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Canine Parvovirus (CPV) Vaccination: Comparison of Neutralizing Antibody Responses in Pups after Inoculation with CPV2 or CPV2b Modified Live Virus Vaccine

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Canine parvovirus type 2 (CPV2) emerged in 1978 as causative agent of a new disease of dogs. New antigenic variants (biotypes), designated CPV2a and CPV2b, became widespread during 1979 to 1980 and 1984, respectively. At the present time the original CPV2 has disappeared in the dog population and has been replaced by the two new viruses. In the present study the comparison of neutralizing antibody titers in two groups of pups (18 pups in each group) inoculated with CPV2 and CPV2b modified live virus vaccines is reported. Using the hemagglutination inhibition (HI) test, relevant differences between antibody titers, against either the homologous or the heterologous virus, were not constantly observed. Using the neutralization (Nt) test, however, the pups inoculated with CPV2 had antibody titers which were approximately 30 times higher to the homologous virus (mean, 4,732) than to the heterologous virus (CPV2b) (mean, 162). The results of these experiments support two conclusions: (i) the HI test may not always accurately evaluate the true immune status of dogs with respect to CPV, and (ii) dogs inoculated with CPV2 vaccine develop relatively low Nt antibody titers against the heterologous virus (CPV2b). These data may suggest an advantage for new vaccines, considering that most presently licensed vaccines are produced with CPV2, which no longer exists in the dog population.

Canine parvovirus type 2 (CPV2) emerged in 1978, almost simultaneously in Europe and North America, as a new pathogen of dogs that was responsible for myocarditis and hemorrhagic gastroenteritis in pups (2, 7, 11, 12). The close antigenic and genomic relationships that exist between CPV2, feline panleukopenia virus, and mink enteritis virus (18) suggest that CPV2 may have originated by genetic mutation in a wild host receptive to one of the feline panleukopenia virus-like parvoviruses that infected carnivores (19).

By use of monoclonal antibodies, restriction enzyme analysis, and DNA sequencing, Parrish et al. demonstrated that the original antigenic type (CPV2) has been replaced, over the period from 1979 to 1981, by an antigenic variant or biotype (CPV2a) that differs from the original strain in three coding regions of the gene for the VP2 capsid protein (13, 14). A second biotype (CPV2b) appeared around 1984, and the only significant difference from CPV2a was the substitution of one amino acid (Asn→Asp) in the VP2 protein (13, 14). Both of these biotypes have now replaced the original strain CPV2 throughout the canine population worldwide. In particular, in the United Kingdom, Australia, and Italy the CPV2a biotype is more common than the CPV2b biotype; in Germany and Spain the two biotypes appear to be distributed about equally; and, in contrast, CPV2b appears to be more common in the United States (6, 8, 10).

An important question concerns the clinical and immunological significance of the antigenic variation of CPV2. Previously, experiments have not demonstrated any significant relevance of the antigenic changes with respect to the ability of CPV2 vaccines to protect dogs from the infection (1, 9). Furthermore, a preliminary study showed a one-way cross-reactivity (CPV2b→CPV2) of sera from pups inoculated with CPV2 or CPV2b modified live virus vaccines (17).

The aim of this study was to compare the neutralizing antibody titers of two groups of dogs inoculated, respectively, with a CPV2 or CPV2b modified live virus vaccine. Our results pose questions regarding the interpretation of serological data, especially those obtained by hemagglutination inhibition (HI) tests, with respect to the immune status of pups.

MATERIALS AND METHODS

Vaccines. (i) CPV2 vaccine. A modified live CPV2 vaccine (17/80 ISS strain) (3) with a titer of $10^{5.50}$ tissue culture infectious doses ($TCID_{50}$)/ml was used. The virus was cultivated on the canine A-72 cell line grown in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum.

(ii) CPV2b vaccine. A modified live CPV2b vaccine (29/97-40 strain) (5) with a titer of $10^{4.50}$ $TCID_{50}$ was used. The virus was cultivated on the Crandell feline kidney (CrFK) cell line grown in DMEM supplemented with 10% fetal calf serum.

(iii) Virus titrations. The virus titration test was performed in microtiter plates. Tenfold dilutions of each virus were prepared in quadruplicate in DMEM and mixed with 50 μl of a suspension containing 200,000 A-72 cells for CPV2 vaccine and 200,000 CrFK cells for CPV2b vaccine. The plates were incubated at 37°C for 5 days in a humidified CO_2 atmosphere. The plates were then frozen and thawed three times, and the supernatant of each well was tested for CPV hemagglutination (HA) activity using 1% pig erythrocytes. Fifty percent end points were calculated using the Kärber formula.

Experimental procedures. Thirty-six pups, 9 to 10 weeks old, from seven litters were randomly assigned to two groups (A and B) and housed in two separate and isolated facilities. The pups in each group were handled by different workers. All pups were serologically negative to CPV at the time of vaccination, as determined by HI and neutralization (Nt) tests. Group A pups (n = 18) were inoc-

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TABLE 1. Results of HI test on pups (group A) inoculated with CPV2 vaccine

Pup no.	Antibody titer	
	CPV2	CPV2b
1	320	160
2	2,560	1,280
3	2,560	640
4	640	2,560
5	2,560	640
6	2,560	2,560
7	2,560	2,560
8	1,280	2,560
9	10,240	10,240
10	5,120	1,280
11	1,280	1,280
12	1,280	640
13	1,280	640
14	2,560	1,280
15	2,560	320
16	1,280	640
17	2,560	1,280
18	2,560	1,280
Mean	1,950	1,138

TABLE 2. Results of HI test on pups (group B) inoculated with CPV2b vaccine

Pup no.	Antibody titer	
	CPV2	CPV2b
19	2,560	2,560
20	2,560	2,560
21	5,120	2,560
22	10,240	5,120
23	1,280	2,560
24	2,560	2,560
25	2,560	5,120
26	1,280	2,560
27	1,280	2,560
28	1,280	2,560
29	2,560	5,120
30	2,560	5,120
31	5,120	5,120
32	2,560	5,120
33	5,120	5,120
34	5,120	5,120
35	2,560	5,120
36	1,280	1,280
Mean	2,655	3,475

ulated subcutaneously with 1 ml of the CPV2 vaccine, and group B pups ($n = 18$) received 1 ml of the CPV2b vaccine. Thirty days after vaccination, the antibody titer of each pup was evaluated by HI and Nt tests using both CPV2 and CPV2b viruses. No illness was observed in any pup throughout the study.

Serological assays. (i) HI test. HI tests were carried out at 4°C using 1% pig erythrocytes and 8 HA units of either CPV2 (17/80 ISS strain) or CPV2b (29/97-40 strain). Serial twofold serum dilutions were made in phosphate-buffered saline (pH 7.2), starting from a 1:10 dilution. Titers were expressed as the reciprocal of the highest serum dilution that completely inhibited the HA, and the geometric means were also calculated.

(ii) Nt test. Serial twofold dilutions (starting from 1:10) of each serum in DMEM were mixed with 50 μl of a virus suspension containing 100 to 300 $TCID_{50}$ of either CPV2 (17/80 ISS strain) or CPV2b (29/97-40 strain). After 1 h of incubation at room temperature, 100 μl of a suspension containing 200,000 A-72 cells for CPV2 and 200,000 CrFK cells for CPV2b was added to each well. The plates were incubated at 37°C in a humidified CO_2 atmosphere for 5 days, and virus was then detected using the HA test as described above for virus titration. Neutralizing antibody titers were calculated as the reciprocal of the highest serum dilution that completely neutralized the virus (absence of HA activity). The geometric means of each group (A and B) also were calculated.

Statistical analysis. The statistical analysis was performed using the Kruskal-Wallis one-way analysis of variance on ranks.

RESULTS

The results of HI tests carried out on the sera of pups inoculated with CPV2 (group A) or CPV2b (group B) are reported in Tables 1 and 2, respectively. For dogs given CPV2 (group A) (Table 1), the homologous geometric mean antibody titer was 1,950 and the mean heterologous titer was 1,138, and this difference is statistically significant ($P = 0.036$). For dogs inoculated with CPV2b (group B) (Table 2), the homologous and heterologous geometric means were, respectively, 3,475 and 2,655; the difference is not statistically significant ($P = 0.108$).

The Nt antibody titers of pups inoculated with CPV2 (group A) or CPV2b (group B) are shown in Tables 3 and 4, respectively. In general, higher titers were observed in pups inoculated with CPV2 than in pups inoculated with CPV2b. The homologous geometric mean antibody titer for pups in group A (Table 3), inoculated with CPV2, was 4,732, whereas the mean heterologous titer was 162, which is a statistically significant difference ($P < 0.01$). Pups in group B (Table 4), inoculated with CPV2b, had a homologous mean titer of 1,138 and a mean heterologous titer of 940, which is not statistically significant ($P = 0.184$).

DISCUSSION

Effective vaccines are generally available for the prevention of CPV2 infection. Both modified live and inactivated CPV2 vaccines have been demonstrated to have the ability to immu-

TABLE 3. Results of Nt test on pups (group A) inoculated with CPV2 vaccine

Pup no.	Antibody titer	
	CPV2	CPV2b
1	320	<10
2	10,240	160
3	2,560	160
4	2,560	160
5	20,480	160
6	10,240	320
7	2,560	320
8	1,280	640
9	20,480	1,280
10	5,120	160
11	10,240	320
12	5,120	160
13	2,560	40
14	5,120	160
15	2,560	40
16	10,240	320
17	10,240	160
18	5,120	320
Mean	4,732	162

TABLE 4. Results of Nt test on pups (group B) inoculated with CPV2b vaccine

Pup no.	Antibody titer	
	CPV2	CPV2b
19	1,280	640
20	640	640
21	640	640
22	1,280	1,280
23	1,280	640
24	5,120	2,560
25	640	2,560
26	1,280	1,280
27	2,560	640
28	2,560	640
29	640	1,280
30	640	1,280
31	640	1,280
32	640	1,280
33	640	2,560
34	320	1,280
35	640	1,280
36	640	1,280
Mean	940	1,138

nize pups efficiently. Inactivated vaccines, however, provide only a short immunity to the infection. Although dogs may be protected for several months against disease, they may have subclinical infection (15). In contrast, modified live vaccines have been shown to protect dogs for at least 2 to 3 years.

Previous studies (4, 15, 16) have demonstrated that pups with HI titers of >1:80 are immune to oronasal CPV2 challenge. In those studies, clinical illness was not observed, virus was not recovered from fecal specimens, and active serological responses were not observed after challenge. On the other hand, pups with HI titers of <1:40 become subclinically infected, with virus shedding and antibody responses indicative of active infection.

The results of this study raise puzzling questions about the clinical significance of the antigenic CPV biotypes regarding (i) the serological tests presently used for diagnosis and (ii) the actual immunity of vaccinated dogs, considering that most of the licensed CPV vaccines are presently produced from virus (CPV2) with the original antigenic structure.

The HI test using CPV2 as the antigen is presently employed by most investigators to measure antibody titers to CPV, since it has been correlated with the level of pup immunity to CPV2. However, the results presented here and our field experience indicate that the HI test, unlike the Nt test, does not always detect differences in amounts of antibody against either the homologous (CPV2) or the heterologous (CPV2b) virus. In contrast, using the Nt test, the antibody titers of pups vaccinated with CPV2 were much higher to the homologous virus (CPV2) than to the heterologous virus (CPV2b). These findings suggest that HI antibody values using a CPV2 antigen may not allow a true prediction of the earliest age at which pups would be expected to become susceptible to CPV infection or amenable to immunization. In several cases which we investigated, pups with HI of titers 1:160 to 1:320 to CPV2 were not vaccinated because they were considered still protected, yet they developed parvoviral disease caused by CPV2b shortly after serological evaluation was done (C. Buonavoglia, unpublished data). Such findings prompted this study.

Another question concerns the real immunity conferred by CPV2 vaccines against the CPV2a and CPV2b biotypes now circulating in the dog population. Our results (obtained by serological tests and without challenge trials) revealed that pups inoculated with CPV2 vaccine had significantly higher Nt antibody titers to the homologous virus (CPV2) than to the heterologous virus (CPV2b). In contrast, pups inoculated with CPV2b vaccine had similar Nt antibody titers to both viruses.

Despite the serological problem noted here, the problem may not be critical because, in the field, documented parvoviral infections due to CPV2 vaccine failures as a consequence of a low level of immunity in dogs against the heterologous viruses are rare. Pups vaccinated with CPV2 possess antibodies against the heterologous virus which probably are at levels which still provide complete or partial immunity to young pups at 2 to 4 months of age, a time when they are more susceptible to CPV infection. Nevertheless, there is evidence to suggest that optimal protection may not be provided if CPV2 vaccines are used, considering that (i) the original CPV2 has disappeared from the canine population worldwide and (ii) the CPV2 vaccine appears to confer a somewhat lower and shorter immunity against the CPV2b biotype. As suggested in 1982, "more effective vaccines that induce longer lived immunity to infection should be sought to control the spread of canine parvovirus" (15).

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