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Abstract
The advent of technologies to express heterologous proteins in planta has led to the proposition that plants may be engineered to be safe, inexpensive vehicles for the production of vaccines and possibly even vectors for their delivery. The immunogenicity of a variety of antigens of relevance to vaccination expressed in different plants has been assessed. The purpose of this article is to examine the utility of plant-expression systems in vaccine development from an immunological perspective.

Introduction

From its genesis, vaccine development has primarily been an empirical science; originating with the chance discovery of Jenner that a relatively benign cowpox infection protected an individual against the considerably more deadly smallpox (Jenner 1798). Based on this observation and his own work on the germ theory of disease, Louis Pasteur pioneered the classic approach to vaccine development: attenuating the pathogenic agent such that its capacity to cause disease was limited but its antigenic structure unchanged (Pasteur 2002). During the heyday of vaccine development that followed, it was recognized that certain diseases caused by proteins, elaborated by bacteria and toxins, could be rendered apathogenic and used to vaccinate against the disease (Ramon and Zoeller 1927). Thus, by the beginning of the twentieth century, the basic attributes of a successful vaccine had been established. Essentially, the causative agent of a disease was modified to dissociate its ability to induce a protective immune response from its pathogenicity. Many of the vaccines for infectious diseases commonly in use today still consist of preparations of attenuated viruses or inactivated viruses and toxins (CDC 2002). The use of such reagents is termed active vaccination because their success is dependent upon the induction of an immune response in the recipient. This contrasts with passive immunization where the administration of preformed antibodies is used to confer immunity, as naturally occurs between the mother and offspring during pregnancy and early development.

Vaccination by infection with an attenuated variant of a pathogen should provide the best long-term protection by eliciting the full range of immune effectors and immunological memory, the capacity to rapidly mount a recall response to an antigen. Nevertheless, even a vaccine that induces an incomplete immune response may be considered successful if it prevents disease, the primary objective of

vaccination. For example, passive vaccination can be very effective at neutralizing a disease-causing toxin but does not induce immunological memory. Nevertheless, in rabies postexposure prophylaxis the passive administration of virus-neutralizing antibodies provides an underlying active immune response the additional time required to develop and clear the infection so that rabies with its lethal outcome is avoided (Hanlon et al. 2001). Similarly, active vaccination with a noninfectious vaccine is unlikely to generate a cytotoxic CD8 T cell response, which generally requires infection of target cells, but can be efficacious in protecting against an intracellular pathogen. In this case, the response would limit the capacity of an infectious agent to invade (antibody) as well as provide the immunological memory (T helper cells) that would accelerate the induction of the CD8 T cells or other cytotoxic effectors required for clearance. Under normal circumstances, vaccination is not expected to prevent subsequent infection with a pathogen, as this would require the maintenance of high levels of pathogen-neutralizing antibodies at the point of entry. While this is theoretically possible through IgA secretion in the gut, in practice even the clearance of an enteric virus does not prevent subclinical infection with the same virus (Weinstein and Cebra 1991). Thus the objective of vaccination is to elicit an antibody response that interferes with the invasion of the target pathogen and to prime T helper cells such that the generation of a complete response encompassing humoral and cellular immunity is enhanced during the natural infection.

As knowledge of immune function as well as the pathogenesis of different diseases advanced the uses of vaccination expanded into areas distinct from protecting against infection. One of these areas is immunomodulation. An example of this would be passive immunization with Rh-specific antibodies to prevent sensitization of Rh-negative mothers against the Rh antigen, which has been used to modulate immunity for nearly 50 years (Kumpel 2002). Recently trials have been conducted to determine if active immunization can be used to treat autoimmune disease (Vandenbark et al. 2001; Cohen-Kaminsky and Jambou 2005; Li et al. 2005). Another rapidly growing use for vaccination is in cancer therapy. With the identification of antigens expressed exclusively or at higher levels than normal by transformed cells, a variety of active and passive anti-cancer vaccines are in development (Dredge et al. 2002; Vichier-Guerre et al. 2003; Lenarczyk et al. 2004; Bodey et al. 2000; Ko et al. 2005).

Molecular technologies enabling both antigens and antibodies to be expressed by plant-based systems have been developed. The purpose of this article is to examine the potential advantages and disadvantages of the in planta production of vaccine reagents from an immunological perspective.

Basic Immunology: Antigens and Immunogenicity

Antigens are the structures that are recognized by T cells, B cells and antibody in an immune response. The capacity of an antigen to induce an immune response is its immunogenicity. Most antigens are protein and not inherently immunogenic. This is likely to be at least partly due to the nature of antigen recognition. While B cells and antibodies can passively interact with intact antigens, T cells recognize antigens that have been processed and presented at the cell surface, an active process. For example, to stimulate the helper T (CD4) cells that promote the expansion and maturation of different immune

effectors, antigen must be taken up, processed into peptides, and presented by specialized antigen-presenting cells (APCs) in the context of Class II major histocompatibility complex (MHC) antigens and second signals (Robinson and Delvig 2002). A protein that does not trigger uptake and presentation by APCs, as is the case for most self-proteins, may be nonimmunogenic yet still possess antigens. Under experimental conditions, these can be revealed by administering the protein together with an adjuvant to stimulate APC function (Schijns 2001). In reality, most natural immune responses are generated against invading pathogens and attributes of the infection likely provide the necessary immunogenic stimuli. Several toxins are among the limited group of noninfectious agents known to be highly immunogenic. Notably with respect to vaccine development, some of these remain immunogenic when their toxicity is attenuated.

Possible Advantages and Limitations of Using Plants to Produce Reagents for Active Vaccination

Plants as Expression Vectors

Edible plants expressing antigens that elicit protective immunity serve as the basis for the ideal vision of a vaccine that is inexpensive to both produce and deliver. While concerns about the impact of engineered plants on the environment and issues of profitability may dictate otherwise, the model plant-based vaccine could be grown locally using existing agricultural methods, harvested and fed to subjects. The plant approach would yield a reagent free of potential contaminants from the originating pathogen and other human or mammalian cell-based expression systems. Characteristics of the plant cells and tissues may be utilized to provide sufficient protection for the vaccine moiety to reach the small intestine for uptake and the induction of an immune response. Moreover, plant systems may be able to produce antigenic structures that are difficult to express in eukaryotic cell culture.

Plant expression systems have been successfully used to produce relatively complex proteins including structurally intact, active antibody molecules. These consist of two heavy and two light chains with a total molecular weight of approximately 150 kDa. Thus antigen complexity is not a concern and, at present, a low-yield and weak immunogenicity are the major limitations of plant antigen expression systems. There are a number of approaches being investigated to increase the yield of foreign proteins expressed in plants (reviewed in Gleba et al. 2005; Ko et al. 2003) and any obstacle here will likely be overcome in the near future. On the other hand, attempts to improve the immunogenicity of antigens expressed by plants have met with only partial success and this objective is not without concerns, as discussed in the following sections.

Plant Viruses and Bacteria as Expression Vectors

In a somewhat different but related approach to the use of engineered plants, vaccine antigens may be expressed by plant viruses or bacteria. In this case, plants are used to produce the agent, which can then be administered in purified form or using the infected plant tissue as a vehicle. The expectation here is that based on their structures, the plant virus or bacterium may share some immunogenic attributes but

none of the pathological properties of human pathogens. While plant bacteria can express relatively complex antigens, the use of plant viruses as expression vectors may be restricted to relatively simple antigenic determinants because of issues with virus assembly. The inability to express intact antigenic structures has repercussions for the utility of the construct. For example, short peptide sequences expressed in an appropriate context may be able to induce a limited T cell response but are unlikely to trigger antibody production. Thus there would be no capacity to neutralize the target pathogen and the protective attributes, if any, would depend on a more rapid induction of a comprehensive immune response when an infection occurred. Yields of the vaccine antigen, in these expression systems only a fraction of the plant virus or bacteria, are also a concern. In addition, it is conceivable that the response to a weak antigen may be negatively impacted by prior tolerogenic exposure to native plant viruses and bacteria naturally present in the diet.

Our experience with the plant bacterium *Clavibacter xyli cynodontis* and an expression system using the coat protein of the alfalfa mosaic virus (AMV) expressed by tobacco mosaic virus highlights some of the differences between these two approaches as well as shared limitations. We were able to express intact rabies virus nucleoprotein (54,000 MW) in *Clavibacter* and use this to readily elicit a nucleoprotein-specific immune response (Fig. 1). On the other hand, we were limited to expressing a peptide (N31D) derived from rabies nucleoprotein and glycoprotein in the AMV, achieving only relatively weak immunity (Yusivov et al. 2002). Nevertheless, both approaches were limited by the difficulty in obtaining high yields of material from infected plant tissues. For our *Clavibacter* experiments, the bacteria was grown in vitro and extensive purification of the virus from infected plant tissues was necessary to obtain a response in the AMV experiments. Any requirement for purification would place an additional financial constraint on the use of plant bacteria and viruses.

Basic Immunology: Routes of Vaccine Administration

Most existing vaccines are administered by injection into the dermis or muscle tissue. This primarily targets T-helper cells and a circulating IgG antibody response, even where a mucosal IgA antibody response may be more appropriate, but circumvents any difficulty with antigen stability in the gut. Vaccination targeting the naso-oropharynx may provide a means of inducing both IgA and IgG responses as well as reduced concerns about antigen stability but presents technical challenges with respect to administration. The oral route is clearly preferred. However, the antigens of an orally administered vaccine that targets the mucosa of the small intestine have to survive the environment of the gut and be taken up in an immunogenic form by the appropriate cells in the small intestine. The nature of the antigen and its carrier both contribute to this process. In our studies of antigens expressed by the plant bacterium *Clavibacter xyli cynodontis* (CXC), we compared the immune responses induced by intraperitoneal (i.p.) versus oral administration of two *Clavibacter* constructs: one expressing rabies virus nucleoprotein (N) and the second expressing *Bacillus thuringiensis* toxin (BT). Both were effective at inducing antibody responses to their respective antigens after i.p. injection but only a BT-specific response was seen after

oral administration (Fig. 1). We expect that the inherently greater immunogenicity of the BT construct as well as the ability of BT antigens to survive the gut are important in this regard.

Plant expression systems may provide a natural means to preventing degradation of antigens by the gut. Antigen expression can be targeted to a variety of locations in different plant species and certain plant structures, plant cell walls, and plant cell organelles all have the potential to protect against antigenic degradation. For example, seeds may be particularly suited to the expression of high levels of antigen in a relatively stable package. While there has been extensive work on the expression of various vaccine antigens by plants, studies of the impact of targeting different plant structures on antigen stability are in early stages. Ideally, a plant-based structure that degrades and releases antigen in the small intestine can be identified and will function as a natural enteric capsule. However, a nonreplicating mucosal vaccine will primarily trigger a local secretory IgA response and T helper cell response. Cytotoxic T cells, an important arm of the immune response against pathogens invading across the mucosa, would not be efficiently induced. It remains to be proven for each pathogen whether or not such a limited response can limit the spread of infection and protect against disease.

Enhancing Immunogenicity of a Potential Plant Vaccine Versus the Risk of Breaking Tolerance to Food Antigens

Strategies to Make Antigens Expressed by Plants More Immunogenic

As a general rule, most noninfectious, nonreplicating antigens are not very immunogenic, the exceptions being structures that have unusual stimulatory properties such as toxins and superantigens. To induce an immune response under experimental conditions, an antigen is administered together with an adjuvant, a substance with properties that enhance the immune response to associated antigens. Adjuvants generally function by stimulating the activity of APCs such that the associated protein is efficiently taken up and presented in an immunogenic fashion to T cells. As our understanding of immunity has improved, a variety of structures that naturally have adjuvant-like properties have been identified including certain toxins (Lyche 2005; Choi et al. 2006), components of bacterial cell walls (Gustafson and Rhodes 1992; Jalava et al. 2003), DNA with CpG motifs (Krieg 2002), double-stranded RNA (Cui and Xiu 2006), and uric acid crystals released by dying cells (Shi et al. 2003). Strategies currently used to make antigens expressed by plants more immunogenic are, for the most part, based on those successful for other nonreplicating antigens, including the incorporation of an apathogenic toxin in the construct (Choe et al. 2006). However, there are other possibilities based on the differences in glycosylation between plants and other antigen expression systems. For example, proteins retained in the endoplasmic reticulum of plants undergo high mannose glycosylation (Ko et al. 2003) and mannose receptors are expressed by dendritic cells, the specialized APCs that drive primary immune responses (Diebold et al. 2002). Thus there is no doubt that the immunogenicity of antigens expressed in plants can be improved by several of the above approaches and an effective injectable vaccine can be produced in planta. However, as is the case for other noninfectious, nonreplicating oral vaccines, enhancing the immunogenicity of a plant-based

oral vaccine is more problematic. The best characterized proteins with adjuvant activity following oral administration are the toxins of cholera and enteropathogenic *Escherichia coli* (Fujihashi et al. 2002). Considerable efforts have been made to dissociate the pathological attributes of cholera and LT toxins from their oral adjuvant properties, with varying success (Fujihashi et al. 2002). Subunits of these toxins can readily be expressed in the context of vaccine antigens in plant systems (Choe et al. 2006). Nevertheless, without other means of enhancing the immunogenicity of nonreplicating oral vaccines, repeat immunization using a toxin subunit may be limited, particularly if the toxin subunit is more immunogenic than the vaccine antigens.

Risks of Breaking Tolerance

Under normal circumstances, an ingested antigen that is taken up in the gut induces tolerance, an immunologically unresponsive state to the antigen. The existence of food allergies attests to the fact that certain food antigens can be taken up intact in the gut and induce an immune response in some individuals. One of the primary concerns of using plant-based systems for vaccination is that the vaccine construct may provoke a response to plant antigens, breaking tolerance and causing a food allergy. This is not simply a matter of a strong immune response occurring in the presence of food antigens or tolerance would be broken whenever someone gets a gastrointestinal infection. The prevalence of allergies to certain foods, for example peanuts, demonstrates that the nature of the antigen is an important risk element for the development of a food allergy. To induce a food allergy in experimental animals, a common food allergen must be administered with cholera toxin, a potent mucosal adjuvant (Helm et al. 2002). Since a nonreplicating oral vaccine likely must have adjuvant properties to be effective, there is a chance that sensitization against plant antigens contained in the vaccine may occur. The possibility that any such response would elicit the IgE antibodies commonly involved in an allergic reaction is more remote. In reality, there is probably as much of a chance that the mechanisms responsible for the maintenance of tolerance to plant-based food antigens may negatively impact the response to antigens expressed by the plant. Experiments with effective plant-based oral-mucosal vaccines are necessary to resolve these issues.

Other Uses of Plant-Based Immunological Reagents

Immunomodulation

While oral tolerance to experimental antigens has been studied for a number of years, relatively little is known about how tolerance to food antigens is generated and maintained. The possibility that the oral administration of antigen may induce tolerance or immune bias and reduce the severity of autoimmune disease has been investigated, with limited success, in multiple sclerosis (Faria and Weiner 2005). However, the possibility that there may be a greater therapeutic effect if such antigens are administered in the context of plant antigens that are a normal part of the diet has not been thoroughly examined. Clearly, the recognition of antigen is required to induce antigen-specific antibodies and, as noted above,

plant expression systems can be developed for enteric delivery of antigens. Further studies are needed to determine whether such systems may have added utility in the modification of aberrant immune responses in autoimmunity and food allergy.

Production of Antibodies for Passive Immunization in Plants

Passive immunization, the rapid provision of protection to an individual by the administration of preformed, antigen-specific antibodies, is extensively used in a variety of situations ranging from the postexposure treatment of infections such as rabies (Hanlon et al. 2001) to the prevention of Rh sensitization after an Rh-negative mother has delivered an Rh-positive child (Kumpel 2002). Passive immunization may prove to be the most effective means of protecting a population after a bioterror attack in that the delay required for the production of protective levels of antibody after active immunization is eliminated and the level of protection provided when necessary is uniform. Extensive stocks of antibodies are required for these applications as well as the use of passive immunization in immunocompromised individuals. At present, most antibodies used for passive immunization are isolated from the sera of immunized individuals, limiting their supply, and presenting a potential risk of contamination with human pathogens. Monoclonal antibodies are now being investigated as replacements for the more crude immunoglobulin preparations for certain applications (Hanlon et al. 2001; Sawyer 2000; Keller and Stiehm 2000). However, the production of monoclonal antibodies using conventional culture technologies is expensive and often does not eliminate the possibility of contamination with a pathogen. As noted above, intact, functional antibody molecules have been successfully expressed in plants (Ko et al. 2003; Verch et al. 1998) and plant-based technologies may prove advantageous over conventional antibody production methods in cost and safety.

Conclusions

There is no doubt that plants can be used as factories to produce immunological reagents and that such products will become widely available in the foreseeable future. Any nonreplicating injectable vaccine could be expressed in planta. However, as is the case for any noninfectious oral vaccine, the prospects of developing plant-based edible vaccines are more difficult to predict. To be successful, several elements of the system must come together: (1) the nature and level of expression of the antigen; (2) the effectiveness of the plant tissues as a delivery vehicle to protect against antigen degradation in the gut; (3) the capacity of the construct to promote antigen uptake in the gut; and (4) the immunogenicity of the construct. Each new construct studied that addresses any of these issues provides us with a better understanding of the complex interactions between the immune system and ingested antigens and closer to the ultimate goal of a safe, inexpensive, and effective plant-based oral vaccine.

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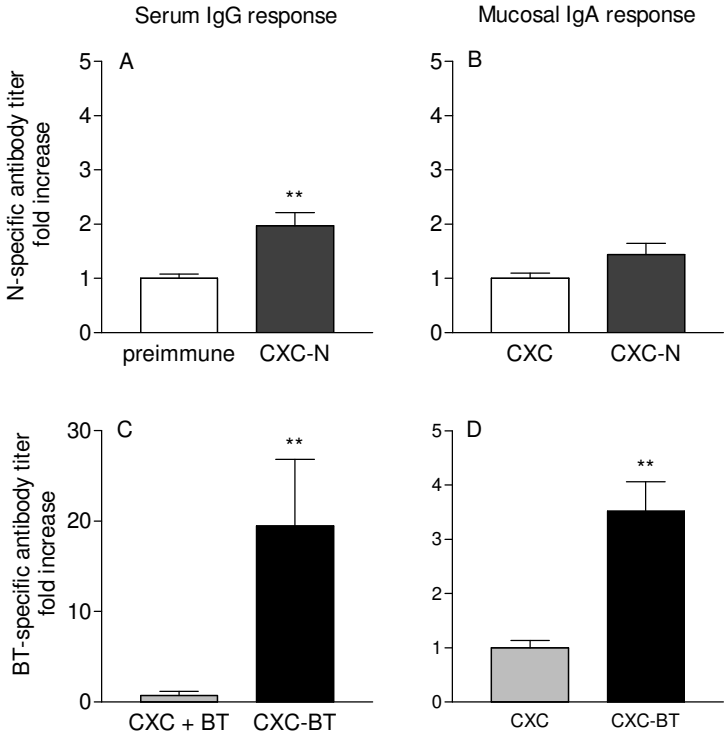
Fig. 1. Comparison of the immunogenicity of different *Clavibacter xyli cynodontis* constructs following parenteral and oral immunization.



Groups of 10 8 week old female Swiss-Webster mice were immunized with *Clavibacter xyli cynodontis* (CXC) engineered to express rabies virus nucleoprotein (N), *Bacillus thuringiensis* toxin (BT), or the native bacterium (CXC). Immunization consisted of a single dose of 5×10^6 CXC in saline intraperitoneally or two doses of 5×10^7 CXC in 10% sucrose at 2-week intervals per os. Serum and fecal pellet antibodies specific for N (**A, B**) and BT (**C, D**) were assessed 21 days after immunization by ELISA using antigen-coated plates and antibody isotype-specific secondary antibodies. The results are expressed as the fold-increase in antibody titers by comparison with preimmune levels. Statistically significant differences between the groups detected by the Mann-Whitney test are denoted by **, $p < 0.01$