INFECTION AND IMMUNITY, Mar. 1981, p. 1177-1183
0019-9567/81/031177-07$02.00/0

Vol. 31, No. 3

In Vitro Identification and Characterization of a Virus Isolated from a Dog with Neurological Dysfunction

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A virus, 78-238, isolated from the cerebrospinal fluid of a dog with neurological dysfunction, was characterized as a paramyxovirus. This conclusion was supported by viral cytopathic effects and morphological appearance of virions and nucleocapsids in infected cells. Nucleocapsids were found in the cytoplasm of all infected cells and in the nuclei of 0.001% of these cells. Growth curves revealed that a high percentage (≥76%) of infectious progeny virus was cell released. Persistent infection of Vero cells with 78-238 showed a consistently high percentage of fluorescence-positive cells and a low proportion of hemadsorption-positive cells. Serological studies indicate that the virus was closely related to Simian virus 5 and reference canine parainfluenza virus.

Simian virus 5 (SV-5) viruses were first isolated from normal rhesus and cynomolgous kidney cell cultures in 1956 (22). In 1959, Schultz and Habel (30) described the isolation of an SV-5-like virus from a human volunteer with a common cold showing acute respiratory symptoms. In the following years, SV-5-like viruses were isolated from multiple tissue sources and from different diseases of humans such as respiratory illness, pemphigus skin lesions, infectious hepatitis, and Jacob-Creutzfeldt disease (21). In spite of the diversity of sources of isolation, there are virtually no significant biological differences among these virus strains (21).

An SV-5-like virus was first isolated from dogs with respiratory disease in 1967 (6). Since that report, additional isolations from the respiratory tracts of dogs with upper respiratory disease have been reported (1, 15, 16, 30). These canine parainfluenza virus (CPI) isolates were found to be closely related to SV-5 (25). Under natural conditions, clinical signs and pathological changes of CPI are thought to be restricted to the respiratory tract (1). Recently, however, a paramyxovirus, 78-238, was isolated from the cerebrospinal fluid of a dog suffering from incoordination and posterior paresis (J. Evermann, J. D. Lincoln, and A. I. McKeirnan, J. Am. Vet. Med. Assoc., in press). The virus, after eight passages in Vero cells and two plaque purifications, was sent to this laboratory to determine its neurological potential in virus-susceptible gnotobiotic dogs. The isolate was capable of inducing acute and chronic neurological disease (W. K. Baumgärtner, S. Krakowka, A. Koestner, and J. Evermann, submitted for publication). As part of our studies, the virus was further propagated in vitro and classified. The objectives of this study were to evaluate in vitro growth properties of the isolate in Vero cells; to determine the relationship between 78-238, reference CPI virus (strain D008), and SV-5; and to determine the relevance of 78-238 infection in selected canine populations by seroepidemiological means.

MATERIALS AND METHODS

Viruses. The virus isolate, 78-238, obtained from the spinal fluid of a dog with neurological dysfunction, was kindly provided by J. Evermann (Department of Veterinary Clinical Medicine and Surgery and Diagnostic Laboratory, Washington State University, Pullman). For comparative purposes, reference SV-5 virus strain 21005-2WR (23) and CPI strain D008 (6) were purchased from the American Type Culture Collection.

Cell cultures and virus titrations. Two continuous cell lines, African green monkey kidney (Vero) and MDCK, were maintained in minimal essential medium with Earle basal salt solution. Cells were found to be mycoplasma-free by the Ohio State University Public Health Laboratory. Growth medium consisted of minimal medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1% antibiotics (100 U of penicillin G and 100 μg of streptomycin per ml), and 1% $NaHCO_3$ (8.8%, wt/vol). D008 was propagated in MDCK cells; SV-5 and 78-238 were propagated in Vero cells. Preconfluent monolayers of Vero cells were infected at a multiplicity of infection (MOI) of 0.01 50% tissue culture infective dose ($TCID_{50}$) per cell. After incubation for 1 h at 34°C, cells were maintained with growth medium at 34°C. D008 and 78-238 were harvested when 80% of the cells showed syncytial giant cells; SV-5 was harvested 8 days postinfection (p.i.). After three cycles of freezing and thawing, the viruses were divided into portions and stored at −70°C. Infected cultures were maintained at either 34 or 37°C

with 5% CO_2. Virus titrations ($TCID_{50}$) were performed in a microtiter system by adding serial 10-fold dilutions of viral suspensions to cells (15,000 cells/well) and incubating for 7 days. Infectivity was calculated by the formula of Spearman-Karber (19).

Growth curves. Preconfluent monolayers of Vero cells were infected at an MOI of 0.01 $TCID_{50}$/cell with 78-238 and maintained in 25-cm^2 plastic flasks at both 34 and 37°C. Cells were harvested every 8 h p.i. for 2 days and every 11 h p.i. thereafter. Cells from duplicate flasks were scraped into the medium and centrifuged for 10 min at 200 × g. The supernatant containing the cell-released virus fraction was divided into portions and stored at −70°C until titration. The cellular sediment was resuspended in the original volume of fresh minimal medium, rapidly frozen and thawed three times, and clarified by centrifugation. The clarified supernatant containing the cell-associated virus was divided into portions and stored as described above.

Light microscopy and immunofluorescence (IF). To evaluate viral cytopathic effect (CPE), infected cells were propagated on glass microscope slides (Lab-Tek Products, Westmont, Ill.; four wells). At various intervals after infection, monolayers were fixed in Bouin fixative for 10 min and stained with hematoxylin and eosin.

For detecting viral antigens in infected cells, both direct and indirect IF methods were used. For direct IF, convalescent 78-238 gnotobiotic serum was conjugated to fluorescein isothiocyanate (24) and applied to acetone-fixed cells at a final dilution of 1:4. For indirect IF, immune serum (1:60) was incubated with monolayers for 45 min at room temperature and washed, and then the monolayers were stained with rabbit anticanine immunoglobulin (1:40) (Miles Laboratories, Inc., Elkhart, Ind.). For both methods, preinoculation canine serum and uninfected cell cultures were used as the controls.

Electron microscopy. For electron microscope evaluation of the replication cycle of 78-238, Vero cells infected with an MOI of 1.0 were harvested at the same intervals as described for the growth curves. After the medium was decanted, cultures were washed once, scraped from the plastic surface into 8 ml of phosphate-buffered saline and centrifuged for 10 min at 250 × g. The pelleted cells were suspended in 3% glutaraldehyde in cacodylate buffer for 1 h and postfixed in 1.33% osmium tetroxide in collidine buffer for 1 h. After dehydration in alcohol, the cellular pellets were embedded in Epon 812 (3). Thin sections were stained with uranyl acetate and lead citrate and examined on carbon-coated grids on a Philips 200 electron microscope. For negative staining, the virus was harvested from infected Vero cells by three cycles of rapid freezing and thawing. The crude suspension was clarified by centrifugation at 200 × g for 10 min and 1,000 × g for 30 min, and the final supernatant was ultracentrifuged at 82,000 × g for 40 min. The sediment was suspended in double-distilled water. A carbon-coated grid was put on the top of 1 drop of this suspension, the excess fluid was removed, and the grid was put for 30 s on a drop of 2% potassium phosphotungstic acid (28). Electron micrographs were taken with a Philips 200 electron microscope.

Hemagglutination (HA) and hemagglutination inhibition (HAI) tests and virus neutralization. **(i) Sera.** Immune sera against 78-238 were taken from gnotobiotic dogs experimentally infected with 78-238. Anti-SV-5 serum was purchased from the American Type Culture Collection. The anti-CPI serum (Manhattan strain) was kindly provided by M. J. G. Appel (James A. Baker Institute for Animal Health, Cornell University, Ithaca, N.Y.). All sera were heat inactivated for 30 min at 56°C and treated with potassium periodate solution (KIO_4) for destruction of nonspecific HAI. To 1 volume of serum, 3 volumes of freshly prepared 0.11 M KIO_4 was added, well mixed, and incubated at room temperature for 15 min. Thereafter, 3 volumes of 1% glycerol-saline solution was added to stop excess periodate. The serum starting dilution of 1:10 was reached by adding 3 volumes of saline solution, pH 7.2 (27).

To further determine the specificity of HAI, virus neutralization tests were performed essentially as described by Evermann et al. (in press).

(ii) HA and HAI. Whole blood was collected and gently mixed within an equal volume of Alsever solution. Erythrocytes for HA and HAI were washed three times and stored at 4°C as a 10% packed cell volume stock solution (27). Erythrocytes (0.5%, packed cell volume) from human (type O), canine, guinea pig, and chicken blood were tested for HA activity at three temperatures: 4, 22, and 37°C. The techniques used for hemadsorption, HA, and HAI are described elsewhere (11). To enhance HA titers of viral preparations, virus was treated with Tween 80 and ether (24). Briefly, 5% Tween 80 was added to a virus suspension under shaking at room temperature to a final concentration of 0.125%. Chilled ether (one-half of the virus suspension) was then added and mixed for 15 min at 4°C. After removing the ether by centrifugation and evaporation, the released HA could be stored at −70°C indefinitely or 2 months at 4°C before a decline of the HA titer was noticed. To determine the extent of immunological relatedness among the three strains, the formula of Archetti and Horsefall (2) was used.

Establishment of persistently infected cells. Vero cells were infected with an MOI of 2.0 and maintained at 34°C. Medium was changed every 3 days for 6 weeks. During this time, the cells were subcultured once. Persistently infected Vero cells were monitored for 20 passages for the presence of intracellular antigens by IF, for cell membrane-associated antigens by hemadsorption, and at selected intervals by light and electron microscopy.

Seroepidemiology. To determine the extent of infection within the dog population, a limited seroepidemiological study was performed. Thirty-five sera were collected from dogs entering the veterinary hospital (The Ohio State University, Columbus, Ohio). The dogs varied in age from 3 months to 14 years. According to clinical records, one-half of the dogs had been vaccinated against CPI with a commercial combination vaccine at least once in their lives. Either the remaining dogs were unvaccinated or vaccination histories were not available. A second group of 40 sera was collected from a licensed commercial beagle colony (Laboratory Research Enterprises, Inc., Kalamazoo, Mich. 49009). The sera were collected from bitches in 1978 and 1979. None of these dogs had been vaccinated with CPI. All dogs of both groups were, at the time of serum collection, judged clinically healthy

without involvement of the respiratory tract or nervous system. Serum titers below 1:20 were considered negative.

RESULTS

CPE of 78-238 in Vero cells. The first evidence of 78-238 infection in Vero cells consisted of small, focal, multinucleated syncytical giant cells 48 to 72 h. During the following 72 h, syncytia progressively enlarged to contain 20 to 40 nuclei per focus. Associated with this progressing CPE, individual cells became vacuolated and rounded before detaching from the culture vessel. By 6 days p.i., approximately 90% of the cells were detached from the monolayer. In the late stage of infection, intranuclear inclusion bodies were occasionally observed (Fig. 1). In contrast, the CPE in SV-5-infected Vero cells consisted only of small syncytia (three to four nuclei per cell) without progressive detachment of the monolayer. The CPE of strain D008 was intermediate between that of 78-238 and SV-5 in that both small syncytia and progressive cellular detachment were observed

Growth curves. Figure 1 shows the growth curves of 78-238 at 37°C. Eclipse periods before the appearance of extracellular infectious virus were 16 to 27 h at 34°C and 8 to 16 h at 37°C. At 34°C, peak infectivity ($10^{7.0}$ $TCID_{50}$/0.1 ml) was reached after 109 h. The percentage of cell-associated virus declined from 85% (27 h p.i.) to 15% (67 h p.i.). At 37°C a similar pattern was observed except that titers of cell-released virus exceeded those of cell-associated virus at 24 h and maximal total infectivity ($10^{7.5}$ $TCID_{50}$/0.1 ml) was reached after 24 h.

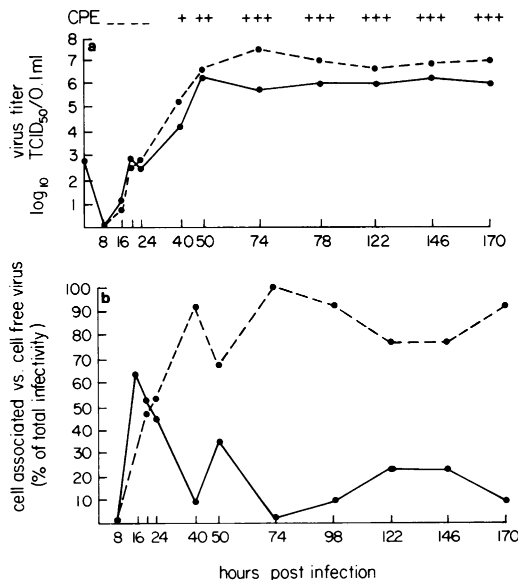

FIG. 1. Growth curves of 78-238 in Vero cells. (a) Virus titers of cell-free (---) and cell-associated (——) virus at 37°C. (b) Percentage of cell-free (---) versus cell-associated (——) virus at 37°C. CPE: ---, none observed; +, a few multinucleated cells; ++, 80% multinucleated cells; +++, giant cells and cell detachment.

IF. At 24 h p.i., virtually all 78-238-infected Vero cells contained focal fluorescent granules. By 3 days p.i. the focal areas had progressed to diffuse cytoplasmic fluorescence in multinucleated cells. At this time, increasing numbers of infected cells had brightening fluorescent-cell membranous spikes (Fig. 2).

Electron microscopy. The first evidence of 78-238 infection in Vero cells was seen 27 h p.i. Viral nucleocapsids in the form of densely packed areas within the cytoplasm consisted of granular and filamentous structures with a smooth surface and a diameter of 17 to 15 nm. By 38 h p.i., viral budding along with thickening of the cellular membrane and alignment of nucleocapsids was observed. Most of the budding particles were spherical and had diameters of 70 to 130 nm, although some measured up to 250 nm. In addition, filamentous viral structures were seen regularly, although they were limited to 5 to 10% of all virus particles produced. The filaments varied in length between 650 and 1,100 nm with a width of 120 nm (Fig. 3). Both spherical and filamentous virions were covered with surface projections about 12 nm long. In the late stage of infection (109 h p.i.), intranuclear inclusion bodies were observed (Fig. 4), although the frequency (1 out of 1,000 infected cells) was low. These inclusions were roughly circular and had an overall diameter of 500 nm. They consisted of tortuous and interwoven nucleocapsid-like particles with granular and filamentous structures of 22 to 27 nm in diameter. The outer edges of the intranuclear nucleocapsid-like particles were less sharply bordered than were the cytoplasmic nucleocapsids. The chromatin in the nuclei of these infected cells frequently showed marginal condensation. The intranuclear inclusions were clearly values separated by an electron-lucent space. Negative staining revealed the shape of spherical particles and a few filaments.

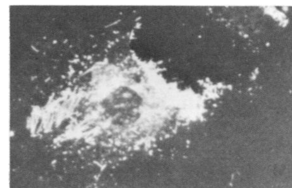

FIG. 2. Vero cell 4 days after infection with 78-238 showing cell membrane spikes with a bright fluorescence representing filamentous virus particles (×250).

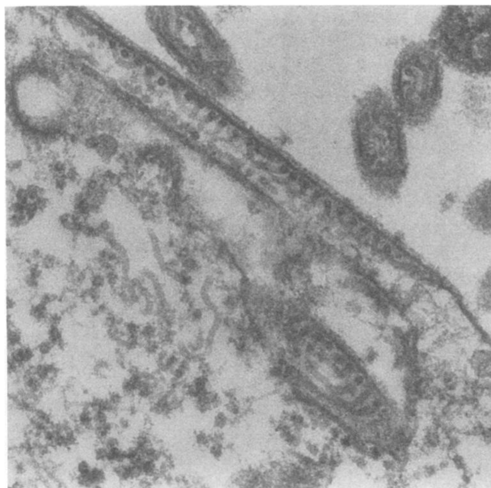

FIG. 3. *Filamentous 78-238 particle budding at the cell surface of a Vero cell. The particle has a length of 790 nm and a width of 124 nm. The virus membrane, underlined by transversal sections of nucleocapsids with a diameter of 17 nm, has projections 11 nm in length (×177,500).*

Virus 78-238 had an envelope with surface projections approximately 12 nm in length. This envelope enclosed a helical internal nucleocapsid with a diameter of 17 nm.

Persistently infected cells. A persistently infected cell line of 78-238 in Vero cells was developed from cells surviving a typical lytic infection. Upon passage, these cells experienced three minor lytic crises before a stable in vitro persistent infection was achieved. The persistently infected Vero cells could not be distinguished from noninfected cells by examining unstained cultures with a light microscope. A constantly high percentage of fluorescence-positive cells was revealed by fluorescence microscopy: between 85 and 100% after five passages. Of these cells, 1 to 15% were hemadsorption positive (Fig. 5). Ultrastructural observation revealed a high percentage of cells with cytoplasmic nucleocapsids. The smooth cytoplasmic nucleocapsids were dispersed over the cytoplasm, and each was more elongate than the cytoplasmic nucleocapsids of lytic infection. Intranuclear inclusions were not observed. Virus budding was observed in only a few cells.

Antigenic relationship of 78-238 to other CPI. Tween 80 and ether-treated viral preparations were tested for HA activity at 4, 22, and 37°C with different erythrocytes (Table 1). None showed HA at 37°C, whereas agglutination with D008 and SV-5 was observed only after 90 min at 22°C with all species of erythrocytes. Thirty minutes after the HA titers were read, the erythrocytes were eluted. At 4°C all three isolates agglutinated canine, guinea pig, human, and chicken erythrocytes, in that order of sensitivity.

Based on these results, subsequent HAI assays were performed with canine erythrocytes at 4°C. The degree of antigenic relatedness among 78-238, SV-5, and D008 was determined by cross-HAI tests (Table 2). All three antisera reacted against homologous and heterologous HA to some extent, with the homologous titers always higher except in one instance (anti-D008 versus 78-238 HA). The computed R values indicated that 78-238 is more closely related to D008 than to SV-5.

Serological studies in normal dogs. Approximately 60% of sera from both sources contained antibodies to 78-238 as determined by HAI (Table 3). Thirty-eight percent of the vaccinated dogs were seronegative. It appeared that, depending on the individual dog, a loss of HAI antibodies occurred 4 to 10 months after vaccination. The high incidence of positive HAI titers in dogs not vaccinated with CPI indicated that the virus is widespread in certain canine populations.

DISCUSSION

The objectives of this study were fourfold: to determine whether 78-238 is an SV-5-like virus; to determine the relationship of 78-238 to reference CPI (D008) and SV-5 strains; to determine in vitro various morphological properties of 78-238 infection in Vero cells; and to determine, by serological means, the prevalence of CPI infection in two separate canine populations.

By light microscopy, developing CPE (i.e., multinucleated syncytial giant cell formation, eosinophilic cytoplasmic inclusion body formation, and progressive cellular degeneration) was typical of that reported for parainfluenza virus (11). Electron microscope evaluation of 78-238 revealed that the virus replication cycle had ultrastructural features consistent with those of other characterized parainfluenza viruses (12–14). Early in the course of infection, cytoplasmic accumulations of viral nucleocapsids were observed. The replication cycle was completed by nucleocapsid alignment, spiking, and virus budding. Two forms of virions were observed. The spherical form covered externally with submembranous spikes predominated. The filamentous form occurred frequently (5 to 10%) and was consistently present in all stages of infection. Infected cells, particularly in the later stages of infection, showed degenerative changes compatible with a direct sublethal viral effect.

Intranuclear inclusion bodies in infected Vero cells were found infrequently but regularly.

FIG. 4. *Vero cell nucleus 109 hours after infection with 78-238, showing marginal chromatin condensation and an inclusion body with an overall diameter of 500 nm (×42,500). (Insert) Intranuclear nucleocapsid structures with a diameter of 22 to 27 nm (×102,500).*

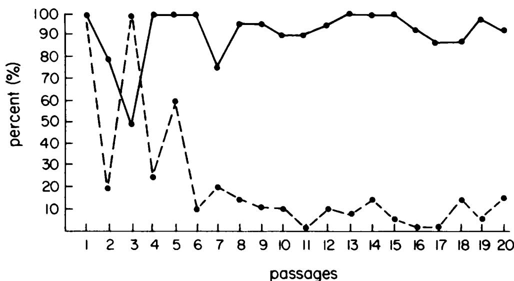

FIG. 5. *Percentage of IF-positive cells (solid line) and of cells showing hemadsorption (broken line) in Vero cells persistently infected with 78-238.*

Their paucity plus small size (500 nm) made it difficult, but not impossible, to demonstrate them by conventional light microscopy. The presence of such inclusions is not considered typical of parainfluenza viruses (12). To date, only bovine parainfluenza virus (SV-4) has been shown to produce intranuclear inclusions (27).

The virus, as indicated by infectivity titers, readily replicated in susceptible Vero cells. The optimal temperatures mentioned in the literature for propagating CPI in vitro range from 35 to 36°C (4-6, 8, 10, 14, 16, 17, 29). Growth curve analyses performed at both 34 and 37°C indicate that temperature influences the rate of virus production and the overall infectious titer. Propagation of the virus at 37°C resulted in a more rapid progression of the infection. Throughout these experiments, no overt differences in CPE were observed between cultures maintained at 34 and 37°C.

The infection was readily converted from a lytic to a persistent type by maintaining cells that survived a lytic crisis. Unlike lytically in-

TABLE 1. *Influence of temperature on the HA titers of Tween 80- and ether-treated 78-238, SV-5, and D008 with various erythrocytes*

Source of erythrocytes	HA titer								
	D008			SV-5			78-238		
	4°C	22°C	37°C	4°C	22°C	37°C	4°C	22°C	37°C
Dog	1,280	320	<2	1,280	160	<2	320	<2	<2
Guinea pig	640	320	<2	320	160	<2	320	<2	<2
Chicken	320	80	<2	320	80	<2	20	<2	<2
Human type O	320	160	<2	320	160	<2	160	<2	<2

TABLE 2. *Cross-HAI reactions among 78-238, SV-5, and CPI and extent of immunological relatedness*

Antiserum to:	78-238		CPI (D008)		SV-5	
	Titer[a]	Relatedness[b]	Titer	Relatedness	Titer	Relatedness
78-238[c]	800	1.00	320		320	
78-238[d]	4,388	1.00	1,828		2,195	
CPI (Manhattan)[e]	1,760	0.68	1,473	1.00	7,286	
SV-5	3,840	0.44	3,840	0.65	7,680	1.00

[a] Expressed as reciprocal of last dilution of serum that completely inhibited virus HA; Mean titers of 10 separate determinations over 2 months.

[b] Expressed as immunological relatedness: $R = \sqrt{r_a \cdot r_b}$, where r_a = (titer of antiserum A with heterologous virus B)/(titer of antiserum A with homologous virus A) and r_b is (titer of antiserum B with heterologous virus A)/(titer of antiserum B with homologous virus B).

[c] Serum obtained 3 months p.i.

[d] Serum obtained 1 week p.i.

[e] Manhattan strain and strain D008 are identical (M. Appel, personal communication).

TABLE 3. *Incidence of HAI antibodies to 78-238 in dogs*

Titer[a]	No. positive			
	40 Bitches	35 Routine admission dogs		
		Vaccinated (no.)	Unvaccinated (no.)	Total %
<20	17 (42.5)[b]	5	3	40.0
20	3 (7.5)	2	1	8.6
40	1 (2.5)	1		2.8
80	3 (7.5)	4	3	20.0
160	9 (22.5)	2	4	17.2
320	5 (12.5)		3	8.6
640	2 (5.0)		1	2.8
Incidence of positive sera[c]	23/40 (57.5)	21/35 (60.0)		
Mean titer of positive sera	1:203	1:139		

[a] Expressed as the reciprocal of the last dilution of serum that inhibited virus HA.

[b] Percentage of total is given in parentheses.

[c] Number positive/total.

fected cells, persistently infected cultures did not display CPE, nor could they be distinguished from uninfected control cells by patterns of growth or examination of unstained monolayers. Between 85 and 100% of the cells contained viral antigen by IF determination; a lower (≤15%) expressed a membrane-associated HA. The infection in these cells was not defective in that viral budding was observed. No intranuclear inclusions were observed in persistently infected cells.

The extent of the serological relatedness among 78-238 and other CPI isolates was determined by virus neutralization and cross-HAI experiments with canine erythrocytes. The isolate 78-238, though related to both SV-5 and D008 strains, is apparently more similar to D008. It can, however, be distinguished from both strains by its lack of HA ability with erythrocytes at 22°C. Espmark (18) evaluated the differences between two strains of SV-5. The strains differed in their HA properties and in vitro plaque morphology but not significantly as antigens in HAI or plaque neutralization assays.

The limited seroepidemiological study revealed that approximately 60% of the dogs tested had HAI antibodies to 78-238. Since the results of the HAI test demonstrated that there are significant cross-reactions among various members of CPI, these data indicate that CPI, either as a naturally occurring infection or as a result of vaccination, is widely distributed in the canine population. Ten years ago, an extensive serological study of dogs from various geographic areas

in the United States indicated that the overall attack rate (14%) was actually quite low (9). Other studies revealed that the SV-5 infections in dogs appear to occur in widely separated parts of the United States, and various percentages of dogs with seropositive titers were found (5, 7, 8, 20).

In vivo studies with this virus in seronegative gnotobiotic dogs has revealed that 78-238 can produce encephalitis and internal hydrocephalus in addition to respiratory involvement (Baumgärtner et al., submitted for publication). Further investigation is necessary of the biological properties of other CPI isolates, the frequency of central nervous system involvement and, therefore, the significance of this virus as a systemic disease of dogs.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Addajane L. Wallace, David Long, and Nancy J. Austin.

This work was supported by Public Health Service grant A109022 from the National Institutes of Health and by the State of Ohio Canine Research Funds. W.B. was supported by a scholarship from the Deutsche Akademische Austauschdienst, Bonn, West Germany. A.E.M. was supported by fellowships from the Schweizerische Stiftung fur Medizinisch-Biologische Stipendien, Basel, and the Janggen-Pohn Stiftung, St. Gallen, Switzerland.

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