Canine parvovirus empty capsids produced by expression in a baculovirus vector: use in analysis of viral properties and immunization of dogs

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The VP-2 genes of canine parvovirus (CPV) and a recombinant consisting of CPV and feline panleukopenia virus (FPV) sequences were cloned into baculovirus expression vectors, fused to the baculovirus polyhedrin promoter. Recombinant baculoviruses were prepared and the properties of the parvovirus proteins expressed in insect cells examined. The proteins produced were the same size as the authentic CPV VP-2 protein, and were produced late after infection; the quantity of proteins recovered from the insect cell cultures was similar to those produced in CPV infections. Parvovirus particles formed had the haemagglutination (HA), sedimentation and buoyant density properties of authentic CPV capsids. Both the CPV capsids and the CPV–FPV recombinant capsids from the baculovirus system expressed the same epitopes as those seen in the viable parvoviruses when tested with a panel of antiparvovirus monoclonal antibodies. Lysates of recombinant baculovirus-infected cells were inoculated into dogs, giving rise to serum neutralizing and HA-inhibiting antibodies, and the immunized dogs were protected from clinical disease upon challenge with a virulent isolate of the most recent antigenic type of CPV.

Introduction

The capsids of the autonomous paroviruses are composed of two or three structural proteins, VP-1, VP-2 and VP-3, of apparent sizes of 82-3K, 67-3K and 63-5K respectively in canine parvovirus (CPV) (Cotmore & Tattersall, 1987; Paradiso et al., 1982). The VP-1 and VP-2 proteins are derived from differentially spliced mRNAs, and the VP-1 protein contains the entire sequence of the VP-2 protein, as well as a unique peptide of about 15K at its N terminus which contains many basic amino acids (Reed et al., 1988). Studies of several paroviruses have shown that both full (DNA-containing) and empty (non-DNA-containing) particles contain, on average, about 10 copies of VP-1 and about 50 copies of VP-2 protein (Cotmore & Tattersall, 1987; Tattersall et al., 1976). Some of the VP-2 protein of full capsids may be proteolytically cleaved to the 63-5K VP-3 protein by the removal of 12 to 15 amino acids from its N terminus (Paradiso et al., 1982). The recently determined structure of CPV shows that the icosahedral shell of the capsid is assembled from 60 VP-1 or VP-2 molecules by interactions between the regions of the capsid proteins in common between the three virus structural polypeptides (Tsao et al., 1991). Studies of adeno-associated viruses have shown that mutant genomes in which VP-1 protein is not present do not package ssDNA efficiently or form infectious particles (Hermonat et al., 1984; Tratschin et al., 1984; Smuda & Carter, 1991).

Vaccines against CPV have been developed from attenuated live CPV strains (Bass et al., 1982; Carmichael et al., 1981, 1983) and inactivated CPV (Eugster, 1980; Pollock & Carmichael, 1982). Other vaccines have included use of live feline panleukopenia virus (FPV) (Pollock & Carmichael, 1983), or expression of the complete CPV VP-1 and VP-2 genes from a bovine papillomavirus vector (Mazzara et al., 1987). Expression of the human parovirus B19 VP-1 and/or VP-2 genes from recombinant baculoviruses gives rise to virus particles with many of the properties of authentic B19 particles (Brown et al., 1990; Kajigaya et al., 1991).

Here we use the baculovirus expression system to produce CPV VP-2 protein in insect cells for use as a potential vaccine antigen, and to allow the genetic
analysis of CPV and FPV capsid structures without producing viable parvoviruses. The correct assembly of the VP-2 protein into virus particles was demonstrated, and protect them against challenge with virulent CPV was examined.

**Methods**

**Viruses and cells.** The CPV-d isolate from 1979 (type-2 antigenic type) (Parrish et al., 1982) was used in most experiments, whereas the CPV-39 strain used for challenge was a 1984 isolate (Parrish et al., 1985; Parrish, 1991). The vBl260 virus was a recombinant between CPV and FPV prepared in vitro, and had the 59 to 73 map unit (m.u.) region of the CPV-d genome replaced by the equivalent region of FPV-b (Parrish, 1991). Feline NLFK cells (Crandell et al., 1973) were grown in a 50% mixture of McCoy's 5A and Liebovitz L15 medium with 5% foetal bovine serum (FBS). Cells were seeded thinly with 5% foetal bovine serum (FBS). Cells were seeded thinly with 5% foetal bovine serum (FBS). Cells were seeded thinly with 5% foetal bovine serum (FBS).

**Plasmid cloning.** DNA clones of CPV-d (Parrish et al., 1982) and a recombinant baculovirus (vBl260) containing the PstI-PstII region of FPV-d replacing the same region from CPV-d were prepared from replicative form (RF) DNA and cloned into plasmids as previously described (Parrish, 1991). Clones in baculovirus expression vectors were prepared as shown in Fig. 1. DNA was digested at an 1:3 x 10^4 cells/cm^2 in Ta MFH medium (Hink, 1970) with 10% FBS. After 1 h at 27 °C, the medium was removed and cells were infected with virus (10 p.f.u./cell) in serum-free medium or were mock-infected. After 1 h of adsorption at 27 °C, the inoculum was removed and complete growth medium was added.

**Preparation and analysis of recombinant baculoviruses.** Plasmids were used to transfet SF21 cells along with isolated AcNPV DNA using standard procedures (Burand et al., 1980). Recombinant baculoviruses were selected by picking polyhedrin-negative plaques or by staining plaques for β-galactosidase with X-gal (Viallard et al., 1990). One virus stock of each recombinant baculovirus was prepared after repeated plaque cloning.

For protein analysis, inoculated and mock-inoculated SF21 cells were lysed at various intervals post-infection with 1/3 NP40 in 30 mM-Tris-HCl pH 7.5 and 10 mm-magnesium acetate, as described by Summers & Smith (1987). To optimize protein harvest, infected cell supernatants were centrifuged and the pellet of dislodged cells was lysed along with the remaining cell monolayer. Control or CPV-infected NLFK cells were lysed with 2% (w/v) SDS, 10 mM-Tris-HCl pH 7.5. Cell lysates were precipitated with 80% acetone, dried and electrophoresed in 8% discontinuous SDS-polyacylamide gels (Laemmli, 1970). Separate proteins were transferred onto nitrocellulose (NC) paper following standard procedures (Towbin et al., 1979). NC strips were reacted with a 1:1000 dilution of a canine post-infection serum against CPV, and then with an 125I-labelled Protein A conjugate at 1 μCi/ml. After washing, label was detected by exposing NC strips to X-ray film with an intensifying screen at -70 °C. For quantification of the amount of VP-2 protein synthesized in insect cells relative to mammalian cells, the exposed bands corresponding to VP-2 protein were scanned using an LKB Ultrascan XL laser densitometer.

For analysis of capsids, viruses were banded in 10 to 40% (w/v) sucrose gradients for 6.5 h at 100000 g following standard procedures (Paradiso et al., 1982; Parrish & Carmichael, 1983; Parrish et al., 1982). Fractions collected were tested for virus by haemagglutination (HA) of rhesus macaque erythrocytes in barbital/acetate-buffered saline pH 6.2.
copy (Fig. 3e, J). Viruses from the same fractions were examined for antibodies (MAbs) prepared against CPV type-2 (Parrish antigenic type by HA inhibition (HAI) using a panel of monoclonal particles in peak fractions from the CsCl gradients were negatively containing the same sequences prepared from infected NLFK cells (Parrish, 1991) (Table 1).

**Immunization of dogs.** Baculo-BVr-infected Sf21 cells were frozen and thawed once, mixed with a carbopol-containing adjuvant and used as a vaccine. Three 14-week-old beagle dogs were inoculated subcutaneously with a 1 ml dose ($10^7$ infected cells) of baculo-BVr vaccine, then reinoculated with a second dose 3 weeks later. Two groups of three animals were either vaccinated once with $10^5.5$ TCID$_{50}$ per dog of the modified live virus (MLV) CPV vaccine (94LP) (Carmichael et al., 1981, 1983), or were not vaccinated. All dogs were challenged 9 weeks after the first vaccination by oro-nasal inoculation with 2 ml of CPV-39.

After challenge, dogs were monitored daily for clinical signs, including high temperature, loose stools or diarrhoea, and their total white blood cell count was determined. Serology was performed by HAI tests using 4 HA units (HAU) of the viral antigen and feline erythrocytes, basically as described previously (Carmichael et al., 1980; Parrish et al., 1982). For serum neutralization (SN) tests, 0.05 ml of serum diluted in culture medium was mixed with 100 TCID$_{50}$ virus (0.05 ml), incubated for 1 h at 37 °C and the mixtures were used to inoculate suspension cultures of feline kidney cells. Cultures were seeded into culture trays and incubated for 6 days, then virus replication was assayed by titration of the HA activity of cell cultures after extraction with 0.2 M-glycine buffer (Hallauer et al., 1971).

**Results**

Western blot analysis of infected cell lysates revealed that insect cells inoculated with recombinant baculoviruses expressed proteins that were the same size as the VP-2 protein in purified CPV preparations and in CPV-infected cells (Fig. 2). The baculo-349- and baculo-BVr-infected cell lysates lacked the VP-1 protein present in CPV preparations and infected cells, and also lacked the NS-1 protein present in lysates of infected cells. The two clones in the E10 and pJVP10Z vectors gave about the same level of expression 3 days after infection (Fig. 2a). No proteins were detected in Sf21 or NLFK control cells by Western blotting. The VP-2 protein was expressed in greatest amounts in recombinant baculovirus-infected Sf21 cells between 3 and 4 days after infection (Fig. 2b), as expected for the polyhedrin promoter (Summers & Smith, 1978, 1987). The level of expression, about 14 µg/cm² of cell culture, was about the same as that observed in CPV-infected NLFK cells. The amount of CPV protein produced by baculo-BVr infection from $10^6$ cells was about 10-fold lower than that produced from

CPV-infected NLFK cells (9 µg versus 94 µg). Reports of expression of other parvovirus proteins in insect cells using baculovirus vectors have indicated amounts around 40 µg/$10^6$ cells (Brown et al., 1990), so the amounts produced here were low.

When lysates of baculo-BVr-infected cells were analysed by sucrose gradient centrifugation, virus particles having a sedimentation coefficient identical to that of authentic CPV empty particles were detected (Fig. 3a, b). A more rapidly sedimenting peak of DNA-containing particles in the preparations from CPV-infected cells was not present in the recombinant baculovirus-infected Sf21 cells. On isopycnic CsCl gradients the baculovirus-expressed particles and empty CPV particles banded as single peaks at 1.31 to 1.32 g/ml (Fig. 3c, d). Electron microscopy showed that the particles in both preparations had the same overall morphology with diameters of 21 to 25 nm (Fig. 3e, f), as expected for CPV particles (Paradiso et al., 1982).

Antigenic analysis of the capsids by reactivity with MAbs in the HAI test showed that the two different baculovirus-expressed genes produced capsids identical to those produced from the viable parvoviruses containing the same VP-2 gene sequences (Table 1). Whereas baculo-349 capsids were identical to the wild-type
CPV-d capsids, the baculo-BVr recombinant capsids lacked one CPV-specific epitope recognized by MAb 7, 14 and 13, and also expressed an FPV-specific epitope recognized by MAb H. The pH dependence of HA of the parvovirus- and baculovirus-expressed capsids was also seen to be the same (results not shown). This indicates that the capsids composed only of VP-2 protein had all the defined properties of the capsids containing both VP-1 and VP-2 proteins from viable parvoviruses, and also shows that all the MAbs in our various panels are directed against VP-2 amino acid sequences, and presumably the region of the VP-1 protein in common with VP-2 protein.

Antiviral antibodies were detected by HAI 10 days after inoculation of dogs with adjuvant–VP-2 protein expressed by baculo-BVr in insect cells, and increased after the second vaccination (Fig. 4). Neutralizing antibody titres in baculo-BVr-vaccinated dogs showed a mean titre of 1:1150 on day 63 and increased to over 1:50200 14 days after challenge. Both SN and HAI titres (Fig. 4) stayed constant during the same period in MLV-vaccinated dogs, the SN titre averaging 1:11500 in these dogs. Therefore, the anti-BVr serological responses are similar to those reported previously for inactivated CPV or FPV vaccines, the antibody titres being about 10-fold lower than those generated after vaccination with MLV (Fig. 4) (Bass et al., 1982; Carmichael et al., 1981, 1983; Eugster, 1980).

After challenge, all dogs vaccinated with either the baculo-BVr capsids or MLV were protected against disease (as demonstrated by absence of clinical signs),

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**Table 1. Antigenic typing of purified capsids using various MAbs**

<table>
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<th>Antibody</th>
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* Specific MAbs react only with CPV, FPV or CPV-2a isolates, whereas common MAbs react with isolates of FPV, mink enteritis virus and raccoon parvovirus, as well as CPV.
† HAI titres are expressed as the reciprocal of the last dilution of the MAb that inhibited HA by between 4 and 8 HAU of antigen.
were vaccinated only on day 1, and controls were not vaccinated (O). All dogs were challenged on day 63.

Vaccinated on day 1 and again on day 21; MLV-vaccinated dogs (11) showing that the challenge virus most likely replicated, whereas non-vaccinated dogs suffered from severe loss between 4 and 8 days post-challenge (results not shown). Upon challenge, the dogs vaccinated with baculo-BVr mounted a strong antibody response, indicating that the challenge virus most likely replicated, whereas the antibody titres of the MLV-immunized dogs did not change (Fig. 4).

Discussion

Here we demonstrate the efficient expression of CPV VP-2 protein from a recombinant baculovirus, and also show that the VP-2 protein expressed alone assembles into empty capsids which are antigenically indistinguishable from authentic empty capsids produced from viable parvoviruses of the same genetic type.

This will be a useful method for studying virus capsid structure and function. At present the mechanisms of assembly of parvovirus capsids are poorly understood. It is known that the expression of VP-1 and VP-2 proteins from a number of systems produces empty particles containing both proteins (Kajigaya et al., 1989; Labieniec-Pintel & Pintel, 1986; Mazzara et al., 1987). Since VP-1 molecules are present in parvovirus capsids in about the proportion at which they are synthesized (Cotmore & Tattersall, 1987; Tattersall et al., 1976), it appears that VP-1 molecules are incorporated into capsids in a fashion equivalent to VP-2 molecules, and that the unique N-terminal portion of VP-1 is not required for capsid assembly. This is also suggested by the crystallographic structure of CPV, which indicates that the resolved polypeptide involved in the assembly of the capsid is the region after residue 38 in the VP-2 molecule, and the N-terminal regions of the VP-2 protein (or VP-1) are not resolved (Tsao et al., 1991). The structural proteins of several other viruses, including the human parvovirus B19, have been shown to self-assemble into virus-like structures when expressed by baculovirus vectors in insect cells (Brown et al., 1990; French et al., 1990; Roosien et al., 1990; Luo et al., 1990; Kajigaya et al., 1991).

The level of protection conferred by baculo-BVr when used to immunize dogs appeared equivalent to that achieved with CPV live vaccine under the conditions used. As the empty capsids contain the VP-2 protein in a native form they allow the preparation of an effective inactivated vaccine. Since the capsids produced are non-viable there is no need to inactivate the parvovirus, with the attendant possibility of altering antigenic epitopes. This ability to make immunogenic empty capsids of CPV will allow the preparation of inactivated vaccines, and will also permit the further analysis of those VP-2 protein sequences important for capsid assembly, antigenic epitopes and receptor-binding sites of the capsid.

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References


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