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Potato-derived Antigens for use as a Vaccine against Alzheimer's Disease

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ABSTRACT

An alternative vaccine, developed from plant-derived antigens of transgenic crops, is considered to be a good candidate for the next generation of vaccines. A plant-derived vaccine could be orally administered by consumption of the antigen-transformed plant product, ultimately inducing an immune response in the recipient. In the past ten years, a number of antigens have been successfully expressed in plants and orally delivered to animals, where they elicit an immune response. To date, efforts to produce antigen proteins in plants have focused on potato, tobacco, alfalfa, and maize hosts. The choice of plant species is extremely important, and is generally determined by how the vaccine is to be applied in the future. Potato tubers are likely to have fewer phenolic compounds and a less complex mixture of proteins and lipids than green leaves, which might be an advantage during purification. Another advantage of tubers is their ability to be stored for long periods of time. Also, the transformation of potatoes is technically easy and the expression of foreign genes is relatively stable, and the deletion of transgenes is rare since potatoes are propagated vegetatively. The successful development of a potato-derived vaccine would allow manufactures to meet huge, steady demands. Studies have focused on the generation of transgenic potatoes expressing the main antigens related to Alzheimer's disease (AD) and an analysis of potatoes. AD is a neurodegenerative disorder in the elderly characterized by memory loss and cognitive impairment. As AD progresses, insoluble amyloid plaques forms are deposited in the brain. Suppressing the generation of beta-amyloid (Aβ) is considered an effective strategy for preventing and treating AD. Here, we report on the feasibility of potato-derived antigens for use as a vaccine against AD.

Keywords: beta-amyloid, edible plant vaccine, immunogenicity, transgenic potato

CONTENTS

INTRODUCTION

Since the early 1970s, plant biotechnology has been used to solve several agricultural problems. Continued advances in the field of biotechnology have addressed stress tolerance, quality improvement, and mass production of useful proteins. The main proteins produced include antigens, antibodies, enzymes that are of immense importance in therapeutics, and proteins for various pharmaceutical and industrial applications (Giddings *et al*. 2000; Daniell *et al*. 2001). Though many of these proteins are made in bacterial, fungal, or animal systems, plants are now preferred for manufacturing these proteins. There are many advantages to producing useful human proteins in plants. Plant systems provide an opportunity for high production with decreased production costs; they also eliminate the risk of contamination from potential mammalian pathogens, and there is no need for cold storage of the recombinant proteins (Korban *et al*. 2002).

PLANT-DERIVED ANTIGENS

Transgene expression and recombinant protein accumulation, stability, and processing in plants has resulted in the development of novel strategies, such as the use of edible plants for the delivery of antigens. In 1990, the World Health Organization emphasized the need for new technologies to advance immunization programs. The mission was that new technologies would produce vaccines for diseases with limited preventative options, and improve existing vaccines by reducing production costs and resolving logistic difficulties. In particular, new technologies might eliminate the need for needles during immunization, possibly generating heat stabile, oral, multi-component vaccines that require reduced or one-time administration. The development of needle-free vaccines is considered one of the "grand challenges in global health" which was recently defined by Varmus *et al*. (2003).

Curtiss and Cardineau began pioneering experiments using transgenic plants to produce vaccines (Curtiss and Cardineau 1990). The concept of plant-derived vaccines

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was realized when Hepatitis B surface antigen was expressed in tobacco (*Nicotiana tabacum* cv. 'Samsun') by Mason *et al*. (1992). Over the past decade, a number of antigens have been successfully expressed in plants and orally delivered to animals, providing immunogenicity. These antigens include the disease-related virus capsid and surface proteins, and pathogenic bacterial antigens (Haq *et al*. 1995; Mason *et al*. 1996; Arakawa *et al*. 1997).

The Hepatitis B virus vaccine is based on the highly immunogenic surface antigen, HBsAg which has been expressed in tobacco and potato (*Solanum tuberosum* L.) (Mason *et al*. 1992; Richter *et al*. 2000). The HBsAg extracted from transgenic tobacco leaves evoked an immune response when administered parenterally (Mason *et al*. 1992; Thanavala *et al*. 1995). Oral feeding studies using HBsAg-expressing potato tubers with a mucosal adjuvant demonstrated the possibility of obtaining both a primary immune response and a strong and sustained secondary immune response when the immunized mice were boosted with yeast-derived recombinant HBsAg antigen. Loss of immunogenicity upon cooking of potatoes was reported, and the requirement for an adjuvant for mucosal immunity was also reported.

Also, a non-toxic, immunogenic B subunit (CTB) of enterotoxin that helps the toxin bind to gut cells has been expressed in potatoes (Arakawa *et al*. 1997, 1998a) and tomatoes (Jani *et al*. 2002). Mice administered transgenic potato tubers expressing CTB fused with the endoplasmic reticulum (ER) retention signal showed induction of both serum and intestinal CTB-specific antibodies (Arakawa *et al*. 1998a). For expression in potato tubers, synthetic heat labile enterotoxin (LT-B) subunit of *E. coli* was expressed under a class I tuber-specific patatin promoter. The extracted protein from transgenic potato was used to immunize mice. The recombinant protein was immunogenic and could elicit local and systemic IgA responses in parenterally primed mice (Lauterslager *et al*. 2001).

Antigens for some of the enteric viral pathogens, such as Norwalk virus and rotaviruses, were expressed in potato (Mason *et al*. 1996; Yu and Langridge 2003). Mice immunized orally by feeding on potato tubers expressing recombinant Norwalk virus-like particles (rNV) developed serum IgG specific for rNV (Mason *et al*. 1996). Yu and Langridge (2003) produced transgenic potatoes expressing the capsid structural protein VP6 of the murine rotavirus. Oral immunization of mice with these transformed potato tuber tissues generated detectable antibody responses against the rotavirus capsid protein. The success in mouse trials led to the testing in human trials. Human volunteers orally immunized with either bacterial (LT-B) or viral (Norwalk virus capsid protein) protein developed specific serum and immune responses against these antigens (Tacket *et al*. 1998, 2000).

The concept of edible vaccines has been extended to various other diseases. Human papilloma virus (HPV) infection has been associated with cervical cancer. It was reported that VLPs were the most attractive candidates for developing a prophylactic vaccine against HPV infections, and L1 protein of papilloma virus types 11 or 16 can be expressed in transgenic plants to form immunologically functional VLPs (Varsani *et al*. 2003; Warzecha *et al*. 2003; Liu *et al*. 2005; Fernández-San *et al*. 2008). The immunogenicity test of this recombinant protein, performed by feeding transgenic potato tubers to mice, showed an anti-L1 response in about 50% of the animals (Biemelt *et al*. 2003).

Antigens for diabetes mellitus, human GAD65, and human insulin have been engineered for expression in transgenic carrots (*Daucus carota* cv. 'Berlicum'), tobacco, and potatoes, respectively (Arakawa *et al*. 1998b; Porceddu *et al*. 1999; Avesani *et al*. 2003). Oral administration of transgenic potatoes to diabetic mice resulted in a substantial reduction in insulitis and a delay in the progression of clinical diabetes. These results indicate the feasibility of oral delivery of plant-derived antigens for imparting immune tolerance against this T cell-mediated autoimmune disease (Arakawa *et al*. 1998b). Also, Webster and colleagues (Huang *et al*. 2001) expressed the measles virus hemagglutinin protein (MV-H) in plants, and mice were immunized by oral gavage leading to the induction of a low titer of MV-specific neutralizing antibodies. In a subsequent study, this group tested the plant-derived MV-H protein in prime-boost experiments. MV-specific serum IgG titers were greater in mice boosted with MV-H plant extract compared with those boosted with a control-plant extract (Webster *et al*. 2002, 2006). This research represents a significant step towards the development of a measles vaccine formulation that is effective, temperature-stable, easy to administer in a resource-poor setting, and amenable to large scale manufacturing.

There are various groups involved in making edible vaccines for animals as well. One example is a vaccine against the spike (S) protein of transmissible gastroenteritis virus (TGEV), which Tuboly *et al*. (2000) expressed in tobacco plants (2000). Pigs were immunized with the plant leaf extracts mixed with an adjuvant, but low levels of virus-neutralizing antibody were detected. In two independent studies, vaccine antigens for hemorrhagic disease in rabbits and infectious bronchitis (IB) in chickens have been expressed in transgenic potatoes and were successfully tested as an oral immunogen on rabbits and chickens, respectively (Martín-Alonso *et al*. 2003; Zhou *et al*. 2004).

Although pre-clinical trials for plant-based vaccines have been performed (Tacket *et al*. 1998, 2000), many challenges, including optimization of the expression level to suit the dosage requirements and stabilization during post harvest storage, need to be addressed. The first clinical trial was presented by Tacket *et al*. (1998) who tested the immunogenicity of bacterial antigens expressed in transgenic potatoes. In this study, 14 volunteers ingested transgenic potatoes or control wild-type potatoes. On day 28, serum antibodies were detected in ten of the volunteers.

In another trial, twenty human volunteers received 2-3 doses of transgenic potato expressing the Norwalk virus capsid protein (Tacket *et al*. 2000). Each dose consisted of 150 g of raw, peeled, diced potatoes. Nineteen (95%) of 20 volunteers who ingested transgenic potatoes developed significant increases in the numbers of specific IgA antibody–secreting cells. Four (20%) of 20 volunteers developed specific serum IgG, and 6 (30%) of 20 volunteers developed specific stool IgA. Tacket and colleagues (2004) also reported on the immunogenicity of LT-B delivered to humans via transgenic corn (*Zea may* L.). Volunteers were received three doses of defatted LT-B corn germ meal from transgenic corn or wild-type corn. Seven (78%) of the nine volunteers developed rises in both serum IgG anti-LT and numbers of specific antibody secreting cells after vaccination. Four (44%) of nine volunteers also developed stool IgA. These trials served as proof that humans can develop serum and mucosal immune responses to antigens delivered in transgenic plants products, although the level of serum antibody increases was modest.

The low expression level of protein is one of the remaining problems before commercial application is feasible. The 'magnifection' system and 'chloroplast expression' system show the highest expression levels of recombinant proteins to date. Magnifection provides up to 5 g of recombinant protein per kg of fresh leaf biomass, with the relative recombinant protein yield accounting for up to 80% of the total soluble protein (Gleba *et al*. 2005). Chloroplast genetic engineering offers up to 46.1% of the total leaf protein in the expression of *Bacillus thuringiensis* cry2Aa2 protein (Daniell *et al*. 2005). However, neither system is a normal and routine method applied since both have potential limitations. It is important to choose optimal techniques for each situation.

Although plant-derived vaccines offer many advantages, putative disadvantages are as follows; long development times to establish the platform technology, plant production is restricted to regulated field areas or greenhouses, negative public opinion regarding genetically modified (GM) plants, potential for induction of oral tolerance and aller-

genicity, and no approved oral adjuvants to aid in product development. Robert and Kirk (2006) explored the ethical and pragmatic dimensions of these assumptions.

VACCINES FOR ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a neurodegenerative disease which progresses over a prolonged period, and is the most common cause of dementia. As societies age and life expectancy increases in modern societies, the burden of caring for AD patients will increase. At the present time, neither effective treatments nor preventive vaccines are available for AD. The pathological hallmarks of AD are neuritic plaques and neurofibrillary tangles. Neuritic plaques are extracellular deposits of fibrils and amorphous aggregates of beta-amyloid (Aβ) peptides. Neurofibrillary tangles are intracellular fibrillar aggregates of the microtubule-associated protein tau that exhibit hyperphosphorylation and oxidative modifications (Taylor *et al*. 2002). Genetic and pathological evidence strongly supports the amyloid cascade hypothesis of AD (Hardy and Selkoe 2002). The accumulation and aggregation of Aβ appears in damaged neurons, which lead to the degeneration of synapses and eventually neuronal death. Namely, it is believed to be caused by the accumulation of Aβ, a toxic protein in the brain. Therefore, an effective strategy for the prevention and treatment of the underlying causes of AD would be the development of an agent that inhibits the degeneration of the nervous system by suppressing Aβ generation, thus circumventing its associated toxicity.

One promising approach to prevent and treat AD is based on stimulating the immune system to remove Aβ from the brain (Schenk *et al*. 1999). Several strategies, including active and passive immunization, have been examined. The initial reports that immunization with aggregates Aβ1-42 or passive immunization with anti-Aβ antibodies resulted in the clearance of Aβ plaques from the brains of Amyloid Precursor Protein (APP)-mutant transgenic mice were followed by several reports found that such immunizations could restore cognitive deficits in transgenic mice (Morgan *et al*. 2000; McLaurin *et al*. 2002; Orgogozo *et al*. 2003).

In a recent study, a variety of vaccination advances were made using the Aβ peptide. Schenk *et al*. (1999) and Morgan *et al*. (2000) reported that immunization of PDAPP (different transgenic strain) mice with synthetic, preaggregated Aβ42 raised an antibody against the 42-amino acid form of Aβ and reduced the extent and progression of AD pathology. Although immunization studies showed promising results in APP transgenic mice without detectable adverse effects, the recent extension of this approach to AD patients resulted in the development of meningoencephalitis, a potentially deadly inflammation of the brain and surrounding membrane, in a small but unacceptable number of patients (Orgogozo *et al*. 2003). This called AD researchers' attention to the issue of refining immunization methods. After stopping the vaccine-derived approach against Aβ, much progress has been made in designing a vaccine which is appropriate for human use (McLaurin *et al*. 2002; Wilcock *et al*. 2004). Nonetheless, an effective vaccine would be the ultimate solution to AD, and is currently one of the most exciting fields in AD research.

AD is characterized by the progressive formation of insoluble amyloid plaques and vascular deposits consisting of the 4-kD Aβ in the brain. For this reason, the enzymes responsible for the generation of these peptides are considered therapeutic targets important in the treatment of AD (Pastarino *et al*. 2004). Beta-site amyloid cleaving enzyme (BACE) has been characterized as the major β-secretase activity *in vivo* and *in vitro*. Formation of Aβ requires proteolytic cleavage of a large type-1 transmembrane protein, the APP, which is constitutively expressed in many cell types. BACE1 (β-secretase) inhibitors may prove beneficial in reducing the production of Aβ, because BACE1 knockout mice showed reduced Aβ production and do not exhibit any abnormal phenotypes (Luo *et al*. 2001). Hussain *et al*. (2007) demonstrated that oral administration of a BACE inhibitor can result in significant lowering of brain Aβ in APP transgenic mice.

WHY POTATOES?

Vegetables, especially potatoes and tomatoes, are good candidates for oral immunization. Potatoes are the fourth most important food crop consumed worldwide, and a prime example of a vegetatively propagated storage organ. The tubers are derived from underground stems or stolons which enlarge under favorable conditions to form tubers. The potato is propagated through seeds or cuttings, and grown in a controlled culture room or greenhouse or produced through tissue culture methods. The desired tissues are harvested from the plant and processed by grinding or freeze-drying to extract the total protein for delivery to humans. Potatoes are also facile and efficient transformation systems, with *in vitro* tuber production for quick assays, low potential for outcrossing in the field, and industrial tuber processing is already well established (Mason *et al*. 2002).

The potential advantages of producing vaccines through plant-systems are no injection requirements, which help reduce the cost of needles and necessity for highly trained staff, no contamination with blood or culture products, and stability at ambient temperatures for several years which means no refrigeration costs during distribution, and reduced waste of the product by heat or contamination. The successful development of a potato-derived vaccine would allow manufacturers to meet huge, steady demands.

Various crops have been used to develop plant-derived antigens. The choice of plant species (and tissue in which the protein accumulates) is important, and is usually determined by how the vaccine is to be used in the future. Plants such as tobacco and alfalfa (*Medicago sativa* L.) often produce proteins in their green leafy tissues with sheer productivity, but these tend to contain high concentrations of phenolic and other potentially toxic compounds (Daniell *et al*. 2001). Seeds are likely to have fewer phenolic compounds and a less complex mixture of proteins and lipids than green leaves, which can be an advantage during purification. Another advantage of seeds or tubers is their ability to be stored for long periods of time. Levels of single-chain Fv (scFv) in rice seeds did not significantly decline after storage at room temperature (Stöger *et al*. 2000), also in potato tubers stored in cold storage for 18 months lost only 50% of their functional activity (Artsaenko *et al*. 1998). In conclusion, potatoes are a good system in which to test the use of edible vaccines.

POTATO-DERIVED ALZHEIMER'S VACCINES

It is difficult to produce Aβ through mammalian expression systems because the protein is toxic to these cells. When the idea that Aβ could be used as a vaccine first emerged, there was concern that the injection of a peptide found in the body could induce an autoimmune response. This concern seemed to be supported when phase α clinical trials of a vaccine-directed approach against Aβ were stopped after several people developed symptoms of central nervous system inflammation. For this reason, the possibility of Aβ expression in plant cells for oral ingestion was investigated. Kim *et al*. (2003) reported, as a first step, the production of transgenic potato plants by introducing the gene encoding Aβ. They confirmed that it is feasible to produce Aβ protein in transgenic potatoes and that the protein product obtained exhibits identical morphological characteristics as the Aβ protein obtained by other methods. The morphological characteristics, including shoot growth and tuberization, were investigated with both non-transgenic and transgenic potato plants. No significant differences in shoot growth and tuberization were observed between the non-transgenic and transgenic plants.

Subsequently, Szabo *et al*. (2004) also reported a high

level expression of Green Fluorescent Protein (GFP)-Aβ1-40 and 1-42 peptides in pepper (*Capsicum annum*) using a new tomato mosaic tobamovirus-based hybrid replication vector. The fusion protein accumulated at up to 10% of the fresh weight of the tobacco, making it possible to test whether oral immunization by feeding plant samples could stimulate antibody production against Aβ peptides. Recently, Youm and colleagues (2005) carried out the cloning and expression of multimeric forms of human Aβ in potato plants to increase their expression level and detection efficiency. Moreover, they tested the ability of transgenic potato tubers expressing the tandemly repeated Aβ gene to serve as a novel delivery system for the oral immunization of BALB/c mice or transgenic mice, Tg 2576. Immunized mice produced serum antibodies against the Aβ1-42 antigen. In addition, Tg 2576 mice that were immunized with plant-derived Aβ showed a decrease in Aβ levels, as well as the number of plaques in their brains, without noxious side effects.

It is also known that transgene deletion is rare, since potatoes are propagated vegetatively. Transgene stability in *Agrobacterium*-transformed potato was seen among three generation of tuber-derived progeny plants. However, gene silencing or deletion of introduced transgenic DNA is also an inevitable phenomenon in nature. To provide potato-derived 5Aβ as vaccine material, a stable yield of 5Aβ protein through constant expression of the introduced transgene is the most important factor. In a recent study, we confirmed that the 5Aβ42 transgene was inherited and stably expressed through *in vitro* vegetative propagation over a period of 3 years without silencing. The 5Aβ42 protein, too, was well expressed in transgenic potatoes without change in its antigenicity. Stable storage conditions were vital in maintaining the stability of the potato-derived 5Aβ42 protein, and the expression level of the protein was not affected after three months storage at low temperature.

Until recently, pathogen related infectious diseases are introduced into plants for using as edible vaccines. In conclusion, here, we introduce examples of a human-originated disease, AD, and confirm the possibility of developing transgenic potatoes as vaccine material. This study is an example of active immunization for AD with unique approach, and we recognize that there are many ways to achieve this success. However, there remain long-standing challenges include understanding the potential for inducing oral immunologic tolerance, developing oral adjuvants which may be necessary to increase vaccine potency, controlling the dose for self-administered oral vaccines, determining the impact of plant glycosylation patterns on immunogenicity, and identifying the optimal plant expression system.

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