JOURNAL OF VIROLOGY, May 1992, p. 2748–2753
0022-538X/92/052748-06$02.00/0

Vol. 66, No. 5

Recombinant Vaccine for Canine Parvovirus in Dogs

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Received 3 October 1991/Accepted 7 February 1992

VP2 is the major component of canine parvovirus (CPV) capsids. The VP2-coding gene was engineered to be expressed by a recombinant baculovirus under the control of the polyhedrin promoter. A transfer vector that contains the *lacZ* gene under the control of the p10 promoter was used in order to facilitate the selection of recombinants. The expressed VP2 was found to be structurally and immunologically indistinguishable from authentic VP2. The recombinant VP2 shows also the capability to self-assemble, forming viruslike particles similar in size and appearance to CPV virions. These viruslike particles have been used to immunize dogs in different doses and combinations of adjuvants, and the anti-CPV responses have been measured by enzyme-linked immunosorbent assay, monolayer protection assays, and an assay for the inhibition of hemagglutination. A dose of ca. 10 μg of VP2 was able to elicit a good protective response, higher than that obtained with a commercially available, inactivated vaccine. The results indicate that these viruslike particles can be used to protect dogs from CPV infection.

Canine parvovirus (CPV), a member of the autonomous parvoviruses, is the cause of an important disease in dogs. It was first identified in 1978 (1, 4), and it is now endemic around the world. CPV is very related genetically and antigenically to feline panleukopenia virus and mink enteritis virus (5, 14, 15, 18, 24).

Nonenveloped icosahedral capsids of autonomous parvoviruses have a diameter of ≈25 nm and contain a single-stranded DNA of ≈5,000 bases. CPV capsids contain three structural proteins (VP1, VP2, and VP3). VP2 (64 kDa) is an NH_2-terminally truncated form of VP1 (84 kDa) and is the major component of the capsid. VP3 is derived from VP2 by posttranslational proteolytic cleavage and is present only in complete (DNA-containing) virions. Full capsids contain 60 copies of a combination of VP2 and VP3 and contain some VP1 (25). Empty particles do not contain VP3. Trypsin treatment of full particles cleaves VP2 to VP3-like protein (22). There is some evidence that the VP1 terminus is internal and may help neutralize the DNA (25).

Since VP2 in the CPV capsids is far more abundant than the other two components, it is reasonable to think that VP2 alone could be able to self-assemble to make viruslike particles (VLPs). Previously, Mazzara et al. (13) had reported the synthesis of empty capsids using VP1 and VP2 coexpressed in a chimeric papillomavirus expression system. When this paper was in preparation a paper by Brown et al. (3) describing the preparation of VP2 capsids for human parvovirus B19 was published.

In order to get high-reliability and high-level expression of CPV VP2, we have chosen the baculovirus system. Baculovirus has been successfully used to express large amounts of foreign antigens (for a review, see reference 11). A number of VLPs have been obtained for several different viruses, e.g., hepatitis B virus (21), poliovirus (26), bluetongue virus (8), cauliflower mosaic virus (29), and rotavirus (17), among others. The assembly of these particles allows the study of viral morphogenesis and the potential use of these structures as putative vaccines. These vaccines will allow, among other advantages, (i) elimination of viral reservoirs used for the manufacturing of conventional vaccines, (ii) avoidance of new outbreaks due to viral escape during vaccine production, and (iii) facilitation of the eradication of the diseases.

In this paper we report the ability of the CPV coat VP2 protein to form particles by using a recombinant baculovirus. A dose-related study of the immunogenic properties of these particles in dogs, the natural host of CPV, is presented. The potential of these recombinant subunit vaccines is discussed.

MATERIALS AND METHODS

Cells and virus. The *Spodoptera frugiperda* cell line Sf9 (ATCC CRL 1711) was used to propagate wild-type (wt) and recombinant baculoviruses. Sf9 cells were grown in suspension or as monolayer cultures in TNM-FH medium (20) supplemented with 10% fetal calf serum plus antibiotics. The E2 strain of the nuclear polyhedrosis virus of *Autographa californica* (AcNPV) (19), kindly provided by M. Summers (Texas A&M University), was used as the wt virus.

Competent cells of *Escherichia coli* DH5α were used in plasmid DNA transformation according to standard protocols (9).

DNA manipulations. Plasmid pCPV12 containing fragment *Hpa*II-A (≈2.0 kbp) of CPV (10) was used as the parent vector. In the transfer vector pJVP10Z, kindly provided by C. Richardson (National Research Council of Canada), the polyhedrin-coding region has been removed and a unique cloning site, *Nhe*I, has been created (27). This transfer vector also contains a copy of the *lacZ* gene as a reporter under the control of the p10 promoter.

To produce a VP2 fragment with flanking *Xba*I sites, pCPV12 was *Hpa*II digested. A 2.0-kb fragment was isolated on low-melting-point gel agarose and cloned into the *Acc*I sites of pMTL24, yielding pCPV13. VP2 was excised from pCPV13 by digestion with *Xba*I. This fragment was ligated into *Nhe*I-digested, phosphatase-treated pJVP10Z to give transfer vector pCPVEx17. Plasmid DNA was recovered from transformed *E. coli* cells (DH5α) by the alkaline lysis

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method of Birnboim and Doly (2) followed by CsCl centrifugation.

Transfection and selection. Sf9 cells in T-25 culture flasks were cotransfected with wt AcNPV DNA and plasmid pCPVEx17 by a modification of the calcium phosphate precipitation technique as described previously (20). Transfection supernatants were plated and recombinant plaques were detected after addition of 0.1 ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (25 μg/ml), 4 days after infection. Recombinant viruses that developed a blue color upon addition of X-Gal were subjected to three rounds of plaque purification before preparation of high-titer viral stocks.

Dot blot analysis. Culture supernatants of infected cells were diluted with phosphate-buffered saline (PBS), adjusted to 0.1 M NaOH, boiled for 5 min, neutralized with 0.1 M $NaPO_4H_2$, adjusted to 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate), and blotted on nitrocellulose sheets by using a dot blot apparatus. Filters were dried at 37°C, and nucleic acids were fixed to the filter by UV irradiation for 3 min.

CPV-specific probe was prepared by 5′-terminal labelling with T4 polynucleotide kinase and [γ-^{32}P]ATP of a 24-mer VP2-specific oligonucleotide, spanning nucleotides 2787 to 2810 of the CPV genome (18), according to standard protocols (12). The sequence of the oligonucleotide is ATGAGT GATGGAGCAGTTCAACCA. Filters were hybridized with ^{32}P-labelled oligonucleotide in 6× SSC–10× Denhardt's solution–0.2% sodium dodecyl sulfate (SDS) at 37°C overnight. The membrane was washed three times with 1× SSC containing 0.1% SDS at 37°C, dried, and autoradiographed.

Preparation of cellular extracts and purification of the recombinant proteins. Sf9 cells were grown in monolayers or as suspension cultures and infected with the recombinant baculovirus at a multiplicity of 2 to 5 PFU per cell. Cells were harvested at 48 to 72 h postinfection, centrifuged for 10 min at 200 × *g*, washed with PBS, and resuspended in 25 mM HNa_2CO_3, pH 8.3, at a density of 2 × 10^7 cells per ml. Infected cells were lysed by sonication and centrifuged for 15 min at 15,000 rpm in an SS34 rotor. The supernatant, containing the expressed recombinant protein, was harvested and stored at 4°C.

Sonication supernatants were precipitated with a saturated solution of ammonium sulfate until a final concentration of 20% was reached. The resultant pellet, containing most of the VP2 protein, was resuspended in PBS (10^7 cells per ml) and filtered through a Sephadex G50-80 spun column (12) to desalt it.

β-Galactosidase protein was removed by affinity chromatography. Anti-β-galactosidase immunoglobulin G was purified from a polyclonal rabbit antiserum with protein A-Sepharose. Fractions containing immunoglobulin G were pooled, concentrated, and linked to CNBr-activated Sepharose. VP2 preparations were adsorbed onto this column to remove the β-galactosidase.

Polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. Recombinant proteins were analyzed on a Phast System device (Pharmacia) with 10 to 15% gradient SDS-polyacrylamide gels and were Coomassie stained. Proteins were transferred to nitrocellulose membranes as previously described (23). Protein transfer was done on a Phast System device (Pharmacia). Usually 25 mA per gel was used for 10 to 15 min. The nitrocellulose was blocked with 3% nonfat dried milk in 20 mM Tris-HCl (pH 7.5)–500 mM NaCl (TBS) for 30 min at room temperature. Filters were then incubated for 1 h at room temperature with a cocktail of four CPV-specific monoclonal antibodies (10), washed with TBS–0.05% Tween 20 for 15 min at room temperature, and incubated with a biotin-labelled rabbit anti-mouse immunoglobulin G serum (dilution, 1:1,000). Strips were washed again and incubated with streptavidin-peroxidase at a 1:2,000 dilution. After washing, strips were developed with a solution containing 0.5 mg of 4-chloro-1-naphthol (Sigma) per ml, 17% (vol/vol) methanol, and 0.015% hydrogen peroxide in TBS until visible bands appeared. The reaction was stopped by rinsing the strips with distilled water.

Immunization of animals. Beagles, 45 days old, were immunized subcutaneously with two doses of semipurified (β-galactosidase-containing) VP2 preparations. The concentration of protein was estimated by the Bradford assay. The booster was given 28 days after the first immunization. The antigen was adjuvanted with alumina (Alhydrogel; Superfos, Vedbaek, Denmark), Quil A (Superfos) (50 μg per dog) (7), or a combination of both. Dogs were bled twice a week until 2 months after the first injection. Two dogs were immunized with an inactivated commercially available vaccine. An unvaccinated sentinel dog was used as a negative control to check for any accidental exposure to CPV.

In vitro protection assays. To determine the ability of the dog serum to neutralize the virus in vitro, a monolayer protection assay was performed as previously described (10).

HA assays. Hemagglutination (HA) and inhibition of HA (IHA) titers were determined as previously described (6).

CPV challenge. All of the dogs were challenged by oronasal inoculation with 1 ml of infective dog feces diluted twice in PBS containing virulent CPV at day 42 after the second dose of vaccine. Clinical manifestations of the disease were monitored for 17 days postchallenge.

RESULTS

Construction of transfer vector pCPVEx17. Transfer vector pCPVEx17 was obtained after a two-step cloning procedure (Fig. 1). First, the VP2-coding gene was obtained as a 2-kbp *Hpa*II-A fragment from plasmid pCPV12 (10) and ligated into the *Acc*I-duplicated cloning site of pMTL24, to give plasmid pCPV13. This plasmid allowed us to obtain the VP2-coding gene flanked by two *Xba*I sites. This fragment was then directly ligated to the compatible *Nhe*I unique site of the vector pJVP10Z, under control of the polyhedrin promoter, resulting in transfer vector pCPVEx17.

Transfer vector pCPVEx17 contains the VP2 gene plus 40 nucleotides upstream of the initial codon ATG. The use of pJVP10Z facilitates the selection of recombinant viruses, since the *lacZ* gene is expressed under control of the strong p10 promoter of AcNPV, by the presence of blue coloration in combination with polyhedron-negative plaques.

DNA analysis of recombinants. Sf9 cells were cotransfected with pCPVEx17 and wt AcNPV DNA. Recombinant viruses were selected by their polyhedron-negative appearance and blue coloration. Three putative recombinant viruses were plaque purified at least three times. The recombinant baculovirus was named AcCPV17.

The identity of CPV-specific inserts was confirmed by dot blot hybridization. DNA isolated from recombinant baculovirus AcCPV17 present in the supernatant of lysed cells was shown to be CPV specific by positive hybridization to a ^{32}P-labelled oligonucleotide probe. The CPV probe did not hybridize to DNA isolated from wt baculovirus-infected cells (data not shown).

Protein analysis of recombinants. A protein similar in size

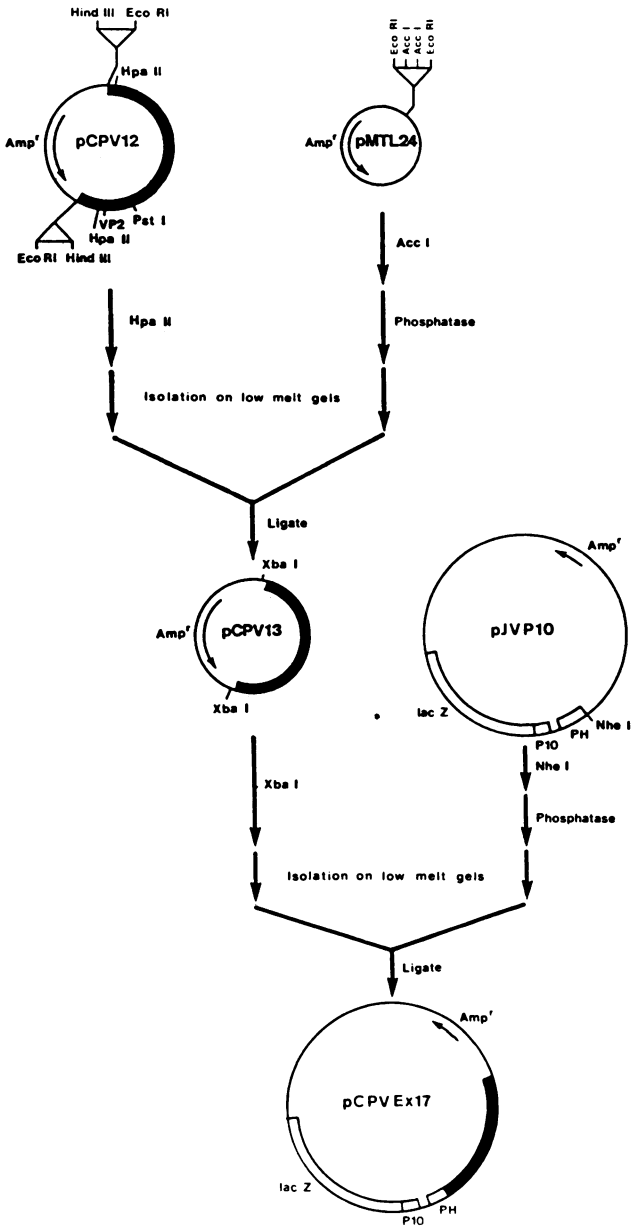

FIG. 1. Construction scheme of the baculovirus expression transfer vector containing the VP2 gene of CPV.

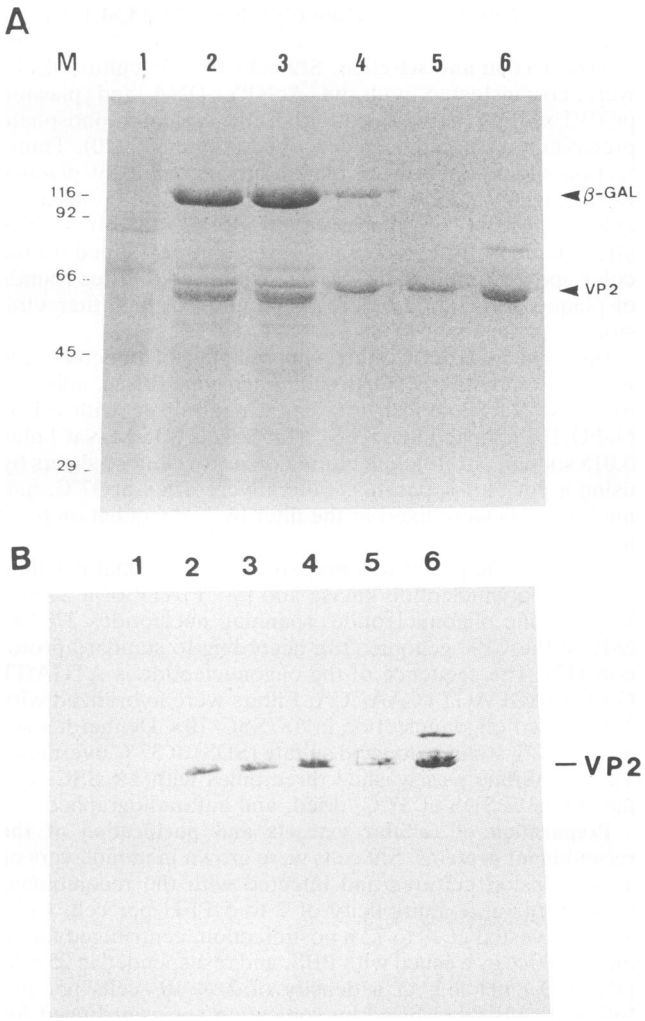

FIG. 2. Expression, purification, and analysis by Western blot of the major CPV coat protein VP2. M, molecular weight markers. Lanes 1, mock-infected cells used as a negative control; lanes 2, *S. frugiperda* cells infected at a multiplicity of 1 PFU per cell with the recombinant baculovirus expressing VP2; lanes 3, supernatant from infected cells, lysed by sonication; lanes 4, ammonium sulfate-precipitated supernatant; lanes 5, affinity chromatography-purified VP2; lanes 6, purified CPV virions. Proteins were separated by SDS-PAGE and stained with Coomassie blue (A) or were blotted on nitrocellulose membranes and made to react with a cocktail of four anti-CPV monoclonal antibodies (B). Bound antibody was detected with a peroxidase conjugate by standard methods.

to that expected for VP2 was readily observed when recombinant AcNPV-infected Sf9 cells at 72 h postinfection were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie blue (Fig. 2A). Identity of this protein was confirmed as VP2 in immunoblot analysis (Fig. 2B) using CPV-specific monoclonal antibodies (10). A protein of 116 kDa (Fig. 2A) was also detected in the lane corresponding to the recombinant viruses; this protein was identified as β-galactosidase by immunoblot using a specific rabbit polyclonal serum (data not shown). The amount of recombinant protein expressed was two to three times smaller than the amount of β-galactosidase, which is produced under control of the p10 promoter.

Purification of the recombinant VP2 protein. Recombinant protein VP2 was found to be present on the cytoplasmic fraction of Sf9 cells infected with AcCPV17, after lysis of the cells by sonication. After supernatant precipitation by addition of 20% ammonium sulfate, we got a material that contained mostly VP2 and some β-galactosidase. The identity of the 64-kDa protein as the CPV VP2 protein was confirmed by Western blot (immunoblot) analysis using a cocktail of four anti-CPV monoclonal antibodies (Fig. 2B).

β-Galactosidase was removed by affinity chromatography using anti-β-galactosidase immunoglobulin G Sepharose conjugated as shown in Fig. 2A.

Electron microscopy of VP2 preparations. Figure 3 (left) shows an electron micrograph of VP2 preparations showing a large number of aggregates. The aggregates consisted of VLPs with a morphology similar to that of the authentic

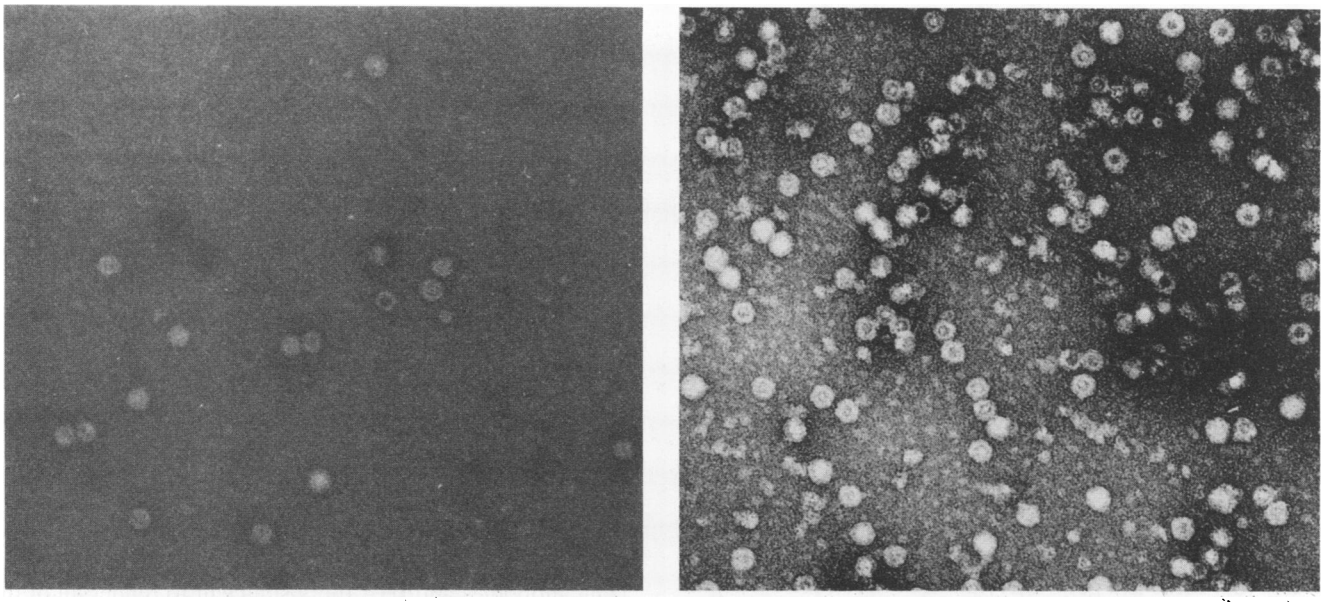

FIG. 3. Electron micrographs of recombinant VLPs (left) and sucrose-CsCl gradient-purified CPV (right). Preparations were negatively stained with 2% uranyl acetate. One centimeter equals 100 nm at the actual magnification (40,000 × 2.5).

virus. The diameter of the particles was estimated to be around 20 nm, i.e., comparable to that of CPV (Fig. 3, right). To investigate the equilibrium density of these VLPs, they were centrifuged on self-forming CsCl gradients, banding at a density of 1.33 g/cm^3, a density similar to that of empty capsids.

HA and immunogenicity of expressed VLPs. The presence of VLPs analogous to authentic virus was confirmed by HA analysis. These particles showed the same or higher hemagglutinating titer (up to 5×10^5 HA U/ml) than viral preparations.

To investigate the immunogenicity of these VLPs, four groups of 10 beagles were immunized subcutaneously with the semipurified preparations of VLPs (containing β-galactosidase). Booster doses were administered on day 28 (see Table 1). The dogs in group I received approximately 100 μg of VP2 antigen per inoculation. Groups II, III, and IV received approximately 50, 25, and 10 μg, respectively, per inoculation.

To find an adequate adjuvant, two types of adjuvants were evaluated, alumina and Quil A. Oil emulsions, such as Freund's adjuvant, were not used because of the great hypersensitivity of dogs to these emulsions.

Serum samples were collected twice a week between days 3 and 62 postimmunization and tested for the presence of CPV-specific antibodies by an enzyme-linked immunosorbent assay, a monolayer protection assay, and an assay for the ability to inhibit the viral HA. All of the dogs immunized with our antigen exhibited CPV-specific antibodies to various levels with both types of adjuvants (Table 1). However, the highest antibody titers were obtained when Quil A was included in the preparation (Table 1) alone or in combination with alumina. This combination was more effective with low doses (10 μg) of VP2 antigen.

TABLE 1. Serum IHA titers of dogs inoculated with recombinant VLPs

Day	Serum IHA titer of indicated dog group (dose)[a]:											
	VAC[b]	I (100 μg)		II (50 μg)			III (25 μg)			IV (10 μg)		
		Quil A + alumina	Alumina	Quil A	Quil A + alumina	Alumina	Quil A	Quil A + alumina	Alumina	Quil A	Quil A + alumina	Alumina
0	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20
7	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20
10	≤20	50	120	≤20	25	25	50	50	≤20	≤20	25	≤20
18	40	160	240	≤20	≤20	≤20	25	25	≤20	≤20	≤20	≤20
35	320	3,000	1,500	1,200	1,200	300	600	3,000	120	800	3,000	80
40	640	2,000	2,000	600	2,400	600	1,200	1,200	300	600	2,000	150
49	200	1,600	ND[c]	600	800	75	500	500	100	300	900	50
56	50	400	ND	300	300	25	250	125	25	100	250	25
62	50	400	ND	200	200	≤20	125	125	25	250	75	≤20

[a] All dogs were inoculated on days 0 and 28 with the dose and adjuvant indicated. Each value represents the average value for two dogs inoculated under the same conditions.

[b] VAC, dogs vaccinated with commercially available inactivated vaccine. All values for the negative control were ≤20.

[c] ND, not done.

TABLE 2. Serum IHA titers of dogs challenged with virulent CPV

Dog type[a]	Titer at indicated day postchallenge:			
	0	7	10	17
1		400	3,200	1,600
2		1,600	3,200	1,600
3	400	400	400	400
4	400	400	400	400
5	400	200	200	200
6	400	200	200	200

[a] The dogs used in the challenge experiment were as follows: 1, sentinel dog; 2, dog vaccinated with a commercially available vaccine; 3, dog vaccinated with 50 μg of VLPs adjuvanted with Quil A; 4, dog vaccinated with 50 μg of VLPs adjuvanted with alumina plus Quil A; 5, dog vaccinated with 25 μg of VLPs adjuvanted with alumina plus Quil A; 6, dog vaccinated with 10 μg of VLPs adjuvanted with alumina plus Quil A.

IHA and neutralization assays were used to evaluate the ability of these preparations to induce protection in dogs against CPV. Pollock and Carmichael (16) showed previously that there is a good correlation between IHA titer and protection; dogs showing IHA titers of ≥80 are considered refractory to infection by CPV. All of the dogs vaccinated with our preparation showed high IHA titers, between 150 and 5,120 (Table 1). Good IHA titers persisted for more than 2 months. A good correlation between IHA and neutralization titers by monolayer protection assays was found (data not shown). In both cases, titers obtained are far superior, even at very low doses (10 μg per dog), to those necessary to obtain a protective response. The titers were very similar to that obtained with sera from dogs recovered from CPV infection (data not shown) and much higher than those obtained with the vaccinated control. No CPV-specific antibodies were detected in the sera of dogs not inoculated.

Protection against viral infection. To evaluate the ability of the recombinant VLPs to elicit a protective immunity, six immunized dogs were challenged 6 weeks after the booster by oronasal inoculation (16) with infective dog feces containing virulent CPV. From day 3 postchallenge, rectal temperatures were recorded daily and the dogs were checked for clinical manifestations of parvovirus disease. Blood samples were collected at intervals after the virus challenge during 20 days and were screened for the presence of antibodies against the virus and for viremia by HA and tissue culture infection (Table 2). The recovered virus was identified as CPV. All of the dogs infected with the VLP antigen were immune to the viral challenge. None of them developed any clinical symptoms of disease or demonstrable viremia. The sentinel dog and the vaccinated dog, on the other hand, showed a large antibody response indicative of virus replication.

DISCUSSION

Because of the high level and faithful expression of foreign proteins characteristic of the baculovirus system, it is possible to think of the commercial exploitation of recombinant subunit vaccines based on the use of recombinant baculovirus (for a review, see reference 28). In most of the cases described, the products of the baculovirus expression are chemically, antigenically, functionally, and immunologically identical to their native counterparts. Until now, replacement of the polyhedrin by foreign genes has been the basis for the use of baculoviruses as expression vectors. We have used this approach, in combination with the use of the *lacZ* gene as a reporter gene, to express the major capsid protein of CPV, VP2, in insect cells by the recombinant baculovirus AcCPV17. The expression of the CPV coat VP2 protein results in the synthesis of a protein nearly identical to the authentic protein and with the ability to self-assemble in VLPs of the same size and appearance as authentic CPV capsids. The synthesis of these particles seems to be independent of the presence of VP1 and/or viral DNA. During the assembly of the capsid, the presence of VP3 cannot be observed. This result confirms the necessity of viral DNA to process the VP2 to VP3. VP1 does not seem to be relevant to the formation of parvovirus capsids, and its role seems to be confined to the stabilization of the DNA. It would be interesting to investigate the changes in morphology with respect to authentic viral capsids by three-dimensional analysis and find the exposed regions in the capsids in order to use the potential of these particles, not only as vaccines for CPV as we have shown in this work but also as a carrier for foreign epitopes in order to make polyvalent vaccines.

With regard to the use of these VLPs as vaccines, doses containing only 10 μg of protein were able to elicit neutralizing antibodies and IHA titers sufficient to render all of the immunized animals protected. These results confirm our previous observations that VP2 is the main determinant of the neutralization-specific immune response in CPV (10) and that it is able to induce protection. The minimal amount of protein required to elicit protection in animals still needs to be determined, although theoretical estimations, based on the amount of hemagglutinating units included by manufacturers in commercial inactivated vaccines, could fix the amount of the dose as low as 1 μg per dog. The choice of adjuvant is also crucial in vaccine development. Oil adjuvants are absolutely excluded, first, because given the high sensitivity of dogs to these type of adjuvants they are not acceptable, and second, because they tend to mask the actual immunogenicity of the preparations. Combinations of Quil A and alumina or Quil A alone has been more effective than alumina alone as an adjuvant.

The presence of β-galactosidase as a contaminant does not seem to affect the immunogenicity of the preparations very much. However, to avoid this contamination, a new construction is being built by using transfer vector pAcDZ1 (29), in which the *lacZ* gene is under the control of the weaker *Drosophila hsp-70* promoter, which is constitutively expressed in insect cells. Also, experiments are in progress to try to improve the VP2 expression by deleting the 40 nucleotides upstream of the initiation codon.

Because of the significant genetic and immunological relationship among CPV, feline panleukopenia virus (cats), and mink enteritis virus (minks), it is reasonable to think that the same VP2 particles will be useful to immunize cats and minks against parvovirus, as happens with conventional vaccines. Experiments along this line are in progress.

ACKNOWLEDGMENTS

Electron micrographs were kindly prepared by J. Nowicki and K. Dalsgaard (SVIV) and C. Sanmartín (CBM). We thank Mar Pitarch for technical help with the manuscript.

We thank ERCROS S.A. and C.D.T.I. (grant P-900078) for financial support.

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