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Isolation of a Virus Closely Related to Feline Panleukopenia Virus from Dogs with Diarrhea

By

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With 5 figures and 2 tables

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Introduction

The first isolation of a parvovirus from dogs, the Minute Virus of Canines (MVC) dates from 1970 (3). The virus which was isolated from stools of healthy dogs did not appear to be associated with disease symptoms and no antigenic relationship with any of the known parvoviruses including feline panleukopenia virus (FPV) could be demonstrated (3, 15).

During the last two years parvovirus-like particles were shown to be present in feces of dogs involved in several outbreaks of a contagious diarrhea often accompanied with vomiting, in the USA, Canada and Australia (1, 4, 5, 6, 8, 10). In many cases intestinal lesions were very similar to those of feline panleukopenia (1, 4, 10). A virus isolated from dogs in these outbreaks was shown to be antigenically related to FPV on the basis of a cross-reactivity in immunofluorescence (IF) tests (2, 4) and in serum neutralization tests (4).

In the present paper we describe the demonstration of parvovirus-like particles in stools of dogs in outbreaks of contagious diarrhea often associated with vomiting in the Netherlands. Mortality rates were high, especially among dogs under one year of age. Intestinal lesions very similar to those of feline panleukopenia were often observed in these dogs (7). The virus isolated from stools of an affected dog showed a close antigenic relationship with FPV.

Material and Methods

Animals

Two non-vaccinated female SPF kittens of about five months old from an SPF colony were obtained as a gift from Dr. A. F. ANGULO (Centraal Proefdierenbedrijf TNO,

Zeist, The Netherlands). No indications of the presence of known feline viruses have ever been found in regular screening procedures in this surgically derived colony (OSTERHAUS, unpublished observations). The animals were kept in a pressurized glove box throughout the experiments.

Two non-vaccinated conventionally reared male dogs about four months old were also made available by Dr. A. F. ANGULO. They were kept in an isolated room throughout the experiments.

Feline panleukopenia virus (FPV)

A vaccine strain of FPV ("Dohyvac P") together with the Crandell Feline Kidney (CrFK) cell line were kindly provided by Dr. C. FOLKERS (Philips-Duphar, Weesp, The Netherlands). The virus was propagated in monolayer cultures of these cells in Leighton tubes or in Roux bottles by adding the virus suspensions to cell suspensions or to settled monolayers respectively. The medium used was similar to the medium used for virus isolation procedures (see below). After an incubation period of two to three days at 37 °C, the Leighton tube cultures were used for indirect IF studies and the bottle cultures were centrifuged at low speed after three cycles of freezing and thawing; the supernatants were used for hemagglutination (HA), (immune) electron microscopy (IEM) and serum neutralization (SN) studies.

Sampling of stools and sera

Stools were taken from dogs involved in the outbreaks of diarrhea in the Netherlands either in a breeding colony for experimental animals, or in boarding kennels. Serum samples were collected from dogs in the breeding colony after the outbreaks had started. In addition sera were tested from dogs of the breeding colony and from the open population in the Netherlands, which had been collected about four years ago (11).

Electron microscopy (EM) and Immune electron microscopy (IEM)

Fecal samples were homogenized in 10 volumes of PBS and centrifuged at 10,000 g. for 5 minutes. Material from the supernatant-pellet interface was used as a fecal extract for EM and IEM.

For negative contrast EM the fecal extracts were applied to carbon-coated grids. After 10 minutes the grids were rinsed with distilled water and contrasted with a solution of 2 % phosphotungstic acid, containing 0.01 % bacitracin and adjusted to pH 6.2 with KOH.

For IEM the samples were mixed with equal volumes of 1 : 10 diluted immune sera or control sera and incubated for two hours at 37 °C. The reactant mixtures were applied to carbon-coated grids and negatively contrasted as described above. Grids were examined at a magnification of 50,000 using a JEOL 100 C electron microscope.

Virus isolation

Fecal samples were diluted 1 : 10 in PBS and shaken for 15 minutes with chloroform at 4 °C. After low speed centrifugation 0.15 ml. volumes of the aqueous phases were mixed in triplicate with 1.5 ml. volumes of a freshly trypsinized suspension of Madin Darby Canine Kidney (MDCK) cells. The cells were permitted to settle and grow on cover slips in Leighton tubes. The suspension contained about 2×10^5 cells per ml. in Eagles MEM-lactalbumin hydrolysate, supplemented with 10 % fetal calf serum and antibiotics. After an incubation period of two or three days the cultures were examined for cytopathic changes. One tube per sample was used for haematoxylin and eosin (HE) staining (standard procedure), one for possible demonstration of viral antigen in an indirect IF test and one for subsequent passaging. For reisolation the procedure used was similar to the original isolation procedure. However, the material was passed through a 220 nm filter (Millipore®) after treatment with chloroform.

Hemagglutination and hemagglutination inhibition (HAI) tests

HA and HAI tests were carried out in a microtiter system (Cooke Engineering Co.) essentially as described previously (11) using veronal gelatin buffer containing 0.15 % bovine serum albumin as a diluent. Hemagglutinating antigen was prepared from a canine parvovirus-like virus (CPV), isolated during the course of these studies, by centrifuging infected Roux bottle cultures three days post-infection (p. i.) at low speed after three cycles

of freezing and thawing. The supernatant was used as CPV hemagglutinating antigen. Likewise, the supernatant from FPV infected CrFK cell cultures treated in the same way were used as antigen for HA and HAI tests. HA tests were carried out by adding a 0.75 % suspension from African green monkey (AGM), dog, cat, swine, mouse, rat, guinea pig, Chinese hamster or chicken erythrocytes, to serial twofold dilutions of the antigen preparations. After an incubation time of two hours at 4 °C the tests could be read. For HAI tests serial twofold dilutions of kaolin treated (14) antisera were mixed with four hemagglutinating units (HAU). After an incubation period of one hour at 20 °C, a 0.75 % erythrocyte suspension was added and the tests were further incubated and read as the HA tests. Titers < 20 were regarded as non-specific.

Indirect immunofluorescence tests

Two or three days p. i. indirect IF tests were performed on Leighton tube cultures of MDCK or CrFK cultures after cold (— 70 °C) ethanol fixation, to demonstrate CPV or FPV antigen. The tests were essentially carried out as described previously for a coronavirus system (12). A rabbit anti-dog IgG and a rabbit anti-cat IgG preparation kindly provided by Dr. J. NAGEL (Natl. Inst. Publ. Hlth.), both labelled with FITC, were used as conjugates. When canine sera were used on MDCK cells or when feline sera were used on CrFK cells a FITC labelled protein-A preparation, also provided by Dr. J. NAGEL, was used instead of the anti-IgG conjugates.

Serum neutralization tests

Neutralization of CPV or FPV with inactivated (30 min 56 °C) sera was demonstrated by determining the neutralization indices in MDCK or CrFK Leighton tube cultures respectively: Semi log 10 dilutions of the virus suspensions were mixed with equal volumes of 1 : 25 diluted sera and incubated for one hour at 20 °C. Volumes of 0.5 ml. of these mixtures were added simultaneously to Leighton tube cultures with MDCK or CrFK cell suspensions. Three tubes were inoculated with each virus-serum mixture. After two or three days the cultures were fixed in 4 % formaldehyde and stained with HE and examined for the presence of intranuclear inclusion bodies. From these results neutralization indices were calculated.

Animal inoculation

After collecting pre-inoculation serum samples of the two cats, one was vaccinated with two doses of a formaldehyde-inactivated panleukopenia vaccine ("Felidovac" Behringwerke AG, Marburg, GDR). After four weeks this cat was boosted with another two doses of the same vaccine and serum samples were collected from both cats one week later (post-vaccination and second serum sample respectively). Two weeks after the second vaccination, both cats were challenged with CPV, isolated during the course of these experiments: 1 ml. of a CPV suspension, containing about 10^4 $TCID_{50}$ was inoculated intranasally and 1 ml. subcutaneously in both cats. The cats were checked for clinical signs daily, which included measuring of rectal temperature. White blood cell (WBC) counts were made every second day.

After collecting pre-inoculation serum samples, the dogs were infected by application of about 10^4 $TCID_{50}$ CPV directly into their stomachs using an oesophageal tube. The dogs were checked daily for overt clinical signs. Post-infection sera were collected from the dogs 21days after experimental infection.

Results

Demonstration of virus-like particles in stools

Stools from eight dogs in the breeding colony and from eight dogs in boarding kennels, which all died with symptoms of acute diarrhea, were examined in negative contrast electron microscopy. In seven samples of the first group and in six samples of the second group parvovirus-like particles were observed. Both empty and full particles were found and the diameter measured 23 ± 2 nm. (n. = 29). In some fecal extracts the particles appeared aggregated, but in general they were randomly distributed over the grid.

Virus isolation from stools

The same stool suspensions used in the EM studies were inoculated onto MDCK cell cultures after chloroform treatment. A cytopathic agent was isolated from the stools of one dog in the breeding colony, although cytopathic changes were minor and disappeared during prolonged incubation periods and in subsequent passages. Intranuclear inclusion bodies very similar to those described for the later stages of FPV infected cell cultures (9) were observed in the four subsequent passages of infected MDCK cells which had been stained with HE (Fig. 1). Electron microscopic examination of water-disrupted, infected MDCK cells revealed the same CPV particles that were found in fecal samples. The virus, which apparently passed a 220 nm. filter, could be reisolated from the same sample.

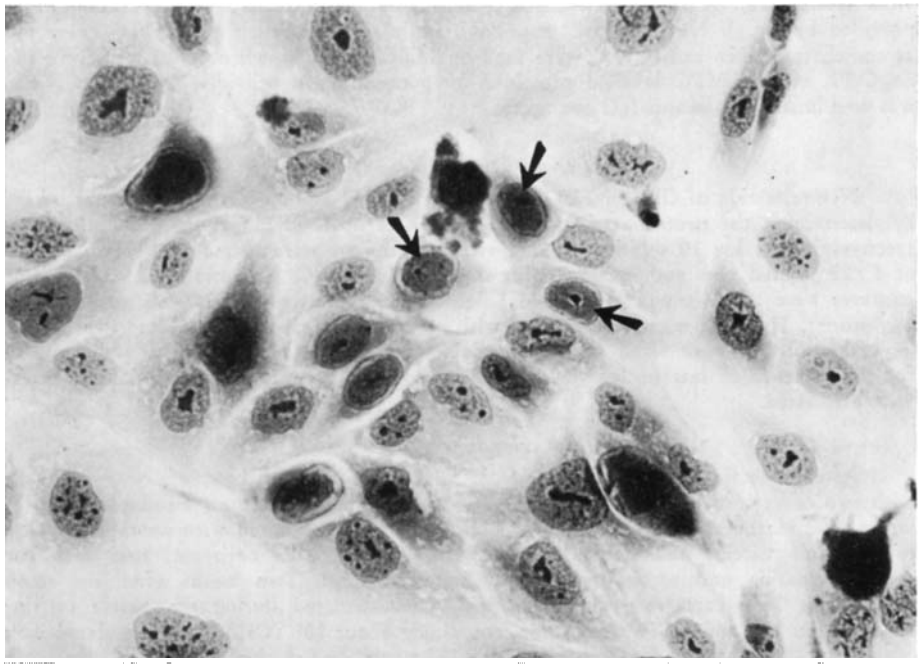


Fig. 1. HE stained MDCK cell culture, 48 hours after infection with CPV (2nd passage in MDCK cells). Note intranuclear inclusion bodies (e. g. arrows)

Hemagglutination by CPV and FPV antigen

CPV and FPV antigens were prepared from infected MDCK (second passage) and CrFK cell cultures respectively as described above and tested for hemagglutinating activity at 4 °C with erythrocyte suspensions from different animal species. The results of these tests are summarized in Table 1: CPV antigen agglutinated AGM, cat and swine erythrocytes, whereas FPV antigen only caused agglutination of swine erythrocytes. Specificity of these reactions could be assessed in HAI tests with specific antisera produced in the experimentally infected dogs and cats as described below. Erythrocytes from the other animal species were not agglutinated by CPV or FPV antigen, although some inconsistent results were obtained with rat erythrocytes. Control antigen preparations, prepared similarly from non-infected MDCK or CrFK cultures did not show hemagglutinating activity.

Table 1

Hemagglutinating activity of CPV and FPV antigen per 25 μl. with 0.75 % erythrocyte suspensions from different animal species. The tests were read after an incubation period of two hours at 4 °C

animal species	HA titer CPV antigen	HA titer FPV antigen
human 0	–*)	–
African green monkey (AGM)	256	–
dog	–	–
cat	128	–
swine	256	128
mouse	–	–
rat	(–)	(–)
guinea pig	–	–
Chinese hamster	–	–
chicken	–	–

Antigenic relationship between CPV and FPV: 1. Homologous and heterologous HAI tests

In HAI tests the pre-inoculation sera of the experimentally infected dogs and cats, the post-infection sera of these dogs, the post-vaccination serum of the cat (before challenge), the second serum of the non-vaccinated cat and the post-challenge sera of both cats were tested with the CPV and FPV antigen (Tab. 2). The tests were carried out with AGM, cat and swine erythrocytes in the CPV-HAI test and with swine erythrocytes in the FPV-HAI test. No HAI could be demonstrated with any of the presera, nor with the second serum of the non-vaccinated cat. The post-infection sera of the dogs, the post-vaccination serum of the cat and the post-challenge sera of both cats all showed titers $\geqq$ 1024 using 4 HAU of CPV or FPV antigen, regardless of the erythrocyte species used (Tab. 2).

Table 2

Hemagglutination inhibition (HAI), immune electron microscopy (IEM), immunofluorescence (IF) and serum neutralization (SN) tests, performed with CPV or FPV antigen or virus, using pre-inoculation sera of the non-vaccinated (NV) and the vaccinated (V) cat, the second and post-vaccination sera of the respective cats, the post-challenge sera of the cats, the pre-infection sera of dog I and II and the post-infection sera of both dogs. Positive reactions are marked with + and negative reactions with –. ND = not done

	HAI		IEM		IF		SN	
	CPV	FPV	CPV	FPV	CPV	FPV	CPV	FPV
sera CAT NV								
pre	–	–	–	–	–	–	–	–
second	–	–	–	–	–	–	–	–
post challenge	+	+	ND	ND	ND	ND	ND	ND
sera CAT V								
pre	–	–	–	–	–	–	–	–
post vaccination	+	+	+	ND	+	+	+	+
post challenge	+	+	ND	ND	ND	ND	ND	ND
sera DOG I								
pre	–	–	–	–	–	–	ND	ND
post	+	+	+	+	+	+	+	+
sera DOG II								
pre	–	–	–	–	–	–	ND	ND
post	+	+	+	+	+	+	+	+

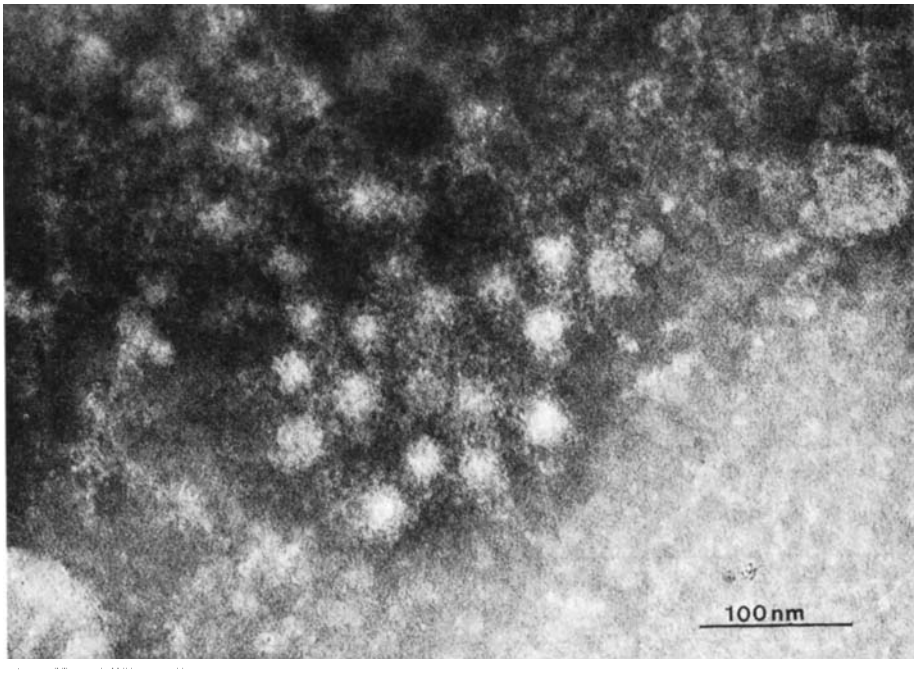
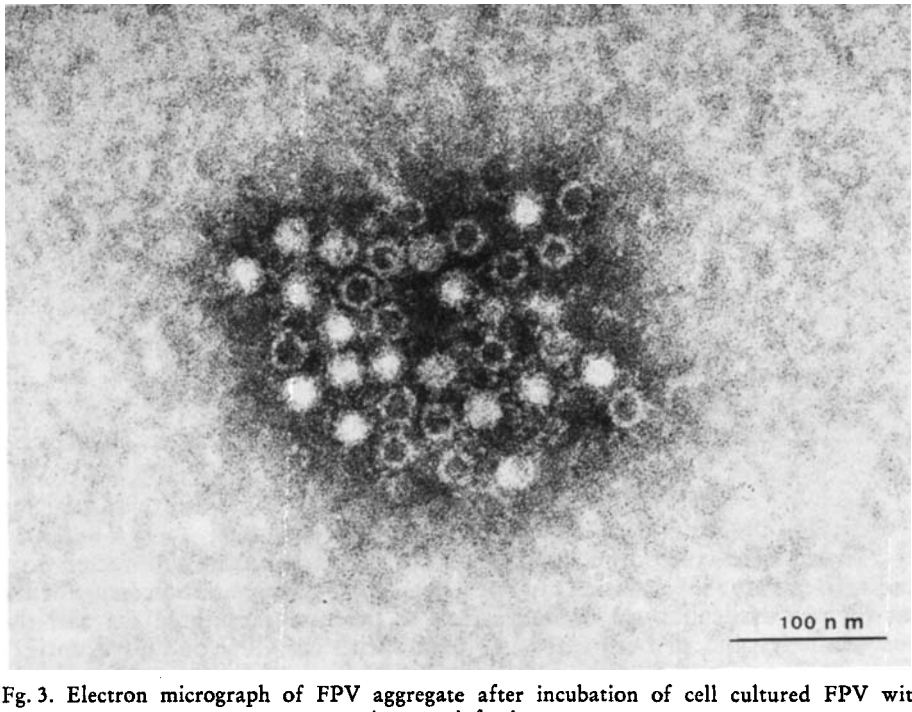


Fig. 2. Electron micrograph of a CPV aggregate, after incubation of a fecal extract with the feline post-vaccination serum



Fg. 3. Electron micrograph of FPV aggregate after incubation of cell cultured FPV with a canine post-infection serum

2. Homologous and heterologous IEM tests

IEM test were performed with the post-vaccination (anti-FPV) serum of the cat (before experimental infection) on fecal samples of the dog from which CPV was isolated and of another dog showing CPV particles in its stools. With both fecal samples clear immune aggregates could be produced using this specific anti-FPV serum (Fig. 2). In addition the same serum-virus combinations used in the homologous and heterologous HAI tests were tested in the IEM tests, with the exceptions shown in Table 2. The results were essentially the same as in the HAI tests: a two-way cross-reaction between both viruses was demonstrated in these IEM tests with the sera which were also positive in the HAI test, while all presera failed to produce any aggregation (Tab. 2, Fig. 3).

3. Homologous and heterologous IF tests

Indirect IF tests were performed in Leighton tube MDCK and CrFK cell cultures, three days after infection with the third passage of CPV and FPV respectively, using the same serum-viral antigen combinations as used in the previous tests with the exceptions shown in Table 2. In homologous and heterologous systems a clear, predominantly nuclear, immunofluorescence was observed (Fig. 4) in all combinations, which were also positive in the two previous tests. Inclusion bodies could be shown to consist of accumulations of virus-specific antigen with these tests (Fig. 4). The negative combinations in the previous tests also proved to be negative in the corresponding IF tests (Tab. 2).

4. Homologous and heterologous SN tests

Minimal neutralization indices of sera used in the previous three tests were determined for CPV and FPV in Leighton tube cultures, using the presence of intranuclear inclusion bodies in HE stained cultures as a criterion

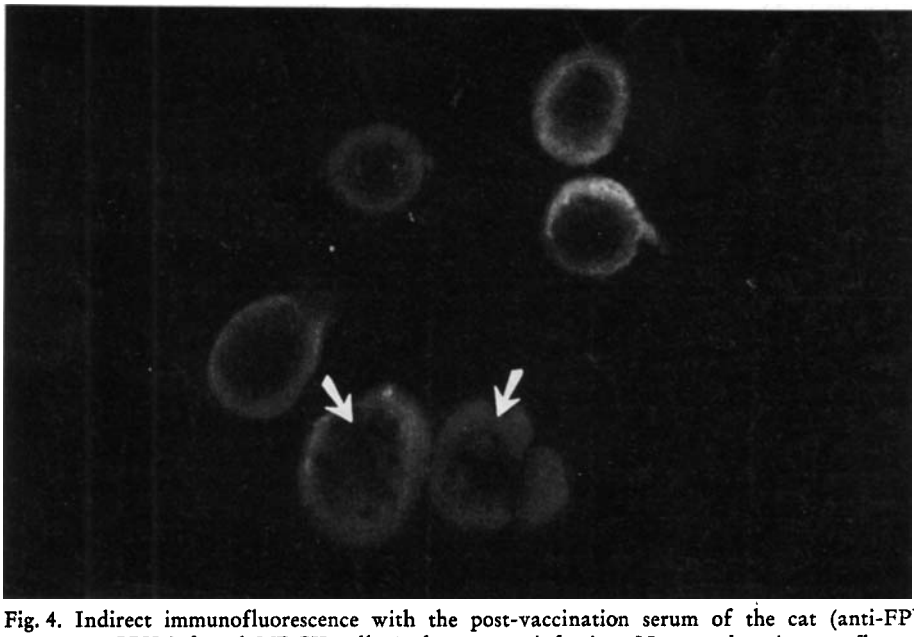


Fig. 4. Indirect immunofluorescence with the post-vaccination serum of the cat (anti-FPV serum) on CPV infected MDCK cells 48 hours post-infection. Note nuclear immunofluorescence and fluorescent inclusion bodies

for virus multiplication. The serum-virus combinations which were tested are shown in Table 2. All combinations showing positive reactions in the previous tests were also positive in the SN test: all sera with neutralization indices $\geqq 2$ are marked with + in Table 2, whereas the sera marked with − did not show neutralization (index < 0.5) in this system.

Serologic screening

In order to estimate the frequency of CPV infections in the breeding colony, 38 sera from randomly sampled dogs in the colony were tested in HAI tests, using 4—8 HAU of CPV antigen and feline erythrocytes. In the same test 62 sera from dogs in the same colony collected about four years earlier and 50 sera collected also about four years earlier from dogs of the open population in the Netherlands were tested. All except four of the recently collected sera of the breeding colony showed high anti-CPV titers (> 64). In contrast no antibodies were found in the sera of this colony collected earlier, suggesting that the infection must have started during the last few years. Also the open population sera collected about four years earlier were all negative.

Experimental infection of cats and dogs with CPV

The non-vaccinated cat showed a slight temperature rise (up to 40 °C) on the second day p. i. and a gradual fall in total WBC counts from 15.2×10^9 to 8.5×10^9 cells/l. on the fourth day p. i. (Fig. 5). In the vaccinated cat no temperature rise was observed and the WBC fall was less pronounced, but initial counts were already low (Fig. 5). No further clinical signs were observed in either of the cats. In dogs no clinical signs were observed whatsoever. All three non-vaccinated animals showed seroconversion after experimental infection with CPV, as is shown in Table 2.

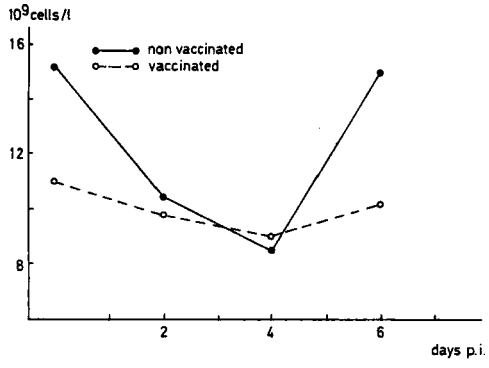


Fig. 5. White blood cell counts of the FPV-vaccinated and the non-vaccinated cat during six days post-infection

Discussion

In the present paper we describe the isolation in the Netherlands of a virus from dogs with acute symptoms of diarrhea and with pathological lesions very similar to those of feline panleukopenia. A close antigenic relationship between this virus and FPV, a parvovirus of the cat, could be demonstrated. On the basis of this relationship, size and morphology of the particles, resistance to chloroform and the nature of the cytopathic changes caused in cell cultures, CPV appears to be a parvovirus. Using pre- and post-inoculation sera from an FPV-vaccinated and a non-vaccinated cat and also from non-vaccinated dogs, which were all experimentally infected with CPV, we were able to demonstrate a two-way cross-reactivity between CPV and FPV in HAI, IEM, IF and SN tests. Although CPV and FPV are closely related, they agglutinate a different spectrum of erythrocytes: CPV agglutinates AGM, cat and swine erythrocytes at 4 °C and FPV only agglutinates swine

erythrocytes at 4 °C. Nevertheless, the cat anti-FPV serum (post-vaccination serum) inhibited HA of AGM, and cat erythrocytes by CPV. These results which coincide with those of others who demonstrated HA activity directly in fecal suspensions (8), probably also distinguish CPV from the previously isolated canine parvovirus MVC, which failed to show cross-reactivity with FPV in HAI tests (15). Another difference between CPV and FPV may be the cell spectrum in which both viruses multiply: CPV did multiply in both MDCK and CrFK cells, although higher titers of infectivity were found in the homologous system, whereas FPV could only be propagated in the latter (OSTERHAUS, unpublished observations). However, this could be a matter of adaptation. A practical implication of the close antigenic relationship between CPV and FPV might be the protection of dogs from CPV infections by vaccination with FPV vaccines. Apart from a mild leukopenia and temperature rise disease symptoms resembling feline panleukopenia were absent in the non-vaccinated cat after experimental infection with cell culture propagated CPV. It is not unlikely that environmental conditions and secondary bacterial infections may be important in the pathogenesis as was shown for feline panleukopenia (13). This is also suggested by the fact that the two dogs did not develop any overt clinical symptoms after experimental infection with CPV, although also in these animals virus multiplication was evidenced by seroconversion. However, the potential role played by this virus in the etiology of contagious diarrhea is suggested by various reports from all over the world, associating a similar virus with outbreaks of diarrhea in the dog. Moreover, in a large number of fecal samples from dogs in the Netherlands, screened during the last few years no parvovirus-like particles were observed (unpublished observations), whereas almost all stools collected from dogs involved in the outbreaks described above were positive. As in mink virus enteritis (for review see 15), a virus closely related to FPV seems to cause a canine viral enteritis. It is still an open question why infections with CPV occurred apparently suddenly in many parts of the world. Serological and clinical data from the Netherlands, and also from the USA (4), suggest that infections date only from the last few years.

Summary

Parvovirus-like particles were demonstrated by negative contrast electron microscopy in feces of dogs involved in outbreaks of a contagious diarrhea in the Netherlands. The virus was isolated in a continuous cell line in which it produced intranuclear inclusion bodies. It showed a close antigenic relationship with feline panleukopenia virus: a two-way cross-reaction was observed in hemagglutination inhibition, immune electron microscopy, immunofluorescence and serum neutralization tests.

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Zusammenfassung

Isolierung eines mit dem Virus der felinen Panleukopenie verwandten Virus von Hunden mit Diarrhoe

Parvovirus-ähnliche Partikel wurden mit Hilfe der Negativkontrast-Elektronenmikroskopie während eines Ausbruches von kontagiöser Diarrhoe

in Fäces von Hunden in den Niederlanden nachgewiesen. Das Virus wurde in einer permanenten Zell-Linie isoliert, in der intranukleäre Einschlußkörperchen nachgewiesen werden konnten. Es zeigte eine nahe Antigenverwandtschaft mit dem Virus der felinen Panleukopenie. Eine beiderseitige Kreuzreaktion wurde beobachtet in der Hämagglutinationshemmung, Immunelektronenmikroskopie, Immunofluoreszenz und im Serumneutralisations-Tests.

Résumé

Isolement d'un virus très proche au virus de la panleucopénie féline chez des chiens diarhéiques

Des particules ressemblant aux parvovirions ont été démontrées au microscope électronique (contraste-négatif) dans les matières fécales des chiens diarrhéiques aux Pays-Bas. Le virus était isolé sur une ligne cellulaire continue dans laquelle il produisait des corpuscules d'inclusion intranucléairs. Le virus manifestait un parenté antigénique avec le virus de la panleucopénie féline: des réactions croisées dans les deux sens étaient observées, utilisant des méthodes d'inhibition d'hémagglutination, immunomicroscopie électronique, immunofluorescence et séroneutralisation.

Resumen

Aislamiento de un virus estrechamente relacionado con el virus de la panleucopenia felina en perros con diarrea

Partículas semejantes a parvoviriones fueron demostrados por medio de microscopía electrónica (contraste negativo) en muestras fecales de perros durante endemias de diarrea infecciosa en los Paises Bajos. Se mostró el aislamiento del virus en una línea celular continua en donde producía corpúsculos de inclusión intranucleares. El virus tiene una relación antigénica estrecha con el virus de la panleucopenia felina: reacciones cruzadas en ambos sentidos fueron observadas por medio de pruebas de inhibición de la hemaglutinación, inmunomicroscopía electrónica, inmunofluorescencia y sueroneutralización.

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