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Isolation and Characterization of a Parvovirus of Rabbits

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The isolation and characterization of a new virus from rabbit stool are described. The virus replicated in rabbit kidney cell cultures and agglutinated human group O erythrocytes at 4°C. It was stable at acid pH and resistant to chloroform and heat treatment. The growth of the virus was inhibited by 5-iodo-2-deoxyuridine, and virions were stained red with acridine orange, suggesting that they contain single-stranded deoxyribonucleic acid. The density of virions was 1.41 to 1.44 g/ml in CsCl, and the sedimentation value was 137S in sucrose at 4°C. The infectious particles had cubic symmetry and were 27 to 28 nm in diameter by electron microscopy. By these properties this virus can be classified as a member of the parvovirus group. Antibody response was demonstrated in the rabbit from which this virus was recovered. A number of rabbits from a commercial source were found to contain hemagglutination-inhibiting antibody to this virus.

Since the first isolation of rat virus by Kilham and Oliver (9), parvoviruses, the smallest animal viruses containing single-stranded deoxyribonucleic acid (DNA), have been found in virus stocks (3, 5, 12, 13), tissue cultures (6), and feces (1, 4) in many species of animals. However, parvoviruses of rabbits had not been described before 1972 (2).

During experimental infection of rabbits with herpesvirus cuniculi (HCV), one of the authors isolated two unidentified viruses from rabbit feces (10). These viruses grew in rabbit kidney (RK) cell culture and produced cytopathic effects (CPE) that differed from those of HCV. They were distinguished from HCV by their physicochemical properties and by neutralization tests (10).

This paper reports the morphological, physical, and chemical properties of the virus by which it was determined to be a new parvovirus of rabbits.

MATERIALS AND METHODS

Cells. Primary RK cells were prepared from trypsinized kidney tissues of 2-week-old rabbits as described previously (10). The primary culture and the subcultures of 2nd and 8th to 30th passages were used. Cells were grown in Eagle minimal essential medium supplemented with calf serum (8%), penicillin (100 U/ml), and streptomycin (50 μg/ml). For maintenance, calf serum was reduced to 3%. RK-13 cells, RL-33 cells (16), and HeLa cells were also used.

Virus isolation and infectivity assay. Methods for virus isolation have been described previously (10). In short, four rabbits were inoculated with HCV intravenously or intradermally, and stool specimens were collected every day of the first week and every other day of the following week. Whole blood samples were also obtained at appropriate intervals. All specimens were stored at −60°C until used. Stool suspensions at 10% were made with minimal essential medium and centrifuged at 10,000 × *g* for 30 min. The supernatants were removed and inoculated onto RK cell cultures. Blood specimens were directly inoculated onto RK cell cultures. After the virus was isolated, stock virus was prepared after three to four passages in RK cell cultures. Infectivity was assayed in RK cell cultures by observation of the CPE. The end point of virus titrations was obtained 12 to 14 days after virus inoculation.

Physicochemical characterization. Acid stability and chloroform sensitivity were tested by the methods of Hamparian et al. (8) by using herpes simplex virus as a reference. Temperature stability was tested at 56°C for 60 min and at 60°C for 30 min. To determine the nucleic acid type, the inhibiting effect of IUdR (5-iodo-2-deoxyuridine) on virus replication was tested and acridine orange staining of purified virions was performed. For IUdR inhibition, 10^{-4} mol of IUdR per ml was added to maintenance medium of infectivity assay cells. For acridine orange staining, purified virions were fixed with Carnoy fluid and stained with 0.01% acridine orange at pH 4.0 according to the method of Mayor (11). Virions of bacteriophage ϕX-174 (single-stranded DNA), adenovirus type 3 (double-stranded DNA), and echovirus type 7 (single-stranded ribonucleic acid) were also examined as controls. Virions were treated with deoxyribonuclease (DNase) (100 μg/ml) or ribonuclease (RNase) (100 μg/ml) for 1 h at 37°C and then fixed and stained. Stained virion preparations were examined with a fluorescence microscope (Tiyoda, FM 200A).

HA test. Hemagglutination (HA) was performed on U-type microplates (Cooke Engineering Co., Alexandria, Va.) at 4°C, room temperature, and 37°C with 0.4% erythrocytes of human group O, guinea pig, or

African green monkey. Dulbecco PBS-A containing gelatin (0.005%) and bovine plasma albumin (0.0025%) was used as a diluent. Titers were expressed as a reciprocal of the highest dilution of hemagglutinin that showed complete agglutination.

Purification of virus. Three- to four-day-old cultures of RK cells were inoculated with the F-7-9 isolate at a multiplicity of infection of about 0.1 to 1.0. When 50% or more cells showed CPE, cells were harvested by trypsinization, disrupted by repeated freezing and thawing, and then treated with receptor-destroying enzyme (Takeda Chemical Industries Ltd., Osaka, Japan) and sodium deoxycholate (0.1%). Cell debris was removed by low-speed centrifugation, and the supernatant was layered over a preformed 25 to 45% (wt/wt) CsCl gradient in STE buffer [0.02 M tris-(hydroxy-methyl)aminomethane-hydrochloride–0.1 M NaCl–0.001 M ethylenediaminetetraacetic acid, pH 7.6] and centrifuged at 35,000 rpm (152,000 × g) for 4 h at 4°C with a Hitachi RPS 40T rotor. Fractions were collected from the bottoms of the centrifuge tubes, and each fraction was assayed for HA activity. Two HA peaks (heavy and light) were pooled and recentrifuged in a CsCl gradient, as above. After fractionation, HA activity, complement fixation (CF) antigenicity, and infectivity were titrated in each fraction. Density was determined by direct weighing of a 50-μl portion of each fraction. For estimation of the sedimentation coefficient, the CsCl-purified heavy or light HA peak was mixed with [^{14}C]uridine-labeled virions and [^{3}H]leucine-labeled procapsids of poliovirus as reference. The mixture was then layered on the top of a 15 to 30% (wt/wt) sucrose gradient and centrifuged at 28,500 rpm (70,000 × g) for 3 h at 4°C in a Hitachi RPS 40 rotor.

Electron microscopy. The heavy and light HA peaks obtained by equilibrium density gradient centrifugation were negatively stained with 2% potassium phosphotungstate (pH 7.2) or 2% uranyl acetate (pH 6.3) and examined with a JEM 100U electron microscope.

Sera. Sera of HCV-inoculated rabbits were obtained weekly for 5 weeks after inoculation and once a month thereafter. Normal rabbit sera were collected from healthy rabbits, which were obtained from several farms between 1970 and 1974. Antisera against purified virions of the isolated virus (F-7-9) were obtained from guinea pigs whose hind footpads had been injected with 0.2 ml of CsCl-purified virus (10,000 HA units) mixed with Freund incomplete adjuvant (Difco). Guinea pigs were boostered 3 weeks later in the same manner as in the first injection and then bled 4 days after the second inoculation. Anti-Kilham rat virus (KRV) sera and KRV hemagglutinin were supplied by A. Sasagawa in this laboratory.

Serological tests. Neutralizing antibody titer was expressed as a reciprocal of the serum dilution that neutralized a virus mean tissue culture infective dose of 100. Hemagglutination inhibition (HI) tests were performed as follows: serum specimens were treated overnight with RDE at 37°C and then inactivated at 56°C for 30 min. In some cases, sera were absorbed with human group O erythrocytes. Serial twofold dilutions of sera were mixed with an equal volume of hemagglutinin containing 8 HA units, incubated for 60 min at room temperature, and then mixed with 2 volumes of 0.4% erythrocyte suspension. After incubation at 4°C for 2 or more h, the reciprocal of the highest dilution of sera that inhibited HA was determined as the HI titer. CF tests were done in microplates by using 4 U of antigen (for antibody assay) or 4 U of antiserum (for antigen assay) with 5 $C'H_{50}$ units of complement. For the CF tests, rabbit serum was inactivated by heating at 60°C for 20 min, and guinea pig serum was inactivated at 56°C for 30 min.

RESULTS

Virus isolation. Forty-seven stool suspensions and eight whole blood specimens from four rabbits were inoculated onto RK cell cultures. Cytopathic agents were isolated from the stools of one rabbit (rabbit no. 7) on days 10 and 14 after HCV inoculation, and they were designated as the F-7-9 and F-7-11 isolates, respectively. Both isolates produced similar CPE in RK cell cultures. Figure 1B shows cytopathic changes of RK cells infected with the F-7-9 isolate. Intranuclear inclusion bodies were observed in the infected RK cells that had been stained with May-Grünwald-Giemsa solution (Fig. 1C). Both isolates were not neutralized by anti-HCV serum but were neutralized by the serum of rabbit no. 7 from which they had been recovered. Since the infectivity of the F-7-11 isolate was neutralized by anti-F-7-9 guinea pig serum, both isolates were considered to be the same virus, and therefore the F-7-9 isolate was mainly examined in the following experiments. Antibody response to the F-7-9 isolate was demonstrated in rabbit no. 7 by neutralizing antibody, HI, and CF tests after virus recovery (Table 1).

Host range. Susceptibility of RK cells to the F-7-9 isolate was extremely low in the primary or secondary cultures, but highly susceptible cells were obtained at the 8th to 30th passage of one RK cell culture. These cells were 10,000 times more sensitive to this virus than the primary or secondary cell cultures. (The titer of a virus sample was determined to be $10^{7.0}$ $TCID_{50}$ per 0.2 ml in the former cells and $10^{3.0}$ $TCID_{50}$ per 0.2 ml in the latter cells.)

In the two rabbit cell lines (RK-13 and RL-33) tested, the F-7-9 isolate replicated but showed very mild CPE. The virus did not replicate in HeLa cells.

Chemical and physical properties. As shown in Table 2, the F-7-9 isolate was resistant to chloroform and acid treatment. Infectivity was not reduced by incubation at 56°C for 60 min or at 60°C for 30 min. Replication of the F-7-9 isolate seemed to be inhibited by IUdR since the titer obtained in the IUdR-treated cells was $10^{3.5}$ times lower than that obtained from untreated cells.

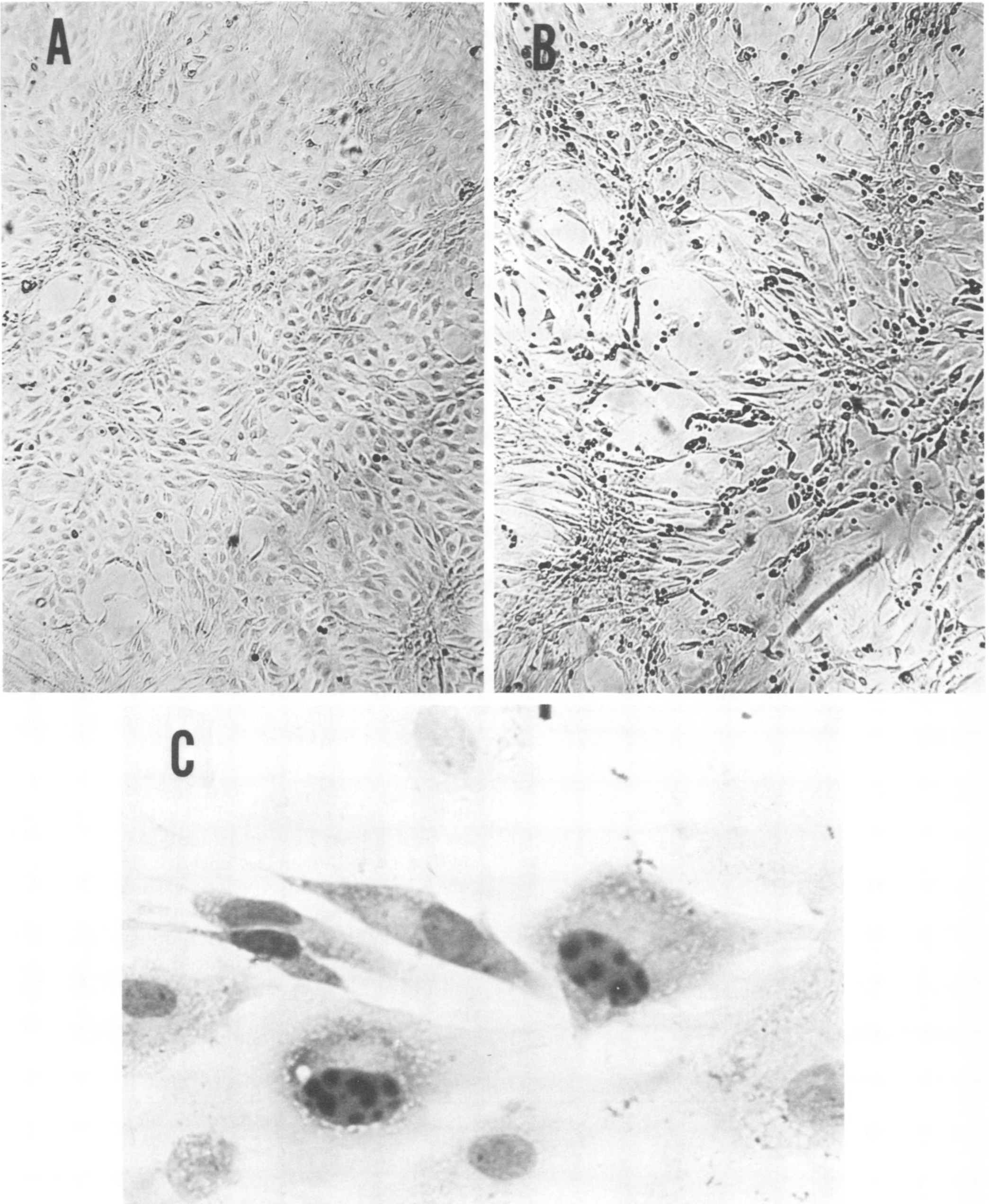

FIG. 1. *Secondary cultures of RK cells infected with the F-7-9 isolate. (A) Uninfected control cells. (B) Infected cells showing CPE. (C) Intranuclear inclusion bodies observed in the infected cells (May-Grünwald-Giemsa staining).* ×1,300.

HA test. In early experiments, infected cells together with the culture fluid were frozen and thawed three times, and, after removal of cell debris by low-speed centrifugation, the HA activity of the supernatant was assayed. Only low HA titers were obtained by this method. Therefore, infected cells were treated in the following way: collection by trypsinization and disruption by freezing and thawing followed by incubation with receptor-destroying enzyme and sodium de-

oxycholate. The disrupted cells were then centrifuged at 2,500 rpm for 10 min. A respectable HA titer was obtained when the supernatant was tested with human group O erythrocytes at 4°C. HA occurred neither at room temperature nor at 37°C. When HA-positive microplates were transferred to 37°C, HA was diminished, but after mixing them well and bringing them back to 4°C, HA appeared again. Similarly, guinea pig erythrocytes were not agglutinated by the virus at room temperature or at 37°C. The HA titer obtained at 4°C with guinea pig erythrocytes was about 1/32 that obtained with human erythrocytes. With African green monkey erythrocytes, HA titer was 1/8 to 1/16 that obtained with human erythrocytes.

Purification of virus. When the F-7-9 isolate was centrifuged in a CsCl density gradient, HA and CF antigens were banded in two major peaks. Peaks from several tubes were pooled and recentrifuged in CsCl. The distribution of HA and CF antigens and infectivity are shown in Fig. 2. The buoyant density of the heavy peak was 1.41 to 1.44 g/ml, and that of the light peak was 1.31 to 1.33 g/ml; another small peak was detected at a density of 1.36 g/ml when assayed by the CF test. This intermediate peak coincided with a shoulder of HA activity. More than 99% of the total infectivity was associated with the heavy peak.

The sedimentation value in sucrose of the heavy particles was estimated as 137S compared with the values of virions (160S) and procapsids (80S) of poliovirus. Light particles had a broad distribution with a mean value of 63S.

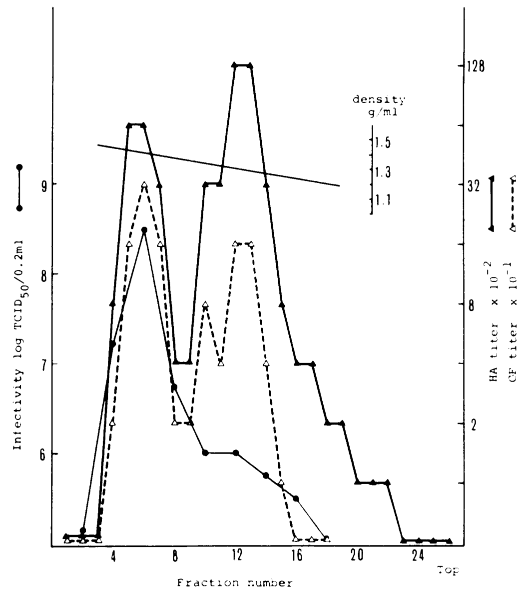

FIG. 2. *Distribution of HA and CF antigens and infectivity of the F-7-9 isolate in a CsCl density gradient. A 4-ml portion of the mixture of heavy and light HA peaks obtained by isopycnic centrifugation of the infected cell extracts was layered on the top of 8 ml of preformed CsCl gradient (45 to 25% [wt/wt] CsCl in STE buffer) and recentrifuged at 152,000 × g for 4 h. A total of 26 fractions were collected and assayed.*

TABLE 1. *Antibody response in rabbit no. 7 to the F-7-9 isolate*[a]

Serum no.	Time after HCV inoculation	Antibody titer		
		NT[b]	CF	HI
S-7-0	Before inoculation	<4	ND[c]	ND
S-7-2	7 days	<4	<4	<8
S-7-3	14 days	≤4	4	<8
S-7-4	21 days	128	ND	128
S-7-5	28 days	64	32	1,024
S-7-19	14 months	320	ND	256

[a] The F-7-9 isolate was obtained from rabbit no. 7 on day 10 after HCV inoculation.
[b] NT, Neutralizing antibody.
[c] ND, Not done.

TABLE 2. *Physicochemical properties of the F-7-9 isolate*

Treatment	Virus	Infectivity (log $TCID_{50}$/0.2 ml)		
		Untreated (A)	Treated (B)	A − B
Acid	F-7-9	2.5	2.5	0.0
(pH 3; RT,[a] 3 h)	HSV (VR no. 3)	4.5	<0.5	>4.0
Chloroform	F-7-9	2.5	2.5	0.0
(4°C, 3 h)	HSV (VR no. 3)	3.5	<0.5	>3.0
56°C, 60 min	F-7-9	6.5	6.5	0.0
60°C, 30 min	F-7-9	6.5	6.5	0.0
IUdR[b]	F-7-9	6.5	3.0	3.5
(10^{-4} mol/ml)	HSV (VR no. 3)	4.5	2.5	2.0

[a] RT, Room temperature.
[b] Infectivity was assayed in RK cell cultures in the presence (B) or absence (A) of IUdR.

Electron microscopy. Figure 3 shows an electron micrograph of heavy and light particles purified by isopycnic centrifugation in a CsCl density gradient. The heavy particles consist of intact virions (Fig. 3A), and the light particles consist of empty capsids (Fig. 3B). The particles had cubic symmetry and were 27 to 28 nm in diameter. Capsomeres were demonstrated in some particles.

Acridine orange staining of virions. Virions of adenovirus type 3, echovirus type 7, bacteriophage ϕX-174, and the F-7-9 isolate were purified by banding in a CsCl density gradient and then dialyzed against PBS-A. Smears were made on cover slips, dried at 37°C, and then fixed and stained as described above. Adeno-3 virions were stained yellow-green, and echo-7 and ϕX-174 virions were stained red. Under the same conditions, the F-7-9 virions were stained red. The staining character of the F-7-9 and ϕX-174 virions was susceptible to DNase, but not RNase, treatment. These results along with the IUdR sensitivity of viral replication suggest that the F-7-9 virions contain single-stranded DNA.

Cross-reaction with KRV. Considering the characters described above, the F-7-9 isolate is classified as a parvovirus. To test the immunological relationship of the F-7-9 isolate with another parvovirus, cross-reactivity of the F-7-9 isolate and an available strain of KRV was examined by the HI test. The HA and HI tests of KRV were performed at room temperature with 0.4% guinea pig erythrocytes. No cross-reactivity was observed between KRV and the F-7-9 isolate (Table 3).

Serological surveillance. A total of 90 sera obtained from commercial rabbits were tested for the presence of HI antibody to the F-7-9 isolate. As shown in Table 4, the antibody was demonstrated in 42 rabbits (46.7%). The mean HI titer of antibody-positive sera was 1:256.

DISCUSSION

This is the first report of the successful isolation of a parvovirus from rabbits. The size of the virions was somewhat larger, but all other

TABLE 3. *Immunological comparison of the F-7-9 isolate and KRV*[a]

Serum	HI titer against:	
	F-7-9	KRV
Anti-F-7-9 rabbit serum		
S-7-4	128	<4
S-7-7	128	<4
S-7-11	256	<8
Anti-KRV rat serum		
No. 1	<4	128
No. 2	<4	256
No. 3	<4	2,048

[a] HI tests were performed with human group O erythrocytes at 4°C for the F-7-9 isolate and with guinea pig erythrocytes at room temperature for KRV. Antibody titer is expressed as a reciprocal of the highest dilution of serum that inhibited 8 U of hemagglutinin.

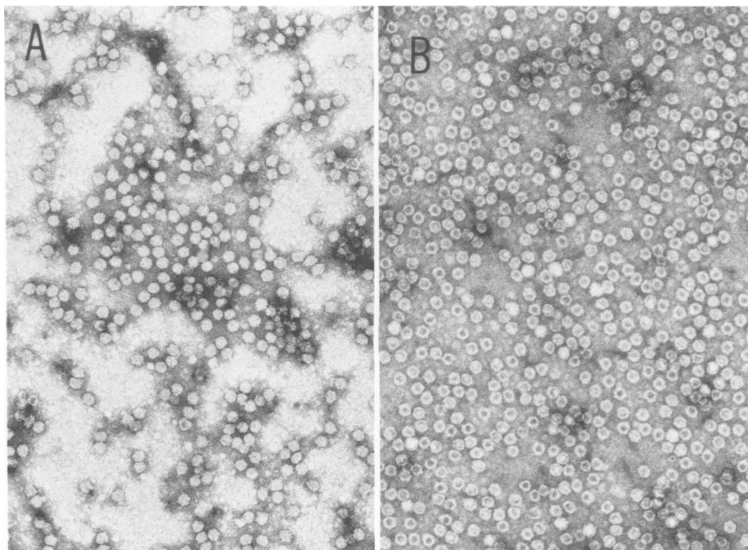

FIG. 3. *Negatively stained preparation of CsCl-banded F-7-9 isolate showing heavy (A) and light (B) peaks. The heavy peak contains full particles and the light peak contains mainly empty particles.* ×64,500.

TABLE 4. *Distribution of the HI antibody titer to the F-7-9 isolate among 90 normal rabbits*

HI titer[a]	No. of rabbits (%)	
<16	48	(53.3)
16	0	42[b] (46.7)
32	3	
64	3	
128	7	
256	11	
512	14	
≥1,024	4	

[a] Antibody titer was expressed as a reciprocal of the highest dilution of serum which inhibited 8 U of HA.

[b] Number of HI antibody-positive rabbits.

properties reported in this communication are identical to those of parvoviruses (15). Immunological cross-reactivity was not demonstrated between KRV and the F-7-9 isolate by the HI tests. The HA reaction of this virus was different from that of rodent and porcine parvoviruses (7, 14) in its low HA activity with guinea pig erythrocytes and nonreactivity at room temperature, and it was also distinguished from minute virus of canines (4) by agglutinating activity with human group O erythrocytes.

The virus was recovered from feces of rabbits that had been inoculated with HCV (10). Consequently, there arose the question of whether the virus was present as a contaminant in HCV stocks and coinoculated into rabbits or whether the rabbit had been contingently infected with this virus before HCV inoculation and had excreted the virus in feces after a latent period. The former possibility is unlikely because the virus was isolated from only one of four HCV-inoculated rabbits, and it has never been recovered from tissue cultures inoculated with the same stocks of HCV. However, the fact that the specific antibodies to this virus were not demonstrated in the rabbit before HCV infection supports the possibility of contamination of the HCV stocks with this parvovirus. Further experiments are expected to clarify this issue and to determine the latent period of infection in rabbits. The possibility that HCV inoculation activates a preexisting parvovirus infection in rabbits will also have to be investigated.

To our knowledge, no parvovirus has been isolated from rabbits, although a number of parvoviruses have been reported from other species of animals (2). One reason that rabbit parvoviruses have not been previously described is that primary or secondary RK cell cultures (which are usually used for virus isolation) are not very sensitive to this rabbit virus. A very careful and lengthy observation period is required to detect CPE in these cells.

Interestingly, it was found that the antibody to this virus was present in a high proportion of rabbits obtained from a commercial source. This parvovirus of rabbits may be distributed widely in Japan. Since rabbits are extensively used in the laboratory for preparing immune serum and RK cell cultures and for other purposes, special caution must be taken against the possible modification of experimental results by this virus.

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