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Immunology

Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice

(edible vaccine/foreign genes/plants)

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ABSTRACT **Alternatives to cell culture systems for production of recombinant proteins could make very safe vaccines at a lower cost. We have used genetically engineered plants for expression of candidate vaccine antigens with the goal of using the edible plant organs for economical delivery of oral vaccines. Transgenic tobacco and potato plants were created that express the capsid protein of Norwalk virus, a calicivirus that causes epidemic acute gastroenteritis in humans. The capsid protein could be extracted from tobacco leaves in the form of 38-nm Norwalk virus-like particles. Recombinant Norwalk virus-like particle (rNV) was previously recovered when the same gene was expressed in recombinant baculovirus-infected insect cells. The capsid protein expressed in tobacco leaves and potato tubers cosedimented in sucrose gradients with insect cell-derived rNV and appeared identical to insect cell-derived rNV on immunoblots of SDS/polyacrylamide gels. The plant-expressed rNV was orally immunogenic in mice. Extracts of tobacco leaf expressing rNV were given to CD1 mice by gavage, and the treated mice developed both serum IgG and secretory IgA specific for rNV. Furthermore, when potato tubers expressing rNV were fed directly to mice, they developed serum IgG specific for rNV. These results indicate the potential usefulness of plants for production and delivery of edible vaccines. This is an appropriate technology for developing countries where vaccines are urgently needed.**

Norwalk virus is a member of the *Caliciviridae* family and causes epidemic acute gastroenteritis in humans (1, 2). Previous studies estimated that ≈42% of outbreaks of acute epidemic gastroenteritis in the United States are caused by Norwalk and Norwalk-like viruses (3). Recent advances in cloning the Norwalk virus genome (1) and expression of the capsid protein in recombinant baculovirus-infected insect cells (4) have facilitated the study of the virus and the development of a candidate vaccine (5).

Expression of the Norwalk virus capsid protein (NVCP) in insect cells yields a protein with an apparent M_r of 58,000 that self-assembles into insect cell-derived Norwalk virus-like particles (i-rNVs) lacking viral RNA, which are reactive with sera from Norwalk virus-infected humans (4). Electron cryomicroscopy of i-rNV shows that the 38-nm empty capsid is composed of 90 dimers of NVCP that form arch-like capsomeres (6). The particles are morphologically and antigenically similar to authentic virus particles, stable on storage at 4°C, stable after lyophilization, and resistant to pH 3.0 treatment (4, 7). These qualities make i-rNV attractive for use as a potential vaccine against Norwalk virus. Recent studies showed that oral immunization of mice with as little as 50 μg of i-rNV per dose resulted in the production of serum and mucosal antibodies against NVCP (5). This result is striking in view of the fact that i-rNV is a nonreplicating vaccine and no cholera toxin (CT) adjuvant is needed to achieve immunization.

We have experimented with the use of plants as an economical alternative for expression and delivery of recombinant vaccines (8–10). Hepatitis B surface antigen (HBsAg) expressed in tobacco leaves forms subviral particles (8) that are similar to the recombinant yeast-derived antigen, which is licensed for parenteral immunization (11). The plant-derived HBsAg retains both B- and T-cell epitopes when studied in a mouse model (10). Furthermore, the *Escherichia coli* heat-labile enterotoxin B-subunit expressed in potato tubers and fed to mice without preparation (other than slicing) stimulates serum and gut mucosal antibodies against *E. coli* labile enterotoxin B-subunit (9). These studies provide proof that recombinant antigens can be produced in transgenic plants, and, at least in some cases, these antigens are orally immunogenic.

We report here the expression of recombinant NVCP in transgenic tobacco leaves and potato tubers. The NVCP from tobacco leaves self-assembles into tobacco-derived virus-like particles (t-rNVs) that are morphologically and physically similar to i-rNVs. Further, we show that either partially purified t-rNV given orally or potato tubers expressing NVCP fed directly to mice stimulate the production of antibodies against NVCP. We conclude that a plant-derived edible vaccine for Norwalk virus is feasible. Together with our previous studies, these findings bolster the concept of using transgenic plants for a novel, safe vaccine production and delivery system for developing countries.

MATERIALS AND METHODS

Construction of Plant Expression Vectors. A 2.4-kbp DNA fragment containing the gene encoding NVCP was obtained by partial *Eco*RI digestion of pUCNV4145 (1) and subcloned into pBluescript-KS (Stratagene) at the *Eco*RI site. One clone (pKSNV2.4) was digested with *Sma*I and *Sst*I; the resulting 1.9-kbp fragment was ligated with pBI121 (Clontech; ref. 12) at *Sma*I/*Sst*I. This gave pNV101, a binary vector for expression of NVCP in plants using the cauliflower mosaic virus 35S promoter to drive transcription and the nopaline synthase terminator (Fig. 1).

pNV102 was constructed as follows. pUCNV4145 was digested with *Hin*dIII followed by blunt-ending with mung bean

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Abbreviations: NVCP, Norwalk virus capsid protein; CT, cholera toxin; HBsAg, hepatitis B surface antigen; rNV, recombinant Norwalk virus-like particle; i-rNV, insect cell-derived rNV; t-rNV, tobacco-derived rNV; TEV, tobacco etch virus; UTR, untranslated region; DM/PBS, nonfat dry milk in PBS.

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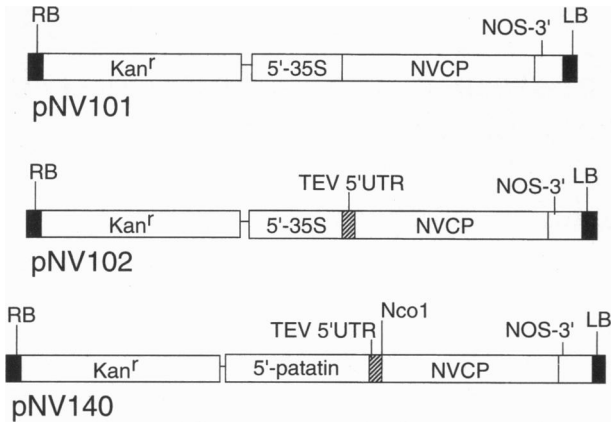

FIG. 1. Plasmids used for expression of rNV in plants. Shown are the transferred DNA regions of binary vectors used for *Agrobacterium*-mediated transformation of tobacco and potato plants. RB and LB indicate the right and left transferred DNA borders that delineate the region transferred into plant cells and inserted into the nuclear DNA. Each plasmid contains a *nptII* expression cassette for selection of plant transformants using kanamycin. pNV101 and pNV102 use the cauliflower mosaic virus 35S promoter to drive transcription, while pNV140 uses the patatin promoter for tuber-specific expression. pNV102 and pNV140 also contain the TEV 5′-UTR for stimulation of translation initiation. pNV140 contains a NVCP coding region modified to contain a *Nco*I site surrounding the translation initiation codon.

nuclease, and then it was digested with *Xba*I to give a 1.3-kbp fragment containing the 3′-terminal 1125 bp of NVCP coding region and 178 bp of noncoding region. This fragment was ligated with pBI201 (Clontech) that had been digested with *Sst*I, blunt-ended with mung bean nuclease, and then digested with *Xba*I to give pNV3. A 900-bp fragment containing a cauliflower mosaic virus 35S promoter with duplicated enhancer region fused to the tobacco etch virus (TEV) 5′-untranslated region (UTR) was obtained by digestion of pRTL2-GUS (13) with *Nco*I, followed by blunt-ending with mung bean nuclease and *Hin*dIII. This fragment was ligated with pNV3 digested with *Pst*I, followed by blunt-ending with mung bean nuclease and *Hin*dIII to give pNV4. The 471-bp 5′-terminal NVCP coding region was obtained by digestion of pUCNV4145 with *Eco*RI followed by mung bean nuclease and then *Xba*I, and then it was ligated with pNV4 digested with *Sal*I followed by mung bean nuclease and *Xba*I to give pNV202. Finally, the *Hin*dIII/*Eco*RI fragment from pNV202 was ligated with pBI121 at *Hin*dIII/*Eco*RI to give pNV102 (Fig. 1).

pNV140, a vector using the patatin promoter (14) to drive expression of NVCP, was constructed as follows. A *Nco*I site was created at the third in-frame ATG codon of NVCP (15) with PCR using the mutagenic primer 5′-AAAACCATGGCGTCTAAGGAC-3′ along with the downstream primer 5′-CTGCTACAGGATCCA-3′. The resulting fragment of 106 bp was digested with *Nco*I and *Bam*HI and ligated with the 1765-bp *Bam*HI/*Sst*I fragment from pUCNV4145, and the expression vector pIBT210 (9) that was digested with *Nco*I and *Sst*I to give pNV210. The NVCP coding region with the modified 5′ end fused to the TEV 5′-UTR was obtained by digestion of pNV210 with *Xho*I, partial filling with Klenow fragment and dCTP/dTTP, and digestion with *Sst*I. This 2-kbp fragment was ligated with pPS20A-G (14) that was digested with *Bam*HI, partially filled with Klenow fragment and dGTP/dATP, and digested with *Sst*I to give pNV140 (Fig. 1).

Plant Transformation. Plasmids NV101, NV102, and NV140 were mobilized into *Agrobacterium tumefaciens* LBA4404 by the freeze–thaw method (16), using plasmids cloned in *E. coli* DH5α. The structures of plasmids in transformed LBA4404 lines were verified by restriction digestion. Tobacco (*Nicotiana tabacum* "Samsun") (8) and potato [*Solanum tuberosum* "Frito-Lay (FL) 1607"] (9, 14) were transformed by modified leaf-disc cocultivation methods using the *Agrobacterium* strains described above. Kanamycin-resistant transformants were screened for NVCP expression by RNA hybridization and ELISA (below). Tissue culture microtubers and soil-grown tubers from transgenic potato plants were developed as described (9).

NVCP ELISA. NVCP in plant extracts was quantified by ELISA as described (4, 17). Rabbit anti-(i-rNV) serum diluted 1:10,000 in 0.01 M phosphate-buffered saline (PBS; 50 μl per well) was bound to 96-well polyvinylchloride microtiter plates for 4 h at 23°C, and the plates were blocked with 5% nonfat dry milk in PBS (DM/PBS) for 1 h at 37°C. After washing the wells three times with PBS with 0.05% Tween 20, samples (50 μl per well) diluted in PBS were added and incubated 16 h at 4°C. The wells were washed and incubated in succession with guinea pig anti-(i-rNV) serum and goat anti-guinea pig IgG–horseradish peroxidase conjugate, each diluted 1:5000 in 2% DM/PBS, for 2 h at 37°C. The plate was developed with Slow TMB substrate (Pierce) for 15–20 min at 23°C, the reaction was ended by addition of an equal volume of 0.5 M H_2SO_4, and the absorbance was read at 450 nm. For a standard curve, i-rNV was diluted with PBS to concentrations between 1.4 and 45 ng/ml and processed as above.

RNA Extraction and Hybridization. Total RNA was extracted from leaves of tobacco, fractionated on formaldehyde gels, and blotted to nylon membranes as described (8). The membranes were hybridized with ^{32}P-labeled random-primed DNA made using the 1.3-kbp *Bam*HI/*Eco*RI fragment from pUCNV4145 as template as described (8), and signals were quantified with a PhosphorImager (Molecular Dynamics).

Extraction and Purification of Recombinant Norwalk Virus-Like Particle (rNV) from Plant Tissues. Microtubers were homogenized in extraction buffer (PBS, pH 7.2/50 mM sodium ascorbate/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.2% Triton X-100) by crushing with a "pellet pestle" (Fisher Scientific) in 1.5-ml microcentrifuge tubes. Soil-grown tubers were weighed, skinned, and sliced before homogenization in extraction buffer with a Ten-Broek (Fisher) apparatus (clearance 0.15 mm). Homogenates were microcentrifuged 5 min at 14,000 × *g* at 4°C, and supernatants were tested for NVCP by ELISA and for total protein by the Coomassie blue dye binding assay (Bio-Rad), with BSA as a standard.

Tobacco leaves (10–20 cm long) were harvested, frozen in liquid N_2, and ground to a fine powder in a stainless steel blender precooled with liquid N_2. Approximately 250 g of leaf powder was suspended in 500 ml of extraction buffer and stirred at 4°C for 4–16 h. The slurry was centrifuged at 3,000 × *g* for 20 min at 4°C in a Sorvall GS-3 rotor, and the supernatant was centrifuged again at 15,000 × *g* for 40 min in a Sorvall F16/250 rotor. The rNV particles were pelleted from the resulting supernatant by centrifugation at 100,000 × *g* for 2 h in a Beckman SW 28 rotor. The pellet was suspended in PBS, and cleared by centrifugation at 10,000 × *g* for 5 min in a Sorvall F18/50 rotor. Four milliliters of the cleared suspension was loaded on a 32-ml discontinuous sucrose gradient made by layering 7 ml each of 50% and 40% sucrose in PBS and 6 ml each of 30%, 20%, and 10% sucrose in PBS. The gradients were centrifuged at 53,000 × *g* for 14 h at 4°C in a Beckman SW 28 rotor, and fractions were analyzed by ELISA or SDS/PAGE and Western blot. Fractions that cosedimented with i-rNV particles were combined and concentrated, and buffer (PBS) was exchanged by centrifugal ultrafiltration with a Centriprep-30 (Amicon).

Analytical Anion Exchange Chromatography and Electron Microscopy of rNV. Eight milligrams of total protein prepared by sucrose gradient as described above for t-rNV or as described for i-rNV (4) was loaded on a Pharmacia Mono-Q HR 5/5 column in 5 ml of buffer A (20 mM Tris·HCl, pH 7.5). The column was eluted with a continuous gradient of 0–100%

buffer B (500 mM NaCl in buffer A) at a flow rate of 1 ml/min. Fractions were collected and tested for NVCP by ELISA. Peak fractions were pelleted by centrifugation at 100,000 × *g* for 2 h, resuspended in 1% ammonium molybdate (pH 6.0), and examined by transmission electron microscopy on a Phillips CM10 microscope (Phillips Electronic Instruments, Mahwah, NJ).

SDS/PAGE and Western Blotting. Tobacco leaf extracts were fractionated on 10% or 12% polyacrylamide gels using the buffer system of Laemmli (18), and proteins were transferred to nitrocellulose in Towbin buffer (19) using a semidry blotter (Bio-Rad) at 20 V for 20 min. The membranes were blocked with 5% DM/PBS for 1 h at 23°C and probed in succession with guinea pig anti-(i-rNV) (4) diluted 1:2000 in 2% DM/PBS and goat anti-guinea pig IgG-horseradish peroxidase conjugate (Sigma) diluted 1:3000 in 2% DM/PBS. Bound antibody was detected with ECL reagent (Amersham).

Immunogenicity Testing in Mice. Tobacco leaf extracts prepared by sucrose gradient sedimentation as described above were administered to CD1 mice by gastric intubation (gavage). Various doses (10–80 μg of t-rNV) or an equivalent dose of an extract of nontransformed control tobacco leaves were given with or without CT on days 1, 2, 11, and 28. For tuber feeding, mice were fasted overnight before consuming ≈4 g of transgenic tuber (40–80 μg of rNV by ELISA) on days 1, 2, 11, and 28. Tubers were prepared for feeding by peeling and slicing, and individual mice were monitored to verify consumption of the entire dose. In some cases, 10 μg per dose of CT was added to the sliced tubers. Tail blood was obtained before immunization and on days as shown in Fig. 5 and tested for anti-NVCP immunoglobulins by ELISA as described (4, 17). Fecal samples were collected on days 6, 19, and 37 and tested for total IgA and anti-NVCP IgA by a modification (5) of a method described (20).

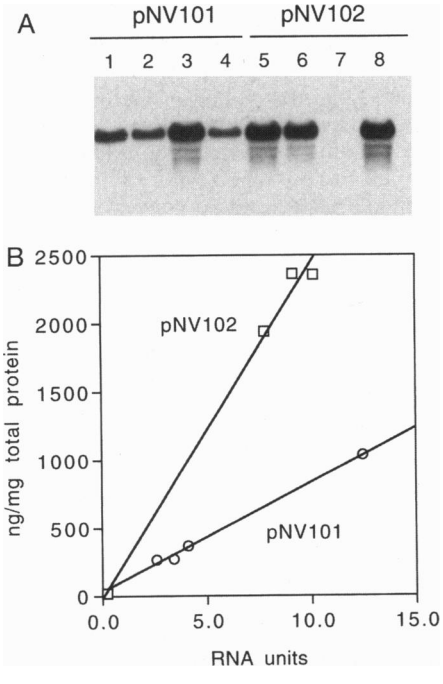

FIG. 2. Effect of TEV 5′-UTR on expression of NVCP in tobacco leaves. (*A*) Four independent transformants with pNV101 (lacking TEV 5′-UTR) and pNV102 (containing TEV 5′-UTR) were analyzed for NVCP-specific mRNA by RNA blot hybridization. Five micrograms of total leaf RNA was fractionated, blotted, and hybridized with NVCP-specific probe. Lanes 1–4, pNV101 transformants; and lanes 5–8, pNV102 transformants. (*B*) The same leaf tissue analyzed in *A* was assayed for NVCP and total protein. The NVCP (in ng/mg of total protein) was plotted versus the relative RNA abundance from blot *A*, and regression lines were calculated are shown.

RESULTS

Expression Vectors and Selection of Transformed Plants. The plasmids for expression of NVCP in plants (Fig. 1) allow selection of transformants on media containing kanamycin. The right and left borders flank the expression cassettes and delineate the transferred DNA, which is stably integrated into nuclear chromosomal DNA at random sites and mediated by *Agrobacterium* infection (21). pNV101 and pNV102 use the cauliflower mosaic virus 35S promoter to drive nominally constitutive transcription. pNV102 differs in the insertion of the TEV 5′-UTR, which stimulates translation by a cap-independent mechanism (22), a duplicated enhancer region in the 35S promoter (13), and the removal of part of the 3′-UTR derived from the NVCP clone pUCNV4145 (1). pNV140 uses the promoter from the major potato tuber storage protein, patatin (14), to drive transcription and contains the TEV translational enhancer fused to a translation initiation site modified to remove the two ATG codons immediately preceding the NVCP initiation codon. The patatin promoter is active preferentially in tubers (14).

NVCP Expression and the Effect of TEV 5′-UTR. Because the site of insertion of the transferred DNA into the cellular chromosomal DNA is random, different levels of foreign

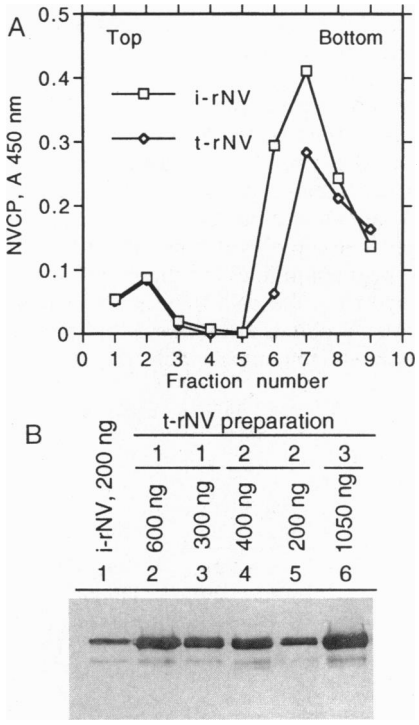

FIG. 3. (*A*) Sucrose gradient sedimentation of i-rNV and t-rNV. Approximately 3 μg of t-rNV prepared by sucrose gradient sedimentation or 4.5 μg of i-rNV expressed in recombinant baculovirus-infected insect cells (4) in a sample volume of 0.1 ml was sedimented in discontinuous sucrose gradients (0.95 ml each of 10%, 20%, 30%, 40%, and 50% sucrose in PBS) at 50,000 rpm at 4°C for 3.5 h in a Beckman SW 55Ti rotor. Fractions were tested for NVCP by ELISA (A_{450}). (*B*) SDS/PAGE and Western blot of i-rNV and sucrose gradient-purified t-rNV. The three different preparations of t-rNV were obtained by sucrose gradient, and they are the same preparations used for mouse feeding studies. Lane 1, 200 ng of i-rNV; lane 2, 600 ng of t-rNV preparation 1; lane 3, 300 ng of t-rNV preparation 1; lane 4, 400 ng of t-rNV preparation 2; lane 5, 200 ng of t-rNV preparation 2; and lane 6, 1050 ng of t-rNV preparation 3.

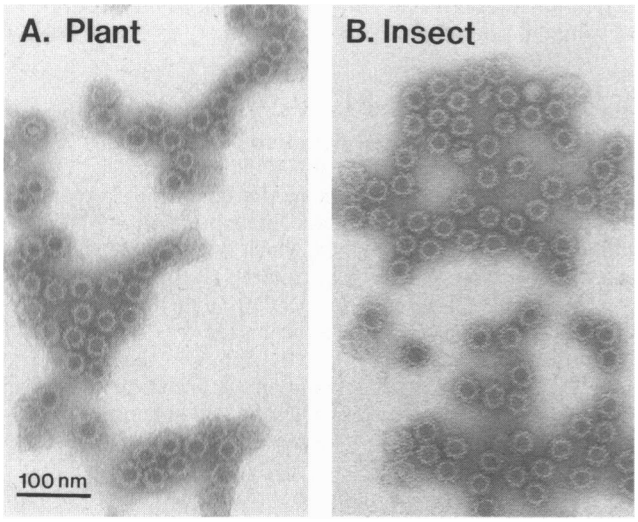

FIG. 4. i-rNV and t-rNV particles visualized by negative staining and electron microscopy. The peak fractions from ion exchange chromatography of t-rNV (*A*) or i-rNV (*B*) were pooled and pelleted at 100,000 × *g*, and processed for negative staining and electron microscopy. (Bar = 100 nm.)

transcripts in independent transformants is expected. This finding is illustrated in Fig. 2*A*, which is a blot of total leaf RNA probed with NVCP coding sequences. Varying levels of NVCP-specific RNA among the independent transformants were apparent. In those showing higher levels of NVCP mRNA, two smaller transcripts appeared, perhaps as a result of alternative 3′ end processing or splicing.

To assess the effect of the TEV 5′-UTR on translational efficiency of NVCP mRNA, we measured mRNA and antigen levels on the same leaf samples. NVCP antigen levels plotted versus RNA abundance in Fig. 2*B* showed a substantial difference between the pNV101 and pNV102 constructs. The slopes of the regression lines for these data indicate that the mRNA derived from the pNV102 cassette, which carries the TEV translational enhancer, allows 3-fold higher accumulation of NVCP antigen, presumably due to more efficient initiation of translation (22). We therefore used the TEV 5′-UTR in pNV140 (Fig. 1).

The maximum level of NVCP accumulation in the leaves of tobacco transformants was 0.23% of the total soluble protein. Similar accumulation levels were obtained with tubers of potato plants transformed with pNV140, using the patatin promoter fused to the TEV 5′-UTR. For mouse feeding studies, we used tubers that were in the size range of 4–20 g and averaged ≈10–20 μg of rNV per g of tuber weight.

NVCP from Tobacco and Potato Sediments as rNV Particles. To test whether the NVCP produced in tobacco leaves forms virus-like particles, we sedimented partially purified extracts and i-rNV on sucrose gradients and monitored the antigen in gradient fractions. Fig. 3*A* shows that NVCP in tobacco leaf extracts (t-rNV) cosedimented with i-rNV. A peak near the top of the gradient indicates that a small proportion of NVCP is present as soluble protein or smaller aggregates. Sucrose gradients on crude 10,000 × *g* supernatants from tobacco leaves and potato tubers expressing NVCP showed that ≈50–60% of the ELISA reactive material cosediments with i-rNV (data not shown). Further characterization of t-rNV by isopycnic CsCl gradient banding showed that the ELISA-positive material had a buoyant density of 1.31 g/ml (data not shown), which is consistent with that observed for i-rNV (4).

t-rNV Comigrates with i-rNV on SDS/PAGE. The t-rNV prepared by sucrose gradient constituted 20–30% of the total protein in the fraction, as judged by Coomassie blue staining of SDS/polyacrylamide gels (data not shown). The Western blot in Fig. 3*B* shows that t-rNV comigrated with i-rNV at 58 kDa. A minor band was observed in both i-rNV and t-rNV preparations at ≈55 kDa. Although not observed in this blot, other t-rNV blots sometimes showed a smaller reactive fragment at ≈34 kDa, which might represent one of several proteolytic cleavage products of the 58-kDa protein (4, 23).

NVCP from Tobacco Forms Virus-Like Particles. Further purification of i-rNV and t-rNV by anion exchange chromatography showed parallel elution of ELISA-positive material on the NaCl gradient (data not shown). We prepared i-rNV and t-rNV by pelleting the peak fractions from anion exchange chromatography and examined the resulting protein by negative staining and electron microscopy (Fig. 4). This analysis

Table 1. Immune response of CD1 mice fed t-rNV and potato-expressed rNV

Group	rNV* μg	Adjuvant†	Expression/delivery	Serum antibody‡ No. positive total	Serum antibody‡ ELISA titers, range	Fecal IgA‡ No. positive/ total
1	10	10 μg CT	tobacco/oral	4/4	100–800	1/4
2	50	10 μg CT	tobacco/oral	9/10	50–3200	5/9
3	50	None	tobacco/oral	8/9	50–800	5/8
4	80	10 μg CT	tobacco/oral	3/3	1600–25,600	ND
5	0	None	control tobacco/oral	0/5	<25	ND
6	40–80§	10 μg CT	potato/eaten	7/10	50–200	0/10
7	40–80§	None	potato/eaten	4/10	50–200	1/10
8	0	None	control potato/eaten	0/6	<25	ND

ND, not determined.

*rNV particles were expressed in tobacco leaves or in potato tubers. The concentration of the expressed rNV particles in the partially purified tobacco extracts and in tuber tissue was determined by ELISA as described. Transformed and untransformed (control) tobacco extracts were administered to CD1 mice by gavage; transformed and untransformed potato tubers were peeled, cut into small pieces, and administered by feeding.

†Ten micrograms of CT was mixed with tobacco extracts or added directly to tuber slices before administration.

‡rNV-specific serum antibody and fecal IgA titers at day 40 (see Fig. 5) were determined by ELISA as described. A reaction was considered to be positive if the titer was 4-fold greater than preimmunization titer.

§The expression levels of rNV varied between 10 and 20 μg per g of tuber tissue; mice were fed 4 g tuber per dose.

revealed empty virus-like particles virtually indistinguishable from i-rNV (4) in both size and appearance.

Oral Immunogenicity of Tobacco and Potato rNV in Mice. We used partially purified t-rNV from sucrose gradients and potato rNV in sliced tubers to test the oral immunogenicity of plant-derived rNV in mice. When CT was given with 50 μg per dose of t-rNV, 9 of 10 mice responded with measurable serum anti-NVCP; when the same dose of t-rNV was given without CT, eight of nine mice also responded with serum anti-NVCP, although the titers were lower than those given CT (Table 1). CT stimulated higher geometric mean titers during earlier phases of immunization, but at the final time point (40 days after the first immunization), the geometric mean titers for mice with and without CT were very similar (Fig. 5*A*). As little as 10 μg t-rNV per dose (with CT) stimulated serum and fecal antibodies, and much higher serum titers were seen with 80 μg t-rNV (Table 1). These data are consistent with those showing successful oral immunization in mice and humans using i-rNV (5) and indicate that plant-derived rNV is an orally active immunogen.

Intestinal rNV-specific and total IgA levels in the mice of Fig. 5*A* were assayed by ELISA. Of the mice that had a positive serum antibody response at a dose of 50 μg of t-rNV, five of nine (with CT) and five of eight (without CT) also had measurable NVCP-specific intestinal IgA. Specific IgA levels ranged from 0.33 to 4.12 ng/μg (with CT) and from 0.08 to 4.21 ng/μg (without CT).

Mice were fed potato tubers expressing NVCP at 40–80 μg per dose with or without CT at 10 μg/dose. Only about half of the NVCP in potato tubers was in particle form. When the material was fed with CT, 7 of 10 mice responded with serum anti-NVCP antibody, and 4 of 10 responded when CT was omitted (Fig. 5*B* and Table 1). The serum titers were lower than those of mice gavaged with gradient-purified t-rNV at a similar dose (Fig. 5*A*). Of the 11 serum responders, only 1 mouse had measurable intestinal IgA (Table 1).

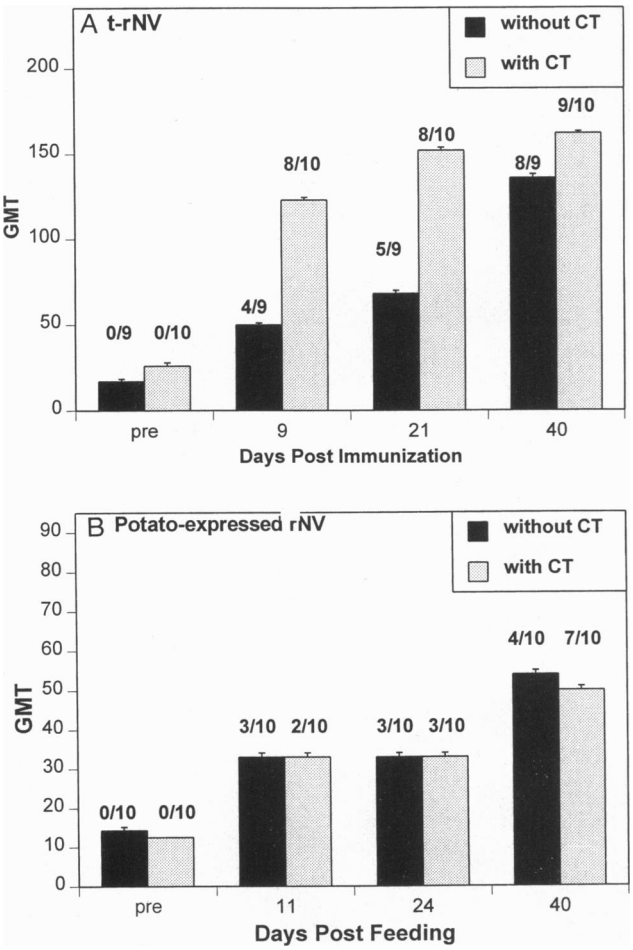

FIG. 5. Kinetics of serum antibody responses in mice immunized with rNV expressed in tobacco (*A*) or potato (*B*). (*A*) Ten CD1 mice each were gavaged with 50 μg of t-rNV with or without 10 μg of CT on days 1, 2, 11, and 28. (*B*) Ten CD1 mice each were fed 4 g of transgenic potato tuber expressing rNV (10–20 μg/g tuber) with or without 10 μg of CT on days 1, 2, 11, and 28. Tail blood was sampled on days shown, and serum was assayed by ELISA for anti-NVCP. The ELISA geometric mean titers are shown on the ordinate, and the time (in days) is shown on the abscissa. Fractions above data bars indicate number of responder mice per total number of mice tested.

DISCUSSION

Our previous work with HBsAg (8, 10) and *E. coli* labile enterotoxin B-subunit (9) showed that plants can produce recombinant viral and bacterial antigens that retain immunogenic epitopes and assemble into the proper quaternary configurations required for maximal functionality. The present work shows similar data for a different viral antigen, NVCP. Expression of rNV in tobacco leaves resulted in the production of virus-like particles that were similar to i-rNV, supporting the idea that plant cells can manufacture functional capsids of animal viruses.

We obtained rNV expression levels of up to 0.23% of total soluble protein in tobacco leaves, and up to 0.37% in potato tubers (34 μg per g of tuber weight). These data represent a substantial increase over that obtained for HBsAg in tobacco leaves, which gave a maximum of 0.01% total soluble protein (8). The reasons for the great difference are unclear but may be related to the subcellular localization of each peptide. HBsAg is a glycoprotein targeted to the endoplasmic reticulum in liver cells by an internal, uncleaved signal sequence (24), whereas Norwalk virions likely accumulate in the cytoplasm of infected cells. Thus it is possible that rNV accumulates more efficiently in plant cells because it is cytoplasmic and requires no subcellular targeting. Studies on plant expression of HBsAg and NVCP with plant-derived N-terminal signal sequence fusions may provide information on the importance of efficient ER-targeting for accumulation of foreign proteins.

Plant-derived rNV delivered orally to mice stimulated the production of humoral and mucosal antibody responses (Fig. 5 and Table 1). This confirms the similar observations on oral delivery of i-rNV (5), but further shows that consumption of recombinant plant material (potato tuber) by normal feeding allows immunogenic responses. The immune response to rNV delivered as food in transgenic potato tubers was lower than that obtained by gavage with a similar dose (measured by ELISA) of partially purified t-rNV, perhaps because the tuber material was consumed more slowly and was less pure. Further, while the t-rNV purified by sucrose gradient sedimentation contained mostly virus-like particles, only about half of the ELISA-reactive material extracted from tubers was in the particle form, as judged by analytical sucrose gradients. The particle form should be more stable in the stomach (4) and more readily taken up by M cells in the gut for presentation to lymphocytes in the Peyer's Patches (25); therefore, the functionally active (particulate) rNV in potato tubers may be only half the concentration that we measure by ELISA, or about 20–40 μg per dose.

CONCLUSIONS

Taken together, the results presented here add further evidence to support the concept (8, 9) of edible vaccines: oral consumption of recombinant plant tissues expressing NVCP evokes specific immunoglobulin production. Although immune responses to dietary antigens, in the form of either active

antibody production or oral tolerance, have been known for some time (25), the intentional stimulation of immunity via ingestion of recombinant plant material has only recently been shown (9). While it may not be surprising to see stimulation of immunity to fed antigens, it was by no means a foregone conclusion and required these seminal experiments to show a proof of the concept. We have not shown neutralizing activity in this report because no assay is yet available to assess Norwalk virus neutralization. However, we anticipate conducting human feeding trials for the direct assessment of protection afforded by ingestion of recombinant potato tubers against Norwalk virus infection.

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