

Edible genetically modified microorganisms and plants for improved health

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The development of new strategies for the delivery of vaccine antigens or immune modulators to the mucosal tissue includes innovative approaches such as the use of genetically modified food microorganisms and plants. Even though the 'proof-of-concept' has recently been established for these two systems, key questions mainly related to efficacy and risk of breaking oral tolerance remain to be critically addressed in the immediate future.

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Abbreviations

CTB	cholera toxin B subunit
ETEC	enterotoxigenic <i>Escherichia coli</i>
HbsAg	hepatitis B virus major surface antigen
LAB	lactic acid bacteria
LTB	heat-labile enterotoxin B subunit
RSV	respiratory syncytial virus
TTC	non-toxic binding subunit C of tetanus toxin

Introduction

The gastrointestinal tract of humans and animals corresponds to a very complex ecosystem the equilibrium of which is critical to health and well-being. On the one hand, it is the place where ingested foods are digested and processed for the nutritional benefit of the host. On the other hand, the huge mucosal surface ($\approx 250 \text{ m}^2/\text{adult}$) associated with this organ is permanently challenged by exogenous substances, including the numerous pathogenic microorganisms associated with common infectious diseases. As a consequence, the digestive tract has evolved into a sophisticated system that is able to discriminate between 'self' and 'non-self' antigens and to elicit either tolerogenic or immunogenic reactions in response to specific compounds. It is now accepted that the constant cross-talk between the gut-associated immune system and the intestinal microflora plays a major role in the maintenance of the homeostatic state of this complex ecosystem. In general, healthy individuals generate low level or undetectable responses to food antigens and commensal bacteria, a status that is known as 'oral tolerance'. This does not prevent the host from mounting active immune defences when challenged by a pathogenic microorganism.

For several immunological and practical reasons, the design of efficient oral, and more recently edible, vaccines represents a challenging alternative to the traditional injectable ones [1]. Their ease of production, handling and administration represent 'low-budget vaccines' and their development has been proposed especially for third world countries. The non-invasive method of application may decrease potential adverse effects, increase patients' compliance and render them 'attractive' even to individuals living in industrialized nations. More importantly, oral immunization is the most efficient means to induce protection at the site of pathogen contact.

Oral delivery of particulate or replicating antigens usually leads to the induction of a protective immune response, whereas ingestion of soluble proteins generally results in immunological tolerance [2–4]. Therefore, the challenge in designing oral vaccines is to find the most appropriate vehicle to produce and mucosally deliver protective antigens or immune modulators such as interleukins. In this context, both recombinant food-grade bacteria and transgenic plants have been intensely developed as carriers over the past decade. This review will focus on the recent progress towards the production of oral or edible vaccines based on dietary lactic acid bacteria (LAB) and transgenic vegetables and fruits [5,6].

Plant foods as edible vaccines

The use of transgenic vegetables and fruits for the expression and delivery of recombinant protein antigens as edible vaccines has become an attractive topic for plant molecular biologists [5]. For almost a decade the production of oral vaccines in transgenic plants, preferably in their edible tissues, has focused on viral and bacterial subunit antigens. Viral antigens included the hepatitis B surface antigen (detailed later), the Norwalk virus capsid protein [7], the rabies virus [8] and the respiratory syncytial virus (detailed later). Bacterial antigens were mainly limited to enteropathogen toxin subunits.

Viral antigens

Hepatitis B surface antigen

The concept of producing a subunit vaccine in transgenic plants was first described in 1992 by Mason and co-workers [9] who integrated the DNA coding for the hepatitis B virus major surface antigen (HBsAg) into tobacco plants by *Agrobacterium*-mediated transformation. HBsAg levels in leaf extracts only reached low levels (2–6 $\mu\text{g/g}$ of the soluble leaf protein) at first, but were further increased to 66 $\mu\text{g/g}$ (0.01%). HBsAg produced in tobacco retained the capacity for self-association, and was antigenically and physically similar to the HBsAg particles derived from

human serum and recombinant yeast. The proof of equivalent immunogenicity for a plant-produced subunit vaccine compared with a commercially available yeast-derived vaccine was partly provided by Thanavala and colleagues [10]. The authors immunized mice intraperitoneally with the two forms of recombinant HbsAg using different adjuvants, and observed systemic immune responses in both cases. However, from this experiment it was not predictable whether an oral immunization or ingestion of the plant-derived antigen would be as effective in protection as the parenteral immunization.

In 1999, Kapusta and co-workers [11] obtained transgenic lettuce from cotyledons by inoculation with *Agrobacterium tumefaciens* containing expression plasmids for HBsAg. Three adult volunteers received transgenic lettuce, 200 g at first, and within two months another 150 g. Two control individuals were given the same amount of non-transgenic lettuce. The concentration of HBsAg was 0.1–0.5 µg/100 g of fresh tissue, corresponding to 1–5.5 ng/g fresh weight. Based on the fact that the dose of HBsAg used for this oral immunization was about 20 times lower than the parenteral dose normally used for immunization of adults and applied without an adjuvant, the outcome of the vaccination — namely, serum antibody levels of minimal protection level (10 IU/l) — could be regarded as successful. However, only two out of three volunteers responded to the vaccine, while the third was a ‘non-responder’. The fact that the immune response declined four weeks after the last ingestion might indicate the development of oral tolerance following a transient immunological responsiveness to the antigen, as often described for other mucosally applied antigens [12].

In 2000, Richter and co-workers [13••] produced transgenic potatoes that expressed HBsAg at 1.1 µg/g fresh tuber, and co-administered a mucosal adjuvant along with this edible vaccine. For each experiment 10 BALB/c mice were fed either transgenic potato tuber containing a total of 5.5 µg HBsAg per dose (or control non-transgenic tuber) plus 10 µg CT (cholera toxin) added as adjuvant, once a week for three consecutive weeks. The primary serum-antibody response peaked at 73 IU/l three weeks after the last dose, then declined over the next three weeks. Intraperitoneal injection of a subimmunogenic dose (0.5 µg/mouse) of the yeast-derived commercial vaccine (Recombivax, Merck Sharp & Dome, Harlow, UK) resulted in an immediate high-level recall antibody response (1679 IU/l). Mice fed control tubers with CT showed no primary or booster responses. From these data, the authors concluded that memory cells were established following oral delivery of HBsAg in potato tuber and a recall response was elicited by parenteral immunization. The combination of oral and parenteral application seems to be a good solution for reaching high systemic, and probably also local, immune responses. It appears that the additional use of a mucosal adjuvant can sufficiently enhance the immune response and probably even prevent induction of

oral tolerance towards the antigen. An important aspect, which needs to be taken into consideration, is whether the co-administration of a mucosal adjuvant will simultaneously induce an immune response to the food antigen. Such a T helper 2 cell (Th2)-biased immune response [14,15] against a food antigen is, however, undesirable and was not investigated in this study. The authors suggest that higher antigen doses could help avoid the use of oral adjuvants.

HBsAg was also produced in the fruit of transgenic tomato at a level such that 100 g of ripe fruit contained ~9 µg of HBsAg. The authors envisage that vaccine administration could be accomplished by a human drinking tomato juice produced from homogenized fruit or by consuming a large quantity of the fruit [16].

Respiratory syncytial virus

Respiratory syncytial virus (RSV) infections are very common in infancy and early childhood, causing serious lower respiratory tract diseases with mortality rates up to 2.5%. So far no successful vaccine that leads to long lasting protection against RSV is available. With respect to the infection mode and the site of pathogen contact, the development of a mucosal (oral or intranasal) vaccine has high priority. For the first time, a fruit-based edible subunit vaccine against RSV was developed by expressing the RSV fusion (F) protein in transgenic tomato plants [17••]. The highest expression level of RSV-F antigen in transgenic cherry tomatoes was 32.5 µg/g fruit fresh weight. Twenty-five female BALB/c mice were fed 4–5 g ripe transgenic fruit tissue on days 0, 4, 14, 18 and 28. Five control mice received wild-type cherry tomatoes. Sera collected on day 32 showed significant serum anti-RSV-F IgG and IgA responses that were not detectable in control mice. A booster immunization on day 33 with an inactivated RSV virus showed significantly increased immune responses. Intestinal extracts from mice on day 39 showed significant RSV-F-specific IgA titers, which were not detectable in control animals. The next logical step will be to analyze the protection rate and duration of protection of the test animals against an RSV infection.

Bacterial antigens

The B subunits of the *Escherichia coli* heat-labile enterotoxin LT and the *Vibrio cholerae* enterotoxin CT

Following the first experiments of Haq *et al.* [18], Mason *et al.* [19] recently demonstrated that the *E. coli* heat-labile enterotoxin B subunit (LTB) expressed in plants has the potential to be used as a vaccine component or as a booster vaccine against ETEC (enterotoxigenic *E. coli*) and cholera. Potato plants were transformed with a plant-optimized synthetic gene encoding *E. coli* LTB. Potato tubers were fed to BALB/c mice at weekly intervals for three weeks delivering ~20 µg or ~50 µg LTB per feeding. Animals from the positive control group were gavaged with 5 µg of purified LTB from recombinant *E. coli*. Mice that ingested three doses of transformed tubers developed anti-LTB fecal IgA and anti-LTB serum IgG antibody

responses equivalent or greater than responses developed by mice gavaged orally with 5 µg purified LTb. Tubers containing the higher levels of LTb produced significantly higher anti-LTb fecal IgA responses. Control mice that were fed untransformed potatoes had no detectable anti-LTb fecal or serum IgG responses.

In experiments comparable to those performed by Haq *et al.* [18], similar results were obtained with potato plants expressing the B subunit of the cholera toxin (CTb) in both microtuber and leaf tissues where it formed up to 0.3% of the total soluble protein [20].

Subsequently, the United States Food and Drug Administration gave their approval to conduct the first human clinical trials with transgenic potato tubers that had been transformed with the optimized LTb gene [21]. Fourteen healthy adults ingested three doses of either 100 g of transgenic potato (six subjects), 50 g of transgenic potato (five subjects) or 50 g of wild-type potato (three subjects). The amount of LTb ingested ranged from ~0.4–1.1 mg/dose (mean level of 0.75 mg/dose). The presence of IgA and IgG anti-LT antibody secreting B cells seven days after immunization reflected immunological priming of the gut mucosal immune system. The IgG antibodies in all responders remained elevated when measured 59 days after ingestion of the first dose. In a toxin neutralization assay, 73% of the volunteers (8 out of 11) who had ingested transgenic potatoes developed neutralization titers >1:100 for 100 pg toxin at some point after immunization. In addition, 50% of the subjects (5 out of 10) developed a fourfold rise in secretory IgA.

The concept of oral vaccination is particularly applicable against diarrheal diseases, and edible vaccines could provide a reasonable strategy. Regarding these publications, it is questionable whether protective immunity can only be reached by antitoxin antibodies. In the case of ETEC diarrhea, a few epidemiologic studies provide evidence that acquired protective immunity is largely directed against fimbrial colonization factors of ETEC [22,23]. Moreover, LTb and CTb do not only function as immunogens but also as adjuvants for co-administered antigens [24,25]. Therefore, one major point of concern that has not been addressed in the mentioned studies, is the possibility that an immune response of unwanted intensity might also be raised against the food vehicle.

Dietary lactic acid bacteria as oral delivery systems for antigens

Live bacterial vectors have been used as carriers for the production and delivery of protective antigens at mucosal surfaces. Until recently, these candidate oral vaccines were mostly derived from pathogenic or invasive bacteria. This made it necessary to select or construct strains that are largely attenuated but still retain their immunogenicity [26]. As an alternative to this strategy, non-pathogenic commensal or food starter strains have been evaluated as mucosal (oral)

delivery systems. This approach covers the use of dairy or meat starter cultures such as *Lactococcus lactis* or *Staphylococcus carnosus*, and a number of *Lactobacillus* species from environmental, food or human sources. In addition to their 'generally recognised as safe' status, these bacteria offer a number of advantages as live carriers [6,27–29].

The first proof that non-invasive Gram-positive bacteria could act as an efficient live mucosal vaccine was obtained with a commensal of the human oral cavity, *Streptococcus gordonii* [30]. Since then, several publications have reported the successful use of LAB as expression hosts for a variety of bacterial and viral antigens. However, examples of detailed immunization studies remain relatively limited (for recent reviews see [6,31–35]). Although the majority of studies have been conducted in mouse models, one paper has reported immunization of monkeys [36]. In general, the immunogenicity of recombinant LAB strains was established by systemic administrations before testing the nasal, oronasal or intragastric routes. In a limited number of cases, vaginal immunizations were also described [37].

The most complete immunological studies have been performed with the non-toxic binding subunit C of the tetanus toxin (TTFC). This 47 kDa antigen was chosen as it allows the protective ability of the induced circulating antibodies to be measured by performing seroneutralization and lethal challenge assays [38]. Using this model antigen and common biological tools, a controlled comparison of different LAB carriers was achieved (reviewed in [6]). This family of microorganisms is very large, including over 200 species, which vary substantially in their physiological and genetic properties. Individual species or strains differ by their capacity to survive and persist *in vivo* and by their intrinsic immunomodulatory properties [6,29,39] — as a consequence, the choice of the candidate vaccine strain might, in principle, lead to different delivery systems [6,31,32,35].

The three LAB systems studied in most detail include the cheese starter derivative *Lc. lactis* MG1363, the human commensal *S. gordonii* and different lactobacilli, mainly belonging to the species *Lactobacillus plantarum* (e.g. the human isolate NCIMB8826 and the silage strain 256) and *Lactobacillus casei/zeae* (e.g. strain ATCC 393) [27,32–35,40,41]. The synthesis of TTFC was targeted to the cytoplasm or to the cell wall of each of these carriers, usually leading to higher production levels (≥ 1 –10% total protein content) in the cytoplasm. Although cell-surface presentation ($\geq 10^3$ – 10^4 TTFC molecules/bacterial cell) seemed to be more immunogenic at equal antigen doses, some of the corresponding recombinant strains were found to be quite unstable, which would hamper their use on a larger scale (N Reveneau, P Chagnaud, A Mercenier, unpublished results). This was not the case for *S. gordonii*, however, which proved to be remarkably flexible in terms of the length (sequences of 15–441 amino acids) and the nature (bacterial, viral or eukaryotic origin) of the epitopes or antigens that could be exposed on its cell surface without affecting

viability or persistence of the recombinant strains in the oral or vaginal cavities [32,33,37]. Interestingly, concomitant expression of the mucosal adjuvant LTB and an antigen was reported for this host [42] (G Pozzi, personal communication).

The work achieved with TTFC as a comprehensive model antigen has proven that harmless Gram-positive bacteria are able to efficiently present antigens at the mucosa-associated lymphoid tissue (MALT). In the best mucosal immunization schemes, TTFC-specific systemic IgG and local IgA were elicited. Dominant serum isotypes included IgG1, IgG2a and IgG2b indicating that both the Th1 and Th2 cellular subtypes were stimulated, independently of the route of administration (intranasal, oronasal or intragastric). The serum IgG responses were very high, reaching end-point titers up to 10^5 – 10^6 or 10^4 – 10^5 after nasal or intragastric immunizations, respectively. When tested, these responses were shown to be protective. For lactococci and lactobacilli, successful intragastric administration required more frequent and higher antigen doses than nasal administration. By contrast, as *S. gordonii* is able to persist in the oropharyngeal and vaginal cavities of mice for several weeks, a single dose of live carriers was usually sufficient to perform immunization by these routes. It is difficult to ascertain if this difference between *Lc. lactis* and *Lactobacillus*, and *S. gordonii* results from their respective capacity to replicate (rather than persist) *in vivo* or from their intrinsic immunomodulation capacities. Although lactococci and lactobacilli have been shown to be relatively poor antigenic carriers, little information is available concerning the intrinsic immunogenicity of *S. gordonii*.

The strong seric immune responses described previously were usually accompanied by the production of significant local IgA in broncho-alveolar or intestinal lavages, in faecal pellets or in vaginal washes depending on the immunization route. TTFC-specific T-cell proliferation was also observed in draining lymph nodes after nasal administration [27,32,33,35,40,41].

In most immunization protocols described so far, live bacterial cells were administered to mice. This seems to be a critical issue in the case of vaginal administration of *S. gordonii* [43]. By contrast, live and UV- or mytomycin C-inactivated *Lc. lactis* MG1363 and *L. plantarum* NCIMB8826 were shown to elicit almost equivalent antibody titers after nasal administration; however, the live bacteria gave superior protection [35,40]. Live carriers were absolutely necessary for the delivery of biologically active interleukins (IL-2, IL-6 or IL-10) by *Lc. lactis* [44,45••]. This was also found to be the case when recombinant *S. gordonii* synthesizing a microbicidal antibody was used to treat experimental vaginitis in rats [46••]. In both cases, it was necessary to secrete the biotherapeutic agent to optimally achieve the targeted biological effect.

Maassen *et al.* [47] also proposed that lactobacilli manipulated to secrete an autoantigen into the gastrointestinal

tract might induce tolerance to that immunogen instead of an adaptive immune response. However, definitive proof that this approach can be followed to treat autoimmune or allergic diseases remains to be established; this is presently a very active area of research.

Although for these specific applications secretion of the heterologous molecules seems to be necessary, antigens were usually presented at the cell surface when using *S. gordonii* as a carrier. For lactococci and lactobacilli, most of the immunological studies were performed with recombinant strains producing antigen in the bacterial cytoplasm. In this case, successful immunization was found to strongly depend on the antigen dose delivered to the nasopharyngeal- or gut-associated lymphoid tissue. Therefore, a variety of expression systems (including food-grade vectors and chromosomal integration technology) were developed for these hosts, which allowed high intracellular production of the antigen [6,35]. Different strategies, including the construction of engineered leaky lactococcal strains [48], are currently being developed to increase the efficiency and the safety of LAB as live vaccine vectors [35].

An original alternative to the systems described has recently been designed by Leenhouts and colleagues (Leenhouts K, Yasawardena S, Zomer A, Venema G, Kok J, Ramasamy R, Abstract, EuroLAB Conference, 3–6 July 2001, Cork, Ireland). To reduce the risk of transfer of heterologous DNA to other microorganisms, these authors have developed a technology to efficiently bind protein hybrids of the lactococcal autolysin AcmA and the protective antigen/epitope, in a non-chemical non-covalent way to the cell surface of non-recombinant LAB. Nasal and intragastric administration of a model *L. lactis* strain (carrying a fusion between AcmA and the MSA2 surface protein of *Plasmodium falciparum*) showed that it was as immunogenic as a recombinant strain displaying the corresponding MSA2–AcmA hybrid at the cell surface. Even though the full potential of this system needs to be further evaluated, it might represent an elegant means to prevent the use of live recombinant strains.

Conclusions

For infectious diseases where local protective immunity is as important as a systemic immune response, oral or edible vaccines could become the strategy of choice. Because of the possible degradation in the gut, presentation of protective antigens by the oral route might require higher doses of immunogen and/or other adjuvants than parenteral delivery. Therefore, when developing new mucosal vaccine formulations based on the use of recombinant plants or LAB, it should be taken into account that the induced immune response could lead to inflammatory or allergic reactions resulting in a breakdown of the physiological tolerance towards the food antigen.

Although genetically modified (GM) plants are being developed to become edible vaccines, the research conducted with LAB has not currently been extended to the

preparation of 'vaccines based on fermented foods'. Although this option is not excluded, detailed studies, probably on plants, to evaluate the potential side-effects of edible vaccines will have to be conducted first.

Transgenic plants and bacteria offer advantages and drawbacks as vaccine carriers. To name only a few, GM plants cause, in principle, less problems in terms of confinement whereas gene technology and genomic analysis remain much better controlled in GM microbes. Nevertheless, the present state of knowledge does not allow us to predict which of the two discussed vaccine carriers will be the safest or the most efficient.

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