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Isolation and Characterization of Sialodacryoadenitis Virus (Coronavirus) from Rats by Established Cell Line LBC

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With 2 figures and 3 tables

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Summary

The outbreak of sialoadenitis occurred in a laboratory rat colony and the causative agent was isolated from the affected salivary glands of diseased rats using the established cell line LBC. The isolate readily multiplied, producing clear cytopathic effects with syncytium formation, and it was identified virologically and serologically as rat sialodacryoadenitis virus. In attempts to isolate the virus by primary rat kidney (PRK) cells and suckling mice as well as LBC cells, the LBC cells showed higher susceptibility for the virus growth as compared with PRK cells or the brain of suckling mice. The isolation rate of virus was 100 % (5/5) in LBC, 40 % (2/5) in PRK cells and 60 % (3/5) in suckling mice.

After four passages in the LBC cells, the virus did not produce disease in adult rats, while the mouse brain-passaged virus did.

Introduction

Sialodacryoadenitis virus (SDAV) (BHATT et al., 1972) is a member of the coronavirus group (SIDDEL et al., 1983), and is distributed world wide in rat colonies. The outbreak of SDAV infection has been reported in many rat colonies (UTSUMI et al., 1978, 1980; KOJIMA et al., 1980; MARU and SATO, 1982; YAMAGUCHI et al., 1982). The virus shares common antigen with rat coronavirus (RCV) (PARKER et al., 1970) and mouse hepatitis virus (MHV) (BHATT et al., 1972; SIDDEL et al., 1983). Seromonitoring is widely conducted for detection of infections in breeding and experimental colonies of laboratory rats and mice (UTSUMI et al., 1978, 1980; FUJIWARA et al., 1980; NAKAGAWA et al., 1984; MACHII et al., 1988), suggesting that inapparent infection with SDAV, RCV or MHV might be perpetuating in rat colonies (UTSUMI et al., 1978, 1980; KOJIMA et al., 1980; MARU and SATO, 1982; YAMAGUCHI et al., 1982). From affected rats SDAV has been isolated by inoculation into the brain of suckling mice (KOJIMA et al., 1980; MARU and SATO, 1982; YAMAGUCHI et al., 1982) or PRK cell cultures (KOJIMA et al., 1980).

The authors previously reported a successful propagation and plaque formation of RCV and SDAV on the rat cell line LBC that had been established from a mammary tumour of Lewis rat (HIRANO et al., 1985, 1986b, 1986c; HIRANO and ONO, 1990).

In October 1989, the outbreak of SDAV infection occurred in the National Institute of Physiological Science (NIPS) in Okazaki, Japan. This study deals with the isolation and characterization of SDAV using LBC cell cultures.

Materials and Methods

Cell cultures

The LBC cells (HIRANO et al., 1985) and CatS + L − cells (HIRANO et al., 1986a) were grown at 37 °C in Eagle's minimum essential medium (MEM) containing 10 % fetal calf serum (FCS) and kanamycin (0.06 mg/ml). The primary rat kidney (PRK) cells were prepared from 4-week-old Wistar rats by a conventional method and were grown at 37 °C in MEM containing 5 % FCS. For maintaining cells and harvesting the virus, FCS concentration was reduced to half.

Virus isolation

A 1:10 homogenate of salivary glands was prepared in MEM containing penicillin G (2000 U/ml) and streptomycin (2000 μg/ml), and half volumes of homogenates were stored at −70 °C for further studies. After centrifugation at 700 × *g* for 10 min, the supernatant was inoculated into LBC cell cultures. The inoculated cultures were incubated at 37 °C for 60 min for virus absorption, and were fed maintenance medium and kept at 37 °C.

Plaque assay

Isolates were assayed for infectivity by a plaque method described previously (HIRANO and ONO, 1990). As references, SDAV 930-10 (SDAV-930) (KOJIMA et al., 1980), feline calicivirus (FCV) A423 and feline herpesvirus (FHV) 406 (HIRANO et al., 1986a) strains were used. For the plaque assay of FCV and FHV, CatS + L − cells were used as described previously (HIRANO et al., 1986a). The infectivity was expressed in plaque-forming units (PFU).

Physicochemical properties of the isolate

Sensitivity to ether and chloroform, effect of 5-iodo-2-deoxyuridine (IUDR) on virus growth, pH 3.0 stability, heating at 50 °C and filtration test were examined as described previously on MHV-2 strain (HIRANO et al., 1978). Sensitivity to ether or chloroform was tested by mixing with an equal volume of virus material and organic solvent and by incubating at 37 °C for 10 min. To see the effect of IUDR on virus growth, LBC and CatS + L − cell cultures were pretreated with IUDR (50 μg/ml) at 37 °C for 2 h and inoculated with the viruses at an input of 1 PFU per cell. After virus adsorption at 37 °C for 60 min, the cultures were incubated at 37 °C for 36 h in a maintenance medium containing 50 μg/ml IUDR. Cultures without drug served as control. Stability at pH 3.0 was examined by incubating at 37 °C for 60 min. Sensitivity at 50 °C was examined by heating for 60 min. A filtration test was made by passing through 50–200 nm membrane filters (Sartorius, Göttingen, Germany).

Electron microscopy

The supernatant of infected culture fluid was negatively stained with 2 % uranyl acetate and examined by electron microscopy (Hitachi H-600A, Tokyo, Japan).

Animal inoculation

Specific pathogen-free pregnant ICR mice and 15-week-old male Wistar rats were obtained from a commercial breeder colony (Japan SLC, Hamamatsu, Japan) which documented to be free from MHV and SDAV infections. The suckling mice were nursed by their dams and were intracerebrally (i.c.) inoculated with virus material (0.02 ml). Rats were inoculated intranasally (i.n.) with virus material (0.2 ml) and were observed for 14 days. Animal experiments were performed humanely in accordance with the guidelines of animal experimentation of Iwate University.

Antiserum preparation

Rats aged 15 weeks were inoculated i.n. with the isolate (10^6 PFU/0.2 ml), and inoculated twice intraperitoneally (i.p.) with the virus at an interval of 7 days. Rats were sacrificed for

collecting blood at 7 days after the last inoculation. All the animals were kept in the vinyl isolators and given the autoclaved commercial pellets and water. Antiserum against SDAV-930 was prepared in the same manner as described previously (HIRANO and ONO, 1990). Prior to i.n. inoculation, some rats were bled for collecting blood from the heart and were checked free from infection of SDAV, RCV or MHV by serology.

Immunofluorescence (IF)

The indirect IF was carried out as described previously (HIRANO et al., 1986a, 1986b; HIRANO and ONO, 1990). The infected LBC cell cultures grown on coverslips were washed once with phosphate buffered saline (PBS, pH 7.2) and fixed with cold acetone, and then treated with rat antiserum at 37 °C for 60 min. After washing three times with cold PBS, the infected cell cultures were stained with fluorescein isothiocyanate conjugated anti-rat IgG rabbit serum (Miles Biochemical, Kantakee, USA) diluted 1 : 20 in PBS at 37 °C for 60 min. Non-infected cell cultures were used as control. The antibody titre was expressed as the highest dilution of serum showing positive fluorescence.

Neutralization (NT) test

The test was carried out by 50 % plaque reduction method. The virus sample (200 PFU/0.2 ml) was incubated with rat antiserum of varying concentration at room temperature for 30 min. The mixtures (0.2 ml) were inoculated into LBC cell cultures and the number of plaques was assayed as described above. The antibody titre was expressed as the highest dilution of antiserum inhibiting 50 % plaque formation.

Results

Outbreak of sialoadenitis (SDA) in rats and isolation of causative agent

In October 1989, ocular discharges and swelling of salivary glands strongly indicative of SDAV infection were detected in three of five male Wistar-Imamichi rats (about 300 g) and seven of 15 male Wistar rats (about 200 g) in NIPS. All rats were killed in the room, which was then disinfected with formaldehyde. The frozen salivary glands of five affected rats were sent to the laboratory and the supernatant of tissue homogenates (1 : 10) was inoculated into LBC cell cultures. All the inoculated cultures showed cytopathic changes in 2 days by cell rounding and syncytium formation, as seen with RCV and SDAV infection in LBC cell cultures (HIRANO et al., 1978, 1986b, 1986c). The inoculated cell monolayers prepared on coverslips were fixed with cold acetone at 10 h post-inoculation (p.i.). and subjected to indirect IF using antiserum to SDAV-930. As shown in Figure 1, SDAV antigen was detected in the cytoplasm of LBC cells. These isolates were designated as L-1 to L-5, which were readily passaged in LBC cell cultures at intervals of 2 days. Cytopathic changes appeared earlier after several passages, showing virus titres of more than 1×10^6 PFU/0.2 ml within 24 h p.i. The isolates produced clear plaques 2.5–3.0 mm in diameter by the agar overlay method described previously (HIRANO and ONO, 1990).

Attempts to isolate the virus were made by PRK cells and suckling mice as well as LBC cells. The affected salivary gland homogenates stocked at −70 °C were inoculated into LBC, PRK cell cultures and 2-day-old mice. The inoculated LBC cells developed cytopathic effects (CPE) within 2 days. In PRK cell cultures, CPE appeared in two of five experiments on day 3. After inoculation into suckling mice, central nervous system (CNS) symptoms developed on days 5–7 in three of five experiments. From the brains of mice showing CNS signs, cytopathic agents were isolated on LBC cells, and they were positive for SDAV by IF. The isolation rate of virus was five of five in LBC, two of five in PRK cells and three of five in suckling mice as shown in Table 1. In inoculation into LBC and PRK cells with L-1 isolate at an input of 1 PFU per cell, the infectivity titres of the isolate was 5×10^4 and 2×10^5 PFU in PRK and 7×10^5 and 5×10^6 PFU in LBC cell cultures at 24 and 36 h p.i., respectively. The virus yield in LBC cell cultures was more than 10-fold higher than that in PRK ones.

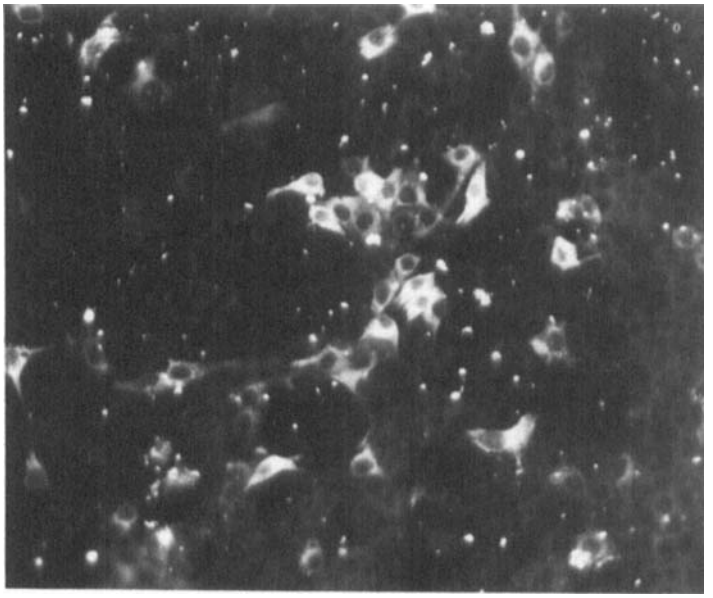

Fig. 1. Viral specific fluorescence in the cytoplasm of LBC cells, 7 h p.i.

Table 1. Comparative susceptibility of LBC or PRK cell cultures and suckling mice for SDAV isolation

	Isolation by		
Virus sample[1]	LBC[2]	PRK[2]	Sucklings[3]
1	+ (L-1)[4]	+ (L-1P)	+ (L-1m)
2	+ (L-2)	+ (L-2P)	+ (L-2m)
3	+ (L-3)	–	–
4	+ (L-4)	–	–
5	+ (L-5)	–	+ (L-5m)
Isolated/tested	5/5	2/5	3/5

[1] Frozen homogenates of affected salivary glands stored at −70 °C.
[2] LBC and PRK cell cultures prepared in 50 ml plastic bottles inoculated with 0.2 ml virus material, which were kept at 37 °C for 60 min, were given maintenance medium and incubated at 37 °C. Culture fluids on days 2 and 4 were inoculated into LBC cells and tested for infectivity and viral antigen (IF)
[3] The same virus materials were i.c. inoculated into five 2-day-old mice, which were nursed by their dams for 14 days. Brain homogenates (1 : 10) were inoculated into LBC cells and tested for infectivity and viral antigen (IF)
[4] Designation of isolates

Physicochemical properties of the isolates

The physicochemical properties of the L-1 to L-5 were compared with those of SDAV-930, FCV and FHV. As shown in Table 2, all the isolates, SDAV-930 and FHV were sensitive to ether and chloroform. While virus growth of FHV was significantly affected by IUDR, that of the isolates as well as SDAV-930 and FCV was not affected. The isolates and SDAV-930 were resistant to pH 3.0 treatment inactivating FCV and FHV. The titres of the isolates decreased to 1 : 10 000 after heating at 50 °C for 60 min. The isolates were passed through 100 and 200 nm membrane filters but not through a 50 nm filter.

Table 2. Physicochemical properties

Treatment	Infectivity titre[1] Isolate L-1	SDAV-930	FCV	FHV
Control	6.5	6.7	7.5	6.3
Ether	0	0	7.7	0
Chloroform	0	0	7.5	0
Control	7.3	6.7	7.0	6.7
IUDR (50 µg/ml)	7.0	6.7	7.7	2.0
pH 3.0	5.7	5.5	0	0
pH 7.0	6.7	7.0	7.0	4.5
50 °C				
0 min	6.7	7.0	NT[2]	NT
60 min	3.0	3.0	NT	NT
Filtration				
200 nm	6.5	6.7	7.7	6.3
100 nm	5.3	5.7	NT	2.0
50 nm	0	0	7.3	0

[1] Infectivity titre (log PFU/0.2 ml)
[2] NT = not tested

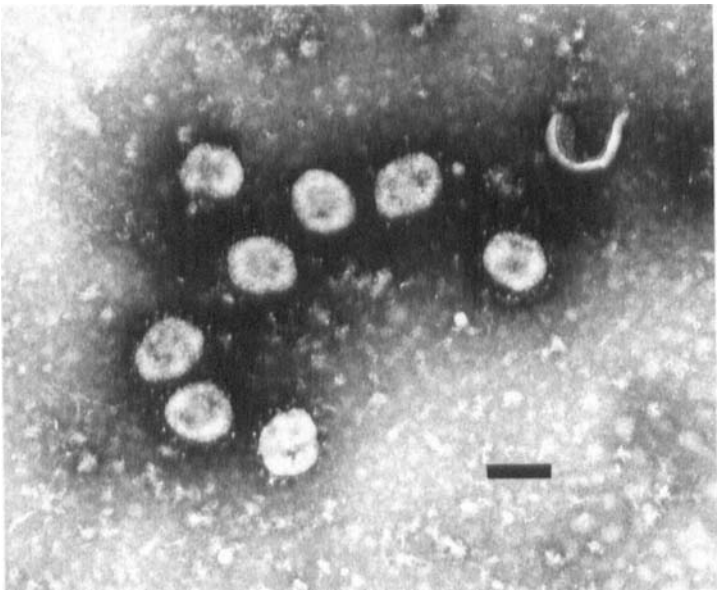

Fig. 2. Negatively stained virus particles of L-1 strain from the infected culture fluid of LBC cells (× 100 000). Bar indicates 100 nm

Electron microscopy

As shown in Figure 2, numerous virions, 110–150 nm in diameter, with characteristic spikes, were shown in the infected culture fluids of LBC cells with the L-1 isolate.

Table 3. Relative antigenicity of isolate L-1 and SDAV-930 by indirect IF and NT test

Virus	IF		NT	
	Anti-L-1	Anti-SDAV-930	Anti-L-1	Anti-SDAV-930
L-1	12 800[1]	3200	1280[2]	1280
SDAV-930	3200	6400	640	5120

[1] Reciprocal highest dilution of antiserum demonstrating specific viral antigen
[2] Highest dilution of antiserum showing 50 % plaque reduction

Serology

In indirect IF, anti-SDAV-930 and anti-L-1 sera stained the cytoplasm of LBC cells infected with both viruses. Non-infected cells were negative. In the cross-NT test, there were cross-reactions between the SDAV-930 and the L-1 isolate and their respective antisera as shown in Table 3.

Pathogenicity for rats

Rats aged 8 weeks, inoculated i.n. with 1×10^6 PFU of the isolate L-1 at the sixth-passage level on LBC cells, were apparently normal for 14 days without submaxillary swelling. In the next experiment, five 15-week-old rats were inoculated i.n. with 1×10^6 PFU of isolate L-1m at the fifth-passage level in suckling mice. At 5–7 days p.i. three of five rats showed submaxillary swelling and virus was recovered from the three affected rats at autopsy on day 7. After inoculation of the L-1 virus at the fourth-passage level (1×10^6 PFU) into 15-week-old rats, no disease was produced, but serum NT antibody titres of 1:64 to 1:512 were detected in the non-diseased animals.

Discussion

The outbreaks of SDAV infection have been reported in many rat breeding and experimental colonies, causing serious problems (NUNOYA et al., 1977; UTSUMI et al., 1978, 1980; FUJIWARA et al., 1980; KOJIMA et al., 1980; MARU and SATO, 1982; YAMAGUCHI et al., 1982; NAKAGAWA et al., 1984; MACHII et al., 1988), and SDAV has been isolated from the affected rats by inoculation of suckling mice (KOJIMA et al., 1980; MARU and SATO, 1982; YAMAGUCHI et al., 1982) and PRK cells (KOJIMA et al., 1980). However, there has been no report on SDAV isolation using established cell lines. Growth of SDAV as well as RCV in cell line LBC has previously been reported (HIRANO et al., 1985, 1986b, 1986c; HIRANO and ONO, 1990), and it seems that LBC cells might be useful for isolating and studying wild as well as laboratory strains of SDAV. In this study, within 2 days after inoculation SDAV was isolated from the affected salivary glands by LBC cell cultures, and it grew well in LBC cells with syncytium formation as seen in cases of RCV and SDAV (HIRANO et al., 1985, 1986b, 1986c).

These isolates were serologically identified as SDAV by indirect IF and cross-NT test using anti-SDAV-930 rat serum. The physicochemical properties of the isolates were similar to those of SDAV-930 and other SDAV strains (BHATT et al., 1972; KOJIMA et al., 1980). Negatively stained virions of the L-1 isolate had a medium size and characteristic spikes similar to those of RCV and SDAV 681 (BHATT et al., 1972) grown in LBC cells (HIRANO et al., 1985, 1986c).

For routine virus isolation, suckling mice and PRK cells have been used (KOJIMA et al., 1980; MARU and SATO, 1982; YAMAGUCHI et al., 1982) but LBC cells were proved to be more useful, showing the highest isolation rate and virus yield, whereas

CPE developed a little slowly. The PRK cells and suckling mice were also susceptible to SDAV, but there might be risk of contamination with rat or murine viruses.

The L-1 isolate was shown to share common antigen with SDAV-930 by indirect IF and cross-NT tests as reported by KOJIMA et al. (1980) on SDAV 681 strain and SDAV-930.

An animal-passaged SDAV was reported to produce SDA in rats (JACOBY et al., 1975) and older rats were reported to be more susceptible for the virus (UTSUMI et al., 1980). The LBC-passaged isolate did not produce any disease in 8- and 15-week-old rats, whereas the mouse-passaged isolate produced submaxillary swelling in 15-week-old rats. PERCY et al., (1989) reported that the virus at 25th-passage level failed to produce typical lesions in salivary glands. KOJIMA et al., (1980) reported that no clinical signs of SDA developed in 8-week-old rats inoculated with SDAV-930 grown in PRK cell cultures. MARU and SATO (1982) described that 8-week-old rats developed SDA well after inoculation with the homogenate of affected salivary gland, while the virulence lowered with mouse passages. YAMAGUCHI et al., (1982) reported that mouse-passaged TG and KA strains of SDAV caused submaxillary swelling milder than the wild virus in 10-week-old rats, and that the severities of clinical signs and histopathological changes were higher with rat-passaged virus.

The LBC cells provide a sensitive, reproducible and practical assay method as well as a useful source of SDAV for studying rat coronaviruses.

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