Studies on Canine Parvovirus Isolation, Experimental Infection and Serologic Survey

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Abstract. Nineteen strains of canine parvovirus (CPV) were isolated in primary feline kidney cell and/or Crandell feline kidney (CRFK) cell cultures from 52 dogs clinically suspected of the CPV infection in 1979/80. They were derived from feces and various tissues of dogs, regardless of age, and were serologically indistinguishable from feline panleukopenia virus. Of three dogs exposed to experimental infection, one dog manifested severe symptoms, which were quite similar to those in natural infection, and died. The first clinical sign was vomiting, followed by hemorrhagic diarrhea, fever and remarkable leukopenia. Histopathologic changes were extensive loss of mucosal epithelium of the small intestine, villous atrophy, and dilatation of crypts devoid of epithelial cells. A serologic survey was carried out on CPV infection in 369 dogs by the hemagglutination-inhibition test. Antibody-positive canine sera first appeared in January, 1979. Since then, serologically positive dogs have been increasing in number in Japan.

Canine parvovirus (CPV) was isolated from feces of nonfatal diarrheal dogs in 1977 [8]. A similar virus, which was called “minute virus of canines” (MVC), had been isolated in 1970 from feces of normal dogs. The pathogenicity of MVC was uncertain [3], though MVC antibodies were common in dogs [24].

Then, in the summer of 1978, an acute infectious disease of dogs with vomiting, hemorrhagic diarrhea and an extreme decrease of leukocytes was reported in the U.S.A., where CPV was isolated [1, 2, 4, 7]. Outbreaks of a similar disease occurred almost at the same time in Australia [15, 16], United Kingdom [13, 19], Canada [9, 26], New Zealand [10], Belgium [5] and the Netherlands [20].

In all of them, clinical and pathologic findings were similar to those of feline panleukopenia virus (FPLV) infection. Then CPV was indistinguishable from FPLV in serologic tests [4, 6, 15, 20].

A serologic survey on CPV infection was carried out only in the U.S.A. [6] and Australia [25, 27].

In Japan, similar outbreaks have occurred since March, 1979, prevailing all over the country.

The present report describes the first isolation of CPV, the production of severe disease in experimental dogs, and a serologic survey on CPV infection conducted in Japan.

Materials and Methods

Specimens: Specimens were collected from 52 dogs in 11 veterinary hospitals. These dogs had died from hemorrhagic diarrhea or had been clinically suspected of the CPV infection. Tissue and fecal materials were collected from them and made to 10% (w/v) suspensions in Hanks’ balanced salt solution. After centrifugation at 2,000 rpm for 10
minutes, the supernatant fluid was collected. To it were added antibiotics (as final concentration, 1,000 units of penicillin G potassium, 1,000 μg of streptomycin sulfate, 200 μg of kanamycin and 200 units of nystatin per ml) and/or it was made to pass through a cellulose filter 450 nm in pore size. Then 0.2 ml aliquots were inoculated into Leighton tubes with freshly seeded cells, as well as monolayer cell cultures. On postinoculation day (PID) 1, 3, 5, 7 and 14, the coverslips were removed and stained with May-Grünwald-Giemsa (MGG) solution, and examined for the presence of intranuclear inclusion bodies. The remaining fluid and cells were frozen and thawed, and blind-passaged at least three times to verify that they contained no intranuclear inclusion bodies.

Cell cultures and media: In the first group of 10 dogs, the following cells were used for virus isolation; primary canine kidney (PCK) cells, Madin-Darby canine kidney (MDCK) cells, primary feline kidney (PFK) cells, Crandell feline kidney (CRFK) cells and Vero cells grown in Leighton tubes containing glass coverslips. In the second group of 42 dogs, only CRFK cells were used for virus isolation.

The growth medium used was Eagle's minimum essential medium (MEM) with 10% tryptose phosphate broth and 10% fetal bovine serum. To it were added final concentrations of 200 units of penicillin G potassium, 200 μg of streptomycin sulfate, 100 μg of kanamycin and 50 units of nystatin per ml.

Stock viruses: Stock viruses of CPV and FPLV-TU 1 [18] were produced in CRFK cells grown in Roux bottles. On PID 4, the cells were frozen and thawed, and all the fluid in the bottle was harvested and centrifuged at 2,000 rpm for 10 minutes. The supernatant fluid was stored at −70°C until use.

Virus titration: CPV and FPLV were assayed by determination of the 50% tissue culture infective dose (TCID₅₀) calculated by the method of Reed and Muench [22]. Two-tenth ml of each tenfold dilution of virus material was inoculated into each of four CRFK cultures in Leighton tubes. After incubation at 37°C for 4 days, coverslips were stained with MGG stain and at least 2,000 cells examined for inclusion bodies. The highest dilution of virus producing at least one typical intranuclear inclusion body (Fig. 2) was taken as an end point.

Physicochemical properties of the virus: The Cp 49 strain was used for experiments. Determination of nucleic acid type was performed by titrating the virus in CRFK cell cultures in the presence or absence of 10⁻⁵.₅ M/ml of 5-ido-2'-deoxyuridine (IUDR) in growth medium. The virus was examined for sensitivity to ether, low pH (3.0) and heating (at 50°C for 30 minutes) by the standard procedures. Virus particle size was measured by Millipore filters 450, 220, 100, 50 and 25 nm in pore size.

Hemagglutinating (HA) activity: The HA test was performed by essentially the same tube method as described previously [18] with 1% erythrocyte suspension. To establish a routine procedure, the following conditions were investigated: erythrocytes from different animal species (cattle, horse, pig, sheep, dog and chicken); concentration of erythrocyte suspension (1.0 and 0.5%); temperature (4°C, room temperature and 37°C); pH (6.8–8.0).

Hemagglutination titers was expressed as the reciprocal of the highest dilution of antigen giving complete HA.

Immune sera: Immune sera were prepared against the Cp 49 strain. Two rabbits were inoculated intravenously with 2 ml and four guinea pigs intraperitoneally with 1 ml of the Cp 49 strain at weekly intervals for 4 weeks. One week after the last inoculation, serum samples were collected from all the animals and stored at −20°C. Immune sera to the FPLV-TU 1 strain were also prepared identically. All the immune sera were inactivated by heating at 56°C for 30 minutes.

Serum neutralization (SN) test: The SN test was performed by the serum dilution method. Serial serum dilutions were mixed with an equal volume of virus suspension containing 100 to 320 TCID₅₀/0.1 ml. After incubation at 37°C for 1 hour, 0.2 ml of the mixture was inoculated into each of four Leighton tubes of CRFK cells. After incubation at 37°C for 4 days, the tubes were examined by MGG staining. The SN titer was expressed as the reciprocal of the serum dilution at which 50% of the cultures lacked in viral inclusion bodies. It was calculated by the methods of Reed and Muench [22].

Hemagglutination-inhibition (HI) test: In the HI test, phosphate-buffered saline (PBS) at pH 6.8 supplemented with 0.1% bovine serum albumin was used as a diluent for serum and virus. A quarter ml of twofold serum dilution was mixed with an equal volume of virus suspension containing 4 HA units and placed at room temperature for 1 hour. Then two volumes of 1% porcine erythrocyte suspension in PBS at pH 6.8 were added. Reading was usually made after incubation at 4°C for 18 hours. The HI titer was expressed as the reciprocal of the highest dilution of serum giving a complete HI pattern.

Complement fixation (CF) test: The technique used was identical with that of the modification of
Kolmer's method. The CF titer was expressed as the reciprocal of the highest dilution of serum giving at least 50% fixation.

Experimental infection: Three apparently healthy dogs were used. Of them, two (Nos. 1 and 3) were sero-negative and approximately 8 and 2 months old, and one (No. 2) was sero-positive approximately 8 months old. They were inoculated per os with 4 ml (approximately 10^6.0 TCID_{50}) of the Cp 49 strain that had completed the fifth passage in CRFK cell cultures. During the observation period after inoculation, rectal temperature, clinical signs, especially vomiting and diarrhea, and general clinical conditions were recorded daily, and attempts made to recover the virus from the blood and feces.

Sera from the experimental dogs were inactivated by heating at 56°C for 30 minutes before serologic examination, which was carried out by the SN test and the HI test in a microtiter system (Cooke Laboratory Products). In the HI test, acid-washed kaolin (Fisher Scientific Company) in PBS was used for removing non-specific serum inhibitors. The test serum was diluted to 1:4 in PBS and an equal volume of 25% kaolin suspension added. The mixture was incubated at room temperature for 20 minutes. The kaolin was removed by centrifugation at 2,500 rpm for 10 minutes. Then one-eighth volume of 50% porcine erythrocytes in PBS was added to the serum. The mixture was placed at 4°C for 2-3 hours for adsorption of natural hemagglutinins. The erythrocytes were removed by centrifugation at 2,000 rpm for 10 minutes.

Serologic survey on CPV infection: A total of 369 samples of canine serum were collected mainly in the Tokyo and Saitama areas over a period from 1973 to 1980. All the samples collected before 1979 were derived from dogs without clinical records of parvovirus infection. In 1979 and 1980, 27 and 63 samples, respectively, were collected from dogs suspected of parvovirus infection clinically or in contact with suspected dogs.

The SN test was performed by the same method as described above and sera were diluted by the fourfold step. In the HI test, sera were treated by the same method as described above. The HI test was performed after Appel et al. [2], using 4 to 8 HA units and PBS with 0.1% bovine serum albumin at pH 6.8.

**Results**

Virus isolation: Transmissible agents were recovered from four of ten dogs (strain numbers are Cp 49, Cp 52, P 1 and V 1) in PFK and CRFK cells, but not in any other cell. Then, only CRFK cells were used for virus isolation from the other specimens. Viruses were recovered from 15 of 42 dogs (Table 1). On PID 3 or 5 intranuclear inclusion bodies were evident in these cells. They were not found in cultures of any other cell. When a large size of viral inoculum was applied, cell debris increased remarkably and cells were detached from

![Table 1. Canine parvovirus isolation from dogs clinically suspected of the disease in 1979/80](image-url)
the glass surface in virus-inoculated cultures (Figs. 3, 4). In addition to feces, CPV was isolated from cerebrum, lung, heart, liver, spleen, mesenteric lymph node, pancreas, kidney, small intestine and large intestine. The results of CPV isolation in dogs at various ages are summarized in Table 2. The virus was isolated equally from young, adult, and aged dogs.

Physicochemical properties: As shown in Table 3, the Cp 49 strain was stable to ether, acid and heating. Treatment with IUdR (10⁻⁸.⁵ M/ml) inhibited the growth of the virus. The size of the virus was determined to be less than 25 nm by Millipore membrane filtration.

Hemagglutinating activity: The Cp 49 strain agglutinated porcine erythrocytes only. It failed to agglutinate cattle, horse, sheep, dog, and chicken erythrocytes. Hemagglutination occurred to both 0.5% and 1% erythrocyte suspensions at 4°C. HA titer was little influenced with pH within a range from 6.8 to 8.0.

Immunologic relationship between CPV and FPLV and among CPV strains: As rabbit antiserum contained no non-specific HA inhibitor, it was used in the HI test without any special treatment. In the CF test, guinea pig antiserum was used. The relationships between CPV and FPLV are shown in Table 4. In HI and SN tests, both viruses were closely related with each other, but reacted predominantly to homologous antiserum. In the HI test, all the 19 strains of CPV isolated so far were similar in antigenicity against anti-Cp 49 strain serum.

Experimental infection: Clinical signs, antibody response and virus recovery from
two dogs inoculated experimentally are illustrated in Fig. 1. One of these dogs (No. 1) developed a severe enteric disease characterized by vomiting, diarrhea, fever and leukopenia, and died on PID 6. It closely resembled a natural case in which death occurred after manifestation of severe symptoms. At necropsy, it was found to have lesions in the intestinal tract. The whole serosal surface of this tract was hemorrhagic or congested. The mesenteric lymph nodes were enlarged and congested. The mucosal surface of the intestine was hemorrhagic. There were no gross lesions in any other organ.

Main histopathologic changes were an extensive loss of the mucosal epithelium of the small intestine, villous atrophy, and dilatation of crypts squamatized or devoid of epithelial cells. The other histopathologic changes were necrosis of epithelial cells and the lamina propria in the large intestine and an outstanding decrease in leukocytes in the bone marrow. Intranuclear inclusion bodies were observed in crypts of the small intestine (Fig. 5). Viruses were recovered from blood on PID 2 and from feces on PID 2 to 4.

Serologically, both SN and HI titers began to be elevated on PID 4 to reach 1:722 and 1:2,048 eventually. No CF antibody was detected.

Dog No. 2 presented no clinical signs. In it no viruses were recovered or no significant lesions observed at necropsy.

Dog No. 3 presented no clinical signs either, but the virus was recovered from blood on PID 3 and from feces on PID 2 to 8, except PID 3. SN and HI titers were elevated and CF antibody was also detected. Necropsy revealed lesions of slight proliferation of reticuloendothelial cells in the spleen. No virus was recovered from any organ.

Serologic survey on CPV infection: In the SN test, sera collected over a period from 1977 to 1980 were examined. Positive sera were found first among those taken from dogs without clinical manifestations in January, 1979. All the positive sera had an SN titer of 1:256 or more (Table 5).

In the HI test, serum samples collected over a period from 1973 to 1980 were examined. The results obtained are summarized in Table 6. As in the SN test, positive samples first appeared in January, 1979. They were derived from dogs without clinical manifestations.

Table 7 presents the summarized results of the HI test performed on samples col-
### Table 1: Symptoms of Experimental Infection

<table>
<thead>
<tr>
<th>Dog No. (age) dose and route</th>
<th>Symptom</th>
<th>Days after inoculation</th>
<th>Remarks</th>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>WBC</td>
<td>15000</td>
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<td></td>
<td>Vomiting</td>
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<td></td>
<td>Diarrhea</td>
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<tr>
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<td>HI</td>
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<td>NT</td>
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<td>Vir. rec.</td>
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<tr>
<td></td>
<td>Virus rec.</td>
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#### Table 2: Symptoms of Experimental Infection (continued)

<table>
<thead>
<tr>
<th>Dog No. (age) dose and route</th>
<th>Symptom</th>
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<td></td>
<td>Virus rec.</td>
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#### Figure 1: Experimental infection of dogs with Cp 49 strain

1) Inoculation per os; 2) Positive clinical sign; 3) Died; 4) Neutralization test; 5) Hemagglutination-inhibition test; 6) Complement fixation test.
CANINE PARVOVIRUS

Table 5. Prevalence of SN antibody against canine parvovirus in canine sera (collected 1977–1980)

<table>
<thead>
<tr>
<th>Year</th>
<th>SN titer*</th>
<th>Positive/Tested</th>
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<tr>
<td></td>
<td>&lt; 8</td>
<td>8 — 128 256 512 1024 2048 4096</td>
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<tr>
<td>1977</td>
<td>2**</td>
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<td>1978</td>
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<tr>
<td>1980</td>
<td>3***</td>
<td>1 2 1 4 4 7</td>
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</table>

* Expressed as the reciprocal of the serum dilution at 50% end point.
** No. of dogs without clinical manifestations.
*** No. of dogs suspected of parvovirus infection or having got in contact with suspected dogs.

Table 6. Prevalence of HI antibody against canine parvovirus in canine sera (collected 1973–1980)

<table>
<thead>
<tr>
<th>Year</th>
<th>No. examined</th>
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<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>1024</th>
<th>2048</th>
<th>4096</th>
<th>≥8192</th>
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<td>27***</td>
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<td>1980</td>
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<td>3</td>
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<td>4</td>
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</table>

* Expressed as the reciprocal of the highest serum dilution giving complete HI pattern.
** Without clinical manifestations.
*** Suspected of parvovirus infection or having got in contact with suspected dogs.

Table 7. Percentage of HI positive dogs in 1979/80

<table>
<thead>
<tr>
<th>Year</th>
<th>No. examined</th>
<th>No. positive*</th>
<th>Positive %</th>
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<tr>
<td>1979</td>
<td>(41)**</td>
<td>7</td>
<td>17</td>
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<td></td>
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<td></td>
<td>63</td>
<td>46</td>
<td>73</td>
</tr>
</tbody>
</table>

* ≥1:128.
** Without clinical manifestations.
*** Suspected of parvovirus infection or having got in contact with suspected dogs.

lected in 1979 and 1980. These samples were classified into two groups. One group was derived from dogs with clinical manifestations of the parvovirus infection, and the other from dogs without such manifestations. In both years, the positive rate of the infection was very high among the dogs. In the second group, it was higher in 1980 than in 1979.

Discussion

The present paper deals with the first
isolation of CPV in Japan from dogs with hemorrhagic enteritis and those subjected to experimental infection and a serologic survey on CPV infection.

The Cp 49 strain was identified as *Parvovirus* by its physicochemical characteristics. Eugster and Nairn [8], Osterhaus et al. [20], and Gagnon and Povey [9] isolated CPV in various canine cells. On the other hand, McCandlish et al. [19] isolated CPV in a canine cell culture, but mentioned that the virus appeared to grow better in a feline embryonic cell culture. In the present study, CPV was isolated in PFK and CRFK cell cultures only when the specimen was inoculated immediately after cell seeding.

CPV was isolated from not only feces but also various tissues of dogs, and at an especially high rate from the spleen. In addition to enteritis, myocarditis was reported in CPV infection [11, 12, 14, 17]. In this study, myocarditis was not observed even in dogs from which CPV had been isolated from the heart. It seemed that CPV might have spread to all organs through blood and that CPV might be infective to dogs of all ages.

It has been reported that CPV is serologically indistinguishable from FPLV [4, 6, 15, 20]. In this study, it also appeared that CPV was quite similar antigenically to FPLV. These findings are interesting, because it has been known that FPLV has no pathogenicity for dogs. Then CPV may differ from FPLV in host cell range and hemagglutination spectrum.

Mild diseases were reported in dogs experimentally infected with CPV [2, 6, 20]. In the present study, however, a severe disease was observed among such dogs. When dogs were inoculated per os with the CPV Cp 49 strain, rectal temperature began to elevate on PID 2 and reached a maximum of 40.6°C in one dog. The leukocyte count began to decrease also on PID 2 and reached 1,600 per mm³. Vomiting and diarrhea with hemorrhage were seen on PID 2 and 3, when the virus was recovered from feces was 3 days. This finding explains why the isolation rate of CPV was low in dogs many of which were positive for antibody.

In dogs which had presented a very rapid and clear antibody response, SN titer was 1:96 and HI titer 1:256 on PID 4. This parallelism in the appearance and titer between both antibodies is an interesting finding by itself.

It has been known that the pathologic changes in CPV infection are quite similar to those in feline panleukopenia infection [21]. In the present experimental CPV infection, lesions were found only in the intestinal tract. The whole serosal surface of the intestine was dark red because of hemorrhage or congestion, and the mucosal surface also hemorrhagic. The mesenteric lymph nodes were enlarged and congested. Histopathologically conspicuous were loss of the mucosal epithelium of the small intestine, villous atrophy, and dilatation of the crypts devoid of epithelial cells. Intracellular inclusion bodies were observed in the crypts of the small intestine.

Of the other dogs, Dog No. 2, which was sero-positive (with an HI titer of 1:1,024), presented neither clinical signs nor virus shedding, whereas Dog No. 3, which was sero-negative (with an HI titer of 1:16), presented no clinical signs either, but was positive for virus recovery from blood and feces, with an antibody titer elevated.

Thus, the occurrence of secondary bacterial infection must be a cause of the above-mentioned difference, because numerous hemolytic *Escherichia coli* were isolated from the small intestine of Dog No. 1 at
necropsy.

There are a few reports on the serologic survey of the canine parvovirus infection [6, 25, 27]. In experimental inoculation studies, SN antibody was parallel with HI antibody in the time of appearance, development pattern, and titer (Fig. 1). All the sera positive for the SN test had an HI titer of 1:128 or more. Judging from these results, as well as those of previous reports [6, 27], it seems reasonable in the present report that an HI titer of 1:128 or more was regarded as positive in the HI test. Walker et al. [27] proposed, however, that an HI titer of 1:256 or more should be regarded as positive, and Carmichael et al. [6] an HI titer of 1:320 or more. They [6, 27] used methods different from that used by the authors in details and mentioned nothing clearly about background for deciding an antibody titer to be positive. So there still remains a question about the HI-positive antibody titer.

According to the authors' criteria, first positive samples were taken in January, 1979. Some samples taken before that time, however, had HI titers of 1:8 to 1:32. They must have been discarded as non-specific reactors. Then the virus must have appeared first in Japan around January, 1979, as the etiology of a new disease.

Carmichael et al. [6] submitted first positive samples during the last week in June, 1978, and Walker et al. [27] in May, 1978. As compared with these results, first positive samples were taken in Japan about half a year later than in the U.S.A. and Australia. Although the origin of the virus is unknown as yet, this gap of time of about half a year suggests that the virus may have been imported into Japan.

In 1979 and 1980, the rate of positive serum samples was so high among dogs suspected of the disease or having got in contact with suspected dogs. It seems to reflect the morbidity of the disease. There were many sero-positive dogs which seemed to be involved in subclinical infection. In one colony of 19 dogs without history of the disease, one died of parvoviral enteritis-like disease and all the remained dogs, except one, became sero-positive without presenting any typical clinical sign. Similar results were also obtained from one veterinary hospital. So, severe fatal infection was considered to have occurred in only a few cases. Judging from the present and some previous reports on experimental inoculation with the virus [2, 6, 9, 11, 20, 23], at least another factor may be involved in the pathogenetic mechanism of the infection.

In Japan, CPV is widely spread, but no vaccine is on the way of development as yet. So, much attention should be paid to CPV infection.

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References


要約

イヌ・バラボウイルスの分離、実験感染および血清学的研究：倉野政行・平沢　勉・小西信一郎・尾形　学（農学部家畜微生物学教室）——出血性腸炎で発死、または臨床的にイヌ・バラボウイルス感染症を疑われた犬32頭のうち19頭から、イヌ・バラボウイルスがネコ腫脹代細胞（CRFK細胞）および同初代細胞培養で分離された。本ウイルスは粪便および種々の臓器から分離され、年齢的には必ずしも幼犬に限らず、成・老犬からも分離され、犬は本ウイルスに対して年齢に関わりなく感受性を有するものと考えられた。本ウイルスは血清学的にはネコ・バラボウイルスと全く区別できなかった。また健康な犬3頭における感染実験では、抗体陰性であった犬の1頭が、自然感染における重症発症例と極めて類似した症状を呈し、6日目に発症した。剖検所見では、腸管のほぼ全長にわたる激しい粘膜・粘膜の出血が認められ、病理組織学的には、小腸粘膜上皮の広汎な剝脱・新生に隆の篭張と上皮の剝脱および核内変性体が観られた。369頭の犬血清について本ウイルスのH1抗体を、一部は中和抗体を実施した結果、1978年以降のものは陰性であり、1979年以降の例では、本ウイルス感染症を疑わせるもの、およびそれとの接触があったものでは極めて高率に陽性であるのみならず、このような経歴のないものにおいても陽性例が認められた。

Explanation of Figures

Fig. 2. Typical intranuclear inclusion bodies in CRFK cell inoculated with the Cp 49 strain 5 days after inoculation. May-Grunwald Giemsa (MGG) staining, ×400.

Fig. 3. Normal CRFK cell monolayer 5 days after seeding. MGG staining, ×100.

Fig. 4. Cytopathic changes of CRFK cells by the Cp 49 strain 5 days after inoculation. MGG staining, ×100.

Fig. 5. Intranuclear inclusion bodies (arrows) are seen in the epithelial cells of a crypt in the small intestine. Dog No.1. Hematoxylin and eosin staining, ×400.