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Replication and plaque formation of swine hemagglutinating encephalomyelitis virus (67N) in swine cell line, SK-K culture

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Summary

Swine hemagglutinating encephalomyelitis virus (HEV), 67N strain, adapted to suckling mouse brain, grew readily in a porcine cell line, SK-K cell culture with cytopathic effect (CPE) consisting of syncytium formation and detachment of fused cells and round cells from glass surface. After further passages in SK-K cell monolayers with undiluted culture fluid, CPE developed earlier and became complete within 48 h postinoculation (p.i.). Viral specific antigen was detected in the cytoplasm of the infected SK-K cells by indirect immunofluorescence using rabbit antiserum against the mouse-passaged virus. The SK-K-passaged virus as well as the original mouse-passaged virus formed clear plaques on SK-K cell monolayers under simple overlay medium. The plaque assay system for HEV 67N was established by studying various factors influencing the plaque formation in the SK-K cell cultures. By this system more than 10^6 PFU/0.2 ml of the virus yield was detected in the fluid phase of the infected cultures at 48 h p.i. The SK-K-passaged virus caused fatal infection in 4-week-old mice by intracerebral inoculation, but was inhibited by rabbit antiserum against the mouse-passaged virus. Plaque formation and hemagglutinating activity of the virus were specifically inhibited by antisera against the mouse-passaged and SK-K-passaged 67N virus.

Swine hemagglutinating encephalomyelitis virus; Coronavirus; Replication; Plaque assay

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Introduction

Swine hemagglutinating encephalomyelitis virus (HEV) is a coronavirus which causes vomiting and wasting syndrome of young piglets (Roe and Alexander, 1958; Siddell et al., 1983). The growth of HEV has been reported in primary and secondary cell cultures of swine kidney (Greig and Girard, 1963; Mengeling et al., 1972) and thyroid (Mengeling, 1973), and in suckling mouse brain (Kaye et al., 1977).

In established cell lines, however, only Sato et al. (1983) reported replication and plaque formation of 67N strain of HEV (Mengeling et al., 1972) in ESK cells derived from embryonic swine kidney with incorporation of diethylaminoethyl (DEAE)-dextran in maintenance medium and agar overlay medium, whereas no cytopathic changes and no plaque formation were observed without DEAE-dextran. However, CPE and plaques on ESK cells produced by the virus were not so clear that consistent work with HEV still remain difficult. It is essential for studying HEV infection to find the susceptible cell lines showing clear CPE and plaques with the virus growth.

Recently, we found that HEV 67N strain replicated readily in SK-K cells, an established cell line from swine kidney (Yamagishi et al., 1981), with marked cytopathic effect and produced very clear plaques under simple overlay medium without DEAE-dextran. The present paper discusses the successful replication and plaque formation of the virus in the SK-K cell cultures.

Materials and Methods

Virus

The HEV 67N strain adapted to suckling mouse brain (Yagami et al., 1987) was passaged in brains of suckling ICR mice, which were obtained from a commercial breeding colony free from mouse hepatitis virus (MHV) infection. The infected brains were stocked at -70°C until use. The brains were homogenized in Dulbecco's phosphate buffered saline (PBS, pH 7.2) and centrifuged to obtain the supernatant as virus material.

Cells and cell culture

Seven cell lines susceptible to different coronavirus were used. They were SK-K, CPK (swine kidney), STR (swine thyroid), PK15 (swine kidney), BEK-1 (bovine embryonic kidney) (Hirano et al., 1985), DBT (delayed brain tumor of CDF1 mouse) (Hirano et al., 1974) and LBC (mammary tumor of Lewis rat) (Hirano et al., 1985, 1986). The SK-K, CPK and STR cells were kindly supplied by Dr Yamagishi, Research Center for Veterinary Science, Kitasato Institute, Kashiwa, Japan. These cells were grown at 37°C in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS), 10% tryptose phosphate broth (TPB) and 0.06 mg/ml kanamycin.

Immunofluorescence

The indirect immunofluorescence was carried out in the same manner as described previously (Yagami et al., 1987). The infected cell cultures grown on coverslips, washed once with Dulbecco's phosphate-buffered saline (PBS, pH 7.2), were fixed with cold acetone, and then treated with anti-67N rabbit serum diluted 1:20 in PBS at 37°C for 60 min. After washing 3 times with cold PBS, the cells were stained with fluorescein isothiocyanate-conjugated anti-rabbit IgG goat serum (Miles Biochemicals, U.S.A.) diluted 1:10 in PBS at 37°C for 60 min. Non-infected cell cultures were treated in the same manner as the controls.

Plaque assay

The following method of plaque assay was established on the basis of the results obtained in this study. Monolayer cultures were prepared in 60-mm plastic Petri dishes (Terumo, Japan) by seeding with 1×10^6 cells suspended in 5 ml of growth medium and by incubating at 37°C for 2 days in an atmosphere of 5% CO₂ in air. Confluent cell monolayer in dishes were washed 3 times with MEM and inoculated with 0.2 ml of virus dilutions in MEM. After virus adsorption at 37°C for 90 min, the inoculated cultures were overlaid with 5 ml of agar overlay medium which consisted of 0.8% Agar Noble (Difco, U.S.A.) in MEM containing 10% TPB and kanamycin (0.06 mg/ml). The inoculated cultures were incubated in a CO₂ incubator at 37°C for 3 days, and stained by incubating at 37°C for 6 to 8 h under a second overlay medium containing 0.01% neutral red. The infectious titre was expressed in plaque-forming units (PFU).

Animal test

Four-week-old male ICR mice were obtained from a commercial breeder free from MHV infection. Animals were inoculated intracerebrally (i.c.) with 0.02 ml of virus material and observed for 7 days.

Antiserum preparation against mouse- and SK-K-passaged virus

Four-week-old ICR mice were inoculated intraperitoneally twice with 0.2 ml of each virus material at an interval of 2 weeks. At 1 week after the last inoculation mice were killed to collect the blood. The antiserum was heated at 56°C for 30 min for serological tests.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests

HA and HI tests were carried out by the microtitre method using 0.5% chicken red blood cells as described by Hirai et al. (1974).

Results

Replication of HEV 67N in SK-K cells

The cell cultures of SK-K, CPK, STR, PK15, BEK-1, DBT and LBC cells grown in 50 ml culture bottles were washed 3 times with MEM and inoculated with 0.2 ml of the virus material prepared from infected mouse brains. After virus adsorption at 37°C for 60 min, the inoculated cultures were fed maintenance medium consisting of MEM and 10% TPB and incubated at 37°C. Cytopathic changes were observed only in SK-K cell monolayers in 72 h p.i. and were characterized with round cells, syncytium formation and cell detachment from glass surface. Round cells and fused cells were scattered over the cell sheet and eventually detached from glass surface, on which a few cells remained at 96 h p.i. No visible changes were found in other 6 cell cultures at 7 days p.i. and also in the blindly passaged cell cultures. Further passages of the virus in SK-K cell cultures were readily accomplished with undiluted culture fluid at intervals of 3 days. After a few passages, the CPE began to appear within 24 h p.i. and was complete within 48 h p.i. (Fig. 1).

Immunofluorescence

The SK-K cell monolayers grown on coverslips were inoculated with the virus passaged 6 times in SK-K cells for detection of virus specific antigen and incubated

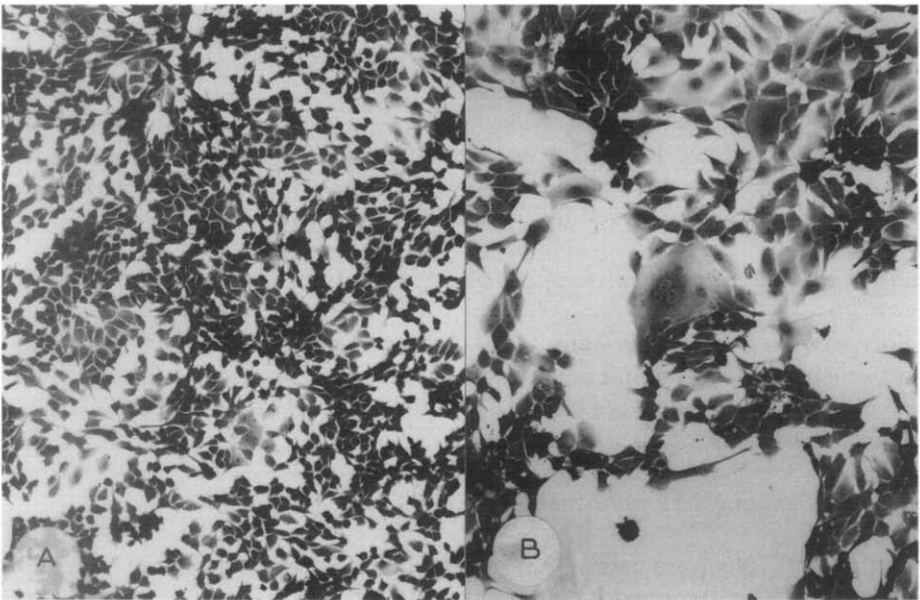


Fig. 1. Cytopathic change of SK-K cell culture infected with HEV 67N strain. Cells uninfected (A), and 24 h after infection with the virus (B).

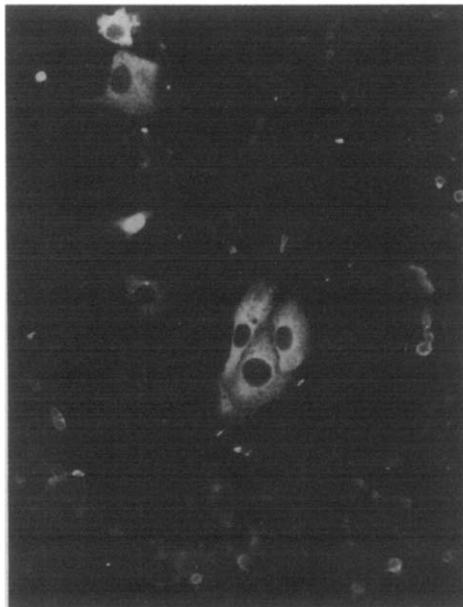


Fig. 2. Immunofluorescence of SK-K cells 6 h after infection with HEV 67N.

at 37°C in maintenance medium after virus adsorption at 37°C for 60 min. The infected cell cultures were examined for virus antigen by an indirect method using anti-HEV 67 rabbit serum. Specific viral antigen was first observed at 6 h p.i. as perinuclear, fluorescent granules in the cytoplasm (Fig. 2), and the positive cells to viral antigen increased in number with the progress of incubation and reached 100% at 24 h p.i. The viral antigen was not detected in non-infected cells.

Plaque formation of the virus

The SK-K-passaged virus as well as the original mouse-passaged virus formed plaques on SK-K cell monolayers prepared in plastic dishes by agar overlay method. The cell cultures inoculated with 0.2 ml of infectious material were incubated at 37°C for 90 min for virus adsorption, and then overlaid with 5 ml of MEM containing 0.8% Agar Noble and 10% TPB. After incubation at 37°C for 48 h, small plaques became visible without staining. At 72 h p.i., the inoculated cultures were overlaid with agar medium containing neutral red. The clear plaques were produced by both viruses. Various factors influencing plaque formation were examined in order to establish a routine assay for 67N virus.

Factors influencing plaque formation

Comparison of adsorption medium

Effect of adsorption medium on plaque formation was tested by diluting the virus with the following media; MEM, PBS and LE (Earle's solution containing 0.5% lactalbumin hydrolysate). Infectious culture fluid was diluted 1000-fold with the diluent indicated described above. No significant difference in plaque count was found between MEM, PBS and LE. From these results, MEM was selected for following tests as the adsorption medium.

Effect of adsorption time on plaque formation. In order to determine experimental conditions for routine assay, the kinetics of virus adsorption onto SK-K cell monolayers in MEM were investigated. SK-K cell monolayers were inoculated with the diluted virus, incubated at 37°C for 30, 60, 90 and 120 min for virus adsorption and overlaid with agar medium immediately after virus adsorption or after washing 3 times with MEM. The number of plaques in unwashed cultures was about 3-fold higher than the washed ones, and showed a maximum both at 90 min of incubation in both unwashed and washed ones. For the routine procedure, the inoculated cultures are incubated at 37°C for 90 min, and overlaid with agar medium without washing.

Effect of composition of overlay medium on plaque formation. As shown in Table 1, the effect of composition of the agar overlay medium on plaque formation was tested. Addition of 10% TPB to the agar medium exerted a marked effect on plaque formation. However, overlay medium supplemented with 5% FCS produced a small number of ill-defined plaques whether or not the media contained TPB. As agar overlay medium, MEM containing 0.8% Agar Noble and 10% TPB was selected.

Effect of incubation time on plaque formation. Readily countable, clear plaques with a well-defined circular margin, 0.5 to 0.7 mm across, were obtained after incubation at 37°C for 48 h before staining by second overlay with neutral red, whereas no plaques were formed after incubation for 24 h. Incubation for 72 h increased the plaque size and number. The plaque size was 2.0–2.5 mm in diameter (Fig. 3). Further incubation resulted in the degeneration of the uninfected cell

TABLE 1

Effect of composition of overlay medium on plaque formation

Overlay medium containing ^a	Plaque number ^b
TPB	49.0
TPB + FCS	28.0
FCS	30.7

^aMEM containing 0.8% Agar Noble was supplemented with 10% TPB, or 5% FCS or both.

^bAverage of 3 dishes.

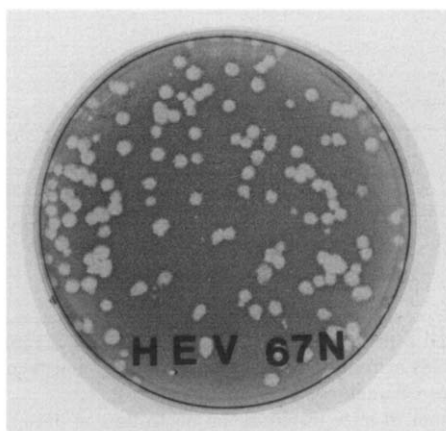


Fig. 3. Plaque produced 72 h after inoculation with the SK-K-passaged virus (67N).

monolayers and decrease of plaque count. Incubation for 72 h was therefore adopted as a standard procedure.

Effect of DEAE-dextran and trypsin in diluent and overlay medium on plaque formation. As shown in Table 2, the addition of DEAE-dextran (50 $\mu\text{g/ml}$) to the virus adsorption medium and overlay medium increased the plaque count by 50% but the condition of the SK-K cell cultures became worse in comparison to the control cultures without DEAE-dextran. When trypsin was added, no significant difference in the size and number of plaques was found between added and control cultures, but the cells were damaged by trypsin. The addition of both DEAE-dextran and trypsin enhanced damage of the cells. Based on these results, the standard technique of plaque assay was established as described in Materials and Methods. Using this technique, the plaque count was shown to be directly proportional to virus concentration. In the serial passages of the virus in SK-K cells described above, the virus yield in the fluid phase ranged from $10^{6.0}$ to $10^{6.3}$ PFU/0.2 ml after 2 days of incubation, although the titers in the early passages were lower.

TABLE 2

Effect of DEAE-dextran and trypsin on plaque formation

Viral diluent and agar medium ^a	Plaque number ^b
-	37.7
DEAE-dextran	59.3
Trypsin	37.0

^aViral diluent (MEM) and agar overlay medium containing DEAE-dextran (50 $\mu\text{g/ml}$) or trypsin (5 $\mu\text{g/ml}$).

^bAverage of 3 dishes.

TABLE 3

Pathogenicity of SK-K-passaged virus for mice

Virus (PFU/0.02 ml)	Inoculated with ^a	
	MEM	Antiserum
2.0×10^4	4/4 (4.0) ^b	1/4 (5.0)
2.0×10^3	4/4 (3.3)	0/4
2.0×10^2	4/4 (4.5)	0/4
2.0×10	4/4 (5.0)	0/4

^aVirus material was incubated with an equal volume of either MEM or rabbit antiserum against mouse-passaged virus (HI titer 1:128) at room temperature for 30 min, and then 0.02 ml of the mixture was inoculated intracerebrally into 4-week-old ICR mice.

^bNumber of mice dead/number of mice tested; mean death time in days was shown in parenthesis.

Pathogenicity of the SK-K-passaged virus for mice. Since the 67N virus was neuropathogenic for mice (Yagami et al., 1987), the SK-K-passaged virus at the 9th passage level was examined for pathogenicity for 4-week-old ICR mice by intracerebral inoculation. The mice, inoculated with the virus of 10 to 10^4 PFU/0.02 ml, died within 7 days with severe symptoms in the central nervous systems. Preincubation of the virus with rabbit antiserum against mouse-passaged virus prevented fatal infection in mice (Table 3).

HA and HI tests

HA activity was demonstrated in the infected culture fluid by microtiter method. When the SK-K cell cultures developed confluent CPE at 48 h after inoculation with the 10th passaged virus, the cultures were frozen and thawed 3 times. The supernatant of whole cultures showed HA activity 1:8 to 1:16 in titer. Cross-HI tests were carried out between SK-K- and mouse-passaged viruses and antisera against both viruses in order to identify the SK-K-passaged virus as 67N virus. As shown in Table 4, no antigenic discrepancy existed between both viruses.

TABLE 4

Cross-HI test and cross-neutralization test by mouse- and SK-K-passaged viruses and mouse antisera against these viruses

Virus passaged in	Antiserum against the virus passaged in	
	Mouse	SK-K
Mouse	1:5120 ^a (1:51200) ^b	1:1280 (1:51200)
SK-K	1:5120 (1:12800)	1:2560 (1:25600)

^aHighest antiserum dilution showing complete HI.

^bHighest antiserum dilution showing 50% plaque reduction.

Plaque neutralization test

The final identification of the SK-K-passaged virus was established by cross-neutralization test using hyperimmune mouse sera against the mouse-passaged and SK-K-passaged viruses. The tests were carried out by 50% plaque reduction method. Virus sample (200 PFU/0.2 ml) was incubated at room temperature for 30 min with respective antiserum of varying concentrations. There was no antigenic discrepancy between both viruses, identifying the SK-K-passaged virus as HEV 67N (Table 4).

Discussion

In the present study, SK-K cells, a stable cell line derived from a swine kidney was shown to be more sensitive to HEV 67N and found satisfactory for viral production and plaque formation of the virus.

Compared with the tissue culture methods described previously for replication and infectivity assay of HEV 67N (Greig and Girard, 1962; Mengeling et al., 1972; Mengeling, 1973; Sato et al., 1983), the SK-K cell cultures have several advantages. The cells can be grown and handled easily. There is no risk of contamination with HEV and other porcine viruses, which may occur with the use of primary cultures of porcine tissues. After inoculation of 67N virus into the SK-K cell cultures, CPE developed earlier and more clearly than in other cell cultures reported previously (Greig and Girard, 1963; Mengeling et al., 1972; Mengeling, 1973; Sato et al., 1983). Indirect immunofluorescence using rabbit antiserum against mouse-passaged virus demonstrated viral specific antigen in the cytoplasm of fused SK-K cells, the same as reported on 67N infection of cell cultures of embryonic porcine kidney (Mengeling et al., 1982; Mengeling, 1983).

The standard technique of plaque assay, as described in Materials and Methods was developed on the basis of the findings presented in this paper and fits the requirement that a routine procedure should be as simple as possible. On plaque formation of HEV in established cell line, Sato et al. (1983) first demonstrated plaque formation in ESK cell cultures under the agar overlay medium containing DEAE-dextran. In the SK-K cell system, DEAE-dextran did not show the significant enhancing effect on plaque formation, and plaques developed earlier and clearer than in ESK cell system. FCS is known to inhibit adsorption of coronaviruses onto cell membrane (Gerna et al., 1980). In this study, the same effect was observed with plaque formation by the 67N virus because the addition of FCS to the agar medium reduced the number of plaques.

The SK-K-passaged virus caused fatal infection in mice, as observed with the mouse-passaged virus. Preincubation with rabbit antiserum against mouse-passaged virus prevented fatal infection in mice. The final identification of the SK-K-passaged virus as HEV 67N was established by cross-HI and cross-neutralization tests, using hyperimmune sera both against the mouse-passaged virus and the SK-K-passaged virus.

Thus, SK-K cells provide a satisfactory source of the virus, and a sensitive, practical, reproducible plaque assay method for viral infectivity and neutralizing antibody in HEV infection. Further investigation of other HEV strains as well as wild strains would be of interest.

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