Use of Plant Viruses for Delivery of Vaccine Epitopes[a]

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Certain plant viruses have the potential to be developed for the synthesis and accumulation of proteins and epitopes that are inducers of protective immunity against human and animal diseases. Such plant viruses should replicate to high levels in infected plant tissues, be readily purified, be stable *in vivo* and *in vitro*, and be sufficiently characterized both genetically and structurally to allow for directed molecular modifications. Viruses that have been used specifically to produce vaccine epitopes include tobacco mosaic tobamovirus (TMV), cowpea mosaic comovirus (CPMV), and johnsongrass mosaic potyvirus (JMV). Discussion in this paper is limited to generic modification of replicating viruses that lead to production and accumulation of virions that display unique sequences on the surface of the virus particle.

The earliest report of research to modify a plant virus capsid (coat) protein to carry a foreign epitope described genetic modification to cause the addition of eight amino acids derived from a poliovirus capsid protein to the coat protein of TMV and expression of the modified protein in *Escherichia coli*. Injecting the assembled protein subunits of TMV to mice caused production of antibody against the poliovirus epitope.[1] Recently a similar approach was taken with JMV to demonstrate that the coat protein of this potyvirus could assemble to form virus-like particles in *E. coli* when up to 26 kD of foreign protein sequences were added to, or replaced, the amino terminal sequence of the coat protein.[2]

Recently there have been a number of reports on the construction of cloned cDNAs that represent the full-length genome(s) of viral RNAs from which infectious viral RNAs can be transcribed *in vitro*. The infectious cloned cDNAs (the term used to describe such constructs) of several plant viruses have been subjected to *in vitro* mutagenesis so that they encode modified coat proteins during virus replication, including proteins that can serve as immunogenic epitopes. Furthermore, knowledge of the crystal structures of TMV and CPMV makes it possible to predict the impact of foreign sequences on capsid and virion structures. These advances allow the design of strategies to develop these viruses as delivery vehicles for vaccine epitopes.

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DEVELOPING TOBACCO MOSAIC VIRUS FOR DELIVERY OF VACCINE EPITOPES

Tobacco mosaic virus is a rod-shaped particle composed of ≈2,100 capsid molecules that are arranged in an elongated helix; intersubunit interactions are stabilized by the presence of single-stranded viral RNA in the core of the assembled coat protein (CP) molecules.[3] Both amino- and carboxy-termini are exposed on the surface of the virion.

Several different approaches have been taken to develop TMV as a vaccine, while attempting to ensure that the modified CP molecules retain the capacity to assemble and to produce virus particles while maintaining, if possible, the ability to cause a systemic infection in the host. In one such example, Clark *et al.*[4] demonstrated that adding sequences from the CP of sunn hemp mosaic virus to the COOH-terminus of the TMV CP did not reduce virion assembly or the ability of the virus to cause a systemic infection. In other cases, however, protein fusions limited systemic infection or caused local necrosis to which virus infection was limited. Because necrotic reactions and other limiting local or systemic infections reduced dramatically the amount of virus that is produced, several different approaches have been taken.

One approach was to use two mutant viruses, one of which encoded wild-type CP but did not produce movement protein and was dependent on infection by the second virus, which produced movement protein in addition to modified CP (J. H. Fitchen and R. N. Beachy, unpublished data). Such an infection would produce virions comprised of both types of CP while limiting the infection by the virus that produces wild-type CP; furthermore, in those cases in which the epitope prevented efficient local and/or system infection, sufficient levels of wild-type CP accumulate to ensure spread of the infection (as demonstrated in FIG. 2). A second

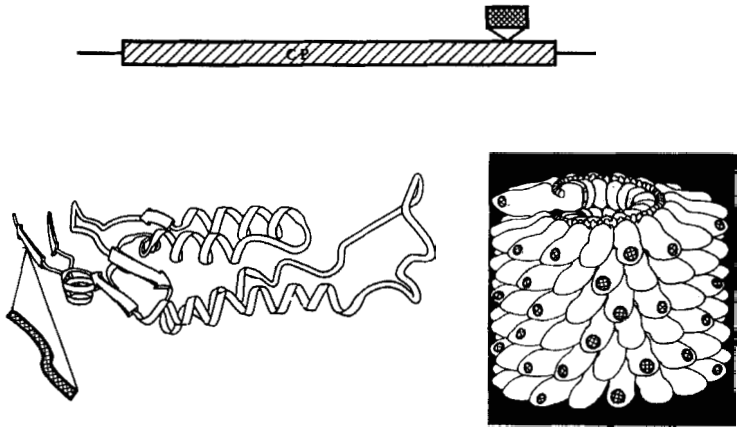

FIGURE 1. Developing TMV (tobacco mosaic virus) as a carrier of vaccine epitopes. **(Top)** Linear diagram of the coat protein (CP) of TMV showing the approximate position at which additional amino acids are inserted. **(Bottom)** Structure of TMV CP derived from X-ray crystallography and presented as a ribbon diagram.[3] Insertion of new amino acids (as above) is between amino acids 154 and 155. **(Bottom right)** Diagrammatic representation of TMV showing some of the CP molecules modified by additional sequences (*hatched region*).

TABLE 1. Amino Acid Sequences Added to Modify the Coat Protein (CP) of Tobacco Mosaic Virus (TMV) and Symptoms Induced on *N. tabacum* Plants upon Infection by the Modified Virus

Origin of Sequence	Epitope	No. of Amino Acids Added	Site of Insertion in TMV CP	Symptoms in *N. tabacum*
HIV	gp120 loop III	10, 15, 15, 20	154	Necrosis (ts), chlorosis
Influenza	Hemagglutinin 12CA5	9	1, 154, 158	Mild chlorosis, necrosis (ts)
Human *c-myc*	*c-myc* 9E10	10	155	Mild chlorosis
Murine zona pellucida	ZP3	13, 25*	154	None, chlorotic mottle in upper leaves

approach was to construct the virus so that a "leaky" termination codon separates the CP from a COOH-terminal peptide; at a frequency determined by the infected cell some of the coat protein is wild type, while the remainder is modified by an extension at the COOH-terminus.[5-7] This results in virus particles that contain both mutant and wild-type CP.

We have adopted a third strategy that involves inserting epitope sequences at a site in the coat protein that is less likely to interfere with virus assembly or systemic infection. As shown in FIGURE 1, the site of insertion was selected to be outside of the sequences that are required for intramolecular structure or intersubunit assembly[3]; epitopes are generally inserted between amino acids 154 and 155. In most cases, this leads to the accumulation solely of modified coat proteins in sufficiently large amounts for large scale purification. TABLE 1 presents the results of some studies of sequences that were added as COOH-terminal extensions of the CP or between amino acids 154 and 155. As indicated in the right-hand column, the various sequences induced different types of symptoms in the host. At the current time, we are unable to predict if specific sequences will or will not induce chlorosis or necrosis in *Nicotiana tabacum*.

Plants that are infected with TMV produce large amounts of CP, most or all of which is assembled with viral RNA to produce progeny virions. In chronically infected tobacco leaves, 10–40% of the leaf protein can be TMV CP. Likewise, plants infected with modified virus can produce large amounts of the modified CP. As shown in FIGURE 2 SDS-PAGE analysis of the proteins extracted from leaves infected with TMV in which the coat protein is modified by insertion of nucleotides that encode the 9 amino acid sequence derived from the hemagglutinin of influenza virus revealed a prominent protein band representing the modified coat protein (lanes 2, 3, 4, and 5). Furthermore, the virus was readily purified by PEG precipitation, effecting rapid and easy enrichment of the virions (FIG. 2, lanes 3–5). To date, the sequences added to the coat protein were retained during virus replication, local and systemic spread, and purification. However, it is likely that not all sequences will be equally stable when added to the TMV coat protein.

We selected for further studies a sequence derived from the murine zona pellucida 3. ZP3 is one of the three glycoproteins that comprise the covering of unfertilized eggs and is the primary binding site for sperm during fertilization.[8] It was previously demonstrated that an epitope comprising amino acids 336–343,

a B-cell epitope, is responsible for inducing antibody-mediated contraception.[9] DNA sequence encoding these amino acids was placed between codons 154 and 155 in the TMV CP gene on the infectious cloned cDNA, and plants were infected with the modified virus.[10] Enriched preparations of virus particles isolated from these plants or unmodified virus were parenterally administered to mice: both wild-type and modified TMV induced antibodies against the coat protein of TMV, and the modified virus also induced antibodies that recognized ZP3 on ELISA. Histologic and immunologic analyses of ovaries collected from immunized mice showed that after treatment with the modified virus, antibodies were recruited and bound to the zona pellucida. These studies demonstrated that the TMV-borne epitope can induce a B-cell–mediated response. Immunized and control mice were then used in fecundity assays to determine if immunization regimens had a positive or a negative impact on fertility of the mice. However, in these studies it was not possible to assess the fertility of the mice, and additional studies are needed.

Other studies are in progress to determine if TMV can be used effectively to carry an epitope derived from a mouse hepatitis virus, a coronavirus. In the near future we will determine if TMV can be used to confer protective immunity against this virus (J. H. Fitchen, work in progress).

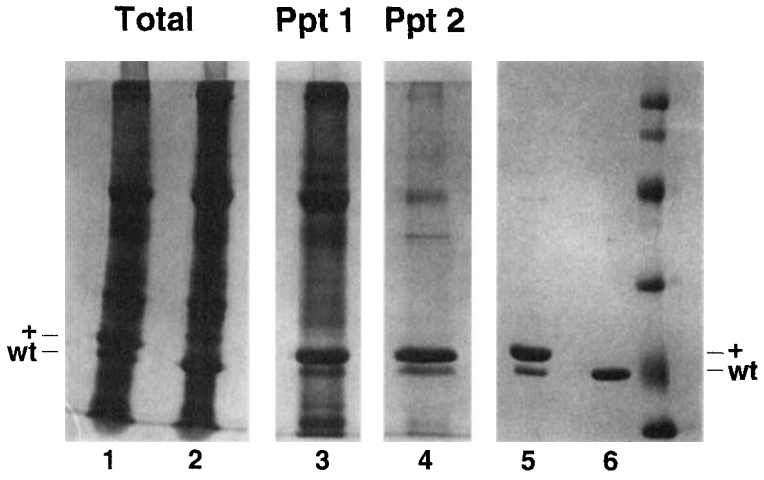

FIGURE 2. Accumulation of wild-type and modified TMV from infected leaves of *N. tabacum*. Tobacco plants were inoculated with TMV modified to carry 9 amino acids of the hemagglutinin of influenza A between amino acids 154 and 155. Modified virus was co-inoculated with a TMV mutant that lacked a functional movement protein (TMV-MPΔ 3-5).[15] Proteins were analyzed by standard SDS-PAGE, using 12% acrylamide in the separation gene. Total soluble proteins extracted from leaves infected with modified TMV plus TMV: MPΔ 3-5 (*lane 1*) or wild-type TMV (*lane 2*). Precipitates were collected after the first (*lane 3*) and second (*lane 4*) step of PEG-NaCl precipitations as described by Asselin and Zaitlin.[16] Following resuspension of ppt 2 (as in *lane 4*) in buffer containing Triton X-100, virus was collected by ultracentrifugation and protein released from the mixed infection (*lane 5*) or wild-type TMV (*lane 6*) was subjected to SDS-PAGE. *Lane 7*, molecular weight standards. Wt = wild-type TMV CP, M_r = 17.5 kD; TMV-CP modified by 9 amino acid insertion.

DEVELOPING JMV POTYVIRUS FOR DELIVERY OF VACCINE EPITOPES

Potyviruses, like TMV, are comprised of single-stranded (+) sense RNA, and they assemble to form elongated helical rod-shaped particles. However, potyviruses are flexuous rather than rigid rods due to the nature of the capsid protein molecule. Like TMV, the NH_2- and COOH-termini of the CP are exposed on the surface of the virus particle: the exposed amino terminal amino acid sequence can be greater than 30 amino acids. A single report describes the results of studies with sequences fused to coat proteins of johnsongrass mosaic potyvirus (JMV).[2] Fusion proteins were made at the amino terminus of the CP of JMV with an octapeptide derived from *Plasmodium falciparum* or the 10 amino acid luteinizing hormone releasing factor. In another construct, the 26-kD protein Sj-26-glutathione S-transferase from *Shistosoma japonica* replaced 62 amino acids at the NH_2-terminus of the CP. Fusion proteins comprising the CP plus added sequences accumulated in *E. coli* and assembled to form virus-like particles. Injection of the Sj-26:CP fusion to mice elicited antibody response to Sj-26. These studies demonstrate the capacity of modified potyvirus CP to assemble to form virus-like particles (VLP), including very large proteins. However, it is unknown if virions encapsidated by these mutant CP molecules can cause local and systemic infections in plants.

DEVELOPING CPMV FOR DELIVERY OF VACCINE EPITOPES

The structure of CPMV, determined to the atomic level in the late 1980s (reviewed by Chen *et al.*[11]), revealed that the virus surface includes several exposed loops. Of particular interest for developing CPMV as a vaccine is a loop made of amino acids 20–27 of the S capsid subunit; the loop lies on the surface of the virus and is not directly involved in intersubunit interactions. Usha *et al.*[12] inserted into the infectious cloned cDNA of CPMV DNA sequences to encode amino acids 136–160 from VP1 of foot and mouth disease virus for expression between amino acids 18 and 19 of the S-peptide. Transcripts derived from the modified clone were inoculated into protoplasts and whole plants. Chimeric virus particles accumulated in protoplasts and inoculated leaves but not in upper leaves; the added sequence is likely responsible for restricting systemic spread of the virus. Virus particles that were recovered reacted with antibodies raised against FMDV, confirming that the FMDV sequence was retained in the virus capsid.

It was subsequently found that the FMDV sequences inserted in CPMV were rapidly lost upon serial passaging of the virus, a result that led the investigators to redesign the chimeric protein in order to stabilize inserted sequences. Porta *et al.*[13] subsequently carried out mutagenesis reactions that led to the insertion of foreign sequences between amino acids 22 and 23 of the S-protein. Sequences that were stable included amino acids 141–159 of VP1 of FDMV, amino acids 85–98 of human rhinovirus 14 (HRV-14), and amino acids 731–752 of gp41 from human immunodeficiency virus III (HIV-III). These viruses were reactive with antibodies directed against the specific peptides, and in the case of the HRV-14 epitope, the virus was active as a vaccine in injected rabbits and induced antibodies directed against HRV-14.

Modified CPMV that contained the sequences from gp41 of HIV-III was used to inject C57/BL6 mice. Antibodies recovered from injected mice reacted with purified peptide and in *in vitro* assays neutralized HIV-1 strain IIIB as well as

HIV-I strains RF and $SF2^{14}$ (McLain and Dimmock, 1994). Titers of neutralizing antibodies declined to undetectable levels several weeks after the second injection but were stimulated upon reinjection. The results of these studies demonstrated the use of CPMV as a carrier of vaccine epitopes and indicate that this virus may be especially well suited for presenting epitopes that are structured as a loop rather than as a linear array or random coil.

CONCLUSIONS

The results of studies in structural biology, virology, and immunology completed during the recent 20 years have made it possible to contemplate the development of plant viruses as carriers of vaccine epitopes. As described herein, well-characterized epitopes can be added to the capsid proteins of plant viruses, recovered in large quantities, and injected to induce immune responses in animals. Whereas early studies have concentrated on confirming the stability of the modified viruses and their capacity to induce the predicted antibody response, ongoing studies will determine whether the induced antibodies provide protective immunity against disease agents. Studies in this laboratory have used TMV to carry an epitope from mouse hepatitis coronavirus (J. H. Fitchen, M. Buchmeier, and R. N. Beachy, work in progress). Future research goals include the development of a better understanding of how modified, nonreplicating viruses, such as those described here, induce B-cell and T-cell responses, and the induction and maintenance of protective immunity. Although an important future goal is to deliver plant virus-based vaccines as a component of food, considerable experimentation will be required before we can understand how such immunization can be accomplished and how oral tolerance can be avoided in the process.

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