J. Vet. Med. B 37, 91–96 (1990)
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ISSN 0931–1793

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Plaque Assay and Propagation in Rat Cell Line LBC Cells of Rat Coronavirus and 5 Strains of Sialodacryoadenitis Virus

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With 3 figures and 3 tables

(Received for publication June 28, 1989)

Summary

Various factors influencing the plaque formation of rat coronavirus (RCV) and sialodacryoadenitis virus (SDAV) in LBC cell monolayers were studied to develop the practical method for plaque assay. By this method, 4 Japanese isolates of SDAV also produced clear plaques. In one-step growth experiments of these viruses, newly formed virus was first recognized within 7.5 h postinfection and showed subsequently a rapid exponential increase. The virus was released rapidly from the infected cells. By indirect immunofluorescence virus specific antigen was detected as perinuclear granules in the cytoplasm of the cells at 5–6 h postinfection, and all the cells revealed fluorescence at 12 h postinfection.

Key words: Rat coronavirus, Sialodacryoadenitis virus, LBC cells, Plaque assay, Replication

Introduction

Rat coronavirus (RCV) (6) and sialodacryoadenitis virus (SDAV) (1) have been reported to propagate with cytopathic effect (CPE) on primary cultures of rat kidney but not on any established cell lines (7). In addition, no adequate plaque assay for RCV and SDAV has been described.

Recently, the authors introduced a rat cell line, LBC, as a useful medium for propagation of RCV 8190 strain (2) and SDAV 618 strain (3), and reported successful propagation of 4 Japanese isolates of SDAV in LBC cell cultures (4). Of several advantages of this cell system over the primary culture of rat kidney, the most salient is the fact that LBC cells are very sensitive to the virus and yield high-titered active virus. In addition, the cells can be grown and handled easily, and provide a sensitive, reproducible assay method for infectivity.

The present paper deals with investigation of various factors influencing plaque formation in LBC cells with RCV and SDAV, and a standard technique of plaque assay and the growth characteristics of RCV and 5 strains of SDAV including Japanese isolates in LBC cell monolayers.

Material and Methods

Cell culture: LBC cells, derived from mammary tumor of Lewis rat, were grown at 37 °C in Eagle's minimum essential medium (MEM) containing 10 % fetal calf serum (FCS) and kanamycin

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(0.06 mg/ml). For maintaining the cells and harvesting the virus, FCS concentration was reduced to 5 %.

Virus: RCV strain 8190 (6), SDAV 681 strain (SDAV-681) (1), strains 930-10 (SDAV-930) and M (SDAV-M) (5), TG (SDAV-TG) and KA (SDAV-KA) (8) were passaged on LBC cells as described in our previous papers (2—4) and used at the 20 to 30th passage levels in this study. The seed viruses used were supernatant of culture fluid harvested from the infected LBC cell cultures after incubation at 37 °C for 36 h postinoculation (p. i.).

Plaque assay: The following method was established on the bases of the results obtained in this study. LBC cell monolayer cultures were prepared in 60 mm plastic dishes (Terumo, Japan) by seeding $1.0-1.5 \times 10^6$ cells in 5 ml of growth medium and incubating at 37 °C for 2 days in an atmosphere of 5 % CO_2 in air. The cultures were once washed with MEM, and inoculated with 0.2 ml of virus materials diluted in MEM containing 2 % FCS. After virus adsorption at 37 °C for 90 min, the inoculated cultures were overlaid with 5 ml of MEM containing 0.9 % Agar Noble (Difco, U. S. A.) and 5 % FCS, and incubated at 37 °C for 72 h in CO_2 incubator. The infected cultures were then stained with 5 ml of agar medium containing 1 : 10,000 neutral red at 37 °C for 6 to 8 h to count plaques. The infectious titers were expressed in plaque-forming units (PFU).

Growth curve experiment: The experiments were carried out by the same method previously described on SDAV-681 strain (4).

Antiserum preparation: Four-week-old male Wistar rats were obtained from commercial breeding colony, which was free from RCV and SDAV infections. They were inoculated intranasally with SDAV-930 (10^5 PFU / 0.25 ml), and inoculated twice intraperitoneally with the virus at intervals of one week. At 7 days after the last inoculation, rats were sacrificed for collecting blood. These animals were kept in the vinyl isolators throughout the immunization.

Indirect immunofluorescence: Infected LBC cell cultures on coverslips were fixed with cold acetone and stained by using anti-SDAV-930 rat serum and fluorescein isothiocyanate-conjugated rabbit antibody against rat IgG (Miles Biochem., U. S. A.).

Results

RCV and SDAV-681 readily formed plaques in LBC cell monolayers under the agar overlay medium. To establish the routine plaque assay system, various factors influencing plaque formation were examined for LBC cells with RCV and SDAV-681.

Effect of adsorption medium on plaque formation: As shown in Table 1, various media were tested for effects on virus adsorption. Among media tested, MEM gave the highest plaque count of both RCV and SDAV-681 with or without 2 % FCS, while addition of 2 % FCS increased plaque count in MEM, PBS and PBS containing Ca and Mg ions. From these results, MEM containing 2 % FCS was selected as medium for virus adsorption.

Effect of adsorption times on plaque formation: In order to determine experimental conditions for routine assay, kinetics of virus adsorption onto LBC cell monolayers were investigated. RCV and SDAV-681 were adsorbed onto LBC cell monolayers at 37 °C for

Table 1. Effect of adsorption medium on plaque formation

Virus	Medium	Number of plaques[a] FCS (−)	FCS (+)
RCV	MEM	66.7	71.3
	PBS	54.7	60.3
	PBS (+)	63.0	68.3
SDAV-681	MEM	76.3	83.0
	PBS	31.3	51.3
	PBS (+)	51.3	71.7

The virus material was diluted appropriately with the following diluents, and inoculated into LBC cell cultures. The diluents used were MEM, PBS and PBS (+) (PBS containing 0.1 mg/ml $MgCl_2\,6\,H_2O$ and 0.1 mg/ml $CaCl_2$) and those containing 2 % FCS. [a]: Average of 3 dishes; Plaques were counted 72 h postinoculation.

Table 2. Effect of adsorption times on plaque formation

Virus	Adsorption time[a] (min)	Plaque number[b] Unwashed	Washed[c]
RCV	30	34.7	16.0
	60	46.0	21.7
	90	51.3	29.0
	120	43.3	22.7
SDAV-681	30	90.0	29.7
	60	98.0	59.0
	90	100.3	62.0
	120	80.0	61.0

[a]: Adsorption at 37 °C. [b]: Average of 3 dishes. [c]: Washed 3 times with MEM.

30, 60, 90 and 120 min. After virus adsorption half the inoculated cultures were washed 3 times with MEM for removing free virus, and then overlaid with agar medium and incubated at 37 °C for 72 h. After adsorption for 90 min at 37 °C, the number of plaques obtained was largest in both unwashed and washed cultures inoculating with RCV and SDAV-681 as shown in Table 2. Further incubation for up to 120 min reduced the plaque counts in both virus groups. Washing with MEM significantly reduced the number of plaques. In the routine procedure, the inoculated cultures are incubated at 37 °C for 90 min, and are overlaid with agar medium without washing.

Effect of incubation time on plaque formation: The inoculated cultures were examined for size and number of plaques by incubating for 48, 72 and 96 h. As shown in Table 3, readily countable, clear plaques with a well-defined circular margin, 1.0—2.0 mm and 0.8—1.2 mm in diameter were obtained in the RCV and SDAV-681 inoculated cultures, respectively, by incubation at 37 °C for 48 h. Incubation for 72 h increased the plaque size and number. Further incubation for up to 96 h increased the plaque size but not number because the adjacent plaques were fused, and because the uninfected cells became degenerated. Therefore, incubation for 72 h before application of the second overlay for staining was adopted as a standard procedure.

Plaque formation of other SDAV strains: By this technique, SDAV-930, SDAV-M, SDAV-TG and SDAV-KA produced also clear plaques on the LBC cell monolayers as well as RCV and SDAV-681. Figure 1 represents plaques formed by RCV and 5 SDAV strains. Of the 6 strains, RCV, SDAV-681 and SDAV-930 produced the largest plaques 2.5—5.0 mm in diameter, and SDAV-M produced the smallest ones 2.0—2.5 mm in diameter. However, there was no significant difference in plaque morphology among the strains.

Relationship of plaque number and virus concentration: RCV and 5 SDAV strains were examined. In the 6 strains the plaque count was shown to be directly proportional to

Table 3. Incubation time and plaque formation

Virus	Incubation[a] (h)	Plaque Size	Number[b]
RCV	48	1.0—2.0 mm	48.3
	72	3.0—5.0 mm	61.3
	96	5.0—8.0 mm	51.7
SDAV-681	48	0.8—1.2 mm	79.0
	72	2.5—5.0 mm	101.0
	96	4.0—7.0 mm	71.3

[a]: After virus adsorption and agar overlaying. [b]: Average of 3 dishes.

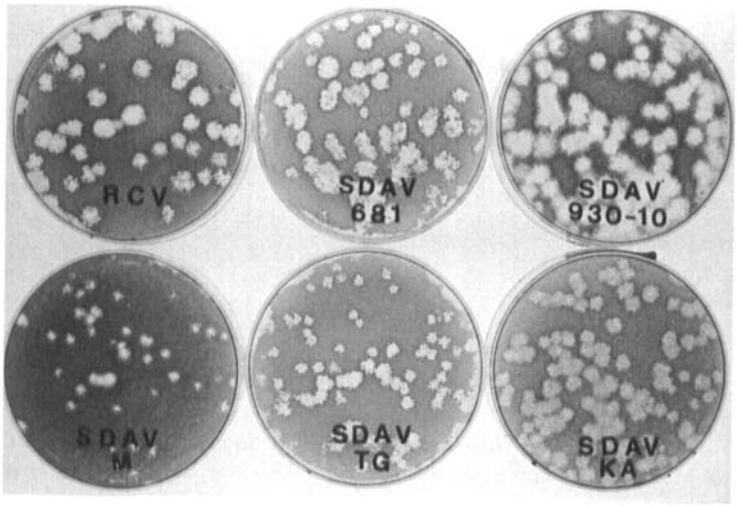

Fig. 1. Plaques produced 72 h after inoculation with RCV, SDAV-681, SDAV-930, SDAV-M, SDAV-TG and SDAV-KA

virus concentration, indicating that infection with one virus particle is sufficient to produce one plaque. Based on these results hitherto presented the standard technique of plaque assay was established as described in Material and Methods.

Replication of RCV and 5 strains of SDAV: Using this plaque assay, replication of RCV and 5 SDAV strains in LBC cells was examined. Figure 2 illustrates representative viral growth curves which were obtained in LBC cell cultures infected at input multiplicity of 10 PFU per cell. These curves were considered to present one-step growth, since all the cells were shown to be infected by immunofluorescence. CPE was first observed at 9—12 h p. i. and became complete in the infected cultures at 15—18 h p. i.

Titer of cell-associated virus declined gradually during the initial 6 h of incubation and showed a rise within 7.5 h p. i. followed by an exponential increase up to 12—15 h p. i., reaching $10^{6-6.5}$ PFU / 0.2 ml at 15—24 h p. i. Titer of extracellular virus was higher than that of the cell-associated virus and reached a plateau of $10^{6.5}$ PFU / 0.2 ml at 15—18 h p. i. The growth experiments showed that a release of newly produced virus from the cells was dependent upon the progress of CPE.

In the experiments of one-step viral growth, viral antigen demonstrable by indirect immunofluorescence was first detected at 5 h p. i. as perinuclear granules in the cytoplasm of less than 1 % of the cells infected with SDAV-930 (Fig. 3), and about 10 % of the cells infected with RCV and SDAV-681. In the cases of SDAV-M, SDAV-TG and SDAV-KA, cytoplasmic fluorescence was detected at 6 h p. i. At 12—15 h p. i. all the cells manifested cytoplasmic fluorescence.

Discussion

In the present study the LBC cell culture was shown to provide a useful medium for plaque assay and propagation of RCV and SDAV strains including Japanese isolates. The standard technique of plaque assay, as described in Material and Methods, was worked out on the basis of the findings presented in this paper and fits requirement that a routine procedure should be as simple as possible.

The one-step viral growth curves, illustrated in Fig. 2, showed that the initial virus titer rose within 7.5 h p. i. The progressive decrease of the residual titer of cell-associated virus during first 5 h of incubation seemed to be due to eclipsing of virus attached to the cells. The growth curves showing the production of cell-associated and extracellular viruses

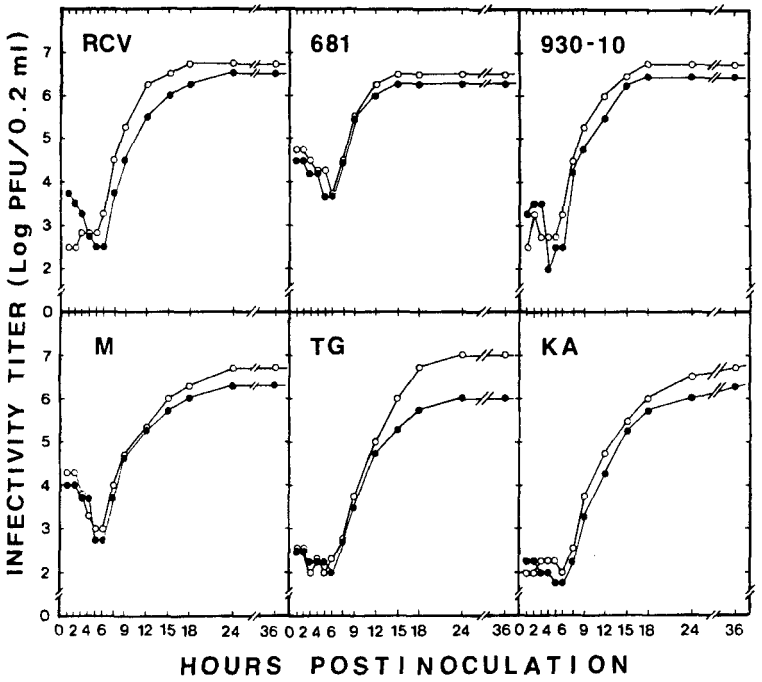

Fig. 2. Growth curves of RCV and 5 SDAV strains in the LBC cell cultures. Cell cultures prepared in 50-mm bottles (1.0×10^6 cells) were infected with 10 PFU per cell of the each virus strain. After virus adsorption at 37 °C for 90 min, the inoculated cultures were washed 3 times with MEM, given 5 ml of maintenance medium and incubated at 37 °C. At intervals, infectivity was determined with culture fluid and cells pooled from two bottles. For the assay, the cells were washed 3 times with MEM and frozen and thawed in 5 ml of fresh medium. Those materials were centrifuged at 800 × g for 10 min before infectivity assay

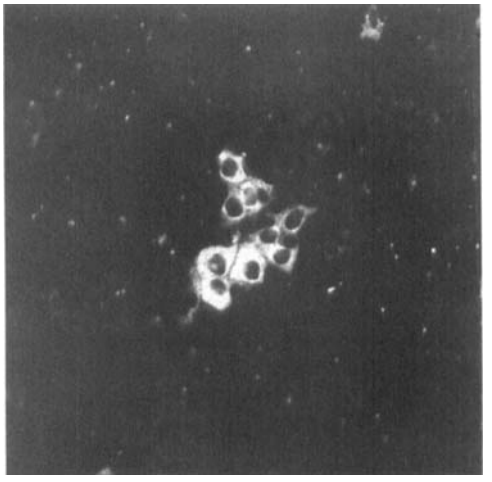

Fig. 3. Virus specific antigen is shown as perinuclear, fluorescent granules 5 h after inoculation with SDAV-930

(Fig. 2) indicated that the production of new virus was very rapid in an exponential phase and newly produced virus is released rapidly from the cell. These findings were similar to the previous observation with SDAV-681 (4).

By immunofluorescent antibody study, the viral specific antigen of RCV and 5 strains of SDAV was detected only as cytoplasmic fluorescence, as previously reported (2—4).

The LBC cells provide satisfactory source of the virus, and a sensitive, practical, reproducible plaque assay system for viral infectivity of RCV and SDAV. In addition to these strains examined, some SDAV strains also showed to be propagated in LBC cell cultures (unpublished observation). Further investigation should be expected for usefulness and application to the other RCV and SDAV strains as well as wild ones.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (62580031) from the Ministry of Education, Science and Culture of Japan.

Zusammenfassung

Entwicklung eines Plaquetests und Vermehrung von Rattencoronavirus und 5 Sialodacryoadenitisvirusstämmen in einer Rattenzellinie, LBC

Verschiedene Faktoren, die die Plaquebildung von Rattencoronavirus und Sialodacryoadenitisvirus (SDAV) in LBC Zellenmonoschichten beeinflussen, wurden untersucht. Aufgrund dieser Ergebnisse wurde eine praktische Plaque-assay-Methode entwickelt, mit der auch 4 Japanische SDAV-Isolate dann eindeutige Plaques bildeten. In einem Vermehrungsversuch, der im Einzelschritt durchgeführt wurde, wurde neugebildetes Virus zuerst 7,5 Stunden nach der Infektion nachgewiesen und vermehrte sich in der anschließenden Exponentialphase sehr schnell. Das Virus wurde von den infizierten Zellen rasch freigesetzt. Mittels indirekter Immunofluoreszenz konnte virusspezifisches Antigen 5—6 Stunden p. i. als perinukleäre Körnchen im Zytoplasma der Zellen nachgewiesen werden und 12 Stunden p. i. waren alle Zellen fluoreszierend.

References

1. BHATT, P.N., D.H. PERCY, and A.M. JONAS, 1972: Characterization of the virus of sialodacryoadenitis of rat: a member of the coronavirus group. J. Inf. Dis. **126**, 123—130.
2. HIRANO, N., K. ONO, Y. SADA, A. INOUE, T. MURAKAMI, and H. TAKAMARU, 1985: Replication of rat coronavirus in a rat cell line LBC. Arch. Virol. **85**, 301—304.
3. HIRANO, N., Y. SUZUKI, K. ONO, T. MURAKAMI, and K. FUJIWARA, 1986: Growth of rat sialodacryoadenitis viruses in LBC cell culture. Jpn. J. Vet. Sci. **48**, 193—195.
4. HIRANO, N., H. TAKAMARU, K. ONO, T. MURAKAMI, and K. FUJIWARA, 1986: Replication of sialodacryoadenitis virus of rat in LBC cell culture. Arch. Virol. **85**, 301—304.
5. KOJIMA, A., F. FUJINAMI, K. DOI, A. YASOSHIMA, and A. OKANIWA, 1980: Isolation and properties of sialodacryoadenitis virus of rat. Exp. Anim. **29**, 408—418.
6. PARKER, J.C., S.S. CROSS, and W.P. ROWE, 1970: Rat coronavirus (RCV): a prevalent, naturally occurring pneumotropic virus in rats. Arch. Ges. Virusforsch. **31**, 293—302.
7. SIDDEL, S.G., A. ANDERSON, D. CAVANAGH, K. FUJIWARA, H.D. KLENK, M.R. MACNAUGHTON, M. PANSAERT, S.A. STOHLMAN, L. STURMAN, and B.A.M. VAN DER ZEIJST, 1983: Coronaviridae. Intervirology **20**, 181—189.
8. YAMAGUCHI, R., F. TAGUCHI, A. YAMADA, K. UTSUMI, and K. FUJIWARA, 1982: Pathogenicity of sialodacryoadenitis virus for rats after brain passages in suckling mice. Jpn. J. Exp. Med. **52**, 45—48.