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Canine Hemorrhagic Enteritis: Detection of Viral Particles by Electron Microscopy

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With 6 Figures

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Summary

At necropsy, several dogs which died showing symptoms of hemorrhagic diarrhea, had significant lesions of the mucosa that were found especially in the duodenum and upper part of the small bowel.

Study of ultrathin sections from the diseased mucosa revealed particles resembling parvoviruses in altered nuclei of cells of the intestinal crypts.

Electron microscopic examination of intestinal contents by negative staining has shown the presence of many viral particles which have a diameter of 24 nm and whose profile is consistent with an icosahedral shape. These virions float at a density of 1.43 g/cm^3 in cesium chloride and agglutinate rhesus monkey and swine red blood cells at 4° C.

A possible etiological role in discussed.

This virus is compared with the minute virus of canines and the Feline Panleukopenia virus.

Introduction

During this last year, among the dogs sent for autopsy to the school of veterinary medicine, seven cases, diagnosed as having fatal hemorrhagic enteritis, showed some interesting similarities.

They all died after an episode of two or three days with vomiting and a bloody diarrhea. These animals were mature dogs coming from different breeding and sheltering centers. In each case the disease had occurred during the week of their arrival at the kennel.

At necropsy the duodenum and proximal part of the small bowel appeared very congested. Histological examination of these organs showed necrosis of the

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villi and profound abnormalities of the crypts. The lumens of the crypts were enlarged, sometimes filled with debris, and their epithelium, often discontinuous, was composed of flat cells with a clear swollen nucleus (6).

No bacterial pathogen was consistently found in these cases nor could any poisonous substance be identified as the cause of death.

In view of the characteristic mucosal lesions (22) a viral etiology was suspected.

To investigate this unusual syndrome we used various technics of electron microscopy which already have been successfully applied to the study of viral diarrheas in man (2, 5, 17) and animals (28).

We describe here the results of direct examination of the feces by negative staining and the analysis of ultrathin sections of the diseased part of the intestinal tract of these dogs.

Materials and Methods

Samples were obtained from 7 dogs which died during an episode of hemorrhagic diarrhea. The cases comprised 1. a four year-old female basset hound, 2. a two year-old male basset hound, 3. a one year-old male german shepherd, 4. a two year-old female basset hound, 5. a five year-old male dalmatian, 6. a one year-old male mongrel dog and 7. a two year-old male poodle.

When it was realized that all these animals had similar histological lesions of the gut, we further analysed the organs which had been removed at the time of autopsy.

Specimens of feces from seven apparently normal dogs were also examined.

Viral Suspension

Portions of intestine from 3 animals (no. 1, 6, 7) had been ligated and maintained at $-20°$ C. After thawing, the contents of each were diluted one to ten in distilled water and clarified by centrifugation at 3000 rpm ($1500 \times g$) for 20 minutes. The supernatant was used for direct examination by E. M., hemagglutination test, equilibrium density gradient centrifugation and virus isolation studies.

Direct Examination by Electron Microscopy (E. M.)

Specimens were prepared according to the pseudo-replica method described by Smith (26).

Briefly, a drop (20 microliters) of viral suspension was placed on a block of special agar Noble (ref. 0/4201 DIFCO Detroit Mi.) at a concentration of 4 per cent in water. The agar block was cut so that there was a 1 cm^2 area on which the drop containing the viruses was evenly spead. After the liquid had penetrated into the gel and after the surface appeared dry, it was covered with a drop of collodion (1 per cent nitrocellulose in amyl acetate; Ernest Fullam Inc.). After drying, the thin layer of collodion was floated off onto an aqueous solution of 1 per cent potassium phosphotungstate at pH 7. The floating membrane entrapped the viral particles which were deposited on the agar. This membrane was then placed on a carbon-coated grid and examined in the E. M. at 80 kV.

The size of the virions was estimated from photographs. A tracing of the profiles of the particles was analyzed by an image analyzer (Quantimet). E. M. Magnification was estimated by using a gold crystal replica (Balzers Union).

Serological Studies

Stagloban (Behring) was used as a commercial source of dog immunoglobulin. We also tested sera from 3 apparently normal dogs living in a kennel where one animal (no. 3) had died with hemorrhagic enteritis several weeks before.

Immunoelectron Microscopy (I.E.M.)

One drop (20 microliters) of a dilution of immunoglobulin solution was added to the same volume of viral suspension. After 1 hour at 37° C, the mixture was left overnight at 4° C and then examined by the pseudoreplica procedure.

Hemagglutination and Hemagglutination Inhibition (H.A. and H.I.)

Tests were performed in microplates (Cooke). Samples were diluted in a saline solution (9 g NaCl, 100 mg $MgCl_2$ and 30 mg $CaCl_2$ in 1 l H_2O) by means of an automatic diluter (Cooke eng. Co. Va. U.S.A.).

Rhesus monkey erythrocytes were obtained from the Pasteur Institut (Paris). Red blood cells were washed three time and adjusted to a final concentration of 5×10^4 cells per mm^3 in the same saline. One drop (20 microliters) of this erythrocyte suspension then was added to each well of the microplate containing the sample dilutions. Hemagglutination titers were read by the pattern method after being left overnight at 4° C.

For hemagglutination inhibition tests, sera were treated with neuraminidase (Microbiological Associates, Inc. Bethesda Md.) and adsorbed by the red cells (24). Four units of antigen were mixed with the serum dilutions and incubated at 37° C for 1 hour. After addition of the red cells, the test was placed at 4° C overnight.

Equilibrium Density Gradient

Three ml of viral suspension were filtered through a filter of 22 micron pore diameter (Millipore filter GSWP 1300). To the filtrate was added 3.174 g of Cesium chloride and distilled water to give a final volume of 6 ml of average density 1.396 g/cm^3. This was centrifuged in a swinging bucket rotor (Christ Ultracentrifuge Omega II) at 4° C and 40,000 rpm ($280{,}000 \times g$) for 40 hours. Twenty one fractions were collected from each sample with a Beckman fraction collector and the density of each estimated from its refractive index.

From each fraction one drop was examined by electron microscopy by negative staining and another for hemagglutinating activity.

Attempted Virus Isolation

The viral suspension was inoculated onto the following cultures: Madin Darby canine kidney, Madin Darby bovine kidney, Primary human embryo kidney cells, Primary Rhesus Monkey kidney cells and Hela cells. The cells were exposed to the virus when the monolayer was about 25 per cent confluent. Inoculated cultures were incubated at 37° C and observed for the development of cytopathic effect.

Samples of the supernatant of these cultures were harvested every day, tested for hemagglutinating activity and examined by E. M. to detect the presence of viral particles.

The virus was also inoculated onto the same type of cells in Leighton tubes—coverslips were fixed in Bouin's solution, stained with hematoxylin eosin and screened for intranuclear inclusions.

Specific pathogen free embryonating eggs were injected by the amniotic or allantoic route.

E.M. Examination of Ultrathin Sections

For each animal, pieces of intestine were kept immersed in 10 per cent formalin saturated with calcium carbonate, at room temperature, for from two weeks to four months. They were then washed three times in phosphate buffer (0.1 M pH 7.2) cut in blocks of roughly 1 mm^3 and fixed with 2.5 per cent glutaraldehyde (LADD) in the same buffer. They were then washed three times, postfixed for one hour in 1 per cent osmium tetroxide, dehydrated and embedded in Epon by a method recommended by DE HARVEN (9).

The zones to be studied were chosen on thick sections (2 microns) stained by toluidine blue (27). Ultrathin sections were made with a L.K.B. ultramicrotome contrasted by means of uranyl acetate and lead citrate (14) and examined in a Philips E M 300 at 60 kV. Photographs were taken on sheet film (Kodak electron microscope film).

Results

Examination of Specimens by Negative Staining

The intestinal contents of 3 dogs (no. 1, 6, 7) were examined and all three were shown to contain many viral particles. The outlines of the particles were often round or polygonal and many of them, almost 20 per cent, were penetrated by the phosphotungstate so that they appeared as empty virions.

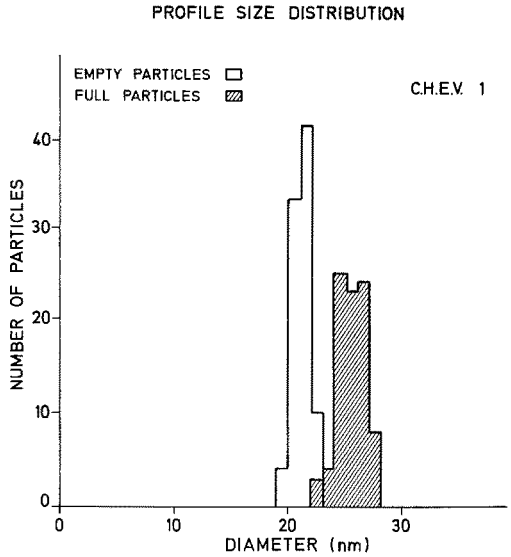

Fig. 1. The profiles of 98 particles found in intestinal content of dog no. 1 (C.H.E.V.) were measured in an image analyzer. Empty particles appear to be smaller

By measuring a large number of profiles it was possible to show that particles which were not penetrated by the stain had a mean diameter of 24.5 nm; with values ranging between 23 and 28 nm. Empty particles were small, their diameters ranging from 19 to 23 nm with a mean of 21 nm (Fig. 1). By immunoelectron microscopy the particles were seen agglutinated in large clumps. This phenomenon was observed using a $^1/_{10}$ dilution of any of the three sera as well as of the commercial dog immunoglobulin (Fig. 2). No such particles were detected in the samples from the healthy animals.

Hemagglutination

The viral suspensions obtained by clarification of the intestinal contents agglutinated rhesus erythrocytes at 4° C and not at 37° C. For dog no. 1 the titer was 12,800, dog no. 6: 8000, and dog no. 7: 128.

The hemagglutination patterns obtained in the cold disappeared at 37° C but agglutinated if the cells were redispersed and returned to 4° C. Swine red blood cells were also agglutinated at 4° C and the titers were similar. Human 0, chicken, pigeon and guinea pig red cells were not agglutinated.

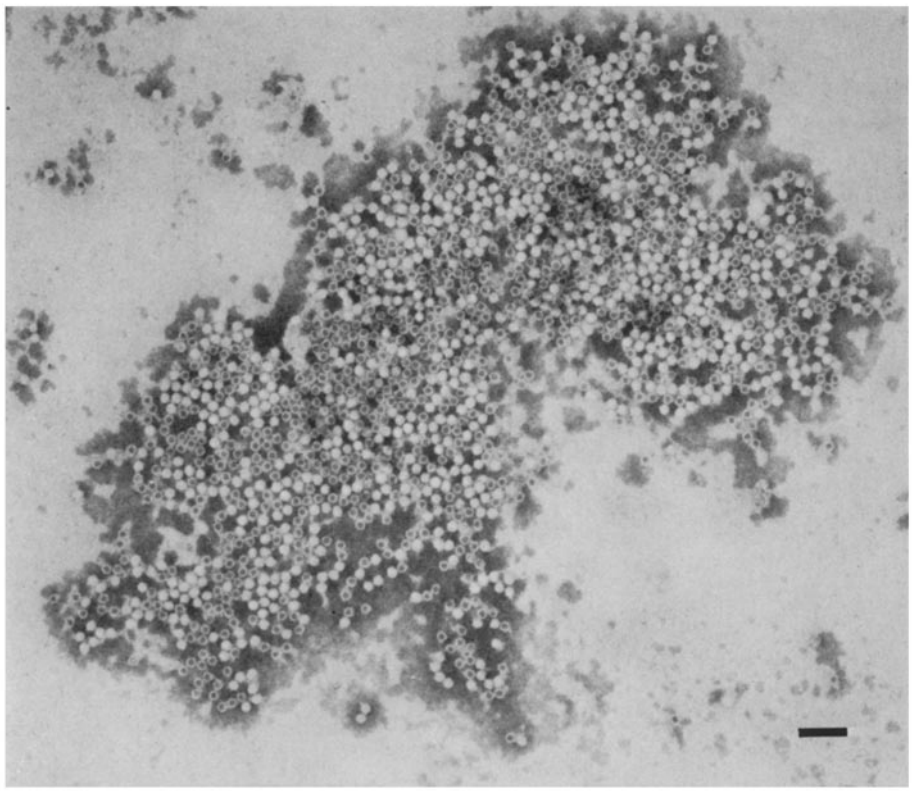

Fig. 2. Particles observed in intestinal content of dog no. 1 after incubation with a 1/10 dilution of dog commercial immunoglobulin (Bar represents 100 nm)

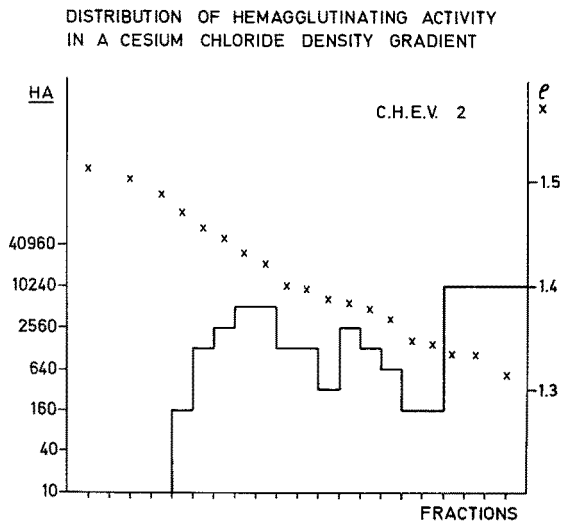

Fig. 3. Viral suspension obtained from dog no. 2 (C.H.E.V. 2) was shown by density gradient to contain three peaks of hemagglutinating activity

When a viral suspension had been filtered through a 22 micron millipore filter, viral particles could still be detected by E.M. but the HA titer was reduced tenfold. The sera of apparently healthy dogs had HI titers greater than 160.

Density Gradient

After centrifugation in cesium chloride the hemagglutinating activity was distributed as shown in Figure 3. The activity was not detected in samples having a density greater than 1.46 but was found in all the remaining fractions with three major peaks at densities of 1.43, 1.38 and 1.34. This was consistently observed in repeated experiments.

The same fractions were examined by immunoelectron microscopy and by this method viruses were also detected in all the fractions with a density less than 1.46.

In the samples corresponding to peak hemagglutinating activity larger numbers of viral particles were also observed by E.M.

At a density of 1.43 only "full" virus particles were seen, at 1.38 equal numbers of "empty" and "full" particles were present, while at a density of 1.34 eighty percent of the virions appeared empty.

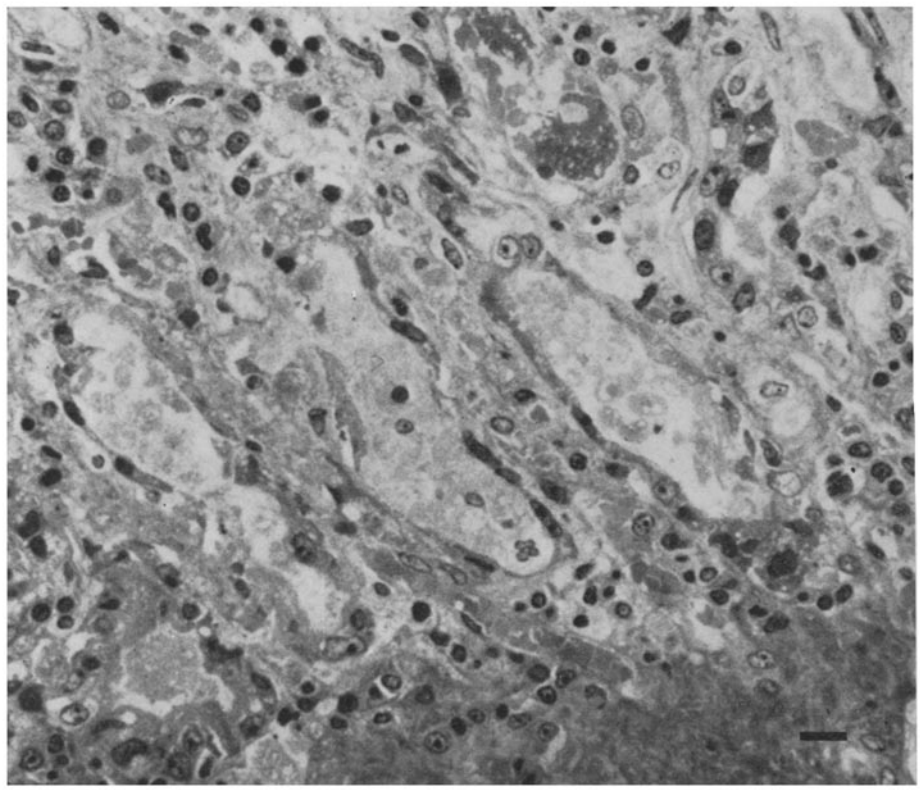

Fig. 4. Section through three ileal crypts which appear to be severely damaged: some swollen nuclei are still visible in the atrophic pattern (Bar represents 20 microns). The semi-thin section of the intestinal mucosa was stainted by Toluidine blue. The area chosen lay between the base of the villi above and the muscularis mucosae below

Tissue Culture

So far attempts to grow this virus in tissue culture have been unsuccessful. No cytopathic effect was observed nor could any intranuclear inclusion be detected. E.M. and hemagglutination test failed to show production of viral particles. No virus was isolated in embryonated hen's eggs.

Examination of Specimens in Thin Section

For every one of the animals, characteristic alterations of the intestinal wall were clearly seen by light microscopy on semi-thin sections stained with toluidine blue (Fig. 4).

Superficial layers of the mucosa were necrotic: the general pattern of the villi was preserved and some cell limits were slightly visible but it was not possible to recognize more detail. However in the deeper layer near the muscularis mucosae the structures were easily distinguished. In this region there was significant infiltration with small mononuclear cells. The lumina of the crypts were swollen and contain debris and vacuolated cells. Their epithelium seemed to be discontinuous. It was composed of flat cells, the nuclei of which frequently appeared filled with abnormal granular material. These areas were selected for ultrathin sections.

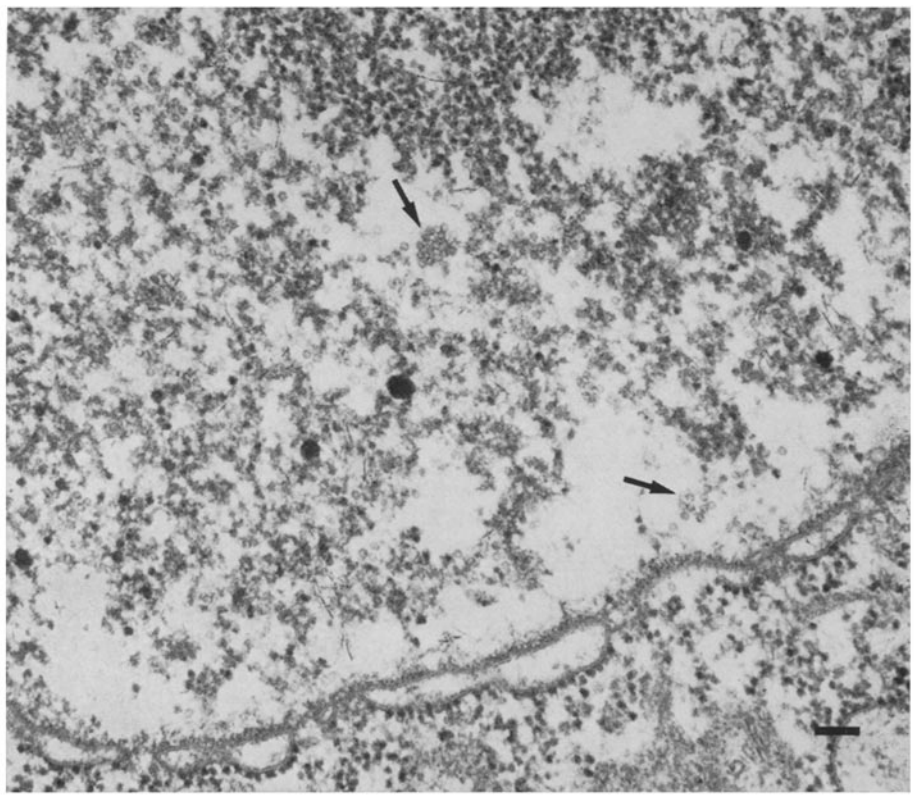

Fig. 5. Presence of round empty particles in the nucleus of an epithelial cell (Bar represents 100 nm)

At the E.M. level most of the cells were severely damaged. Due to the poor quality of preservation of fine structures a precise cytological study was not possible. Nevertheless in cells limiting the crypts viral particles were clearly visible (Fig. 5) and were detected in the nucleus of the epithelial cells. They had a diameter of 20 nm or less. They were circular in outline with a dense margin and a pale center. In a few of them a central dark spot was apparent. Uniformly dense particles with the same dimension and outline were also present and at least some of them were probably complete virions.

In vacuolar cells floating in the lumen of the mucosal crypts many particles were also found, but in the cytoplasmic area and not in the nucleus. In these cells there were large clumps composed of a mixture of full and empty particles often surrounded by a membrane (Fig. 6). In some of these virus containing vacuoles dense granular material and pseudo myelinic patterns were seen.

Similar observations were made in specimens from each of the seven dogs.

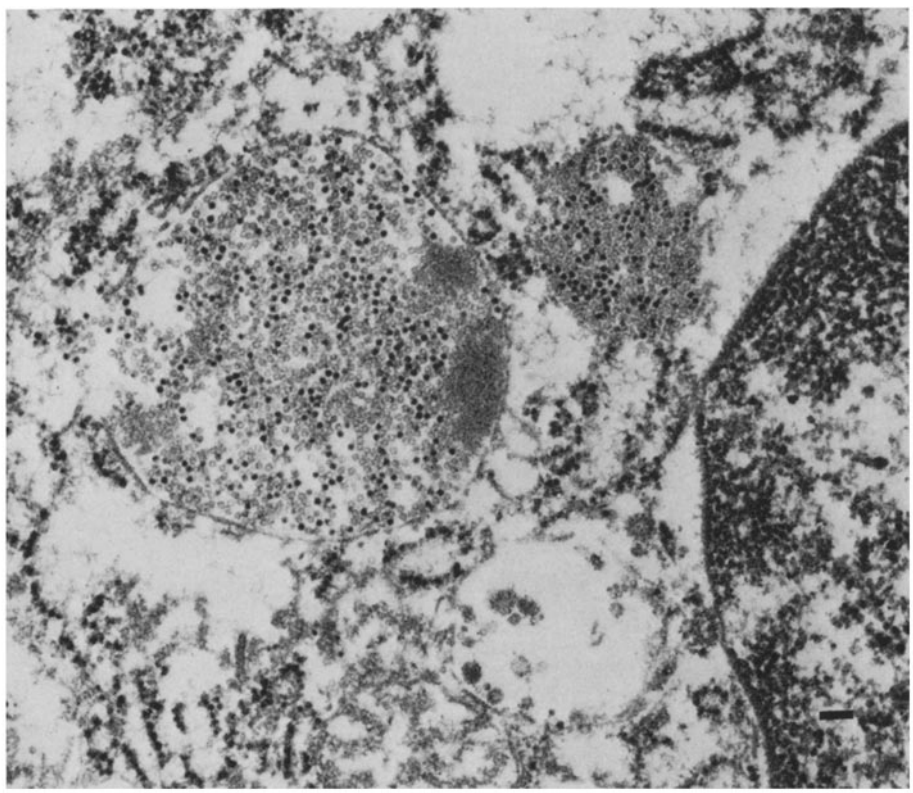

Fig. 6. Clumps of particles in the cytoplasm of a vacuolated cell floating in the lumen of a duodenal crypt. (Bar represents 100 nm)

Discussion

The viruses detected in the fecal material of the dogs with hemorrhagic enteritis closely resemble parvoviruses. The morphological characteristics are similar to those described for other viruses of this family (15, 16, 25). Capsomeres

were not demonstrated but the hexagonal outline of the virions is consistent with icosahedral symmetry (20).

The dimensions found are in the size range given for the group (13). More precise comparison with the data of the literature may not be meaningful since the diameters of viral particles measured by E.M. can depend on working conditions (25). Furthermore in constant and controlled experimental conditions differences have been reported to exist between strains (18). In addition in our measurements full particles appear larger than empty ones. Therefore reliable estimations of differences can only be made by considering size distributions. Even so homogeneous populations of particles taken from some gradient fractions, can only be compared if the error of the estimate has been determined (21) and large number of particles have been measured.

As for buoyant density, the particles present in the dogs intestinal contents were found to distribute in a cesium chloride equilibrium density gradient as described for some parvoviruses (16, 25). For example for minute virus of mice the hemagglutinating activity was shown to peak at exactly the same densities, that is 1.43 and 1.34 with an additional peak at 1.38 (8).

The presence of numerous parvovirus-like particles in the feces fits very well with the histological lesions observed in the same animals by light microscopy. Such crypt injuries are well known in enteritis due to a parvovirus (19). A virus cytopathic effect causing mucosal damage would readily explain the hemorrhagic diarrhea syndrome. This would be supported by the detection of viral particles in the cells (7). We have examined material removed at the time of autopsy even though it had not been fixed for ultrastructural study (10). The fine structures were in fact spoiled either by postmortem artifacts or by poor fixation procedures but the presence of virions was obvious. The particles found in the nuclei of the abnormal epithelial cells of the mucosal crypts were quite similar to what has been described in cells inoculated with a parvovirus (23).

If we assume that the virions of the intestinal content are produced by the infected duodenal cells, the presence of the particles in the nucleus is strong evidence that they belong to the Parvovirus group.

If this dog agent turns out to be a parvovirus it might in fact be the minute virus of canines (M.V.C.) which was proposed ten years ago as a member of the genus Parvoviridae (4). Some evidence to support this idea came from the observation that the canine hemorrhagic enteritis virus (C.H.E.V.) and the minute virus of canines both agglutinated rhesus monkey erythrocytes at 4° C though it was reported that M.V.C. does not agglutinate pig red cells (25).

The M.V.C. was known to grow and produce a cytopathic effect in only one cell line: the Walter Reed canine cell (W.R.C.C.). Since these cells were no longer available (SIEGL, personal communication) it was not possible to determine if the virus found in dog enteritis had this same property. Using cells other than W.R.C.C. we were unable to culture the virus. But such negative results should not be considered as conclusive. Attempts to cultivate the virus are continuing.

The M.V.C., isolated from apparently healthy animals was not thought to be pathogenic (3), though it had been suspected to be related to mild diarrhea in puppies (11). In the cases we have studied we found virions only in animals with a specific intestinal syndrome and not in apparently healthy dogs. But of course

by E.M. or HA it is not possible to detect viral particles except when they are present in large quantities.

Among the other parvoviruses the feline panleukopenia virus has been shown to cause in cats and minks an enteritis with very similar lesions of the intestinal crypts (19). FPLV has been reported to agglutinate swine erythrocytes but rhesus red cells were not tested (25). This hemagglutination occurs at 4° C with the same disaggregation at room temperature and aggregation on returning to the cold.

Little information is available on the importance of this parvoviral infection in dogs. Recently, outbreaks of canine parvovirus-like viral enteritis have been reported in various locations throughout the United States (1, 12).

Until now we have only had the opportunity to examine autopsy material. Investigations in living animals with symptoms of enteritis such as vomiting and diarrhea could be a source of additional information. If many particles are present in feces they could be detected by E.M. or HA.

On the other hand IHA and I.E.M. recognize the presence of antibodies in the serum. In non fatal cases it should be possible to make the diagnosis and to demonstrate a seroconversion. It is conceivable that severe or even fatal disease may occur in adult dogs which lack immunity when they arrive in a new kennel and come in contact with dogs which are immunized but carry the virus.

This is still an hypothesis and needs to be substantiated by further studies.

Acknowledgments

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