The plant as a factory for the production of oral vaccines

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ABSTRACT

In the last years we have been working in the production of plant-derived oral vaccines based on chimeric plant virus particles (CVPs). The CVP technology involves the modification of cowpea mosaic virus (CPMV), which can be easily manipulated to display epitopes from viral pathogens on its surface. These CVPs can be easily obtained in large quantities from infected plants of *Vigna unguiculata* at a very low cost.

Previously, we have identified a linear, 17 amino acid epitope of the VP2 of canine parvovirus, capable of inducing protective immune responses against canine parvovirus and its close relative mink enteritis virus. Chimeric CPMV containing the epitope were propagated in the black-eyed bean and purified to homogeneity. Parenteral immunization of mink with 1 mg of CVPs and dogs with 7.5mg conferred total protection against clinical disease and virus shedding after challenge with virulent MEV and CPV, respectively, demonstrating the efficacy of these plant-derived particles as vaccines.

To test the ability of these chimeric CVPs for oral and nasal immunisation, mice, guinea pigs and rabbits were immunised with two doses of 100µg of antigen, three weeks apart. The responses were stronger after nasal than after oral immunisation, but still low compared to parenteral immunisation. To improve the oral administration, we have used, in two strains of mice, a combination of more immunizations together with a variety of substances for a better formulation in order to improve the antibody response. Some of these combinations have been extremely effective in the induction of peptide-specific antibody responses after oral immunisation, including neutralising antibodies against MEV and CPV. The ability of CPMV carrying the CPV epitope to provoke strong immune responses in mice after oral immunisation holds good promise for the use of this plant-derived virus as oral vaccine.

INTRODUCTION

Vaccination could become as simple as eating a salad or a banana. Edible vaccines constitute one of the first priorities in vaccinology. One of the most logical ways to reach this goal is to produce the vaccines in edible plants or crops. Plant production offers important advantages over conventional methodology. Production of vaccines in plants could be much cheaper that classical fermentation technology. Scaling up would be reduced to add more acres for production. Safety issues are also a plus in plant-derived vaccines. There is no possible contamination with other microorganisms or pathogens. Poor countries on the tropics no longer would need cold-chains for transportation and delivery. No sophisticated equipments would be necessary for production. Vaccinal crops could be grown on the spot and eaten when required.

There exist two alternative approaches for the production of vaccines in plants: i) production of genetically modified plants, which would contain and express the genes encoding for the vaccinal antigen and ii) to put the immunogenic genes into viruses that infect the plant. The best-studied model to this respect is the cowpea mosaic virus that we will comment in this article.

Production of vaccines using plant viruses offers more advantages such as: i) Large amounts of virus can be grown very quickly (<2 weeks) in plants, ii) the virus particles are very stable in any environment and iii) the capsid structure allows for a perfect exposure of the vaccine sequence. All these advantages were used in our project using as disease model the infection for parvoviruses of mink and dogs.

To circumvent the safety concerns of replicating vaccines and to avoid the requirement for peptide synthesis and chemical coupling to protein carriers such as keyhole limpet hemocyanin (KLH), we have been investigating the utility of the plant virus-based technology, which exploits cowpea mosaic virus (CPMV) as a carrier of foreign peptides(Porta *et al*., 1996).

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 (Kammen, 1967). The virus particles comprise 60 copies each of two protein subunits, the L (large) and S (small) proteins, which are arranged with icosahedral symmetry around 28 nm in diameter. The structure of CPMV has been solved to 3 A resolution. The L coat protein consists of two β-barrel domains and the S protein of one β-barrel domain. Peptide sequences can be incorporated into the 8-amino

acid βB-βC loop in the S coat protein (Usha *et al*., 1993), which results in the formation of viable chimeric virus particles (CVPs) carrying 60 copies of the peptide per particle. Pepetides of up to 38 residues can be incorporated into CVPs. A DNA copy of viral RNA, engineered to encode the foreign peptide, is inoculated onto plants. The DNAs are uptaken by the plant and used to initiate generation and replication of the CVP throughout the growing plant. 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). The resultant chimeric virus particles (CVPs) can be propagated to high levels in plants, are easily purified in a cost-effective manner and have been shown to elicit strong humoral immunity (Brennan *et al*., 1999a; Brennan *et al*., 1999b; Dalsgaard *et al*., 1997).

We previously identified a linear peptide epitope (3Ll7) in CPV derived from amino acids 3 to 19 (DGAVQPDGGQPAVRNER) of VP2 (Langeveld *et al*., 1993; Lopez de Turiso *et al*., 1991) capable of inducing protective immune responses against canine parvovirus and its close relative mink enteritis virus. Both diseases are widespread and cause devastating enteric disease in dogs and mink. When coupled to KLH, 3Ll7 elicited peptide-specific neutralizing antibodies and protected dogs (Langeveld *et al*., 1994) and mink (Langeveld *et al*., 1995) from a lethal CPV challenge. In the present study, we summarised the abilities of inactivated CPMV-PARVO1 and 3Ll7-KLH to protect mink and dogs from MEV and CPV challenge, respectively (Dalsgaard *et al*., 1997; Langeveld, 1999).

The purpose of the present study was to investigate further the immunogenicity of this plant virus-derived antigen after oral immunisation of mice. Compared to previous experiments, an enhanced immunisation regime was used, and different substances and formulation were tested in an attempt to further improve the response.

MATERIALS AND METHODS

Animals.

All mink used in the experiments were healthy animals free from Aleutian mink disease-or MEV. Specific-pathogen-free (SPF) beagle dogs, aged 9-12 weeks, were obtained from Harlam (The Netherlands). These animals were specified free from a series of pathogens including canine parvovirus. For the oral immunization of mice, Balb/c and NIH mice housed at SVIV animal facilities were used.

Construct assembly.

A single construction called CPMV-Parvo1 was used throughout all the experiments. CPMV-PARVO1 contains a 17-amino acid peptide (DGAVQPDGGQPAVRNER) corresponding to residues 3 to19 from VP2 of canine parvovirus, inserted between residues 22 and 23 of the S protein of CPMV. 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 and 1 µg chimera contains approximately 20 ng of peptide. Therefore the mink that received 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. Infectious clones pCPl and pCP2 containing the two CPMV cDNAs driven by a cauliflower mosaic virus 35S promoter have been described (Dessens, 1993). The detailed construction was previously described (Dalsgaard *et al.*, 1997). A clone containing the correct insert sequence was designated pCP2-Parvo l.

Plant infection and virus propagation.

Infection of *V. unguículata* leaves was carried out as described (Dessens, 1993) (Dalsgaard *et al.*, 1997) using 10 µg each per plant of PCP1 and pCP2-Parvol linearized with MluI and EcoRI respectively. The infected plants were grown at 25 ºC in a growth chamber for up to 3 weeks post-inoculation before harvesting, and virus was purified as described, 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 characterised as above via sequencing and SDS-PAGE. The characterised chimera was called CPMV-Parvo1.

Production of UV-Inactivated CPMV-PARVO1.

The virus particles were inactivated by irradiation with UV_{254} light during the purification using a stirring platform in a Stratalinker 2400 (Langeveld, 1999). Samples for assay were removed from each aliquot at doses of 0, 300, 600, 900 and 1800 j/m^2 and pooled. The irradiated virus was pooled, filtered through a 0.45 µm membrane and stored at 4ºC.

Appropriate dilutions were made of each pooled sample and the dilutions were then inoculated onto a local lesion host, *Chenopodium amaranticolor* to calculate the inactivation kinetics.

ELISA.

Several types of ELISA have been used according to the hosts used for the immunizations and challenge (mink, dog, rabbit or mice). Details of these procedures have been previously described (Dalsgaard *et al*., 1997; Langeveld *et al*., 1994; Langeveld, 1999; Lopez de Turiso *et al*., 1992).

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.

Mink vaccination and challenge.

For mink, either 100 µg or 1 mg of purified CVP-Parvol 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 aluminium hydroxide gel (Superfos) to make a dose volume of 1 ml.

Two immunization tests were carried out in which six mink per test were immunized with a single dose of 1 ml 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) according to the manufacturer's instructions. As non-vaccinated 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. 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 faecal samples during the critical days indicated using an ELISA method. In addition, the amount of virus particles excreted by the non-immunized controls was sufficient to be confirmed by electron microscopy as previously described. Blood samples were taken before immunization, and also in the low-dose.

Vaccination and challenge of dogs

Either 7.5 mg of CPMV-PARVO1 (containing approximately 150 µg of 3Ll7 peptide) or wild type CPMV, or 1 mg of 3Ll7-KLH was diluted in PBS to a volume of 0.5 ml and mixed with 25 pg of Quil-A. The mixture was added to an equal volume of aluminium hydroxide gel to make a dose volume of 1 ml.

Dogs were kept in four groups of 5 or 6, A-D, each group in a separate room. Groups A, B and C (6 dogs in each) were immunized subcutaneously on days 0 and 28 with either CPMV-PARVO1, 3L17-KLH or wild type CPMV, respectively. A fourth group (D) of 5 dogs was left unvaccinated. Inoculation sites were checked daily for adverse reactions. On day 42, all 23 dogs were challenged with CPV by the oronasal route by distributing CPV onto their eyes, noses and mouths (1.5 ml/dog). Blood was collected prior to immunization, following immunization (days 28 and 38) and following challenge (day 48 and 57) and sera collected and stored at –20ºC. Rectal swabs were stored at –20ºC prior to analysis for the presence of CPV.

Irreversible sick animals were euthanized usually 6 days after challenge to avoid further suffering. Following euthanasia, samples of tissue derived from duodenum, jejunum, ileum, spleen, thymus, tonsil and various lymph nodes were taken from all the dogs and examined macroscopically and microscopically. The presence of CPV antigen was determined by immunohistology using rabbit anti-VP2 antiserum following standard procedures.

Oral immunization experiment

Two different strains of mice were tested: Balb/c and NIH. 14 groups of 8 mice (of each strain) were immunised by feeding antigen formulations as shown in Table 1. Serum and faecal samples were obtained on days 0, day 21, and day 49. In addition, gut and vaginal lavage was performed on selected groups on day 49.

From each group of mice, a pool of serum was prepared, and analysed for antibodies against cowpea mosaic virus and against the inserted peptide. The analysis was performed using ELISA, with CPMV or peptide as coating antigen, respectively. Serum samples were serially diluted, and bound antibody visualised using rabbit-anti-mouse Ig, and OPD as chromogen. Titer was determined as the dilution of serum giving an OD reading of 0,250.

RESULTS

Construction and propagation of CVPs.

Full-length cDNA clones pCPl and pCP2, containing full-length copies of CPMV RNAs 1 and 2, respectively, were previously shown to be directly infectious for cowpea plants (Dessens, 1993). 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-Parvol. Inoculation of this recombinant plasmid (together with pCP1) onto *V. unguiculata* seedlings successfully led to the establishment of infection in 5/5 plants. Viral lesions with CVP-Parvol were observed on the primary leaves within 7 days post-inoculation, and a systemic infection could be observed shortly after the secondary leaf buds opened, approximately 5 days post-inoculation. The yields of virus were 50 to 60 mg from five plants (approximately 50g leaf material). The recombinant virus produced, CVP-Parvol, was genetically stable over at least two passages, as judged by sequencing of reverse transcription polymerase chain reaction (RT-PCR) products from the purified particles.

Characterization of CVPs.

The formation of icosahedral CPMV-like structures was confirmed by negative-staining electron microscopy of purified CVPs. The purity of CVP-Parvol and its subunit profile compared with CPMV was confirmed by SDS-PAGE. 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. CVP-Parvol shows the same L protein band (38kDa), and several bands (26, 20, and 18 kDa) for the S protein (Dalsgaard *et al*., 1997). 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 (Lopez de Turiso *et al*., 1991). The S protein pattern of CVP-Parvo 1 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. N-terminal sequencing of the cleavage products from several of these indicates that this event always occurs in the same location (McLain *et al*., 1995). The same CVPs were used for all the immunization experiments described in this paper.

Immunisation 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 Quil A and adsorbing it to aluminium hydroxide gel. The potential vaccines were applied as a single subcutaneous dose to 6-to 8-month-old mink to mimic a commercially available vaccine that is protective after a single dose using the challenge procedure applied.

The CVPs protect against MEV in a dose-dependent manner (Dalsgaard *et al*., 1997). Although 100 µg CVP protected against clinical disease in five out of six animals, viral shedding was detected for several days. The l-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 the most important.

The non-immunized mink suffered from typical MEV diarrhoea during the observation period, whereas both conventionally and CVP-vaccinated mink showed no signs of diarrhoea (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 post-challenge 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 where prevention of diarrhoea is the primary objective

Immunization of dogs and protection

All dogs immunized with either CPMV-PARVO1 or 3Ll7-KLH were completely protected from lethal challenge with CPV (Table 2). None of the dogs developed any clinical symptoms of disease such as loss of appetite, blood in stool, diarrhea, vomiting, pyrexia or leukopenia. Furthermore, all dogs in these groups showed no or only very low levels of CPV shedding in faeces when examined from day 2 after challenge (titer between 1 to 16). In contrast, dogs immunized with wild-type CPMV or non-immunized dogs developed severe enteritis, approximately 4 days following CPV challenge and all but one had to be euthanised in extreme on day 6 post-challenge (Table 2). High levels of CPV antigen could be detected 4-6 days after challenge in both these control groups until termination of the experiment 6 days post-challenge.

Before vaccination, anti-peptide titers were almost absent. However, following vaccination all dogs given CPMV-PARVO1 developed high titers that rise even more after challenge. The vaccinated dogs also developed antibodies to the whole VP2. VP2-specific antibody was absent in both the wild type CPMV-immunized and unimmunized dogs prior to challenge. However after challenge on day 48 (when severe sickness was evident), titres of VP2-specific antibody developed in these dogs, which was at a comparable level to that present in the vaccinated dogs. Thus, protection from CPV challenge in the groups vaccinated with the 3Ll7 epitope appeared to correlate with the presence, prior to challenge, of high titers of 3Ll7-specific IgG, which also recognized VP2.

Sera from CPMV-PARVO1-immunized dogs neutralized CPV in vitro.

Prior to vaccination, no neutralizing activity was present in the sera of any of the dogs. However, following both immunizations (day 28 and 38), and to a greater extent, challenge (day 48), the sera from all dogs given either CPMV-PARVO1 or 3Ll7-KLH neutralized CPV i*n vitro*. Comparisons of neutralizing titers determined at different time-points indicate a rise in titer, both after the booster vaccination and after challenge. In contrast, pre-challenge sera from both wild type CPMV-immunized dogs and unimmunized dogs failed to neutralize CPV. However, after challenge on day 48, when severe sickness was evident, strong neutralizing activity developed in these groups, which was at a comparable level to that present in the both the CPMV-PARVO1- and 3Ll7-KLH-immunized groups. Although only a low level of virus shedding was observed in dogs vaccinated with either CPMV-PARVO1 or 3Ll7-KLH (Table 2), shedding in individual dogs on the day of highest excretion showed an inverse correlation with the pre-challenge (day 38) titers of anti-3Ll7 antibody and CVP-neutralizing antibody.

Oral immunization of mice and induction of neutralizing antibodies

Initial studies with oral and nasal immunisation of mice, guinea pigs and rabbits, showed that it was possible to induce immune responses in mice using two doses of 100 µg antigen, three weeks apart. The responses were stronger after nasal than after oral administration, but still low compared to conventional, parenteral vaccination.

After this relatively low success, we combined two different approaches: the use of a large collection of substances, which could either facilitate the uptake of the particles through the gastrointestinal route or to exert some kind of adjuvant effect, at the same time a new protocol of immunization was followed. Briefly, it consisted in feeding the animals three

consecutive days, twice, three weeks apart. The different substances are shown in Table 1. For statistical reasons 8 animals were used per group. A total of 14 different groups were made.

Animals were bled at days 27, immediately before the booster, and 49. The results are summarised in figures 1 and 2. The first two columns in each series show the anti-CPMV antibody titer and the next two show the anti-peptide titers, of Balb/c and NIH mice respectively. At day 27, the two types of mice behave in a similar way. Surprisingly, when we look at the antibody response in serum, we realise that there are no major differences between the parenteral immunisation and the oral administration in a number of cases, especially if we look at the anti-cowpea response. Most of the groups gave a vigorous response, even after a single dose. Evidently, the three groups receiving either palmitified or LPS-conjugated antigen respond very poorly. At day 49, there was a strong enhancement of the antibody response in all the responder groups. Titers of circulating antibody against CPMV is in the range of 3,5 - 5 ($\log_{10}$), whereas titers to the peptide are between 2 and 3 ($\log_{10}$). Especially strong was the response in the groups fed with the antigen plus cholera toxin, although the chimeric particles alone were enough to elicit a potent response. Combination of the antigen with salicylate or Quil A were also good responders. Interestingly enough the antipeptide responses increase significantly until titers of 10^3 in the case of the cholera toxin group and very close titers in other groups as native CPMV or salicylate group.

One crucial aspect in the quality of the antibody response is the presence of neutralising antibodies against canine parvovirus, because they determine the protective response in the animals. The neutralisation titers were determined in the mouse sera in selected groups. There was a significant neutralising response in some of the groups and it was particularly high in the cholera toxin group and the leaves group, which gave very similar results to the positive control groups parenterally immunised. This is particularly interesting as it represents the most similar situation to the natural feeding of the animals. These results open therefore the door to the oral immunization with non-replicative particles.

Evaluation of antibody responses in other body fluids.

To determine the presence of antibodies in mucosal-related tissues and samples, antibody titers were determined in the same selected groups as before. Antibodies were determined in intestine (gut lavage), in vaginal lavage and in faeces. The results are shown in the next figures. The antibody titers against CPMV were significantly lower in all these cases, except in vagina were the selected groups gave titers between 10^2 and 10^3. In the gut lavage the highest titers were obtained again in the groups 6 and 12, corresponding to the groups feed

with the CPMV plus leaves or cholera toxin. However, the anti-peptide antibody responses were almost undetectable except in the case of the cholera toxin group. The same situation applies to the antibodies detected in vagina, where these two groups gave the best results and significantly higher titers than the subcutaneously immunised groups. In faeces, the situation was relatively similar showing again that the cholera toxin group was the best adjuvant to elicit a strong antibody response at the mucosal level.

The summary of the results for three selected groups: cholera toxin, native CPMV and subcutaneous immunization plus the negative control group is shown in the next figure. Cholera toxin was the best adjuvant in the four cases, serum antibodies, faeces, intestine and vagina. However, native antigen was very effective to elicit antibodies responses in all the cases, almost as effective as the parenterally-immunised group. Although, significantly lower there was a correlation between the antibody titers obtained at the serum level with those titers obtained in other mucosal sites. The ability of CPMV carrying the CPV epitope to provoke strong immune responses after oral immunisation is surprising and holds good promise for the use of this plant virus as oral vaccine antigen. The next logical step will be to determine the immunogenicity of this antigen in the target animals, i.e. mink and dogs.

DISCUSSION

Production of vaccines in plants offers important advantages over conventional fermentation technology. On top of that if the plants or derived products are palatable and, therefore, edible, it would constitute a historical milestone in vaccinology. In the past years we have been working on the preliminary steps necessary to reach this goal. Chimeric CPMV containing parvovirus epitopes were used to immunize mink and dogs, sequentially. The mink vaccine was the first experimental vaccine produced exclusively by plants that conferred protection against an infectious disease in the target animal. 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 was to test the CVP-Parvol vaccine in dogs for protection against CPV. The vaccination of dogs with the same cowpea-derived vaccine was as successful as the mink experiment. All the vaccinated dogs were protected and clinically healthy. The plant-derived vaccine provided equivalent levels of protection to the KLH-conjugate in that all vaccinated dogs generated high titers of VP2 peptide-specific neutralizing antibody and were completely protected from clinical disease and virus shedding in vivo. Thus, the plant-derived vaccines were extremely

successful by parenteral route. These findings paved the way for further vaccine development based on this technology, which was the major aim of our project: the development of edible vaccines.

After some trials with different protocols of immunization, a protocol based in the ingesta of relative large amounts of antigens in three consecutive days for two periods lead to an strong antibody response against the cowpea particle and the epitope. Moreover, a significant fraction of these antibodies was able to neutralise the canine parvovirus in vitro. These results open the way to the use of non-replicative particles in the production of oral vaccines.

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 parvoviruses, e.g., wild mink, foxes, etc.

Cowpea mosaic virus is not the only choice of plant virus for vaccine development, a full array of plant viruses are being currently studied. Some of them offer even better perspectives from the point of view of oral vaccine development as they grow in more palatable food, as fruits. In this respect, our group has been collaborating with the group of Dr. J. A. Garcia (CNB.Madrid) in the development of Plum Pox Virus, a potivirus that infects bone fruits, as vaccine carrier (Fernandez-Fernandez *et al*., 1998). Similar results to those described with CPMV have been obtained with plum pox virus, indicating a large promise for this approach, with the advantage that plum pox may contain 1600 copies of the epitope in a single virion. Still, many more studies will be necessary before commercial application of this technology become feasible.

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Table 1. Adjuvants used for the oral immunization of mice with CPMV

Group no.	**Formulation (Ag, Antigen)**
1	Ag + 1000µg Na-salicylate
2	Ag + 1000µg Palmitoyl carnithine
3	Ag encapsulated in liposomes
4	Ag + 1000µg Na-desoxycholate
5	Ag mixed with leaves of *V. unguiculata*
6	Ag + 100µg Quil A
7	Ag + 100µg ISCOM matrix
8	Palmitified Ag + 100µg Quil A
9	Palmitified Ag in ISCOMs
10	LPS-conjugated Ag in ISCOMs
11	Ag only
12	Ag + 8µg cholera toxin
13	Subcutan. Immun.: Ag + Quil A
14	Subcutan. Immun.: Ag only

Two different strains of mice were tested: Balb/c and NIH. 14 groups of 8 mice (of each strain) were immunised by feeding with the antigen formulations shown in this table.

Table 2. Protection of dogs from CPV infection by immunization with CPMV-PARVO1

Group	Clinical disease	Protection	Virus shedding[a]
CPMV-PARVO1	0/6	6/6	2± 0.35
3L17-KLH	0/6	6/6	4± 2.2
Wt-CPMV	6/6	1/6	2874± 850
Non-immunized	5/5	0/5	1940±320

a. Titers of virus in faeces are expressed as geometric menas ± SD of the maximun value observed daily in each dog.