Production of Slowly Sedimenting and Rapidly Sedimenting Components Associated with Japanese Encephalitis Virus Envelope Glycoprotein E in Infected Cell Culture Fluids

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Abstract: Production of Japanese encephalitis (JE) virus glycoprotein E into culture fluids of infected mosquito (C6/36) and baby hamster kidney (BHK21) cells at 28 and 37℃ was investigated by the sandwich ELISA using an anti−E monoclonal antibody (MCA). Besides rapidly sedimenting component (RE) at the position of complete virion, slowly sedimenting component (SE) was found near the top of the sucrose gradient sedimentation. Except the first 24 hr of infection when virus production was not so much at 28℃, the peak of SE was more pronounced when the infected cells were incubated at 28 than at 37℃. Production of SE was generally more remarkable in C6/36 than in BHK21 cells at both temperatures. At 28℃, production of both SE and RE continued until 72 hr in C6/36 and 60 hr in BHK21 cells. While, production of both SE and RE declined after 36 hr in both cell lines at 37℃. When SE and RE were recentrifuged, they appeared as independent components. Although both SE and RE were accompanied by the peak of hemagglutination activity (HA), the HA of SE was relatively lower than that of RE by comparing the ELISA color development. Western blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE) showed a single band with apparent Mw of the E protein (54K) for both SE and RE fractions. Electron microscopy (EM) with negative staining revealed spherical particles of 45−55 nm in diameter corresponding to the complete virion together with components of 5−10 nm diameter in RE fractions. While in SE fractions, various components of 5−20 nm in diameter, apparently their aggregates, and membranous structures were observed.

Key words: Japanese encephalitis virus, ELISA antigen, Slowly sedimenting E protein

INTRODUCTION

JE virus belongs to the family Flaviviridae (Westaway *et al.*, 1985) consisting of at least 68 recognized members (Calisher *et al.*, 1989), the majority of which are arthropod-

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borne, and many of them are pathogenic for man and other vertebrates (Monath, 1986). Although JE has been an endemic disease in Asia (Miles, 1960; Umenai *et al.*, 1985; Rosen, 1986), it became a medical problem also in the United States, Europe and Australia because of affected cases among travelers and military personnels (Sabin *et al.*, 1947; Ketel and Ognibene, 1971; Monath, 1988). Several reports have shown that envelope glycoprotein (E) of JE virus carries hemagglutinating activity (HA) and immunogenicity to elicit neutralizing antibodies (Shapiro *et al.*, 1971; Kitano *et al.*, 1974; Takegami *et al.*, 1982; Kimura-Kuroda and Yasui, 1983). Heterogeneity of JE-antigens in virus-infected mouse brain homogenates has been reported as slow-sedimenting hemagglutinin (Hs) and rapid-sedimenting hemagglutinin (Hr) by Igarashi *et al.*, (1963), or slow-sedimenting hemagglutinin and complement-fixing (CF) antigens besides complete and infective virion by Kitaoka and Nishimura (1965). Presence of soluble CF-antigen (SCF) with slowly sedimenting hemagglutinin (SHA) and rapidly sedimenting hamagglutinin (RHA) corresponding to the complete virion of dengue virus, another member of flavivirus, was also reported (Smith *et al.*, 1970; Cardiff *et al.*, 1970., 1971). Later studies showed that SCF is one of the virus-coded nonstructural proteins, NV3 or gp46 (Russell *et al.*, 1980; Smith and Wright, 1985), or NS1 by recent nomenclature of flavivirus proteins (Rice *et al.*, 1985). Slowly sedimenting antigen besides rapidly sedimenting antigen, corresponding to the complete virion, was detected in JE virus-infected C6/36 and BHK21 cell culture fluids and homogenates by the sandwich ELISA using antiflavivirus hyperimmune human sera as the source of catching and detecting antibodies (Bundo-Morita, 1989). Both antigens were apparently accompanied by the HA, and possibly correspond to previously reported Hr and Hs (Igarashi *et al.*, 1963), but the possibility cannot be excluded that the study has measured SCF, or NS1, besides slowly sedimenting component associated with E protein, since anti-dengue human sera were reactive to NS1 (Cardosa *et al.*, 1989).

In this communication, we describe the production of SE and RE in JE virus-infected vertebrate (BHK21) and mosquito (C6/36) cell culture fluids at 28 and 37℃ as measured by the sandwich ELISA using an anti-JE virus E protein monoclonal antibody as the source of catching and detecting antibodies. Our study indicates that the production of SE and RE depends on the host cells as well as incubation temperature after virus infection, and the possibility that the virus-infected cells are shedding SE into their culture fluids, not the break-down product of RE is discussed.

Materials and Methods

Cells: *Aedes albopictus*, clone C6/36, cells (Igarashi, 1978) were grown at 28℃ and BHK21 cells were grown at 37℃ in Roux bottles with 750 cm^3 volume and 150 cm^2 surface area. The growth medium of both cell lines were Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 0.2 mM each of nonessential amino acids (Eagle, 1959).

Virus: The origin of a wild strain of JE virus, JaOArS982 was described by Hori *et al.*, (1986), and seed virus was prepared in C6/36 cells at 28℃.

Preparation of JE virus-infected cell culture fluids and their concentration: Confluent cell monolayers were infected with seed virus (1 ml/bottle) at an input multiplicity of 1 PFU/cell. After 2 hr of virus adsorption at room temperature (RT), 40 ml/bottle of maintenance medium (cell growth medium from which serum concentration was reduced to 2%) was added and the cells were incubated either at 28 or 37℃. Infected culture fluids were differentially harvested at every 12 hr from 24 to 72 hr after post infection (p.i.), and the cultures were supplied by 40 ml/bottle of fresh maintenance medium. The harvested fluid of 40 ml volume each was clarified by low-speed centrifugation (1,500 rpm for 15 min) and the supernatant was concentrated to 1 ml by Centriprep concentrator (Amicon, MA, USA).

Sucrose gradient velocity sedimentation: One-half ml volume of original and concentrated infected culture supernatants were layered on top of the 4.4 ml linear gradients of 15−30% sucrose in STE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4) and was centrifuged at 37,000 rpm for 1 hr at 4℃ in an SW 50.1 rotor of a Beckman model L8−80M ultracentrifuge. Twelve fractions each of 0.4 ml volume was collected by an ISCO density gradient fractionator model 640, and was measured for the presence of E protein by the antigen detection sandwich ELISA.

Monoclonal antibody (MCA) against JE virus E protein: The MCA AI−300 was cross-reactive to the subgroup of flaviviruses including JE, West Nile, Murray Valley encephalitis and St. Louis encephalitis viruses as well as to dengue virus type 2, but reactive only to the E protein of JE virus in the Western blotting after SDS−PAGE both under reducing and nonreducing conditions (Srivastava *et al.*, 1988). The IgG fraction was concentrated and purified from the MCA hybridoma culture fluid by ammonium sulfate precipitation and Affi-Gel Blue column chromatography (Bruck *et al.*, 1982), and concentrated by negative-pressure dialysis to 10 mg/ml. The IgG was used as the catching antibody and to prepare horseradish peroxidase (HRPO)-conjugated detecting antibody in the sandwich ELISA. The conjugation was performed according to Wilson and Nakane (1978).

Detection of JE virus E protein by the sandwich ELISA: The procedure was slightly modified from that described by Voller *et al.*, (1976). Plastic Immulon U−microplate (Sanko Pure Chemical Co. Tokyo, Japan) was coated with the MCA-IgG at 1:100 dilution in ELISA coating buffer (0.05 M carbonate−bicarbonate buffer, pH 9.6) by distributing 100 μl/well and incubating at 4℃ overnight or at 37℃ for 1 hr. The wells of the plate were emptied and washed with PBS−T (0.05% Twen 20 and 0.01% NaN_3 in phosphate buffered saline, pH 7.2) 3 times for 3 min each. Test specimens and 2−fold serially diluted standard antigen (formalin-inactivated and purified JE vaccine concentrate was kindly supplied by The Research Institute for Microbial Diseases of Osaka University) were added to the plate (100 μl/well) and incubated at 37℃ for 1 hr. The plate was emptied, washed as above and reacted with 100 μl/well of the HRPO-conjugated MCA-IgG at 1:400 dilution in PBS−T by incubation at 37℃ for 1 hr. The plate was emptied and washed as above and HRPO-reaction was carried out with 100 μl/well of the substrate solution (0.5 mg/ml of *o*-phenylene diamine 2 HCl and 0.02% H_2O_2 in 0.05 M citrate phosphate buf fer, pH 5.0) at RT in the dark. The reaction was stopped by adding 75 μl/well of 4N

H_2SO_4 and the optical density (OD) at 490 nm of the colored product was measured by an auto ELISA analyzer (Model ETY-96, Toyosokki Co. Tokyo, Japan) using reference wavelength of 630 nm.

Hemagglutination activity determination: The procedure was carried out according to Clarke and casals (1985) using goose red blood cells.

SDS-Polyacrylamide gel electrophoresis: The slab-gel method (Studier, 1973) with discontinuous buffer system (Laemmli, 1970) was used in Mini Gel System (SE8020, Marisol, Co. Tokyo, Japan) with 10% gel (acrylamide: bisacrylamide ratio of 30:0.8). The specimens were solubilized in the sample buffer (0.0625 M Tris−HCl, pH 6.8, 2% SDS, 10% sucrose, 5% 2-mercaptoethanol, and 0.002% bromophenol blue) and boiled at 100℃ for 2 min. After electrophoresis (16 mA/gel for 1.5 hr at RT), protein bands were stained with 0.1% Coomassie Brilliant Blue R250 (CBB) in 10% acetic acid and 30% methanol by diffusion followed by destaining in 10% acetic acid and 10% methanol, or the separated proteins were transferred to nitrocellulose membrane for the Western blotting.

Western blotting: The procedure was carried out according to Towbin *et al.* (1979) and Burnette (1981) with some modifications. The transfer was performed in the buffer containing 0.02 M Tris-HCl, 0.15 M glycine, 0.1% SDS, and 20% methanol, pH 7.4, at 4℃ for 18 hr at 6−8 V/cm in an ETB-15 apparatus (Tohyoh Kagaku Sangyoh, Tokyo, Japan). After transfer, the nitrocellulose membrane was washed for 5 min in TBS buffer (0.02 M Tris-HCl and 0.15 M NaCl, pH 7.4). Effectiveness of the transfer and positions of protein bands on the membrane were tentatively visualized by staining with 0.2% Ponceau S in 2% trichloroacetic acid for 10 min and destained by extensive washing with distilled water (dw) and rinsed with TBS for 10 min. The membrane was inactivated by incubating with 4% bovine serum albumin in TBS or commercial Block Ace (Yukijirushi Nyugyo, Osaka, Japan) for 12−18 hr at 4℃ or 3 hr at RT followed by rinsing with TBS 3 times for 2 min each. The membrane was reacted with anti-JE polyclonal mouse serum at 1:500 dilution in phosphate buffered saline (PBS) containing 0.01% NaN_3 for 1 hr at 37℃. The membrane was washed with TBS as above and reacted with HRPO-conjugated anti-mouse IgG (DAKO Immunoglobulins, Denmark) at 1:500 dilution in PBS. Antigenically active protein bands were visualized by incubation with 0.6 mg/ml of HRPO color development reagent (4-chloro-1-naphthol, Bio-Rad, CA, USA) and 0.03% H_2O_2 in TBS at RT for 10 min. Anti-JE mouse serum was prepared by repeated intraperitoneal inoculation of purified JE virus (Srivastava *et al.*, 1987).

Electron microscopy (EM): The specimen was placed on a sheet of Parafilm (American Can Co. CT, USA), and a Formvar-coated copper grid was soaked in it for 1 min. The grid was washed by dw 3 times for 15 sec each, and the specimen was negatively stained with 4% uranyl acetate for 30 sec and was examined with a JOEL Electron microscope type JEM 100CX at direct magnification of 72,000 with an acceleration voltage of 80 kV.

RESULTS

Production of SE and RE of JE virus in infected C6/36 and BHK21 cell culture fluids at 28 and 37℃: Fig. 1 shows the sucrose gradient sedimentation profile of JE virus E protein detected by the sandwich ELISA for concentrated culture fluids from infected C6/36 and BHK21 cells incubated at 28 or 37℃ and differentially

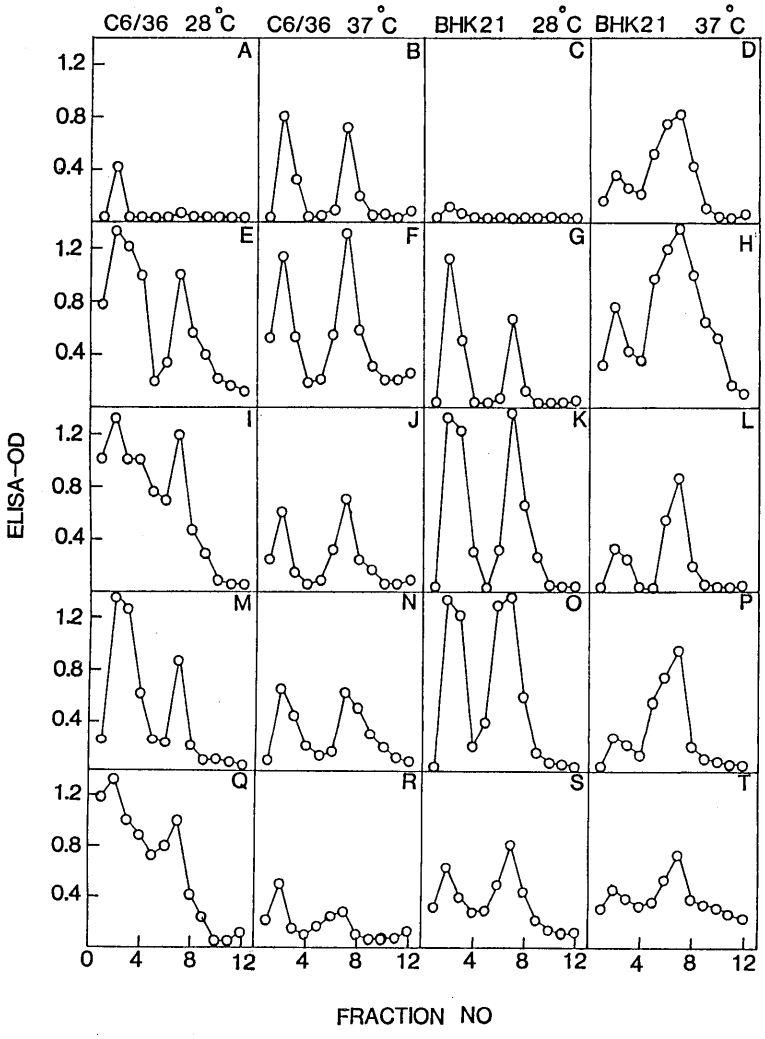

Fig. 1. Density gradient sedimentation profile of components associated with JE virus E protein in the infected and concentrated C6/36 and BHK21 cell culture fluids. Infected culture fluids were differentially collected every 12 hr after 24 hr (A to D), 36 hr (E to H), 48 hr (I to L), 60hr (M to P), and 72 hr (Q to T) of p. i. from infected C6/36 (A, B, E, F, I, J, M, N, Q, R) and BHK21 (C, D, G, H, K, L, O, P, S, T) cells, incubated at 28℃ (A, C, E, G, I, K, M, O, Q, S) or at 37℃ (B, D, F, H, J, L, N, P, R, T). After clarification and concentration, samples were fractionated by sucrose gradient sedimentation to measure JE virus E protein by the sandwich ELISA.

harvested at every 12 hr from 24 to 72 hr of p.i. Two peaks of ELISA-OD (SE and RE) were identified for almost all specimens, except those harvested at 24 hr of p.i. from both cell lines incubated at 28℃. In these specimens only a small peak of SE was detected. No significant ELISA-OD peak was detected for the specimens before Amicon concentration (data not shown). The ELISA-OD for the peaks of SE and RE of each specimen in Fig. 1 was plotted against the time of harvest as shown in Fig. 2. The amount of SE was higher when the infected cells were incubated at 28 than at 37℃ for both cell lines, except the first 24 hr of p.i. While, the amount of RE was higher when the infected cells were incubated at 37 than at 28℃ until 36 hr of p.i. After this time, production of both SE and RE declined at 37℃, but their production at 28℃ continued until 72 hr in C6/36 and 60 hr in BHK−21 cells, respectively. In C6/36 cells, the amount of SE was always higher than that of RE at 28℃, while their amounts were similar at 37℃. In BHK−21 cells, on the other hand, the peak of RE was always higher than that of SE at 37℃, and their amounts were almost similar at 28℃.

In order to see whether SE and RE are independent components or SE was obtained as degradation product of RE during the sucrose gradient sedimentation, recentrifugation experiment was performed. The specimen collected at 36 hr of p.i. from C6/36 cells incubated at 37℃ was fractionated by the sucrose gradient sedimentation, and peak fractions of SE and RE were diluted 2−fold with STE and recentrifuged under the same condition as in the original specimen. The result in Fig. 3 shows that SE and RE distributed in

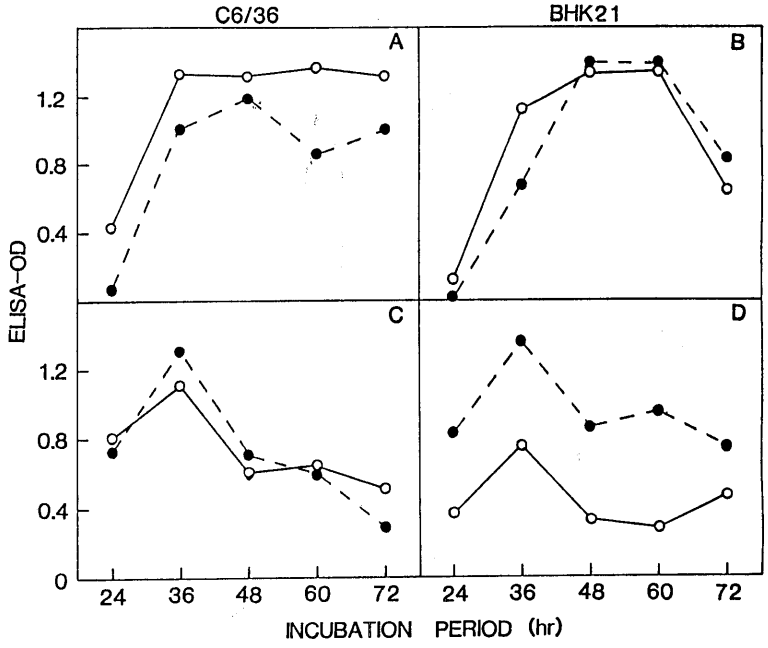

Fig. 2. Time course of SE and RE production into JE virus infected C6/36 and BHK21 cells. ELISA−OD of peak fractions of SE (○——○) and RE (●·····●) in Fig. 1 was plotted against the time of harvesting of infected culture fluid. Host cells were C6/36 (A, C) or BHK21 (B, D), incubated at 28℃ (A, B) or at 37℃ (C, D).

each corresponding position of the original specimen, indicating that they were independent components and SE was not produced by degradation of RE during sucrose gradient sedimentation.

Hemagglutination activity of SE and RE: In roder to see whether SE and RE were associated with HA, the specimens harvested at 36 hr of p.i. from C6/36 and BHK21 cells incubated at 37℃ were fractionated as above, ELISA and HA of each fraction was measured. The result in Fig. 4 shows that both SE and RE were accompanied by the peak of HA, but the HA of SE was relatively lower than that of RE when their HA titers were compared with their ELISA color development. No significant difference was observed in the optimal pH of HA for both SE and RE (pH 6.6).

SDS-PAGE and Western blotting of SE and RE: Since SE and RE were independent components and HA of SE was relatively lower than that of RE compared with their ELISA, we tried to see whether Mw of E protein in SE and RE may show some differences by SDS-PAGE and Western blotting. The CBB-staining of SE after SDS-PAGE showed dense band corresponding to albumin with a faint band at the Mw of 54K corresponding to E protein, together with many other bands. The CBB-stained RE fraction also showed a band corresponding to albumin with faint band at the position of E protein and several accessory bands (data not shown). Since CBB-staining could not clearly identify E protein in both SE and RE fractions, Western blotting was performed and the result was shown in Fig. 5. A single band was visualized at the position of E protein of

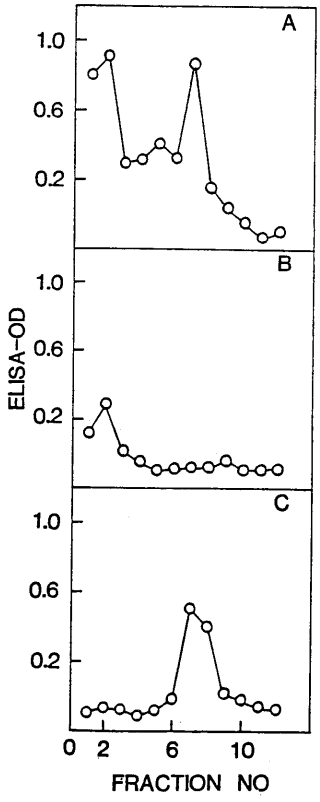

Fig. 3. Recentrifugation of SE and RE in sucrose gradient sedimentation. Clarified and concentrated infected culture fluid harvested from C6/36 cells incubated at 37℃ at 36 hr of p. i. was fractionated by sucrose gradient sedimentation (A), and peak fractions of ELISA−OD corresponding to SE (B) and RE (C) were recentrifuged under the same condition as in A, to assay JE virus E protein by the sandwich ELISA.

concentrated and purified JE virus in both SE and RE specimens as well as their mixture.

Morphological observation of SE and RE fractions by EM: The SE and RE fractions from both C6/36 and BHK21 cells incubated at 37℃ were observed under the EM as described in the Materials and Methods. The negatively stained SE fractions from both cell lines showed various particles of 5−10 nm in diameter, apparently their aggregates, and some membranous structures (Fig. 6A and B). In RE fractions, on the other hand, spherical enveloped particles of 45−55 nm in diameter corresponding to the mature virion were observed together with 5−10 nm components (Fig. 6C and D).

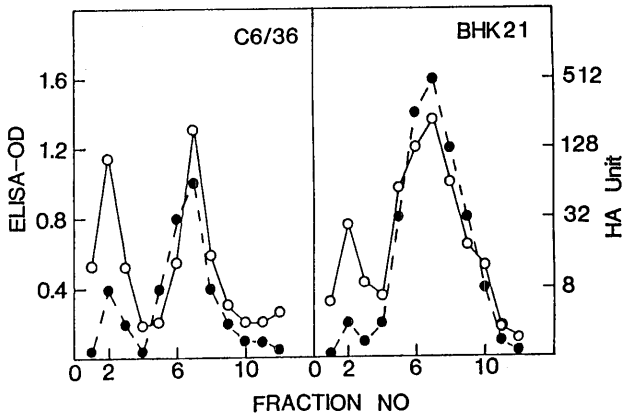

Fig. 4. Sucrose density gradient sedimentation of ELISA and HA in JE virus-infected C6/36 and BHK21 cell culture fluid. Infected culture fluids were collected 36 hr of p. i. from JE virus-infected C6/36 or BHK21 cells incubated at 37℃. After clarification and concentration, the specimens were fractionated by sucrose gradient sedimentation to measure ELISA (○——○) and HA (●·····●) in each fraction.

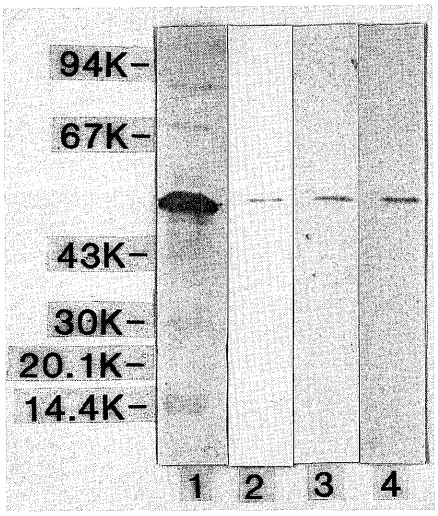

Fig. 5. Western blot profile of SE and RE fractions after SDS−PAGE. Lane 1 concentrated and purified JE virion as positive control, lane 2 SE, lane 3 RE, and lane 4 mixture of SE and RE. Infected culture fluid was collected at 36 hr of p. i. from C6/36 cells incubated at 37℃ to prepare the specimens for this experiment. The numbers on the left show molecular weights of standard marker proteins.

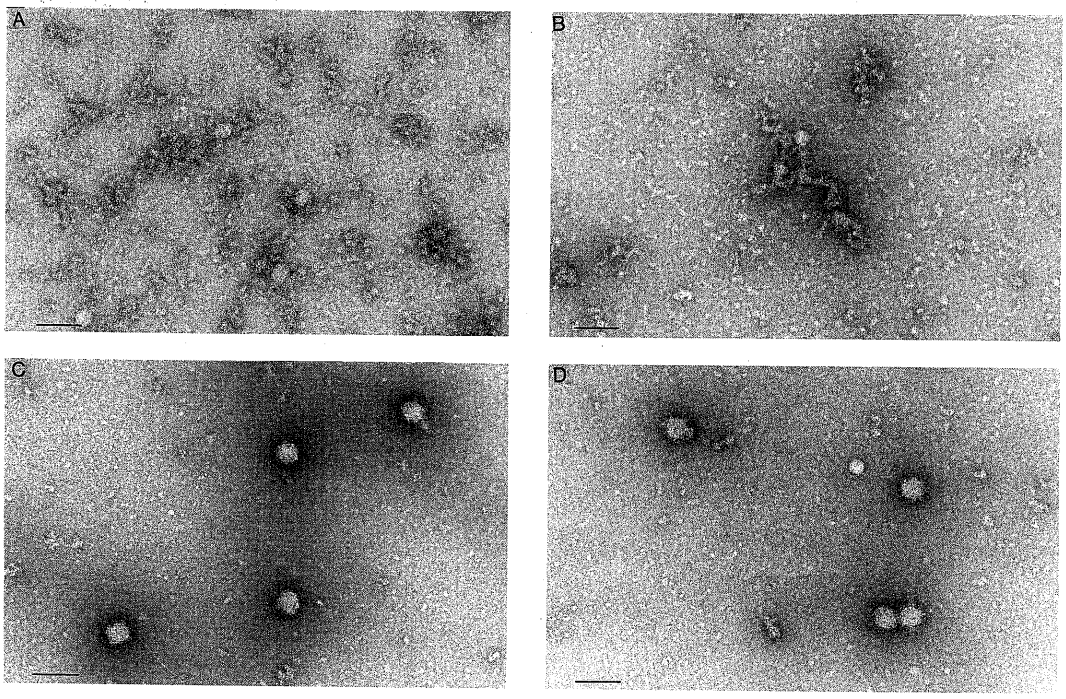

Fig. 6. Negatively stained EM of SE and RE fractions from C6/36 and BHK21 cell lines. Specimens of SE (A, B) and RE (C, D) were prepared by sucrose density gradient sedimentation of clarified and concentrated culture fluid from JE virus-infected C6/36 (A, C) and BHK21 (B, D) cells incubated at 37℃ and collected at 36 hr of p. i. Bar markers represent 100 nm.

DISCUSSION

Production of SE as a separate component from RE into JE virus-infected C6/36 and BHK21 cell culture fluids was shown in this study. The amounts of SE and RE were dependent on the host cells as well as the incubation temperature after virus infection, and C6/36 cells at 28℃ was most suitable for the production of SE for prolonged period of time. While, production of RE was better at 37 than at 28℃ until 36 hr of p.i. especially in BHK21 cells, but the production was shut-off after 36 hr of infection at 37℃ for both cell lines. This result is compatible with our previous report showing the shut-off of infective virus production at 37℃ with this particular strain of JE virus, JaOArS982 (Shameem *et al.*, 1988). The level of virus produced in suspension culture of C6/36 cells was different among several flaviviruses examined, and the phenomenon was referred to some host cell factor(s) regulating the virus production in this cell line (Igarashi and Srivastava, 1988).

The observation of a single band reactive to anti-JE mouse serum with apparently same Mw in RE and SE fractions indicated that relatively low HA of SE compared with its ELISA, in contrast to RE, connot be explained by the different molecular species of E

protein in these RE and SE fractions. Therefore, the phenomenon may be related with the different sizes of components carrying E proteins or different configuration of E protein in these 2 fractions. RE fraction apparently contained complete virion together with some smaller-size components, while SE fraction consisted of various components but their sizes were generally smaller than complete virion. It would be necessary to see the immunogenicity of RE and SE in terms of their eliciting HA-inhibiting, ELISA and neutralizing antibodies. Kitano *et al.,* (1974) reported amorphous substance, probably composed of viral lipid envelope and a part of hemagglutinin, at the top of CsCl density gradient centrifugation of JE virus. Srivastava and Igarashi (1987) isolated the E protein fraction associated with HA from purified JE virus by Triton X−100 treatment. It is still an open question that these components obtained from purified virion have any relationships with some particulate or membranous structures observed in SE fraction by EM.

Shedding of soluble macromolecules from the cell surface is a common phenomenon of viable cells including malignant, non-malignant and virus-infected cells (Black, 1980; Bolognesi *et al.,* 1975; Kang and Prevec, 1971). The mechanism of this secretion of cellular products from the cell surface into the extracellular medium is different from exocytosis (Black, 1980). Several reports described the synthesis and secretion of soluble macromolecules from virus-infected cell surface (Smith *et al.,* 1970; Kang and Prevec, 1971; Chatis and Morrison, 1983). The possibility remains that the SE described in this study is a kind of viral macromolecule shed from JE virus-infected cell surface and may be related to or is independent from the formation of complete virion.

Acknowledgments

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