To complement the efforts with HBV vaccine, the Arntzen and Mason group explored plant expression of other vaccine candidates—the heat-labile toxin B subunit (LT-B) of enterotoxigenic *E. coli* (ETEC) and the capsid protein of Norwalk virus (NVCP) (17–21). These antigens of two important enteric pathogens may represent examples of the ideal oral subunit vaccine candidate. Both are oligomers: LT-B configures as a pentamer, which has a high affinity to GM_1 gangliosides present on mucosal cells (17), while NVCP can form VLPs (20). Furthermore, both have evolved to survive the extreme conditions of the stomach and infect [in the case of Norwalk virus (NV)] or colonize (in the case of *E. coli*) the gut epithelium.

Another apparent advantage associated with plant expression of these proteins compared with HBsAg was accumulation to high levels in potato tuber. Significantly, both antigens assembled correctly into functional oligomers that could elicit oral immune responses in animals (18,20) and humans (19,21). The phase I clinical trial with potato tubers expressing LT-B not only provided the proof of concept that orally delivered plant-based vaccines could result in an immune response in humans but was also one of the first experiments of a pharmaceutical product derived from a transgenic plant conducted in humans.

The clinical trials to date have examined both the safety and immunogenicity of plant-produced LT-B, NVCP, HBsAg, and rabies glycoprotein (12,16,19,21–23,34). In all these trials, individuals who consumed raw potato tubers or lettuce leaves containing approximately 0.3 to 1.0 mg of the antigens developed antibody responses. It is important to note that these antigens represent viral (NV, HBV, and rabies), bacterial (*E. coli*), enteric (NV and *E.* coli), as well as nonenteric (HBV and rabies) organisms. The titers of mucosal and systemic antibodies in some of the test subjects suggest that they would be protected from infection (19,21) and provide the justification for wider-scale clinical trials with these antigens.

PLANT-DERIVED VACCINES CAN PROVIDE PROTECTION AGAINST A PATHOGEN CHALLENGE

The initial successes of early clinical trials encouraged other groups to explore the ability of plants to produce, fold, and assemble other vaccine candidates for the prevention of human and animal diseases. Various laboratories have reported efforts to produce transgenic plant-based vaccines for oral delivery to protect against human pathogens such as rabies (24), respiratory syncytial virus (RSV) (25), measles (45), rotavirus (46–48), and hepatitis B (22), and human cytomegalovirus (49,50), cholera (26,48,51,52), ETEC (27,48), and others have been reported. Plant-derived oral vaccines for veterinary use are aimed at foot and mouth disease virus (FMDV) (28–30,53), swine-transmissible gastroenteritis virus (TGEV) (10,54–56), rabbit hemorrhagic disease virus (31), and *Mannheimia haemolytica*, the bacterial agent that causes shipping fever (32).

While high titers of secretory and circulating antibodies following oral vaccination with plant-derived vaccines are important evidence of immunity, proof of efficacy requires that vaccination result in immune responses that are protective against a pathogen challenge. Veterinary vaccines provide an opportunity to assess the degree of immune protection directly. The series of papers published by the Borca group (28–30) serve as an excellent example of this methodological approach in a veterinary context. Their first report described the use of a model plant system (*Arabidopsis thaliana*) for the expression of the VP1 protein of FMDV (28). Plant extracts containing VP1 provided full protection in mice after parenteral delivery and constituted the first demonstration of protection by a recombinant vaccine candidate produced in transgenic plants. Further studies using a larger number of mice immunized with extracts from transgenic potatoes (26) corroborated the initial work. They next expressed VP1 in alfalfa and delivered the transgenic plant material orally to mice. Despite low antigen expression, they achieved 70% protection against a virulent challenge after repeated oral boosting (30).

The use of viral vectors has been used to produce a candidate vaccine to protect against a possible biothreat agent *Yersinia pestis* (39). Genes encoding the F1 and V antigens and the derived protein fusion F1-V were introduced into tobamovirus-based system vectors, which allowed very rapid and extremely high levels of expression (up to 1–2 g of antigen per kg of plant tissue). All three of the plant-derived purified antigens, administered subcutaneously to guinea pigs, generated systemic immune responses and provided protection against an aerosol challenge of virulent *Y. pestis*.

FORMING MULTIVALENT AND MULTICOMPONENT VACCINES

Vaccines designed to stimulate several facets of the immune system such as induction of strong humoral, mucosal, and cellular immune responses are highly desirable. Similarly, combination vaccines targeting multiple pathogens in one formulation are often desirable. Therefore, developing both multivalent and multicomponent plant-based vaccines would provide for both efficacious and cost-effective immunization strategies. Plants harboring transgenes encoding the antigens of several pathogens, either by direct transformation or through sexual crosses of individually transformed lines, or the blending of separately transformed plant tissues would easily fulfill this need.

An alternative approach to achieve the same goal was taken by Yu and Langridge (48). They described a recombinant multicomponent vaccine based on cholera toxin (CT). They fused peptides containing important protective epitopes derived from two other enteric pathogens, ETEC, which causes bacterial traveler's diarrhea, and rotavirus, which causes acute viral gastroenteritis, to the CT-A2 and CT-B subunits of CT, respectively. The two recombinant CT subunit fusions were expressed from a single bidirectional promoter, ensuring a coordinated expression pattern for the two gene fusions and potentially facilitating the assembly of the chimeric holotoxin. In this approach, CT provides a scaffold for presentation of the protective epitopes, acts as a mucosal targeting molecule without toxic effect due to use of the nontoxic CT-A2 and B subunits, and is itself a vaccine candidate. The recombinant protein represents a trivalent vaccine that can elicit significant mucosal and humoral responses against *Vibrio cholerae*, ETEC, and rotavirus. Mice, orally immunized with potatoes expressing these recombinant antigens, developed immune memory B cells as well as helper T-cell type 1 (T_h1) responses, which are indicators of successful immunization. Further, pups of immunized dams were protected from challenge with rotavirus, with a significantly lower morbidity rate compared with controls. These results provide convincing evidence supporting a vaccine strategy employing chimeric proteins expressed in plants.