Isolation and Properties of Sialodacryoadenitis Virus of Rats

Akihiro KOJIMA, Fujio FUJINAMI, Kunio DOI, Akira YASOSHIMA
and Azusa OKANIWA

Pharmacological Research Laboratory, Tanabe Seiyaku Co., Ltd.,
Kawagishi, Toda-shi, Saitama 335, Japan

(Received for publication: February 1, 1980)

Pathogenic agents were isolated from affected salivary glands of rats with sialodacryoadenitis (SDA) by means of intracerebral inoculation to newborn mice and inoculation to primary rat kidney cell cultures. The properties of these isolates, irrespective of the isolation techniques, were in general agreement with that of SDA virus strain 681, and they were identified as SDA virus. In addition to the known properties, following characteristics were noticed: (1) Particles of SDA virus bore two different long and short surface projections. (2) Some antigenic differences were observed between the present isolate 930-10 and the strain 681. (3) It was suggested that SDA virus grew better at 35°C than at 31°C, 33°C, 37°C or 39°C. (4) SDA virus did not propagate in DBT cells.

Since the successful isolation of a coronavirus from rats with sialodacryoadenitis (SDA) by Bhatt et al. [2], many studies [11, 17] on various aspects of the infection of this virus were reported. However, the knowledge on this virus and its infection obtained up to the present times seems still insufficient to understand properties of the virus and characteristics of its infection. During about 6 months from May to November, 1976, outbreaks of a disease suspected to SDA were noticed among male rats of the Sprague-Dawley strain in a breeding room of the laboratory. This paper deals with the isolation, biological and physicochemical properties of viruses, temporarily designated as strains M and 930-10 of SDA virus.

Materials and Methods

Isolation of virus: Swollen submaxillary glands of specific pathogen free male rats of Sprague-Dawley strain 7 weeks of age suffered from SDA-like disease were made to a 10% emulsion in phosphate buffered saline (PBS) that contained penicillin (500 units/ml), streptomycin (0.5 mg/ml) and amphotericin B (2.5 μg/ml). After centrifugation at 840×g for 15 min, resulting supernatant fluid was collected and used as source of virus.

Two-day-old conventional ddY mice were inoculated intracerebrally with 0.01ml of the above-mentioned tissue emulsion and observed daily for 14 days. Brains of sick mice which exhibited neurologic signs were harvested and subjected to further passage and histopathologic study. Strain M was obtained by means of inoculation into mice and underwent 25 serial passages in mouse brain followed by 6 serial passages in PRK cell cultures including double cloning by the endpoint dilution technique.
Monolayer cultures of primary rat kidney (PRK) cells (see later) were inoculated with the above-mentioned submaxillary gland emulsion, and cytopathic effect (CPE) was observed for 3 days. Two successive blind passages of cultured medium were performed when CPE did not appear. Strains 930-10, 903-2 and 930-12 were obtained by means of inoculation into cultured cells and then subjected to 3 to 4 serial passages in PRK cell cultures.

Reference viruses: Strain 681 of SDA virus and strain S of mouse hepatitis virus (MHV), were kindly supplied by Dr. Kosaku Fujiwara, of the Institute of Medical Science, University of Tokyo, and used as reference strains in the study. Strain S of MHV was grown and assayed on DBT cells.

Cell cultures: PRK cell cultures were prepared from the kidneys of specific-pathogen free rats of the Sprague-Dawley strain 2 weeks of age. These donor rats were consistently negative to neutralizing antibody against SDA virus throughout the experiment. Kidneys were trypsinized in 0.25% trypsin in PBS for 20 to 30 min at 33°C, and resulting cells were seeded at a 0.5% suspension in growth medium in multi-dish trays having 24 wells (Limbro Chemical Co. Inc.), 25cm² TC flasks (Falcon) or 10cm petri dishes depending on the requirement of each experiment. Cell cultures usually were confluent in 3 days and were used for experiment.

Established cell line of SR-CDF1-DBT (DBT) was also kindly supplied by Dr. Fujiwara.

PRK and DBT cells were grown at 37°C in Eagle's minimum essential medium (Nissui, Tokyo) supplemented with 10% fetal calf serum (FCS) and 10% tryptose phosphate broth (TPB), and were maintained in the same medium, except that FCS and TPB supplementalities were reduced to 2 and 5% respectively after virus infection.

Cytopathology: Infected PRK cell cultures grown on Lab-Tek tissue culture chamber slides (Miles, USA) were fixed in Bouin's solution and stained with Mayer's Hematoxylin and eosin (HE) at 14, 16, 18, and 20 hr after infection.

Titration of the virus: Ten-fold dilutions of virus were made in maintenance medium. Each dilution (0.1 ml) was inoculated on each of 4 stationary cultures of PRK cells in multi-dish tray and incubated at 35°C for 4 days.

Physicochemical properties of the virus: The tests of physicochemical properties were carried out in such manners as mentioned previously [5].

Cross-neutralization test: Serial four-fold serum dilutions were mixed with an equal volume of virus suspension containing 100TCID₅₀/0.1 ml. After incubation at room temperature for 1hr, 0.2ml of the mixture was inoculated into four PRK cell culture wells of multi-dish trays. Antiserum against the isolate 930-10 was prepared by intranasal inoculation of the virus into antibody free rats which were bled after two weeks. Antisera against strain 681 of SDA and strain S of MHV were kind gifts of Dr. Fujiwara.

Electron microscopy: Cultured PRK cells were infected with each viral strain and incubated at 35°C for 24hr. The culture medium was harvested and centrifuged at 21,000×g for 90 min. Resulting pellets were resuspended in small volume of 1% ammonium acetate containing 0.5% FCS, and negatively stained with phosphotungstic acid (pH 7.0). Ultra-thin sections were prepared from PRK cell cultures 17 hr after inoculation with strain 930-10 or 681 by the methods described previously [12].

Hemmaglutination (HA): HA test was performed by the microtiter method, using 0.5% erythrocytes from rats and mice. Diluent used was PBS, pH 7.2, containing fetal calf serum or bovine serum albumin fraction V powder to a final concentration of 0.3%. Reading was made after incubation for 60 min, 2, 6 and 24 hr at 4°C.

Inoculation to mice and rats: Male and
Fig. 1 Effect of various temperature on yield of strain 930-10 and SDAV strain 681 grown in PRK cells. ○— m. o.i=0.1, harvested at 21 hr p. i. ●— m. o.i=0.0001, harvested at 39 hr p. i.

Fig. 2 Growth curve of strain 930-10 and SDAV strain 681 in PRK cell cultures. ○— Extracellular virus. ●— Cell-associated virus.

female conventional ddY mice 2 days and 3 weeks of age were obtained from a colony of this institute and used for inoculation experiments. For inoculation experiment to rats, 10 male rats of the Sprague-Dawley strain 8 weeks of age were inoculated intranasally with 0.05 ml (10^5 TCID₅₀/0.1 ml) of strain 930-10. After 5, 7 or 11 days, they were sacrificed for pathological and virological examinations.

Results

1. Isolation of SDA virus

Isolation by means of inoculation into mice and pathogenicity of isolates to the newborn mice: The disease expression associated with positive isolation and successive intracerebral passage was characterized by such neurologic signs as scampering, uncoordination and squeak, followed by paralysis. In this study, each sample was inoculated to one litter of mice, and positive results were obtained in 4 litters inoculated with 4 different samples. In these cases, the incidence of illness was 1/5, 1/7, 1/6 and 2/6. The incidence of illness rose up to 100% on the next and subsequent passages.

Brain emulsion of sick mice inoculated
with one of the above-mentioned isolates designated as M strain was inoculated to the 2-day-old mice by various routes. Intranasal inoculation was similarly effective to cause illness as the intracerebral one, while intraperitoneal inoculation did not produce any sign of illness. The viral titer of infected brain between the second and the 25th intracerebral passage in newborn mice remained relatively stable and ranged always from $10^{2.5}$ to $10^{3.5}$ MLD$_{50}$/0.01 ml. Three-week-old ddY mice exhibited no sign of disease irrespective of inoculation route.

Histopathological examination of the brain of newborn mice sacrificed on the fifth day after inoculation with strain M revealed focal neuronal degeneration and necrosis in both cerebral hemispheres especially in the external germinal layer. The affected areas exhibited spongy appearance by marked loss in nerve cells, and occasional round cell infiltration was seen in the meninge. The other parts of the brain were not involved.

Isolation by means of inoculation into PRK cell culture: Cytopathogenic effect appeared following the two successive blind passages of four submaxillary gland materials in PRK cell cultures. All of these four materials coincided with those produced positive results in above-mentioned mice inoculation test.

2. Properties of the virus

Cytopathogenic effect: PRK cells grown in Lab-Tek chamber slides were inoculated with 930-10 strain at an m. o. i. 0.3 and incubated at 35°C. Syncytial formation (Fig. 3) appeared as CPE at 18 hr postinoculation, developing further thereafter.

In the course of CPE development, characteristic morphological changes were observed in the stained preparations of infected cells. The first evidence of infection was formation of small syncytial cells (<10 nuclei/cell) (Fig. 4), which appeared at 14 hr postinfection (p. i.). By 16 hr p. i., numerous syncytial cells containing 40 or more nuclei were seen (Fig. 5). Characteristic syncytial cells (Fig. 6), observed by 18 hr p. i., had a large number of nuclei, which arranged like as a circle leaving structureless area of cytoplasm inside them. No inclusion bodies were observed in these syncytial cells. Cytopathological changes seen in PRK cells infected with strain 681 were similar to those observed in PRK cells inoculated with strain 930-10.

Optimal growth temperature: PRK cell cultures were inoculated with strain 930-10 or 681 at an m. o. i. 0.1 or 0.0001, and incubated in water baths at 31°C, 33°C, 35°C, 37°C or 39°C for 21 or 39 hr. Cultures were frozen-thawed three times and virus yields were determined. As shown in Fig. 1, the highest yield of both strains 930-10 and 681 was obtained at 35°C.

Virus growth curve: PRK cell cultures were inoculated with strain 930-10 or 681 at an m. o. i. 0.5 and incubated at 35°C. Growth curve was obtained by the method described by Hirano [7]. Fig. 2 shows the growth curve of strains 930-10 and 681 in PRK cells. The virus titer in the fluid phase began to increase 6 hr p. i. and reached a plateau of $10^{5.7}$ (strain 930-10) or $10^{4.7}$ (strain 681) TCID$_{50}$/0.1 ml by 15 hr p. i. By 30 hr p. i., a gradual decrease in the titer occurred, resulting a virus titer about $10^{3.7}$ TCID$_{50}$/0.1 ml after 48 hr p. i. The titer of cell-associated virus tended to run in parallel with that of the fluid phase, but the former was always lower about one log than the latter.

Inoculation into DBT cells: Since coronavirus of mice, MHV, was reported to grow well in DBT cells [7], susceptibility of DBT cells to strains M, 930-10 and 681 was examined. Materials of the first to 7th or the 12 and 25th mouse brain passage of strain M were inoculated into DBT cell cultures. Any sign of CPE was not observed during the observation period of 7 days. In several blind passage experiment with strain M on DBT cells, no CPE was observed. Moreover, inoculation of strain S of MHV to these DBT cells, which had
Table 1. Titer of virus strains following chemical and physical treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Titer of virus*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
</tr>
<tr>
<td>IUDR, $10^{-4}$ M/ml</td>
<td>4.7</td>
</tr>
<tr>
<td>BUDR, $10^{-4}$ M/ml</td>
<td>4.8</td>
</tr>
<tr>
<td>Control</td>
<td>5.3</td>
</tr>
<tr>
<td>20% ether, 4°C, 18 hr</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>5.3</td>
</tr>
<tr>
<td>10% chloroform, 20°C, 15min</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>5.0</td>
</tr>
<tr>
<td>pH 3.0, 20°C, 5 hr</td>
<td>4.0</td>
</tr>
<tr>
<td>pH 7.0, 20°C, 5 hr</td>
<td>5.0</td>
</tr>
<tr>
<td>Filtration, 200 nm</td>
<td>5.3</td>
</tr>
<tr>
<td>100 nm</td>
<td>5.7</td>
</tr>
<tr>
<td>50 nm</td>
<td>0</td>
</tr>
<tr>
<td>37°C, 2 hr</td>
<td>5.7</td>
</tr>
<tr>
<td>5 hr</td>
<td>5.3</td>
</tr>
<tr>
<td>24 hr</td>
<td>1.0</td>
</tr>
<tr>
<td>56°C, 30 min</td>
<td>0</td>
</tr>
</tbody>
</table>

Remarks.  * : Infective titer (log TCID<sub>50</sub>/0.1 ml).

Table 2. Cross-neutralization tests among the strain 930-10, 681 and S of MHV

<table>
<thead>
<tr>
<th>Viurs</th>
<th>Antiserum titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-903-10</td>
</tr>
<tr>
<td>930-10</td>
<td>802</td>
</tr>
<tr>
<td>681</td>
<td>320</td>
</tr>
<tr>
<td>S</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Remarks.  * : Reciprocal of a serum dilution causing 50% inhibition of 100 TCID<sub>50</sub> of each strain.

been subjected to above mentioned blind-passage after inoculation with strain M, permitted to develop a characteristic CPE of strain S of MHV, suggesting no presence of interference. In another experiment, the viral titers of the supernatant fluid or DBT cells inoculated with strain 930-10 or 681 approximately m.o.i. 1.0 and incubated for 2 days at 35°C were lower than $10^{1.0}$ TCID<sub>50</sub>/0.1ml on PRK cells in both cases.

HA activity of the isolates: The supernatant of PRK cell culture harvested at 24 hr after infection with strains 930-10 and 681 (titer : $10^{6}$ TCID<sub>50</sub>/0.1ml) exhibited no HA activity irrespective of centrifugal concentration and/or sonication of the materials.

Physicochemical properties: As shown in Table 1, strains M and 930-10 were sensitive to ether and chloroform, acid stable and thermo-labile. These strains passed through a 200-nm, and 100-nm but not a 50-nm Millipore filter. IUDR or BUDR did not prevent the development of CPE caused by these isolates.

Antigenic properties: Serological relationships among strains 930-10, 681 and S of MHV were examined by the serum-neutralization test. As shown in Table 2, there is cross-neutralization, but some antigenic discrepancy exists between strains 930-10 and 681. The titer of heterologous antisem-
rum is two to four fold lower than that of homologous antiserum. Strains 930-10 and 681 were also weakly neutralized by antiserum against the strain S of MHV. Immune sera to strains 930-10 and 681 had no titer of neutralizing antibody for strain S of MHV.

Morphological characteristics: Negatively stained preparations of strains M, 930-10, 903-2 and 930-12 showed that the virions of these strains were pleomorphic in shape and were mostly round. The diameter of virions, including the surface projections, ranged from 131 to 209 nm, or 161 nm on the average. A characteristic feature was bilaminate fringe surrounding the surface of the particles (Fig. 7). It attributed to the presence of two different surface projections, that is, the widely spaced club- or petal-shaped long projections with a length of 19.2 to 20.7 nm (mean 20.5 nm) and the short projections situated between the formers with a length of 7.2 to 7.7 nm (mean 7.5 nm).

In ultra-thin section preparations of infected cells with strain 930-10, virus particles were observed on the cellular surface and within cytoplasmic vesicles and in the lumen of rough surfaced endoplasmic reticulum. Pictures suggesting budding of the virus particles from the wall of endoplasmic reticulum were also occasionally observed. Occurrence of virus particles in the lysosomal granules was common findings (Fig. 8). The virus particles in ultra-thin sections were generally circular and the maximum diameter was in the range of 100 to 121 nm. The virions of strain 681 had similar morphological characteristics in common with that of strain 930-10.

Experimental infection in the rat: No detectable clinical disease and gross changes were observed in all the treated rats during the 11 days observation. Virus was only recovered from the trachea of both of two animals examined by day 5. Neutralizing antibody against strain 930-10 was not detectable until day 7 after infection, but thereafter the level of serum antibody rapidly increased. Histologically, changes were found only in the parotid gland of one rat killed on day 11 that developed low antibody titer as compared with that of the other two by day 11. In this animal, focal lesion consisted of degeneration of ductular epithelial cells and mild periductular round cell infiltration was observed in the intercalated portion of the salivary duct.

Discussion

Pathogenic agents were successfully isolated from rats with SDA by means of inoculation to PRK cells as effectively as intracerebral inoculation to newborn mice. Both strains M and 930-10, the former was isolated by intracerebral inoculation to newborn mice and the latter by inoculation into PRK cells, were found to have morphological and physicochemical properties in common eith the viruses of the coronavirus group. The isolates meet the first 5 of the following 6 criteria for coronavirus: (1) characteristic surface structure, (2) size, (3) replication within cytoplasmic vesicles, (4) ribonucleic acid content, (5) presence of an essential lipid envelope, and (6) low particle density [16]. They were also related antigenically and biologically to SDA virus strain 681 [2] which grew in brains of infant mice and in PRK cell cultures, serologically related to MHV, and had no HA activity for mice and rats erythrocytes. Pathological changes found in rats collected from the same outbreaks with the present investigation coincided with those of SDA of rats [6]. Based on these evidences, the isolates were identified as the virus of SDA of rats.

Concerning the characteristics of SDA virus, the results of the present study are in general agreement with the properties of SDA virus as described previously [1,2]. In addition to the known properties, the following findings were noticed as the characteristics of SDA virus. Namely,
presence of two different surface projections as surface components of SDA virus. Concerning the structures like this, Sugiyama et al. [14] reported the presence of two types of surface projections on the surface of a corona-like virus causing diarrhea in infant mice (DVIM). Stair et al. [13] observed bilaminate structure on the surface of coronavirus-like agent isolated from neonatal diarrhea in calves. On the other hand, such structure have never been observed in most of the other coronaviruses [10]. Recently, Davies and Macnaughton [4] examined the morphology of three representative coronaviruses in detail and observed only one kind of long surface projections on the surface of avian infectious bronchitis virus strain Connecticut, human coronavirus strain 229 E and MHV strain 3. It will be the subject for future studies to elucidate the morphology of virused of coronavirus group.

Results of the present preliminary neutralization test suggested that SDA virus is not homogeneous in antigenicity and presence of several serotypes, similar to other member of coronaviruses [3, 8], were supposed.

The optimal growth temperature of SDA virus was suggested to be 35°C in the present study. Concerning the propagation of SDA virus in experimentally infected rats, Jacoby et al. [9] demonstrated that viral growth occurred satisfactorily in the tissues which located at the surface of the body and supposed to be lower in temperature than that of the deep part of the body. It has been known that the optimal growth temperature for many respiratory viruses including members of coronaviruses is generally lower than for many other viruses and somewhat low as compared with body temperature of host animals [15].

In stained preparations of infected PRK cell cultures, characteristic large syncytial cells, having large structureless area surrounded by annularly arranged nuclei, were observed. Similar syncytial cells were described to occur in DBT cells inoculated with MHV [7].

Different from MHV [7], SDA virus exhibited no signs of propagation in DBT cells. This difference is considered to be useful in distinguishing between MHV and SDA virus.

With regard to the pathogenicity of strains isolated in the present experiment, strain 930-10 was lowly virulent and inoculation of this strain to 8-week-old rats did not reproduce clinical disease of SDA as seen in natural outbreaks. These results may relate to both of the factors of virus and animals. The reason why no rats developed clinical disease of SDA remains unclear. One possible explanation is that propagation in cell-culture systems may result in loss of virulence, and some serial passages in host animals is necessary for virus to regain virulence to the rat (Taguchi, F. 1979, personal communication). A detailed experiment, however, is needed for further discussion on this problem.

Acknowledgments

The authors wish to thank Prof. Dr. K. Fujiwara, Department of Animal Pathology, Institute of Medical Science, University of Tokyo, for supply of the viruses, cell line and antisera used. They are also grateful to Dr. A. Kiyomoto, director of their laboratory, and Dr. T. Fujita, chief of their section, for their encouragement and support.

References


ラット唾液腺腫腺炎ウイルスの分離と性状

小島 明広・藤波不二雄・土井 邦雄
八島 昭・岡 庭 樹

田辺製薬株式会社薬理研究所

唾液腺腫腺炎（SDA）様疾患罹患ラットの頸下腺組織から、マウス脳内接種法によりM株、およびラット腎培養細胞（PRK）接種法により930-10株を得、これらの分離株につき、ウイルス学的に検索を加えた。その結果、これらの分離株がコロナウイルス群に属し、ラット唾液腺腫腺炎ウイルスと同一のものであることが明らかとなった。分離株の諸性状は、既知のSDAウイルスのそれと概ね一致した。さらに今回の検索において、下記のような知見が得られた。すなわち、1）SDAウイルスのenvelopeには短2種類のSurface projectionを持つ。2）われわれの分離株である930-10株とSDAV681株の間に抗原性の違いがあることが交互中和反応で明らかにされた。3）SDAウイルスの増殖には35℃が適していた。4）DBT細胞では増殖しないため、などの点である。
Explanation of Figures

Fig. 3 Cytopathic effects of strain 930-10 in PRK cell culture 20 hr after inoculation with 10^5 TCID_50/0.1 ml. Unstained, × 150.

Figs. 4 to 6. Virus-induced syncytium in PRK cell culture infected with strain 930-10. Hematoxylin and eosin staining, × 300 except Fig. 6 (×150).

Fig. 4: Cells 14 hours after inoculation. Fig. 5: Cells 16 hours after inoculation. Fig. 6: Cells 18 hours after inoculation.

Fig. 7 Particles of the isolate M, negatively stained with phosphotungstic acid. The bar in figure represents 100nm. × 150,000.

Fig. 8 Viral particles in the rough-surfaced endoplasmic reticulum, cytoplasmic vesicles and lysosomal granules of PRK cell inoculated with strain 930-10. × 40,000. Inserted shows pictures suggesting budding of the virus. × 60,000. Key to abbreviations in figure.

Ly Lysosome
M Mitochondrion
ER Endoplasmic reticulum
Vp Virus particle