Plant-based Multicomponent Vaccines against Enteric Diseases

Jie Yu

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LOMA LINDA UNIVERSITY

Graduate School

Plant-based Multicomponent Vaccines against Enteric Diseases

by

Jie Yu

A Dissertation submitted in partial satisfaction of the Requirements for the degree of Doctor of Philosophy in Biochemistry

March 2001
Each person whose signature appears below certifies that this dissertation in his / her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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<td>adenine</td>
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<td>ANOVA</td>
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<td>ganglioside monosulfate one</td>
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<td>gut-associated lymphoid tissue</td>
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<td>Intraepithelial T lymphocytes</td>
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<tr>
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<td>lamina propria lymphocytes</td>
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<td>LT</td>
<td>heat-labile enterotoxin</td>
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<td>major histocompatibility complex</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MW</td>
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<td>NSP</td>
<td>non-structural protein</td>
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<td>OD</td>
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<td>Ori</td>
<td>origin of replication</td>
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<td>polymerase chain reaction</td>
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<td>RT-PCR</td>
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<td>RLU</td>
<td>relative light units</td>
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<td>TBS</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TNF</td>
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<tr>
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<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SCID</td>
<td>severe combined immunodeficiency syndrome</td>
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<td>slgA</td>
<td>secretory immunoglobulin A</td>
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<td>Th</td>
<td>T helper lymphocyte</td>
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<td>VLPs</td>
<td>virus like particles</td>
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<td>WBC</td>
<td>white blood cell</td>
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ABSTRACT OF DISSERTATION

Plant-Based Multicomponent Vaccines against Enteric Diseases

by

Jie Yu

Doctor of Philosophy, Graduate Program in Biochemistry
Loma Linda University, March 2001
Dr. William H.R. Langridge, Chairperson

To generate a plant-based multicomponent oral vaccine against cholera, rotavirus and enterotoxigenic *E. coli* (ETEC), we have constructed transgenic potato plants that synthesize the cholera toxin B and A2 subunits, the rotavirus enterotoxin NSP4 22 amino acid epitope and the ETEC fimbrial antigen CFA/I. To use cholera toxin as a carrier molecule for delivery of multiple antigens to the gut-associated lymphoid tissues, the rotavirus NSP4 epitope was linked to the cholera toxin B subunit (CTB) and the ETEC fimbrial antigen CFA/I was linked to the cholera toxin A2 subunit. Cholera toxin-antigen fusion proteins were detected in transformed tuber tissues by immunoblot assay and quantitated by enzyme linked immunosorbent assay (ELISA). The CTB-NSP4 protein expression level was found to be approximately 0.01% of total soluble plant protein (TSP) and the CFA/I-CTA2 protein expression level was about 0.002% of TSP. Assembly of the CFA/I-CTA2 subunit into the CTB-NSP4 pentamer was detected by immunoblot and $G_{M1}$ ELISA assays. The CFA/I-CTA2-CTB-NSP4 hetero-hexamer fusion protein complex retained its binding affinity for $G_{M1}$ ganglioside enterocyte membrane receptors. Serum and intestinal
antibodies against NSP4, CFA/I and CTB were detected in orally immunized mice. Analysis of IL-2, IL-4 and IFNγ cytokine levels in splenocytes isolated from immunized mice indicated the presence of a strong Th1 immune response to the plant synthesized antigens. Fluorescent antibody based flow cytometry analysis of immunized mouse spleen cells showed an increase in CD4+ T –helper cell populations. Following rotavirus challenge, passively immunized mouse pups showed a 50% reduction in diarrhea symptoms.

To generate a plant-based subunit vaccine against rotavirus, we have expressed the gene encoding rotavirus capsid protein VP6 in transgenic potato plants. The plant produced VP6 protein has a molecular mass similar to VP6 purified from virus and partially formed VP6 trimers. Mice fed transformed potato tubers containing VP6 generated serum and intestinal antibodies against VP6. The results of these experiments indicate that food plants can serve as safe and inexpensive vaccine production and distribution systems for simultaneous immunization against several diseases.
CHAPTER ONE
INTRODUCTION

1. General

The vaccine concept was first introduced by the English physician Edward Jenner in the late 18th century when he inoculated an 8-year-old boy, James Phipps with cowpox virus isolated from a milkmaid. This procedure called vaccination helped the patient develop resistance to smallpox. After more than a hundred years development, vaccination has become one of the most cost-effective medical intervention methods available for control and eradication of disease epidemics worldwide. However, implementation of a global vaccination program in developing nations has not been possible due to a combination of poor economic conditions and political unrest resulting in an inadequate health care infrastructure, and a prohibitive cost of essential vaccines.

To rectify this situation changes are required in the way vaccines are produced, distributed and administered to make them more effective, less expensive, easier to administer and more widely available. In 1990, the World Health Organization (WHO) launched the Children’s Vaccine Initiative establishing goals for developing vaccines that are safe, inexpensive, easily (orally) administered and widely accessible (Mitchell et al., 1993). Establishment of these goals ultimately led to the idea of producing oral vaccines in transgenic food plants (Mason et al., 1992).

In this dissertation we propose the construction of a multi-component oral vaccine in potato plants for prevention against three infectious enteric diseases: cholera, rotavirus and enterotoxigenic Escherichia coli (ETEC). In our vaccine design, the non-toxic cholera toxin B subunit was employed as a carrier molecule for targeting plant derived
antigens to the intestinal mucosal immune system. Genes encoding antigens or antigen epitopes from rotavirus and ETEC pathogens were fused to the cholera toxin B subunit and the recombinant proteins were synthesized in transformed potato leaf and tuber tissues. The protective efficacy of this multi-component food plant based oral vaccine was tested in mice. The experimental results generated by this project will help us determine the advantages and circumvent the difficulties in establishing food plants as vehicles for the production and delivery of multi-component vaccines for protection against infectious enteric diseases. The results of the proposed experimentation will permit us to improve the first generation plant vaccines to achieve greater targeting ability and higher expression levels for possible use in human vaccine clinical trials.

2. Transgenic Plants as Vaccine Production and Delivery Systems

Traditional methods of plant breeding have been practiced for hundreds of years with great success in improving plant characteristics and agricultural properties, such as improved protein content, resistance to insect and fungal attack and resistance to virus and bacterial disease. However the classical plant breeding approach is slow and imprecise due to the transfer of all the traits of the donor organism, which can alter the expression of the desired gene products. However in the past decade, the advent of modern molecular biology methods, especially recombinant DNA techniques has made a huge impact on plant biotechnology (Schell, 1987). The relatively short time required to identify and clone specific genes encoding desirable traits has made plant systems particularly valuable for production of recombinant molecules (Arntzen et al., 1995, 1997; Hugh et al., 1995). Genetic engineering technology has also been applied to the production of proteins of therapeutic value, especially pharmaceuticals and vaccines.
(Figure 1.). The first reported vaccine candidate expressed in transgenic plants was the cell surface adhesin protein (spa A) from *streptococcus mutans*, a major bacterial cause of tooth decay (Curtiss et al., 1990). The spa A gene was introduced into tobacco by agrobacterium- mediated transformation methods. The protein was expressed at levels of up to 0.02% of total leaf protein. Oral immunization with this plant-synthesized vaccine stimulated the production of protective secretary IgA in saliva. The 185kD size of spa A protein demonstrated the ability of the plant genome to accommodate large foreign gene inserts.

A great advantage of plant genetic engineering is that whole plants can be regenerated from single transformed cells. When a plant is wounded, a patch of fast growing cells called callus tissue grows over the surface of the wound. If a piece of this young callus tissue is removed and placed in a culture medium containing the appropriate nutrients and plant growth hormones, the cells will continue to grow and differentiate into shoots and roots and ultimately a whole flowering plant will be produced. Thus transgenic plants can be conveniently grown to produce stable breeding lines which can be propagated by conventional horticultural techniques and stored and distributed as seeds or vegetative proteins (tubers). Unlike bacterial or animal cells, plant cultivation is straightforward, does not require special media or equipment. Due to their photosynthetic nature, plants require only water, CO₂, soil and sunlight for growth and development. An obvious benefit of plants is the potential for scaled-up production (Tsafrir et al., 1998), in which large amounts of recombinant protein can be produced at a minimal cost. Agracetus, a
HOW TO MAKE AN EDIBLE VACCINE

One way of generating edible vaccines relies on the bacterium Agrobacterium tumefaciens to deliver into plant cells the genetic blueprints for viral or bacterial "antigens"—proteins that elicit a targeted immune response in the recipient. The diagram illustrates the production of vaccine potatoes.

**Fig. 1. Production of oral vaccines in transgenic plants.** (cited from Langridge, *Scientific American* 2000.)
plant biotech company, has developed a strain of corn that can produce 1.5 kg of pharmaceutical quality antibodies per acre. A cost-effective analysis carried out by the Scripps Research Institute showed that the antibodies could be produced at approximately one US dollar per gram (Ma et al., 1999).

To develop a recombinant subunit vaccine, the immunogenicity of the antigen depends on correct protein folding, structure and post-translational modifications. The eukaryotic system of plants provides significant advantages over bacterial or viral expression systems with respect to these issues. With regard to protein folding and structure, small peptides, polypeptides and even fully assembled and functional complex proteins can be expressed in plants. For larger protein molecules, assembly and protein folding are associated with the presence of ER-resident chaperones that are homologous with those involved in protein assembly in mammalian cells. Targeting recombinant proteins for secretion through the ER and Golgi apparatus can be achieved using either native or plant leader sequences (Maarten et al., 1992; Heijine, 1985; Munro et al., 1987). A typical example is the co-expression of antibody heavy and light chains to form a full-length antibody, functionally identical to its mammalian counterpart (Hiatt et al., 1989). Expression of the rabies virus glycoprotein in trangenic tomatoes demonstrated the ability of plant cells to carry out post-translational glycosylation essential for the biological function of proteins of animal origin (McGarvey et al., 1995). The plant glycosylation pattern differs somewhat from mammals in the composition of the complex glycans. The complex glycans in plant proteins tend to be smaller than mammalian complex glycans and differ in the terminal sugar residues. But for recombinant proteins expressed so far, this difference has not led to any detectable loss of structure or function.
Additional advantages of the plant production system include biological safety and ease of purification. Plant viruses do not infect humans. Plant produced vaccines eliminate the contamination of animal viruses. If tissue specific promoters are used, the vaccine antigens can be exclusively expressed in storage organs such as seeds, fruits or tubers (Arakawa et al., 1997; McGarvey et al., 1995; Wenzler et al., 1989). Extraction and purification from these tissues is relatively simple. The idea of an edible plant vaccine is also very tempting from a simplicity perspective. Vaccines produced in transgenic plants can be harvested by conventional machinery and administrated simply by eating the fruit or tubers that contain the appropriate amount of vaccine antigen required for immunization (Arntzen, 1997). To date there are more than 30 antigens from infectious pathogens and auto antigens from auto-immune diseases that have been produced in a variety of plants (Ma et al., 1999). The history and strategies for development of plant-based vaccines will be discussed in more detail in chapter three.

3. Mucosal Immune System

The mammalian mucosal immune system is an integrated network of antigen presenting tissues, lymphoid, cells and effector molecules, substantially different from the peripheral immune system, where lymphoid cells and effector molecules are confined to individual lymph nodes and spleen tissues where intercommunication occurs only by cell trafficking through the lymphatic and blood circulation (Kiyono et al., 1996). Another major difference is that induction of the peripheral immune response does not result in significant mucosal immunity. In contrast, induction of mucosal immune response can result in protective immunity in both the peripheral compartment (blood and lymph) and at mucosal sites such as the lumen of the intestine (Freytag et al., 1999).
When we consider that most infectious agents come in contact with the host at mucosal surfaces, the use of oral vaccines becomes the most attractive approach for immunization. The induction of mucosal immune responses by oral vaccines may not only protect the host from morbidity and mortality due to infection, but possibly prevent infection altogether (Brandtzaeg et al., 1995).

a. The Anatomy of Mucosal Immune System

The respiratory and digestive mucosal surfaces are enormous, approximately 300-4,000 m² in humans. The mucosal immune system comprises over 80% of all immunoglobulin producing cells in human body (Kiyono et al., 1996). The mucosal inductive site of the gastrointestinal tract is the Peyer’s patch which is enriched for macrophages, B and T cells and contains the germinal center for B-cell differentiation. The surface of the Peyer’s patch is covered by a unique epithelium called the follicle associated epithelium (FAE). The intestinal lumen side of the FAE is rich in specialized antigen sampling cells known as M cells, which have thin cytoplasmic extensions surrounding the lymphoid cells (Figure 2.). The M cells are more permeable to the gut contents than other epithelial cells in the villus epithelium (Levine et al., 1983; Neutra et al., 1994). Accumulated experimental results support the concept that antigen uptake by M cells does not result in degradation of the antigen, but rather results in delivery of intact antigens to the underlying lymphoid tissues (Neutra et al., 1987; Giannasca et al., 1994). The production of class II MHC molecules by M cells suggest that they may also play a significant role in presenting antigens to local T cells (Farstad et al., 1994; Allan et al., 1993). Once antigens have traversed the M cells, other antigen presenting cells—(macrophages and dendritic cells) within the Peyer’s patches take them up and present
Fig. 3. Composite model for cholera holotoxin bound to the saccharide moieties of five receptor molecules.

(Modified from *Mucosal Vaccines* 1996, Academic Press.)
them to adjacent regulatory T helper cells (Langhoff and Steinman, 1989), which produce specific cytokines (IL-2, IL-12, IFN-γ) stimulating B cell division and differentiation. The stimulated B cells migrate into the lymphatic and blood circulation and subsequently localize in mucosal effector regions through interaction with specific cellular receptors on endothelial venules. Within the mucosal tissues, e.g., the lamina propria of the gut and the respiratory tract, the sensitized B cells mature into plasma cells and secrete mucosal IgA antibodies into the lumen of the intestine.

b. Secretory Immunoglobulin A

Secretory IgA (sIgA) is a polymeric immunoglobulin, comprising two or more IgA antibody monomers bound together by a polypeptide referred to as the J chain. Approximately 40mg/Kg body weight of IgA is made daily in mucosal tissues. The daily production of IgA exceeds production of immunoglobulins of all other antibody isotypes combined (Mestecky and McGhee, 1987). Protective mucosal immunity is mediated mainly through the local production of sIgA. Secretory IgA has several important biological functions (Childers et al., 1989). Secretory IgA is polymeric, thus it has greater binding capacity than monomeric IgA and is more effective at virus neutralization. The ability of sIgA to neutralize influenza virus was demonstrated by Taylor and Dimmock (Taylor et al., 1985). Binding of influenza virus to a cell monolayer was prevented by sIgA by interfering with virus attachment or internalization, Monomeric IgA or IgG was unable to prevent virus binding to the cells. Intravenously administered polymeric IgA was shown to be transported across the nasal epithelium of mice and provided local protection against influenza virus challenge (Renegar et al., 1991). The multivalence of sIgA also allows the antibodies to more efficiently agglutinate bacteria. A monoclonal
IgA directed against a single surface epitope of *Vibrio cholerae* protected neonatal mice against a normally lethal dose of vibrios (Winner et al., 1989). The antibacterial activity of sIgA is enhanced by its ability to increase the activity of innate antibacterial factors, such as lactoferrin, peroxidases, and lysozyme (Rogers et al., 1978; Tenuovo et al., 1982; Adinolfi et al., 1966). Secretory IgA has also been shown to exert antibacterial activity through an indirect mechanism involving antibody-dependent cell-mediated cytotoxicity (ADCC). For example, T cells bound with IgA have been shown to effectively kill *Salmonella* and *Shigella* (Tagliabue et al., 1989). Immune exclusion is another immune mechanism mediated mainly through sIgA. During immune exclusion sIgA binds to microorganisms, toxins and antigens in the lumen or mucus, preventing their absorption into the blood circulation (Walker et al., 1977). The immune complexes formed with sIgA are more readily cleared from the circulation than antigens alone (Walker and Bloch, 1976). The inhibition of local inflammatory responses by sIgA also prevents the enhanced permeability of the mucosa to bystander antigens.

Cell-mediated immunity of the mucosal immune system is much less studied than antibody responses. The distribution patterns and unusual phenotypes of T lymphocytes suggest the role of regulation of intestinal immunity. Intraepithelial T lymphocytes (IEL) are abundant in the small intestine and are predominantly CD8+ cells, while the lamina propria lymphocytes (LPL) are predominantly CD4+ cells, the IEL may be functional suppressor cells involved in the mediation of oral tolerance. The LPL cells appear to induce isotype switching to IgA plasma cells (Elson et al., 1983).

Many factors affect the mucosal immune response, these include the dose of antigen, the immunization protocol, the type of adjuvant and the nature of the antigen. It has been
shown in many studies that large doses of antigen are required to induce a sIgA response following oral immunization (Michalek et al., 1977). However by using adjuvants and specific targeting strategies, the amount of antigen used can be greatly reduced. The nature of the antigen also plays an important role in mucosal immunity. Secretory IgA is induced most effectively by living rather than killed antigens (Bergmann et al., 1988). Particulate antigens are more immunogenic than soluble antigens (Cox et al., 1984). Intracellular pathogens tend to induce a Th1 cell response, while extracellular pathogens tend to induce Th2 cell responses. The induction of mucosal immunity following oral immunization has been shown to depend upon a number of variables. Consequently, several vaccine delivery strategies may be combined to develop more effective oral vaccines.

4. Immune Modulation by Cholera Toxin

a. Structure and Basis of Cholera Toxin Activity

Cholera toxin (CT) isolated from the gram negative diarrhea causing bacteria *Vibrio cholerae* has been shown to enhance the immunogenicity of mucosal immunogens when mixed with or conjugated to the antigens and given orally (Jaskson et al., 1993; Lycke et al., 1986; Mckenzie et al., 1984). Thus CT and its nontoxic B subunit have generated significant interest as potential adjuvants for oral vaccines. The molecular structure of CT has been studied extensively (Zhang et al., 1995; De Wolf et al., 1981). Cholera toxin is a hetero-hexameric protein in which a single A subunit is associated with a ring-like B pentamer formed by the association of five identical B monomers (Figure 3.). The A subunit is readily cleaved by exogenous proteases to form A1 and A2 peptide fragments, linked via a single disulphide bond (Hol et al., 1995). By a still unclear mechanism the
A1 subunit enters the cell cytosol and catalyses ADP-ribosylation of a Gs protein, which continuously activates adenylate cyclase leading to an elevation in cyclic AMP (cAMP) levels in the cell (Stavric et al., 1978). Raised cAMP levels cause protein kinase A to phosphorylate membrane porin proteins and open the transmembrane Cl⁻ channel. Chloride ion efflux results in the concomitant osmotic movement of water into the gut lumen and characteristic profuse watery diarrhea. The A2 fragment is an adaptor peptide whose major functions are to interact with the B subunit and position the A1 subunit above the B subunit pentamer (Zhang et al., 1995). The C-terminus of the A2 fragment contains a KDEL sequence motif that is associated with retrieval of proteins from the trans-Golgi network to the endoplasmic reticulum (ER). This KDEL sequence is important for delivery of the A1 peptide fragment to the correct cellular compartment, which may have an important role in modulating the immunological effects of the toxin (Hirst and Holmgren, 1987). The B subunit monomers interact with each other through hydrogen bonding of two three-stranded anti-parallel β sheets. This feature, together with the high number of salt bridges between adjacent B monomers makes the CTB pentamer one of the most stable non-covalently associated protein complexes known (Williams et al., 1999). The CTB pentamers are stable in 1mg/ml trypsin or proteinase K, and at acid pH (pH2.0). The CTB pentamers bind to the G_{M1} ganglioside ubiquitously presented on mammalian cell surfaces, including the cell surface of intestinal epithelial cells (Merrit et al., 1994).

b. Cholera Toxin as A Mucosal Adjuvant

The adjuvant activity of CT for orally delivered antigens was first reported by Elson and Ealding in 1984. Cholera toxin was fed to mice either alone or with the unrelated
Fig. 2. The anatomy of mucosal immune system. (cited from *Mucosal Vaccines* 1996, Academic Press.) The follicle-associated epithelium in a rabbit Peyer’s patch contains many M cells (M), identified by their short, sparse microvilli and thin apical cytoplasm, interspersed with enterocytes, identified by their microvillous brush borders (bb). The intraepithelial pockets formed by contiguous M cells are very large and contain B and T lymphocytes and macrophages. The basement membrane (bm) is interrupted to accommodate cellular traffic between the intraepithelial pockets and the subepithelial tissue.
protein antigen, keyhole limpet hemocyanin (KLH). When both proteins were fed together, CT abrogated tolerance to KLH. At the same time, CT also induced an intestinal sIgA response to KLH, which did not occur when KLH was fed alone (Elson and Ealding, 1984). Since these pioneering experiments a variety of antigens have been used to test the adjuvant activity of CT. Cholera toxin has shown adjuvant activity with a remarkably broad array of antigen types, including protein antigens, lipid antigens, carbohydrate antigens, as well as whole viruses, bacteria, and a protozoan. In order for CT to have adjuvant activity it must be administered simultaneously with the antigen by the same immunization route (Lycke and Holmgren, 1986). This suggests that CT acts on mucosal lymphoid tissues in contact with the antigens. The dose of CT required to demonstrate the adjuvant effect for most protein antigens in mice ranges between 1 to 10μg/dose (Lycke and Holmgren, 1986; Kusnecov et al., 1992). Equivalent mucosal adjuvanticity of the holotoxin in mice can be achieved by using the CTB subunit containing trace amounts (0.1-5%) of CT (Wilson et al., 1990; Lee and Chen, 1994). This method is very attractive as it reduces the A subunit toxicity enough to allow use in humans, particularly with the intranasal route of administration. Long-term mucosal and systemic memory B and T-cell responses to co-administered antigens are induced by CT (Jackson et al., 1993). The response is CD4+ T-cell dependent and requires antigen presentation via class II MHC molecules. T-cell responses to CT are predominantly of the Th2 cell type (Williams et al., 1999; Xu-Amano et al., 1993). This finding is supported by the observation that the serum antibody response is dominated by IgG1. Balanced Th1 and Th2 cytokine responses and a predominant Th1 response stimulated by co-administered CT were also reported (Rappuoli et al., 1999). It is important to note that the
adjuvanticity of CT is influenced by genetic background as well as the nature of the co-administered antigen (Hiroshi et al., 1996).

c. Role of CT Subunits in Mucosal Adjuvanticity.

The involvement of the A and B subunits in mediating CT adjuvant activity has been a source of ongoing controversy. Horseradish peroxidase (HRP) chemically conjugated to CTB was found to elicit higher antibody levels in the gut and serum than feeding HRP alone, or an unconjugated mixture of HRP and CTB (McKenzie and Halsey, 1984). This study demonstrated that CTB has intrinsic adjuvant activity. However, the use of CTB conjugates has been reported to be effective in some cases, but not in others. The poor response in other cases may be due to the coupling procedure, which can significantly affect the immunogenicity of protein conjugates. More recently recombinant DNA technology has made it possible to genetically fuse peptides to CTB. Chimeric CTB fusions demonstrated adjuvant activity of CTB, but the activity was not as strong as what was found with the holotoxin in the intestine (Holmgren et al., 1993; Dertzbaugh et al., 1989). The size of the peptide that can be fused to CTB is restricted to about 20 amino acid residues. Larger peptides tend to disrupt the formation of CTB pentamers, abolish the GM1 ganglioside binding activity and eventually impair the adjuvant effect. An alternative solution to this problem was to replace the toxic A1 subunit of CT with relatively large size antigens. The resulting fusion protein containing the A2 subunit reassembled with the CTB pentamer to form a holotoxin-like structure. A CTB fusion protein containing a Streptococcal adhesin molecule (42kD) constructed in this way induced strong sIgA and serum IgG responses in orally immunized mice (Hajishengallis et al., 1995). In another report, mixtures of KLH and CTB were unable to stimulate
immunity to KLH unless a very small amount of holotoxin (<50ng) was added (Lycke and Holmgren, 1986). This leads to the conclusion that adjuvant activity was dependent on ADP-ribosylation by the A subunit. To settle the disparity between studies of the role of the A and B subunits in the immunogenicity and adjuvanticity of CT, mutants that lacked enzymatic and binding ability of both A and B subunit were generated by site-directed mutagenesis. Mice were fed tetanus toxoid (TT) with different combinations of A and B subunits to compare with wild-type CT (Elson et al., 1995). Mice receiving wild type CT had strong responses to both CT and TT. Mice receiving the A mutant or CTB alone had moderate and weak anti-CT responses, but no TT response. Mice given the B mutant or the double A and B mutant had no response to either CT or TT. These results show clearly that both a functional A and B subunit are required for optimal immunogenicity and adjuvanticity of CT in the intestine.

d. Cellular Cargons of CT and CTB Adjuvant Activity

Cholera toxin and the CTB subunit do not alter antigen uptake or processing by antigen-presenting cells (APC) in vitro, nor do they affect macrophage class II MHC expression. However, they do enhance antigen presentation of APC in an indirect way, by increasing the expression of costimulatory cell surface molecules (B7) and costimulatory cytokines (IL-1 and IL-6) (Lycke et al, 1989). Increased B7 and cytokine synthesis result in increased CD4+ T cell priming in mucosal tissues in vivo. Primed CD4+ T cells produce cytokines such as IL-4 (Th2 type) that enhance antibody isotype and subtype switching to IgA and IgG1 respectively (Hornquist et al., 1993). Both CT and CTB can inhibit lymphocyte function in the epithelial layer, particularly CD8+ T cells, which may produce suppressive cytokines (Williams et al., 1999). This inhibition effect abrogates
oral tolerance to co-administered antigens. Cholera toxin does not have direct effects on B cell isotype differentiation in vitro. Cholera toxin enhances the effect of both IL-4 and IL-5 on purified B cells (Wilson et al., 1990; Clarke et al., 1991). In the presence of CT, IL-4 enhanced IgG1 producing B cells three to fourfold, and IL-5 had a similar effect on IgA producing B cells. The mechanisms by which a given antigen triggers a Th1 or Th2 dominated response are at present unclear. Thus, it is important to know what effects CT and CTB have as mucosal adjuvants on Th1 vs Th2 cell responses (Mocci et al., 1997).

5. Infectious Enteric Diseases

Diarrhea is the most prevalent infectious disease in developing countries and ranks second only to respiratory disease in developed countries (Kumate and Isibasi, 1986). The majority of diarrheal illness and deaths occur in children less than 5 years of age. Cholera, enterotoxigenic Escherichia coli (ETEC), rotavirus and Norwalk virus are the four leading causes of diarrhea diseases worldwide.

a. Cholera

Cholera is an acute secretory diarrheal disease. There are approximately 500,000 to 1 million clinical cases of cholera worldwide annually (Waldor et al., 1996). In endemic areas where quick medical treatment (rehydration therapy) is available, mortality from cholera is usually low approximately 1-3%, but in epidemic situations mortality can exceed 50%. In the recent 1994 cholera epidemic in Rwandan refugee camps, an estimated 50,000 cholera fatalities occurred (Bern and Glass, 1994). Until the present there have been seven recorded cholera pandemics. The classical biotype of V.cholerae serogroup O1 is responsible for the first six cholera pandemics. The seventh pandemic of cholera was caused by the El Tor biotype of V.cholerae O1 which emerged in Indonesia
in 1961 and arrived in Peru in 1991 (Kiyono et al., 1996). Since then the El Tor strain has spread throughout Latin America and has given rise to more than a million cases. In 1992 a new serotype O139 \textit{V.cholerae} arose in India, and caused a major cholera epidemic in south Asia. Genetic studies indicated that \textit{V.cholerae} O139 probably arose from the strain \textit{V.cholerae} O1 El Tor, by replacement of a part of the O antigen gene cluster with a non-O1 strain (Holmgren et al., 1996). Although the O139 strain is currently restricted to Asia, there is a great risk that it may follow the tracks of \textit{V.cholerae} O1, reaching Africa and Latin America.

Attempts to make parenteral cholera vaccines began soon after the discovery of \textit{V.cholerae} as the causitive agent of cholera. Field trials revealed a short-lived protective efficacy of the parenteral killed vaccines, usually in the 30-80% protection range in the first 6 months falling to approximately 30% thereafter (Kaper et al., 1984; Clemems et al., 1986). When pathogenesis studies revealed that \textit{V.cholerae} strains are strictly mucosal pathogens, surviving and multiplying on the surface of the epithelium of the small intestine without crossing the epithelial barrier. An oral cholera vaccine composed of killed whole-bacterial cells and nontoxic B subunit (BS-WC) was developed by Svennerholm, Holmgren, and colleagues and tested in Bangladesh. This vaccine provided up to 85% protection against cholera infection in all age groups for the first six months, decreasing to about 51% protection after 36 months (Holmgren et al., 1996). Killed whole-cell (WC) or BS-WC oral cholera vaccines were evaluated in clinical trials in the U.S. and provided 56% and 64% protection against cholera infection respectively (Waldor et al., 1996). Further, BS-WC vaccine gave protection against both cholera and ETEC for the first 3 months of the trial, indicating immunological cross-protection
between the two bacterial pathogens. Recombinant live-attenuated oral cholera vaccines containing a deletion in the toxic A subunit or both A and B subunits were also tested in clinical trials. This vaccine had a protective efficacy of 90%. Safety is a major concern when using recombinant live attenuated vaccines due to the possibility of reactogenicity in vaccinees and reversion of the recombinant strain to the virulent wild-type. Ultimately prevention and eventual eradication of cholera depends on pathogen free water sources and an effective sanitation system. However, the poor economic conditions in developing nations make this goal virtually impossible to achieve. Thus, a cost-effective oral vaccine which can provide repeated protection or long lasting protection has become the most urgent need.

b. Enterotoxigenic Escherichia Coli

Enterotoxigenic E. coli is one of the most common causes of diarrhea and mortality in children in developing countries and continues to be a common cause of traveler’s diarrhea in adults. About one third of all ETEC infections are symptomatic in children, but this is enough to result in at least 650 million episodes of diarrhea and about 800,000 deaths annually in children below the age of 5 years (Black, 1990). Without comparison, ETEC is the most common cause of traveler’s diarrhea. It has been estimated that at least 50% of persons travelling to developing countries experience diarrheal disease, and ETEC is isolated in one third to one half of these episodes. Enterotoxigenic E. coli infects humans by colonization of the small intestine followed by secreting one or more enterotoxins which induce electrolyte and water secretion from the intestine, resulting in diarrhea (Levine et al., 1983). The enterotoxins produced by ETEC include a heat labile enterotoxin (LT) and a heat stable enterotoxin (ST) (Lopez et al.,
1990). The LT toxin is structurally, functionally and immunologically similar to CT. Antibodies against CTB induce significant cross protection against diarrhea caused by LT producing \textit{E.coli} (Svennerholm et al., 1986). In ETEC infection, colonization of the intestine is promoted by distinct attachment factors (Michaud et al., 1990; Buhler et al., 1991). There are three major types of colonization factor antigens (CFAs), referred to as CFA/I, CFA/II or CFA/IV. Colonization factor CFA/I consists of approximately 100 identical 15 kDa subunits. Colonization factor CFA/II consists of three different subcomponents, CS1, CS2 and CS3. Colonization factor CFA/IV is composed of CS4, CS5 and CS6 subunits. Anti-CFA antibodies have been found to cooperate synergistically with anti-LT antibodies in protecting against ETEC infection (Svennerholm et al., 1989). Based on the cross protection between anti-CTB and anti-LTB antibodies, an oral ETEC vaccine was designed. This vaccine contains recombinant CTB and five different \textit{E.coli} strains expressing CFA/I and different fimbrial subcomponents of CFA/II and CFA/IV. This vaccine was found to have a potential protective efficacy of at least 70-80% (Ahren et al., 1993).

c. Rotavirus

Rotavirus is a major cause of diarrhea in young children and animals worldwide. Virtually all children have been infected with rotavirus by the time they are 5 years old (Bishop et al., 1983). The health burden of rotavirus disease is staggering. In developing countries, 20 to 40% of hospitalizations and 870,000 deaths for childhood diarrhea are associated with rotavirus infection each year (Bajolet et al., 1998). In the U.S. about 3.5 million cases occur each year, although the mortality rate is relatively low (75 to 125
deaths each year), the direct and indirect cost of rotavirus infection exceeds one billion dollars.

Rotavirus was first identified in 1958. It is a member of the Reoviridae family. Rotavirus contains 11 segments of dsRNA, each coding for one protein. There are six structural proteins in the virus particle and five nonstructural proteins expressed in infected cells (Figure 4.). Morphologically, rotavirus particles consist of three concentric protein capsid layers. The outer capsid layer is made of VP4 (the spike protein) and VP7 (a glycoprotein). Both capsid proteins induce neutralizing antibodies (Estes, 1996). Capsid protein VP6 is the subgroup antigen, and makes up the intermediate protein shell (Hsu et al., 1997). Capsid protein VP2 forms the inner core of the virus particle. The 11 segments of genomic dsRNA reside inside the VP2 capsid layer. Serotype specificity is defined by a binary classification system referred to as the P (VP4) serotype and the G (VP7) serotype (Mackow et al., 1989). Currently 14 G and 10 P rotavirus serotypes are known. In humans, 9 G and 8 P serotypes have been identified, but the majority of infections are due to rotaviruses of G1-G4 and P4 or P8 serotypes. Overall, the G1 serotype occurs most frequently, but multiple serotypes or strains of rotavirus often circulate in a given community at the same time or within the same year. Antibodies to VP4 and VP7 are each independently associated with protection against rotavirus challenge in various animal models (Ward et al., 1990). Protection against rotavirus is generally serotype specific. The immune response and protection generated by homologous rotaviruses are much stronger than those generated by heterologous rotoviruses.
Fig. 4. Schematic illustration of the rotavirus particle. (cited from Mucosal Vaccines 1996, Academic Press.)
Immune mechanisms for protection against rotavirus infection have not been clearly established. *In vitro* neutralization assays and passive immunization assays with antibodies against different rotavirus components demonstrated that VP4 and VP7 are targets of protective immunity (Ishida et al., 1997). However, John Burn, Harry Greenberg and their colleagues demonstrated in a mouse “backpack tumor” model that immunity to the non-surface capsid protein VP6 has a role in immune protection. The investigators injected hybridoma cells producing anti-VP6 IgA antibody subcutaneously into the back of histocompatible BALB/C mice (John et al., 1996). At the injection site, the mice secreted monoclonal antibodies, which were subsequently transported in the circulatory system and eventually reached the mucosal surfaces by transcytosis. The anti-VP6 IgA antibody completely inhibited rotavirus infection following virus challenge.

Recent identification of a rotavirus nonstructural protein NSP4, which acts as a viral enterotoxin, may provide a new approach to induction of immune protection against clinical rotavirus disease (Ball et al., 1996; Angel et al., 1998). The nonstructural protein NSP4 is an intracellular receptor that mediates acquisition of a transient membrane envelope as subviral particles bud into the endoplasmic reticulum (Superti et al., 1998; Newton et al., 1997; Tian et al., 1996). Baculovirus production of the NSP4 protein caused an increase in intracellular calcium levels in insect host cells (Ball et al., 1996). Purified NSP4 or a peptide corresponding to NSP4 residues 114-135 induced diarrhea in young CD1 mice. Electro-physiologic data from intestinal mucosa showed that the NSP4 peptide potentiates chloride secretion by a calcium dependent signaling pathway. Most importantly antibody against NSP4 significantly reduced diarrheal disease in passive immunized mouse pups (Johansen et al., 1999).
Antibody vs cell mediated immunity in a mouse model has also been investigated. Immunodeficient SCID mice that lack both T and B cells became chronically infected with murine rotavirus. In contrast, β2m knock out mice that lack T cells had delayed virus clearance but were resistant to rotavirus reinfection (Offit, 1996). When B cell deficient JHD knock out mice were challenged with rotavirus, most of them cleared rotavirus infection like control mice. Re-challenge of B cell deficient mice that had completely resolved primary rotavirus infection became reinfected but shed smaller amounts of viral antigens. This series of experiments demonstrated that both T and B cells can clear primary rotavirus infection and protect against reinfection. T cells appear to expedite normal virus clearance while antibodies seem to be the principal factor mediating protection from reinfection.

Rotavirus vaccine development has passed through several developmental stages (Orenstein et al., 1996). First generation rotavirus vaccines used rotavirus strains from animal origins. A live attenuated bovine rotavirus vaccine named RIT4237 was tested in children in Finland, and yielded a 50% protection rate against all rotavirus disease and a 80% protection rate against severe rotavirus disease (Vesikari et al., 1986). However vaccines composed of a single animal origin rotavirus failed to consistently protect infants in repeated clinical trials (Kapikian et al., 1994). Thus second generation rotavirus vaccines composed of animal rotavirus reassortants which also express human rotavirus surface antigens were developed (Glass et al., 1994). A bovine WC3 reassortant vaccine expressing VP7 surface protein of human P1 G1 rotavirus strain was extensively tested in clinical trials (Clark et al., 1988; Bernstein et al., 1990). This vaccine gave 67% protection
against all rotavirus disease and 87% protection against clinically significant rotavirus disease and complete protection against rotavirus diarrhea.

With respect to the use of recombinant protein based vaccines, induction of active immunity with individual rotavirus proteins has so far been unsuccessful. One important reason for the lack of success is that discontinuous epitopes are crucial for induction of protective immunity and native folding of the rotavirus proteins was not achieved with individual soluble rotavirus proteins. Recently promising results have been obtained with recombinant virus-like particles (VLPs) produced by co-expression of rotavirus structural proteins in insect cells (Corsaro et al., 1996; Crawford et al., 1994; Conner et al., 1996). Virus-like particles composed of VP2/6/7 and VP2/4/6/7 administered parenterally to mice and rabbits induced both neutralizing and serotype-specific antibody responses in both serum and intestine (O’Neal et al., 1997). Oral inoculation of mice with rotavirus VP2/6/7 VLPs with cholera toxin as an adjuvant, induced antibodies in both serum and intestine. Multivalent VLP vaccines that display VP7s from two or more serotypes induced neutralizing antibodies to homotypic and heterotypic rotavirus strains.
CHAPTER TWO
MATERIALS AND METHODS

A. Materials

1. Reagents

All restriction endonucleases, T4 DNA polymerase, T4 DNA ligase, alkaline phosphatase were purchased from New England Biolabs (Beverly, MA). QIAquick PCR purification Kit, QIAquick Gel Extraction Kit, QIA quick nucleotide Removal Kit, QIAprep Spin Miniprep Kit, QIAfilter Plasmid Midi Kit, and Omniscript Reverse Transcriptase Kit were purchased from Qiagen Inc. (Valencia, CA). Rabbit anti-mouse IgG, anti-mouse IgA alkaline phosphatase conjugates and mouse anti-rabbit IgG, anti-mouse IgA alkaline phosphatase conjugates were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-mouse CD4, CD8b.2 PE conjugates, anti-mouse CD62L FITC conjugates, and anti-mouse CD44 Cy-chrome conjugates were obtained from (PharMingen, San Diego, CA). Antibiotics gentamycin sulfate, kanamycin monosulfate, rifampicin carbenicillin, and ampicillin were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse interleukin-2, interleukin-4 and interferon gamma ELISA kits were purchased from Endogen Inc. (Woburn, MA).

Oligonucleotides were synthesized using a model 394 DNA/RNA Synthesizer (Applied Biosystems, Inc.) in the DNA Core Facility located in the Center for Molecular Biology and Gene Therapy, Loma Linda University.

Polyclonal anti-CFA/I was generated in rabbits by Biosynthesis Inc. (Lewisville, TX) according the following immunization protocol: two 3 month old female rabbits (New Zealand White, approximately 3-4 kg) were injected intradermally and intramuscularly
with 200-500ug purified CFA/I antigen in a 50/50 emulsion mixture with TiterMax incomplete adjuvant on day 0, 21, 35. The immune response was boosted every two weeks starting from week 6 until the termination of the experiment. Serum was collected on week 6, 8 and 10 followed by ELISA determination of anti-CFA/I titers.

Immuonoaffinity purified SA11NSP4 protein, rabbit anti-NSP4 serum and the rotavirus SA11 strain were provided by Dr. Mary Estes, Baylor College of Medicine. Rabbit anti-rotavirus hyperimmune serum, mouse anti-VP6 serum and the plasmid PKS+ containing murine VP6 CDNA were provided by Dr. H. Greenberg, Stanford University, School of Medicine. Plasmid pPT42 containing the cholera toxin ctxAB operon was provided by Dr. J. Mekalanos, Harvard Medical School.

2. Animals

Adult CD-1 mice (male, female) were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA) at 5-6 weeks of age. The animals were housed in self-contained filter-top cages in a room controlled for temperature, humidity, and a 12 h day-night cycle. Autoclaved bedding, food and water were provided. At the time of sacrifice, rapid CO₂ asphyxiation was performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals. All animal care was performed under the direction of a licensed veterinarian. The animal study was approved by the Loma Linda University Animal Research Committee.

B. Methods

1. Preparation of Electro-competent Cells

A single colony of Escherichia coli strain HB 101 was inoculated into 5ml liquid Luria-Bertani (LB) medium (10 g/l Tryptone, 5 g/l Bacto yeast extract, 10 g/l NaCl, pH
7.3) and was grown at 37°C overnight with vigorous shaking (approximately 250 to 300 rpm). 2.5 ml of the culture was transferred into 500 ml LB medium in a sterile 2 liter flask and was grown at 37°C with shaking to an OD_600 of 0.5 to 0.7. The cells were chilled on ice for 15 minutes, and then were centrifuged at 5000 x g for 20 minutes. The supernatant was removed and the cell pellet was washed twice with 500 ml ice cold distilled water. The cell pellet was washed in 80 ml 10% ice cold glycerol and resuspended in a final volume of 2 ml 10% glycerol. Small aliquots of 50 μl were transferred to pre-chilled microfuge tubes and frozen on dry ice for 20 minutes. Finally, the competent cells were stored at -80°C. For _Agrobacterium tumefaciens_ strain GV3101 pMP90RK, the cells were grown at 28°C with shaking in YEB medium (beef extract 5.0 g/l, Bacto yeast extract 1.0 g/l, Bacto peptone 1.0 g/l, sucrose 5.0 g/l, MgSO4.7H2O 0.1 g/l, pH 7.3) containing the antibiotics rifampicin (100 μg/ml), kanamycin (25 μg/ml), and gentamycin (25 μg/ml).

2. Bacterial Transformation by Electroporation

Plasmid DNA (100-200 ng) was mixed with 50 μl _E.coli_ or _A. tumefaciens_ electro-competent cells. The mixture was transferred to a 0.2 cm electroporation cuvette. The Gene Pulser electroporation apparatus (Bio-Rad, Inc. Hercules, CA) was set at 2.5 kV, 25 uFD, 200 Ω. After electroporation 1 ml SOC medium (2.0% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10mM MgCl2, 10mM MgSO4, 10 mM glucose) was added to the cuvette to suspend the cells. The cell suspension was transferred to a 5ml culture tube, incubated with shaking at 200 rpm for 2 hours. For _E.coli_ 0.1 ml cell culture was plated on LB selection medium containing ampicillin (100μg/ml). Ampicillin resistant colonies were isolated after overnight culture at 37°C. For _A. tumefaciens_ 0.1 ml
cell culture was plated on YEB selection medium containing rifampicin, kanamycin, gentamycin and carbenicillin (100μg/ml). Carbenicillin resistant colonies were isolated after incubation at 28°C for 48 to 72 hours.

3. *A. Tumefaciens* Mediated Plant Transformation

Potato plants *Solanum tuberosum* cv. Bintje were grown in sterile Magenta boxes (Sigma Chemical, St. Louis, Missouri) on Murashige and Skoog (MS) medium (Sigma) containing 3.0% sucrose and 0.2% gelrite. Leaves from 3-4 week old sterile shoots were cut from the plant at the leaf base and transected in the middle of the leaf perpendicular to the midvein. The leaf fragments were immediately transferred and immersed in 10 ml overnight culture of *A. tumefaciens* suspension (2-5 x 10⁹ cells/ml) carrying the plant expression vector with the desired genes inserted between the T-NDA borders. Acetosyringone (370 μM, final concentration) was added to the bacterial suspension to facilitate transformation. The leaf explants were incubated in the bacterial suspension for 5-10 min, blotted damp dry on sterile filter paper and transferred upside down to callus medium (MS medium, 0.1 μg/ml naphthalene acetic acid (NAA), 1.0 μg/ml trans-zeatin, pH 5.7). The leaf explants were incubated for 2-3 days at room temperature to permit the transfer of T-DNA into plant genome. The infected leaf discs were washed in sterile distilled water containing claforan (300 μg/ml), blotted dry on sterile filter paper and placed inverted on callus selection medium (callus medium, kanamycin 100 μg/ml, claforan 400 μg/ml), for selection of transformed plant cells and selection against *A. tumefaciens*. The explants were incubated in the light room for a period of 3-4 days, then transferred to fresh plates and incubated for another 3-4 weeks to permit callus growth at the wound surfaces. Leaf explants containing the green and friable callus tissue were
transferred to shoot growth medium (MS medium, 1.0 μg/ml trans-zeatin, 50 μg/ml kanamycin, 400 μg/ml claforan) and incubated for additional 2-3 weeks to facilitate shoot formation. Potato shoots approximately 0.5 cm long were removed from the callus tissue and transferred to root induction medium (MS, 300 μg/ml claforan) for root formation. The transgenic plantlets were obtained after 4-6 weeks further growth under sterile conditions. The plantlets were transferred to potting soil and grown into mature tuber bearing plants in the greenhouse.

4. Analysis of DNA/RNA from Transformed Plants

Plant genomic DNA was isolated from transformed potato leaf tissues using the Dneasy Plant Mini Kit (Qiagen Inc.) Presence of the gene inserts was determined by Polymerase Chain Reaction (PCR) analysis. The PCR reaction mixture contains: plant genomic DNA (400 ng) as templates, 5’ and 3’ primers (100 pmol each) specific for the gene inserted, 10 μl thermo-buffer, 2 μl dNTP mixture (10 mM each), 0.5 μl (1 unit) Vent DNA polymerase (New England, Biolabs). Distilled water was added to a final volume of 100 μl. The reaction mixture was put under the following PCR conditions: DNA melting: 94°C for 45 sec, primer-DNA annealing: 55°C for 60 sec, and primer extension: 72°C for 60 sec for a total of 30 cycles.

Total RNA from leaves of transformed plants was isolated using Rneasy Plant Mini Kit (Qiagen Inc.) the presence of mRNA transcribed from the inserted genes was determined by reverse transcription PCR (RT-PCR) analysis following the instructions of the Omniscript Reverse Transcirtptase Kit (Qiagen Inc.). The reverse transcription (RT) reaction mixture contains: total RNA extraction (100 ng) from transformed plants as templates, 2 μl RT buffer (Qiagen Inc.), 2μl dNTP (5mM each), 1μl (4 units) reverse
transcriptase, 1µl 3’ primer (1pmol) specific for the gene inserted. RNase-free water was added to make a final volume of 20 µl. The RT reaction mixture was incubated at 37°C for 60 min followed by heat inactivation at 93°C for 5 min. The RT reaction mixture was used as templates in the following PCR reaction: 5’ and 3’ primers (100 pmol each) specific for the gene amplified, 2µl dNTP mixture (10 mM each), 10 µl thermo buffer (Qiagen Inc.), 0.5 µl (1 unit) Taq DNA polymerase (Qiagen Inc.). The reaction was performed in the following PCR cycle: 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec for a total of 34 cycles. This was followed by final extension of 10 min at 72°C. The reaction was then cooled to 4°C. PCR and RT-PCR samples were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

5. Western Blot Analysis of Antigen Gene Expression in Plant Cells

Potato leaves and tuber slices from the transformed plants were incubated at room temperature for 5-7 days on MS medium containing NAA (5.0 mg/l) and 2,4-D (6.0 mg/l). Incubation on high auxin medium for at least 4 days is necessary to detect the maximum amount of gene expression from the mas promoters in potato plant tissues. Approximately 1.0 g of potato tissues were homogenized by grinding in a mortar and pestle on ice in 1 ml extraction buffer (200 mM Tris-HCl, pH8.0, 100mM NaCl, 400mM sucrose, 10mM EDTA, 14 mM 2-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride, 0.05% Tween-20). The tissue homogenates were centrifuged twice at 17,000 x g for 15 min at 4°C to remove insoluble cell debris. An aliquot of 10-20 µl supernatant, containing 100-200 µg of total soluble protein, as determined by Bradford protein assay (Bio-Rad, Inc.), was separated by 10%–12% sodium dedecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 60 min in Tris-glycine buffer (25 mM Tris,
250 mM glycine, pH 8.3, 0.1% SDS). Samples of the plant homogenate were either boiled 5 min prior to electrophoresis or loaded directly on the gel.

The separated protein bands were transferred from the gel to nitrocellulose membrane (MSI Inc.) by electroblotting on a semi-dry blotter (Sigma Chemical Co.) for 90 min at 30 V and 60 mA. Nonspecific antibody binding was blocked by incubation of the membrane in 5% non fat dry milk in TBS buffer (20 mM Tris, pH 7.5, 500 mM NaCl) for 1 hour on a rotary shaker (40 rpm), followed by washing once in TBS buffer for 5 min. The membrane was incubated overnight at room temperature with gentle agitation in 20 ml of a primary antibody in TBST buffer (TBS, 1% non fat dry milk, 0.05% Tween-20) followed by washing three times in TBST buffer. The membrane was incubated for 1 h at room temperature in 20 ml alkaline phosphatase conjugated secondary antibody in TBST buffer. The membrane was then washed three times in TBST buffer and one time with TBS buffer. Finally the membrane was incubated in the substrate BCIP/NPT (Sigma) for 10 min. The color reaction was stopped by washing the membrane several times in distilled water.

6. Chemiluminescent ELISA

The amount of plant-synthesized CTB-NSP4 fusion protein was measured in transformed plant extracts by chemiluminescent G_{M1} ELISA assay. The wells of a 96-well microtiter plate (Dynatech Laboratories) were coated with 100 μl/well of monosialoganglioside G_{M1} (3.0 μg/ml) (Sigma) in pH 9.6 bicarbonate buffer (15 mM Na_{2}CO_{3}, 35 mM NaHCO_{3}), and incubated at 4°C overnight. The plate was washed three times with PBST (phosphate buffered saline (PBS) containing 0.05% Tween-20). The background was blocked by incubation in 1% bovine serum albumin (BSA) in PBS (300
μl/well) at 37°C for 2h followed by washing three times with PBST. The microtiter plate was incubated with 2 μg of transformed plant total soluble protein per well in PBS (100 μl/well). For use as a positive control, a known amount of bacterial CTB (Sigma) plus 2 μg untransformed plant total soluble protein in PBS (100 μl/well) was added to the plate and incubated overnight at 4°C. The plate was washed three times with TBST and then incubated in a 1:8000 dilution of rabbit anti-cholera toxin antibody (Sigma, 100 μl/well) for 2h at 37°C, followed by washing three times with PBST. The plate was incubated with a 1:80,000 dilution of anti-rabbit IgG alkaline phosphatase conjugate (Sigma, 100 μl/well) for 2h at 37°C and washed three times with PBST. The plate was finally incubated with 100 μl/well Lumi-Phos Plus substrate (Lumigen) for 30 min at 37°C. The plate was cooled for 5 min and light emission was measured in a Dynatech 3000 Microtiter Plate Luminometer (Dynatech Laboratories).

The amount of plant-synthesized CFA/I-CTA2 fusion protein in each well was measured by the same chemiluminescent ELISA assay with the exception that 2ug of transformed plant total soluble protein was directly coated on the plate in pH9.6 bicarbonate buffer. The first antibody used was rabbit anti-CFA/I at a 1:3000 dilution. The rest of the assay procedure was identical to the ELISA described above.

The expression level of plant synthesized VP6 was quantified by chemiluminescent sandwich ELISA. A 96 well microtiter plate was coated with mouse anti-VP6 serum diluted 1:1,000 in bicarbonate buffer (pH 9.6) at 4°C overnight. The plate was blocked with 1% BSA in PBS buffer at 37°C for 2 hours. Protein extracts from transformed potato tuber tissues were added to the wells and incubated overnight at 4°C. Known amount of purified virus VP6 mixed with protein extracts from untransformed potato tuber tissues
were added to the wells as standard curves. The plate was incubated with rabbit anti-
rotavirus serum diluted 1:2,000 in PBS buffer and then incubated with mouse anti-rabbit
IgG conjugated with alkaline phosphate in 1:80,000 dilution in PBS buffer. The plate was
finally incubated with chemiluminescent substrate, Lumi-phos Plus (Luminogen, Inc.) for
30 minutes at 37°C, and the plate was read in a Microtiter Plate Luminometer (Dynatech).
Light emission was recorded as relative light units (RLU) over the background.

7. Mice Oral Immunization

Adult CD-1 mice were fasted overnight before each oral immunization. On each
feeding day, 10 μg of purified bacteria cholera toxin (Sigma) was first administered as an
adjuvant to the mice in 0.4 ml sodium bicarbonate buffer (350 mM, pH 8.5) by gastric
intubation (gavage). The mice were then fed 3-5 g (fresh weight) transgenic potato tuber
slices containing 10-20 μg of plant produced VP6, CFA/I or CTB-NSP4 antigens,
previously determined by ELISA assay. A group of CD-1 mice were fed same 3-5 g of
untransformed potato tuber slices to serve as negative control. Mice fed the same amount
of purified rotavirus VP6 or NSP4 proteins served as positive controls. On day 23 after
the final immunization, the mice were euthanized by CO2 anoxia. Blood samples
collected from each mouse were incubated at room temperature for 4-5 hours followed by
overnight incubation at 4°C to retract blood clots. The blood samples were centrifuged at
4°C, 2,800 x g for 10 minutes. The supernatants were removed from the red blood cells
and centrifuged for another 10 minutes at 4°C 4000 x g. NaN3 was added to the obtained
sera to a final concentration of 0.03%. The sera were stored at room temperature. The
small intestines of the sacrificed mice were removed and the lumen of the intestine was
washed with 1 ml PBS buffer, pH 8.5, containing 10 mM PMSF. The intestinal wash was
centrifuged twice at 4°C 5000 x g for 10 minutes. The supernatant was immediately analyzed by ELISA for intestinal antibodies or stored at -80°C.

8. Production of Passively Immunized Mouse Pups

Female CD-1 mice were fed 3-5 g transgenic potato tuber slices, containing plant-synthesized antigens, once a week for four weeks. Immediately following the fourth immunization at maximum antibody titer, each immunized female was paired with one uninfected male. Successful mating was detected by the presence of a copulatory plug in the vagina 24 hours after mating. The male CD-1 mice were separated from the females after mating. After 19-21 days gestation period, mouse pups were born with a litter size of 8-12 pups. The pups remained with the dams until the day of virus inoculation.

9. Rotavirus Challenge of Suckling Mice

Protection of NSP4 antibodies against rotavirus induced diarrhea was evaluated by rotavirus challenge of passively immunized mouse pups. Six day old suckling mice were orally gavaged with SA-11 rotavirus suspension. Each pup received one oral dose that contained 15 x DD₅₀ (virus dose determined empirically to cause diarrhea in 50% of the mouse pups) in 50 μl of endotoxin-free PBS. Virus inocula were not trypsin-activated prior to inoculation. All cages were coded and individual mice were checked for diarrhea daily for 7 days after inoculation by gentle palpation of their abdomen. A diarrhea score and the proportion of mice with diarrhea in each study group was recorded. Stool classification was defined as: 0, no stool; 1, normal stool; 2, normal stool accompanied with yellow pasty stool; 3, all yellow pasty stool; 4, milky-liquid stool. The pups with a stool score ≥ 2 were considered to have diarrhea.
10. Cytokine Analysis in Spleen Cells

Orally immunized mice were killed at three different time points: 13, 34 and 68 days after the final immunization. A spleen cell suspension was prepared by gently pressing spleen tissue through a fine nylon mesh sieve. The suspension was washed in RPMI-1640 medium once by centrifuging at 800 x g, room temperature. The cell pellet was resuspended in complete media (RPMI-1640, 10% fetal calf serum and 1% penicillin/streptomycin), counted with a Unopette hemocytometer (Becton Dickinson, NJ) and put into 24 well tissue culture plates (3 x 10^6 cells/well) in duplicate samples. After incubation for 72 h at 37°C in a humidified, 5% CO2 incubator, supernatants from the spleen cell cultures were collected for assessment of IL-2, IL-4 and IFNγ secretion. Assay procedures followed the instructions of the cytokine ELISA kits (ENDOGEN, MA). Briefly, standard sandwich ELISA technique utilizing capture antibody and enzyme-conjugated detection antibody were employed. Cytokines of known concentrations were included in each assay to produce standard curves. Standards and samples were assayed in duplicates to assure reproducibility. Plates were read in a Mark II Plate Reader (Dynex Technologies Inc, VA).

11. Flow Cytometry Analysis

Spleen lymphocytes were stained with fluorochrome-labeled monoclonal antibodies (mAb) for immunophenotyping. Two monoclonal antibody panels were constructed for three color analysis (fluoresceinisothiocanate (FITC), phycoerythrin (PE), and Cy-Chrome). The first combination used, CD62L*FITC/CD4*PE/CD44*Cy-Chrome, designates naïve and memory T helper cells. The second combination,
CD62L*FITC/CD8b.2*PE/CD44*Cy-Chrome, designates naïve and memory cytotoxic T cells.

Spleen cells were adjusted to 1x 10^6/ml or 3x 10^6/ml. One hundred microliters of cells were aliquotted into 5 ml tubes and incubated 15 minutes on ice with the desired monoclonal antibody. Samples were lysed with 2 ml lysing buffer (0.8% Tris ammonium chloride) while vortexing, then incubated 6 minutes at room temperature. Samples were spun at 400 g for 5 minutes, aspirated, resuspended in 200 μl PBS (Cellgro, San Diego, CA) and placed on ice until acquisition in the flow cytometer. Cells were acquired and analyzed on a Fluorescence-Activated Cell Sorter with Consort 32 software (FACSort, Becton Dickinson, San Jose, CA). A minimum of 1000 events was acquired for analysis using quadrant plots. Analytical controls included unstained cells to assess the degree of autofluorescence.

3. Statistical Analysis

The data were analyzed using one-way ANOVA and student’s t test. A P value of < 0.05% was considered to indicate significant differences among different immunization groups.
CHAPTER THREE

NOVEL APPROACHES TO ORAL VACCINES – DELIVERY OF ANTIGENS THROUGH EDIBLE PLANTS

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Abstract

Advances in genetic engineering throughout the past decade have accelerated the expression of foreign proteins with industrial and pharmaceutical value in plants. Antigens obtained from infectious bacterial or viral diseases have been introduced into plants through plant virus mediated transient transformation or Agrobacterium tumefaciens mediated stable transformation methods. The plant produced vaccine antigens were found to retain their antigenicity. Oral immunization with transgenic plant tissues containing vaccine antigen proteins stimulates both systemic and mucosal immune responses in animals. Plant-based vaccines can provide significant levels of protection against challenge by viral or bacterial pathogens.
1. Introduction

The engineering of plants, traditional sources of food, fiber and energy has been practiced for decades, and has led to major accomplishments in the improvement of agricultural properties such as insect and disease resistance, tolerance to high salinity, drought, increased tolerance to pesticides and herbicides and extraction of toxic metals from the soil. Recent advances in molecular biology have facilitated the production in plants of foreign proteins of high therapeutic value. In 1992, Arntzen and his colleagues introduced the concept of transgenic plants as vaccine production systems [1]. Additional experiments in several laboratories have demonstrated that bacterial and viral antigens can be expressed in a variety of plant species [Table 1]. Oral or parenteral administration of plant derived antigens generated systemic and mucosal immune responses indicating that transgenic plants have great potential for the development of oral vaccines [2-7].

2. Advantages of Plant Vaccines

Plant-based vaccines have attracted the attention of the pharmaceutical industry for safety and economic reasons. For example, proteins isolated from plant tissues are free from animal pathogenic contaminants and plant pathogens are not infectious for humans. In addition, plants do not generate similar metabolic products, which can co-purify with the transgene product of interest. Plant cultivation is straightforward and inexpensive making use of indigenous agricultural techniques and available agricultural machinery. Scale up for agricultural production can be achieved at minimal cost compared with conventional fermentation methods. Of greatest importance, plant cells like other eukaryotic cells, can perform post-translational modifications e.g.,
glycosylation and phosphorylation essential for the biological activity of proteins of animal origin [8]. Transgenic plants can be conveniently stored and distributed in the form of tubers and seeds. Administration of plant vaccines can be as sample as eating, requires no needles or trained personnel. All these benefits may make it possible for global vaccination programs to effectively control endemic diseases that take a huge toll of life, mostly children, in rural populations of the developing world.

3. Production of Antibodies in Transgenic Plants for Passive Immunization

The first case of antibody production in transgenic plants was demonstrated by Hiatt in 1989 [9]. The gamma and kappa chains of immunoglobulin were expressed separately in tobacco plants. Crossing the two plants resulted in simultaneous expression of both chains, which self assembled into functional antibodies. The presence of a leader sequence increased the accumulation of functional antibodies to 1.3% of total leaf protein. Various strategies have been applied to improve antibody yields in plants. Genetic fusion of the endoplasmic- reticulum retention signal KDEL to antibody fragments increased the single chain Fv protein yield up to 4%-6.8% of total soluble protein [10]. So far, the most complex antibody assembled in plants was the recombinant secretory immunoglobulin, Guy’s 13 [11]. This secretory antibody is composed of a murine joining (J) chain, a rabbit secretory protein component (SC), a kappa protein chain and a hybrid IgA-G antibody heavy chain. The Guy’s 13 secretory antibody recognizes the streptococcal adhesion molecule (SAI/II) of Streptococcus mutans, which is the major cause of dental caries. In his experiments, Julian Ma generated four transgenic Nicotiana tabacum plants, each of which expressed one of the four different
parts of Guy’s 13 secretory antibody. A series of sexual crosses between these plants and filial recombinants generated a plant progeny that expressed all four protein chains simultaneously [11]. Efficient assembly of these immunoglobulin chains produced a functional secretory antibody with a molecular weight of approximately 470 kDa. Enzyme linked immunosorbent assay (ELISA) showed its antibody function to specifically recognize the SAI/II antigen. Production of a complex secretory antibody in a plant and retention of biological function demonstrate the amazing capability that plants have to synthesize and assemble complex biomolecules for human applications. The plant produced SIgA has great applications for passive immunotherapy.

4. **Plant-based Vaccines for Protection against Infectious Diseases**

Many infectious agents come in contact with the host at the surface of epithelial membranes. The mucosal immune response in the gastrointestinal, respiratory and urogenital tracts form the first line of defense against a variety of infectious diseases. The mammalian mucosal immune system is an integrated network of tissues, cells and macromolecules. Secretory IgA as an effector molecule plays an important role in neutralizing bacterial or viral toxins, inhibiting pathogen colonization and eradicating bacterial or viral antigens from the circulatory system. Vaccination by oral administration of an antigen protein is more efficient than parenteral inoculation for induction of mucosal immune responses. Attempts to induce mucosal immunity by oral delivery of live attenuated bacterial or viral strains have met with limited success. Plant vaccines, which protect antigens from intestinal digestion, offer a safe and effective alternative. Two strategies have been pursued to develop plants as vaccine production and delivery
systems. The first is the Agrobacterium tumefaciens mediated stable transformation system, which is based on stable integration of foreign DNAs into the plant genome. The integrated genes are passed from one generation to another at expression levels ranging generally from 0.01% to 0.4% of total plant soluble protein. The second method for synthesizing vaccine proteins in plant cells is based on plant virus mediated infection, which provides a higher yield of vaccine antigens, up to 2.0% of total plant soluble protein within weeks after infection rather than months required for stable transformation methods. Virus infection kills the plants which requires reinfection with virus for each vaccine harvest. The viral products can’t be used directly for vaccination but require purification from infected plant tissues.

5. Transient Antigen Expression in Plants

Plant viruses can replicate to high levels in infected plant tissues, up to 50% of total soluble plant proteins. There are generally two approaches in which modified plant viruses have been used for synthesis and accumulation of recombinant proteins in plants. In the first case, the foreign genes are transcribed from viral promoters, which produce soluble antigen proteins during virus infection. The second case involved the modification of viral capsid proteins to carry vaccine epitopes. Due to the expression of multiple copies of antigen on the surface of a virus particle, this method has been the most used one.
a. **Tobacco Mosaic Virus (TMV)**

TMV is a rod-shaped RNA plant virus composed of approximately 2100 identical capsid proteins. Structural studies of the virus particle identified loop regions on the capsid protein that are unlikely to interfere with virus assembly or viral infection. These loops were targeted with foreign antigens. In one example, an HIV epitope of 20 amino acid residues was inserted into the TMV capsid protein loop. Infection of *N. tabacum* plants with the modified virus led to accumulation of the chimeric capsid proteins [12]. Immunoblot and ELISA data indicated that the correct expression of the epitopes is located at the surface of the viral particle. Turpen et al. (1995) described the expression of a malarial sporozoite antigen epitope in TMV using the same strategy [13].

b. **Cowpea Mosaic Virus (CPMV)**

CPMV is an RNA virus containing 60 copies of a large (L) and a small (S) protein subunit. Potential insertion sites of foreign protein sequences were determined by extensive analysis of the 3-dimensional structure of CPMV. An epitope derived from VP1 of foot and mouth disease virus (FMDV) was cloned into one particular loop region of the S protein [14,15]. The chimeric RNAs were able to replicate in cowpea protoplasts. FMDV-specific antiserum recognized the modified S protein. Immunosorbent electron microscopy identified the assembly of the virus particle in cowpea plant leaf tissues. However, serial passage of recombinant CPMV revealed rapid loss of the inserted sequence. Genetically more stable chimeric CPMV particles that express epitopes derived from human rhinovirus 14 and human immunodeficiency virus (HIV-1) were described by Porta et al. in 1994 [16]. The inserted epitopes were immunogenic in rabbits. The
CPMV expression system was successfully used for animal vaccination. Dalsgaard and his colleagues reported insertion of a linear epitope from the VP2 capsid protein of mink enteritis virus (MEV) into CPMV particles [17]. Virus particles harvested from black-eyed bean plants were used for subcutaneous injection in mink. A considerable reduction of virus shedding was detected after challenge with virulent MEV. This was the first experiment to demonstrate that a plant vaccine protects against an infectious disease in target animals.

TMV and CPMV are the most commonly used viruses for the generation of plant-based vaccines due to their wide host range. Research on other plant viruses has also showed promising results. For example, the coat protein of alfalfa mosaic virus was fused with peptides from rabies virus and HIV [18]. The plant produced antigens elicited specific neutralizing antibodies against rabies virus and HIV in immunized mice. In their study 40% of the mice were protected against a lethal dose challenge of rabies virus. However, despite the high vaccine protein yield of the virus system the size of the antigenic epitope that can be attached to or inserted into the virus capsid protein is small. Less than an 80 amino acid sequence has been inserted successfully into the virus without disrupting the viral particle formation. From a clinical perspective, expression of a single epitope in a plant may not provide full protection against the infectious disease.

c. Stable Expression of Antigens in Plants

The surface antigen (spa-A) from Streptococcus mutans was the first vaccine candidate to be stably expressed in plants [19]. The spa-A protein is 18.5 kDa in size and is composed of over 1500 amino acids. In comparison with the virus expression systems,
*Agrobacterium* mediated transformation overcomes the size limitation of recombinant proteins which can be synthesized in plant cells. One of the first vaccines generated by stable transformation methods in plants was the Hepatitis B virus, a major cause of human acute and chronic Hepatitis. The Hepatitis B surface antigen (HBsAg) was expressed in tobacco plants at levels about 0.01% of total soluble protein [1]. The tobacco-derived HbsAg forms subviral particles of 22 nm in diameter in the plant, which are the size found in infected human serum. Parenteral immunization of purified antigen stimulated both B and T cell immune responses in mice, similar to that found for commercial Hepatitis B vaccine derived from yeast [20]. These experimental results proved the concept of low cost plant vaccines.

Driven by the need to develop cheap oral vaccines applicable for the developing world, current plant vaccine research is targeting pathogens that cause infectious enteric diseases. Enterotoxigenic *E.coli* (ETEC), cholera, rotavirus and Norwalk virus are the four leading causes of epidemic gastroenteritis in humans. Diarrhea caused by these bacteria and viruses accounts for approximately three million infant deaths per year, mostly in the developing world. In United States alone, the direct and indirect health costs exceed one billion dollars per year.

Cholera is a devastating diarrheal disease that has caused recurrent pandemics throughout the world since 1871. The cholera toxin (CT) from *Vibrio cholerae* is one of the most potent mucosal immunogens yet identified. This enterotoxin is composed of one A subunit (CTA) and five identical B subunits (CTB) [21]. The A subunit harbors ADP transferase activity. The CTA subunit binds to the adenylate cyclase in the cell resulting in massive secretion of sodium, chloride and water from the blood into the
intestinal lumen. Five copies of the B subunit form a pentameric ring like structure, which binds to GM1 gangloside receptors present in the gut epithelial cell membranes. In 1998, Arakawa et al. expressed the CTB subunit in potato plants [22]. An ER retention signal (SEKDEL) fused to the 3' end of the CTB gene facilitated accumulation of CTB gene products in the plant cells. The expression level was 0.3% of total soluble protein. The synthesized CTB subunits formed CTB pentamers, which retained GM1 ganglioside binding affinity. Oral feeding of 3.0g of transgenic potato tubers induced both serum and intestinal CTB-specific antibodies in mice [23]. More importantly, the immune response against CTB could be boosted significantly by a single additional oral dose. Ileal loop assays demonstrated a 60% reduction in fluid accumulation in the small intestine of immunized mice. In addition to its mucosal immunogenicity, CTB has mucosal adjuvant activity, which is largely attributed to its cell binding properties [24,25]. More recently, CTB was found to be useful as a carrier molecule for specific targeting to the gut associated lymphoid tissues (GALT). A CTB-insulin fusion protein produced in transgenic potato plants successfully protected nonobese diabetic mice from development of antoimmune diabetes mellitus Type I [26].

Enterotoxigenic E. coli (ETEC), is the most common causative agent of travelers' diarrhea. Present estimations indicate that 50% of persons traveling to developing countries experience diarrheal disease and ETEC has been isolated in 33 to 50% of these episodes. ETEC strains possess two pathogenic traits: adhesion to the small intestine and production of a heat labile enterotoxin (LT). The LT is structurally, functionally and antigenically similar to CT. Both toxins share 80% homology in DNA sequence [21]. The binding subunit of the ETEC heat-labile enterotoxin (LTB) has been expressed at low
levels, 0.01% of total protein in potato plants [27]. A plant-optimized synthetic gene encoding the LTB gene has been introduced into potato plants. The expression level was raised to 0.15% of total soluble plant protein. As expected, the plant derived LTB stimulated a mucosal immune response in mice. The immunized mice were partially protected against bacterial LT challenge. The plant vaccine test was later moved into human clinical trials [28]. Raw potato tubers (50g to 100g) containing 0.5 or 1.0mg plant produced LTB were consumed by volunteers on day 0, 7 and 21. The volunteers developed both serum and mucosal immune response against LTB. These results demonstrated for the first time that food plant-based vaccines are immunogenic in humans.

Norwalk virus is known to cause acute gastroenteritis in humans. The virus particle is composed of a single capsid protein. The capsid proteins expressed in tobacco and potato plants self assembled into virus like particles (VLP) [29]. The plant derived VLPs were morphologically and antigenically similar to authentic virus particles. Mice fed 50ug of plant produced NVCP developed serum IgG specific for Norwalk virus-like particles. These results point the way to a new strategy for development of plant vaccines. Since individual soluble protein antigens are often ineffective for oral immunization. This result is probably due to intestinal digestion and lack of tropism for gut associated lymphoid tissues. VLPs are stable in the acidic environments of the stomach, and resistant to enzyme digestion in the small intestine. Of most importance, the VLPs preserve conformational epitopes located on the surface of virus particles which can be recognized by the host immune system. In comparison with other viral antigens, the plant
produced VLP may be the most effective antigen found for protection against infectious enteric viral diseases.

6. The Future of Plant-based Vaccines

Research efforts focused on plant vaccine development have expanded over the past ten years. Bacterial and viral antigens have been successfully synthesized in an increasing number of plant species. Preclinical trials have demonstrated that mammals can be significantly protected against infectious diseases by plant synthesized vaccines. However, many basic questions have not been adequately addressed. Low expression levels of antigen proteins in edible plants remain as one persistent obstacle to successful plant-based vaccine application. A variety of strategies are available to solve this problem. For example, genes encoding vaccine antigens can be optimized for maximum plant codon usage [28], plant promoters can be engineered to increase transcription levels; RNA splice sites and intron sequences can be removed, 5' and 3' untranslated regions that increase mRNA stability can be used; 5' leader sequences and 3' ER and vacuolar retention signals can be added to increase accumulation of antigen proteins in specific cell organelles.

Constitutive expression of foreign genes may lead to accumulation of foreign proteins to toxic levels in the plant. Alternatively, inducible promoters, which stimulate gene expression at specific times during plant development, may not only prevent accumulation of toxic levels of the foreign gene product but will also conserve the plants photosynthate for generation of maximum plant growth resulting in higher yields of recombinant proteins [22,26]. Thus, organ and tissue specific promoters activated only in
the fruit, tubers or seeds must be explored [30]. The cholera toxin B subunit, which targets GM1 ganglioside receptors on the epithelial cell surface, can be used as a carrier molecule for delivery of antigens to the gut associated lymphoid tissues [31]. Due to its size, plant genomes can accommodate large numbers of foreign genes. Therefore it may be possible to express several bacterial and viral antigens in one transgenic plant. Multi-component edible plant vaccines providing immunological protection simultaneously against several infectious diseases are currently under construction in our laboratory at Loma Linda University. However the protective efficacy of these vaccines against multiple antigens is still unknown.

Many pathogenic enteric viruses are composed of more than one capsid protein. The correct assembly of double or triple layered VLPs has been achieved in bacterial or insect cell systems. However, assembly of multi-layered VLPs in plants remains to be detected. The production of complex VLPs in transgenic plants will provide conformational epitopes with superior protective efficacy against pathogen challenge. The area of plant vaccine development is in its infancy and many research experiments remain to be done to move from the “proof of principle” stage to successful commercialization.
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Table 1. Plant Produced Recombinant Proteins

<table>
<thead>
<tr>
<th>Recombinant proteins</th>
<th>Plant host</th>
<th>Transformation vector</th>
<th>Expression level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spa A of <em>S. mutans</em></td>
<td>Tobacco</td>
<td><em>agrobacterium</em></td>
<td>0.02%</td>
</tr>
<tr>
<td>Hepatitis B surface antigen</td>
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<td><em>agrobacterium</em></td>
<td>0.01%</td>
</tr>
<tr>
<td>Secretory Ig A</td>
<td>tobacco</td>
<td><em>agrobacterium</em></td>
<td>200-500 ug/g</td>
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<td><em>E. coli</em> LTB</td>
<td>Potato</td>
<td><em>agrobacterium</em></td>
<td>0.01%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.15%</td>
</tr>
<tr>
<td>Cholera toxin B subunit</td>
<td>Potato</td>
<td><em>agrobacterium</em></td>
<td>0.3%</td>
</tr>
<tr>
<td>Norwalk virus capsid protein</td>
<td>Tobacco</td>
<td><em>agrobacterium</em></td>
<td>0.23%</td>
</tr>
<tr>
<td>Rabies glycoprotein</td>
<td>Tomato</td>
<td><em>agrobacterium</em></td>
<td>0.001%</td>
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<tr>
<td>FMDV (foot and mouth disease virus), VP1</td>
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<td><em>agrobacterium</em></td>
<td>NA</td>
</tr>
<tr>
<td>FMDV, VP1</td>
<td>Cowpea</td>
<td>Cowpea mosaic virus (CPMV)</td>
<td>NA</td>
</tr>
<tr>
<td>Rabies virus epitope</td>
<td>Spinach</td>
<td>Tobacco mosaic virus (TMV)</td>
<td>NA</td>
</tr>
<tr>
<td>MEV (mink enteritis virus), VP2</td>
<td>Black-eyed bean</td>
<td>CPMV</td>
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<td>HIV, gp120</td>
<td>Tobacco</td>
<td>TMV</td>
<td>10-40%*</td>
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<tr>
<td>rhinovirus14, VP1</td>
<td>Cowpea</td>
<td>CPMV</td>
<td>1.2-1.5 mg/g*</td>
</tr>
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</table>

NA Not applicable
* Yield of chimeric virus
CHAPTER FOUR
ASSEMBLY OF CHOLERA TOXIN-ANTIGEN FUSION PROTEINS IN TRANSGENIC POTATO

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Key Words
Trangenic Plant, Cholera Toxin, Fusion Protein, Mucosal Immunization

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Abstract

One of the most significant problems challenging the protective efficacy of plant delivered oral vaccines is the relatively low level of antigenic proteins generally synthesized in plant tissues. To circumvent this difficulty, we explored the feasibility of using cholera toxin as an enterocyte targeted carrier molecule for delivery of multiple antigens to the mucosal immune system. Potato plants were engineered to synthesize two cholera toxin fusion proteins. The cholera toxin B subunit (CTB) was linked to a rotavirus enterotoxin NSP4 epitope and the cholera toxin A2 subunit (CTA2) was fused to the enterotoxigenic E.coli (ETEC) colonization factor CFA/I. The fusion proteins were detected in transformed tuber tissues by immunoblot assay and quantitated by enzyme linked immunosorbent assay (ELISA). The CTB-NSP4 protein expression level was found to be approximately 0.01% of total soluble plant protein (TSP) and the CFA/I-CTA2 protein expression level was about 0.002% of TSP. Assembly of the CFA/I-CTA2 subunit with the CTB-NSP4 pentamer was detected by immunoblot and G_{M1} ELISA methods. The CFA/I-CTA2-CTB-NSP4 pentamer fusion protein complex retained its binding affinity for G_{M1} ganglioside enterocyte membrane receptors. Our experimental results demonstrate for the first time that cholera toxin A and B subunit fusion proteins synthesized in planta interact in a coordinated fashion to generate a multimeric carrier complex for targeted delivery of multiple antigens to the mucosal surface.
1. Introduction

Cholera, rotavirus and ETEC are three major causes of acute gastroenteritis worldwide [1]. Vaccination and eventually eradication of these endemic diseases depends on the development of inexpensive oral vaccines accessible to the developing world. Recombinant DNA technology has now made it possible to express foreign antigens in plants for creating food plant based vaccines [2,3,4,5]. Recent examples are the expression of hepatitis B surface antigens in potatoes and tobaccos [6,7], heat sensitive enterotoxin (LTB) expression in potatoes [8,], and Norwalk virus capsid protein in both tobacco and potatoes [9]. However, oral immunization requires a relatively large dose of antigen to stimulate an efficient immune response due to intestinal digestion and dilution of the antigen by food and mucosal secretion [10]. The generally low expression level of foreign proteins in plants is currently a limiting factor in practical application of transgenic plants as production and delivery vehicles for oral vaccines. One attractive solution to the necessity for antigen overproduction in plants is the development of carrier molecules, which can target fusion antigens to the mucosal immune system. Cholera toxin is such a carrier molecule, which possesses strong immunogenicity as well as strong adjuvant activity [11,12,13]. Cholera toxin is made of 5 copies of the CTB subunit and one copy of the CTA subunit. The CTB subunits assemble to form pentameric structures, which bind to GM1 ganglioside receptors embedded in the surface of intestinal epithelial cells. The CTA subunit is postranslationally cleaved into A1 and A2 subunits. The small helical CTA2 subunit is inserted into the central core of the CTB pentamer and becomes linked to the CTA1 subunit by hydrogen and disulfide bonds. Chemically or genetically fusing small molecules to the CTB subunit was found to
stimulate antibody production against both antigens [14,15]. Alternatively, replacement of CTA1 with streptococcal adhesion protein was found to stimulate an immune response against the fusion protein [16]. In this report we demonstrate the expression of two fusion protein genes in potato plants: a twenty two amino acid epitope of rotavirus nonstructural enterotoxin protein NSP4 linked to the cholera toxin CTB subunit, and the ETEC fimbrial colonization factor CFA/I fused to the cholera toxin CTA2 subunit. Further, we demonstrate the assembly of the two fusion proteins in the plant to generate a multicomponent cholera toxin-like structure.

2. Materials and Methods

a. Construction of Plant Expression Vectors

In our first plant expression vector construct, the CTB gene including its leader peptide was PCR amplified from the ctxAB operon in plasmid pPT42 (provided by J. Mekalanos, Harvard Medical School). The 3’ primer was designed to contain an oligonucleotide encoding the endoplasmic reticulum (ER)-retention signal in frame downstream of the CTB gene. The amplified DNA fragment encoding the CTB:SEKDEL fusion gene was inserted into the plant expression vector pPCV701 under control of the mannopine synthase (mas) P2 promoter. The plant expression vector was designated pPCV701-CTB. In a second construct, the CTB gene and its leader peptide was generated by PCR amplification of the same ctxAB operon. The 3’ primer was designed to contain a nucleotide sequence encoding the tetrapeptide hinge (Gly-Pro-Gly-Pro) to increase the flexibility of the conjugated protein. The CTB:hinge sequence was inserted into plant transformation vector pPCV701 under control of the mas P2 promoter. The plant
expression vector was designated pPCV701 CTBH. A double stranded DNA fragment encoding the rotavirus enterotoxin NSP4 (114-135) epitope was synthesized in a model 394 DNA/RNA Synthesizer (Applied Biosystems, Inc.). The 3’end of the NSP4 sequence contains the ER retention signal, SEKDEL. The NSP4:SEKDEL sequence was cloned into plant expression vector pPCV701CTBh, immediately downstream of the CTB: hinge sequence to create the fusion gene CTB:hinge-NSP4:SEKDEL. The plant expression vector was designated pPCV701CTB-NSP4.

In a third construct, The CTA leader sequence and CTA2 gene, which contains its own KDEL signal sequence was amplified by PCR from the ctxAB operon, and inserted into the vector pPCV701 CTB-NSP4 under control of the mas P1 promoter. A DNA fragment encoding the ETEC colonization factor CFA/I was amplified from plasmid pIGx15A (provided by Dr. M. Levine, University of Maryland), and was inserted in frame between the CTA leader sequence and the CTA2 gene. The resultant plant expression vector was designated pPCV701CFA/I-CT-NSP4 and contains the CTA2-CFA/I fusion gene driven by the mas P1 promoter and the CTBh-NSP4 fusion gene driven by the mas P2 promoter.

b. Plant Transformation

Plant expression vectors pPCV701-CTB, pPCV701CTB-NSP4 and pPCV701CFA/I-CT-NSP4 were introduced into Agrobacterium tumefaciens strain GV3101 pMP90RK by electroporation [17]. Potato Solanum tuberosum cv. Bintje leaf explants were transformed with A. tumefaciens harboring the plant expression vectors and transgenic potato plants were regenerated as described [18].
c. Analysis of DNA/RNA from Transformed Potato

Plant genomic DNA was isolated from transformed potato leaf tissues using a DNeasy Plant Mini Kit (Qiagen Inc.). Presence of the CTB, NSP4 and CFA/I genes were determined by PCR analysis using transformed plant genomic DNA (500ng) as a template and primers specific for each gene. The PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide to visualize the bands. Total RNA from transformed plant leaves was isolated using an RNeasy Plant Mini kit (Qiagen Inc.). The presence of CFA/I mRNA was determined by RT-PCR analysis following the instructions included in the Omniscript Reverse Transcriptase kit (Qiagen Inc.). The RT-PCR samples were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

d. Immunoblot Analysis of Protein from Transformed Potato

Approximately 1.0 g of leaf tissue was homogenized on ice in 1ml of extraction buffer (200mM Tris-HCl, pH 8.0, 100 mMNaCl, 400mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1mM phenyl-methylsulfonyl fluoride, 0.05% Tween-20). The tissue homogenates were centrifuged twice at 17,000 × g for 15min at 4°C to remove insoluble debris. A 10-20 ul aliquot of supernatant fluid containing 100-200 µg of total soluble protein determined by protein assay (Bio-Rad Inc.), was analyzed by 12% SDS-PAGE. Plant homogenate samples were either boiled 5 minutes prior to electrophoresis or loaded directly on the gel. The expression of CTB, NSP4 and CFA/I protein were detected by rabbit anti-CTB antiserum (Sigma1:5000 dilution), rabbit anti-NSP4(114-135) antiserum (provided by M. Estes, Baylor College of Medicine, at a dilution 1:1000) or rabbit anti
CFA/I antiserum (1:1000 dilution). The second antibody used for band detection was an alkaline phosphatase-conjugated mouse antirabbit IgG (Sigma, at 1:10,000 dilution).

e. Quantification of CTB-NSP4, CFA/I-CTA2 Proteins' levels in Transformed Potato

The amount of plant-synthesized CTB-NSP4 fusion protein was measured in transformed plant extracts by chemiluminescent G₉ ELISA assay [17]. Briefly, the wells of a 96-well microtiter plate (Dynatech Laboratories) were coated with 100ul/well of monosialoganglioside G₉ (3.0ug/ml) (Sigma) in pH9.6 bicarbonate buffer (15mM Na₂CO₃, 35mM NaHCO₃), and incubated at 4°C overnight. The plate was washed three times with PBST (phosphate buffered saline (PBS) containing 0.05% Tween-20). The background was blocked by incubation in 1% bovine serum albumin (BSA) in PBS (300ul/well) at 37°C for 2h followed by washing three times with PBST. The microtiter plate was incubated with 2ug of transformed plant total soluble protein per well in PBS (100ul/well). For use as a positive control, a known amount of bacterial CTB (Sigma) plus 2ug untransformed plant total soluble protein in PBS (100ul/well) was added to the plate and incubated overnight at 4°C. The plate was washed three times with TBST and then incubated in a 1:8000 dilution of rabbit anti-cholera toxin antibody (Sigma, 100ul/well) for 2h at 37°C, followed by washing three times with PBST. The plate was incubated with a 1:80,000 dilution of anti-rabbit IgG alkaline phosphatase conjugate (Sigma, 100ul/well) for 2h at 37°C and washed three times with PBST. The plate was finally incubated with 100ul/well Lumi-Phos Plus substrate (Lumigen) for 30 min at 37°C. The plate was cooled for 5 min and light emission was measured in a Dynatech 3000
Microtiter Plate Luminometer (Dynatech Laboratories). The amount of plant-synthesized CFA/I-CTA2 fusion protein in each well was measured by the same chemiluminescent ELISA assay with the exception that 2ug of transformed plant total soluble protein was directly coated on the plate in pH9.6 bicarbonate buffer. The first antibody used was rabbit anti-CFA/I at a 1:3000 dilution. The rest of the assay procedure was identical to the ELISA described above.

f. Detection of CFA/I-CTA2 and CTB-NSP4 Assembly in Plant Tissues

The assembly of CFA/I-CTA2 subunit with the CTB-NSP4 pentamer in transformed plant tissues was detected by the chemiluminescent Gm1 ELISA assay procedure. The analysis procedures were the same as for the Gm1 ELISA for quantification of CTB-NSP4 fusion protein with the exception that the first antibody used was rabbit anti-CFA/I at a 1:3000 dilution.

3. Results and Discussion

a. Construction of Plant Expression Vectors

The plant expression vector pPCV701 CFA/I-CT-NSP4 (Figure 1.) was generated by inserting the CTB-NSP4 gene following the mas P2 promoter and inserting the CFA/I-CTA2 gene 3' adjacent to the mas P1 promoter. The mas P1,P2 promoter is a bidirectional promoter capable of simultaneous expression of two genes in opposite directions. A glycine-proline hinge region (GPGP), was added separating the CTB peptide and the NSP4 epitope to increase the flexibility of the fusion peptide. The CTA2 gene was fused to the 5' end of the CFA/I gene as a linker to facilitate association of the
CFA/I-CTA2 fusion peptide with CTB pentamer structure. Each fusion gene retains its bacterial leader sequence and an endoplasmic retention (ER) retention signal at the 3' end. For use as controls, plant expression vectors containing CTB or CTB-NSP4 fusion genes under control of the mas P2 promoter were included in the transformation experiments. Fifteen kanamycin resistant transgenic potato plants were regenerated following A. tumefaciens mediated transformation of potato leaf explants.

b. DNA/RNA Analysis of Transformed Plants

The transformed potato plants were analyzed by PCR for the presence of the CTB-NSP4 and the CFA/I-CTA2 gene fusions (Figure 2.). A 560 bp DNA fragment which included both the CTB and the NSP4 DNA sequences was amplified from the transformed potato genomic DNA. No nonspecific amplification was observed in untransformed plant samples, indicating the high specificity of the 5'CTB and the 3'NSP4 primers. Using primers including a 5' flanking sequence of the CFA/I gene and a 3' flanking sequence of the CTA2 gene, we detected amplification of a single 590bp DNA fragment, which is the correct size for the CFA/I-CTA2 fusion gene. Since the P1 promoter is often considered to be 5 to 10 times weaker than P2 promoter, we chose to study the mRNA transcription of CFA/I gene at the P1 side of the mas promoter. Both 5' and 3' CFA/I primers were used in the RT-PCR assay. A single 430 bp mRNA fragment was detected, which is the exact size of CFA/I cDNA. Samples without incubation with reverse transcriptase showed no band, which indicated that no DNA contamination was present in the total RNA sample.
c. Detection of Foreign Proteins in Transformed Plants

The presence of the CTB gene product was identified by immunoblot analysis using antiserum against cholera toxin (Figure 3A.). Bacterial CTB formed a 45 kD pentamer structure which was partially dissociated into 11 kD monomers. SDS-PAGE gel electrophoresis results indicated that the plant derived CTB pentamers were approximately 50 kD, higher in molecular weight than the bacterial CTB. The approximately 5 kD molecular mass increase can be attributed to the presence of the additional SEKDEL sequence and the retention of the bacterial CTB leader sequence. The plant derived CTB-NSP4 fusion protein also formed an oligomeric structure about the same size as the plant derived CTB pentamer. Immuno-stained electrophoretically separated extracts of transgenic plants containing the CFA/I-CTA2 and CTB-NSP4 genes displayed a 70 kD band, which was significantly higher in molecular weight than the plant synthesized CTB-NSP4 pentamer. The additional increase in molecular mass can be attributed to the presence of CFA/I-CTA2 fusion protein. Heat treatment resulted in dissociation of the multimeric structures into monomers. Plant derived CTB-NSP4 fusion protein dissociated into a 18 kD monomer. Based on preservation of it’s pentamer or oligomeric structure in transformed plant extracts, the CTB-NSP4 fusion protein appears to have retained the conformation required for maintenance of its carrier function. The bacterial colonization factor CFA/I is made up of 6000 copies of a 20 kD monomer. A multimer ladder of bacterial CFA/I was detected after SDS-PAGE (Figure 3B, lane 1). Anti-CFA/I antiserum was found to react with the same 70 kD band detected by anti-CT antiserum from the plant transformed with the CFA/I-CTA2:CTB-NSP4 fusion genes. Protein extracts of control plants, transformed only with the CTB-NSP4 gene did not
cross react with anti-CFA/I antiserum (Figure 3B lanes 5 and 6). The cholera toxin A2 subunit is inserted into the central pore of the B pentamer as a continuous helix. Hydrophobic interactions between amino acids of the A2 and B subunits contributes to stabilization of the B pentamer structure. The presence of CFA/I-CTA2 peptide appears to have made the pentamer more resistant to heat degradation (Figure 3B, lanes 3 and 4). Immunoblot analysis using antiserum against the NSP4 peptide detected the NSP4 moiety of the CTB-NSP4 fusion protein in monomer form. The reaction was specific and did not cross react with plant derived CTB protein. The inability to detect the small NSP4 22 amino acid moiety in its CTB-NSP4 multimeric form on immunoblots may be due to inclusion of the small NSP4 peptide within the CTB pentameric structure.

d. Quantitation of Protein Levels

The CTB-NSP4 fusion protein expression levels in transformed potato leaf and tuber tissues were determined by quantitative chemiluminescent ELISA. The amount of plant CTB-NSP4 protein was measured in comparison with relative light units (RLU) emitted from a known amount of bacterial CTB protein (Figure 4). The amount of CTB-NSP4 detected in transformed potato tissues was expressed as a percentage of the total soluble plant protein (TSP) measured in tuber samples. Plant #15 was found to have the highest expression level equivalent to approximately 0.01% of TSP and is roughly equivalent to 5-10ug of CTB-NSP4 protein in 3.0g of transgenic potato tuber tissues. Plant #3 was found to have the lowest CTB-NSP4 expression level of approximately 0.001% of TSP. Five minutes immersion in boiling water dissociated the CTB multimers into monomers. However, we were unable to detect light emission from CTB monomers in the boiled
samples. The expression levels of CFA/I-CTA2 fusion protein ranged from 0.0003% to 0.002% of TSP in transformed plants (Figure 5.). The maximum expression level of CFA/I-CTA2 fusion protein (0.002% TSP) from the P1 mas promoter is 5 times lower than that of the CTB-NSP4 fusion protein (0.01%) obtained from the mas P2 promoter.

e. Assembly of Subunit Foreign Proteins

Association of the CFA/I-CTA2 fusion protein with the CTB-NSP4 pentamer was further demonstrated by results obtained from the G_{M1} ELISA using anti-CFA/I antibody as the primary antibody. Transgenic plants #1 and #2 transformed with CTB and CTB-NSP4 genes respectively were used as control plants. Transformed plants #3 through #7 contained the CFA/I-CTA2:CTB:NSP4 genes (Figure 6.A). Statistical analysis using the Student’s t-test revealed significant differences among the control plants and the CFA/I-CTA2 transformed plants (p<0.05). When the amount of total soluble protein was increased from 20 μg to 60 μg, the RLU signal emitted from the CTB-NSP4 control plant remained unchanged at the background level (Figure 6.B). However, in comparison with the control plants, extracts from the plant transformed with CFA/I showed an increase in slope proportional with increased tissue amounts that was statistically significant above the control plant tissues (p<0.05). In Figure 6, panel C, CFA/I peptide was detected in samples from transformed plants containing the CFA/I-CTA2:CTB:NSP4 genes (Figure 6.C, #1). Boiling dissociated the CTB-NSP4 pentamer. The CFA/I-CTA2 protein was no longer able to associate with the CTB-NSP4 protein (Figure 6.C, #2). Addition of purified CFA/I protein to the transformed plant samples containing CTB-NSP4 protein did not show any positive CFA/I signal indicating that CFA/I protein did not bind
unspecifically with CTB-NSP4 multimers unless the CTA2 peptide was linked (Figure 6.C, #3 and #4). The CFA/I singals were not detected in untransformed plant homogenates with the addition of purified CFA/I and NSP4 proteins indicating no unspecific binding of CFA/I or NSP4 protein with GM1 ganglioside (Figure 6.C, #5 and #6).

The mas dual promoters are stimulated by the plant hormone indole acetic acid [19]. Following auxin induction, the expression levels are equivalent to or greater than constitutively expressed promoters such as the cauliflower mosaic virus 35S promoter [6,8], the patatin promoter [20]. The bidirectional nature of the mas promoters permit simultaneous expression of two foreign genes. This advantage may be a benefit for the production of multi-subunit proteins in plants. Coordinate expression levels between the mas P1 and P2 promoters provide options for the production of different amounts of subunits, which may in turn, achieve optimal ratios for subunit association. Assembly of the CFA/I-CTA2 peptide with the CTB-NSP4 pentamer to form a multimeric structure in plants, similar to the natural form of cholera toxin demonstrates the utility of the mas dual promoters for expression of multiple subunits of the cholera toxin. Assembly of the cholera toxin subunits in edible plant tissues provides us with the opportunity to generate fusion proteins with the cholera subunit peptides. Thus, the cholera toxin-like multisinubunit molecules can be used as an enterocyte targeted carrier molecules to transport multiple antigens to the surface of the mucosal immune system. The expression of three antigens CFA/I, CTB, NSP4 from different infectious diseases in one transgenic plant opens the way for development of multi-component oral vaccines in edible plants.
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References


Figure 1. Plant expression vectors. Construct (3), pPCV701CFA/I-CT-NSP4 contains all Four genes located within the transferred DNA (T-DNA) sequence flanked by the right and left border (RB and LB), 25 bp direct repeats required for integration of the T-DNA into plant genome: 1) CTBH:NSP4:SEKDEL fusion gene is driven by the mas P2 promoter; 2) CFA/I:CTA2 fusion gene is driven by the mas P1 promoter; 3) The neomycin phosphotransferase (NPT II) expression cassette provides resistance to kanamycin for selection of transformed plants; 4) Bla, the β-lactamase cassette generates resistance to ampicillin in E.coli and carbenicillin in A.tumefaciens. g7pA, g4pA and OcspA are polyadenylation signals from A.tumefaciens T-DNA gene 7, gene 4 and the octopine synthase gene respectively; Pnos is the promoter of the nopaline synthase gene; Ori pBR is the replication origin of pBR322 for maintenance of the plasmid in E.coli. Construct (2), pPCV701CTB-NSP4 which has only CTBH:NSP4:SEKDEL under control of the mas P2 promoter, and construct (1), pPCV701CTB which has only CTB:SEKDEL under control of the mas P2 promoter, were included as controls.
Figure 2. DNA/RNA analysis of transformed potato. Panel A. PCR detection of CFA/I-CTA2 cDNA in potato genomic DNA: Lane 1, 1 kb molecular weight marker; Lane 2, genomic DNA from untransformed potato leaf tissues; Lanes 3 through 5, genomic DNA from leaf tissues of potato plants transformed with plant expression vector pPCV701CFA-CT-NSP4. Panel B. PCR detection of CTBH-NSP4 cDNA in potato genomic DNA: Lane 1, 1 kb molecular weight marker; Lanes 2 through 5, genomic DNA from leaf tissues of potato plants transformed with plant expression vector pPCV701CFA-CT-NSP4; Lane 6, genomic DNA from untransformed potato plant leaf tissues. Panel C. RT-PCR detection of CFA/I mRNA: Lane 1, 100 bp molecular weight marker; Lanes 2 and 3, total RNA preparations from untransformed potato leaf tissues; Lanes 4 through 7, total RNA preparations from potato leaf tissues transformed with plant expression vector pPCV701 CFA-CT-NSP4; Lanes 4 and 6, PCR reaction without reverse transcriptase.
Figure 3. Immunoblot detection of CTB-NSP4 and CFA/I-CTA2 fusion proteins in transformed potato tuber tissues. **Panel A.** Immunoblot with anti-CT antiserum: Lane 1, molecular weight markers; Lanes 2 through 5, unboiled samples; Lanes 6 through 9, boiled potato samples; Lanes 2 and 6, 100 ng bacterial CTB; Lanes 3 and 7, 100 μg total soluble protein from potato plants transformed with the CTB gene; Lanes 4 and 8, 100μg total soluble protein from potato plants transformed with the CTB-NSP4 fusion gene; Lanes 5 and 9, 100 μg total soluble protein from potato plant transformed with CFA/I-CTA2 and CTB-NSP4 fusion genes. **Panel B.** Immunoblot with anti-CFA/I antiserum. Lane 1, molecular weight markers; lane 2, 100 ng boiled bacterial CFA/I protein; Lanes 3 and 4, unboiled and boiled 200 μg total soluble protein from potato plants transformed with CFA/I-CTA2:CTB-NSP4 double fusion genes; Lane 5, unboiled and Lane 6, boiled potato tuber tissues containing the CTB-NSP4 gene (200 μg total soluble protein each lane). **Panel C.** Immunoblot using anti-NSP4 antiserum as the primary antibody. Lanes 1 through 3, unboiled tuber tissue samples; lanes 4 through 6, boiled tuber tissue samples. Lanes 1 and 4, 200 μg total soluble protein from potato tubers transformed with the CTB gene; lanes 2 and 5, 200 μg total soluble protein from potato plants transformed with the CTB-NSP4 fusion gene; lanes 3 and 6, 200 μg total soluble protein from potato plants transformed with the CFA/I-CTA2:CTB-NSP4 fusion genes.
Figure 4. Quantitation of CTB-NSP4 fusion protein levels in transformed potato tissues. Anti-CT antiserum was used as the primary antibody in a GM1 ELISA assay. Known amounts of bacterial CTB protein plus 2ug total soluble protein from untransformed potato tissues were used to generate the standard curve. A 2ug amount of total soluble protein (TSP) from transformed potato plants #3 and #15 were either boiled or directly used for GM1 ELISA assay. An untransformed plant was used as the negative control. The relative light units (RLU) obtained from the sample assay was measured in comparison with the standard curve to calculate CTB-NSP4 fusion protein expression levels.
Figure 5. Quantitation of CFA/I-CTA2 fusion protein levels in transformed potato tissues by chemiluminescent ELISA. A 2 μg amount of total soluble protein from centrifuged clarified extracts of transformed plants (#1 through #15) was used to directly coat each well of a 96 well microtiter plate for the chemiluminescent ELISA. Anti-CFA/I antiserum was used as the primary antibody. A transformed plant that expressed only the CTB-NSP4 fusion protein was used as a negative control (C). The expression levels of CFA/I-CTA2 fusion protein were presented as % of total soluble plant proteins (TSP).
Figure 6. Detection of CFA/I-CTA2 and CTB-NSP4 fusion protein assembly in transformed potato plants. **Panel A.** A chemiluminescent GM1 ELISA was performed with plant homogenates containing 20 μg of total soluble protein (TSP). Rabbit anti-CFA/I antiserum was used as the primary antibody. Sample #1 is an extract of a control plant transformed with the CTB gene only. Sample #2 is an extract from a control plant transformed with only the CTB-NSP4 gene. Samples #3 through #7 are extracts from plants transformed with the CFA/I-CTA2:CTB-NSP4 fusion protein genes. **Panel B.** Presents a chemiluminescent GM1 ELISA performed with increasing amounts of total soluble protein from transformed plant tissues. Rabbit anti-CFA/I antiserum was used as the primary antibody. Line #1 represents the RLU values obtained from a control plant transformed with the CTB-NSP4 gene only. Line #2 represents the RLU values obtained from a plant transformed with the CFA/I-CTA2:CTB-NSP4 fusion genes. **Panel C.** A chemiluminescent GM1 ELISA was performed with plant homogenates mixed with purified CFA/I or NSP4 proteins. Rabbit anti-CFA/I antiserum was used as the primary antibody. Sample #1 and #2 are unboiled and boiled extracts from plants transformed with both CFA/I-CTA2 and CTB-NSP4 fusion genes respectively. Sample #3 and #4 are unboiled and boiled extracts from plants transformed with the CTB-NSP4 fusion gene mixed with 200 ng purified CFA/I protein. Sample #5 is untransformed plant homogenate mixed with 200 ng purified CFA/I. Sample #6 is untransformed plant homogenate mixed with 200 ng purified NSP4 proteins.
A

RLU

20 ug TSP

B

RLU

20 ug  40 ug  60 ug TSP

C

RLU

#1  #2  #3  #4  #5  #6
CHAPTER FIVE
A PLANT-BASED MULTICOMPONENT VACCINE PROTECTS MICE FROM ENTERIC DISEASES

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Key Words
Trangenic Plant, Cholera Toxin, Rotavirus, *E.coli*. Fimbrial Antigen, Oral vaccine

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Abstract

To generate a plant based multicomponent oral vaccine against cholera, rotavirus and enterotoxigenic *E.coli* (ETEC), we have constructed transgenic potato plants that synthesize the cholera toxin B and A2 subunits, the rotavirus enterotoxin NSP4 22 amino acid epitope and the ETEC fimbrial antigen CFA/I. To facilitate delivery of the plant synthesized antigens to the gut-associated lymphoid tissues, the rotavirus NSP4 epitope was linked to the cholera toxin B subunit (CTB) and the ETEC fimbrial antigen CFA/I was linked to the cholera toxin A2 subunit. The cholera toxin fusion proteins expressed in transformed potato tuber tissues assembled into a cholera holo-toxin-like oligomeric structure, which retained enterocyte membrane receptor G_{M1}-ganglioside binding affinity. Both serum and intestinal antibodies against NSP4, CFA/I and CTB were induced in orally immunized mice. Analysis of IL-2, IL-4 and INFγ cytokine levels in spleen cells isolated from immunized mice indicated the presence of a strong Th1 immune response to the plant synthesized antigens. Fluorescent antibody based flow cytometry analysis of immunized mouse spleen cells showed an increase in CD4+ but not CD8+ memory cell populations. Following rotavirus challenge, passively immunized mouse pups showed a 50% reduction of diarrhea symptoms. The experimental results indicate that food plants can serve as vehicles for production and distribution of multiple antigens for simultaneous immunization against several enteric diseases.
1. Introduction.

Acute gastroenteritis is second only to acute respiratory disease as a cause of death worldwide (Kiyono, et al., 1996). Cholera, rotavirus and ETEC are the three major causative agents of acute infectious enteric diseases. Recently, the successful utilization of plants as production and delivery systems for pathogen antigens has been reported (Mason et al., 1992, 1996; Arakawa et al., 1997; Modelska et al., 1998) The plant expressed antigens were able to generate protective antibodies in orally immunized animals (Thanavala et al., 1995; Haq et al., 1995; Arakawa et al., 1998). However the relatively low expression levels of foreign antigens synthesized in transgenic plants coupled with the modest immune response following oral immunization remain limiting factors for development of an effective plant-based vaccine. The recent expression and successful assembly of cholera toxin B subunits into biologically active oligomeric structures in transgenic potato plants (Arakawa et al., 1997) provides a valuable approach for targeting plant synthesized antigens to the gut associated lymphoid tissues (GALT). Cholera toxin possesses distinct carrier and adjuvant properties. Antigens genetically fused to cholera toxin A or B subunit were found to stimulate strong immune response in orally immunized animals (Czerkinsky et al., 1989; Holmgren et al., 1993). In this report, we have expressed a cholera toxin fusion with two different disease antigens in transformed potatoes. In a C-terminal fusion, we linked the twenty two amino acid immunodominant epitope of the murine rotavirus enterotoxin NSP4 to the cholera toxin B subunit (CTB), and in a N-terminal fusion we fused the ETEC fimbrial colonization factor CFA/I to the cholera toxin A2 subunit (CTA2). Following A.tumefaciens transformation of potato leaf tissue with a plant expression vector carrying the two
cholera toxin fusion protein genes we regenerated transformed plants. Female CD-1 mice were immunized by feeding transformed potato tuber tissues containing the cholera toxin fusion proteins. The titers of serum IgG and intestinal IgG and IgA antibodies against NSP4, CTB and CFA/I were measured in the immunized mice. Spleen cells from immunized mice were analyzed for IL-2, IL-4 and IFNγ cytokine secretion and T lymphocyte subpopulations. Passively immunized mouse pups from orally immunized dams were challenged with SA-11 rotavirus to determine the level of protection against rotavirus infection delivered by the plant-based vaccine.

2. Experimental Protocol

a. Plant Expression Vector Construction and Plant Transformation

The plant expression vector pPCV701CFA/I-CTB-NSP4 was assembled from the parental plasmid pPCV701 in the following manner. A nucleotide sequence encoding the endoplasmic reticulum (ER) retention signal, SEKDEL was first cloned into the plant expression vector pPCV701 on the P2 site of the mannopine synthase (mas) dual P1, P2 promoter. The CTB gene and its leader sequence were amplified by polymerase chain reaction (PCR) from the cholera toxin (ctxAB) operon in plasmid pPT42 (provided by J. Mekalanos, Harvard University, Medical School). The CTB 3’ primer was designed to contain an oligonucleotide encoding the tetrapeptide hinge (Gly-Pro-Gly-Pro) to incorporate a degree of flexibility between the CTB and NSP4 peptides. A synthesized DNA fragment encoding the rotavirus enterotoxin NSP4 (114-135) epitope was inserted in frame between the CTB-hinge and the SEKDEL sequences. The CTA leader sequence and the CTA2 gene were amplified by PCR from the ctxAB operon and cloned into
pPCv701 downstream of the mas P1 promoter region. A DNA fragment encoding the ETEC colonization factor CFA/I (431bp) was amplified from plasmid pIGx15A (provided by Dr. M. Levine, University of Maryland), and was inserted in frame between the CTA leader sequence and the CTA2 gene. The resultant plant expression vector pPCV701CFA/I-CTB-NSP4 was introduced into Agrobacterium tumefaciens strain GV3101 pMP90RK. From sterile plants grown in culture medium in a light room, potato (Solanum tuberosum cv. Bintje) leaf tissue explants were transformed with A. tumefaciens harboring the plant expression vector pPCV701 CFA/I-CTB-NSP4. Transformed plants were regenerated from the explants on selection medium containing kanamycin. Prior to analysis of antigen gene expression transgenic tubers were stimulated to produce high levels of the antigen proteins by incubation of tuber slices on growth medium containing auxin 2,4-D (2,4 dichlorophenoxy acetic acid) for 4 days at room temperature.

b. Immunoblot Detection of the CFA/I and CTB-NSP4 Fusion Proteins

Protein extracts from auxin stimulated transformed potato tubers containing 100 μg of total soluble protein (TSP) were loaded on a 10-15% SDS-PAGE gel with or without 5 min boiling prior to electrophoresis. The separated protein bands were transferred to nitrocellulose membrane by electroblotting on a semi-dry blotter (Sigma) at 30V, 60mA for one and a half hours. The location of CTB, NSP4 and CFA/I proteins were identified by incubation of the blot in rabbit anti-CTB antiserum (Sigma 1:5000 dilution) overnight at room temperature followed by incubation in alkaline phosphatase-conjugated mouse anti-rabbit IgG (Sigma, at 1:10,000 dilution) for 2 hours at room temperature. Finally the
membrane was incubated in the substrate BCIP/NPT (Sigma) for 10 min. The color reaction was stopped by washing the membrane several times in distilled water.

c. Mouse Immunization and Cell Cultures

A group of 10 CD-1 female mice each were fed 3 g transgenic potato tuber tissues containing a total of 7 μg of the recombinant fusion proteins previously determined by chemiluminescent ELISA on day 0, 5, 15, 23 and 56. Using the same feeding schedule, a group of 5 CD-1 mice each were fed 3 g of untransformed potato tuber tissues as a negative control. To evaluate the adjuvant effect of the CTB protein in the CTB-NSP4 fusion, CD1 mice (5 per group) were gavaged with pure NSP4 peptide with or without pure bacterial CTB (adjuvant) according to the same oral inoculation schedule. On day 13 after the final immunization, blood was taken from each mouse for serum antibody titer determination. Three mice per group were euthanized at three different time points: 13, 34 and 68 days after the fifth immunization. Intestinal washing were collected for mucosal antibody detection. Spleen cells from both immunized and negative control mice (3 x 10^6 cells/well) were suspended in RPMI 1640 medium containing 10% fetal calf serum in duplicate samples, in 24 well tissue culture plates. After incubation for 72 h at 37°C in a humidified, 5% CO₂ incubator, supernatants from the spleen cell cultures were collected for assessment of IL-2, IL-4 and IFNγ secretion.
d. Cytokine and Flow Cytometric Analysis of T Lymphocyte Populations in Immunized Mice

IL-2, IL-4 and IFNγ produced in the spleen cell culture supernatants were assayed by ELISA (kits supplied by Endogen, Inc.). Spleen lymphocytes were stained with fluorochrome-labeled monoclonal antibodies (mAb) for immunophenotyping. Two monoclonal antibody panels were constructed for three color analysis (fluoresceinisothiocanate (FITC), phycoerythrin (PE), and Cy-Chrome). The first combination used, CD62L*FITC/CD4*PE/CD44*Cy-Chrome designates naïve and memory T helper cells. The second combination, CD62L*FITC/CD8b.2*PE/CD44*Cy-Chrome designates naïve and memory cytotoxic T cells. The spleen cells were resuspended at 10^6 cells/ml in PBS and stained with fluorochrome-labeled mAbs. The labeled cells were analyzed by flow cytometry to determine the T lymphocyte memory cell sub-populations.

e. Evaluation of Protection against Rotavirus Challenge

Adult female CD-1 mice (five per group) were fed 3 g of untransformed or transgenic potato tuber slices once a week for four weeks. Immediately following the fourth immunization at maximum anti-NSP4 antibody titer, the mice were mated with uninfected males. After a 19-20 day gestation period, mouse pups were born to the immunized dams. On day 6 post parturition, each pup received one oral dose of simian rotavirus SA-11 in 50 µl PBS that contained 15 DD_{50} (the virus dose determined empirically to cause diarrhea in 50% of the mouse pups). The mice were examined for the presence of diarrhea daily for 5 days following inoculation by gentle palpation of
their abdomen to produce fecal pellets. The diarrhea score and the proportion of mice showing diarrhea symptoms in each study group were recorded.

3. Results and Discussion

a. Expression of CFA/I-CTA2 and CTB-NSP4 Fusion Genes

The CTB-NSP4 fusion gene was cloned into plant expression vector pPCV701 under control of the mas P2 promoter. The CFA/I-CTA2 fusion gene was cloned downstream of mas P1 promoter. Each cholera toxin fusion gene contains its own leader sequence and an ER retention signal. To increase the flexibility of the fusion protein, a four amino acid glycine–proline (GPGP) hinge region was inserted between the CTB and NSP4 peptides (Figure 1). Transformed potato plants were generated from leaf explants following an A. tumefaciens mediated stable transformation method (Arakawa et al., 1997).

Bacterial CTB (Figure 2, panel A, lane1) assembled into an oligomeric structure with a molecular weight of 45 kD, characteristic of the CTB pentamer. Plant produced CTB-NSP4 fusion peptide (Figure 2, panel A, lane2) formed a 50 kD oligomeric structure. The 5 kD increase in molecular mass is consistent with the presence of the additional NSP4 peptide and the 6 amino acid SEKDEL signal. The plant sample containing both CFA/I-CTA2 and CTB-NSP4 fusion proteins (Figure 2, panel A, lane3) showed the presence of a 70 kD protein band, indicative of the insertion of CFA/I-CTA2 peptide into the CTB-NSP4 pentamer. The untransformed plant (Figure 2, panel A, lane 4) showed no cross reaction with the cholera toxin antibody. Immersion of the samples in boiling water for 5 min resulted in dissociation of the multimeric structures into monomers (Figure 2, panel B). The bacterial CTB monomer has a molecular mass of 11
kD. The plant derived CTB-NSP4 multimer dissociated into an 18 kD monomer which is the consistent with the molecular mass of CTB plus NSP4.

b. Induction of Serum and Intestinal Antibodies in Orally Immunized Mice

Following five oral inoculations with transgenic potato tuber tissues, blood samples were collected and the serum anti CTB, NSP4 and CFA/I IgG titers were measured by ELISA methods. Out of 10 mice, 8 generated serum IgG against CTB with a mean titer of 312.5±81.3. Of the 10 immunized mice, 8 developed serum IgG against NSP4 with a mean titer of 125±61.23. Out of the 10 immunized mice, 10 developed serum IgG against CFA/I with a mean titer of 84±44.2 (Figure 3, panel A).

Intestinal IgG and IgA antibody titers against the three antigens were analyzed by a chemiluminescent ELISA method. Out of 10 immunized mice, 5 generated measurable intestinal anti CTB antibody titers; Out of 10 immunized mice, 5 were found to have measurable intestinal antiNSP4 antibody titers and 6 out of 10 mice were found to have significant intestinal anti CFA/I antibody titers (Figure 3, panel B). The intestinal antibody titers were relatively low in comparison with serum IgG levels. Negative control mice fed untransformed potato tuber tissues did not develop detectable specific serum or mucosal antibodies (Figure 3, panel A, B). The induction of both systemic and mucosal antibodies in the immunized mice indicated the successful delivery of the cholera toxin fusion proteins to the GALT.

Adjuvant and carrier functions of CTB in the CTB-NSP4 fusion protein were determined by measuring serum anti-NSP4 antibody titers in mice from different vaccination groups. Mice fed the NSP4 peptide alone generated the lowest anti-NSP4
titer. Immunization with 7 μg of bacterial CTB (the same amount detected in the plant
derived CTB-NSP4 fusion protein) increased the serum anti-NSP4 IgG titer
approximately two folds. Mice fed 3 g transformed potato tuber tissues containing the
CTB-NSP4 fusion protein developed the highest anti-NSP4 titer (Figure 4). Small soluble
proteins like the NSP4 22 amino acid epitope that are highly immunogetic by parenteral
routes are frequently ineffective when administered orally unless a large dose of the
protein is used (Bloom et al., 1996; Cox et al., 1984). This result can be attributed to
intestinal digestion and lack of tropism of the peptide for the gut associated lymphoid
tissues. Either cholera holotoxin or the CTB subunit, which function as mucosal
adjuvants can stimulate an immune response against co-administered protein antigens
(Lycke et al., 1986; Mckenzie and Halsey, 1984; Sun et al., 1994; Jackson et al., 1993).
Directly linking small antigens with CTB subunit not only results in specific targeting of
the antigens to the mucosal immune system via specific enterocyte attachment but also
increases the local antigen concentration at the mucosal surface, which may explain our
detection of the strongest immune response directed against the CTB-NSP4 fusion
protein.

c. Cytokine Assays and Detection of CD4 Memory Cells

Following multiple oral immunizations, the IL-2 and the IFNγ expression levels in
spleen cells dramatically increased, reaching the highest level 34 days after the fifth
immunization and decreasing to basal levels by 68 days after vaccination. Throughout
this time period IL-4 levels remained low, equivalent to that found in unimmunized mice
(Figure 5). Thus, cytokine expression pattern clearly indicated a Th1 lymphocyte
mediated immune response generated by feeding mice the plant derived cholera toxin fusion antigens. Both cholera toxin and the CTB subunit function as potent adjuvants that can promote antigen priming of both Th1 and Th2 type of CD4+ T precursor cells (Marinaro et al., 1999; Williams et al., 1999). The pattern of the T helper lymphocyte response may depend on the nature of the co-administered antigens (Xu-Amano et al., 1994). Oral immunization with CTB-insulin or CTB-GAD fusion proteins generate a Th2 response while oral immunization with recombinant E.coli CFA/I stimulated a biphasic Th response; a predominant Th2 response at the early stage and then Th1 cell dominant response 4 weeks after immunization (Arakawa et al., 1998; Pascual et al., 1999). The rotavirus non-structural protein NSP4, which acts as a viral enterotoxin, stimulated increased production of IL-2 and IFNγ in humans (Ball et al., 1994). Therefore we were not surprised to see the overall cytokine secretion pattern of this multicomponent plant vaccine in the mouse model to be indicative of a strong Th1 response. Flow cytometry analysis of spleen cells collected on day 13, 34 and 68 after the last vaccination showed an elevated population of CD4+ memory cells in comparison with the unimmunized mice through the two months after immunization (Figure 6, panel C). The distribution of CD4+ T cell subsets is shown in Figure 6. The CD4+ memory cell subpopulation (CD62− CD44+, gate R4) detected in the immunized mice (panel A) was observed to be significantly higher than the CD4+ memory cell subset in unimmunized mice (panel B). Thus, the generation of a significantly increased T helper memory cell population in the immunized mice indicated successful protective immunization mediated by the plant delivered antigens. The existence of increased numbers of memory cells provided the ability to mount a strong immune response following a second encounter with the same
pathogen. The CD8+ memory cell population detected in immunized mice did not show any significant increase over the unimmunized mouse negative control group (data not shown). This result may be explained by the temporary weak cytotoxic immune response often characteristic of oral immunization (Svennerholm et al., 1984; Czerkinsky et al., 1993).

d. Rotavirus Challenge and Diarrhea Reduction

Anti-NSP4 antibody protection against rotavirus induced diarrhea was evaluated by virus challenge of pups born to dams previously immunized with the plant derived CTB-NSP4 fusion protein (Horie et al., 1999). The number of pups which developed diarrhea symptoms and the duration of the diarrhea was significantly reduced in the pups passively immunized with CTB-NSP4 fusion protein in comparison with pups born to unimmunized dams. On day 3 after rotavirus challenge a 50% reduction of diarrhea symptoms was detected in the immunized pups. Complete resolution of diarrhea symptoms occurred 4 days after virus challenge in pups from immunized dams (Figure 7, the third column in each group). To exclude the possibility of diarrhea reduction due to the presence of anti-CTB antibodies, pups born to dams immunized with plant derived CTB only were also challenged with an identical dose of rotavirus SA11 (Figure 7, second column in each group). No reduction of diarrhea symptoms was detected in mice immunized with plant derived CTB alone. This experiment demonstrated that anti-NSP4 antibodies generated in orally immunized mice were passed on to the pups and protected them from the onset of rotavirus infection as well as significantly reducing the duration of the virus infection.
e. Development of Plant-based Multicomponent Oral Vaccines

Mucosal vaccines capable of inducing both humoral and mucosal immunity would provide the most ideal defense against infectious enteric diseases. However most soluble protein antigens are sensitive to PH and enzymatic digestion in the stomach and intestinal tract (Kiyono et al., 1996). However, cholera toxin is a remarkably stable protein and a potent immungen and has been successfully synthesized in bacterial or viral systems as a carrier molecule for the transfer of chemically or genetically fused antigens (Hajishengallis et al., 1995; Dertzbaugh et al., 1990,1993). Initial mucosal immunization experiments in which synthesis of the CTB subunit in transgenic potato, which was followed by oral immunization of mice with transformed plant tissues demonstrated a significant protective effect of the plant-based vaccine and has opened the way for development of effective mucosal vaccines in stably transformed plants (Arakawa et al., 1998). Based on this result, cholera toxin fusion proteins with other antigens expressed in plants became the next logical focus for the development of food plant based oral vaccines. Thus, the additional finding that the cholera toxin A2 subunit-CFA/I fusion protein associates with the cholera toxin B subunits to form a holotoxin like oligomer capable of binding to gangliosides present in the enterocyte membrane now provides us with the opportunity to generate multicomponent vaccines in plants in which several antigens can be targeted to the mucosal immune system for greater protection efficacy. Oral inoculation of CD1 mice with the multicomponent plant vaccine induced significant levels of humoral and mucosal antibodies against CTB, NSP4 and CFA/I antigens.
Further, the anti-NSP4 antibodies in passively immunized mouse pups offered protection against rotavirus challenge.

Cholera toxin neutralization assays and mouse ileal loop ligation assays previously performed in our laboratory on mice immunized with potato synthesized CTB provided a significant protective effect against diarrhea generated by cholera toxin (Arakawa et al., 1998). The protective efficacy studies with cholera toxin were not repeated because in our present animal immunization study the anti-CTB antibody titer detected was 5 fold higher than obtained in our previous experiments.

T helper lymphocytes play important roles in generating antigen specific humoral and cell mediated immune responses. Serum and mucosal antibodies can be induced either through Th1 or Th2 cell responses depending on the nature of the antigen peptide presented to lymphocyte in the lamina propria of the intestinal tract (Xu-Amano et al., 1998). Cytokine assays performed on spleen cells isolated from immunized mice indicated the presence of a strong Th1 lymphocyte response, possibly including cytolytic T cells. Detection of a CTL response may be due to the presence of a viral antigen NSP4 in the CTB fusion protein (Angel et al., 1998). The observed Th1 response correlated with the appearance of modest titers of mucosal antibodies. Detection of lymphocyte populations from immunized mice by flow cytometry analysis showed a stable T helper memory cell population two months after vaccination. Following immunization the CD8+ cell population remained unchanged in the spleen cell flow cytometry analysis. Previous cell sorting experiments indicate that the cytotoxic T cell response in the spleen may not be a good indicator of the localized cytotoxic T cell response at the mucosal surface (Ishida et al., 1997). The expression of multiple antigens in individual transgenic plants
and the significant antibody titer and protective immune response generated against all of the antigens by the mammalian mucosal immune system in response to mucosal immunization with transformed plant tissues opens the way for production of food plant based vaccines with increased protective efficacy against individual virus or bacterial pathogens (multivalent vaccine) or multiple pathogens (multicomponent vaccines).
Acknowledgements

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Figure 1. Plant expression vector pPCV701 CFA/I-CT-NSP4. Plant expression vector pPCV701CFA/I-CT-NSP4 contains all four genes located within the transferred DNA (T-DNA) sequence which is flanked by the right and left border (RB and LB), 25bp direct repeats required for integration of the T-DNA sequence into plant genomic DNA. The CTBH:NSP4 (114-135):SEKDEL coding sequence is under control of the mas P2 promoter. The CFA/I:CTA2 coding sequence is under control of the mas P1 promoter. The NPT II gene expression cassette was included in the T-DNA to provide resistance to kanamycin for selection of transformed plants. A β-lactamase gene cassette was included to provide resistance to ampicillin for selection in E.coli and carbenicillin for selection in A.tumefaciens. The g7pA polyadenylation signal is from the A.tumefaciens T_L-DNA gene 7 and the OcspA polyadenylation signal is from the octopine synthase gene.
T-DNA

RB OcspA P1 P2 g7pA LB

NPTII mas CTB

mas 480 bp

KDEL CTA2 CFA/I leader 160 bp 431 bp

leader CTB GPGP NSP4(114-135) SEKDEL 309 bp 66 bp
Figure 2. Immunoblot detection of CTB-NSP4 and CFA/I-CTA2 fusion proteins in transgenic potato tuber tissues. Panel A. The oligomeric CTB-NSP4 fusion protein was detected with anti-CT antibody as the primary antibody and anti-mouse IgG conjugated to alkaline phosphatase as the secondary antibody. Lane 1 = 100 ng commercial bacterial CTB pentamer; Lane 2 = 100 μg total soluble protein (TSP) from potato plants transformed with the CTB-NSP4 fusion gene; Lane 3 = 100 μg TSP from potato plants transformed with both CTB-NSP4 and CFA/I-CTA2 fusion genes; Lane 4 = 100 μg TSP from an untransformed potato plant. Panel B. Immunoblot detection of monomeric CTB-NSP4 fusion protein with anti-CT antiserum after boiling the plant homogenates for 5 minutes in SDS containing buffer. Lane1 = 100 ng boiled commercial bacterial CTB monomer; Lane2 = 100 μg boiled TSP from an untransformed potato plant; Lane 3 = 100 μg of boiled TSP from potato plants transformed with the CTB-NSP4 gene; Lane 4 = 100 μg boiled TSP from potato plants transformed with both CTB-NSP4 and CFA/I-CTA2 genes.
Figure 3. Measurement of anti-CTB, NSP4 and CFA/I antibody titers in mice after oral immunization with transgenic potato tuber tissues. CD-1 mice were fed transgenic potato tissues containing 7 μg of CTB-NSP4 and CFA/I-CTA2 fusion proteins five times at one week intervals. Mice fed equal amounts of untransformed potato tissues served as a negative control. Panel A. serum IgG antibody titers against CTB, NSP4 and CFA/I proteins. Panel B. Intestinal IgG and IgA antibody titers against CTB, NSP4 and CFA/I proteins. Antibody titers were determined by chemiluminescent ELISA method. The antibody titer was defined as the reciprocal of the highest dilution of the serum or intestinal contents sample that generated a luminescence signal above the background levels.
A

Immunization⁺  Immunization⁻

<table>
<thead>
<tr>
<th>Serum</th>
<th>IgG Titer</th>
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<tr>
<td>CTB</td>
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B

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108
Figure 4. The adjuvant and carrier functions of CTB in CTB-NSP4 fusion protein. Serum IgG antibody titers against the NSP4 peptide were measured following mucosal immunization of mice from selected vaccination groups. From left to right group one was made of mice fed 3g of untransformed potato tuber tissues. Group two was made of mice fed 7 µg of synthetic 22 amino acid NSP4 peptide. Group three consisted of mice fed 7 µg NSP4 synthetic peptide mixed with 7ug of commercial bacterial CTB. Group four was made of mice fed 3g transgenic potato tuber tissues containing approximately 7 µg CTB-NSP4 fusion protein. Responders: The number of mice which developed a detectable serum IgG titer against NSP4 peptide in each group divided by the total number mice immunized.
Responders:

- untransformed plant: 0/5
- NSP4 peptide: 2/5
- CTB + NSP4 peptide: 4/10
- CTB-NSP4 plant: 8/10
Figure 5. Measurement of cytokine levels in spleen cells from mice immunized with potato tissues containing CTB, NSP4 and CFA/I proteins. Spleen cells were isolated from unimmunized CD-1 mice and mice immunized with potato tuber tissues containing CTB-NSP4 and CFA/I-CTA2 antigens. Supernatants from the spleen cell culture were assayed for the presence of IL-2, IL-4, and IFNγ levels on day 13, 34 and 68 after the final oral immunization. The levels of IL-2, IFNγ and IL-4 were defined as the amount of the cytokines produced in 1ml spleen cell culture (3 x 10^6 cells). Grey bars represent the cytokine levels of unimmunized mice. Black bars represent the cytokine levels of mice orally immunized with tuber tissues containing CTB-NSP4 and CFA/I-CTA2 antigens.
IFN-\(\gamma\) pg/ml

IL-2 pg/ml

IL-4 pg/ml

Days After Immunization

13 34 68
**Figure 6. Detection of CD4+ memory cells in spleen cells from mice immunized with transgenic potato tissues.** Panels A and B are flow cytometry analysis of CD4+ T cell sub-populations isolated from the spleen of unimmunized CD-1 mice and mice immunized with potato tissues containing CTB-NSP4 and CFA/I-CTA2 fusion proteins. Cells were stained with FITC-conjugated anti-CD62 and CYC-conjugated anti-CD44 monoclonal antibodies as described in methods. The cells were recovered, washed by centrifugation, and analyzed by flow cytometry. The chart scale is log10 of the fluorescence intensity. Panel A represents CD4+ spleen cells from a mouse fed untransformed potato tissues. Panel B, CD4+ spleen cells from a mouse fed transgenic potato tissues. Panel C is the analysis of CD4+ memory cells in the spleen of immunized mice. Spleen cells were collected at 13 days, 34 days and 68 days following the last immunization. The first column in each group (gray) represents the % of memory cells in mice fed untransformed potato tissues. The second column in each group (black) indicates the % of memory cells in mice fed transgenic potato tissues.
Days After Immunization

CD4⁺ memory %

13  34  68
Figure 7. Protection against rotavirus infection in passively immunized mouse pups. Mouse pups born to CD-1 mouse dams orally immunized with transformed potato tubers containing the CTB-NSP4 fusion protein were challenged with 15 x DD₅₀ dose of rotavirus strain SA11. The axis X represents the percentage of mouse pups, which developed diarrhea from day one to day four following virus challenge. The number of pups that developed diarrhea in comparison to the total number of pups in each group is indicated on top of each column. The first column in each group (gray) indicates the pups born to dams fed untransformed potato tissues. The second column (black) represents the pups born to dams fed transgenic potato tissues containing CTB protein only. The third column (white) represents the pups born to dams fed transgenic potato tissues containing the CTB-NSP4 fusion protein.
Days After Challenge

- Column 1 (gray): unimmunized mice
- Column 2 (black): mice immunized with CTB plant
- Column 3 (white): mice immunized with CTB-NSP4 plant
CHAPTER SIX

EXPRESSION OF ROTAVIRUS CAPSID PROTEIN VP6 IN TRANSGENIC POTATO AND ITS ORAL IMMUNOGENICITY IN MICE

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Key Words

Transgenic potato, Rotavirus, Capsid protein VP6, Oral immunization

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Abstract

We have expressed the gene encoding rotavirus capsid protein VP6 in transgenic potato plants. The plant produced VP6 protein has a molecular mass similar to purified virus VP6 and partially formed VP6 trimers. Mice orally immunized with transformed potato tuber tissues containing VP6 protein generated significant serum and intestinal antibody titers against VP6 capsid protein. This work represents the first successful step in development of an edible plant-based vaccine against rotavirus.
1. Introduction

Rotaviruses are the leading cause of viral gastroenteritis in young children and animals worldwide. Rotavirus infection leads to about 870,000 deaths annually in developing countries (Bishop et al., 1983). The health burden of rotavirus disease in the United States is comparable to measles, pertussis and varicella prior to universal use of vaccines. A cost-effectiveness analysis of a rotavirus immunization program in the U.S. indicated that a vaccine efficacy rate of 50% could yield net savings of $79 million to health care and $466 million to society (Bajolet et al., 1998).

Rotavirus particles consist of 11 dsDNA genomic segments surrounded by three concentric capsid protein layers: an inner VP2 layer, an intermediate VP6 layer and an outer layer made up of capsid proteins VP7 and VP4. The VP7 capsid protein is a glycoprotein that defines the G serotype of the rotavirus strain (Mackow et al., 1989). The capsid protein VP4, a viral hemagglutinin is an important determinant of virulence. Both VP4 and VP7 induce neutralizing antibodies (Estes, 1996). Serum IgG antibodies against VP4 and VP7 protect mice against rotavirus infection when administered orally (Ward et al., 1990; Ishida et al., 1997). The capsid protein VP6 is the major structural protein of rotavirus, makes up about 50% of virion mass (Hsu et al., 1997). Antibody to VP6 does not have neutralizing activity in vitro. However, IgA antibodies against VP6 transferred into the intestine via transcytosis (backpack tumor model) protected mice against virus challenge (John et al., 1996). This result indicates that immunity to internal proteins may also play a significant role in immunological protection.

Rotavirus capsid proteins produced in insect cells were found to assemble into virus-like particles (VLPs) and induced protective immunity against virus challenge (Corsaro et
al., 1996; Crawford et al., 1994; Conner et al., 1996). In the present study we have expressed the rotavirus capsid protein VP6 in transgenic potato plants to demonstrate that plants can be used as a safe and economical alternative for production and delivery of recombinant rotavirus proteins for protection against rotavirus infection.

2. Materials and Methods

a. Construction of Plant Expression Vector and Plant Transformation

A 1.2 kbp DNA fragment containing the gene encoding murine rotavirus VP6 was obtained by BamHI digestion of plasmid pKS+VP6 (kindly provided by Dr. H.B. Greenberg, Stanford University, School of Medicine) and cloned into plant expression vector pPCV701 at the BamHI site under the control of the mas P2 promoter. The orientation and sequence of the VP6 gene was confirmed by DNA sequence analysis. The resultant plasmid pPCV701VP6 was transferred into A.tumeficiens strain GV3101pMP90RK by electroporation. The structure of the plasmid in the transformed GV3101 strain was verified by restriction endonuclease digestion of the plasmid DNA and analysis of the DNA fragment size and pattern by agarose gel electrophoresis. Potato (S. tuberosum cv. Bintje) leaf explants were transformed with A.tumefaciens strain GV3101pMP90RK harboring the plant expression vector pPCV701VP6. Transformed plants were regenerated from transformed leaf explants grown on MS medium containing kanamycin (100 μg/ml) for selection of transformants. Prior to analysis of VP6 gene expression, transformed potato tubers were stimulated to produce maximum levels of VP6 protein by incubation of tuber slices on solid growth medium containing auxin 2,4-D.
(2,4 dichlorophenoxy acetic acid, 5 mg/l) for 4 days in the growth room at room temperature.

b. Immunoblot Detection of VP6 Protein

Potato tubers induced by auxin 2,4-D on MS medium were homogenized with a mortar and a pestle in extraction buffer containing 200 mM Tris-Cl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Tween 20. The tissue homogenates were clarified by centrifugation 2 times at 17,000 x g for 15 minutes at 4°C. The total soluble protein present in the clear supernatants was determined by Bio-Rad protein assay. Tuber extracts containing 100 µg total soluble protein were loaded per lane and separated by SDS/PAGE gel electrophoresis. The separated protein bands were transferred from the gel to a nitrocellulose membrane by electroblotting on a semi-dry blotter (Sigma) at 30V, 60mA for one and a half hours. The membrane was blocked in 5% dry milk in TBS buffer (20mM Tris, pH7.5, 500mM NaCl). The membrane was first probed with rabbit anti-rotavirus serum diluted 1: 500 in 1% dry milk/ TBS buffer and second with alkaline phosphatase conjugated mouse anti-rabbit IgG(Sigma) at a 1:10,000 dilution in 1% dry milk/ TBS buffer. Finally the membrane was incubated in the chemiluminescent substrate BCIP/NBT (Sigma) for 10 minutes. The reaction was stopped by washing the membrane in distilled water.
c. Quantification of VP6 Expression Levels by Chemiluminescent ELISA

A 96 well microtiter plate was coated with mouse anti-VP6 serum diluted 1:1,000 in bicarbonate buffer (pH 9.6) at 4°C overnight. The plate was blocked with 1% BSA in PBS buffer at 37°C for 2 hours. Protein extracts from transformed potato tuber tissues were added to the microtiter plate wells and incubated overnight at 4°C. Known amounts of purified virus VP6 were mixed with protein extracts from untransformed potato tuber tissues to generate a standard curve. The plate was first incubated with rabbit anti-rotavirus serum diluted 1:2,000 in PBS buffer at 37°C for 2 hours and followed by incubation with a second antibody mouse anti-rabbit IgG conjugated with alkaline phosphate at a 1:80,000 dilution in PBS buffer at 37°C for 2 hours. The plate was finally incubated with chemiluminescent substrate, Lumi-phos Plus (Luminogen, Inc.) for 30 minutes at 37°C, and the plate was read in Microtiter Plate Luminometer (Dynatech). The light emissions were recorded as relative light units (RLU) over the background.

d. Mouse Oral Immunization

In our immunization regimen, transgenic potato tuber slices were fed a total of four times on days 0, 7, 17, and 24 of the experiment, to a group of 10 female CD-1 mice. On each antigen inoculation day, 10μg of purified bacterial cholera toxin (Sigma) was first administered as an adjuvant to the mice in 0.4 ml sodium bicarbonate buffer (350 mM), pH 8.5 by gastric intubation (gavage). The mice were then fed 5 g of transgenic tuber slices containing approximately 15 μg of VP6 protein as previously determined by ELISA and immunoblot assays. Following the same feeding protocol, a group of 5 CD-1 mice were each fed 5 g of untransformed potato tuber slices to serve as a negative
control. Another group of 10 mice was fed the same amount of purified bovine rotavirus VP6 protein (15 μg) mixed with 10 μg cholera toxin by gavage as a positive control. On day 23 after the fourth feeding the mice were sacrificed. Blood was collected from each CO₂ anaesthetized mouse by heart puncture. The small intestines of the sacrificed mice were removed and the lumen of the intestine was washed with 1 ml PBS buffer, pH 8.5, containing 10 mM PMSF to collect any mucosal antibodies. Both blood and intestinal samples were tested for serum IgG and mucosal IgG and IgA titers by chemiluminescent ELISA methods.

3. Results

a. Plant Expression Vector and Transformation of Potato Plants

Plant expression vector pPCV701VP6 contains a 430 bp DNA fragment encoding the mannopine synthase dual promoters (mas P1, P2). The mas P2 promoter controls the expression of the rotavirus VP6 gene. In addition, the plant expression vector contains the β-lactamase gene for ampicillin resistance in E.coli and carbenicillin resistance in A. tumefaciens. The neomycin phosphotransferase II gene (NPTII) cassette provides kanamycin selection in transformed plants (Figure 1). The T-DNA sequence containing the VP6 gene located between the left and right borders (RB and LB) became randomly integrated into the plant genome during A.tumefaciens mediated stable transformation used in our laboratory. A total of five transformed VP6 producing plants were regenerated.
b. Immunoblot Detection of VP6

Tuber and leaf tissues from the transformed plants were analyzed for the presence of VP6 protein by immunoblot assay. Transformed plant produced VP6 protein was identified by rabbit anti-rotavirus serum (Figure 2, lane 4) and thus retained the antigenicity of the native viral VP6 protein. The molecular size of the plant synthesized VP6 protein was 45 kD, which is the approximate size of the native bovine virus VP6 (Figure 2, lane 2). Like the bovine virus VP6, plant produced VP6 underwent partial assembly into 120 kD VP6 trimers detected by unreduced SDS/PAGE gel electrophoresis. The formation of VP6 multimers may be essential for retaining immuno-dominant conformational epitopes that stimulate protective immune response. Untransformed tuber tissue samples (Figure 2, lane 3) did not react with the anti-rotavirus antiserum.

c. Quantification of VP6 in Transformed Plants

The VP6 protein expression levels in transformed potato leaf and tuber tissues were measured by quantitative chemiluminescent ELISA. Two standard curves (0.1% and 0.01%) VP6 protein were generated based on chemiluminescent signals provided by the anti-VP6 antibody complex (Figure 3). The relative light units (RLU) values obtained by measurement of serial dilutions of transgenic plant homogenates were plotted against the amount of total soluble protein in the transformed tuber extracts. The transgenic tuber tissues were found to produce approximately 0.01% VP6 of total soluble protein (Figure 3). The ability of plant synthesized VP6 to bind two different anti-VP6 sera in a sandwich
ELISA confirmed the native structure and biological activity of the plant synthesized VP6 protein.

d. Immunogenicity of the Plant Produced Rotavirus VP6 Protein

CD-1 mice were fed transgenic potato tubers to test the oral immunogenicity of plant derived VP6. When tuber slices containing 15 μg VP6 protein were fed concomitantly with 10 μg CT, 10 out of 10 mice developed detectable serum IgG levels against VP6 with anti-VP6 titers ranging from 1:80 to 1:1280. When the same amount of purified virus VP6 protein was fed along with 10 μg CT as adjuvant, 10 out of 10 mice responded by producing serum anti-VP6 IgG titers ranging from 1:500 to 1:16,000. The titers of antibodies raised against pure VP6 protein were higher than titers raised against plant derived VP6. In addition to significant serum IgG titers, plant expressed VP6 also stimulated a detectable intestinal anti-VP6 IgA response in 7 out of 10 mice. Rotavirus VP6 protein stimulated the production of intestinal IgA in 6 out of 10 immunized mice. The percentage of mice responding to VP6 challenge and the intestinal IgA titers obtained following immunization with plant produced VP6 was comparable to the results found from oral immunization with pure virus VP6 protein (Table 1). Both IgG and IgA antibodies generated against VP6 protein were detected in serum and intestinal samples taken from CD-1 mice immunized with plant produced VP6 (Figure 4). Serum IgG demonstrated the highest titers, which indicated successful stimulation of the systemic immune system. The presence of measurable mucosal antibody titers indicated a successful immune response at the local site. Most IgA antibody presents at the mucosal surface in a form of secretory IgA, the function of the small amount of serum IgA is
unknown. It probably exists in a form of precursor IgA before it is transferred to the mucosal surface by the transcytosis mechanism. Mice fed untransformed tuber tissues did not develop any specific anti-VP6 antibodies.

4. Discussion

Plants can produce viral and bacterial antigens that retain their biological activities and functions essential for plant based oral vaccine development (Mason et al., 1992, 1996; Haq et al., 1995; Modelska et al., 1998). Expression of individual rotavirus antigens in plants allows us to determine which rotavirus proteins may be most active in protection against virus infection and to determine the role of non-neutralizing antibodies in disease prevention (Ishida et al., 1997; Ball et al., 1996). The rotavirus capsid protein VP6 is a virus structural protein that is highly immunogenic. Specific antibodies against VP6 can be detected in the sera of clinical patients (Burns et al., 1995; Eydelloth et al., 1984). The role of VP6 antibody in protection against disease onset has already been demonstrated in several different aspects. A VP6 DNA vaccine was shown to protect mice from rotavirus challenge. Hybridoma cells secreting anti-VP6 IgA in a “back pack” mouse model provided protection from rotavirus infection by an IgA transcytosis mechanism (John et al., 1996). Dr.Estes and her colleagues also showed that VP2/VP6 VLPs achieved the same level of protective efficacy as obtained by immunization with 2/6/7 VLPs without requirement for the neutralization antigens VP7 and VP4 (Conner et al., 1996). In the present study, we have demonstrated that VP6 protein produced in transgenic potatoes retained its antigenicity and partially folded into multimers, which may be important for the formation of conformational epitopes to provide increased
immunogenicity. Oral immunization in mice stimulated both humoral and mucosal antibody production, indicated a successful delivery of the VP6 antigens to the mucosal immune system. The presence of intestinal IgA, which is strongly correlated with protection in vivo indicated a protective immune response was generated by the plant produced VP6 (Hoshino and Kapikian, 1994).

The *A. tumefaciens* mannopine synthase promoter is a dual promoter providing the advantage of coordinate expression of two foreign genes simultaneously in transformed plant tissues (Langridge et al., 1989). Since the P2 promoter is 2-10 times more active than P1 promoter, we selected the *mas* P2 promoter to drive the expression of the VP6 gene. In plant homogenates of transformed tuber tissues we were unable to detect VP6 VLPs by transmission electron microscopy. However rod-like structures were detected present in transformed tissue homogenates only which may be due to the aggregation of VP6 proteins (data not shown) (Ready and Sabara, 1987). The availability of the *mas* P1 promoter in our plant expression vector allows us to clone additional rotavirus capsid genes into potato such as the VP2 gene. This will provide us with the opportunity to generate VP2/VP6 VLPs in transgenic plants to provide greater protective efficacy to the present plant-based vaccines. The production of the mammalian rotavirus VP6 protein monomers and aggregates in potato plants and demonstration of oral immunogenicity in CD-1 mice is the first successful step toward the development of a protective rotavirus subunit vaccine in plants.
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Figure 1. Plant expression vector pPCV701VP6. Figure 1. Shows the transferred DNA (T-DNA) sequence of the plant expression vector pPCV701VP6 used for transformation of potato plants. RB and LB are the right and left borders required for integration of the T-DNA into the plant genomic DNA. The C-DNA of rotavirus VP6 was cloned into P2 side of the mannopine synthase (mas) dual promoter. A β-lactamase gene cassette (Bla) was included in the T-DNA to provide resistance against ampicillin for selection in E.coli and carbenicillin for selection in A.tumefaciens. The NPTII gene expression cassette was included to provide resistance to kanamycin for selection of transformed plants.
Figure 2. Immunoblot detection of VP6 protein in transgenic potato tuber tissues. The VP6 protein was detected with anti-VP6 antibody as the primary antibody and alkaline phosphatase conjugated anti-rabbit IgG as the second antibody. Lane 1, molecular weight markers; Lane 2, 100 ng purified bovine rotavirus VP6 protein; Lane 3, 100 μg total soluble protein (TSP) from an untransformed potato plant; Lane 4, 100 μg TSP from potato plants transformed with VP6 gene.
Figure 3. Determination of VP6 protein levels in transformed potato plants. The VP6 protein levels in transformed potato tissues were measured by chemiluminescent ELISA. Known amounts of purified rotavirus VP6 protein were mixed with plant homogenates from untransformed potato tissue to generate two standard curves (0.1% and 0.01% VP6 protein). Homogenates from untransformed plants were used as negative controls. The relative light units (RLU) obtained from the samples were measured in comparison with the standard curves to calculate VP6 protein expression levels.
Graph showing the relationship between Plant homogenate (µg) and RLU for different treatments:

- 0.1% VP6 protein
- VP6 plant
- 0.01% VP6 protein
- Untransformed plant
Figure 4. Measurement of anti-VP6 antibody titers in mice after oral immunization with transgenic potato tuber tissues. CD-1 mice were fed transgenic potato tubers containing 15 μg of VP6 protein with 10 μg of cholera toxin as adjuvant. Mice fed untransformed potato tubers served as a negative control. Serum and intestinal IgG and IgA titers against VP6 protein were determined by a chemiluminescent ELISA as described in the methods section. The antibody titer was defined as the reciprocal of the highest dilution of the serum sample or intestinal contents that generated a luminescence signal above background levels.
Table 1. Immune response of CD1 mice fed VP6 protein

<table>
<thead>
<tr>
<th>Group</th>
<th>VP6 protein (ug)</th>
<th>VP6 protein origin</th>
<th>Delivery</th>
<th>Adjuvant</th>
<th>Serum IgG</th>
<th>Intestinal IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. positive / total</td>
<td>Titer, range</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>virus</td>
<td>gavage</td>
<td>10ug CT</td>
<td>10/10</td>
<td>500-16,000</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>tuber</td>
<td>feed</td>
<td>10ug CT</td>
<td>10/10</td>
<td>80-1280</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>tuber</td>
<td>feed</td>
<td>none</td>
<td>0/5</td>
<td>&lt;4</td>
</tr>
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</table>

Serum and intestinal anti-VP6 IgG and IgA titers were measured by chemiluminescent ELISA methods. The antibody was defined as the reciprocal of the highest dilution of the serum or intestinal sample that generated luminescence Signal above background levels.
CHAPTER SEVEN

CONCLUSIONS AND FUTURE PERSPECTIVES

1. Plants as Vaccine Production and Delivery Systems

Plants are traditional sources of food, fiber and medicinal compounds. Recent developments in plant biotechnology have facilitated the production of a variety of economically or pharmaceutically important recombinant proteins in transgenic plants (Hein et al., 1996, Hiatt et al., 1989, McGarvey et al., 1995). An increasing number of investigators have demonstrated plant production of antigenic proteins from viral and bacterial pathogens and that these proteins elicit immune responses and even protection against pathogen challenge, by oral and/or parenteral administration (Dalsgard et al., 1997, Haq et al., 1995; Modelska et al., 1998). Plant-based vaccine production and delivery systems have attracted the interest of governmental agencies, Agri-business and pharmaceutical companies for commercialization of vaccines (Ma and Vine, 1999; Mason et al., 1995). However, prior to commercialization the potential of plant vaccines to generate protection against individual diseases must be developed in animal model systems (preclinical trials) followed by toxicity and efficacy testing in human phase I and II clinical trials (Moffat, 1995; Mor et al., 1998).

a. Multicomponent Vaccines

From a clinical perspective, it would be very useful to express antigens of multiple pathogens in one plant. Protection against several infectious diseases could be achieved by oral administration of a multi-component food plant-based vaccine. Stimulated by the World Health Organizaiton’s request for a simple vaccine for protection against severe enteric pathogens we have produced multiple antigen proteins (CTB, NSP4, CFA/I) for
protection against severe enteric diseases (cholera, ETEC, rotavirus) in a single potato plant. The disease antigen proteins synthesized in the plant have retained their antigenicity. Oral inoculation of mice with potato tuber tissues containing the three antigens stimulated both humoral and mucosal immune responses against the three antigens, which provided protection against both viral and bacterial pathogen challenge. Thus, we were able to demonstrate for the first time the protective efficacy of a multi-component food plant based vaccine.

The CTB subunit expressed in the transformed potato tubers not only functioned as a vaccine for protection against the diarrhea caused by cholera toxin, but in addition the CTB subunit was found to function well as a carrier molecule for specific targeting to enterocyte cells of the mucosal epithelium and as an immune modulation molecule (adjuvant) for sensitizing and stimulating the mucosal immune system. The novel approach of targeting the disease antigen to the GALT via CTB-antigen fusion protein has greatly reduced the amount of the antigen required for the development of significant mucosal immune response. The recent discovery that the CTA2 subunit can be inserted into the CTB pentamer in plant cells forms the foundation of the carrier system capable of targeting additional antigens to the mucosal immune system. In the dissertation we have demonstrated that five copies of a small peptide such as NSP4 containing approximately 20 amino acid residues can be genetically fused to the CTB pentamer; while one copy of large peptide up to 50 kD such as CFA/I can be linked to the CTA2 subunit and inserted stably into the CTB pentamer without disrupting the pentamer structure. The resultant double fusion protein structurally and functionally resembles the cholera holotoxin with the further attribute of delivery additional antigen to the GALT. The design of the CTA2-
CTB hetero-hexameric enterocyte targeted carrier system has opened the way for the expression of a variety of antigen combinations from diverse origins for control of infectious diseases.

b. Multivalent Vaccine

Vaccines composed of single antigen are able to confer a limited degree of immunity against a single pathogen. A more ideal vaccine will be one that is able to induce antibody protection against bacterial or viral pathogen structural proteins in addition to the enterotoxins they secrete. Based on this concept we have designed a multivalent plant-based vaccine against rotavirus disease utilizing the CTA2-CTB carrier system. A DNA fragment encoding the rotavirus capsid protein VP6 gene was genetically linked with CTA2 sequence and a nucleotide sequence encoding the CTB gene linked to the rotavirus enterotoxin NSP4 epitope (114-135). A plant expression vector containing the two rotavirus fusion genes was transferred into *A. tumefaciens*. Transformed potato plants were generated by *A. tumefaciens* mediated stable transformation methods used in our laboratory. Identification of the plant synthesized rotavirus antigens is currently in progress. By taking advantage of the CTA2-CTB system, we expect to develop a plant-based multivalent vaccine against rotavirus with enhanced protective efficacy against rotavirus challenge.

c. Plant-based VLPs Vaccines

In 1996, Mason and his colleagues reported the expression of Norwalk virus capsid protein in transformed tobacco and potato tissues (Mason et al., 1996). These capsid proteins formed single layered, empty virus like particles (VLPs), which were morphologically and immunogenically identical to mature virus particles. Containing
four capsid proteins, rotavirus is structurally more complex than Norwalk virus. In our attempt to create a plant VLPs vaccine against rotavirus, we first expressed the most abundant rotavirus capsid protein VP6 in transgenic potatoes. Plant-produced VP6 proteins with a molecular mass similar to their virus VP6 counterpart. In addition, plant synthesized VP6 was able to react with anti-VP6 antiserum and VP6 protein partially assembled into trimers, which indicated the presence of molecular structure responsible for the capsid protein folding. Rod shaped structures were detected by transmission electron-microscopy in extracts of transgenic potato tissues, which were not detected in untransformed potato samples (data not shown). Previous transmission electron microscopic studies of purified bovine rotavirus VP6 protein demonstrated the presence of tubular particles which were formed between pH 5.0 and 9.0. Spherical particles resembling single-shelled virus were formed at pH 4.0, while sheet-like structures formed between pH 6.0 to pH 4.0 (Ready and Sabara, 1987). Thus, the rod shaped structures that we observed in Tris-HCl buffer extracts of transformed potato leaves (pH 8.0) may be due to partial assembly of the VP6 capsid protein. In the future we will purify the plant synthesized VP6 proteins from plant homogenates by glucose gradient centrifugation and try to identify the VP6 protein assembly into single shelled virus like particles at appropriate pH conditions.

The mas dual promoter in the plant expression vector (pPCV701) is bi-directional, and can drive expression of two genes simultaneously in opposite directions (Langridge et al, 1989). Taking advantage of this unique characteristic, we cloned the rotavirus VP2 gene down stream of the mas P2 promoter and the VP6 gene downstream of the mas P1 promoter. Transformed potato plants expressing both VP2 and VP6 capsid proteins were
regenerated. Co-expression of VP2 and VP6 proteins was detected on immunoblots, Detection of double layered VP2/6 VLPs in plant tissues by transmission electron microscopy remains to be attempted.

2. The Role of Cholera Toxin in Immune Modulation

a. Adjuvanticity of CT and the CTB Subunit

Cholera toxin is one of the most potent mucosal immunogens yet identified. The holotoxin induces strong intestinal S-IgA and serum IgG responses following oral adminsitration (Elson and Ealding, 1984, Fujita et al., 1972). Despite its remarkable properties as a mucosal immunogen, the mucosal immune response to CT follows rules applicable to most conventional protein antigens in that the immune response is CD4+ T cell dependent and requires antigen presentation via class II MHC molecules (Jackson et al., 1993). The adjuvanticity of CT appears to be linked to its immunogenicity (Freytag and Clement, 1999). The effectiveness of the plant-produced CTB subunit as a mucosal adjuvant was clearly demonstrated by the immune response detected following oral immunization with the plant synthesized CTB-NSP4 fusion protein. When the NSP4 peptide was genetically conjugated to CTB it elicited higher antibody levels in serum than feeding either NSP4 alone or an unconjugated mixture of NSP4 and CTB proteins. Protein fusions based on the GM1 ganglioside binding activity of CTB subunit greatly reduced the amount of antigen protein required to stimulate a mucosal immune response (Holmgren et al., 1996). Oral administration of plant-produced CFA/I-CTA2 and CTB-NSP4 fusion proteins as low as 7 µg/ dose was able to generate measurable titers of both serum and intestinal antibodies. The long term T cell response induced by the plant-derived cholera toxin fusion protein was demonstrated by flow cytometry assay.
Increased CD4+ memory T cell populations were detected in spleen cells from orally immunized mice as early as 14 days after vaccination and persisted for two months following immunization.

The mechanism of CTB enhancement of the mucosal immune response has not been clearly determined. Previous experiments have demonstrated that many cellular events are triggered by the CTB-GM1 ganglioside receptor interaction (Lycke and Holmgren, 1986). Intestinal epithelial cells are recognized as active antigen presenting cells of the mucosal immune system. They express both class I and class II MHC molecules, produce certain cytokines and respond to an even larger array of cytokines. Cholera toxin stimulates the production of IL-6 by the epithelial cell in vitro. Cholera toxin also stimulates the production of IL-1 and IL-6 by macrophages, which provide costimulatory signals to T helper cells. Increased T cell priming was observed in mucosal tissues in vivo when CT was used as an adjuvant.

b. The Th1 vs Th2 Lymphocyte Response

Murine CD4+ helper T cells have been subdivided into Th1 and Th2 subtypes based on the pattern of cytokines which they secrete (Mosmann and Coffman, 1989). The Th1-type helper cells secrete IL-2 and IFNγ whereas Th2-type helper cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Kjerrulf et al., 1998). Several of the Th2 cytokines have been shown to be important in generating IgA responses and this Th2 subtype may be preferentially expressed in mucosal follicles such as Peyer’s patches (Lebman et al., 1988; Lycke et al., 1983). In mice the IgG2a isotype antibody response is Th1 dependent, whereas IgG1 isotype and IgE antibody responses are Th2 dependent.
The mechanisms by which a given antigen triggers a Th1 or Th2 lymphocyte dominated response are at present unclear. The CTB subunit tends to stimulate a Th2 immune response (Marinaro et al., 1999; Xu-Amano et al., 1994). Binding of the CTB pentamer to intestinal epithelium cell surface ganglioside receptors results in induction of CD25 on macrophage and lymphocyte populations. Such activation is associated with subsequent apoptotic cell death of CD8+ T cells, enhanced expression of MHC class II on B cells and CD40L on CD4+ T cells (Hornquist and Lycke, 1993). Macrophage activation is associated with release of IL-10. The Th1 cytokine release is down-regulated as a result of decreased IFNγ production, through loss of CD8+ T cells and IL-10 mediated suppression of Th1 activation by antigen-presenting cells (Romagnani, 1994). Concomitantly, enhanced T cell-B cell interactions leading to up-regulation of B7, CD40, and ICAM-1 on B cells and CD40L on CD4+ cells increases the commitment towards Th2 cytokine production.

Based on cytokine analysis we detected a Th1 type immune response in mice orally immunized with plant-derived CFA/I-CTA2 and CTB-NSP4 fusion proteins. Previous reports showed that CTB fusion proteins favor a Th2 type immune response (Marinaro et al., 1999; Xu-Amano et al., 1994). The disparity between our experimental results and previously reported studies may be explained by the following reasons. Antigens coupled to the CTB subunit may influence the type of immune response being induced. A Th2 cell response is induced by CTB fusion with either self antigens such as insulin, glutamic acid decarboxylase (GAD), myelin basic protein (MBP) or with nonpathogenic antigens such as ovalbumin (OVA), bovine gamma globulin (BGG) (Khoury et al, 1990; Melamed and Friedman, 1993; Miller et al., 1991). Bacterial or viral antigens such as inactivated
repiratory syncytical virus and tetanus toxoid fragment C plus CT as an adjuvant induce a Th1 cell response. When purified CFA/I or NSP4 antigen proteins were added to spleen cell cultures, we detected increased expression levels of IL-2 and IFNγ, which indicated the presence of a Th1 cell response. Thus, it was not unexpected to see a Th1 immune response in mice fed trangenic potato tissues containing the CTA2-CFA/I and CTB-NSP4 double fusion proteins.

c. Immune Response vs Immune Tolerance

When CT is used as a mucosal adjuvant, it has the potential to stimulate the synthesis of a broad range of cytokines such as IL-1, IL-2, IL-4, IL5, IL6, IL10, and IFNγ accompanied by an enhanced proliferative response of both Th1 and Th2 type CD4+ T precursor cells. When an antigen (autoantigen) which stimulates a Th2 cell response is conjugated with CTB, it selectively activates suppressor T-cells (Arakawa et al., 1998; Sun et al., 1994; Trentham et al., 1993). The suppressor T cells migrate from the GALT into the systemic circulation. When the suppressor T cells encounter the autoantigen at the target organ, they secrete suppressor cytokines such as IL-4, IL-10 and transforming growth factor (TGF)-β which results in a down-regulation of the autoreactive T-cells (Czerkinsky et al., 1995; Trentham et al., 1993). When a Th1 cell-inducing antigen (viral or intracellular bacterial antigens) is encountered, IL-2, IFNγ levels are up-regulated accompanied by the activation of cytotoxic T lymphocytes. Prolonged CT-antigen/autoantigen feeding studies will be necessary to provide a more complete understanding of the immune modulation effects of CT which will help us to further our understanding of the mechanisms involved in the regulation of mucosal immunity.
3. Plant Vaccines' Future Perspectives

Plants are one of the most inexpensive sources of protein and therefore, potentially also one of the cheapest sources of recombinant proteins, even if purification of the protein is required. The potential for production scale-up can make plant vaccines the most cost effective vaccines yet developed (Arntzen, 1997, 1998). However some basic questions must be addressed. One of the biggest obstacles to an effective plant-based vaccine remains the low level of accumulation of foreign antigens in plant tissues. This problem becomes even more acute for less immunogenic antigens. Manipulation of antigen encoding genes at both the transcriptional and translational levels may increase the expression levels of recombinant protein in the plant. Such strategies include addition of transcriptional and translational enhancer sequence, addition of leader peptides, addition of ER retention signals (Schouten et al., 1996), application of constitutively expressed promoters, adjustment of the codon usage to favor plant gene expression, and removal of RNA splice sequences and intron sequences (Ma et al., 1999; Tsafrir et al., 1998). However there may be limitations to how much foreign proteins can synthesized or accumulated in plant cells. Alternative solutions to increased production of antigen proteins in plants include improved targeting of antigen molecules to the mucosal immune system, which could considerably reduce the amount of plant-produced antigen protein required for successful oral immunization (Arakawa et al., 1998). An accumulating body of evidence indicates that the cholera toxin B subunit is an effective carrier molecule for targeting antigens specifically to receptors on the intestinal epithelial cell membrane. The HIV transactivation sequence (TAT) is another non-specific targeting protein under active investigation in our laboratory (Barka et al., 2000).
Mutated cholera toxin A subunit that has no toxicity, yet maintains strong adjuvant activity, can also be employed to increase the mucosal immune response. Another possible solution to accumulate plant-produced antigen protein might be the addition of a plant secretion signal to the antigen protein. Recombinant proteins produced by the plant will be continuously secreted into the liquid culture media, never reaching a toxic level that might compromise the fitness of the transgenic plant. Simple purification steps may harvest large amounts of recombinant proteins accumulated in liquid culture media. Under sterile environmental conditions of optimal temperature and light, transgenic plants can produce foreign proteins approaching 24 hours a day. The expression levels of antigen protein in the plant may influence the dose of plant vaccine delivered for immunization. This source of antigen variability might be reduced by minimal processing of the plant material, for example, freeze drying followed by grinding and mixing the dried tissues to create uniform batches of plant-based antigen. Foreign proteins are frequently subjected to post-translational modifications in the plant cells following synthesis (McGarvey et al., 1995). Post-translational processes such as correct protein folding, glycosylation and proteolysis may greatly affect antigen stability and immunogenicity. While post-translational modification in plants is similar to that found in animal cells, small but critical changes may occur. Therefore, efforts to study the structure and function of plant-produced antigen proteins must be emphasized. Further, the issue of biological safety must also be addressed. Regulations for the use of transgenic plant tissues for vaccine purposes must be established before human clinical trials are initiated. Finally the environmental impact of growing transgenic plants in the field must be assessed to settle issues of public safety. Thus, considerable research and education of
the public concerning appropriate applications of plant-based vaccines still needs to be accomplished before children can be simply vaccinated with a glass of tomato juice or a slice of transgenic banana.
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