Microbiol. Immunol.
Vol. 24 (11), 1023–1033, 1980

Mode of Subacute Sclerosing Panencephalitis (SSPE) Virus Infection in Tissue Culture Cells

II. Cell-Free Viruses in Cell Cultures Infected with Kitaken-1 and Biken Strains of SSPE Virus

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(Accepted for publication, August 25, 1980)

Abstract Cell-free infectious viruses were successfully recovered by the aid of freezing and thawing from cultures infected with the Kitaken-1 and Biken strains of subacute sclerosing panencephalitis (SSPE) virus. Our results including those in a previous report which dealt with the Niigata-1 strain of SSPE virus show that cell-free viruses can be detected from all of the SSPE virus-carrying cultures established in Japan. It was also found that cell-free infectious viruses can be recovered efficiently by dispersing the virus-carrying cultures with EDTA. The inclusion of trypsin in the EDTA solution, however, caused a poor recovery of the infectious viruses. Infection of cells with the cell-free viruses readily established the virus-carrying cultures that have characteristics comparable to those of their original cultures. The culture infected with the Kitaken-1 strain produced infectious viruses in about ten times the amount of the other two infected cultures. The buoyant densities of the cell-free infectious viruses were almost the same among the three strains, the values being 1.120 to 1.132, but significantly less than that of 1.164 of measles virus. The low density can be ascribed to one of the characteristics of these SSPE viruses.

Isolation of measles virus-like agents from brains of patients with subacute sclerosing panencephalitis (SSPE) by cocultivation of the brain cells with tissue culture cells susceptible to measles virus has been successful (1, 2, 4–7, 11, 12, 14, 16, 21, 24). These isolated strains of the SSPE agent are maintained by cocultivation of the infected cells with various kinds of uninfected cells. The virus-carrying cultures can be divided into three groups according to their ability to produce cell-free viruses. The first group produces a relatively large amount of cell-free virus, like conventional measles virus (10). The second group releases only a small amount of virus and most of the infectious agents remain cell associated (20). The third group, in which the original properties of SSPE virus seem to have been well preserved (21, 23, 26), is thought to be defective in the production of cell-free virus (4, 6, 7, 14, 18, 22, 24). The Niigata-1, Kitaken-1, and Biken strains which cover

all of the isolates in Japan have been regarded as members of the third group (6, 14, 24).

In the previous study, however, we demonstrated in a culture system with the Niigata-1 strain that cell-free infectious viruses could be detected with the aid of freezing and thawing or hypotonic treatment of the cells (15). In the present study, we report the presence of cell-free viruses in two other culture systems infected with the Kitaken-1 and Biken strains. The differences in the growth characteristics among these viruses and in the buoyant density of SSPE and measles viruses are also described.

MATERIALS AND METHODS

Cells and SSPE virus-carrying cultures. Vero cells infected with the Kitaken-1 strain of the SSPE agent (Vero/SSPE-K1) at the 104th passage level and uninfected Vero cells were kindly supplied by Dr. S. Makino, Kitasato Institute, Tokyo. Human embryonic lung (HEL) cells and HEL cells infected with the Biken strain of the SSPE agent (HEL/SSPE-B) at the 12th passage level were kindly supplied by Dr. S. Ueda, Hyogo College of Medicine, Kobe. Vero cells infeced with the Niigata-1 strain of SSPE virus (Vero/SSPE-N1) which was kindly supplied by Dr. T. Sato, Niigata University, are described in the first report of this series (15). Since the Vero cell lines supplied by the Kitasato Institute and Niigata University are slightly different from each other in morphology and biological properties, they will be referred to as Vero-K and Vero-N, respectively. HEL-R66 cell, an established line of human embryonic lung, was kindly supplied by Dr. F. Taguchi, Kitasato University (19). These cells were grown at 37 C in Eagle's minimum essential medium (MEM) supplemented with 10% bovine serum and maintained in a medium in which the bovine serum was reduced to 2%. Subpassages of the virus-carrying cultures were made every 5 to 7 days by mixing the infected and uninfected cell suspensions after each was dispersed by trypsin and EDTA solution.

Detection of cell-free viruses by freezing and thawing. The method of detection of cell-free viruses in the SSPE virus-carrying cultures was described previously (15). Briefly, an SSPE virus-carrying culture in a 50- $\times$100-mm culture bottle was subjected to three cycles of freezing and thawing in fresh maintenance medium and the resulting cell homogenate was centrifuged at 2,000$\times g$ for 10 min at 4 C to sediment the cell debris. The cell homogenate and the supernatant fluid after centrifugation were assayed for infectivity by inoculation onto a monolayer culture of the cells prepared in a 40- $\times$65-mm plaque bottle. After adsorption for 2 hr at 37 C, the culture was washed and incubated with fresh maintenance medium at 37 C, and the syncytial plaques were counted under a microscope on the 3rd day after inoculation.

Neutralization test. The neutralization test was performed with heat-inactivated hyperimmune rabbit serum to measles virus as described previously (15).

Determination of buoyant density of the cell-free viruses. Cell-free viruses suspended in maintenance medium were subjected to potassium tartrate density gradient cen-

trifugation at 160,000 × *g* for 100 min and their buoyant densities were determined as described previously (15).

RESULTS

Recovery of Cell-Free Viruses from Cultures Infected with Kitaken-1 and Biken Strains of SSPE Virus

Vero/SSPE-K1 cultures at the 104th passage and HEL/SSPE-B cultures at the 15th passage were subjected to three cycles of freezing and thawing, 3 and 2 days after seeding the cells, respectively. The cell homogenates and the supernatant fluids after centrifugation, as well as the culture fluids collected before freezing and thawing of the cells, were assayed for infectivity by the method described in "MATERIALS AND METHODS." The infectivity of the specimens derived from the Vero/SSPE-K1 culture was measured in Vero-K cells while the infectivity of those derived from the HEL/SSPE-B culture was measured in Vero-N cells. In both cases, syncytia began to appear on the 2nd day after infection and their numbers reached a plateau on the 3rd day. Recovery of the infectivity from the cell homogenates and their supernatant fluids after centrifugation, but not from the culture fluids was evident in both cultures (Table 1). The infectivity was completely neutralized by the antiserum against measles virus. In addition, when cultures of Vero-K and HEL cells were infected with the supernatant of the cell homogenates of Vero/SSPE-K1 or HEL/SSPE-B respectively, virus-cell interactions identical to those of the original virus-carrying cultures were established.

When a culture of HEL cells was infected primarily with the cell-free viruses from any of the SSPE virus-carrying cultures, no visible syncytia appeared during the incubation period of 7 days. Nevertheless, many syncytia appeared on the next day after only a single passage of the cells (Table 2). In contrast, when a culture of HEL cells was infected with the Edmonston strain of measles virus, syncytia readily developed without passage of the cells (data not shown). Thus the phenomenon observed may be a characteristic of the SSPE virus strains used in the present study.

Table 1. Recovery of cell-free viruses from Vero/SSPE-K1 and HEL/SSPE-B cultures[a)]

Specimens	Infectivity recovered from[b)]	
	Vero/SSPE-K1	HEL/SSPE-B
Culture fluid	0	0
Frozen and thawed cell homogenate	15,800	940
Supernatant fluid of the cell homogenate	3,100	150

[a)] Cultures of Vero/SSPE-K1 and HEL/SSPE-B cells containing about 1,500 and 600 syncytia, respectively, were used.

[b)] Plaque-forming units per culture. The infectivity of the specimens from Vero/SSPE-K1 and HEL/SSPE-B cells was assayed on Vero-K and Vero-N cells, respectively.

Table 2. Syncytium formation in HEL cell cultures inoculated with cell-free SSPE viruses

Source of cell-free viruses	Titer of inoculum[a]	Number of syncytial plaques[b]	
		Before passage	After passage
HEL/SSPE-B	400	0	120
Vero/SSPE-Nl	3,150	0	1,750
Vero/SSPE-Kl	2,000	0	25

[a] Plaque-forming units. The viruses from HEL/SSPE-B and Vero/SSPE-Nl cultures were assayed on Vero-N cells and the viruses from Vero/SSPE-Kl cultures on Vero-K cells.

[b] The cultures of HEL cells were infected with cell-free viruses derived from each virus-carrying culture and the plaques were counted just before cell passage 7 days after infection, and 2 days after the cell passage.

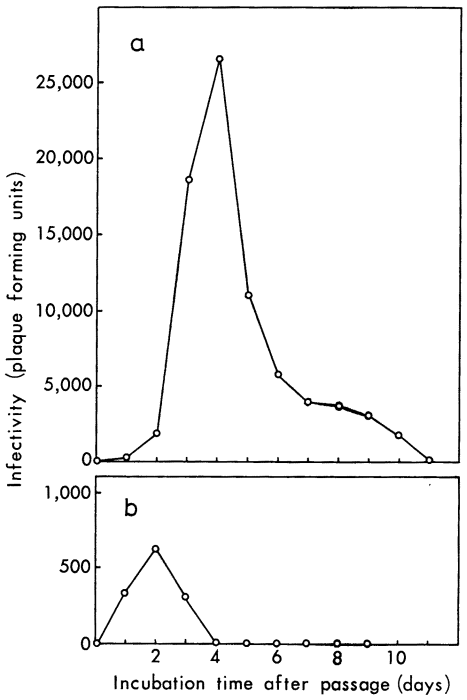

Fig. 1. Time course of the production of cell-free viruses in Vero/SSPE-Kl and HEL/SSPE-B cultures. Replicate cultures were prepared in plaque bottles by mixing the cells from Vero/SSPE-Kl (a) and HEL/SSPE-B (b) cultures with normal Vero and HEL cells, respectively, and cultivated at 37 C. Culture bottles were removed daily and the infectivity was assayed by the method described in "MATERIALS AND METHODS."

In the following experiment, the time course of the production of the cell-free viruses in Vero/SSPE-K1 and HEL/SSPE-B cultures was investigated. The virus-carrying cultures were prepared in plaque bottles. A bottle from each culture group was removed each day and the cell homogenates after freezing and thawing were measured for infectivity. In the culture of Vero/SSPE-K1 cells, the cell-free viruses were first detected on the lst day after passage and the titer reached a maximum on the 4th day and decreased thereafter (Fig. 1a). On the other hand, in the culture of HEL/SSPE-B cells, the cell-free viruses were detectable for only 3

days after passage (Fig. 1b). The production of cell-free viruses in Vero/SSPE-K1 cultures always lasted longer than in HEL/SSPE-B cultures.

Recovery of Cell-Free Viruses with the Aid of EDTA Treatment

The facts that infectious virus could seldom be detected in the culture fluids but became detectable after freezing and thawing of the SSPE virus-carrying cultures may suggest that the infectious virus exists intercellularly by being bound tightly to the cell membrane. If this is the case, the infectious virus should be recovered by simply dispersing the virus-carrying cultures instead of disrupting the cells by freezing and thawing. Duplicate cultures of Vero/SSPE-N1, Vero/SSPE-K1 and HEL/SSPE-B cells were prepared in plaque bottles. Two days later, half of the cultures were washed once with PBS and immersed in PBS containing 0.05% EDTA at 37 C for 5 min. After removal of the EDTA solution, the cultures were then dispersed into fresh maintenance medium by vigorous manual shaking and centrifuged at $2,000 \times g$ for 10 min to sediment the cells, and the supernatant fluids were assayed for infectivity. The remaining half of the cultures were frozen and thawed in fresh maintenance medium and the cell homogenates were assayed for infectivity. The treatment of the cultures with EDTA facilitated liberation of the cell-free viruses as efficiently as disruption of the cells by freezing and thawing, with the exception of HEL/SSPE-B in which the cells were only slightly dispersed by the EDTA treatment (Table 3).

In Table 4, an example of the recovery of infectious viruses at every step of the above procedure is presented. A large number of cell-free viruses were recovered after vigorous shaking of the EDTA-dispersed cells but there were only a limited number in the EDTA washings. A considerable amount of the virus still remained cell associated but the viruses were readily freed from the cells by freezing and thawing. It was evident that the freezing and thawing applied to the singly dispersed cells after EDTA treatment was more efficient for recovery of the

Table 3. Recovery of infectious viruses by dispersing the virus-carrying cultures with EDTA

Specimen	Infectivity recovered from[a]		
	Vero/SSPE-N1	Vero/SSPE-K1[d]	HEL/SSPE-B
Frozen and thawed cell homogenate[b]	1,210	1,110	510
Supernatant of the EDTA-dispersed cell suspension[c]	1,518	2,925	160

[a] Plaque-froming units assayed on Vero-N cells with Biken and Niigata-1 strains and on Vero-K cells with Kitaken-1 strain.

[b] Cultures were frozen and thawed three times.

[c] Cultures were treated with 0.05% EDTA solution and suspended in fresh maintenance medium by vigorous manual shaking. The cell suspensions were centrifuged at $2,000 \times g$ for 10 min and the supernatants were assayed for infectivity.

[d] The same culture was used for the experiment shown in Table 4.

Table 4. Effect of EDTA treatment on the recovery of infectious viruses from the culture of Vero/SSPE-K1 cells

Specimen[a]	Infectivity[b]
EDTA washings	15
Supernatant of the EDTA-dispersed cells	2,925
Frozen and thawed homogenate of the EDTA-dispersed cells	4,005

[a] Culture fluid from 2-day-old Vero/SSPE-K1 cells was discarded and the culture was immersed in a 0.05% EDTA solution for 5 min at 37 C. The EDTA solution was withdrawn carefully, clarified by light centrifugation at 2,000 × *g* for 10 min and the supernatant fluid (EDTA washings) was used for the test. The culture then received maintenance medium, was shaken vigorously to disperse the cells, and centrifuged at 2,000 × *g* for 10 min. After removal of the supernatant fluid (Supernatant of the EDTA-dispersed cells), the sedimented cells were resuspended in fresh maintenance medium and subjected to freezing and thawing three times (Frozen and thawed homogenate of the EDTA-dispersed cells).

[b] Plaque-forming units in each whole specimen.

cell-free infectious viruses than that applied directly to the cell monolayers (see Table 3).

From the technical viewpoint, cell density of the culture seemed responsible for efficiency of the recovery of the infectious viruses by EDTA treatment, *i.e.*, the recovery of the infectious viruses from old dense cultures was poor, probably because they were difficult to disperse with the aid of EDTA alone. The incorporation of trypsin into the EDTA solution at a concentration of 0.05% for dispersion of the cells, however, drastically reduced the recovery of the infectious viruses (data not shown). This reduction might be caused by inactivation of the viruses by trypsin.

Comparison of Virus Production among the Cells Infected with SSPE Virus Strains

The quantities of cell-free virus recovered from the virus-carrying cultures varied among the three strains of SSPE virus, a fact which might be a reflection of the difference in the properties of the viruses. This view, however, is only conjectural because each virus strain had been passaged in a different cell line. Therefore, we attempted to compare the three strains of the viruses in their ability to produce cell-free viruses in the same host cell system.

Separate cultures of HEL-R66 cells were infected with the SSPE viruses derived from cultures of Vero/SSPE-N1, Vero/SSPE-K1, and HEL/SSPE-B cells. The infected cells were dispersed by EDTA 4 days after infection and the cell-free virus preparations were made by the method described in the footnotes to Table 3. To

compare the virus production directly, approximately 500 plaque-forming units of the SSPE virus of the three strains were inoculated separately into plaque bottle cultures of HEL-R66 cells. On the 4th day, after the syncytial plaques were counted, all of the infected cultures were subjected to freezing and thawing and the amount of the infectious virus produced in each culture was assayed by inoculating HEL-R66 cell cultures with the cell homogenate.

As shown in Table 5, the Kitaken-1 strain was highly productive, whereas both Niigata-1 and Biken strains produced fewer cell-free viruses. Although the yields of the infectious viruses easily fluctuated from one experiment to another, the Kitaken-1 strain was always the most productive.

Comparison of Buoyant Density of SSPE Viruses

To compare the buoyant densities of the cell-free viruses of the Kitaken-1 and Biken strains with those of the Niigata-1 and Edmonston strains, Kitaken-1 and

Table 5. Comparison of virus production in HEL-R66 cells infected with various SSPE virus strains

SSPE virus strain	Inoculum[a]	Number of cell-free viruses produced[b]
Niigata-1	555	2,000
Kitaken-1	490	23,000
Biken	460	1,800

[a] Number of syncytia formed in HEL-R66 cells 4 days after infection with the indicated strain of virus.

[b] After the syncytia were counted, the infected HEL-R66 cells were frozen and thawed three times, and the homogenates were measured for infectivity in HEL-R66 cells.

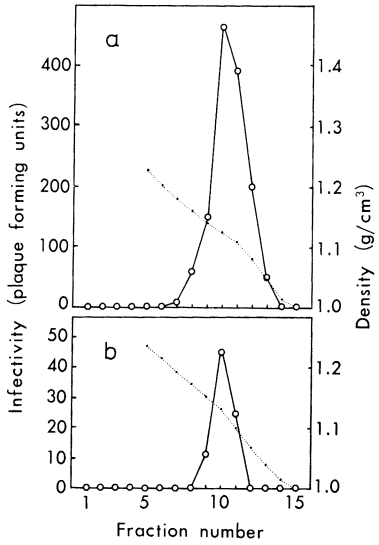

Fig. 2. Equilibrium density gradient centrifugation of the infectious cell-free viruses of Kitaken-1 and Biken strains of SSPE virus. A culture of Vero-N cells infected with either the Kitaken-1 (a) or Biken strain (b) of SSPE virus was prepared as described in the text. The cell-free viruses were prepared by treatment of the culture with the EDTA solution as described in the footnotes to Table 3 and centrifuged on a potassium tartrate density gradient (5 to 60%) at 16,000×*g* for 100 min at 4 C. Fractionation and assay for infectivity were as described previously (15). Dotted lines indicate the density of the fractions.

Biken strain viruses were prepared from Vero-N cells, in the same way as the Niigata-1 and Edmonston strains (15). The cell-free viruses to be tested were obtained by dispersing the infected cells with EDTA 1 day after cell passage and subjected to potassium tartrate density gradient centrifugation as described in "MATERIALS AND METHODS." As shown in Fig. 2, the infectious viruses of the Kitaken-1 and Biken strains were banded at the same position, with the density of 1.120 which is very close to the value of 1.132 for the Niigata-1 strain but significantly lower than the value of 1.164 for the Edmonston strain of measles virus (15).

DISCUSSION

Our results demonstrate the production of cell-free infectious viruses in Vero/SSPE-K1, HEL/SSPE-B and Vero/SSPE-N1 cell cultures, which include all of the SSPE virus-carrying cultures in Japan and have been regarded as non-virus producers (6, 14, 24). Thormar et al also found some syncytiogenic activity in Millipore filtered (0.45 μ in pore size) supernates of frozen and thawed suspensions of cells infected with the Biken strain as well as the D.R., LEC, and IP-3 strains of SSPE virus; however they claimed that the activity was mostly associated with living infected cells (23). Although the time course of the production of virus in the cells and the properties of the viruses were not described in detail, their cell-free syncytiogenic activity could be due to a principle comparable to our cell-free infectious virus. Accordingly, with regard to these results, it is necessary to reinvestigate whether or not infectious viruses are produced in other SSPE virus-carrying cultures that are thought to be non-virus producers.

Failure of the recovery of infectious virus from Vero/SSPE-K1 cultures was reported by Makino et al (14) and conflicts with our present finding. As the production of infectious virus was related closely to the formation of syncytia (15), poor development of the syncytia in their experiments might have caused this discrepancy, *i.e.,* their syncytia did not grow beyond 1–2 mm in diameter but ours continued to grow to 5 mm in diameter (data not shown). What causes this difference in the development of syncytia, however, remains to be determined.

Working with the HEL/SSPE-B culture, Ueda et al failed to detect infectious virus (24), in disagreement with our present finding. Retrospectively, the HEL cells they used for detection of the infectious virus were not suitable for that purpose because our experiment showed that HEL cells primarily infected with cell-free SSPE viruses could not form a syncytium until another passage had been made (Table 2).

When infectious viruses of both Kitaken-1 and Biken strains were recovered by the aid of EDTA treatment, they were both sedimented at a density of 1.120 on a potassium tartrate gradient. In the first report of this series, the cell-free viruses of the Niigata-1 strain which were recovered by freezing and thawing banded at two positions, at densities of 1.132 and 1.200, and the latter band was shown to be the viruses attached to the aggregate of the cell debris (15). This was also the case with the Kitaken-1 and Biken strain viruses whenever they were prepared by

means of freezing and thawing instead of by EDTA treatment (data not shown). These results indicate that EDTA is much more effective for liberating SSPE viruses freed from the cell debris than is freezing and thawing.

The buoyant density of the viruses of the Kitaken-1 and Biken strains was much the same, 1.120, and was very close to that of the Niigata-1 strain, 1.132 (15). Since these values are significantly lower than that of the Edmonston strain of measles virus, 1.164 (15), it is important to determine whether or not every SSPE virus has a lower density than measles virus and what causes the lower density. Recently, Kendal et al demonstrated that influenza virions with reduced amounts of M protein had a lower buoyant density than normal virions (13). It is probable that our SSPE viruses have smaller amounts of M protein than measles virus.

Since production of the virus in none of the virus-carrying cultures in the present study could be enhanced by lowering the incubation temperature to 33 C (data not shown), the restriction in the virus production in these systems might not be a temperature-dependent event. Recently, Hall and Choppin reported that M polypeptide was not detected in the cultured brain cells from a patient with SSPE in spite of the presence of all other viral polypeptides and they thought that lack of the synthesis of M protein could explain their failure to detect cell-free viruses in the culture (8). The above observation is supported by the fact that patients with SSPE selectively lack the antibody to M protein of measles virus (9, 25). On the other hand, Breschkin et al reported that M protein as well as other viral proteins was synthesized normally in BSC-1 cells infected with the Biken strain of SSPE virus but they claimed that failure of the insertion of the hemagglutinin (HA) protein into the cell membrane could account for the failure of the release of infectious viruses (3). Whether or not every SSPE virus has the same defective site should be determined. It should also be determined whether the lack of synthesis of M protein or of the function of HA would cause complete inhibition of the formation of infectious virions, because Kendal et al obtained influenza virions with reduced M protein content (13), whereas Portner et al obtained Sendai virus entirely devoid of hemagglutinin-neuraminidase protein (17). It is possible that, in the SSPE virus-carrying cultures, the synthesis of M protein at a reduced rate causes a restriction in the virus morphogenesis rather than a complete cessation. The cell-free SSPE viruses thus formed may have a reduced amount of M protein and therefore have a lower density than measles virions.

This work was supported in part by research grants from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare.

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(Received for publication, March 13, 1980)