DEVELOPMENT OF A GENETICALLY-ENGINEERED, CANDIDATE POLIO VACCINE EMPLOYING THE SELF-ASSEMBLING PROPERTIES OF THE TOBACCO MOSAIC VIRUS COAT PROTEIN

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A synthetic gene coding for the coat protein of tobacco mosaic virus (TMVCP) was expressed in *E. coli* under the direction of the *lac*UV5 promoter. Modification of the 3′ end of the TMVCP gene by insertion of a region coding for an antigenic epitope from poliovirus type 3 resulted in the production of a hybrid TMVCP (TMVCP-polio 3). Both the *E. coli*-produced TMVCP and TMVCP-polio 3 were shown to assemble into virus-like rods under acidic conditions in *E. coli* extracts. Their purification was accomplished in a single step by chromatography on Sepharose 6B. TMVCP-polio 3 induced the formation of poliovirus neutralizing antibodies following injection into rats. The level of immune response was related to the degree of polymerization of the TMVCP-polio 3 preparations.

A recent application of biotechnology to the production of a new generation of safer and more effective vaccines has focused on the use of synthetic oligopeptides coupled to carrier molecules[1–10]. Although the coupling of a peptide to a carrier molecule usually results in an enhancement of the peptide's immunogenicity, no vaccines suitable for human use have yet been produced by this technology.

We have extended the concept of peptide carriers to include the use of a self-assembling carrier molecule containing an antigenic epitope linked to a specific attachment site. The *in vitro* assembly of such a molecule into a high molecular weight complex in the size range of many viruses is expected to result in the creation of a highly immunogenic particle containing the antigenic epitope repeated many times on its surface.

One example of a self-assembling carrier molecule is the coat protein of tobacco mosaic virus (TMVCP), which polymerizes into high molecular weight, helical rods in acidic solutions[11]. The C-terminus of the TMVCP is exposed to solvent on both the intact virion and the protein polymers, suggesting that this would be an ideal location for the attachment of foreign antigenic epitopes.

In this report, we describe the synthesis of a modifiable TMVCP gene, which can direct the expression of TMVCPs containing C-terminal extensions. We show that a modified TMVCP, containing a poliovirus type 3 antigenic epitope, can be expressed in *E. coli*, and polymerized *in vitro*. This hybrid molecule induces the production of anti-poliovirus neutralizing antibodies following injection into rats.

RESULTS

Synthesis of the TMV coat protein gene. By synthesizing DNA containing the TMVCP coding sequence it was possible to produce a gene having both a convenient arrangement of restriction enzyme cleavage sites and an "optimized" set of codons for efficient translation in a prokaryotic system[12]. The synthetic coding sequence spec-

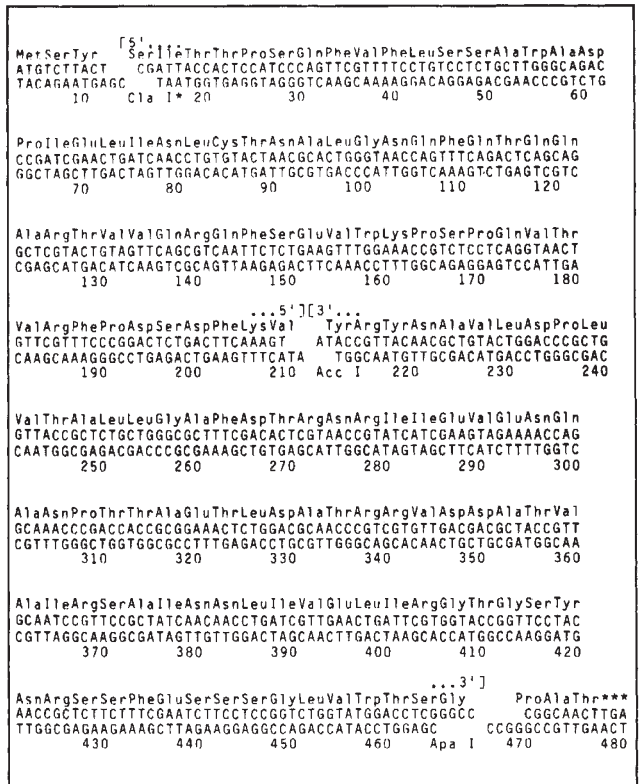

FIGURE 1 Sequence of the synthetic TMVCP coding region. The synthetic TMVCP coding sequence was designed to contain predominantly preferred codons for translation in a prokaryotic host while at the same time coding for the authentic amino acid sequence of the coat protein from TMV Vulgare. The regions marked 5′ and 3′ were separately synthesized and cloned. It should be noted that the ClaI site indicated at the proximal end of the 5′ region is destroyed after ligation to the translation initiation linker in the final expression construct (see Fig. 2).

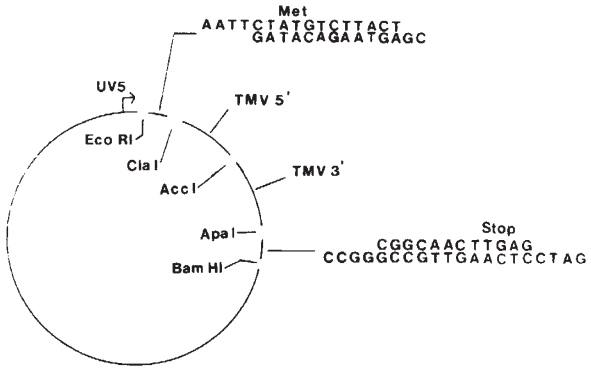

FIGURE 2 Construction of the pTMVCP expression plasmid. The TMV 5′ and TMV 3′ fragments were inserted into a vector containing the *lac*UV5 promoter using two double stranded linkers which adapted the restriction ends and supplied the necessary translation initiation and termination codons.

ifying the coat protein of TMV Vulgare[13], is shown in Figure 1, and was synthesized as two halves, which were separately cloned for propagation in bacteria.

The insertion of the 5′ and 3′ halves of the TMVCP coding sequence into the final bacterial expression vector is shown in Figure 2. This construction utilized two double stranded linkers to adapt the restriction ends and to supply the remaining nucleotide sequences needed for the N-terminal and C-terminal amino acids. The final construct (pTMVCP) contained the entire TMVCP coding sequence located downstream from the *lac*UV5 promoter[14].

Insertion of the poliovirus type 3 epitope. The synthetic TMVCP gene was designed to facilitate 3′ end modifications to produce TMVCP's with predetermined C-terminal extensions. Evans et al.,[15] described the location of an epitope on the VP1 of poliovirus type 3 consisting of eight amino acids. To express this epitope on the C-terminus of the TMV coat protein, the pTMVCP expression plasmid was cleaved with ApaI and BamHI to remove the small fragment coding for the two C-terminal amino acids (Fig. 2). This fragment was replaced with a double stranded ApaI-BamHI linker coding for the two C-terminal amino acids, plus two glycine residues to serve as a spacer, plus the eight amino acids of the poliovirus epitope (Glu Gln Pro Thr Thr Arg Ala Gln). Thus the final construct, pTMVCP-polio 3, specified the expression of a modified TMV coat protein molecule containing an additional 10 residues at its C-terminus.

Expression of TMVCP and TMVCP-polio 3 in *E. coli*. Western blot analysis demonstrated expression of TMVCP and TMVCP-polio 3. Bacterial lysates of cultures containing either the pTMVCP or pTMVCP-polio 3 expression plasmids were electrophoresed on an SDS-polyacrylamide gel (SDS-PAGE). Proteins were transferred to nitrocellulose and incubated sequentially with anti-TMV antiserum and radiolabeled protein-A. Autoradiography revealed a product reacting with anti-TMV antisera in cells containing the pTMVCP expression plasmid, which comigrated with the authentic TMVCP from TMV virions (Fig. 3a). A related product, which showed a reduced relative mobility, also reacted with anti-TMV antisera in the sample from cells containing the pTMVCP-polio 3 expression plasmid. The latter also bound antisera against poliovirus type 3 vaccine confirming the presence of a poliovirus epitope on the TMVCP-polio 3 product (Fig. 3b).

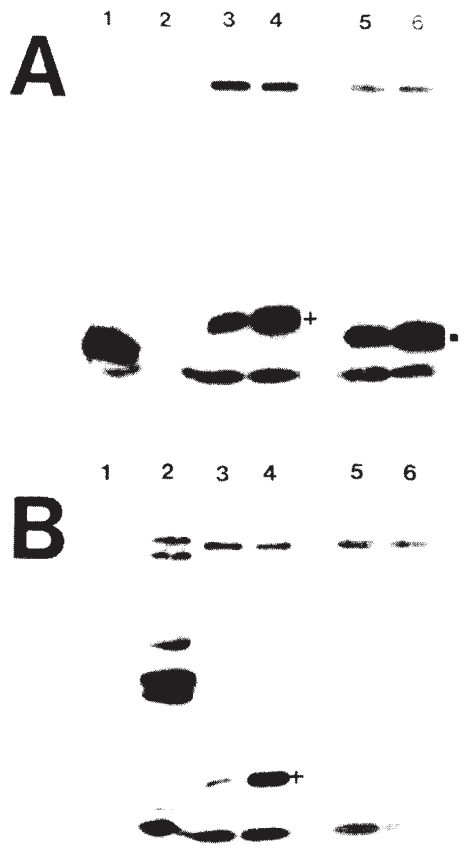

FIGURE 3 Western blot analysis of TMVCP and TMVCP-polio 3 in bacterial extracts. Cultures of *E. coli* containing either the pTMVCP or pTMVCP-polio 3 expression plasmids were grown with and without IPTG as an inducer of the *lac*UV5 promoter. Culture samples were lysed by boiling with SDS and electrophoresed on a 12.5% SDS-gel. Replicate blots were reacted with either anti-TMV (blot A) or anti-poliovirus type 3 (blot B) antisera. Lane 1, TMV virus positive control; lane 2, poliovirus type 3 positive control; lane 3, pTMVCP-polio 3 not induced; lane 4, pTMVCP-polio 3 induced; lane 5, pTMVCP not induced; lane 6, pTMVCP induced. The dot indicates the TMVCP product while the cross indicates the TMVCP-polio 3 product. The two bands located at the extreme top and bottom of lanes 3-6 represent *E. coli* products which nonspecifically react with the radiolabeled protein-A.

Purification of TMVCP and TMVCP-polio 3 from *E. coli*. Purification of TMVCP and TMVCP-polio 3 products was based on their self-assembling properties. Sonicated lysates of both pTMVCP and pTMVCP-polio 3 containing bacteria were dialyzed overnight (pH 5.0) to induce polymerization of the TMVCP products. Following concentration by ultrafiltration, the samples were each chromatographed on Sepharose 6B and the fractions analyzed by SDS-PAGE. The peaks at the void volume contained only TMVCP or TMVCP-polio 3, indicating that the bacterially-produced TMVCP products polymerized in the extracts to such an extent that they each exhibited the highest molecular weight of any protein present (data not shown).

Characterization of the polymeric TMVCP products from *E. coli*. Electron micrographs of TMVCP and TMVCP-polio 3 (Fig. 4) demonstrate the presence of rods and disks of the type previously observed for natural TMVCP[16,17]. The TMVCP-polio 3 appeared to polymer-

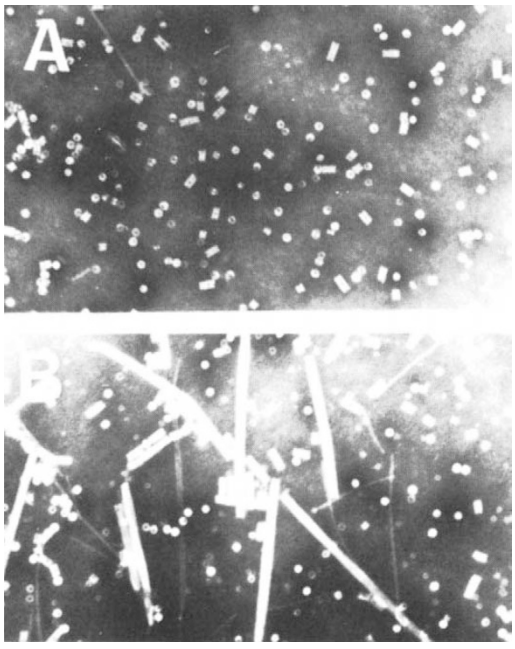

FIGURE 4 Electron microscopic analysis of pH 5.0 assembled TMVCP and TMVCP-polio 3. Samples of TMVCP and TMVCP-polio 3 purified from a pH 5.0 Sepharose 6B column and were concentrated to 0.1 mg per ml, for electron microscopic analysis. Magnifications were at ×176,000. Micrograph A, TMVCP; micrograph B, TMVCP-polio 3.

ize more efficiently than the bacterially produced TMVCP. Electron microscopic data showed longer rods in the TMVCP-polio 3 sample (Fig. 3B), and in size exclusion columns the TMVCP-polio 3 reproducibly eluted ahead of the TMVCP.

Immunogenicity studies. An initial immunization experiment indicated that the TMVCP-polio 3 product could indeed induce an anti-poliovirus neutralizing response after injection into rats (data not shown). However, these data did not indicate whether the observed response was directed against the aggregated form of the TMVCP-polio 3 or against material which had disaggregated soon after injection. To investigate this point, a second immunization was performed in which we compared the immune responses to three forms of TMVCP-polio 3: pH 5.0 polymerized, pH 8.0 disaggregated, and material assembled in the presence of TMV genomic RNA at neutral pH. The latter product was produced by reacting TMVCP-polio 3 with TMV genomic RNA *in vitro* to form virus-like rods stable over a broad pH range. This reaction was monitored by electron microscopy (data not shown).

Table 1a presents the results of this experiment showing that the pH 5.0 assembled TMVCP-polio 3 elicited a higher neutralizing response than the pH 8.0 disassembled sample. Moreover, the pH stable, RNA-assembled TMVCP-polio 3 did not induce a greater response indicating that the pH 5.0 sample remained aggregated after injection.

The difference in the levels of neutralizing antibody induced by the pH 5.0 and pH 8.0 TMVCP-polio 3 samples was shown to be reproducible in a further immunization study employing five rats per group (Table 1b).

TABLE 1 Immunogenicity testing.

A. Comparison of immune responses to three forms of the TMVCP-polio 3.

Inoculum	Rat	Neutralizing Antibody Titers (Log_2)			
		1 week after 2nd injection	1 week after 3rd injection	1 week after 4th injection	3 weeks after 4th injection
TMVCP-polio 3	1	7	9	10	11
ph 5.0	2	3	6	8	8
CFA	3	—	—	3	3
TMVCP-polio 3	4	—	2	4	1
pH 8.0	5	3	4	6	4
CFA	6	—	—	dead	dead
TMVCP-polio 3	7	2	7	4	9
RNA-assembled	8	—	—	1	6
CFA	9	2	2	5	5

B. Comparison of immune responses to TMVCP-polio 3 at pH 5.0 and pH 8.0.

Inoculum	Rat	Neutralizing Antibody Titers (Log_2)	
		1 week after 2nd injection	1 week after 3rd injection
TMVCP-polio 3	1	7	7
pH 5.0	2	6	7
CFA	3	1	3
	4	2	3
	5	—	3
TMVCP-polio 3	1	1	4
pH 8.0	2	1	2
CFA	3	—	—
	4	—	3
	5	2	1

—indicates no significant response. CFA, complete Freund's adjuvant.

These immunization data support the idea that the molecular weight of the TMVCP-polio 3 antigen has a definite influence on the efficiency of the immune response.

It should be noted that significant variations were observed in the levels of the immune responses between individual rats in a given injection group. We attribute this variation to the immunization regime employed (see Experimental Protocol). Rats injected with inactivated Salk vaccine in a similar manner usually show significant variations in their immune responses (data not shown).

DISCUSSION

We have described the development of a genetically engineered, self-assembling, peptide-carrier vaccine system combining the tobacco mosaic virus coat protein with a poliovirus epitope. It is difficult to compare the immunization results for the TMVCP-polio 3 with those previously reported for the same epitope attached to bovine thyroglobulin[3]. This is due to differences in the animal species used for immunization and in the actual amounts of antigenic peptide injected per animal. In the present studies, rats injected with 200 μg of TMVCP-polio 3 received approximately 10 μg of epitope peptide, while in the typical conjugated carrier-peptide experiments, the equivalent of several hundred micrograms of peptide was injected per animal. Thus, the data do not allow a valid comparison of the immune responses for the two systems.

It should be noted that the levels of neutralizing antibody shown in Table 1 were dependent upon the use of complete Freund's adjuvant (CFA). With the use of adjuvant for four injections, it was possible to achieve a neutralizing antibody titer, in some rats, as high as that observed from a single injection of Salk vaccine. Using aluminum phosphate as an adjuvant, the TMVCP-polio 3 still induced neutralizing responses in some rats but the levels were significantly reduced (data not shown). We have recently shown that multiple TMVCPs, containing different epitopes, can be copolymerized *in vitro* to produce multispecific heteropolymers. It will be interesting to determine if heteropolymers containing multiple epitopes from poliovirus type 3 show enhanced immunogenicity.

A related system has recently been described by Valenzuela et al.[18], in which they reported the use of the hepatitis B surface antigen (HBsAg) as a carrier of antigenic epitopes. A recombinant HBsAg gene containing a herpes simplex glycoprotein D coding segment inserted into its pre-S region was expressed in yeast resulting in the formation of HBsAg-lipoprotein particles containing a foreign epitope. A number of differences exist between the HBsAg system and the one described here. It is likely that the HBsAg molecule will be capable of carrying significantly larger epitope regions than the TMV coat protein. The former has been shown to assemble *in vivo* with a 300 amino acid insertion in its pre-S region. Preliminary results with the TMVCP system suggest that epitopes of this size no longer permit assembly. This difference may not prove to be a disadvantage for the TMVCP carrier as it appears capable of carrying extensions long enough to contain the type of epitopes defined by experiments with synthetic peptides. A second difference is that while the hybrid HBsAg products are aggregated *in vivo*, the TMVCP-epitope products can be reversibly assembled *in vitro* by pH shifts. This will permit various TMVCP-epitope products to be mixed and assembled at predetermined ratios with or without the unmodified subunit to optimize the density of epitopes and to create multi-specific vaccines.

A final point to consider is the effect a particular carrier may have after it has been presented to the immune system by repeated vaccinations. Will repeated exposure to the TMVCP carrier produce a subsequent enhancement or inhibition of the immune response to various haptens? In at least one case, the use of the diphtheria toxoid as a carrier for the capsular polysaccharide of *Haemophilus influenzae* type B was shown to have a stimulatory effect[19]. In immunizations using the polysaccharide antigen coupled to diphtheria toxoid, it was found that preimmunization with the toxoid alone significantly enhanced the subsequent immune response to the conjugated hapten. On the other hand, a recent report describing the use of the tetanus toxoid as a carrier has shown that preimmunity to the carrier molecule has a suppressive effect on the subsequent immune response to coupled peptides[20]. Detailed immunization experiments will have to be performed for all carriers in order to determine their potential usefulness for vaccine production.

EXPERIMENTAL PROTOCOL

Synthesis of the TMVCP gene. Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer and were purified by electrophoresis on preparative DNA sequencing gels[21]. Bands were visualized by UV imaging[22]. The region of the TMVCP coding sequence in Figure 1 marked 5′ was assembled from a collection of 13 overlapping oligonucleotides whereas the region marked 3′ was assembled from a collection of 17 oligonucleotides. The ligations were performed in two stages in which groups of 4 to 5 complementary oligonucleotides were initially annealed and ligated. The products of these reactions were pooled for the final ligations yielding the 199 bp 5′ fragment (ClaI-AccI) and the approximately 250 bp 3′ fragment (AccI-ApaI). The 5′ and 3′ fragments were separately cloned into pBR322 for propagation in *E. coli*. Assembly of the pTMVCP and pTMVCP-polio 3 expression plasmids is described in the text.

Western blotting of *E. coli* produced TMVCP products. Confirmation of the presence of TMVCP products in *E. coli* clones containing the pTMVCP or the pTMVCP-polio 3 plasmids was obtained by Western blot analysis. Cultures of *E. coli* strain JM103 containing the respective plasmids were inoculated 1:100 into L-broth containing 1mM isopropyl beta-D-thiogalactopyranoside (IPTG) and 50 μg ampicillin per ml. Cultures were grown to an OD_{575} of 1.0 after which 0.25 ml of each culture was centrifuged and resuspended in SDS-PAGE sample buffer[23] and lysed by incubation in a boiling water bath for 5 minutes. Samples were electrophoresed on a 12.5% SDS-polyacrylamide gel[23] after which the proteins were transferred to nitrocellulose[24]. Nitrocellulose blots were reacted with either anti-TMV antisera or anti-poliovirus type 3 vaccine antisera diluted 1:100 in incubation buffer[24]. Following binding with I^{125}-labeled protein-A (Amersham), the blots were washed and autoradiographed.

Purification of TMVCP products from *E. coli*. Purification of TMVCP products from *E. coli* was carried out by growing 3 liter cultures of pTMVCP or pTMVCP-polio 3 containing bacteria as described in the previous section. At an OD_{575} of 1.0-2.0 the bacteria were chilled on ice and harvested by centrifugation. Bacteria were resuspended in 60 ml of 0.3 M sucrose, 0.2 M NaCl, 0.1 M tris, pH 7.9, 50 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride and sonicated twice at an amplitude of 18 microns for 90 seconds with a 1 minute pause between sonications (MSE Soniprep 150). After removal of cell debris by centrifugation at 10,000 RPM in a Sorvall SS34 rotor the supernatant was dialyzed overnight at room temperature against 0.1 M sodium acetate, pH 5.0, to induce TMVCP aggregation. Following dialysis the precipitate was removed by centrifugation as described above and the supernatant was concentrated to a volume of 50 ml using an Amicon ultrafiltration membrane with a molecular weight cutoff of 10,000 daltons. The concentrate was chromatographed on a Sepharose 6B column (5 × 30 cm) in 0.1 M sodium acetate, pH 5.0. Fractions containing TMVCP products were identified by PAGE analysis.

Electron microscopy of TMVCP-epitope products. TMVCP and TMVCP-polio 3 preparations were prepared for electron microscopy by applying samples to formvar-carbon coated grids and blotting off excess sample after 30–60 seconds. Grids were negatively stained with 2% phosphotungstic acid for 30–60 seconds after which the excess was removed by blotting and the grids were allowed to dry. Samples were examined on a Phillips 200 electron microscope.

Immunogenicity testing. TMVCP-polio 3 was tested for immunogenicity using coronavirus free, Wistar rats as suggested by

van Steenis et al.[25] Antigen, at a concentration of 0.2 mg per ml, was mixed with an equal volume of complete Freund's adjuvant prior to each injection. The first and second injections were spaced three weeks apart while the remaining injections were two weeks apart. For a given injection, each rat received 1 ml i.p. and 1 ml i.d. (multiple sites). Rats were bled seven days following each injection. Sera were tested for neutralizing antibodies by a metabolic inhibition test using Vero cells in 96 well microplates. Equal volumes of serum dilutions (25 µl) and live virus (20–50 $TCID_{50}$) were mixed and incubated for 3 hours at 37°C and 12 hours at 4°C in microplates after which cells were added to the wells. After 7 days incubation at 37°C the presence or absence of virus activity was determined by a metabolic inhibition assay. A pH of less than 7.2 indicated cell growth and absence of virus activity while a pH of greater than 7.2 indicated cell death and residual virus activity due to a lack of complete virus neutralization. The pH 5.0 TMVCP-polio 3 samples (0.1 sodium acetate) were obtained from the void fractions of the preparative Sepharose 6B column runs (see above). The pH 8.0 TMVCP-polio samples were obtained by dialyzing the pH 5.0 samples against 30 mM Tris-HCl, pH 8.0, overnight at 4°C. RNA-assembled TMVCP-polio 3 used in the second immunization experiment was prepared by dialyzing a preparation of TMVCP-polio 3 at 0.4 mg/ml against 0.1 M sodium pyrophosphate (pH 7.0) for two days at room temperature in order to induce disk formation. Assembly was accomplished by adding dithiothreitol to 1 mM and placental ribonuclease inhibitor to 75 units per ml after which a 1.3 fold stoichiometric excess of TMV genomic RNA was added. Incubation was continued for 18 hours at 25°C after which successful assembly was assayed by electron microscopy as described above.

Acknowledgments

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