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PURIFICATION OF JAPANESE ENCEPHALITIS VIRUS GROWN IN BHK21 AND SINGH'S *AEDES ALBOPICTUS* CELLS BY POLYETHYLENE GLYCOL PRECIPITATION

AKIRA IGARASHI, TAKAHISA FUKUOKA[1], FUYOKO SASAO, SURANGA SURIMARUT[2] and KONOSUKE FUKAI

Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka

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SUMMARY Japanese encephalitis virus (JEV) grown in BHK21 or Singh's *Aedes albopictus* (SA) cells was concentrated by polyethylene glycol precipitation. Recovery of hemagglutinating activity (HA) was complete, and that of infectivity (PFU) was 30–75%, with 3–4% protein due to impurities, giving purification factors (PF) of 30–40 for HA and 10–30 for PFU. The material was then centrifuged on sucrose cushions, with recovery of almost 100% HA, 5–6% PFU and 0.1% protein due to impurities, giving PF of more than 800 for HA and 30–70 for PFU. The specimen was concentrated and then fractionated by sucrose gradient sedimentation, giving purified JEV. No significant differences were observed between JEV grown in BHK21 and in SA cells in respect to buoyant density, structural polypeptides, optimal pH for HA, antigenicities or gross size or structure of the virions, except that particles grown in SA cells appeared more smooth under an electron microscope than those grown in BHK21 cells.

INTRODUCTION

In the preceding paper (Igarashi et al., 1973), a certain strain of Japanese encephalitis virus (JEV), JaOH-0566 which had been passaged in MK and BHK21 cells, was reported to give a high yield of virus in Singh's *Aedes albopictus* (SA) cells (Singh, 1967). Although JEV is known to grow in some lines of established insect cells (Suitor, 1966; Řeháček, 1968a, b; Singh and Paul, 1968; Banerjee and Singh, 1968), little is known about the properties of progeny virus grown in arthropod cells. In order to understand the events occurring in JEV-infected mosquito cells, it seemed necessary to see whether JEV grown in mosquito cells had the same properties as that grown in mammalian cells.

1 Present address: Hitachi Ltd. Naka Works, Department of Applied Techniques. No. 882, Ichige, Katsuta-shi, Ibaraki, Japan.

2 Trainee from the Virus Research Institute, Bangkok, Thailand, under the Colombo plan.

MATERIALS AND METHODS

1. *Japanese encephalitis virus (JEV)*

The JaOH-0566 strain of JEV was passaged 3 times in suckling mouse brains, and 62 times in MK cells and 14 times in BHK21 cells with 3 plaque isolations (Igarashi et al., 1973). Then it was stored at −70 C. Before inoculating the virus onto cultured cells, it was grown in BHK21 cells at 37 C for 20 hr in Eagle's minimal essential medium (MEM, Eagle, 1959) supplemented with 2% calf serum. All sera used in tissue culture were free from JEV antibody.

2. *Cells*

An established line of Singh's *Aedes albopictus* (SA) cells (Singh, 1967) was originally supplied from Dr. S. Makino, Department of Microbiology, Kobe University, and was grown in a 1 : 1 mixture of MEM and Mitsuhashi-Maramorosch's (MM) medium (Mitsuhashi and Maramorosch, 1964) supplemented with 10% calf serum at 28 C. An established line of baby-hamster kidney-cells, BHK21 clone 13 (MacPherson and Stoker, 1962) was supplied by Dr. S. Hotta, Department of Microbiology, Kobe University, and was grown at 37 C in MEM supplemented with 10% calf serum.

3. *Inoculation and concentration of JEV*

Growth media were removed from confluent sheets of SA or BHK21 cells in large 1600 ml Roux bottles, and 2 ml of seed virus were inoculated into each bottle. During the adsorption period of 2 hr, at 28 C for SA and at 37 C for BHK21, virus was spread every 30 min. Then 60 ml of virus phase medium, consisting of 2% calf serum in a 1 : 20 mixture of MM and MEM, were added. After incubation at 28 C for 40 hr for SA or at 37 C for 20 hr for BHK, infected fluids were collected and virus was concentrated as follows. Infected fluid (F) was clarified by centrifugation at $8,500 \times g$ for 15 min. To the supernatant (S_1), NaCl and polyethylene glycol 6000 were added to concentrations of 0.5 M and 8%, respectively. When both were dissolved, the solution was centrifuged at $8,500 \times g$ for 15 min. The supernatant was removed and the precipitate was dissolved in a volume of $S_1/20$ of STE (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6). The specimen (P_2) was centrifuged at $700 \times g$ for 10 min. The supernatant (S_3) was layered on 10 ml cushions of 50% and 20% sucrose in STE, and centrifuged in an SW25.2 rotor at 25,000 rev/min for 2 hr. The visible band (SB) of partially purified JEV at the interphase of the two sucrose layers was taken out.

4. *Infectivity and neutralization of JEV*

The infectivity of JEV was assayed by plaque titration on BHK21 cells as described before (Igarashi et al., 1973). Titers were expressed as plaque forming units (PFU) per ml.

The neutralization titer (N) of antiserum was assayed by the 50% plaque reduction method as described before (Igarashi, Nithiuthai and Rojanasuphot, 1970). Antiserum against JEV was prepared in adult mice by 5 successive intraperitoneal inoculations of 0.1 ml of live virus at weekly intervals, using successively 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} dilutions, and then undiluted culture fluid of JEV-infected BHK21 cells. Mice were bled one month after the last inoculation.

5. *Hemagglutination (HA) and hemagglutination-inhibition (HI) of JEV*

The techniques of Clarke and Casals (1958) using goose red blood cells were followed with a microtiter method (Sever, 1962).

6. *Protein determination and purification factors (PF)*

Protein was determined as described by Lowry et al. (1951) with bovine serum albumin as a standard. Purification factors (PF) were calculated by dividing the specific activity (HA/protein or PFU/protein) of a given specimen by that of the starting material (F).

7. *Sucrose gradient sedimentation*

Specimens were applied to 4.5 ml columns containing 10–35% sucrose gradients in STE, and centrifuged in an SW50L rotor of a Beckman, model L2–65B, ultracentrifuge at 35,000 rev/min for 60 min at 4 C. Fractions were collected dropwise from the bottom of the tube.

8. *Potassium tartrate isopycnic density gradient centrifugation*

Specimens were layered onto 4.5 ml columns containing gradients of 16–28% potassium tartrate in 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6, and centrifuged in an SW50L rotor of a Beckman model L2–65B ultracentrifuge at 35,000 rev/min for 14 hr.

Fractions were collected dropwise from the bottom of the tube. The densities (ρ^{20}) of fractions were estimated from their refractive indices (n_D^{20}) using the following formula obtained experimentally with standard solutions: $\rho^{20}=5.425\, n_D^{20}-6.276$.

9. *Polyacrylamide gel electrophoresis*

The methods of Summers et al. (1965) and Strauss et al. (1968) were followed with slight modifications. The gel mixture consisted of 7.5% acrylamide, 0.5% *N*, *N'* methylene-bisacrylamide, 0.1% sodium dodecyl sulfate (SDS), 0.1 M sodium phosphate, pH 7.0, 0.034% *N*, *N*, *N'*, *N'* tetramethylethylene diamine and 0.07% ammonium persulfate. Gel columns of 5×50 mm were prepared in 5×80 mm glass tubes, and were prerun for 2 hr at room temperature at 5 mamp/gel with electrophoresis buffer of 0.1 M sodium phosphate, pH 7.0, containing 0.1% SDS. Specimens were dissolved in 1% SDS and 1% 2-mercaptoethanol by heating at 100 C for 1 min, and then dialyzed overnight against 0.01 M sodium phosphate, pH 7.0, containing 0.1% SDS and 0.1% 2-mercaptoethanol at room temperature. One-fifth volume of 60% sucrose containing bromophenol blue was mixed with the dialyzed specimen, and 0.1 ml of the mixture was applied on the gel column. Electrophoresis was performed at room temperature for 4 hr at 5 mamp/gel. After the run, gels were removed from the glass tubes, fixed in 20% sulfosalicylic acid, and stained with Coomassie brilliant blue R250 and then destained in 7% acetic acid, as described by Shapiro, Viñuela and Maizel (1967).

10. *Electron microscopy*

Test specimens were dialyzed overnight against 1000 volumes of an aqueous solution of 1% ammonium acetate, pH 7.2, at 4 C. One drop of the dialyzed specimen was mixed with one drop of 2% silicotungstic acid, adjusted to pH 7.2 with 1M KOH, or with one drop of saturated uranyl acetate solution, pH 4.3. The mixture was applied to a carbon-coated microgrid. Excess fluid was removed with filter paper, and the specimen was air dried and observed under a Hitachi, model HU-11Ds, electron microscope at an acceleration voltage of 75 kv and an instrument magnification of 47,000–50,000.

RESULTS

1. *Concentration and purification of JEV*

Attempts to concentrate JEV by precipitation with ultracentrifugation or with inorganic salts gave rather poor recoveries of virus activity. Better and reproducible results were obtained by precipitation with polyethylene glycol (Yamamoto et al., 1970; McSharry and Benzinger, 1970). Average values of 4 experiments starting from JEV-infected SA cells and of 5 experiments using JEV-infected BHK21 cells are summarized in Table 1.

TABLE 1. *Concentration and partial purification of JEV*

Host cell	Sample	Volume (ml)	Protein		HA			PFU		
			mg/ml	R[a]	log	R[a]	PF[b]	log	R[a]	PF[b]
SA	F	600	3.43	100	1.58	100	1	8.76	100	1
	S_1	600	3.32	96	1.51	88	1	8.58	70	0.8
	P_2	30	2.03	4	2.58	91	19	9.59	29	11
	S_3	30	2.02	4	2.58	133	35	9.64	29	12
	SB	3	0.49	0.1	3.77	152	1116	9.69	6	77
BHK	F	600	2.10	100	0.96	100	1	8.35	100	1
	S_1	600	1.76	84	0.90	90	1	8.25	86	1
	P_2	30	1.70	4	1.56	83	22	9.51	78	24
	S_3	30	1.28	3	2.19	127	44	9.50	75	27
	SB	3	0.44	0.1	2.86	75	817	8.72	6	30

a Recovery %.
b Purification factor.

On precipitation with polyethylene glycol, complete recovery of HA and 30–40% recovery of PFU were observed with 3–4% protein due to impurities. Thus the PF were 30–40 for HA and 10–30 for PFU. The material (S_3) was centrifuged on cushions of sucrose, and the visible band (SB) at the interphase of the two sucrose layers contained almost 100% of the HA and 5–6% of the PFU of the starting material (F), with 0.1% of protein due to impurities, so that PF were more than 800 for HA and 30–70 for PFU.

The partially purified JEV (SB) was still found to contain an appreciable amount of non-viral proteins on analysis by polyacrylamide gel electrophoresis. To achieve further purification, the specimen (SB) was diluted 4 fold with STE and an equal volume of saturated ammonium sulfate, pH 7.2, was added. The mixture was kept for 15 min in an ice bath, and then centrifuged at 20,000 $\times g$ for 15 min at 4 C. The supernatant was discarded and the precipitate was dissolved in 1 ml of STE and purified by sucrose gradient sedimentation. Fig. 1 shows that the HA and PFU of the virus sedimented as a single peak leaving most of the contaminating protein near the top of the gradient. The sedimentation pattern was essentially the same with JEV specimens obtained from SA and from BHK21 cells.

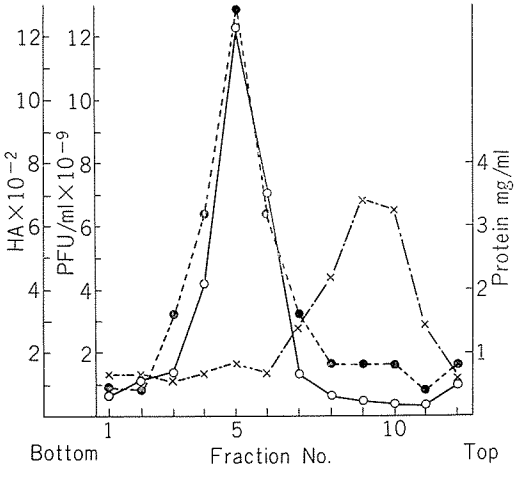

FIGURE 1. *Sucrose gradient sedimentation of partially purified JEV grown in BHK21 cells.* ●----●, *HA;* ○——○, *PFU;* ×—·—·—×, *protein.*

2. *Properties of purified JEV*

The fraction containing the peak of JEV-HA and PFU obtained by sucrose gradient sedimentation (Fig. 1) was used as the purified preparation of JEV. On centrifugation through a potassium tartrate density gradient, both the HA and PFU of JEV formed a single peak at a density of 1.20 g/cc (Fig. 2). No significant difference was observed between the densities of JEV obtained from SA cells and from BHK21 cells.

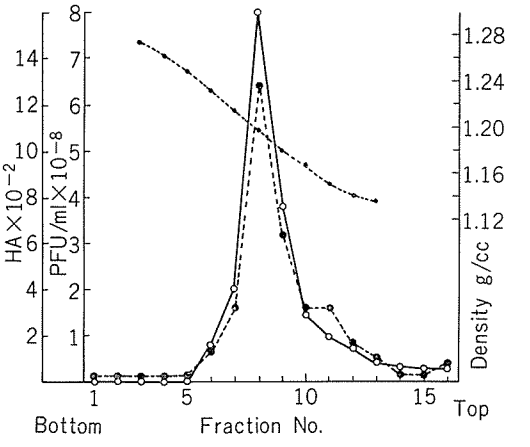

FIGURE 2. *Potassium tartrate isopycnic density gradient sedimentation of purified JEV grown in BHK21 cells.* ●----●, *HA;* ○——○, *PFU;* •-·-•-·-•, *density.*

The structural protein of purified JEV was analyzed by SDS-polyacrylamide gel electrophoresis. The results in Fig. 3 show that JEV specimens obtained from both infected SA and BHK21 cells had 3 polypeptides, as already concluded from results on radioisotope-labeling (Shapiro et al., 1971) or staining (Kitano, 1971). No significant difference was observed between the mobilities of the polypeptides of JEV from SA and from BHK cells.

The optimal pH values for HA for these 2 JEV specimens were also the same (Table 2),

as were their antigenicities analyzed by HI or the neutralization test (Table 3).

Electron microscopic examination showed

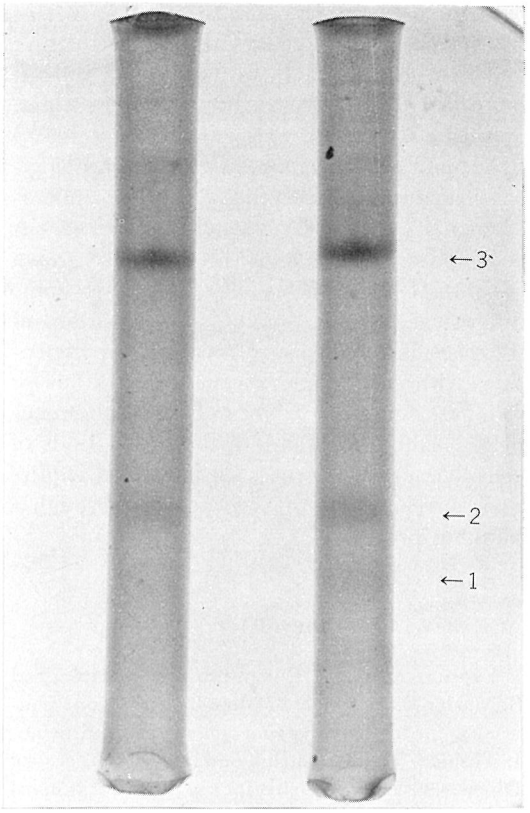

FIGURE 3. *Protein components of purified JEV analyzed by SDS-polyacrylamide gel electrophoresis and staining. (A) JEV grown in SA cells. (B) JEV grown in BHK21 cells.*

TABLE 2. *Optimal pH for HA*

VAD	HA titer of JEV grown in	
	SA	BHK 21
6.0	160	320
6.2	640	640
6.4	320	320
6.6	80	80
6.8	≤20	≤20
7.0	<20	<20

TABLE 3. *Antigenicities of JEV*

Host		HI	N[a] (log)
SA	Exp. 1	640	3.49
	Exp. 2	1280	3.69
BHK	Exp. 1	1280	3.35
	Exp. 2	1280	3.57

a Dilution of anti-JEV serum reducing the plaque number to 50% of the control.

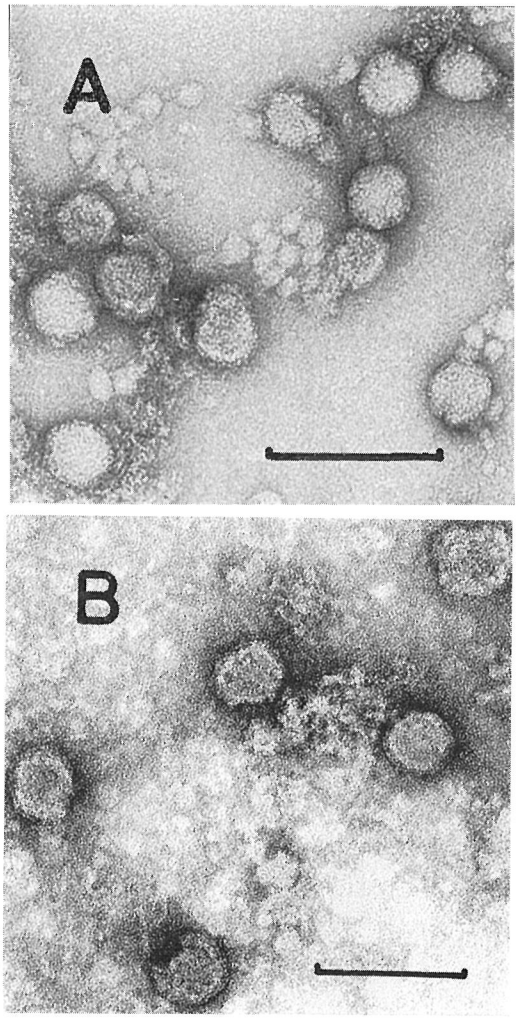

FIGURE 4. *Electron micrographs of purified JEV. scale: 100 nm. (A) JEV grown in SA cells, magnification: 230,000. (B) JEV grown in BHK21 cells, magnification: 200,000.*

that the JEV specimens obtained from SA and BHK21 cells both contained spherical particles of 40–45 nm diameter. These particles consisted of a spherical core surrounded by a membranous structure with short projections (Fig. 4). However the JEV particles from SA cells appeared more smooth than those from BHK21 cells.

DISCUSSION

There are several reports that JEV can grow in certain lines of insect cells, as mentioned in the introduction. Moreover spherical particles similar to those observed in infected mammalian cells or tissues were demonstrated by electron microscopy using the thin sectioning technique in cultured mosquito cells infected with JEV (Filshie and Rehacek, 1968). However there are no reports of comparative studies on the exact nature of JEV particles grown in insect cells and in mammalian cells. Using the JaOH–0566 strain and the Singh's *Aedes albopictus* cell line (Igarashi et al., 1973), we could study this because of the high virus yield.

In this work JEV was grown in SA and in BHK21 cells. Particles were concentrated by polyethylene glycol precipitation and sedimentation on sucrose cushions. Then they were precipitated with ammonium sulfate and finally purified by sucrose gradient sedimentation. On polyacrylamide gel electrophoresis, the resulting preparations contained no detectable polypeptides other than the 3 known structural polypeptides of JEV.

We could not detect any significant differences between JEV preparations grown in SA and in BHK21 cells with respect to their apparent sedimentation in a sucrose gradient, buoyant density, structural polypeptides, optimal pH for HA, antigenicities as revealed by HI and neutralization, or gross morphological structure as observed by electron microscopy. However JEV particles grown in SA cells appeared more smooth than those grown in BHK21 cells. This may be due to some differences in the mode of association of polypeptide 3, the major membrane polypeptide, with membranes of these two kinds of virions. And this might reflect a difference between the membranes of these two kinds of cells, because arboviruses are known to acquire their envelopes from the host cells through a budding process.

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