hardly the mucosal immune system [4]. Vaccines administered by the mucosal route can induce both immune systems [2,5-8].

The mucosal tissues represent the interface between the host and its environment and mucosal vaccines can be applied at any point of the mucosal system. From a practical point of view, intranasal and oral vaccinations are most attractive, but intravaginal and intrarectal vaccinations are considered also (e.g. for protection against HIV [9]). All

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Box 1.1   The mucosal immune system

The mucosal immune system (MIS) is stimulated by uptake of antigens (micro-organisms and particles) and initiates an immunological cascade that primes the mucosa-associated lymphoid tissue (MALT). The MALT represents a compartmentalised, interconnected system of lymphoid tissue with various induction and effector sites, like the lamina propria of the upper and lower respiratory tract, the intestine, the genitourinary tract, and the salivary, mammary and lacrimal glands [2]. The inductive sites of the gut-associated lymphoid tissue (GALT) are the Peyer's patches (PP). PPs are found on the follicle-associated epithelium (FAE) and are covered with specialised cells called microfold or M-cells. M-cells sample luminal antigens by receptor-mediated uptake and transcytose them for presentation by antigen presenting cells (APC), which are located in the dome-area of the follicle [64]. Mucosal epithelial cells express major histocompatibility complex (MHC) class-I molecules and low levels of MHC-class II molecules and can also present antigen to CD4$^+$ and CD8$^+$ T-cells [1]. The dome area contains mainly B-cells and CD4$^+$ T-cells. The MALT is best primed by local application of antigen. Once the mucosal immune system has been triggered, oral boost immunisations induce more vigorous responses than parenteral boost immunisations [69].

Upon stimulation, primed antigen-specific B- and T-cells migrate via the thoracic duct to the draining mesenteric lymphnodes (MLN) and spleen where clonal expansion and isotype-switch takes place [65]. The most important effector molecule of the MIS is secreted dimeric immunoglobulin of the IgA isotype, sIgA. sIgA is produced in large quantities in both animals and humans (~ 3 g/day in human) [66,67], which is more than all other immunoglobulins together [30].

Proliferating B- and T-cells enter the circulation and home preferentially to all mucosal effector sites and also to peripheral lymphoid organs where, in human, they can differentiate into IgA- and, to a lesser extent, IgG-secreting plasma cells [8,13,14]. The interconnection between all mucosal tissues is also known as the common mucosal immune system (CMIS) [6,13,65]. The secretion of IgA across intestinal mucous membranes is excellently reviewed by MacPherson *et al.* and Mestecky *et al.* [67-68].

mucosal tissues are connected via the common mucosal immune system (CMIS) (box 1.1). Live mucosal vaccines based on invading microorganisms are able to penetrate the mucosal tissues due their biological characteristics [10]. Non-living vaccines lack the feature of passing the physical barrier and are presented by MHC class-II instead of MHC class-I like live vaccines. Irrespective of the type of mucosal vaccine, the major obstacle in their development is their low and short lasting efficacy [6,11,12].

Oral vaccines

Oral vaccination is the most attractive route of mucosal vaccination because of its simple way of administration. In theory, oral vaccines are capable of inducing both mucosal and systemic immune responses [13,14]. Progress in the development of oral vaccines is, however, limited. At present, there are a few commercially oral vaccines available. In the Netherlands, a polio (OPV, Sabin®) and a typhoid vaccine (Ty21a, Vivotif Berna®) are the only prescribed oral vaccines. Both are composed of live, attenuated microorganisms. Non-living oral vaccines are still in phase of research and development and in the last decade, no major breakthrough has been reported.

Oral immunisation
Besides the nature of the antigen (living/non-living) [12,15], there are several other factors affecting the outcome of oral immunisation. Factors that may be of influence in the induction of oral immune responses or oral tolerance instead, are the dose of antigen, frequency of administration and immunisation protocol, age of first exposure, immune status of the host towards the specific antigen, species, delivery systems and use of adjuvants [4]. Non-living oral vaccines preferably must be designed according to the following criteria: 1) protection of the antigen from enzymatic digestion. Exposure of the vaccine to low pH and proteolytic enzymes during passage through the GI tract results in degradation of the antigen or loss of conformation [11]; 2) enhancement of antigen uptake by M-cells or epithelial cells in the GI tract and facilitation of passage through the epithelial barrier; 3) activation of the innate and/or specific immune system instead of induction of oral tolerance; 4) induction of immunological memory [16,17]. For a detailed overview of the pro's and con's of oral vaccines we refer to an excellent textbook [4] and recent publications [1,2].

Oral tolerance
Oral tolerance is the phenomenon of systemic immunological unresponsiveness that occurs after oral intake of antigens. This subject has been recently reviewed by Iijima *et*

al. [18]. The primary task of the GI tract is absorption of nutrition out of food and under normal conditions and the body is tolerant against dietary antigens. Immunological reactions are preferably prevented [7]. Perturbation of the mucosal immune response may lead to unwanted diseases like food allergy and coeliac disease as a result of breakdown in oral tolerance [19]. There are several possible mechanisms for the induction of mucosally induced tolerance. High doses of antigen can induce clonal deletion and anergy of T-cells whereas low doses of antigen can generate regulatory cytokines with suppressive effects on many aspects of the immune response [2,18-20]. Both mechanisms lead to suppression of antigen-specific immune responses. Recently, reports have been published which revealed possible roles of $\gamma\delta$T-cells, dendritic cells and intestinal epithelial cells (IECs) in the induction of oral tolerance. The exact mechanisms by which these cells establish oral tolerance are still under investigation [18,19,21]. Once a T-cell is tolerised, tolerance can be spread via the cognate interaction between antigen presenting cells (APC) and/or T-cells. It has been proposed that tolerised cells may mediate their suppressive effects directly via the production of inhibitory cytokines or indirectly by competing for growth factors, MHC-peptide complexes, or co-stimulatory molecules on APC and thus pass on tolerance [19].

For oral vaccination, it is important that the immune system can distinguish the proteins within a vaccine from common dietary proteins and reacting with an immune response against the former. When this distinction cannot be made, oral tolerance will be broken and the immune system will react toward all orally administered antigens.

Mucosal adjuvants and delivery systems

The first obstacle mucosally administered antigens encounter is the non-specific defence mechanisms along the mucosal surfaces (e.g. cilia in the nasal system, low pH in the stomach, and proteolytic enzymes in the gut). Then, the antigens have to pass a thick mucosal layer and the epithelial barrier before they finally reach the immune system. Microbial pathogens that colonise the host through mucosal surfaces have evolved strategies to cross these physiological and chemical barriers [12]. Vaccines composed of such microorganisms are more successful in surviving the passage through the GI tract and are efficiently taken up. Live recombinant microorganisms (e.g. Lactobacteria and Salmonella) can thus be appropriate vectors for oral uptake of various proteins or peptides [22]. Virus receptor binding of live vaccines with retained ability to invade the host probably provides a danger signal [23]. This actively stimulates the MIS. In addition, live vaccines form a constant source of antigen and can stimulate the MIS constantly. Non-living vaccines do not have these features and are therefore less successful [10,15,24].

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The immune response can be enhanced by use of appropriate adjuvants and delivery systems. Until today few mucosal adjuvants are known. Delivery vehicles can protect oral antigens from degradation and can enhance vaccine uptake. Commonly used delivery vehicles are microparticles, liposomes, immune stimulating complexes (ISCOMS), and carrier-molecules, which have been reviewed recently [1,25]. This introduction will only summarise the main characteristics of the most thoroughly studied adjuvants.

LT and CT

Escherichia coli heat-labile enterotoxin (LT) and the highly homologous *Vibrio cholerae* toxin (CT) are powerful mucosal immunogens and can also act as powerful adjuvants. Their immunogenicity and adjuvant activity against co-delivered antigens are well documented [6,26,27]. Orally administered LT and CT predominantly induce T-cell responses with Th2-associated cytokines (box 1.1), and IgG1 and IgA antibodies. However, Th1-associated responses like CTL activation are also induced. Both toxins are composed of two structurally and functionally separate A and B subunits [1].

The toxic A-subunit induces ADP-ribosylation, which causes the disease symptoms [28,29]. It is highly immunogenic and can act as an adjuvant towards co-administered antigens. Due to the toxicity, however, LT and CT and their A-subunits are regarded as too toxic for clinical use. Non-toxic forms with retained mucosal adjuvanticity have been created by site-directed mutagenesis [30-35]. These mutant toxins demonstrated that the ADP-ribosylating activity of LT and CT was not a prerequisite for their adjuvant effects since enzymatically inactive mutant toxins retain adjuvant activity after intranasal administration [35]. Nevertheless, ADP-ribosylation appears to enhance the immunogenic and adjuvant potency of orally administered toxins [33].

The non-toxic B-subunit binds as a pentamer with high affinity to gangliosides (mainly GM1) [36-38], and induces apoptosis of CD8$^+$ and CD4$^+$ cells [26]. LTB was taken up predominantly by the IEC rather than the M-cells [25]. Conjugated to antigens, it can act as a carrier molecule and induce mucosal and systemic immune responses [26] while mere mixing with antigen elicits weaker responses [36,39,40]. However, many early studies on the adjuvanticity of the B-subunits have proven to be inconsistent as the presence of traces of holotoxin could not be excluded. The use of recombinant LTB and CTB improved the insight in the adjuvanticity of the B-subunit [30,41]. Furthermore, the degree of cross-linking between different conjugate preparations varies, which may have affected the GM1 binding and decrease the immunogenicity of the conjugate [42]. Some of these problems can be overcome by genetic constructs, but the fusion of genes may also affect the pentamer formation and by consequence the affinity for GM1.

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It is important to note that coupling of antigen to LTB or CTB prior to oral delivery can dramatically decrease the dose required to stimulate tolerance. LTB/CTB then acts as a carrier that shuttle antigen into a tolerance-inducing pathway associated with the gut mucosa, but the precise mechanisms are unknown [36]. This other immunological property of the B subunit can be used as effective therapy against certain diseases. For example, oral administration of CTB-insulin conjugates to nonobese diabetic (NOD) mice could suppress type I diabetes, a model of spontaneous autoimmune disease [12].

This suggests LT and CT have distinct immunological activities and that there is some sort of deviation involved that determines the outcome of oral vaccination: an immune response or tolerance.

Microparticles

Microparticles are spheres of polymers with diameters ranging from nanometers up to several micrometers. They may be built from different polymers and are easy to produce and are stable. Depending on their size, they are taken up by M-cells (box 1.1) or even epithelial cells [1,43]. When composed of biodegradable and biocompatible poly (lactide-co-glycolide) (PLG) polymers, microparticles can controllably release incorporated antigens. Incorporation of additional immunomodulators or carrier molecules can improve their potency. There are obvious differences between soluble and particulate antigens. In general, soluble antigens are less immunogenic and tend to induce tolerance rather than immune system activation [4]. Particle size is crucial to its immunogenicity, since small particles are non-specifically taken up by epithelial cells [43,44].

Liposomes

Liposomes are composed of phospholipids and cholesterol. Antigens can be incorporated into liposomes, which are stabile in acidic solutions, bile and pancreatin solutions. Like microparticles, they are actively taken up due to their small size and particulate formulation. Their efficacy can be improved by adding immunomodulatory or carrier molecules [1].

ISCOMS

Immunostimulatory fractions of *Quillaja saponica* (Quil A) have been incorporated into lipid particles and form immune stimulating complexes (ISCOMS). Hydrophobic or membrane-associated proteins are able to incorporate spontaneously into the ISCOMS when present during their assembly. Incorporation of non-hydrophobic proteins is more difficult but also possible. ISCOMS are resistant to both temperature and low

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pH. Their particulate formulation facilitates uptake by M- or epithelial cells. ISCOMS can induce CTL and are able to stimulate cells of the innate immune system [1,4,45].

Lectins and ligands

Interaction of antigen with mucosal tissues can be improved by binding to lectin-like structures on IECs. Ligands with affinity for these structures include pili, viral haemagglutinins, many bacterial toxins, lectins, plant toxins and bacterial invasins. These ligands can act as carrier (or transport) molecules or as targeting device to antigens. [1]. Direct targeting to M-cells (box 1.1) further enhances antigen uptake. A few interesting lectins that bind selectively to M-cells have been identified (e.g. *Euonymus europaeus* in canine and *Ulex europeaus* I and II in mice) [46,47]. After binding they are actively taken up by those cells [47-51]. However, these lectins are species specific and at this moment,

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Box 1.2   Construction of edible vaccines

There are several ways to accomplish recombinant gene expression in plants: plastid transformation via particle bombardment (e.g. chloroplast transformation) [70], transient expression by chimeric plant viruses [56,71,72] and *Agrobacterium*-mediated transformation [73].

Plastid transformation results in high expression levels of recombinant protein, and due to their uniparentally maternal inheritance, escape of recombinant genes through pollen can be avoided. It is appropriate for the production of bacterial antigens but not suitable for the production of glycoproteins like viral surface antigens, because of differences in glycosylation [73].

Transient expression by transformed plant viruses (e.g. tobaco and cowpea mosaic virus) also achieves considerable levels of protein. Immunogenic epitopes can be presented on the surface of plant viruses by making translational fusions within or at the 3' terminus of a coat protein. The recombinant gene is not passed down to following generations since it is not incorporated into the plant genome, which takes extra inoculation steps when producing vaccine. The immunogenic epitopes are expressed in virus-like particles (VLP) or linked to viral proteins [56].

Transformation with *Agrobacterium tumefaciens* is the most commonly used method although expression levels obtained with this method are low. The advantage of this method is that foreign genes are stabile incorporated into the nuclear genome (Figure 1), thus enabling large-scale cloning and maintenance of selected high-expressing lines and the ability to sexually cross transgenic lines to obtain multiple proteins expressed in the same plant. One of the major disadvantages of *Agrobacterium*-mediated transformation of plants is the low expression level of the recombinant protein compared to other methods [74].

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no human M-cell binding lectins are identified. Further studies are involved to prove their carrier function [52], and, to our best knowledge, successful immunisations have not been reported yet.

Miscellaneous

In addition to the vehicles described above, the mucosal immune response upon oral vaccination can be enhanced by adjuvants such as avridine, muramyl dipeptide, saponins or derivatives thereof [53], aluminum salts [54], or cytokines such as IL-12 possibly in combination with a suitable delivery system [8]. Ryan *et al.* gave an excellent overview of the choice of adjuvant or delivery system and their effect on the polarisation of the immune response [1]. The immunostimulatory capacities of unmethylated CpG motifs of bacterial DNA currently receive great attention. CpG oligodeoxynucleotides were shown to enhance the local and systemic antibody responses to oral, intrarectal or intranasal immunisation with tetanus toxoid or influenza virus vaccines [1]. CpG motifs are detected, like bacterial or viral DNA, as a danger signal by the vertebrate immune system [55]. They cause B-cells to proliferate and secrete immunoglobulin, which directly synergise with the antigen-specific effects mediated through the B-cell receptor. In addition, CpGs improve antigen presentation by up regulation of co-stimulatory molecules and MHC class II molecules [54].

Plants as edible vaccines

The easiest way to deliver oral vaccines is by mixing them with food. This is the basic concept of edible vaccines. The increased knowledge on molecular biology made way for a novel type of farming, namely molecular farming, which uses ordinary plants as factories for the production of inexpensive factories for the production of expensive drugs and vaccines. Production of vaccines in edible plants or plant parts thereof gave rise to a new concept: edible vaccines.

An overview of the synthesis methods in edible vaccines is given in box 1.2 and Figure 1.1. Plants have several advantages above traditional productions systems. They only require simple growth circumstances and large-scale production is easy, which makes them inexpensive production factories. As with all oral vaccines, administration of edible vaccines does not require trained personnel and avoids needle-use and its associated risks [56]. Still, degradation in the stomach and gut is a major concern. Transgenic plant tissue can possibly act as a natural delivery system by encapsulation of the vaccine in plant cells with their tough outer wall [3]. Plant tissue may also contain possible molecules with adjuvant capacities (e.g. Quil A) [57].

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Plants can correctly process and express complex foreign proteins (Table 1.1 and 1.2). The complex sIgA molecule has been produced successfully in potato and tobacco plants, including the correct assembly of the two IgA molecules, the J-chain and the
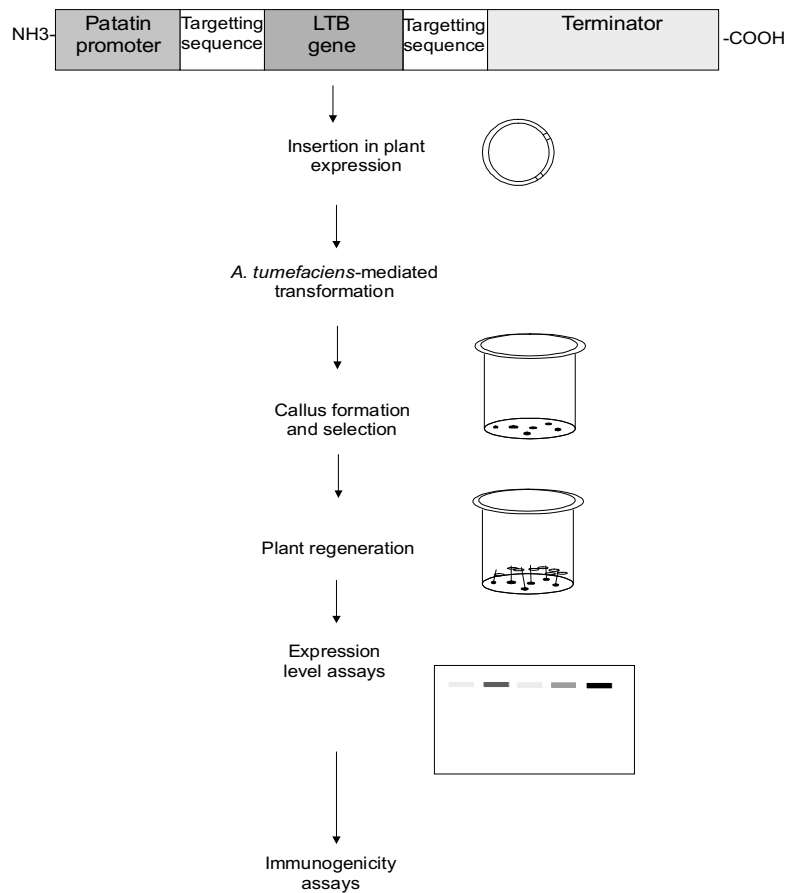
Figure 1.1

Construction of an edible vaccine using *Agrobacterium tumefaciens* mediated transformation.
Gene transfer into a plant genome is mediated by the plant-infecting bacterium *A. tumefaciens*. This bacterium contains a low copy number of a tumour inducing (Ti) plasmid. The Ti-plasmid transfers part of its DNA (T-DNA) stabile into the plant genome via a wounded plant cell and the T-DNA. Callus that is formed is allowed to grow out into mature plants. The LBT-gene is expressed under control of the tuber-specific promoter patatin. After tuber formation, LTB is expressed and transported to the endoplasmatic reticulum.

secretory component [58,59]. Other research groups reported the correct expression of pentameric LTB or CTB with GM1 binding activity [60-62].

Choice of plant species and plant part

The ideal edible vaccine should have high protein contents with high expression levels of the recombinant protein. It should grow fast and should be easy to multiply for bulk production. Finally, the ideal edible vaccine should not be toxic when given at the required amounts and should be edible as raw, uncooked food. At this moment, the list of food plants that have been transformed is rather long (Table 1.1, 1.2, and 1.3) and several plants have been tested already for oral immunisation. From this list the most suitable plant can be chosen from this list.

Bananas are regarded to be ideal vaccine plants as they do not require cooking and can be grown in developing countries. Growth, however, is slow and the fruit decays quite rapidly after ripening. Tomatoes grow faster and can be cultivated throughout the world, but they too decay rapidly. Drying might preserve these foods and overcome decay [3], but may also alter the vaccine or its bioavailability. Other plausible plants are maize, carrots, peanuts, rice, wheat, and soybeans [63].

With the availability of in-depth knowledge on the molecular biology of potatoes and its transformation possibilities within our research group, we decided to use this plant as model. Potato plants can be propagated rapidly from tubers, produced easily and at large scale, and can be stored for long periods without special precautions [3]. The use of potatoes has also several drawbacks. Consumption of raw potatoes might give complications and cooking may denature the antigens. Being a member of the family of solanaceae, they contain several toxic glycoalkaloids of which the highest levels are found in the foliage, blossoms and sprouts, followed by the peel and the tuber flesh.

Aim and outline of this thesis

The goal of the present thesis is to determine the feasibility of edible vaccines for oral immunisation. Can edible vaccines provide immunological protection? What immunisation protocol is most suitable for edible vaccines? Is LTB a suitable adjuvant for edible vaccines? What are the possibilities of potatoes as vaccine-production system? For this purpose, the research was focussed on a model with LTB produced in potato tubers and tested in mice. Protection was determined by measuring systemic and local antibody responses.

Table 1.1 Vaccine proteins

Plant expression system	Source of the protein	Protein/ peptide expressed	Transformation method	Maximum expression level *in planta*	Integrity of the protein	Tested species	Immunogenicity and protective capacity	References
Alfalfa	Footh-and-mouth-disease virus	VP1	tobacco mosaic virus	not known		mice	immunogenic and protective upon injection and oral administration	Wigdorovitz, 1999
Arabidopsis	Footh-and-mouth-disease virus	VP1	not mentioned	not known		??	immunogenic and protective upon injection	Carillo, 1998
Arabidopsis	Transmissible gastro-enteritis coronavirus	Glycoprotein S	not mentioned	0.06% TSP		??	immunogenic upon injection	Gomez, 1998
Lettuce	Hepatitis B virus	surface protein	*Agrobacterium*	< 0.01% FW		mice	orally immunogenic	Kapusta, 1999
Lupin	Hepatitis B virus	surface protein	*Agrobacterium*	< 0.01% FW		mice	orally immunogenic	Kapusta, 1999
Maize	*E. coli*	LTB	not mentioned	not known		mice	orally immunogenic and protective	Streatfield, 2000
Maize	Transmissible gastro-enteritis coronavirus	Glycoprotein S	not mentioned	< 0.01% FW		??	orally protective	Streatfield, 2000
Potato	*E. coli*	LTB	*Agrobacterium*	0.19% TSP	GM1-binding multimers	mice, human	orally immunogenic and protective	Haq, 1995; Mason 1998; Tacket, 1998
Potato	*Vibrio cholerae*	CTB	*Agrobacterium*	0.30% TSP	GM1-binding	??	orally immunogenic and protective	Arakawa, 1997; Arakawa, 1998
Potato	Hepatitis B virus	surface protein	*Agrobacterium*	< 0.01% FW		mice	orally immunogenic	Richter, 2001
Potato	Norwalk virus	capsid protein	*Agrobacterium*	0.37% TSP	VLP form	mice	orally immunogenic	Mason, 1995
Potato	Rabbit hemorrhagic disease virus	VP60	*Agrobacterium*	0.30% TSP		rabbit	immunogenic and protective upon injection	Streatfield, 2001
Tobacco	*E. coli*	LTB	chloroplast	< 0.01% FW	multimers	mice	orally immunogenic	Haq, 1995
Tobacco	Hepatitis B virus	Surface protein	chloroplast	< 0.01% FW	VLP form	mice	immunogenic upon injection	Mason, 1992; Thanavala, 1995
Tobacco	Norwalk virus	capsid protein	not mentioned	0.23% TSP	intact protein and VLP form	mice	orally immunogenic	Mason, 1996
Tobacco	Rabies virus	Glycoprotein	not mentioned	1% TSP	intact protein	??	not known	Streatfield, 2001
Tobacco	Transmissible gastro-enteritis coronavirus	Glycoprotein S	not mentioned	0.20% TSP	intact protein	>>	immunogenic upon injection	Streatfield, 2001

Transformed plants that produce vaccine proteins
(TSP – total soluble protein; FW = fresh weight)

The first part of the thesis describes the efforts to optimise the immunisation protocol for transgenic potatoes, and addresses the following questions: can the immune response be increased by the immunisation schedule? (Chapter 2); can the immune response be increased by modifying the immune status of the host? (Chapter 3); and is the optimised immunisation strategy suitable for an antigen produced in potato tuber? (Chapter 4). Chapter 5 describes the efforts to explore whether LTB-fusion proteins can be produced in potato tubers and whether LTB was a suitable adjuvant in edible vaccines. The final chapters of this thesis discuss the feasibility of edible vaccines. Are the speculations about edible vaccines justified? (Chapter 6) and what are the consequences of feeding a vaccine and are potato tubers suitable vaccine delivery systems?(Chapter 7).

Table 1.2 Biopharmaceuticals and plantibodies

Plant expression system	Application
Arapdopsis	Human enkephalins
Alfalfa	Plantibodies
Canola	Human hirudin
Maize	Human aprotinin
Potato	Human lactoferrin
Rice	Plantibodies; human interferon-α; human-α-1-antitrypsin
Soybean	Plantibodies; human protein C, - somatropin, -erythropoietin, -epidermal growth factor, -interferon-β, -serum albumin, -hemoglobin α/β, -homotrimeric collagen
Tobacco	Angiotensins-converting enzyme, glucocerebrosidase
Tomato	Angiotensins-converting enzyme
Wheat	Plantibodies

Transformed plants that produce biopharmaceuticals and plantibodies (adapted from Streatfield, 2001).

Table 1.3   Various transformed plants

Miscellaneous plant expression systems	
Asparagus	Papaya
Banana	Pea
Barley	Peanut
Cabbage	Pepper
Cantaloupe	Plum
Carrot	Raspberry
Cauliflower	Serviceberry
Cranberry	Squash
Cucumber	Strawberry
Eggplant	Sugar beet
Flax	Sugarcane
Grape	Sunflower
Kiwi	Sweet potato
melon	Walnut

Transformed plants with unknown transformation products
(adapted from Richter, 1999)

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Chapter 2

A multiple dose immunisation protocol suitable for edible vaccines

Tosca Lauterslager and Luuk Hilgers

Chapter 2

Summary

Frequent administration of oral immunisation has proven to be more successful than single administration. The frequency of feeding edible vaccines, however, is limited by the maximal oral intake, the lack of nutritional value and the possible presence of toxic ingredients. Therefore, we designed a protocol in which the animals received multiple immunisations on three alternating days ('triple dose') and the protocol was compared to single immunisations. Mice were immunised via intragastric (IG) gavage with ovalbumin (OVA) mixed with cholera toxin (CT) and the effects on systemical and local immune responses were determined. Serum IgG1 and IgA titres against OVA after oral boost immunisation given three weeks after primary immunisation were significantly higher after 'triple dose' than after 'single dose' immunisation. Faecal IgA was detected only after 'triple dose' boost immunisation. A second boost did not further increase serum IgG1 and faecal IgA. Antibody responses against CT were also elicited and again, boost immunisations did not further increase this response. We concluded that oral immunisation with multiple doses was more effective than 'single dose' immunisation and it seems practical and efficient for edible vaccines.

Introduction

Several factors affect the immune response upon oral administration of antigen and a few can be manipulated [1]. General complicating factors are degradation of the antigen in the gastro-intestinal tract and the induction of a state of oral tolerance [2,3]. Furthermore, the nature of the antigen strongly determines the outcome of oral immunisation. Oral administration of live pathogens revealed in many cases significant mucosal and systemic immune responses [4]. Oral immunisation with non-living pathogens, subunits or peptides, however, is often inefficient and requires multiple administrations with large amounts of antigen and adjuvant [5]. Another important factor is the immunisation schedule. Chalacombe [6] found that a weekly immunisation did not result in significant responses. Serial immunisations on consecutive days, however, induced sIgA. These and other data suggested that frequency and timing of immunisation are important.

Detailed study on differences between single dose immunisation and multiple dose immunisation on the development of IgA and IgG1 antibodies and their course in time after priming and booster immunisation are not described yet. The goal of the present study was to establish an effective oral immunisation protocol applicable for

edible vaccines for which the frequency of feeding is limited by the maximal possible oral intake.

Materials and methods

Mice

Swiss female mice (6 to 8 weeks old) were obtained from Charles River (Sulzfield, Germany) and housed per groups under conventional conditions. All mice were raised and kept on an OVA free diet. All animal experiments were held under auspices of the ID-Lelystad B.V. Animal Experimentation Committee according to the Dutch Law on Animal Experimentation.

Antigen preparation and immunisation

The antigen preparation tested consisted of 10 mg of ovalbumin (OVA; Grade V, A-5503, Sigma) mixed with 5 μg cholera toxin (CT; C-8052, Sigma) dissolved in 0.4 ml saline. Mice fasted overnight (water was provided *ad libitum*) and were immunised orally
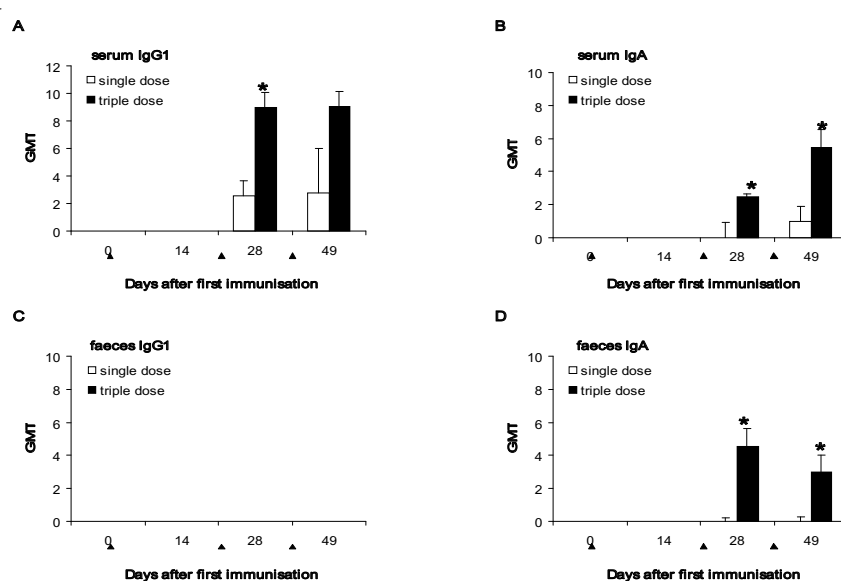
Figure 2.1
Anti-OVA antibody titres after priming and subsequent boost immunisations with 10 mg OVA and 5 μg CT. Arrowheads mark days of immunisation. An asterisk marks statistically significant differences between 'single' and 'triple dose' oral immunisations. The data represent GMTs and SEMs of serum IgG1 (A), serum IgA (B), faeces IgG1 (C) and faeces IgA (D).

by intragastric intubation with OVA plus CT on day 0, 21, and 42 ('single dose') or on day 0, 2, 4, 21, 23, 25, 42, 44, and 46.

Collection of faeces and serum samples

Pre-immune tail blood serum and faeces samples were collected before the first immunisation and on day 14, 35, and 49. Fresh faeces pellets were immediately frozen at –20 °C. Before testing, faeces pellets were treated as described elsewhere to prevent degradation [7].

Detection of anti-OVA and anti-CT antibodies

High binding ELISA plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 100 μg ml^{-1} OVA or 2 μg ml^{-1} CT dissolved in PBS. ELISA was performed as described earlier [7]. 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 and standard error of the mean (SEM) 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. Extinctions below detection limit were considered to have a GMT of -10.

Results

Antibody responses against OVA

Neither 'single dose' nor 'triple dose' oral priming with OVA plus CT resulted in detectable anti-OVA immune responses in serum or faeces (Fig. 2.1). 'Single dose' oral boost immunisations induced anti-OVA IgG1 and IgA titres in serum, and serum IgG1 was significantly higher compared to pre-immune serum on day 28. 'Triple dose' oral boost immunisations induced significantly higher antibody titres in serum (IgG1 and IgA) and in faeces (IgA) compared to pre-immune titres. Second boost immunisations administered on day 42, or at day 42, 44, and 46, further increased serum IgG1 but not serum IgA or faecal IgA, but only after 'triple dose' boost immunisations. Antibody titres were significantly higher (IgG1 on day 28 and IgA on day 49) and in faeces (IgA on day 28) after 'triple dose' immunisation.

The number of responder mice and non-responder mice on day 49 using each immunisation protocol is represented in Table 2.1. Mice were considered to be responding when the GMT titre was at least 1. In serum, 4 out of 4 and 5 out of 5 'triple dose' immunised mice had increased IgG1 and IgA responses, respectively, while 3 out of 5 mice responded with faecal IgA. After 'single dose' immunisation, 4 out of

5 mice had positive serum IgG1 and 3 out of 5 had positive serum IgA responses. None responded with faecal IgA. Also, the GMTs on day 49 of all mice and of only the responding mice are represented in this table. Significantly higher GMTs after 'triple dose' immunisation compared to 'single dose' immunisation are indicated with an [a]. Non-responding 'single dose' immunised mice were not responsible for the differences between the two immunisation protocols.

Antibody responses against CT

Both 'single dose' and 'triple dose' oral priming with OVA plus CT resulted in anti-CT IgG1 in serum (Fig. 2.2), but serum IgA was observed only after 'triple dose' priming. Antibody titres were significantly higher after 'triple dose' prime immunisation. Boost immunisations did not further increase serum IgG1 or IgA and no differences between the immunisation protocols were found after the boost immunisation. Antibody responses in faeces were not determined.

Discussion

After oral immunisation with OVA plus CT, OVA-specific IgG1 and IgA could be measured in serum, and 'triple dose' immunisation revealed significantly higher antibody titres than 'single dose' immunisation, but differences were not always significant (Fig. 2.1). CT was necessary as adjuvant as OVA without CT did not evoke detectable
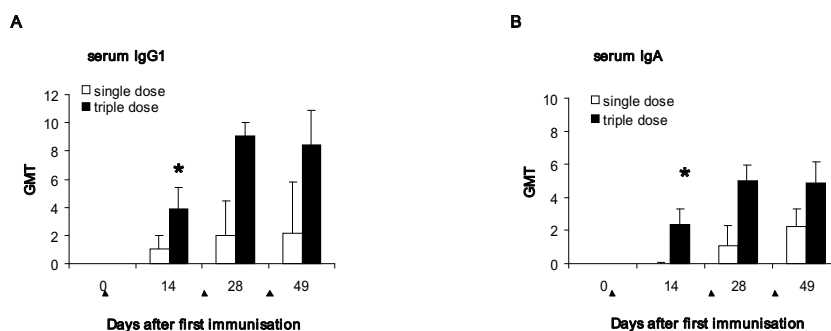

Figure 2.2
Anti-CT antibody titres after priming and subsequent boost immunisations with 10 mg OVA and 5 μg CT. Arrowheads mark days of immunisation. An asterisk marks statistically significant differences between 'single' and 'triple dose' oral immunisations. The data represent GMTs and SEMs of serum IgG1 (A) and serum IgA (B).

Table 2. 1 Responding and non-responding animals on day 49

	immunisation protocol	# of responding mice / # of tested mice	all tested mice			responding mice		
			n	GMT	SEM	n	GMT	SEM
serum IgG1	single dose	4/5	5	2.8	3.2	4	6.0	0.6
	triple dose	4/4	4	9.0	1.1	4	9.0	1.1
serum IgA	single dose	3/5	5	1.0	0.9	3	2.2	0.5
	triple dose	5/5	5	5.5	1.1	5	5.5[a]	1.1
faeces IgA	single dose	0/5	5	-1.0	1.3	0		
	triple dose	3/5	5	3.0[a]	1.1	3	4.6[a]	0.8

The number of responding mice of the total number of tested animals per group is given. Mice were considered to be responding when the antibody titers was > 1. The GMTs and SEMs of the tested mice (responding and non-responding mice) and of the responding mice are represented. Significantly differences between 'triple dose' and 'single dose' immunisations are indicated with an [a].

responses (data not shown). Both 'triple dose' and 'single dose' immunisation induced antibodies to CT, but differences between the protocols were only observed after prime immunisation.

'Triple dose' immunisation was able to induce systemic as well as local antigen-specific antibody responses, whereas 'single dose' immunisation only raised detectable antibodies in serum. In faeces, OVA-specific IgA but not IgG1 was induced. IgA was the predominantly produced immunoglobulin by mucosal tissues. Thus, we expected to find IgA and not IgG1 in faeces. Our results were confirmed by ELISPOT, in which we observed more IgA than IgG1 antibody secreting cells in the lamina propria [unpublished results]. But strong mucosal immunogens like CT and CTB are indeed able to elicit local IgG1 responses [8].

Primary 'triple dose' immunisation was sufficient to induce anti-CT but not anti-OVA antibodies in serum and faeces. Boost immunisations were necessary to induce detectable antibody titres. Second boost immunisations further increased anti-OVA serum IgA titres, but did not further increase serum IgG1 or faeces IgA titres, suggesting that these latter reached a plateau level. Anti-CT titres already reached a plateau after priming. The observation of a plateau suggested that a secondary reaction of the immune system towards boost immunisation with an antigen does not occur. This raised the question if memory is indeed induced in the mucosal immune system. Cebra reported that cells in the germinal centres of Peyers Patches (PP) are transient and that successful secretory IgA responses attenuated the stimulation by secondary mucosal challenge [9]. This might explain why traditional boost responses were not induced after oral immunisation. Our findings indicated that memory cells were formed after 'triple dose' priming, but that the extent of memory triggering was different for each antibody isotype, each compartment of the immune system, and the antigen used. CT is more immunogenic upon oral immunisation compared to OVA and this might explain why differences between 'single -' and triple dose' disappeared after boost immunisations.

Oral boost immunisations were given three and six weeks post-priming. Seven days after the last booster, all 'triple dose' immunised mice responded with anti-OVA IgG1 and IgA titres in serum, while after 'single dose' immunisation, few animals remain non-responding. In faeces, 3 out of 5 of all 'triple dose' immunised mice responded, while in the 'single dose' immunised group no mice responded. The non-responding animals were not responsible for the significant differences between 'single dose' and 'triple dose' immunisation (Table 2.1). Thus, the 'triple dose' immunisation protocol did not only increase the mean antibody titre, but also increased the number of responding animals.

Chapter 2

The 'triple dose' oral immunisation protocol as presented in this paper, resulted in higher antigen-specific antibody titres against non-live antigens than to 'single dose' immunisation, most probably due to the extended exposure of the antigen to the mucosal immune system. A major problem in oral immunisation is the degradation of antigen by the gastrointestinal tract, and prolonged exposure of antigen might give the mucosal immune system more time to respond. Frequent oral administration can enhance the efficacy of edible vaccines. The frequency of feeding edible vaccines, however, is limited by the maximal oral intake, the lack of nutritional value and the possible presence of toxic ingredients. The here proposed 'triple dose' protocol was developed for the use with transgenic potatoes as edible vaccines [7]. Since potato-produced vaccines contain less nutrition than standard food and a fasting period is involved, immunisation on alternating days provides mice 24 hours to recover from immunisation at the disposition of standard food.

In the study presented here, the antigen dose of each immunisation was equal, which meant that the 'triple dose' treated mice received a three times higher priming dose than 'single dose' immunised mice. Future studies must determine whether the antigen dose can be divided over the three immunisations days to diminish the risk of antigen-overdose, like toxicity.

'Triple dose' oral immunisation has been proved to be effective in inducing systemical and mucosal immune responses and can be applied in feasibility studies with edible vaccines and to gain more insights in the various aspects of oral immunisation.

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Chapter 2

Chapter 3

Improvement of the systemic prime/ oral boost stragegy for systemic and local responses

Tosca Lauterslager, Wil Stok and Luuk Hilgers

Chapter 3

Summary

This paper describes oral boost immunisations of primed animals as an alternative oral vaccination strategy. Mice were primed orally (PO), intranasally (IN), subcutaneously (SC), or intraperitoneally (IP) with ovalbumin (OVA) with or without adjuvant. Boost immunisations were given orally with or without cholera toxin (CT) as adjuvant. Prime immunisations induced variable IgA and IgG_1 titres in serum depending on the route. A subsequent oral boost increased these titres. Use of an adjuvant in the priming significantly increased serum IgA and, to a lesser extend, IgG_1. Oral boost immunisation induced significantly higher serum IgA titres in animals primed via the SC, IP and the IN route compared to the PO route. This was independent of the use of CT. Three oral boosts with OVA plus 5 μg CT given in five days to primed mice revealed higher IgA titres compared to single oral boosts and anti-OVA IgA titres in faeces were also detected. Finally, we put together our findings and propose a systemic priming/oral boost strategy in which mice were primed via the SC route with 100 μg OVA plus 50 μg Butyl16-p(AA), and subsequently orally boosted with three doses of 300 μg OVA plus 5 μg CT each.

We concluded that oral immunisation is more effective in IN, SC, or IP primed mice than in PO primed mice, and that the IgA antibody response in serum and faeces can be improved by increasing the immunisation frequency and the use of appropriate adjuvants in primary and boost immunisation. The here-formulated strategy improves the probability of success of oral vaccination. The results are discussed in the light of the development of edible vaccines.

Introduction

Oral vaccination is an attractive but not very efficient way to induce immunity. Despite considerable effort, only few alive oral vaccines are commercially available at this moment. Several studies demonstrated that oral vaccination requires multiple administrations of high doses of antigen, which increases the production costs. Furthermore, oral intake of antigen tends to establish a state of immunotolerance rather than immunity. Obviously, the primary function of the gastro-intestinal tract is not to develop immunological reactions to the various food ingredients entering this organ. In order to obtain significant immune responses, appropriate adjuvants or antigen-delivery systems are used for oral immunisation [1,2]. The co-administration of antigens with bacterial toxins, such as cholera toxin (CT) or heat-labile enterotoxin of *Escherichia coli* (LT) improves the immune response. To deliver non-living antigens to the mucosal immune system, conjugates of antigen and B-subunits of CT or LT, or antigens incorporation into microparticles were used [3-5]. Despite these approaches, oral vaccination with non-living antigen still induces insufficient levels and duration of immunity [1,2,6,7].

Improvement of the systemic prime/oral boost strategy

To improve the efficacy of oral vaccines and to reduce the risk of immunotolerance, we examined the potentials of oral vaccination in mice primed previously by one of the systemic routes. Several authors have shown previously that oral boost immunisation of primed animals is effective in stimulating local and systemic responses. This systemic prime/oral boost strategy was first reported by Pierce and co-workers in 1977 and proved to enhance the enteric immune response to non-replication antigens (CT) and provided long-lasting protection against a subsequent challenge in dogs [8]. The consequences of this immunisation strategy on local and systemic immune responses have been studied further in mice using living antigen [9], OVA [10] and recently using hepatitis B surface antigen [11]. Furthermore, this immunisation strategy has also been applied to birds using replicating antigens [12,13]. In our study, we refined this oral boost concept. Priming conditions such as the type of adjuvant used, route of systemic immunisation, number of immunisations, and dose of antigen were studied. Finally, we present a systemic prime/oral boost strategy with improved probability of successful oral vaccination with non-living antigens.

Materials and methods

Vaccine and adjuvant preparation

Ovalbumin (OVA; A-5503, Grade V, Sigma) was dissolved in saline and mixed with or without one of the following adjuvants:

rCTB: recombinant CTB [10].

Specol: a water-in-mineral oil-emulsion [10,14].

S/W: squalane-in-water emulsion containing 80 g l^{-1} squalane and 20 g l^{-1} Tween 80 [15,16].

SE/S/W: sucrose ester-in-water emulsion containing 8 g l^{-1} sucrose poly fatty acid, 80 g l^{-1} squalane (Merck, Germany), and 20 g l^{-1} Tween 80 (ICI) in phosphate buffered saline (Covaccine BV, The Netherlands).

SL-CD/S/W: sulpholipo-cyclodextrin in a squalane-in-water containing 80 g l^{-1} squalane, 20 g l^{-1} Tween 80, and 8 g l^{-1} SL-CD (Fort Dodge Animal Health, The Netherlands) [16,17].

Butyl16-p(AAA): butyl alkyl-polyacrylate with an esterification grade of 16% (Butyl16-p(AA)) (Fort Dodge Animal Health, The Netherlands) [18].

CT: cholera toxin (CT; C-8052, Sigma).

Animals

Swiss female mice (6 to 8 weeks old) were obtained from Charles River (Sulzfield, Germany) and housed per groups under conventional conditions. All animals were

raised and kept on an OVA free diet. Experimental groups consisted of five to six animals. All animal experiments were held under auspices of the ID-Lelystad BV Animal Experimentation Committee according to the Dutch Law on Animal Experimentation.

Immunisation and sample collection

All oral immunisations (per orally, (PO)) were preceded by overnight fasting of mice (water was provided *ad libitum*) and administered by intragastric intubation of 0.4 ml or 0.5 ml vaccine. Subcutaneous (SC) and intraperitoneal (IP) immunisations were administered in a total volume of 0.1 ml. For intranasal (IN) immunisation, the animals were anaesthetised according to standard methods and 10 μl of vaccine were applied to each nostril.

Experiment 1: Groups of six mice were immunised by the SC or IP route with 100 μg OVA mixed with or without adjuvant. Three or four weeks later, on day 0, mice were given PO boost immunisations of 10,000 μg OVA in 0.5 ml saline. Tail blood serum samples were collected at several time intervals.

Experiment 2: Groups of five mice were immunised by the PO route with 10,000 μg OVA mixed with 5 μg CT in 0.4 ml saline, or by the IN route with 10,000 μg OVA mixed with 5 μg CT in the appropriate volume. Others were immunised by the SC or IP route with 100 μg OVA and 50 μg Butyl16-p(AA) in the appropriate volume. Three weeks later, on Day 0, all mice were given a PO boost with 10,000 μg OVA mixed with 5 μg CT. Boost immunisations were administered according to a 'single dose' schedule (day 21) or a 'triple dose' schedule (day 21, 23, and 25) [19]. Tail blood serum and faeces samples were collected at several time intervals. Fresh faeces pellets were collected and immediately frozen at –20 °C. To prevent degradation by proteases, faeces samples were pre-treated as described earlier [20].

Experiment 3: Groups of five mice were immunised by the SC route with 100 μg OVA and 50 μg Butyl16-p(AA). Three weeks later, on day 0, mice were given a 'triple dose' PO boost with various doses of OVA mixed with 5 μg CT. The doses tested were: 0.01, 0.3, 10, 300, and 10,000 μg. Tail blood serum and faeces samples were collected at several time intervals and processed as described above.

Detection of anti-OVA antibodies

High binding ELISA plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 100 μg ml^{-1} OVA (Sigma) dissolved in PBS. 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 [20].

Statistical analysis

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

Effects of systemic priming with different adjuvants on the IgG$_1$ antibody response in serum after oral boost

Groups of mice were primed by the IP or SC route and three or four weeks later, on day 0, they were boosted by the PO route. In parallel, groups of naïve mice were immunised solely by the PO route on day 0. PO boost immunisation of primed but
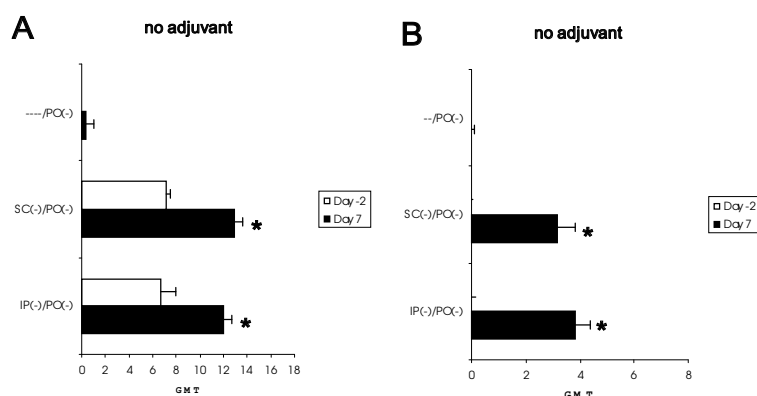

Figure 3.1

Serum IgG$_1$ and IgA after prime and oral boost immunisation without adjuvant.One group of six mice only received an oral boost immunisation (---/PO(-)), other were SC or IP primed. Serum samples were tested undiluted. The data represent IgG$_1$ (A) and IgA (B) in serum after priming (day –2, open bars) and oral boost (day 7, filled bars). The data are shown as GMTs and SD. Statistically significant differences between naïve and primed animals on day 7 are indicated by an asterisk (P < 0.05).

not naïve mice resulted in high antigen-specific IgG$_1$ responses. Without the use of an adjuvant for priming, the PO boost increased the serum GMT 5-fold (Fig. 3.1A).

Systemic priming with adjuvant considerably increased the pre-boost IgG$_1$ titre on day –2 (Fig. 3.2A), except with rCTB and S/W. The titre at 7 days post-boost was considerably increased by PO boosts without adjuvant compared to non-boosted animals, except after SC priming with Specol.
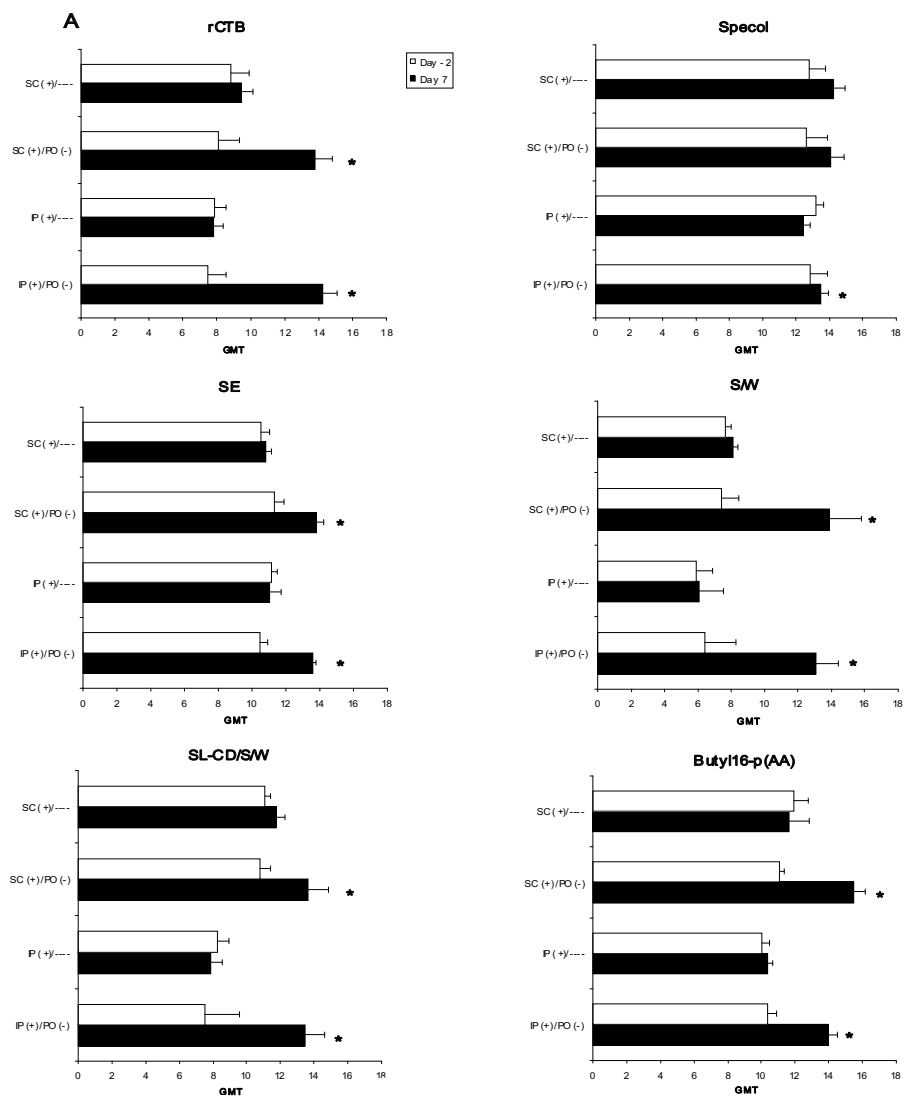
Figure 3.2
Serum IgG$_1$ and IgA after priming and oral boost with adjuvants.
The adjuvants rCTB, Specol, Butyl16-p(AA), SE/S/W, S/W, or SL-CD/S/W were used for the parenteral prime immunisation. Groups of six mice were primed only (SC(+)/-- or IP(+)/--), or primed and boosted (SC(+)/PO(-) or IP(+)/ PO(-)). Serum samples were tested undiluted. The data represent IgG$_1$ (A) and IgA (B) in serum after priming (day −2, open bars) and oral boost (day 7, filled bars). The data are represented as GMTs and SD. Statistically significant differences between antibody titres on day −2 and day 7 are indicated by an asterisk (P < 0.05).
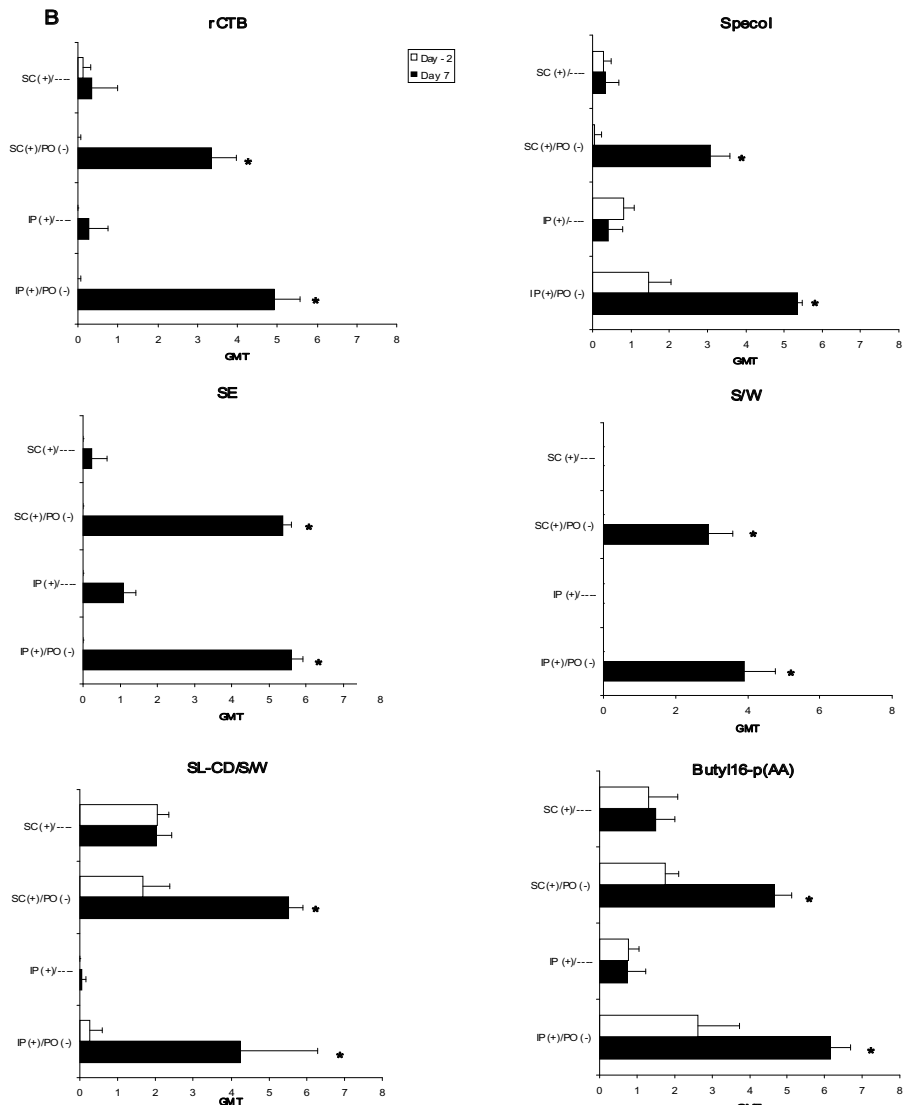
No significant difference between the SC and IP route was observed, except with SL-CD/S/W, which was more effective after SC administration.

The effect of the PO boost was most pronounced in animals with relatively low pre-boost titres. The serum titres at 7 days post-boost were significantly higher in animals primed via the SC or IP route with rCTB, SE/S/W or Butyl16-p(AA), via the SC route with Specol, and via the IP route with SL-CD/S/W.

Effects of systemic priming with different adjuvants on the IgA antibody response in serum after oral boost

PO boost immunisation of primed but not naïve mice resulted in detectable antigen-specific IgA immune responses in serum (Fig. 3.1B). In a few situations, the addition of an adjuvant to the systemic priming increased the pre-boost IgA titre on day -2 (Fig. 3.2B). Priming via the SC route with SL-CD/S/W or Butyl16-p(AA), or via the IP route with Specol resulted in significantly increased IgA titres in serum.

PO boosts significantly increased serum IgA titres after priming via the SC or IP route with SE/S/W or Butyl16-p(AA), via the IP route with rCTB or Specol, and via the SC route with SL-CD/S/W. No difference between the SC and IP route was observed, except for Specol, which gave significantly higher IgA titres in serum after IP priming.

Effects of priming route on the antibody response in primed mice

In another experiment, we examined which prime/boost immunisation protocol resulted in optimal antibody responses. Mice were primed either systemically by the SC or IP route or mucosally by the IN or PO route on day -21. Butyl16-p(AA) was selected as adjuvant for systemic priming as it induced significantly enhanced serum IgG_1 and IgA titres after PO boost immunisation. To maximise the mucosal immune response, 5 µg CT was used as adjuvant for the IN and PO immunisations. Three weeks later, on day 0, PO boosts were given together with 5 µg CT as adjuvant.

The SC, IP, and IN routes were compared to the PO route in their priming efficiency for an subsequent PO boost immunisation. Pre-boost IgG_1 titres in serum were similar after priming by either route (Fig. 3.3A). Pre-boost IgA titres were significantly higher after priming via the SC or IN route than after the PO route (Fig. 3.3B). Post-boost serum IgG_1 titres on day 7, post-boost serum IgG_1 titres were significantly higher after IN priming than after PO priming. Post-boost serum IgA titres were significantly higher after SC, IP or IN priming than after PO priming. SC priming resulted in significant higher post-boost IgA titres than IP priming. No or very low IgG_1 or IgA responses were detected in faeces (data not shown).

Oral boost of primed mice using a 'triple dose' immunisation schedule

Previously, we demonstrated that three immunisations given in a period of five days was more effective than a single dose[19]. In order to improve the efficacy of the oral

boost immunisation, this so-called 'triple dose' immunisation schedule was applied to the systemic priming/oral boost protocol. According to this schedule, PO boosts were administered on three alternating days, i.e. on day 0, 2, and 4. 5 μg CT was used as adjuvant for the PO immunisations. Compared to a single dose, triple dose PO boosts enhanced significantly post-boost serum IgG_1 titres on day 7 in mice primed by the SC but not by the PO or IP route. IgA titres were enhanced in all groups boosted with triple doses (Table 3.1). In faeces, triple dose PO boosts enhanced significantly the IgA response in all mice, and the IgG_1 response in animals primed by the SC but not by the IP or PO route.

Oral boost with graded doses of OVA

So far, the systemic priming/oral boost protocol consists of SC priming with 100 μg OVA mixed with 50 μg Butyl16-p(AA) followed by a triple dose boost with 10 mg OVA mixed with 5 μg CT. To determine the minimal antigen dose for the oral boost, we have boosted mice with graded doses of OVA. OVA was administered according to the triple dose schedule, at doses of 0.01 μg, 0.3 μg, 10 μg, 300 μg or 10,000 μg per administration mixed with 5 μg CT. Control mice were not immunised. In serum (Fig. 3.4A), significant anti-OVA IgG_1 and IgA titres were induced with 300 μg and 10,000 μg OVA. Antibody responses in faeces were low, and titres were only significant with the two highest doses (Fig. 3.4B).

Discussion

The oral vaccines presently available consist of alive microorganisms. Despite considerable research efforts, the use of non-living antigens has not resulted in effective products. Oral administration of non-living antigens can elicit detectable immune responses, but these are often low. Apparently, exposure of the immune system of the gastro-intestinal tract to non-living antigens is not a very efficient way to trigger responses. Here, we examined the efficacy of oral administration of a non-replicating antigen in systemically (parenterally) primed and naïve animals. The effects of adjuvants used for systemic priming, the route of systemic priming, the immunisation schedule, and the dose of antigen on systemic and local antibody titres have been studied.

Repeated oral immunisation with OVA without adjuvant was not strong enough to induce detectable immune responses in serum. When oral immunisation was preceded by systemic immunisation, significant serum responses were detected. IgA was observed only after the oral boost immunisation. The oral booster increased the IgG1 that was elicited by the priming. We concluded that systemic administration of antigen primes the host for a subsequent contact of antigen with the immune system associated with the gastro-intestinal tract. This is in agreement with earlier reports demonstrating the
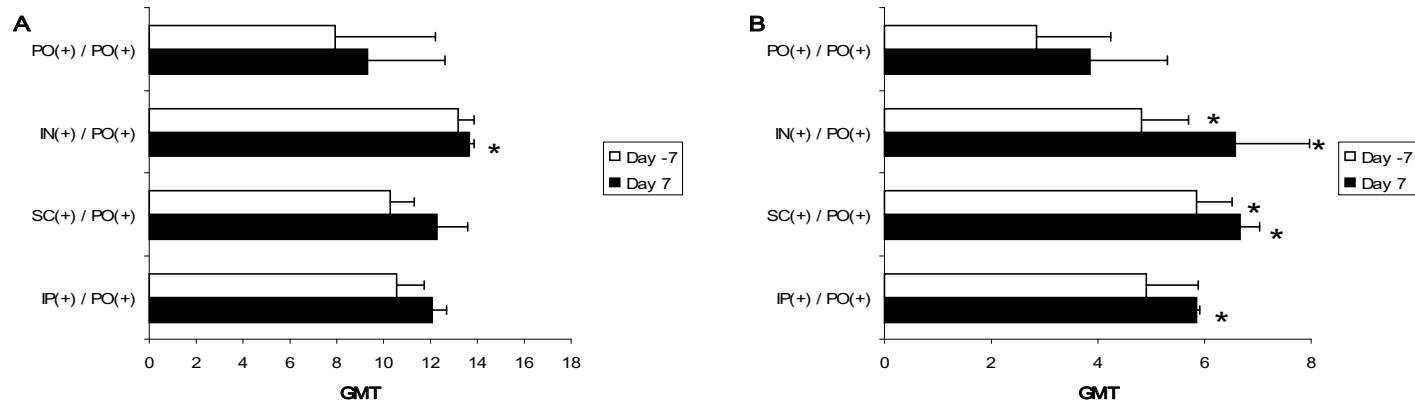
Figure 3.3

Serum IgG$_1$ and IgA after priming via different routes and oral boost immunisation.

Prime immunisations were administered via different parenteral (SC and IP) or mucosal routes (IN and PO) together with an adjuvant. Oral immunisations were given with 5 μg CT. Serum samples were 40 to 400 times diluted for IgG1 measurement and tested undiluted for IgA measurement. All faeces samples were tested undiluted.

The results represented are IgG$_1$ (A) and IgA (B) in serum after priming (day −7, open bars) and oral boost (day 7, filled bars). The data are represented as GMTs ± SD. Statistically significantly higher antibody titres than in PO(+)/PO(+) primed mice are indicated by an asterisk (P < 0.05).

induction of both systemic and mucosal antibody by a combination of systemic and mucosal immunisation [2,8-10,12,13].

Six different adjuvants, which have been proven to be effective for systemic or mucosal responses or both, were selected for systemic priming to evaluate whether the immune response can be further increased. CT and CTB are well-known strong mucosal adjuvants [3]. Specol is a water-in-mineral oil emulsion with a strong activity towards a wide range of antigens. It is similar to Freund's Incomplete Adjuvant, reactogenic and persists at the site of injection [14,21]. S/W [15,16], SL-CD/S/W and SE/S/W are emulsions of squalane-in-water and have considerable adjuvant capacities and low reactogenicity. SE/S/W and SL-CD/S/W are powerful adjuvants with low reactogenicity upon systemic immunisation [16,17]. Butyl16-p(AA) is a water-soluble polymer and has been proved to be effective for both systemic and mucosal immunisation [17,18]. IN immunisation with Butyl16-p(AA) induced high IgA and IgG responses in the lungs and spleen. In general and as expected, the adjuvants enhanced pre-boost serum IgG_1 titres but did not induce detectable IgA responses. Depending on the type of adjuvant and route of priming, oral boosts induced IgA responses in serum and further increased the IgG_1 titre. S/W was the only adjuvant that did not enhance pre-boost IgA titres, but the combination of S/W with the synthetic sulpholipo-derivatives of cyclodextrin (SL-CD/S/W) or with sulpholipo-derivatives of sucrose (SE/S/W) enhanced significantly the immune response after SC administration. SE/S/W and Butyl16-p(AA) were the two adjuvants, which enhanced both IgG_1 and IgA titres in pre- and post-boost serum. Applied in oral vaccines, adjuvants might improve the persistence of the antigen in the gastro-intestinal tract, the targeting of the antigen to the immune system (e.g. by specific binding to epithelial cells or facilitating translocation across epithelial surface), or the molecular context of the antigen or might activate the immune system (e.g. cytokines) [4,22]. However, the exact modes of action of adjuvants are still poorly understood.

The effect of the oral booster was determined on day 7 post-boost. Previous experiments demonstrated that the post-boost antibody titre was maximal 7 days later and did not further increase on day 14 or on day 21 (data not shown). The effect of an oral boost on IgG_1 was evident with all adjuvants, except after IP priming with Specol. The increase in antibody titre was most distinct in animals with relatively low pre-boost IgG_1 titres and not, for example, in animals IP primed with Specol. In the latter cases, the systemic priming revealed high titres and the effect of the oral boost was overshadowed by that of the prime immunisation. Probably, some kind of plateau is reached by strong systemic priming. In addition, mucosal immunisation is not capable of boosting the immune response to high level, but rather to maintain antibody titres at a steady level [23]. Oral immunisation mostly induces T-helper type 2 responses, which was confirmed by our observations that only IgG1 and not IgG2a (data not shown) was induced. Remarkably, IgG2a was even not induced after systemic priming with an adjuvant (data not shown). As compared to IgG_1, the effects of an oral boost on IgA

Table 1: Effects of 'triple dose' oral immunisation

		serum				faeces			
		IgG1		IgA		IgG1		IgA	
priming	booster	Day -7	Day 7	Day -7	Day 7	Day -7	Day 7	Day -7	Day 7
PO(+)	PO(+)	7.9 ± 4.3	9.3 ± 3.3	2.9 ± 1.4	3.8 ± 1.5	< 1	< 1	0.3 ± 2.6	0.0 ± 1.2
SC(+)	PO(+)	10.3 ± 1.0	12.3 ± 1.3	5.9 ± 0.6	6.7 ± 0.3	< 1	< 1	< 1	< 1
IP(+)	PO(+)	10.6 ± 1.2	12.1 ± 0.6	4.9 ± 1.0	5.9 ± 0.1	< 1	< 1	< 1	< 1
3PO(+)	3PO(+)	9.0 ± 1.9	14.1 ± 0.1	3.3 ± 1.1	10.1 ± 1.3[a]	< 1	< 1	< 1	3.4 ± 0.4[a]
SC(+)	3PO(+)	11.5 ± 0.6	14.8 ± 1.9[a]	6.0 ± 0.8	9.9 ± 2.2[a]	< 1	1.8 ± 0.8[a]	< 1	6.0 ± 1.3[a]
IP(+)	3PO(+)	11.0 ± 2.1	15.2 ± 2.3	6.0 ± 1.4	9.0 ± 0.2[a]	< 1	0.1 ± 2.0	< 1	4.9 ± 1.5[a]

The data represent the mean 2-log titre ± SD in serum and faeces 7 days before and 7 days after the oral booster. Significantly higher titres after 'triple dose' immunisation compared to its 'single dose' counterpart
are indicated by an [a].

were more obvious and were found with all adjuvants. This was as expected, since systemic immunisation induces poor IgA responses and the booster immunisations were given via one of the mucosal routes. Because IgA is the most important
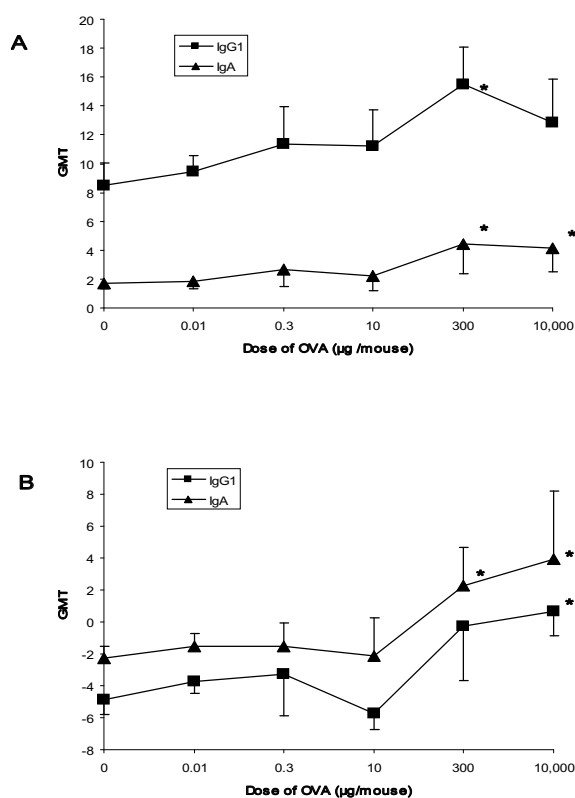
Figure 3.4
The relationship between dose of antigen and IgG_1 and IgA response.
Mice were primed SC with OVA in various doses and Butyl16-p(AA). 'Triple dose' oral boost immunisations were given with 5 µg CT. The data represent the post-boost antibody titres at day 7 in serum (A) and faeces (B) and are depicted against the antigen dose in milligrams per animal. IgG_1 is indicated by squares and IgA by triangles. Statistically significant anti-OVA titres above the background are indicated by an asterisk ($P < 0.05$).

immunoglobulin produced by the mucosal immune system, we focussed our study on this antibody.

Next to the effects of adjuvants, we studied the effects of the route of priming. In order to maximise the immune response, we decided to use Butyl16-p(AA) as adjuvant for the systemic immunisation in the further experiments and CT for the mucosal immunisation. Butyl16-p(AA) was able to stimulate both systemic and mucosal responses and CT is today's best-known adjuvant for oral immunisation. Systemic (SC and IP) and mucosal (IN) priming elicited higher post-boost IgG_1 and IgA antibody responses in serum than PO priming, indicating that solely oral immunisation is less effective than a combination of different routes. All animals were closely observed during immunisation. Nevertheless, swallow of the vaccine after IN immunisation could never be completely excluded. We decided to use the SC route for priming as it is more convenient that IP and better to control.

In contrast to other findings, no antibodies were found in faeces, despite the use of CT as mucosal adjuvant [3]. A single oral boost immunisation with OVA is probably not efficient enough to induce local responses. Manners to further improve mucosal responses in orally boosted animals were considered. Previously, we observed that three oral doses in five days revealed higher responses than single doses [19]. This so-called 'triple dose' immunisation schedule was applied to further increase the effect of the oral boost. As a consequence of this schedule, the antigen dose is three-fold higher but also the exposure of the antigen to the immune system is prolonged. Serum and faeces IgG_1 and IgA titres were significantly higher after 'triple dose' immunisation of naïve and primed mice than after 'single dose' immunisation. Augmentation of IgA by oral boost immunisation was detected in serum and faeces. We concluded that significant local responses can be induced by oral boost immunisation, provided that multiple doses are given, which is in agreement with observations by others [1]. Besides faeces, intestinal scrapings were tested and the number of antibody-secreting cells in the lungs or in the lamina propria of the small intestine were examined (data not shown). No responses could be detected in these samples. Obviously, these samples could only be obtained ate the end of the animal experiments which is may probably be not the optimal time point to measure mucosal immune responses.

The gastro-intestinal tract is originally not designed to react with an immune response towards orally delivered antigens and multiple oral administrations of antigen harbours the risk to establish of a state of immunotolerance [26]. The concept of systemic priming followed by oral boosts prevents the development of oral tolerance. Once the immune system has established an immunological memory to a certain antigen, tolerance is not induced by subsequent feeding of the antigen, not even after increasing the antigen dose and frequency of feeding [27]. Antigen-experienced T cells may be inherently resistant to induction of tolerance, because these cells are less dependent on co-stimulation for their activation than naïve T cells. Primed T cells localise in discrete anatomical

niches where they are capable to sustain their effector functions but are inaccessible for tolerogenic signals [28]. We only examined the humoral responses by determination of the number of antibody-secreting cells in the lamina propria of the small intestine. We did not study the number of T cells in serum or any other compartment of the (mucosal) immune system. These types of studies are of interest in order to elucidate the mechanisms underlying parenteral priming for oral booster immunisation.

The systemic priming/oral boost strategy is already known for several years [8-10,12,13,29] . We attempted to further optimise this concept. The highest systemic and local responses were found after SC priming with 100 μg OVA plus 50 μg Butyl16-p(AA) as adjuvant, and subsequently triple dose boosted with 300 μg OVA plus 5 μg CT. Our refined priming/boost strategy may contribute to the development of more successful oral vaccines.

Chapter 3

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Chapter 3

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*

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[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.
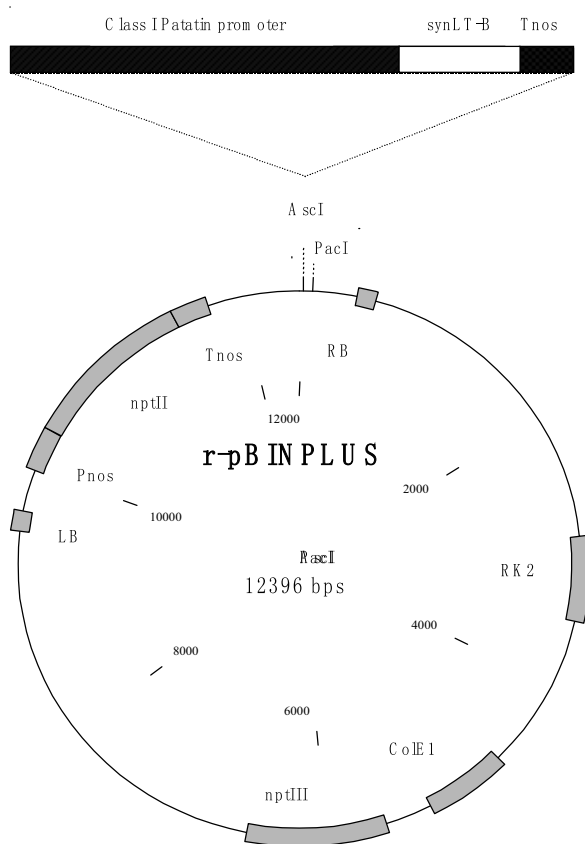
Figure 4.1
Schematic representation of the binary vector pLANTIGEN4 containing the synthetic gene for LT-B. RB, right T-DNA border sequence; Tnos, nopaline synthase terminator; SynLT-B, synthetic gene construct coding for LT-B; nptII, neomycin phosphotransferase II gene, plant selectable kanamycin resistance 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-dried. 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)).

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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.

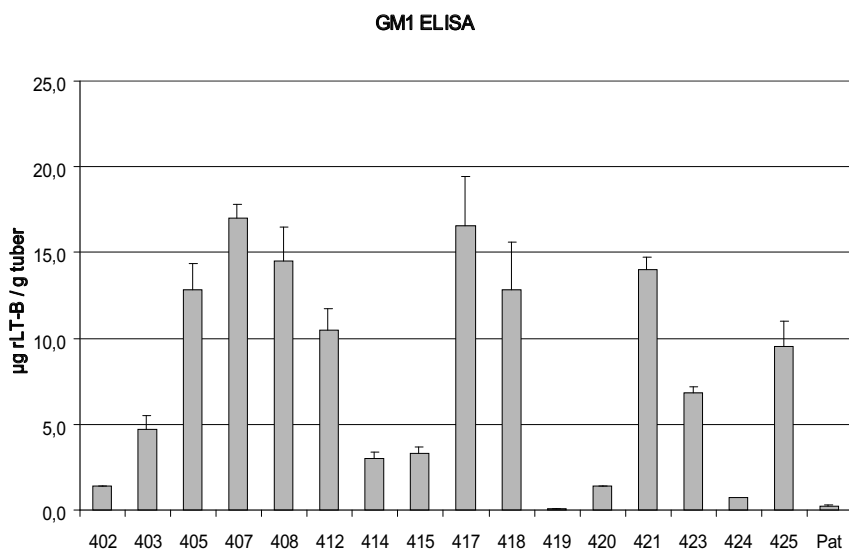**GM1 ELISA**

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.

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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
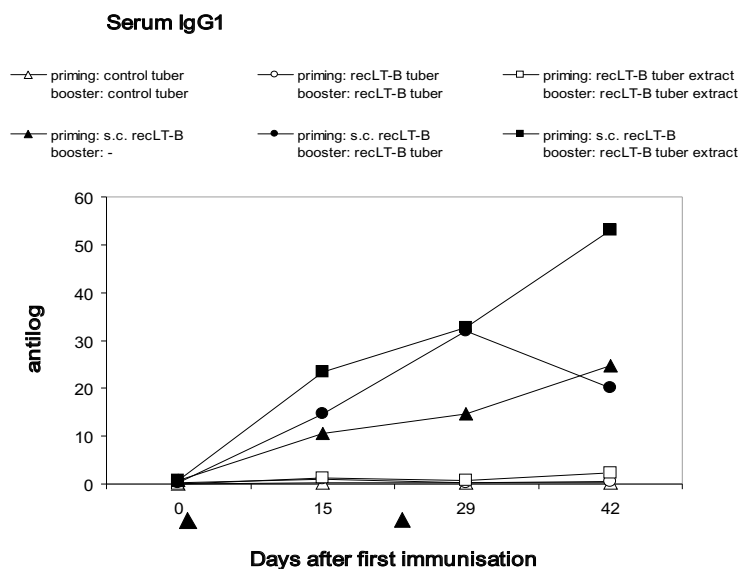
Serum IgG1

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
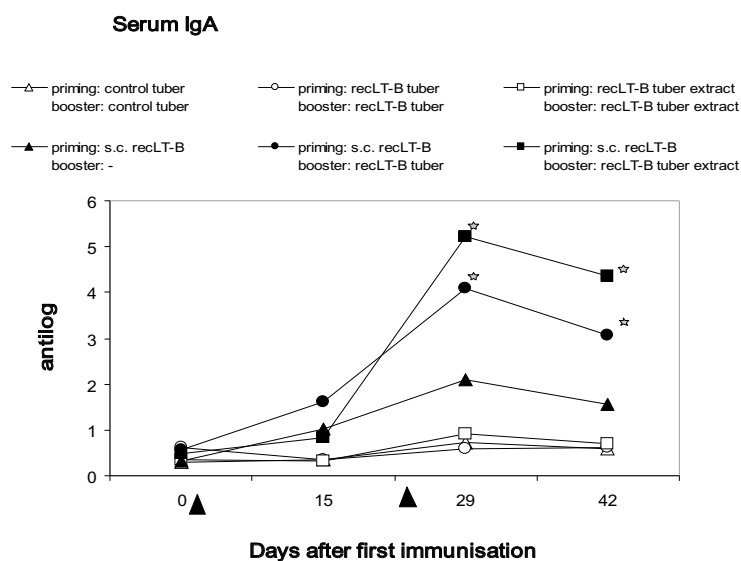
Serum IgA

priming: control tuber / booster: control tuber — priming: recLT-B tuber / booster: recLT-B tuber — priming: recLT-B tuber extract / booster: recLT-B tuber extract

priming: s.c. recLT-B / booster: - — priming: s.c. recLT-B / booster: recLT-B tuber — priming: s.c. recLT-B / booster: recLT-B tuber extract

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).

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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
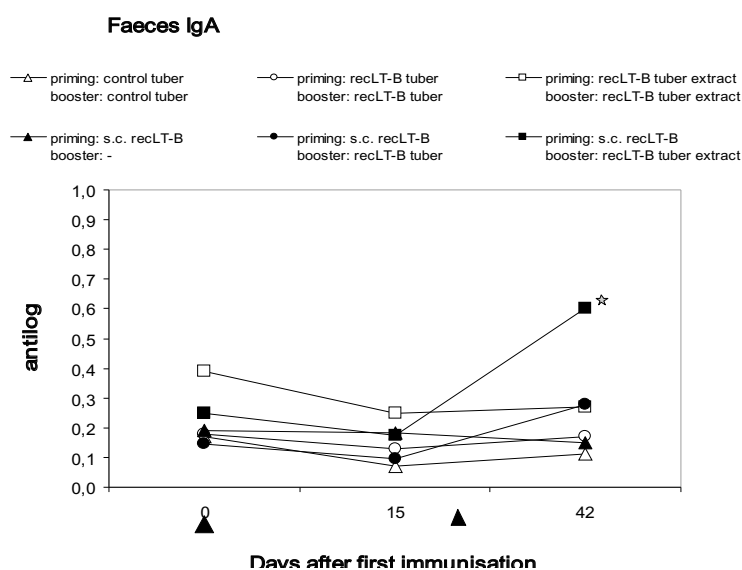
Faeces IgA

| —△— priming: control tuber booster: control tuber | —○— priming: recLT-B tuber booster: recLT-B tuber | —□— priming: recLT-B tuber extract booster: recLT-B tuber extract |
| —▲— priming: s.c. recLT-B booster: - | —●— priming: s.c. recLT-B booster: recLT-B tuber | —■— priming: s.c. recLT-B booster: recLT-B tuber extract |

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 5

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

Tosca Lauterslager, Dion Florack, Dirk Bosch, Rob Moormann and Luuk Hilgers

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.

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.
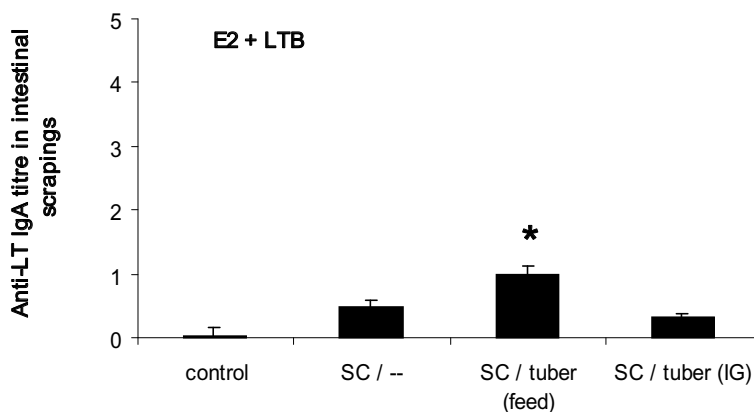

Figure 5.1
Anti-LT IgA antibody titres in intestinal scrapings on day 35, two weeks after oral boost immunisations with E2 + LTB tuber material. Control mice were immunised with tuber material transformed with an empty vector. An asterisk indicates significant responses induced by the oral boost.

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.

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Chapter 6

Efficacy of oral administration and oral intake of edible vaccines

Tosca Lauterslager and Luuk Hilgers

Chapter 6

Summary

To evaluate whether vaccine administration via intragastric gavage is indicative for the outcome of edible vaccines, mice were orally immunised with ovalbumin (OVA) mixed with or without *Vibrio cholerae* toxin (CT) in various compositions via various routes: 1) OVA dissolved in saline and administered intragastrically ('ig'); 2) OVA mixed with food extract and administered ig ('food ig'); 3) food chow absorbed with OVA dissolved in saline and fed to the animals ('food'); and 4) OVA dissolved in saline and administered via drinking bottles ('drinking'). When given to naive mice, 'ig' and 'food ig' but not 'food' or 'drinking' induced anti-OVA IgG1 responses in serum, but oral boost immunisations were necessary. Serum IgA was not induced. Oral boosting of subcutaneously primed mice enhanced the IgG1 and IgA response in serum regardless of the route of immunisation or the vaccine composition. CT did not dramatically enhance the immune response. All immunisation routes except 'drinking' induced antigen-specific IgA antibody secreting cells in the lamina propria of naive mice. But antigen-specific antibody responses in faeces were not observed.

We concluded that oral (ig) administration is distinct from oral intake. The composition of the vaccine (food or saline) did not influence oral administration. We thus suggested that the route of administration greatly influenced the outcome of oral immunisation. Although oral administration is a well-accepted route to test the potentials of oral vaccines, our study demonstrated that it is merely indicative for the effectiveness of edible vaccines. Studies on the feasibility of edible vaccines should thus be performed by eating the vaccine.

Introduction

Oral vaccination is regarded to be a safe and simple alternative for parenteral administered vaccines. When produced in edible plants or parts thereof, the production costs of such a vaccine can be reduced considerably, increasing the availability and use for both human and animals [1]. Furthermore, production of vaccines in plants eliminates the risk of contamination with animal pathogens such as viruses and prion proteins [2]. The first reports on oral vaccination were published when molecular biological techniques were scarcely available. These studies were performed via intragastric (IG) gavage or intraduonenal (ID) immunisation [3,4]. At that time, many researchers already speculated on the development of edible vaccines and the results of IG or ID immunisation were thought to be predictable for the efficacy of edible vaccines. At present, only few oral vaccines are licensed and available and all are based on live microorganisms [5].

The increasing knowledge on the virulence factors of pathogens allows the development of non-living vaccines that are regarded to be safer than live vaccines [2]. In general, non-living vaccines are poor immunogens and adjuvants are required. The

situation becomes even worse when such vaccines are delivered orally since passage through the gastrointestinal (GI) tract involves many degradation steps. Only a fraction of the initially administered material will finally arrive in the gut. It must then still pass the epithelium in order to elicit an immune response. Degradation can be partially overcome by encapsulation of the antigens. In transgenic plants, plant cells can protect the antigen against the acidic environment of the stomach [5,6] and proteolysis in the GI tract.

Recently, we have reported studies on oral immunisation of mice with potato tubers expressing the B-subunit of *Escherichia coli* heat labile toxin (LTB). We observed that feeding intact tuber was less effective than IG gavage of tuber extract containing a similar antigen dose. We then suggested that the route between mouth and stomach or the vaccine formulation is a crucial factor for the outcome of oral immunisation [7]. In the present study, we evaluate both possibilities. Mice were orally immunised with plain ovalbumin (OVA) or OVA incorporated in standard mice chow in order to mimic edible vaccines.

Materials and methods

Mice

Swiss female mice (6 to 8 weeks old) were obtained from Charles River (Sulzfield, Germany). Animals immunised via intragastric (ig) gavage were housed per group under conventional conditions. Animals immunised by food or drinking water were housed individually. All mice were raised and kept on an OVA free diet. All animal experiments were held under auspices of the ID-Lelystad B.V. Animal Experimentation Committee according to the Dutch Law on Animal Experimentation.

Antigen preparation and immunisation

Four antigen preparations were tested:
1) Ovalbumin (OVA; Grade V, A-5503, Sigma) was dissolved in saline at a final concentration of 10 mg per 0.4 ml with or without 5 µg cholera toxin (CT; C-8052, Sigma) and administered via ig gavage ('ig).
2) A food extract of standard food chow dissolved in saline was made. Subsequently, OVA was dissolved in this food extract at a final concentration of 10 mg per 0.4 ml with or without 5 µg CT and administered via ig gavage ('food ig').
3) OVA was dissolved in saline at a final concentration of 10 mg per 0.1 ml with or without 5 µg CT and added to standard food chow of about 1 g until completely absorbed. A single treated chow was given to individual mice (food).
4) OVA was dissolved in saline at a final concentration of 50 mg OVA per 50 ml with or without 25 µg CT. Standard drinking bottles were filled with 50 ml of this

antigen preparation and given to individual mice ('drinking'). The drinking bottles were weighed before and after immunisation.

Mice were fasted overnight before each oral immunisation (water was provided *ad libitum*). The groups immunised via the ig route, received standard food 2 h after gavage. The 'food' and 'drinking' preparations were given for 24 h after which the animals received standard food.

Groups of naive mice were primed orally on day 0, 2, and 4, and boosted orally on day 21, 23, and 25. Other groups were primed subcutaneously (sc) on day 0 with 100 µg OVA and 50 µg Butyl16-p(AA) in 0.1 ml phosphate buffered saline and subsequently boosted orally on day 21, 23, and 25.

Collection of faeces and serum samples

Serum and faeces samples were collected before immunisation and on day 14 and 35. Four to six fresh faeces pellets per mouse were collected and pre-treated as described previously [7].

Detection of antibody secreting cells by ELISPOT

From some groups, lamina propria lymphocytes were isolated from the small intestine and OVA-specific antibody producing cells (APC) were determined by ELISPOT as described before [8].

Detection of anti-OVA antibodies by ELISA

High binding ELISA plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 100 µg ml^{-1} OVA dissolved in PBS (pH = 7.4). ELISA was further performed as described earlier [7]. Antibody titres were expressed as the dilution factor of the sample giving an extinction value of 1.0 above the background. Geometric mean titres (GMT) of individual 2-log titres and standard error of the mean (SEM) 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. Extinctions below detection limit were considered to have a GMT of -10.

Results

Oral immunisation of naive mice

All 'food' immunised mice ate the treated chow within 24 hours. The 'drinking' groups drank 10 ml on average, which corresponded with an oral intake of 10 mg OVA. Oral priming of naive mice did not evoke OVA-specific IgG1 or IgA antibodies in serum. Oral boosting induced OVA-specific IgG1 antibodies on day 35 after 'ig' or 'food ig' administration but not after oral intake of food or drinking water (Fig. 6.1A). The differences between 'ig' and 'food ig' were not significant. Serum IgA antibodies were
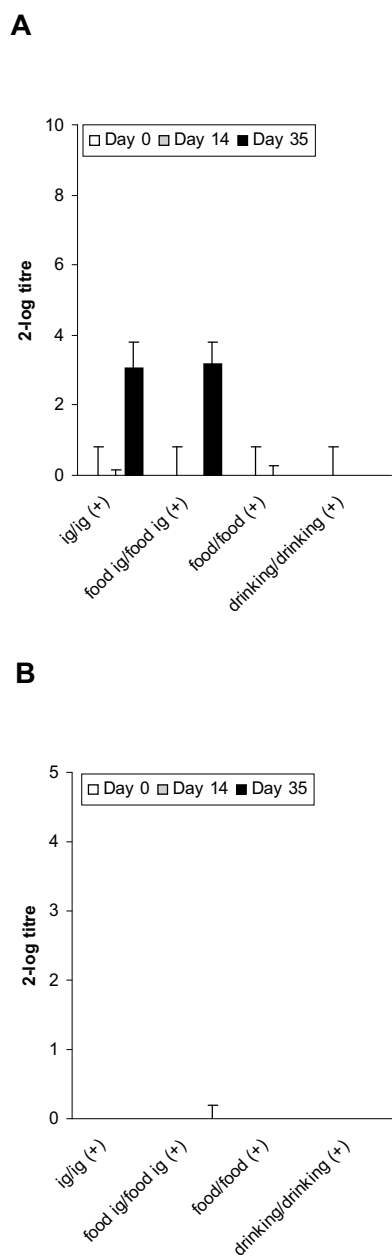
A

B

Figure 6.1
The immune response after oral immunisation of naive mice. Naive mice were primed on day 0 and boosted on day 21. CT was used as adjuvant. Samples were collected prior to immunisation. Anti-OVA specific IgG1 (A) and anti-OVA specific IgA (B) in serum. The mean 2-log titre ± SEM for five mice in each group is shown.

not detected after the boost (Fig. 6.1B). No antibody responses were detectable in faeces (data not shown).

Oral immunisation of primed mice

Subcutaneous (sc) priming of mice with OVA plus Butyl16-p(AA) as adjuvant elicited anti-OVA IgG1 but not IgA in serum (Fig. 6.2A and 6.2B). Subsequent oral boosting significantly increased the IgG1 titre compared to non-boosted animals regardless of the route of immunisation or the vaccine composition. The IgA titre was increased in all groups except in the sc/'food ig'(-) and sc/'food'(+) groups, but the titre was only significant in 'ig' boosted mice. Addition of CT only significantly increased the immune response after 'drinking' immunisation.

ELISPOT analysis of lymphocytes of the lamina propria of the small intestine

The presence of OVA-specific antibody secreting cells (ASCs) in the lamina propria of the small intestine were measured only in orally primed and boosted animals. Anti-OVA IgA but not IgG1 ASCs were observed (Fig. 6.3). The highest number of anti-OVA ASCs were observed after 'food ig' immunisation, but 'ig' and 'food' immunisation also induced significant antigen-specific IgA ASCs.

Discussion

Previously, we demonstrated that antigen-specific immune responses after feeding of LTB-expressing potato tubers were weaker than after intragastric (ig) gavage of tuber extract, although the amount of LTB in intact tubers was 30 times higher [7]. We suggested that the method of administration caused these differences. Protein degradation in the gastrointestinal (GI) tract is an important factor in the outcome of oral vaccination. At the beginning of the GI tract, mastication breaks food into smaller pieces, thereby aided by proteolytic enzymes in the saliva. Subsequently, the food suspension is transported to the stomach with its low pH and finally arrives at the small intestines, where the M-cells reside. In the experiments described here, mice were immunised orally with different formulations of ovalbumin (OVA) via different routes. By addition of a fasting period, we assumed that the length of stay in the stomach was similar in all animals. And antigen administered via food or drinking water but not via ig gavage was subjected to the passage from mouth via oesophagus into the stomach.

In a first experiment ig gavage with OVA dissolved in saline ('ig') was compared to immunisation of mice with chow added with the antigen ('food'). In accordance with our observations with transgenic potatoes [7], we observed that 'ig' immunisation was more effective than 'food' and, in contrast to the transgenic potato study, the antigen doses were similar (data not shown). However, besides the difference in route, the vaccine composition was an additional difference.

A
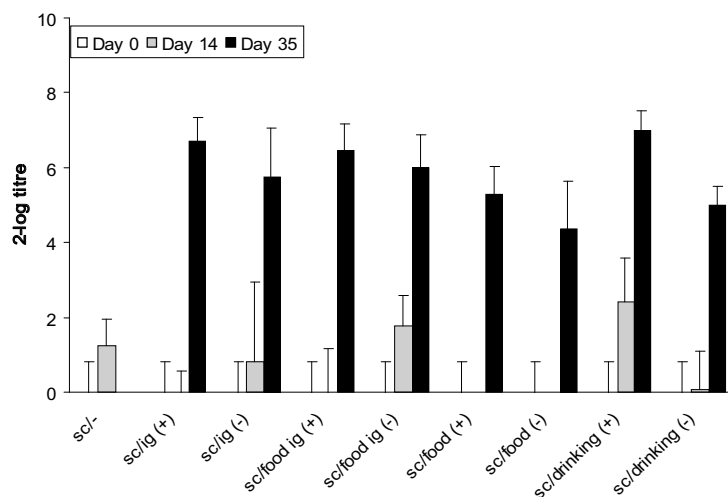
B

Figure 6.2
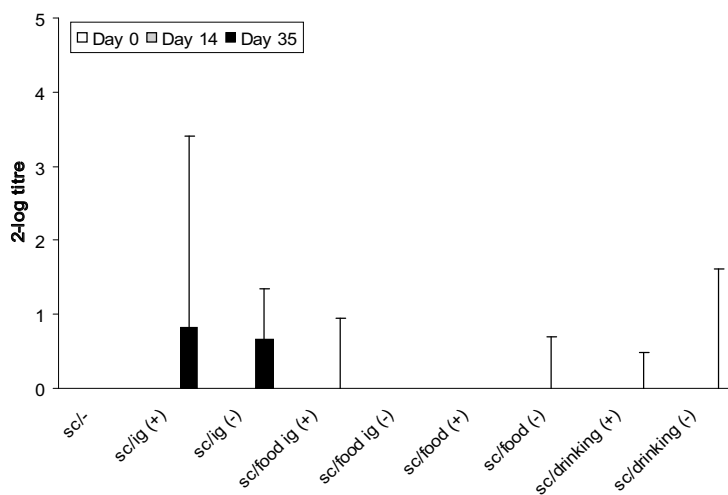The immune response after oral immunisation of primed mice. Mice were primed subcutaneously on day 0 and orally boosted on day 21. Control groups were not boosted (sc/-). CT was used as adjuvant in groups indicated with an +. Samples were collected prior to immunisation. Anti-OVA specific IgG1 (A) and anti-OVA specific IgA (B) in serum. The mean 2-log titre ± SEM for five mice in each group is shown.

Chapter 6

To determine the role of food components in oral immunisation, we repeated the experiment with two additional groups: one group received the antigen via drinking water ('drinking') and another via ig gavage of the antigen dissolved in an extract of mice feed ('food ig'). Again, ig gavage was more effective than 'food' immunisation regardless of the vaccine composition (saline or chow extract). ig gavage was also more effective than 'drinking' immunisation. This corresponded with findings of Felder *et al* who directly injected microparticles into the mouth of pigs instead of IG gavage and observed no immune responses [9].

Surprisingly, in subcutaneously (SC) primed mice, 'drinking' was more effective than 'food' immunisation. This suggested that chow components still might have played a role in the outcome of oral vaccination. The primary task of the GI tract is absorption of nutrition out of food and under normal conditions, the body is tolerant against dietary antigens and immunological reactions are preferably prevented [10]. Perturbation of the balance in the gut may lead to unwanted diseases like food allergy, Crohn's disease, and coeliac disease as a result of breakdown in oral tolerance [11]. Taking the natural function of the GI tract into account, it is not remarkable that OVA mixed with food is less effective in evoking an immune response than OVA dissolved in an aqueous phase. The body probably recognises OVA mixed with food as 'normal' food, and does not react on it. On the other hand, 'food IG' immunisation evoked serum IgG1 and high numbers of IgA ASC. This suggested that orally administered antigens were only recognised as food when it follows all processing steps, including passage through the mouth and oesophagus. The route of administration is probably a crucial factor in the outcome of oral vaccination. 'Food' induced significant numbers of APC, so it is not likely that oral tolerance was induced. Future studies must assess whether 'food' immunisation is not immunogenic or induce oral tolerance instead. Our results corresponded with observations of Klipper *et al.* who reported that administration of bovine serum albumin (BSA) in solution induced immune responses whereas feeding of BSA powder mixed with standard food induced tolerance [12,13]. In contrast to us, Klipper *et al.* concluded that the physical form of an antigen was an important factor for oral immunisation.

We fasted the animals to reduce duration of stay in the stomach. After fasting, food is released by the stomach as a bolus. This might have influenced the 'food IG' and 'food' immunisations, but not the other two. The efficiency of vaccine take up out of a bolus may be more difficult. This may be another explanation of the reduced effectiveness of 'food' immunisation, and must be also taken into account when designing an edible vaccine.

Similar to previous experiments of our group [7], oral immunisation of naive mice was less effective than oral boosting of subcutaneously (SC) primed mice. Although all naive mice were immunised with cholera toxin (CT) as adjuvant. Subcutaneous priming with Butyl16-p(AA) as adjuvant triggered the immune system for an oral booster. When

the immune system was sufficiently triggered, all administration routes boosted the immune response, regardless of the vaccine composition and the use of CT.

Surprisingly, IgA was not the predominant immunoglobulin induced by oral immunisation and IgG1 was also induced. In serum, very low IgA titres were measurable, but that was consistent with the observations that IgA is predominantly present in mucosal secretions. This was confirmed by ELISPOT, which revealed that IgA and not IgG1 antibody secreting cells (ASCs) were present in the lamina propria of the small intestine.

Oral immunisation by oral intake of food an attractive concept because of its ease of administration. Various studies have been reported the use of virus-like-particles (VLPs) or non-living vaccines expressed in plants [7,14,15]. Preliminary human trails have been published [16,17]. However, the development of transgenic plants is laborious and time-consuming. Anticipating to future edible vaccines, studies are performed by oral administration of antigen by IG gavage. The present study demonstrated that oral administration is distinct from oral intake of food and may overestimate the efficacy thereof.
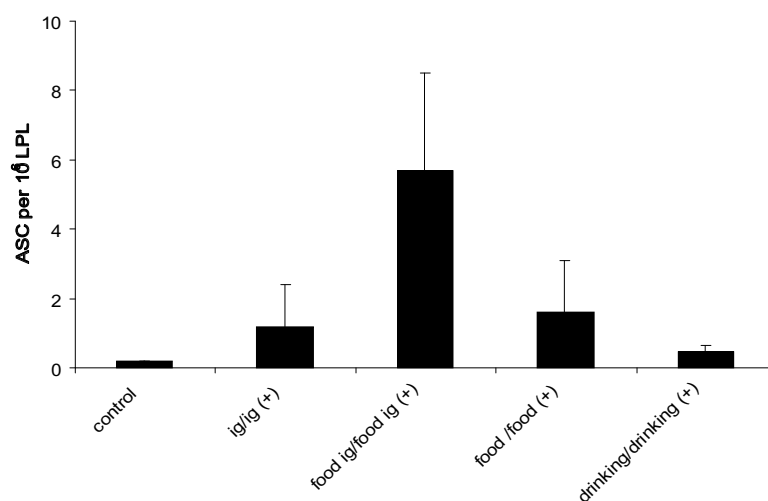

Figure 6.3
Detection of OVA-specific antibody secreting cells (ASC) in the lamina propria lymphocytes of orally immunised naive mice. Control mice were not immunised. The mean number of ASC per 10^6 lamina propria lymphocytes (LPL) $\pm$ SEM are shown.

Chapter 6

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Chapter 7

General discussion

Chapter 7

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

94

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

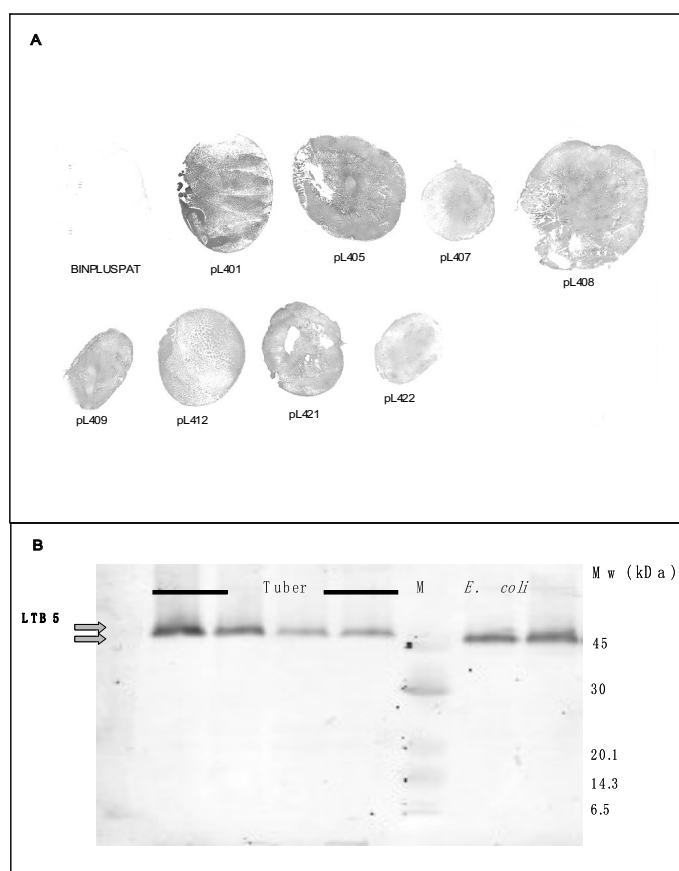**Figure 7.1**
Immunoblots of LTB produced in transgenic potato tubers. Accumulation of LTB in the tuber was demonstrated using a LTB-specific monoclonal antibody on a nitrocellulose tissueblot. BINPLUSPAT contains the empty vector. pL401 until pL422 contain the LTB-coding vector (A). Presence of pentameric 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
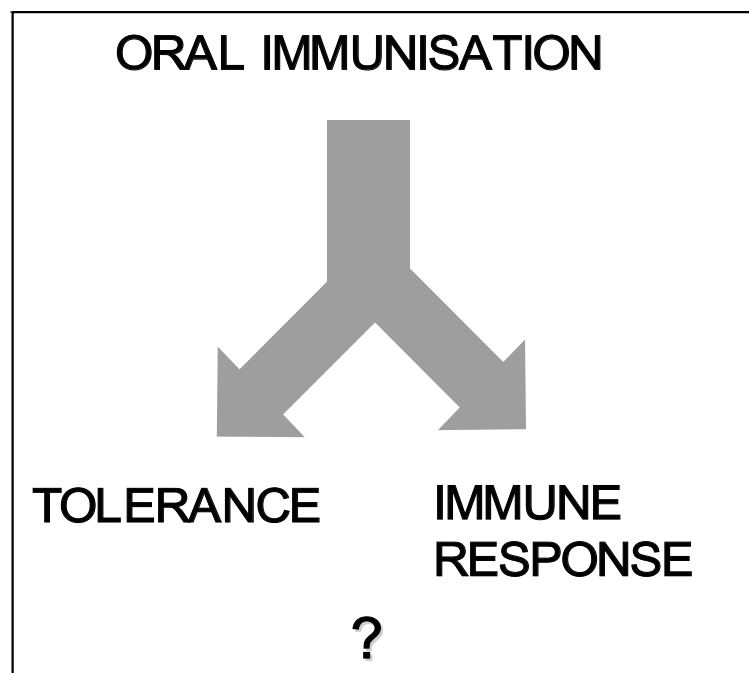

Figure 7.2
The oral vaccination paradox: oral vaccination is intended to active, protective immunity against pathogens invading mucosal tissues, but the GI-tract is programmed to respond with a state of immunotolerance against oral antigens

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
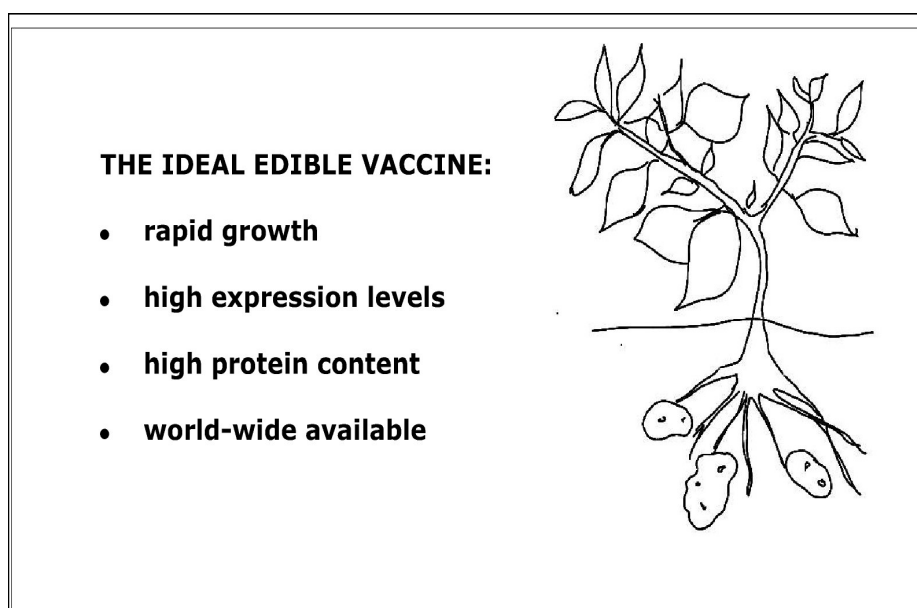

THE IDEAL EDIBLE VACCINE:

- **rapid growth**
- **high expression levels**
- **high protein content**
- **world-wide available**

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.

Chapter 7

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ABBREVIATIONS

APC   antigen presenting cells
ASC   antibody secreting cells
CMIS   common mucosal immune system
CPV   canine parvo virus
CT   *Vibrio cholerae* toxin
CTA   *Vibrio cholerae* toxin subunit A
CTB   *Vibrio cholerae* toxin subunit B
DC   dentritic cells
FAE   follicle associated tissue
GALT   gut-associated lymphoid tissue
GI   gastro-intestinal
IEC   intestinal epithelial cells
ID   intraduonenal(ly)
IG   intragastric(ally)
IN   intranasal(ly)
IP   intraperitoneal(ly)
ISCOMS   immune stimulating complexes
LT   *Escherichia coli* enterotoxin
LTA   *Escherichia coli* enterotoxin subuntit A
LTB   *Escherichia coli* enterotoxin subuntit B
MALT   mucosa-associated lymphoid tissue
MHC   major histocompatibility comples
MIS   mucosal immune system
MLN   mesenteric lymphnode
OVA   ovalbumin
PLG   poly (lactide-co-glycolide)
PO   per oral(ly)
PP   Peyers patches
RHDV   rabbit haemorragic disease virus
SC   subcutaneous(ly)
TSP   total soluble protein
VLP   virus-like particle

Abbreviations

Summary

This thesis describes the research to explore the feasibility of plants for oral vaccination. The research focussed on a model of LTB produced in potato tubers or ovalbumin (OVA) as antigen and tested in mice. A general introduction into the backgrounds of oral immunisation is given in Chapter 1. The next two chapters describe the optimisation of an immunisation protocol for edible vaccines by addressing the following questions: can the immune response be increased by more frequent doses of edible vaccines without negative effects on general health (Chapter 2)?; and can the immune status of the host be modified to react more efficiently to a subsequent oral boost (Chapter 3)?. Ovalbumin (OVA) was used as model antigen and administered via intragastric (IG) gavage to mice. The results of these studies led to a refined immunisation protocol in which one single subcutaneous, adjuvanted priming is followed three weeks later by oral boost immunisations on three alternating days (also referred to as the 'systemic prime/oral boost' protocol).

As a model for edible vaccines, the heat-labile enterotoxin subunit-B (LTB) of *Escherichia coli* was produced in potato tubers. This edible vaccine was either fed or administered orally to mice. Using the optimised immunisation protocol, local and systemic responses against LTB were induced (Chapter 4). Subsequently, the use of LTB as adjuvant for co-expressed antigens in edible vaccines was explored. A glycoprotein (E2) of the classical swine fever virus was co-expressed as fusion protein to LTB or expressed together with LTB in potatoes, resulting in E2-LTB and E2 + LTB potatoes, respectively. LTB fused to these antigens retained its biological activity (GM1-binding). The expression levels of LTB varied from 0.25% in LTB-transgenic plants to 0.01% LTB per total soluble protein in E2-LTB-transgenic plants. The levels of LTB as fusion-proteins were lower than those of LTB alone or co-expressed with E2. LTB, E2 + LTB, and CVP-LTB producing tubers were immunogenic upon subcutaneous immunisation and significant antibody responses against LTB were detected. The response towards the co-expressed antigens were low or undetectable. Probably, the antigen dose of the co-expressed antigen was too low and the adjuvant capacity of LTB was insufficient (Chapter 5). Further research is required to improve the carrier and adjuvant function of LTB. Another point of concern is that besides adjuvant activity, LT,CT and their B-subunits are also known to be capable to induce oral tolerance. Future research must concentrate on more effective carrier-molecules or adjuvants devoid of tolerating properties.

The chapters 4 and 6 clearly demonstrated that IG gavage of tuber or chow extracts induced higher antibody responses than similar doses of antigen taken up with feed or drinking water. It was concluded that the route of oral administration is at least as important than the vaccine composition. The difference between feeding and oral administration must be taken into account when the feasibility of edible vaccines is assessed (Chapter 6).

Summary

In Chapter 7, the general findings of this thesis are discussed. The proposed systemic prime/'triple dose' oral boost protocol appeared to be applicable for oral administration and oral intake of edible vaccines in particular. However, significant adverse effects were observed after tuber intake. The ideal edible vaccine in plants has sufficient levels of antigen, is easy to propagate under a wide range of conditions and is not toxic when given the amounts required. In this respect, the use of potatoes has several drawbacks. Consumption of raw potatoes is not preferable and cooking might denature the antigen. For future edible vaccine studies, a more suitable plant with relatively high expression levels should be chosen (e.g. tomato).

Samenvatting

In de strijd tegen infectieziekten zijn er in de loop der jaren verschillende succesvolle vaccins ontwikkeld. Deze vaccins worden meestal via injecties toegediend, maar hieraan kleven ook enkele nadelen: het gebruik van naalden houdt altijd een risico van besmetting met pathogenen (ziekteverwekkers) in, injecties moeten door speciaal opgeleid personeel worden toegediend en het gebruik van naalden is met name voor kinderen beangstigend.

Bij vaccins die oraal (via de mond) worden toegediend zijn geen naalden meer nodig. De toediening van orale vaccins is verder dusdanig eenvoudig dat, in principe, iedereen ze kan toedienen. Hierdoor zal een grootschalige vaccinatie (zoals de recente meningococcen-vaccinatie campagne) minder tijd kosten en minder angst en stress bij de gevaccineerde opleveren. Verder zijn orale vaccins in staat om zowel in het bloed als in de beschermde slijmlagen van het lichaam (de mucosa) een immunologische reactie op te wekken. Het mucosale immuunsysteem wordt hierbij geactiveerd. Bescherming aan de mucosa is belangrijk, omdat de meeste pathogenen via de mucosa het lichaam van een gastheer binnenkomen. Injecteerbare vaccins brengen met name immunologische bescherming in het bloed teweeg, maar het mucosale immuunsysteem wordt nauwelijks geactiveerd. Een voorbeeld van een succesvol oraal vaccin is het poliovaccin wat een belangrijke bijdrage heeft gehad in de wereldwijde uitroeiing van polio. Momenteel zijn er weinig andere orale vaccins commercieel verkrijgbaar, maar gezien de vele voordelen van orale vaccins wordt er nog steeds intensief onderzoek naar nieuwe orale vaccins uitgevoerd.

Met behulp van de huidige kennis in moleculaire biologie is het mogelijk om vaccins in planten te produceren. Wanneer een oraal vaccin in eetbare delen van een plant wordt gemaakt, kan het vaccin toegediend worden door simpelweg de plant of het eetbare deel ervan op te eten. In dit proefschrift is het onderzoek naar de haalbaarheid van de productie van orale vaccins in planten beschreven. Het onderzoek werd uitgevoerd in muizen die oraal eiwitten toegediend kregen. Als eerste modeleiwit werd ovalbumine (OVA) gebruikt en als tweede modeleiwit werd de B-subunit van het hittelabiele enterotoxine van *Escherichia coli* gebruikt.

Wanneer een pathogeen een gastheer voor de eerste maal binnendringt wordt het immuunsysteem geactiveerd. Bij deze primaire reactie slaat het geactiveerde immuunsysteem herkenningspunten (epitopen) van het pathogeen op in het geheugen. Bij een nieuwe besmetting met hetzelfde pathogeen herkent het immunologisch geheugen van de gastheer dit pathogeen en wordt er sneller op de binnengedrongen ziekteverwekker gereageerd (secundaire reactie). Een vaccin moet een pathogeen kunnen nabootsen en het immuunsysteem dusdanig activeren dat het immuunsysteem in het vervolg het pathogeen herkent. Het vaccin zelf mag de gastheer niet ziek maken. Veel vaccins bestaan uit volledige pathogenen die verzwakt of afgedood zijn. Hierbij bestaat er echter altijd een mogelijk risico op reactivatie van het pathogeen. Daarom wordt er

Samenvatting

veel onderzoek gedaan naar veiliger geachte vaccins. Deze zouden kunnen bestaan uit stukjes van het pathogeen, bijvoorbeeld alleen de voornaamste eiwitten van het pathogeen die een immunologische reactie kunnen veroorzaken. Uit onderzoek is gebleken dat vaccins die bestaan uit delen van het pathogeen minder effectief zijn dan vaccins die uit het complete pathogeen bestaan en moeten daardoor aangevuld worden met een adjuvant, een component die de immunologische reactie versterkt.

In hoofdstuk 1 werd een algemene inleiding over de achtergronden van orale vaccinatie gegeven inclusief een beknopt overzicht van het mucosale immuunsysteem. In de volgende twee hoofdstukken werd de optimalisatie van een immunisatie protocol voor eetbare vaccins beschreven. De effectiviteit van een oraal vaccin bleek te kunnen worden verhoogd door meerdere (multiple) doses van het vaccin te verstrekken. Wanneer deze multiple doses niet meerdere dagen achtereen, maar om de dag werden gegeven, waren er geen nadelige gevolgen voor de gezondheid (hoofdstuk 2). Orale vaccinatie bleek minder effectief te zijn dan injecteerbare vaccinatie. Daarom werd er gekeken of de twee vaccinatie vormen combineerbaar waren en zo bescherming in zowel het bloed als in de mucosa opgewekt kon worden. Door het immuunsysteem eerst met een injecteerbare (subcutane) vaccinatie optimaal te activeren (priming), was het mogelijk om met behulp van orale vaccinaties het immuunsysteem te reactiveren (booster; hoofdstuk 3). Bij deze experimenten werd OVA gebruikt als model eiwit en vloeibaar rechtstreeks in de maag (intragastraal) van muizen toegediend. De resultaten van deze studies hebben geleid tot een immunisatie protocol waarbij één enkele subcutane priming met een antigen in combinatie met een adjuvant drie weken later wordt gevolgd door orale boost immunisaties op alternerende dagen (systemische priming/orale boost protocol). Dit protocol werd toegepast op eetbare vaccins.

Als een model voor eetbare vaccins werd de B-subunit van het hittelabiele enterotoxine (LTB) van *Escherichia coli* geproduceerd in de knollen van transgene aardappelplanten. Stukjes aardappelknol werden gevoerd aan muizen of een aardappelextract werd intragastraal toegediend volgens het systemische priming/orale boost protocol. Specifieke antilichamen tegen LTB waren meetbaar in zowel het bloed als in mucosale secreties zoals de feces of in darmschraapsels (hoofdstuk 4). Vervolgens werd bestudeerd of LTB als adjuvant voor een ander, tegelijkertijd toegediend eiwit gebruikt kon worden. Hiervoor werden transgene aardappelen gemaakt die, naast LTB, een eiwit (E2) van het klassieke varkenspest virus tot expressie brachten. Dit eiwit werd samen met LTB in de aardappelknollen geproduceerd (E2 + LTB), maar werd ook als fusie-eiwit aan LTB geproduceerd (E2-LTB). De expressie niveaus van E2 + LTB en E2-LTB waren aanzienlijk lager dan die van LTB alleen. Dit komt waarschijnlijk omdat E2 + LTB en E2-LTB complexere en grotere moleculen zijn. Dit verklaart mogelijk ook waarom de immunologische reactie tegen deze aardappelen veel lager was. Er werden alleen maar specifieke antilichamen tegen LTB en niet tegen E2 gemeten. LTB geproduceerd in

aardappelknollen kon niet dienen als adjuvant voor tegelijkertijd toegediende eiwitten (hoofdstuk 5). Om de effectiviteit van op LTB gebaseerde vaccins te verhogen moeten allereerst de expressieniveaus van de (fusie)eiwitten worden verhoogd.

Tijdens de experimenten met de aardappelknollen werd aangetoond dat er een verschil bestaat tussen de immunologische reactie na het voeren van aardappelknollen en het intragastraal toedienen van een aardappelextract. In hoofdstuk 6 werd dit uitgebreider bestudeerd. Intragastrale toediening van OVA samen met een opgeloste voedselbrok van muizen wekte sterkere immunologische reacties op dan het direct opeten van een droge voedselbrok met vergelijkbare doses OVA. De route van orale toediening bleek een belangrijke rol te spelen bij orale vaccinatie. Dit maakt experimenten met intragastrale toediening van vaccins minder geschikt om de mogelijkheden van eetbare vaccins te bepalen (hoofdstuk 6).

Het voorgestelde systemische priming/orale boost protocol bleek toepasbaar te zijn voor intragastrale toediening van orale vaccins en het voeren van eetbare vaccins in het bijzonder. Hoewel na het eten van rauwe knollen er niet-wenselijke bijwerkingen bij de muizen zichtbaar waren. Het ideale door planten geproduceerde eetbare vaccin moet het eiwit in voldoende mate tot expressie brengen, moet makkelijk te vermeerderen zijn onder uiteenlopende omstandigheden en mag niet toxisch zijn wanneer het in hoge doses wordt toegediend. Wanneer hiernaar gekeken wordt blijken aardappelen minder geschikt te zijn. Consumptie van rauwe aardappelen is niet aan te raden en koken kan het vaccin denatureren. Voor toekomstige studies naar eetbare vaccins moet een meer geschikte plant gekozen worden (bijvoorbeeld tomaten- of bananenplanten).

Samenvatting

CURRICULUM VITAE

Tosca Lauterslager werd op 10 maart 1971 geboren te Oss. In deze stad heeft zij haar lagere- en middelbare schooltijd doorgebracht. In 1990 werd aan het Maasland College het atheneum-B diploma behaald en in datzelfde jaar werd de studie Biologie aan de Katholieke Universiteit Nijmegen gestart. Ze volgde de studierichting Medische Biologie met hoofdvakstages bij de sectie Medische Parasitologie (afdeling Medische Microbiologie, Academisch Ziekenhuis Nijmegen) en de afdeling Moleculaire Dierfysiologie (Katholieke Universiteit Nijmegen). Een bijvakstage werd vervuld bij de Applied Microbiology Unit in samenwerking met het Muhimbili Medical Centre aan de University of Dar es Salaam, Tanzania. In 1996 werd het doctoraal examen gehaald. Van februari 1997 tot en met september 2001 was zij werkzaam als assistent in opleiding bij de afdeling Virologie van de Universiteit Utrecht. Het onderzoek werd uitgevoerd bij de divisie Dier en Omgeving van ID-Lelystad te Lelystad en staat beschreven in dit proefschrift. Sinds november 2001 is zij als post-doc werkzaam de afdeling Pathologie van het Vrije Universiteit Medisch Centrum te Amsterdam waar zij een bijdrage levert aan het onderzoek naar het Epstein Barr virus.

Curriculum vitae

Dankwoord

Hè, hè, eindelijk ben ik aan dit laatste stuk van mijn proefschrift toe. Een zeer drukke en, met name de laatste jaren, stressvolle tijd sluit ik hiermee af. Ik heb hierin geleerd dat wetenschap zich niet helemaal in 4 jaar laat voorspellen en dat "leuke" resultaten vaak moeizaam tot stand komen. Toch heeft dit mij er niet van weerhouden in het onderzoek te blijven: de uitdagingen die ik hierin dagelijks ondervind, trekken mij nog steeds erg aan. Alle jaren ben ik enorm gesteund door verschillende mensen, zowel binnen als buiten het werk en ik doe een poging mijn dankbaarheid hier te verwoorden.

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De overige leden van het "eetbare vaccins"-team mogen zeker ook niet onvermeld blijven: Thijs, hartelijk bedankt voor de zeer prettige samenwerking (TNT) tijdens de secties, de ELISA's en de overige proeven. Fijn dat je mijn paranimf wilt zijn. Jan L., bedankt voor je steun door de jaren heen. Dirk, Jos, Mieke, Sofia en met name Dion: hartelijk bedankt voor het maken van de aardappelen. Het was altijd leuk om in Wageningen langs te komen. Dion, nog bedankt voor je hulp bij de twee "aardappel-stukken"; we houden contact.

Natuurlijk wil ik "mijn" stagiaires niet vergeten: Rob, Nathaly en Hetty, jullie hebben mij enorm geholpen door in de laatste twee jaren van mijn project, toen alles nog moest gebeuren, gigantisch veel werk te verzetten. Voor een ELISA met meer dan 50 platen zullen wij nooit meer onze hand voor omdraaien! Heel veel tijd is doorgebracht in de stallen bij de muizen en (enkele keren) de konijnen. Hier heb ik altijd veel hulp gehad van Jan, Henk, Johan, Arnold en Wilfred, die altijd voor mij klaarstonden als er weer eens "zo snel mogelijk, want we hebben nog maar weinig tijd" een proef gestart moest worden. Mijn bijna dagelijkse bezoek aan de stallen was hierdoor nooit onprettig.

Dan kom ik zo langzamerhand al aan bij de mensen die niet direct met mijn onderzoek te maken hebben gehad, maar zonder wie mijn tijd in Lelystad heel anders zou zijn geweest. Allereerst mijn andere paranimf, Conny: kamergenootje van het begin. Met

Dankwoord

elkaar hebben we veel lief en leed gedeeld en je hebt mijn jaartje als inwoonster van Lelystad prettig veraangenaamd. Heel veel geluk voor jou, Joris en Anouk!

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Nu wordt het tijd om over te stappen naar mijn "nieuwe" werkgever, de afdeling Pathologie bij het VUMC. Allereerst wil ik prof. Meijer bedanken voor de mogelijkheid die hij me gegeven heeft om, naast mijn werk, mijn promotie af te ronden. Degene die me daarbij het meest gesteund heeft is de grote EBV-baas zelf: Jaap, ontzettend bedankt voor al je geduld als ik met mijn gedachten bij mijn promotie was, terwijl ik geacht werd met EBV bezig te zijn. Ontzettend fijn dat je in mijn leescommissie hebt willen plaatsnemen. Nu kan ik me eindelijk helemaal op het LMP1,2 werk storten!

De rest van de EBV-groep (Elisabeth, Jeffrey, Tabitha, Servi, Josine, Sandra, Tineke, Sander en Wendy), mijn kamergenoten (Erik, Marco, Kirstin, Esther, Janneke en Duco), de apoptose-groep (Saskia en Jettie), de polycomb-groep (Danny, Joost en Cindy), mijn DC-raadgevers (Hetty en Tanja,) en de overige medewerkers van de afdeling pathologie: bedankt voor al jullie belangstelling, luisterend oor en steun in het afgelopen jaar. Rik, bedankt voor het plaatsnemen in de leescommissie.

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Een speciale vermelding wil ik die unieke en (te?) gezellige sportclub geven: SKDO. Zonder karate had ik het reizen nooit vol kunnen houden, kon ik mijn frustraties nooit letterlijk van me afslaan en had ik nooit mijn man ontmoet. Al meer dan 15 jaar maakt karate deel uit van mijn leven en het blijft een zeer belangrijke factor die mij aan Oss bindt. Iedereen hartelijk bedankt (met name mijn trainingsmaatje!) voor alle belangstellende vragen naar mijn onderzoek en naar mijn vorderingen tijdens het schrijven. Nu beland ik onherroepelijk bij mijn familie die voor een (volgens sommigen te) groot deel onlosmakelijk met deze club verbonden is.

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Tosca

Dankwoord