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Plant-derived vaccine protects target animals against a viral disease

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The successful expression of animal or human virus epitopes on the surface of plant viruses has recently been demonstrated. These chimeric virus particles (CVPs) could represent a cost-effective and safe alternative to conventional animal cell-based vaccines. We report the insertion of oligonucleotides coding for a short linear epitope from the VP2 capsid protein of mink enteritis virus (MEV) into an infectious cDNA clone of cowpea mosaic virus and the successful expression of the epitope on the surface of CVPs when propagated in the black-eyed bean, *Vigna unguiculata*. The efficacy of the CVPs was established by the demonstration that one subcutaneous injection of 1 mg of the CVPs in mink conferred protection against clinical disease and virtually abolished shedding of virus after challenge with virulent MEV, demonstrating the potential utility of plant CVPs as the basis for vaccine development. The epitope used occurs in three different virus species—MEV, canine parvovirus, and feline panleukopenia virus—and thus the same vaccine could be used in three economically important viral hosts—mink, dogs, and cats, respectively.

Keywords: cowpea mosaic virus, parvovirus, plant vaccine, protection, peptide epitope, chimeric virus particles

The fine epitope mapping of viruses has led to the identification of short amino acid sequences that are potentially highly immunogenic[1]. Interest in using such sequences as the basis for vaccine development has intensified, and several approaches directed at the mass production of useful epitopes are being investigated. These include chemical synthesis and coupling to carriers, expression in both prokaryotic and eukaryotic cells as well as subcellular organelles, and insertion into live virus vectors. The successful expression of animal or human virus epitopes on the surface of plant viruses has been achieved[2–5].

Cowpea mosaic virus (CPMV) is a positive strand RNA virus with a bipartite genome and is a member of the comovirus group of plant viruses. The virus particles comprise 60 copies each of two protein subunits, the L (large) and S (small) proteins, which are arranged with an icosahedral symmetry. The S protein is subject to C-terminal proteolytic truncation. The structure of CPMV has been solved to 3 Å resolution. The L coat protein consists of two β-barrel domains and the S protein of one β-barrel domain[6,7]. Peptide sequences can be incorporated into the 8-amino acid βB-βC loop in the S coat protein[2], which results in the formation of viable chimeric virus particles (CVPs) carrying 60 copies of the peptide per particle. The exact site of insertion has been further refined allowing the production *in planta* and isolation of large quantities of CVPs containing epitopes from either human rhinovirus 14 (HRV) or human immunodeficiency virus type 1 strain IIIB (HIV)[3]. Purified CVPs containing an HIV epitope have been shown to induce neutralizing antibodies to HIV in mice[8,9].

The autonomous parvoviruses are nonenveloped icosahedral viruses, approximately 25 nm in size. Within the parvovirus group, feline panleukopenia virus (FPLV), canine parvovirus (CPV), and mink enteritis virus (MEV) represent a set of host range variants that infect either cats, dogs, mink, or raccoons and show over 98% homology at the amino acid level[10]. The viruses possess three structural proteins VP1, VP2 (major), and VP3. VP1 and VP2 are different splice products from the same gene[11], and VP3 results from proteolytic removal of the amino terminus from VP2[12]. Only the major protein, VP2, is required for particle formation; it has been shown that VP2 is capable of self-assembling into virus-like particles when expressed in the baculovirus/insect cell system[13,14].

MEV, CPV, and FPLV are viruses of great economic importance in their natural hosts, mink, dogs, and cats, respectively. The major clinical manifestation of MEV is a severe inflammation of the intestine, resulting in profuse diarrhea with gray, white, or creamy feces, often flecked with blood, mucus, fibrin, and desquamated gut epithelium. Anorexia is observed from early on in the disease[15]. CPV infection leads to a similar clinical disease in dogs, while FPLV induces a severe febrile illness in cats. The diseases can be controlled by vaccination. Existing vaccines are either chemically inactivated or live attenuated viruses propagated from cell cultures.

We have identified a linear, neutralizing epitope in the amino-terminal part of the CPV VP2 capsid protein[16,17]. Peptides covering

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the VP2 epitope, when coupled to KLH, are capable of inducing neutralizing antibodies irrespective of the orientation (N- or C-terminal) of the coupled epitope[17]. CPV, MEV, and FPLV all have identical sequences in this region, and peptide vaccines based on the VP2 N-terminal sequences have been shown to induce neutralizing antibodies in guinea pigs and rabbits as well as solid protection against disease in the natural hosts (dogs and mink) after a heavy challenge[18,19].

MEV is relatively difficult to grow in animal cell culture, whereas CPMV and CPMV-derived CVPs can be grown to significant amounts in the plant host, the black-eyed bean *Vigna unguiculata*. CVP yields of up to 1 to 2 g/kg of fresh weight of infected plant tissue can be achieved and purified in a relatively simple and cost-effective way[20].

We show that a plant-derived CVP vaccine containing the VP2 epitope protects against clinical disease and virus shedding in vivo.

Results and discussion

Construction and propagation of CVPs. Full-length cDNA clones pCP1 and pCP2, containing full-length copies of CPMV RNAs 1 and 2, respectively, were previously shown to be directly infectious for cowpea plants[21]. The nucleotide sequence of the VP2 epitope was cloned between nucleotides 2725–2726 in the βB-βC loop of the S protein to produce a plasmid, pCP2-Parvo1. Inoculation of this clone (in conjunction with unmodified pCP1) onto *V. unguiculata* seedlings successfully led to the establishment of infection in 5/5 plants. Viral lesions with this chimera (CVP-Parvo1) were observed on the primary leaves within 7 days postinoculation, and a systemic infection could be observed shortly after the secondary leaf buds opened, approximately 5 days postinoculation. The primary lesions had the same appearance as those found in a wild-type CPMV infection, and the CVP spread systemically throughout the plant producing a severe chlorotic mosaic, also characteristic of a wild-type infection. The yields of virus were 50 to 60 mg from five plants (approximately 50 g leaf material). The recombinant virus produced, CVP-Parvo1, was genetically stable over at least two passages, as judged by sequencing of reverse transcripton polymerase chain reaction (RT-PCR) products from the purified particles.

Characterization of CVPs. The formation of icosahedral CPMV-like structures was confirmed by electron microscopy of purified CVPs, which could be seen in abundant numbers by negative staining (Fig. 1).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) shows the purity of CVP-Parvo1 and its subunit profile compared with CPMV (Fig. 2A). The L protein of wild-type CPMV is present as a single band of 38 kDa. The S coat protein shows two bands at 25 and 22 kDa, the two forms of the S protein generated by proteolytic removal of the C-terminal 24 amino acids[22]. CVP-Parvo1 shows the same L protein band (38 kDa), and several bands (26, 20, and 18 kDa) for the S protein. The 26-kDa S protein contains the 17-amino acid VP2 epitope and reacts with both an antiserum to the VP2 peptide as well as a monoclonal antibody to CPV, 3C9, which has been mapped to this site[16,23] (Figs. 2B and C). The S protein pattern of CVP-Parvo1 (Fig. 2A) is more complex due to the occurrence of an additional cleavage reaction. This cleavage occurs between the penultimate and ultimate amino acids of the VP2 epitope, which, under denaturing conditions, removes all but one amino acid of the epitope and the N-terminal 22 amino acids of the S protein. The small 38-amino acid fragment has been run off the gel. This cleavage event has been observed in most constructs made to date. N-terminal sequencing of the cleavage products from several of these indicates that this event always occurs in the same location[8]. The cleaved fragment remains associated with the virus particle, since the N-terminal 22 amino acids of the S protein are an integral part of the βB sheet. Direct evidence for this exists for another chimera containing a 14-amino acid insert from HRV, for which the crystal structure has recently been solved. In this case all the epitope loops have undergone cleavage[24].

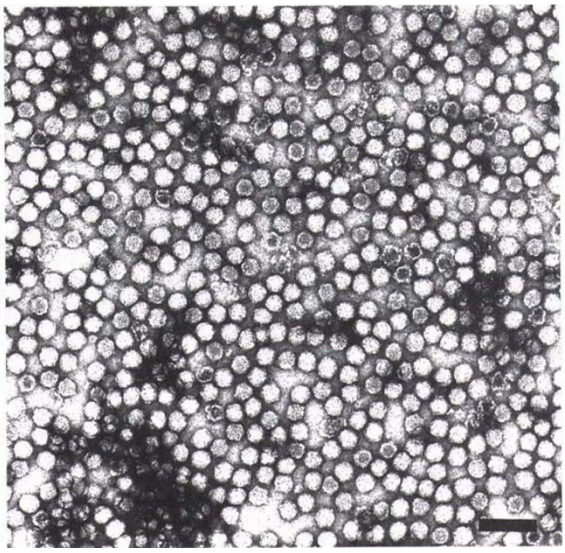

Figure 1. Electron micrograph of CVP-Parvo1 particles after negative staining. The bar represents 100 nm.

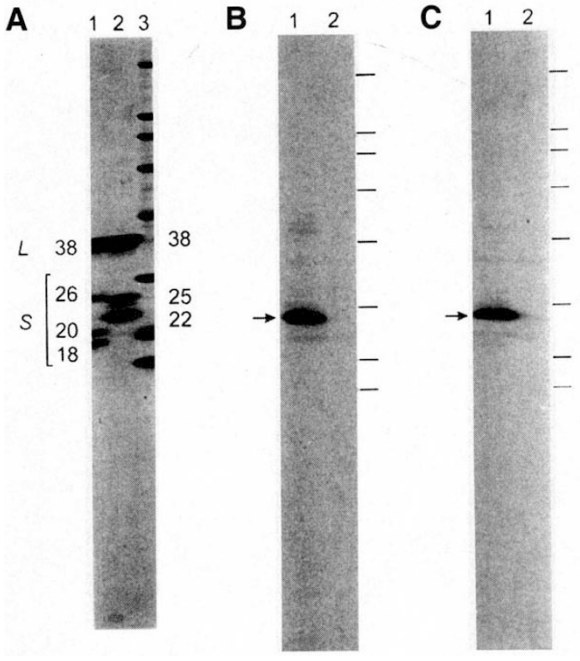

Figure 2. Subunit profile and immunochemical reactivity of CVP-Parvo1 after SDS-PAGE and Western blotting. (A) Gel stained for total protein (10 μg material applied per lane) with Coomassie brilliant blue; (B) and (C) Western blot (1 μg material per lane) immunostained with rabbit antiparvopeptide antiserum and monoclonal anti-CPV antibody 3C9, respectively. Lanes 1 and 2, CVP-Parvo1 and CPMV, respectively; lane 3, molecular weight markers. The molecular weight of the polypeptides of CVP-Parvo1 and CPMV are given in kDa. Position of the molecular weight markers in (B) and (C) is indicated with bars; L and S indicate migration positions of L and S proteins. Arrowhead indicates S protein band containing the epitope.

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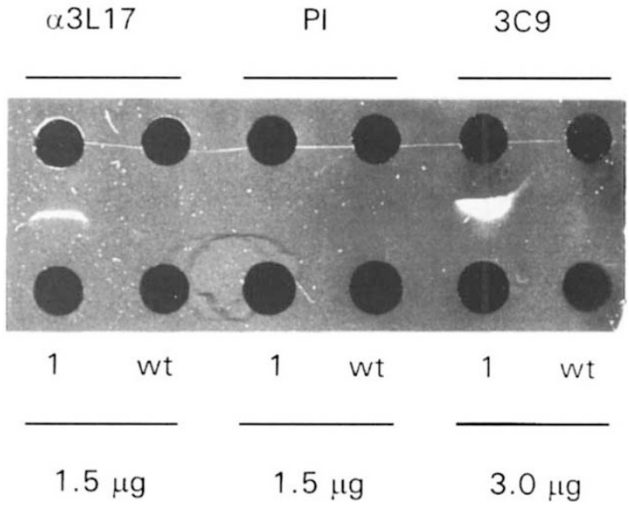

Figure 3. Counter immune electrophoresis of CVP-Parvo1 and CPMV. Top wells: α3L17, rabbit antiparvopeptide antiserum; PI, rabbit preimmune serum; 3C9, mouse anti-CPV monoclonal antibody. Each well contained 15 μl of serum diluted 1:10. Bottom wells: 1, CVP-Parvo1; wt, wild-type CPMV. Each well contained 15 μl of 1.5- to 3-μg particles as indicated.

Verification of the presence of a functional epitope on the surface of the CVP-Parvo1 was obtained by counter immune electrophoresis in an agarose gel. A specific precipitin line was formed both with the rabbit antiparvo peptide antiserum and with the monoclonal antibody 3C9 (Fig. 3).

Immunization of mink and protection against MEV challenge. Two different experiments were performed, with different amounts of antigen in the vaccine. Either 100 μg (low-dose experiment) or 1 mg (high-dose experiment) of purified CVPs were formulated for administration to mink by mixing with the saponin adjuvant Quil A (ref. 25) and adsorbing it to aluminum hydroxide gel. The potential vaccines were applied as a single subcutaneous dose to 6- to 8-month-old mink. The reason for using only a single dose was that a commercially available vaccine is protective after a single dose using the challenge procedure applied. This vaccine consists of inactivated, cell culture-derived virus, adjuvanted with aluminum hydoxide gel.

The CVPs protect against MEV in a dose-dependent manner (Table 1). Although 100 μg CVP protected against clinical disease in five out of six animals, viral shedding was detected for several days. The 1-mg dose, however, not only protected completely against clinical disease but also to a large extent against viral shedding. In the high-dose experiment, viral shedding was assayed only on days 4, 5, 6, and 7 because it has been shown that these days are critical when using this challenge procedure (low-dose experiment[26]).

The nonimmunized mink suffered from typical MEV diarrhea during the observation period, whereas both conventionally and CVP-vaccinated mink showed no signs of diarrhea (except for one animal in the low-dose group), maintained a good appetite, and appeared clinically healthy. Some of the control animals of the low-dose experiment were severely ill, and were euthanized before the end of the experiment on postchallenge days 4 (one animal) and 7 (two animals).

We conclude that both CVP-derived vaccines meet the potency requirements for inactivated MEV vaccines, as defined by the US FDA[27] where prevention of diarrhea is the primary objective. The regulations do not require prevention of viral shedding in feces, which is more difficult to obtain. However, from an epidemiological point of view, prevention of viral shedding is also important, as transmission of MEV occurs primarily via the fecal-oral route.

As the protective VP2 epitope is only 17 amino acids in length, it can be calculated that the 60 copies of the sequence represent only 2.1% of the CVP on a weight basis (this takes into account the contribution of the RNA and assumes a ratio of 1:2:2 for the T, M, and B particle components[20]). Therefore the mink that received 100 μg of CVPs received an equivalent of approximately 2 μg of peptide epitope, and the group receiving 1 mg of CVPs received approximately 20 μg of peptide epitope. Twenty micrograms of peptide is about 3.5 to 7 times lower than the amount used for the synthetic peptide vaccination studies in which a mixture of two overlapping peptides were used[19].

Antibody responses to the VP2 epitope before challenge were not seen in any of the mink except for two animals in the high-dose experiment (Fig. 4B). That a response occurred towards the immunogen was obvious from measurement of anti-CPMV antibodies in the group immunized with the chimeric particle. In the low-dose experiment, four out of six, and in the high-dose experiment, five out of six animals developed detectable anti-CPMV titers. After challenge some additional animals in the CVP-immunized groups developed a response to the peptide and CPMV. This suggests that before challenge the animals had been primed. A similar situation has been described for another autonomous parvovirus, porcine parvovirus. Although significant antibody titers are usually obtained after vaccination with inactivated PPV vaccines, Mengeling et al. reported a case where vaccination of pigs did not induce a measurable antibody response, yet the animals were afforded protection[28]. None of the nonimmunized animals or the mink immunized with commercial MEV vaccine developed antipeptide antibodies in response to challenge. The protective effect of the vaccine most probably is based on low-titer antibody responses as was also observed previously when immunizing with

Table 1. MEV challenge study in mink.

Vaccine	Clinical disease*		Number of mink shedding virus (day after challenge)										
	Diarrhea	Anorexia	2	3	4	5	6	7	8	9	10	11	In total†
Low-dose experiment													
nonvaccinated	4/6	2/6	0	2	4‡	5	4	3‡	1	1	0	0	6/6
CVP (100 μg)	1/6	0/6	0	1	2	4	4	4	1	0	0	0	4/6
Biovac vet. vaccine	0/6	0/6	0	0	0	0	0	0	0	0	0	0	0/6
High-dose experiment													
nonvaccinated	3/4	3/4			2	3	3	3					3/4
CVP (1 mg)	0/6	0/6			0	2	1	0					3/6
Biovac vet. vaccine	0/2	0/2			0	0	0	0					0/6

*Figures indicate numbers of affected animals / number of animals in group. †Number of mink shedding virus at least 1 day during observation period / number of animals per group. ‡One animal was euthanized on day 4 and two more were euthanized on day 7.

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synthetic peptide[19]. Thus, the enzyme-linked immunosorbent assay (ELISA) system used is probably not always sensitive enough to detect these antibody changes.

Due to the relative ease of CVP production, it is feasible to consider commercial development of such a plant-derived vaccine. Further optimization of the chimeras may be possible in the future by additional engineering of the insertion site and/or insertion of dimers of the epitope sequence. It is possible that by using two or more immunizations less CVP would be needed to generate an adequate immune response. However, because mink are difficult to handle, the use of a single-dose vaccine would be preferred.

Oral vaccination using CVP-Parvo1 could be possible, because oral vaccination using antigens produced in transgenic plants has been shown to induce antibodies in mice[29]. The development of oral vaccines is facilitated by the fact that CVP production is cheap and easy, therefore allowing for high dosages. Once developed, oral vaccines could also make possible the vaccination of wild animals susceptible to infection with these viruses, e.g., wild mink.

To our knowledge, this is the first experimental vaccine produced exclusively by plants that confers protection against an infectious disease in the target animal. Thus, these findings show the way for further vaccine development based on this technology. The epitope used in this study is of particular interest as the sequence is identical in MEV, CPV, and FPLV, and for this reason the same vaccine could be protective against all three viruses. Therefore, the next step will be to test the CVP-Parvo1 vaccine in dogs and cats for protection against CPV and FPLV, respectively.

Experimental protocol

Construct assembly. Infectious clones pCP1 and pCP2 containing the two CPMV cDNAs driven by a cauliflower mosaic virus 35S promoter have been described[21]. Plasmid pCP2 was further modified for use as a cloning vector for foreign sequences. The AatII site was removed from the pUC portion of the plasmid by linearization with this enzyme followed by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase I, to eliminate the 3′ overhangs, and religation to produce pCP2-AatII. pCP2-AatII was digested with BamHI and EcoRI to release a 2-kb fragment comprising the sequence of the S protein. This fragment was substituted by that released from similarly treated plasmid pMT7-HRV-II (ref. 3); the latter fragment contains an insertion from HRV-14 within the sequence of the S protein, introduced by cassette mutagenesis between the restriction sites NheI and AatII. The resulting recombinant clone was called pCP2-0.51. After digestion with NheI and AatII, pCP2-0.51 was separated from the HRV-14 sequence by agarose gel electrophoresis and ligated to two pairs of overlapping oligonucleotides coding for the VP2 epitope (sequence: DGAVQPDGGQPAVRNER, corresponding to residues 3 to 19 from VP2 of canine parvovirus) and the appropriate CPMV flanking sequences to place the insert between residues 22 and 23 of the S protein. The ligation mixture was transformed[30] into DH5α (Gibco-BRL, Gaithersburg, MD) and the resultant clones verified by plasmid sequencing using the Sequenase system (Amersham, Buckinghamshire, UK) with plus- and minus-strand primers corresponding to nucleotides 2654-2673 (5′-GCACAAGGACCTGTTTGTGC-3′), and 2806-2786 (5′-CGTATTCCAATTGTCATCACC-3′) respectively. A clone containing the correct insert sequence was designated pCP2-Parvo1.

Plant infection and virus propagation. Infection of *V. unguiculata* leaves was carried out as described[21] using 10 μg each per plant of pCP1 and pCP2-Parvo1 linearized with MluI and EcoRI respectively. The infected plants were grown at 25°C in a growth chamber for up to 3 weeks postinoculation before harvesting, and virus was purified as described[20], except that sedimentation for 4 h at 112,000 G was used in place of sucrose density gradient fractionation. The final pellet was resuspended in 10 mM sodium phosphate buffer pH 7.0, filter sterilized through a 0.2-μm membrane and the particle concentration measured spectrophotometrically using an absorbance of 8.0 (1 mg/ml, 1 cm light path) at 260 nm. Particles isolated from the DNA inoculation were designated as seed stocks and a second round of infection using 50 μl per leaf of a 10 μg/ml solution of these particles was carried out to produce working stocks. The working stocks were characterized as above via sequencing and SDS-PAGE. The characterized

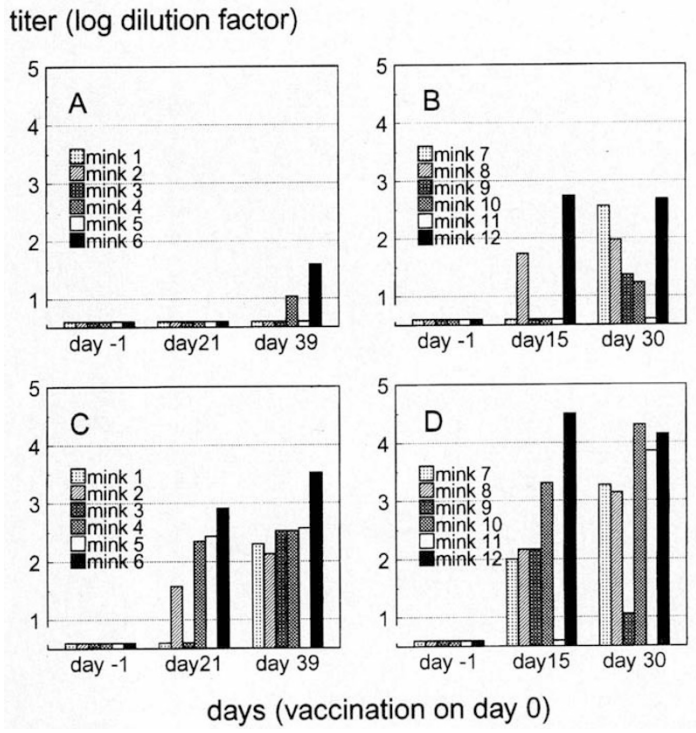

Figure 4. Antibody titers in individual mink after vaccination with low- and high-dose of CVP-Parvo1. (A) and (B), Anti-peptide antibody titers; (C) and (D) Anti-CPMV antibody titers. In (A) and (C), the results from the same six animals in the low-dose experiment are displayed; in (B) and (D) idem for six animals in the high-dose experiment. Vaccination was performed at day 0. Challenge was performed at day 28 and day 23 in the low- and high-dose experiments respectively. All control animals receiving no vaccine or the commercial inactivated vaccine remained negative in these enzyme-linked immunosorbent assays. The lowest serum dilution tested in each animal was 1/10. Titers are presented as log of dilution factor.

chimeric virus particles were designated CVP-Parvo1.

Sequence verification. The viral RNA was characterized by RT-PCR and sequencing of the PCR products. 1-μg particles were heated to 99°C for 5 min in 20 μl 1x M-MLV RT reaction buffer (Gibco-BRL) containing 50 pmol of the minus-strand primer, followed by chilling on ice. 200u M-MLV RT (Gibco-BRL) was added and the reaction incubated at 37°C for 1 h. Two microliters of this reaction were added to a standard PCR reaction[31] containing 50 pmol each of the minus- and plus-strand primers. The reaction was cycled 25 times at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The reaction products were separated on a 4% agarose gel, and the single band at 204 bp was excised from the gel and sequenced using the Sequenase system.

SDS-PAGE, Western blotting, and immunostaining. CVP-Parvo1 and wild-type CPMV were analyzed by electrophoresis under denaturing conditions through 1.5-mm linear gradient (6% to 25% acrylamide) SDS-polyacrylamide slabgels[32]. Samples were heated for 10 min at 95°C in sample buffer in the presence of 2% β-mercaptoethanol. Molecular weight markers used were lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa) (Sigma, St. Louis, MO). Staining for total protein in gels was carried out with Coomassie brilliant blue. Western blotting, followed by total protein staining and destaining with Ponceau S, and immunostaining for the presence of the VP2 epitope was performed according to previously described methods[33]. Rabbit antiparvopeptide (3L17) serum[17] and monoclonal anti-CPV antibody 3C9, which has previously been mapped to the same peptidic antigenic site[16,23] were used for the immunostaining. Peroxidase conjugated antirabbit and antimouse Ig were from DAKO (Denmark).

Counter immune electrophoresis. Electrophoresis was carried out in 0.025 M citrate buffer pH 6.0, high endosmosis agarose (LSA, Litex, Glostrup, Denmark) at 1.5 V/cm for 4 h. The counter immune electrophore-

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sis had to be adjusted to the specific characteristics of the CPMVs, i.e., the low isoelectric point. The CPMVs are too negatively charged at pH 8.6 and therefore citrate buffer was used to lower the pH to 6.0. These conditions allowed the successful precipitation reaction by both the polyclonal and monoclonal antibody. Glass plates 10×10 cm with a layer thickness of 1.5 mm were used. Holes of 4 mm in diameter, accommodating 15-µl samples, were punched in the gel. Precipitin lines were observed and photographed in indirect light[24].

ELISA. MEV was detected in fecal samples by subjecting suspensions (approximately 1:10) in buffer to an antigen ELISA based on capturing the virus to a specific rabbit antibody, and detecting with the same antibody biotinylated and reacted with peroxidase conjugated streptavidin[26]. The level of viral excretion was expressed as the OD-ratio of a positive sample to known virus-negative control samples. Anti-peptide and anti-CPMV antibodies were measured by ELISA using the synthetic peptide or CVP-Parvo1 as coating antigen, respectively. Biotinylated rabbit antibodies to protein-G purified mink Ig (kindly provided by Bent Aasted, Royal Veterinary and Agricultural University, Copenhagen, Denmark) and peroxidase-labeled streptavidin (DAKO) were used to detect bound mink antibodies. After overnight coating, all subsequent incubations (mink sera, biotinylated rabbit anti-mink IgG and peroxidase-labeled streptavidin) were performed for 1 h at 25°C in 0.15 M NaCl, 0.05% (w/v) Tween20, 2% (w/v) bovine serum albumin. Color was developed with hydrogen peroxide/tetramethylbenzidine and stopped with sulphuric acid. Absorbance was read at 450 nm. Titers were calculated, after subtraction of the values found for each individual preimmune sample, as the serum dilution where absorbance amounted to three times the background value (buffer added instead of serum).

Electron microscopy. Purified CVPs were adjusted to 175 µg/ml in distilled water, adsorbed to carbon-coated grids, and stained in 2% uranyl acetate for 2 min. Particles were visualized in a Zeiss (Herts, UK) EM 10.

Vaccine formulation. Either 100 µg or 1 mg of purified CVP-Parvo1 was diluted in phosphate-buffered saline to a volume of 0.5 ml/dose and mixed with 50 µg/dose of the Quil A adjuvant (Superfos, Denmark). This mixture was added to an equal volume of aluminum hydroxide gel (Superfos) to make a dose volume of 1 ml.

Vaccination and challenge. All mink used in the experiments were healthy animals free from Aleutian mink disease—or MEV. Two immunization tests were carried out in which six mink per test were immunized with a single dose of 1 µl containing either 100 µg (low-dose experiment) or 1 mg CVP/dose (high-dose experiment). For comparison, six (low-dose experiment) or two (high-dose experiment) mink were vaccinated using inactivated MEV vaccine (Biovac vet., United Vaccines, Madison, WI) according to the manufacturer's instructions. As nonvaccinated controls, groups of six (low-dose experiment) or four (high-dose experiment) mink were housed together with the vaccinates. Twenty-eight (low-dose experiment) or 23 (high-dose experiment) days after immunization, all mink were challenged by the oronasal route using 0.8 ml of 25% intestinal homogenate from mink suffering from acute MEV infection[14,19]. All mink were euthanized on day 11 (low-dose experiment) or day 7 (high-dose experiment) after challenge for ethical reasons. MEV antigen was assayed in fecal samples during the critical days indicated using an ELISA method[26]. In addition, the amount of virus particles excreted by the nonimmunized controls was sufficient to be confirmed by electron microscopy as previously described[19]. Blood samples were taken before immunization, and also in the low-dose experiment on days 21 and 39, and in the high-dose experiment on days 15 and 30.

Acknowledgments

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