Size and Antigenic Comparisons among the Structural Proteins of Selected Autonomous Parvoviruses

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SUMMARY

The size and antigenic relationships among structural proteins (VPs) of canine parvovirus (CPV), feline parvovirus (FPV), porcine parvovirus (PPV), minute virus of mice (MVM) and bovine parvovirus (BPV) were determined by SDS–PAGE of radiolabelled, purified virus and immunoprecipitated viral proteins. Mature virions of CPV, FPV, PPV and MVM were composed of three VPs designated VP1, VP2 and VP3. The corresponding proteins of each virus were similar in molecular weight [79 000 to 82 500 (VP1), 65 000 to 66 000 (VP2), 62 000 to 63 500 (VP3)]. Additional similarities among VPs were indicated by antigenic relationships which included precipitation of VPs of CPV, FPV and PPV by both homologous antisera and antisera raised to each of the other two viruses and by precipitation of VPs of MVM by cat anti-FPV sera. A non-structural protein identified in lysates of cells infected with FPV and CPV was precipitated by cat anti-FPV and dog anti-CPV sera only. Mature virions of BPV were composed of four VPs [74 500 (VP1), 67 000 (VP2), 60 000 (VP3), 57 500 (VP4)] which were antigenically unrelated to those of the other parvoviruses tested. However, the possibility that swine are sometimes infected with a virus which is antigenically related to BPV was suggested by the finding that sera from conventionally raised swine, irrespective of their serological status for PPV, precipitated VPs of BPV, whereas neither pre-exposure sera nor anti-PPV sera from gnotobiotic pigs did so.

INTRODUCTION

In a previous study we identified a relatively large group of autonomous parvoviruses that share one or more common or similar epitopes (Mengeling et al., 1986). Of nine viruses tested, namely minute virus of mice (MVM), Kilham rat virus, H-l virus (H-l), haemorrhagic encephalopathy of rats virus, porcine parvovirus (PPV), canine parvovirus (CPV), feline parvovirus (FPV), bovine parvovirus (BPV) and goose parvovirus (GPV), all but BPV and GPV were found to be antigenically related when their intracellular antigens were reacted by immunofluorescence microscopy (FA) with homologous and heterologous anti-viral sera.

Although the nature of the relevant antigen(s) has not been fully defined, several lines of evidence suggest that shared epitopes may be associated primarily with non-structural proteins (NSPs) (Cotmore et al., 1983; Mengeling et al., 1983). If such proteins are essential for virus replication as seems likely (Cotmore et al., 1983; Rhode, 1985a), they are probably conserved more or less conformationally as well as functionally among various members of the genus. On the other hand, it is known that at least a few of the autonomous parvoviruses also share epitopes associated with their structural proteins (VPs). Mink enteritis virus, CPV and FPV can be difficult to distinguish even by relatively stringent serological tests that involve only surface antigens of the virion (Johnson, 1967; Johnson & Spradbrow, 1979). These viruses are probably exceptions within the genus, however, and may actually represent host variants of the same virus (Johnson & Spradbrow, 1979; Lenghaus & Studdert, 1980; Moraillon et al., 1980; Wilson, 1980). Evidence for common epitopes among VPs of other autonomous parvoviruses is tenuous (Mengeling et al., 1986), but the extensive genomic homologies among several of the autonomous parvoviruses in portions of their genome that are believed to code for VPs (Banerjee
et al., 1981; McMaster et al., 1981; Mitra et al., 1982; Tratschin et al., 1982; Banerjee et al., 1983; Burd et al., 1983; Cotmore & Tattersall, 1984; Carlson et al., 1985; Rhode, 1985b; Ridpath et al., 1987) suggest that failure to detect such epitopes may be due to the stringency of the serological tests by which the viruses were compared.

The present study was designed to define more clearly the degree of antigenic homology among VPs of five autonomous parvoviruses (CPV, FPV, PPV, MVM, BPV) which were believed to represent a gradient of antigenic continuity (Mengeling et al., 1986). The VPs were identified by SDS-PAGE of radiolabelled, gradient-purified virus and tests for shared epitopes were performed by selective precipitation of radiolabelled, virus-infected cell lysates with antiviral sera. The experimental design also allowed for comparison of the number and Mr values of VPs prepared and tested under similar conditions.

METHODS

Viruses. Viruses included in the study were CPV, FPV, PPV, MVM, H-1 and BPV (Mengeling et al., 1986). Each was used to prepare radiolabelled, infected cell lysates, each, except H-1, was used to prepare antisera and radiolabelled, gradient-purified virus, and each except FPV was used to prepare monoclonal antibodies.

Antisera. Antisera were raised in the natural host of each virus. In general the procedure used for the production of antisera was as follows. Pre-exposure serum was collected and the animal was then exposed to virus oronasally and often also by additional routes. Most animals were exposed to virus at least twice and antisera were collected 2 or more weeks after the last exposure. During the immunization procedure animals were kept in isolation cages or isolation rooms, or both. At least two antisera for each virus were prepared either in animals from a parvovirus-free colony (dogs, cats, mice) or in gnotobiotic animals (pigs, calves). Additional antisera were collected from conventionally raised animals that were either experimentally or naturally exposed to one of the parvoviruses included in the study and from gnotobiotic pigs and calves that had been exposed experimentally to other swine and cattle viruses. Where relevant in subsequent sections of the text, antisera are identified as to whether they were raised in gnotobiotic or conventionally raised animals.

Monoclonal antibodies were raised in mice to CPV, PPV, MVM, H-1 and BPV by procedures described elsewhere (Van Deusen & Whetstone, 1981).

Cell cultures. Cell cultures used for virus propagation were Crandall feline kidney cells for CPV and FPV, foetal porcine kidney cells for PPV, foetal rat fibroblasts for MVM and H-1, and bovine embryonic spleen cells for BPV. Nutrient medium was Eagle's minimum essential medium supplemented with 10% or 20% foetal bovine serum, 0-25% lactalbumin hydrolysate, and gentamicin (50 µg/ml).

Preparation of radiolabelled viral antigens. Procedures were the same for each of the viruses. Cell cultures were grown in 28 cm² Petri dishes kept at 37°C in a humid environment containing 5% CO₂. Nutrient medium was aspirated when monolayers were 50% to 90% confluent and in a state of vigorous mitotic activity as evidenced by numerous cells in metaphase. Virus inoculum was added at an m.o.i. of between 10 and 100 and was kept in contact with the cells for 2 h. The inoculum was then aspirated and fresh medium was added. Six h later (i.e. 8 h after virus was first added to the culture) nutrient medium was replaced with a medium that was similar in composition but was free of lactalbumin hydrolysate and methionine except for 40 µCi/ml of [³⁵S]methionine (> 1000 Ci/mmol, DuPont). Cultures were harvested 16 h later (i.e. 24 h after virus was first added to the culture) by scraping infected cells into their nutrient medium. Non-infected control cell cultures were treated in parallel except that they were exposed to a 'sham' inoculum of nutrient medium during the interval when infected cultures were exposed to virus.

For preparation of radiolabelled, gradient-purified virus, the cell-free virus and infected cells were sedimented by centrifugation at 100000 g for 2 h. The supernatant fluid was discarded and the cells and virus from two Petri dishes were resuspended in 2 ml of high pH buffer (0.5 mM-EDTA, 50 mM-Tris-HCl pH 8.6 at 25°C) to dissociate virus from cell debris (Hallauer & Kronauer, 1965). The cell and virus suspension was then frozen and thawed twice, and filtered (0.22 µm). Radiolabelled virus present in the filtrate was purified by isopycnic centrifugation (Tattersall et al., 1976). Two peaks of radioactivity and virus haemagglutination activity, known from previous experiments to be likely to correspond to populations of virions variously identified as mature, complete, heavy or full (density about 1.33 g/ml and lacking all or most of the viral genome), were collected for each virus.

For preparation of radiolabelled, infected cell lysates, the cells were washed twice by alternate sedimentation (10000 g) and resuspension in wash buffer (150 mM-NaCl, 5 mM-Tris-HCl, 5 mM-EDTA, pH 7.2 at 25°C) and then lysed with lysis buffer (150 mM-NaCl, 50 mM-Tris-HCl, 5 mM-EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0 at 25°C). Lysates were incubated at 40°C for 30 min, 4°C for 2 h, and were then clarified by centrifugation at 100000 g for 30 min.
Radioactivity of purified virus and cell lysates was determined by trichloroacetic acid precipitation of 5 μl aliquots of virus or lysate onto pieces (1 cm²) of filter paper (Whatman No. 3). These pieces were subsequently rinsed in ethanol to remove unincorporated radiolabel and then submerged in scintillation fluid for counting.

**Immunoprecipitation and electrophoresis.** Radiolabelled, infected cell and non-infected cell lysates were diluted to contain a selected amount of radioactivity, usually 250 000 c.p.m., in 100 μl of lysis buffer. Antisera and pre-exposure sera were diluted to 100 μl in lysis buffer that contained an approximately 0.1% suspension of non-radioactive, non-infected cells. The type of cell matched that of the radiolabelled lysate with which the serum was subsequently reacted. The dilution of serum depended on the affinity of its immunoglobulins for Protein A, which varied markedly among species (Richman et al., 1982), and to a lesser extent on individual differences such as the relative concentration of immunoglobulin in a particular serum. Within practical limits the dilution was selected to reduce these differences. Dilutions ranged from 1:50 for some dog, cat and pig sera to 1:4 for mouse sera. Hydrated Protein A–Sepharose (Pharmacia) was diluted 1:10 in lysis buffer containing a 0.1% cell suspension as described above.

Immunoprecipitation was performed by (i) adding 100 μl of radiolabelled cellular lysate with the selected c.p.m. to 100 μl of the selected dilution of serum, (ii) incubating the mixture for 2 h at 37 °C with constant mixing, (iii) adding 100 μl of a 10% suspension of Protein A–Sepharose, (iv) incubating the mixture for 2 h at 37 °C with constant mixing, (v) sedimenting the Protein A–Sepharose–immunoglobulin–antigen (or antigen-free) complex by centrifugation (3000 g) and (vi) washing the complex twice in lysis buffer and twice in distilled water by alternate resuspension and sedimentation (3000 g).

For SDS–PAGE the sedimented complex of step (vi) above was resuspended in 50 μl of sample buffer (67-5 mM-Tris–HCl, 5% 2-mercaptoethanol, 10% glycerol, 2% SDS, 0.015% bromophenol blue, pH 6.8 at 25 °C), heated to 100 °C for 3 min, added (25 μl) to a sample well of a polyacrylamide (4% stacking, 10% resolving) gel and electrophoresed in a vertical gel system (Model SE 400, Hoefer Scientific Instruments, San Francisco, Ca., U.S.A.) until the dye front was eluted from the bottom of the gel (about 18 h at 5 mA). Radiolabelled and unlabelled protein Mr markers were also electrophoresed in each gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue to determine the relative quantity and position of immunoglobulins in each lane of the gel. It was then photographed, impregnated with scintillator (EN³HANCE, DuPont), dried and used to expose X-ray film (X-Omat AR, Eastman Kodak).

Radiolabelled, gradient-purified virions, and infected cell and uninfected cell lysates were electrophoresed in a similar fashion except that only radiolabelled protein Mr markers were run in gels free of immunoglobulins and such gels were not stained with Coomassie Brilliant Blue and photographed before they were dried and used to expose X-ray film.

**Molecular weight markers.** Two sets of Mr markers were included in the study. One set (Bio-Rad) was identified by staining with Coomassie Brilliant Blue. Proteins included in this set and their indicated Mr values were: phosphorylase b, 92 500; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme, 14 400. The other set (Amersham) was identified by radioactivity ([14C]methylated). Radiolabelled proteins and their indicated Mr values were: myosin, 200 000; phosphorylase b, 92 500; bovine serum albumin, 69 000; ovalbumin, 46 000; carbonic anhydrase, 30 000; lysozyme, 14 300. A minor component with an Mr of 100 000, associated with radiolabelled phosphorylase b, is identified in subsequent illustrations. However, neither this nor the lysozyme marker (which was often electrophoresed out of the gel) was used in calculations to estimate the Mr values of VPs. Note that slightly different Mr values were ascribed to several of the proteins common to both sets of markers. To determine whether such values reflected real differences, we electrophoresed both sets of markers in the same lane as well as different lanes of the same gel. By then superimposing fixed points of the fluorograph over the corresponding dried gel, we found that all of the Coomassie Brilliant Blue-stained markers had migrated slightly farther than had the radiolabelled markers regardless of their indicated Mr values.

**Estimation of molecular weights of viral proteins.** The Mr values of VPs of CPV, FPV, PPV, MVM and BPV were estimated by a standard procedure in which the relative distance of migration of VPs and proteins of defined Mr are compared by SDS–PAGE (Laemmli, 1970). Preliminary trials indicated that none of the markers migrated to the same position as any of the VPs and so markers were run with VPs in the same lanes as well as alone in the same gel. The relative positions of most proteins in the gel were determined by densitometric tracing of a film exposed to a dried gel containing radiolabelled VPs and markers. When the position of a VP was not adequately resolved by densitometry because of its proximity to another dense band, i.e. VP2 of BPV, VP3 of CPV, FPV, MVM and VP4 of BPV, the position was determined manually. The myosin marker was used as the point from which all other migrations were measured and the positions of the remaining markers were used to estimate the Mr of VPs by the method of Schäffer & Sederoff (1981); namely, Mr = a + [c/(D – b)] where D is the distance migrated, and a, b and c are least square estimates based on migration of Mr markers. In all cases where the same VP was associated with both mature and immature virions, its Mr was estimated by taking the average of the two values.
RESULTS

Identification of VPs

The number of VPs associated with each of the autonomous parvoviruses tested was dependent on the stage of virion development, i.e. mature or immature, and the virus in question (Fig. 1). Mature virions were composed of either three (CPV, FPV, PPV, MVM) or four (BPV) VPs, whereas immature virions were composed of either two (CPV, FPV, PPV, MVM) or three (BPV) VPs. The $M_r$ values of individual classes of VPs of CPV, FPV, PPV, MVM were similar, ranging from 79000 to 82500 for VP1, 65000 to 66000 for VP2 and 62000 to 63500 for VP3; those of BPV were 74500 for VP1, 67000 for VP2, 60000 for VP3 and 57500 for VP4 (Table 1).

To provide an indication of the accuracy of the applied equation in predicting $M_r$ values and to assess the consistency of protein migration in different lanes of the gel, the markers were treated as unknowns and their $M_r$ was calculated from distance migrated. The mean (and range) of 12 observations, i.e. 12 lanes (Fig. 1), were: phosphorylase b 92428 (91914 to 92890), bovine serum albumin 69388 (68749 to 69664), ovalbumin 45687 (45321 to 45881) and carbonic anhydrase 30069 (29809 to 30516).

Most of the VPs that had been identified by electrophoresis of gradient-purified virus were selectively precipitated from infected cell lysates by monoclonal antibodies that, based on their haemagglutination inhibition (HI) activity, were capable of reacting with epitopes on the
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Fig. 2. Identification of VPs of autonomous parvoviruses with monoclonal antibodies. Lanes 1 and 8, Mr markers. Lanes 2 to 7, immunoprecipitation of lysates of cells infected with BPV, PPV, MVM, CPV, FPV and H-1 respectively. Antigens of FPV were precipitated by mouse monoclonal antibody raised to CPV. All others were precipitated by homologous mouse monoclonal antibody. All monoclonal antibodies were present in ascitic fluid and all had HI titres for the virus with which they were reacted (see text for individual titres).

Fig. 3. Precipitation of VPs of PPV with homologous and heterologous antisera. Lane 1, Mr markers. Lane 2, uninfected cell lysate. Lane 3, PPV-infected cell lysate. Lanes 4 to 11, immunoprecipitation of PPV-infected cell lysate with the following sera: lanes 4 and 5, pig 23 anti-PPV and pre-exposure sera respectively; lanes 6 and 7, cat 302 anti-FPV and pre-exposure sera respectively; lanes 8 and 9, dog 109 anti-CPV and pre-exposure sera respectively; lanes 10 and 11, calf 9605 anti-BPV and pre-exposure sera respectively. The protein of about 45 000 Mr evident in this and other illustrations is presumed to be actin. Similar results (not illustrated) were obtained when CPV- and FPV-infected cell lysates were tested with aliquots of the same sera except that, as with the results with monoclonal antibodies (see Fig. 2), VPs of CPV and FPV were not detected.

surface of the virion. Homologous HI titres were: anti-BPV, 1280; anti-PPV, 2560; anti-MVM, 40960; anti-CPV, 20480; anti-H-1, 2560. However, with the exception of PPV, the VP (VP3 or VP4) associated only with mature virions was absent, probably due to sedimentation of such virus during preparation of the lysate. The VP2 of H-1 migrated to about the same position as VP2s of CPV, FPV, PPV and MVM (Fig. 2).

All of the VP2s (VP3 for BPV) and many of the other VPs identified by electrophoresis of gradient-purified virus and by selective immunoprecipitation with monoclonal antibodies were also evident following side-by-side electrophoresis of non-infected and infected cell lysates (e.g. lanes 2 and 3, Fig. 3). In addition, a poorly defined band of protein(s) with an Mr of about 28000 was identified in each of several BPV-infected cell lysates prepared during the course of the study. A visual comparison of the density of bands of VP2s (VP3 for BPV) in aliquots of the different virus-infected cell lysates run in the same gel indicated that all were of similar concentration and suitable for immunoprecipitation and tests of antigenic relatedness.

Antigenic comparisons among VPs

Tests of infected cell lysates of CPV, FPV, PPV and BPV with antisera raised in the natural host of each of the viruses revealed a reciprocal antigenic relationship among the VPs of CPV, FPV and PPV (Fig. 3). In contrast, precipitation of VPs of BPV with heterologous antisera was weak or absent except when BPV-infected cell lysate was reacted with either pre-exposure or
Fig. 4. Precipitating activity for BPV in pig sera. Lane 1, Mr markers. Lanes 2 to 13, immunoprecipitation. Lanes 2, 4, 6, 8, 10 and 12, BPV-infected cell lysate with calf 9605 anti-BPV serum (lane 2), gnotobiotic calf 26 anti-BPV serum (lane 4), pig 23 anti-PPV serum (lane 6), gnotobiotic pig 2B anti-PPV serum (lane 8), gnotobiotic pig 1A anti-PPV serum (lane 10) and pig 73 anti-PPV serum (lane 12). Lanes 3, 5, 7, 9, 11 and 13, PPV-infected cell lysate with the same sera as indicated for lanes 2, 4, 6, 8, 10 and 12 respectively.

post-exposure (anti-PPV) pig sera. Precipitation of VPs of BPV by pre-exposure pig serum suggested possible exposure of the pig to other cross-reactive antigens in its environment even though it had been derived by hysterectomy, deprived of colostrum and raised in contact only with other pigs treated similarly. To address this possibility in part, BPV-infected cell lysate was reacted with anti-PPV sera collected from two gnotobiotic pigs and from two pigs raised under less defined conditions (one described above and one obtained from a PPV-free farm herd). The VPs of BPV were precipitated by both of the anti-PPV sera from the more or less conventionally raised pigs, but not by either of the anti-PPV sera from gnotobiotic pigs. Conversely, the homologous reactions, i.e. anti-PPV sera with PPV, were at least as marked with the antisera from the gnotobiotic pigs (Fig. 4).

The above findings with BPV antigens led us to test the cross-reactivity of anti-PPV sera raised in gnotobiotic pigs with antigens of other paroviruses. Lysates of cells infected with PPV, CPV, FPV, MVM or BPV were each reacted with each of the two gnotobiotic, anti-PPV sera used in the previous experiment. Both of the sera precipitated VPs of PPV, CPV and FPV, whereas they had little or no precipitating activity for VPs of MVM and BPV (Fig. 5).

Precipitation of VPs of BPV by sera collected from conventionally raised pigs, irrespective of their serological status for PPV, suggested that BPV might share epitopes with some other common porcine virus. To test this hypothesis BPV-infected cell lysate was reacted with antisera raised in gnotobiotic pigs for pseudorabies virus, swine influenza virus, swine enterovirus, reovirus, haemagglutinating encephalomyelitis virus, swine adenovirus, swine transmissible gastroenteritis virus and encephalomyocarditis virus. None of these antisera precipitated VPs of BPV.

The first preparation of anti-MVM serum used in the study was a pool of antisera raised in mice by exposing them oronasally to the virus. When this antisera pool was tested by immunoprecipitation there was no evidence of cross-reactivity with antigens of CPV, FPV, PPV or BPV; however, neither was the homologous reaction quantitatively comparable to those of the other paroviruses previously examined. While the difference could be attributed at least in part to the relatively low affinity of mouse immunoglobulin for Protein A, especially in comparison with dog, cat and pig immunoglobulins, it may also have reflected a poor immune
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Fig. 5. Homologous and heterologous precipitating activity of gnotobiotic pig anti-PPV sera. Lane 1, Mr markers. Lanes 2 to 11, immunoprecipitation. Gnotobiotic pig 2B anti-PPV serum (even-numbered lanes) and gnotobiotic pig 1A anti-PPV serum (odd-numbered lanes) with PPV-infected cell lysate (lanes 2 and 3), CPV-infected cell lysate (lanes 4 and 5), FPV-infected cell lysate (lanes 6 and 7), BPV-infected cell lysate (lanes 8 and 9) and MVM-infected cell lysate (lanes 10 and 11). Inset, Coomassie Brilliant Blue stain of the gel showing precipitated immunoglobulins electrophoresed in lanes 2 to 11.

Table 2. Relative precipitating activity of selected cat anti-FPV sera

<table>
<thead>
<tr>
<th>Antiserum number (cat)</th>
<th>Virus strain*</th>
<th>HI titre</th>
<th>Precipitating activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>ICK-33</td>
<td>1280</td>
<td>MVM VPs + +</td>
</tr>
<tr>
<td>294</td>
<td>ICK-33</td>
<td>1280</td>
<td>FPV NSP + + +</td>
</tr>
<tr>
<td>301</td>
<td>Johnson</td>
<td>1280</td>
<td>MVM VPs + +</td>
</tr>
<tr>
<td>302</td>
<td>Johnson</td>
<td>640</td>
<td>FPV NSP + +</td>
</tr>
</tbody>
</table>

* Strain used for immunization. ICK-33 is virulent, Johnson is attenuated.
† Subjective evaluation of relative precipitating activity; + + + strong to ± barely perceptible (and often absent). All of these sera had strong precipitating activity with the VPs of FPV. Several reactions are also illustrated, see Fig. 6, 7 and 8.

response of the donors with regard to precipitating antibodies. As a consequence, sera from 10 mice of a colony known to be endogenously infected with MVM were tested by immunoprecipitation with MVM-infected cell lysate and two were chosen for subsequent experiments following the finding that they were slightly more effective for immunoprecipitation than was the pool of antisera prepared experimentally. The only cross-reaction subsequently detected with the selected sera was between one of the two sera and the VPs of H-1. To test the reciprocal relationships, MVM-infected cell lysate was reacted with antisera for MVM, CPV, FPV, PPV and BPV. The VPs of MVM were precipitated by both anti-MVM and anti-FPV sera, and to a very minor extent by anti-CPV sera, but not by either anti-PPV or anti-BPV sera.

The unilateral nature of the cross-reactivity between MVM and FPV led us to examine their apparent antigenic relationship in greater detail. Because neither a pool of anti-MVM sera nor either of two individually tested anti-MVM sera had precipitated VPs of FPV in prior experiments, this 'negative' aspect of the relationship was not pursued further. However, three
additional anti-FPV sera were tested for their ability to precipitate VPs of MVM. Although all were essentially indistinguishable on the basis of their relative precipitating activity for VPs of FPV, their effectiveness in precipitating VPs of MVM varied markedly. Precipitating activity did not appear to be directly related to the strain of virus with which the cat had been immunized or to the HI titre of the serum (Table 2). Three additional anti-CPV sera were also tested because of the antigenic similarity of FPV and CPV and the expectation that antisera to either of these two viruses would react similarly with other antigens. However, none of the anti-CPV sera precipitated any appreciable amount of MVM antigen.

Non-structural proteins

The use of several anti-FPV sera to evaluate the cross-reactivity between anti-FPV serum and VPs of MVM revealed that not only did these sera differ in their quantitative capacity to precipitate VPs of MVM but they also differed markedly in their capacity to precipitate what was believed to be a virus-induced NSP (Mr, approx. 85 000) present in FPV-infected cell lysate. The NSP had not been identified definitely in prior experiments, apparently because anti-FPV serum 302 (Table 2) had been used to evaluate antigenic relationships. However, retrospective examination of fluorographs indicated that a barely perceptible amount of NSP may have been occasionally precipitated by this serum. Cross-reactive sera, i.e. dog anti-CPV and pig anti-PPV, appeared to be similar to cat anti-FPV serum 302 in that they efficiently precipitated VPs of FPV but had little or no precipitating activity for NSP. We speculated that with these sera it would be possible to precipitate the VPs from a FPV-infected cell lysate while leaving the NSP
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Fig. 7. Relative precipitating activity of anti-FPV and anti-CPV sera for VPs and NSP. (a) Lanes 1 and 11, Mr markers. Lane 2, FPV-infected cell lysate. Lane 5, uninfected cell lysate. Lane 8, CPV-infected cell lysate. Lane 3, 4, 6, 7, 9 and 10, immunoprecipitation as follows: FPV-infected cell lysate with cat 294 anti-FPV serum (lane 3) and dog 145 anti-CPV serum (lane 4); uninfected cell lysate with cat 294 anti-FPV serum (lane 6) and dog 145 anti-CPV serum (lane 7); CPV-infected cell lysate with cat 294 anti-FPV serum (lane 9) and dog 145 anti-CPV serum (lane 10). (b) Coomassie Brilliant Blue stain of corresponding gel showing precipitated immunoglobulins electrophoresed in lanes 3, 4, 6, 7, 9 and 10. (c) Relevant portion of lanes 9 and 10 containing virus-coded proteins overexposed to show faint bands more clearly; from top to bottom the bands, indicated by arrows, represent (1) NSP, (2) VP-1, (3) an undefined protein Mr approx. 71,500 seen only when some FPV-infected and CPV-infected cell lysates were reacted with either anti-FPV or anti-CPV serum and (4) VP-2. (d) Lanes 3 and 4 treated as in (c).

in suspension. To test this hypothesis aliquots of FPV-infected cell lysate were reacted with anti-FPV serum 302 and with anti-CPV and anti-PPV sera that, based on results of prior experiments, had little or no precipitating activity for NSP. A monoclonal antibody raised to purified CPV and known to precipitate VPs of FPV was run in parallel as a control. The ‘adsorbed’ lysate was then reacted with anti-FPV serum 294 (Table 2) to precipitate the remaining NSP. In all cases the relative concentration of NSP in the lysate was increased by selective precipitation of VPs. The VPs were adsorbed more completely with antisera than they were with monoclonal antibody (Fig. 6).

The difference in the ability of different anti-FPV sera to precipitate NSP from FPV-infected cell lysate led us to examine the same phenomenon with regard to anti-CPV sera and CPV-infected cell lysate. While all of the four dog anti-CPV sera examined had excellent precipitating activity for VPs of CPV, three were only moderately to slightly reactive and one was apparently non-reactive with NSP. By immunoprecipitation of several CPV-infected cell and FPV-infected cell lysates with the same antisera it was evident that the relative concentration of VPs and NSP varied among the different lysates even though they had been prepared in essentially the same manner. For example, a different FPV-infected cell lysate was used for the experiment illustrated in Fig. 6 than was used for those illustrated in Fig. 7 and 8. However, the apparent concentration of NSP in a lysate as determined by immunoprecipitation was, in major part, a reflection of the precipitating activity of a particular serum. When cat anti-FPV and dog anti-
CPV sera (which were judged to be most reactive with homologous NSP from previous results) were run in parallel against both FPV-infected and CPV-infected cell lysates, the cat anti-FPV serum precipitated more NSP from both (Fig. 7).

The ability of cat anti-FPV serum 294 to precipitate NSP efficiently from lysates of FPV-infected and CPV-infected cells led us to test this serum against lysates prepared for all of the parvoviruses included in the study. With the exception of the precipitation of NSP of FPV and CPV, the results with serum 294 (Fig. 8) were the same as those previously obtained with serum 302. Both antisera precipitated the VPs of PPV, MVM, CPV and FPV, but not those of BPV. Antigens of H-1 were not precipitated by antiserum 294; they had not been tested against serum 302.

**DISCUSSION**

Our findings regarding the number of VPs associated with purified virions of CPV, FPV, PPV, MVM and BPV were consistent with those previously reported (Tattersall _et al._, 1976; Carman & Povey, 1983; Lederman _et al._, 1983; Molitor _et al._, 1983; Surleraux _et al._, 1986). The appearance of an additional VP in association with mature virions is characteristic of autonomous parvoviruses and results from the enzymic cleavage of VP2, or VP3 in the case of BPV, to VP3 and VP4, respectively, during the final stages of virus assembly and maturation (Tattersall _et al._, 1976). The apparent absence of these cleavage products in most infected cell lysates can probably be attributed to sedimentation of mature virions by clarification of the lysates at 100 000 g during the final step in their preparation. We are unable to explain, however, why lysates of PPV-infected cells were not affected similarly (e.g. see Fig. 2) unless capsids of PPV are more likely to dissolve in the lysis buffer. This difference between PPV and the other parvoviruses under study was observed with all of several lysates prepared for each of the viruses and when either polyclonal or monoclonal antibodies were used for immunoprecipitation.

In general, our estimates of _M_ values differed somewhat from those of other workers. While our values for VPs of BPV (Table 1) are difficult to reconcile with previous estimates (Lederman _et al._, 1983), namely 80 000 (VP1), 72 000 (VP2), 62 000 (VP3) and 60 000 (VP4), others are likely to be within the limits of statistical error and additional variables associated with experimental procedures and fundamental assumptions. For example, slightly different _M_ values may be ascribed to the same _M_ markers by various suppliers. The same factors probably explain the difference between our estimate of 85 000 _M_ for the NSP associated with lysates of cells infected with CPV and FPV and a previous estimate of 87 000 _M_, for what is likely to be the same protein (Parrish & Carmichael, 1983). However, despite the potential inaccuracies inherent in the method used to determine _M_ values, their assessment under similar conditions was expected to provide a dependable indication of relative size. With this assumption, it appears that the corresponding VPs of CPV, FPV PPV and MVM are much closer in _M_ (Table 1) than suggested by previous reports in which estimates collectively ranged from 77 500 to 86 000 for VP1s, 63 000 to 70 000 for VP2s and 60 000 to 67 000 for VP3s (Tattersall _et al._, 1976; Carman & Povey, 1983; Lederman _et al._, 1983; Molitor _et al._, 1983; Parrish & Carmichael, 1983; Surleraux _et al._, 1986).
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The range of $M_r$ values of VP2s (65000 to 66000) was only about 1.5% of the mean and raised a question as to whether these proteins might be almost identical in $M_r$. To provide an estimate of the variation that might be expected of identical proteins under the conditions of our study, we looked at bovine serum albumin markers in the same lanes. Because the values for VP2s were an average from two lanes, i.e. mature and immature virions (Fig. 1), we took the average of the values for the bovine serum albumin markers in the same lanes and then determined the mean and range of the four averages. Taking the average had no effect since the values were 69479 for both lanes of PPV (lanes 4, 5), CPV (lanes 8, 9) and FPV (lanes 10, 11), and 69664 for both lanes of MVM (lanes 6, 7). These calculations revealed that the range of estimated $M_r$ values for bovine serum albumin (69479 to 69664) was only about 0.3% of the mean (69525) and suggested that the range of values (i.e. 1.5%) for VP2s reflected real differences in $M_r$.

An antigenic relationship among the VPs of CPV, FPV and PPV was established by reciprocal immunoprecipitation. The relationship between CPV and FPV was expected from previous work (Carman & Povey, 1983; Parrish & Carmichael, 1983; Mengeling et al., 1986), whereas their association with PPV was less predictable. We had previously found that pig anti-PPV sera had a low level of serum neutralization (SN) and HI activity for CPV and FPV which suggested cross-reactivity with an epitope on the surface of the virion. However, neither dog anti-CPV nor cat anti-FPV sera had any appreciable level of such activity for PPV (Mengeling et al., 1986). The contrasting finding of this study, that all of these antisera precipitated relatively large amounts of VPs from heterologous lysates, indicates an additional set of epitopes distinct from those involved in SN and HI or more limited functional properties of antibodies in regard to heterologous activity.

We had previously suggested that the cross-reactivity of cat anti-FPV serum with intracellular antigens of MVM by indirect FA might be due to NSP since the same serum had neither SN nor HI activity for MVM (Mengeling et al., 1986). While results of the present study do not exclude the possibility that NSP is involved in the FA reaction, they do at least extend the cross-reactivity to include VPs of MVM. There was no evidence that the same epitope was shared with CPV even though both cat anti-FPV and dog anti-CPV sera react with intracellular antigens of MVM by indirect FA (Mengeling et al., 1986). The question of whether particular strains of FPV and CPV may differ in this respect is addressed in part by considering that the anti-FPV sera that precipitated VPs of MVM were from cats exposed to two strains of FPV (Table 2), and the anti-CPV sera without such activity were from dogs exposed to a designated strain of CPV (KB) and other dogs exposed to two field isolates of CPV. Despite the numerous antigenic similarities between FPV and CPV, other workers have shown that each also possesses unique epitopes (Parrish et al., 1982; Parrish & Carmichael, 1983). We have no explanation as to why mouse anti-MVM sera did not cross-react with antigens of FPV, but suggest that either the mice from which antisera were collected did not respond immunologically to the epitope(s) in question because of lack of recognition or sufficient viral replication, or that the epitopes associated with the FPV antigen were inaccessible to the relevant mouse antibodies. There is a remote possibility that the common epitope(s) associated with FPV antigen was relatively unstable and as a consequence was denatured in lysis buffer. However, mouse anti-MVM serum also failed to react with antigens of FPV when tested by indirect FA (Mengeling et al., 1986). We observed a somewhat analogous situation in another study in which guinea-pigs and mice were immunized with gradient-purified preparations of CPV and PPV (data not shown). Although none of the antisera had heterologous HI activity, anti-CPV and anti-PPV sera raised in guinea-pigs were cross-reactive with intracellular antigens of PPV and CPV, respectively, by indirect FA whereas those raised in any of three strains of mice (BALB/c, DBA, C57) were not. Guinea-pig antisera also had higher homologous HI titres (10240 to 40960) than did mouse antisera (640 to 5120).

Although NSPs were demonstrated for only FPV and CPV, we assume, from the reports of others (Cotmore et al., 1983; Lederman et al., 1984; Molitor et al., 1985), that they were also present in lysates of cells infected with MVM, BPV and PPV. Several observations suggest that their identification by immunoprecipitation depends largely on properties of the antisera with which they are reacted. In an earlier study, NSP of PPV was precipitated by antisera raised to
one strain of PPV, but not to another (Molitor et al., 1985). In this study, precipitation of NSPs of FPV and CPV was also related to the antiserum used. In addition, the ill-defined band of protein(s) with an $M_r$ value of about 28,000 associated with each of three BPV-infected cell lysates prepared during the course of this study may correspond to the 28,000 $M_r$ NSP described for BPV (Lederman et al., 1984), yet none of the three anti-BPV sera we used in the study (two from gnotobiotic calves and one from a conventionally raised calf) precipitated this protein. Without identifying NSPs in each of the infected cell lysates we cannot provide a general assessment of the relative degree of cross-reactivity among parvoviruses in regard to their VP s and NSPs. However, it is obvious from the results with FPV that heterologous, e.g. pig anti-PPV, as well as homologous antisera may be highly reactive with VPs and yet have little or no activity for NSP of the same virus. In contrast, none of the antisera precipitated only NSP.

We did not obtain any conclusive evidence as to why VPs of BPV were precipitated by certain pig sera. The absence of such activity in sera from gnotobiotic pigs exposed to either PPV or any of several other common viruses of pigs seemed to exclude the possibility that the results were due to epitopes shared between BPV and any of the viruses tested. It was also unlikely that the pigs in question had simply responded to BPV since none of their sera had any HI activity for BPV. If there were a tendency for pig immunoglobulins to attach non-specifically to VPs of BPV, then those of gnotobiotic pigs might be expected to behave similarly. For these reasons we suspect that the reactions were due to antibody raised to an, as yet unidentified but probably common, antigen in the pig's environment. It is of related interest that an antibody reactive towards BPV has also been detected in human sera by indirect FA (Mengeling & Paul, 1986) and such sera also immunoprecipitate VPs of BPV (W. L. Mengeling, unpublished). The present results further support the possibility of another antigenically related group of parvoviruses whose members may be difficult to propagate in vitro and thus remain largely unrecognized.

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REFERENCES


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