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Detection of Japanese Encephalitis Virus Antigens by the Sandwich ELISA in Infected Cell Culture Fluid and Cell Homogenates

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Abstract: Sandwich ELISA was applied to detect Japanese encephalitis (JE) virus antigens in culture fluid and homogenate of *Aedes albopictus*, clone C6/36, and BHK21 cells daily after infection with various concentrations of JE virus. In both cell systems, the ELISA antigen in the infected fluid became detectable when infective virus titer rose up to 10^7 PFU/ml, and the ELISA titer remained at similar levels after reaching its plateau. Growth curve experiment of JE virus in both cell systems at high input multiplicity of infection showed that the titers of virus infectivity, hemagglutinating activity (HA), and ELISA antigen rose up almost in parallel in the infected BHK21 cell culture fluid and homogenate as well as in the infected C6/36 cell culture fluid. In the infected C6/36 cell homogenate, however, the HA titer remained at undetectable level while infectivity and ELISA titers rose up. Density gradient sedimentation analysis on the infected BHK21 and C6/36 cell culture fluids and homogenates showed that larger amounts of the virus antigen were detected in the slowly sedimenting light fraction (density 1.12 g/cc in potassium tartrate), with less amounts in the rapidly sedimenting heavy fraction associated with virus infectivity (densty 1.16 g/cc). The detection of JE virus ELISA antigen was applied to the culture fluid of C6/36 cells inoculated with field–caugth *Culex tritaeniorhynchus* mosquito homogenates and maintained at 28℃. The JE–ELISA antigen became detectable between 2 to 5 days after the inoculation for all the 9 pools which eventually turned out to be positive with JE virus isolation by the established method, while all the 11 pools which turned out to be negative with JE viurus isolation did not produce detectable levels of JE–ELISA antigen except a single specimen showing transient and borderline level (1 unit) of the reaction. A total of 256 culture fluids from C6/36 cell cultures inoculated with field–caught *Cx. tritaeniorhynchus* between 1978 to

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1981 were assayed for their JE−ELISA antigen titers. All the 110 fluids, which were positive with JE virus isolation, possessed detectable levels (more than 4 units except a single specimen with 2 units) of JE−ELISA antigen, in contrast to 146 fluids, which were negative with JE virus isolation, with less than 2 units of JE−ELISA antigen titers. By applying the sandwich ELISA, the procedure for screening JE virus isolation by *Aedes albopictus* clone C6/36 cells could be reduced to 5 days in contrast to the previous procedure requiring total 10 days of incubation.

Key words: Japanese encephalitis virus, Antigen detection, ELISA

INTRODUCTION

Japanese encephalitis (JE) is a typical acute viral encephalitis with high mortality and grave sequelae and has been a target with high priority among public health problems in many countries of East, Southeast to South Asia (Umenai *et al.*, 1985). Epidemiological studies on the spread of JE virus in nature has been performed by antibody survey on swine population as well as isolation of JE virus from field−caught mosquitoes. The virus isolation procedure had routinely been performed by inoculating test specimens to the brains of suckling mice, however, the procedure became simpler and economical by using a clone, C6/36, of *Aedes albopictus* cells (Igarashi, 1978; Igarashi *et al.*, 1981a; b; c; d; 1982). The established method, however, requires total 10 days of incubation after inoculation of test specimens before finding positive isolates. Since enzyme−linked immunosorbent assay (ELISA) was introduced to serology with various advantages (Engvall and Perlman, 1971), the method has been used to detect animal (Voller *et al.*, 1976a; Wolters *et al.*, 1976; Yolken *et al.*, 1977; Miranda *et al.*, 1977), plant (Clarke *et al.*, 1976; Clark and Adams, 1977; Lister, 1978), insect (Kelly *et al.*, 1978; Longworth and Carey, 1980; Crook and Payne, 1980), or fish (Dixon and Hill, 1983) viruses. We tried to apply the sandwich ELISA method to detect JE virus antigen in culture fluid of C6/36 cells infected with the virus, hoping that the procedure will reduce the incubation time necessary for the screening of JE virus isolates from field specimens. Studies to analyze the ELISA antigen produced in C6/36 and BHK21 cells were also performed to characterize JE−ELISA antigens.

MATERIALS AND METHODS

Cells: *Ae. albopictus*, clone C6/36, cells were grown at 28℃ with cell growth medium, consisting of Eagle's minimal essential medium (Eagle, 1959) supplemented with 10% heat−inactivated fetal calf serum and 0.2mM each of nonessential amino acids (Igarashi, 1978). BHK21 cells were grown at 37℃ by the same medium.

Virus and assay of its biological activity: The Nakayama strain of JE virus was used throughout. The virus was grown at 28℃ in C6/36 cells to make seed stocks. Infectivity

of the virus was assayed by plaque titration on BHK21 cells (Hashimoto *et al.*, 1971) modified to use 24 multiwell plates, and the titers were shown by plaque-forming units (PFU) per ml. Hemagglutinating activity (HA) of the virus was assayed according to the method of Clarke and Casals (1958) using goose red blood cells.

Virus growth experiment: Replicate cultures of C6/36 or BHK21 cells were prepared in 2-ounce rubber-stoppered bottles. When the cells became semiconfluent, growth medium was removed and seed virus at various dilutions was inoculated using 0.2ml/bottle. Virus adsorption was performed for 2 hours at 28℃ for C6/36 cells and at 37℃ for BHK21 cells spreading the virus every 30 minutes. At the end of adsorption, inoculum virus was removed and the cell sheets were washed twice with PBS. The cells were covered by the maintenance medium (cell growth medium from which serum concentration was reduced to 2%), and incubated at 28℃ for C6/36, and at 37℃ for BHK21 cells, respectively. Every day after virus infection, duplicate bottles were harvested from each series. Infected culture fluid was saved for the virus assay, and the cells were scraped into PBS, homogenized and also saved for virus assay.

Preparation of anti-flavivirus IgG: The procedure was as described before (Bundo and Igarashi, 1985). Sera from dengue hemorrhagic fever patients with broadly reactive high antibody titers were obtained from Virus Research Institute, Bangkok, Thailand. Several sera were pooled, dialyzed against 0.005M phosphate buffer, pH8.0, and then applied on DEAE Sephacel column (16×160mm) equilibrated with the same buffer. IgG-fractions which were not retained by the column were pooled as anti-flavivirus IgG, and concentrated by negative pressure dialysis. A part of the concentrated IgG was used as the catching antibody, and the rest was conjugated with horseradish peroxidase (Sigma, Type IV) by Wilson and Nakane's method (1976) to prepare detecting antibody.

Sandwich ELISA procedure: The method was as described by Voller *et al.* (1976b). U-shaped 96-well plastic plates (Dynatech, USA) were coated with catching antibody diluted in coating buffer using 100 μl/well, and incubated at 37℃ for 1 hour. The plates were washed with PBS-Tween and reacted with test specimens (100 μl/well). Standard JE virus antigen serially diluted by 2-fold steps in PBS-Tween, was also run in parallel. The plates were incubated at 37℃ for 1 hour, washed with PBS-Tween and then reacted with 100 μl/well of the detecting antibody (peroxidase-conjugated anti-flavivirus IgG) appropriately diluted in PBS-Tween. The plates were incubated at 37℃ for 1 hour, washed with PBS-Tween, and then peroxidase reaction was performed by adding 100 μl/well of substrate solution (0.5mg/ml of *o*-phenylene diamine and 0.02% H_2O_2. The plates were incubated at room temparature for 1 hour in the dark, and the reaction was stopped by adding 75 μl/well of 4 N H_2SO_4. Optical density of the colored product was measured by the Micro ELISA Autoreader (Dynatech, USA) at 490 nm with reference wavelength of 630nm. The antigen titers of test specimens were estimated by comparing their color density with those developed by serially diluted standard antigen using a computer system similar for the antibody assay (Morita *et al.*, 1982).

Density gradient centrifugation: Velocity sedimentation was performed in 15-30%

sucrose gradient made in STE buffer (0.1M NaCl, 0.01M Tris−HCl, 0.001M EDTA, pH 7.6) and 0.5ml volume of the specimen was centrifuged in an SW 50.1 rotor at 37,000 rpm for 60min at 4℃. Fractions of 0.4ml volume were collected by ISCO density gradient fractionator model 640. Fractions were assayed for virus infectivity, HA and ELISA antigen titers as described above. Some fractions from sucrose gradient sedimentation were applied on 15−30% potassium tartrate density gradient and centrifuged at 40,000 rpm for 3 hours in an SW 50.1 rotor and fractionated as above to measure ELISA antigen titers. Density in several fractions from potassium tartrate gradient was measured by refractometry using an empirical formula of $\rho=5.4250\ n\ -\ 6.2760$, where n is the refractive index and ρ is the density.

Virus isolation from field−caught Culex tritaeniorhynchus mosquitoes and production of JE− ELISA antigen in the infected C6/36 cell culture fluid: The procedure was described before (Igarashi *et al.*, 1981a; b; c; d; 1982). Mosquitoes were collected at a pig pen in Aino Town, Nagasaki Prefecture, 1982, by the staffs of Nagasaki Prefectural Institute of Environmental Sciences and Public Health using light traps. Female *Cx. tritaeniorhynchus* were pooled so that the number of mosquitoes in each pool does not exceed 100. Each pool was homogenized with 2ml of PBS containing 0.2% bovine plasma albumin and was centrifuged. The resulting supernatant was inoculated into brains of suckling mice using 0.02ml/brain and a litter mice for a pool. The remaining supernatant was filtrated through 0.22 μm filter and 0.1ml of the filtrate was inoculated to a tube culture of C6/36 cells. After 1 hour's adsorption at room temperature, the cells were covered by the maintenance medium and incubated at 28℃ for 7 days. Every day after the specimen inoculation, a part of the maintenance medium was harvested from each tube and assayed for JE−ELISA antigen as described above. After 7 days of incubation, presence of infective virus in the medium was screened by inoculating to C6/36 cells grown on 8−chamber slide (Miles, I 11, USA), which was harvested 3 days later in order to reveal intracellular JE virus antigen by the immunoperoxidase method (Okuno *et al.*, 1977).

Retrospective study on the culture fluid from C6/36 cells inoculated with field−caught Cx. tritaeniorhynchus homogenates: The infected fluids harvested 7 days after specimen inoculation during virus isolation studies from 1978 to 1981 have been kept at −70℃. The JE−ELISA antigen titer in a total of 256 fluids were examined as described above and compared with the results of JE virus isolation (110 positives and 146 negatives).

RESULTS

Production of infective JE virus and virus antigen in infected BHK21 and C6/36 cells

Fig. 1 shows production of infective JE virus and JE−ELISA antigen in culture fluid of BHK21 cells inoculated with serially diluted virus in 10−fold steps and incubated at 37℃. JE−ELISA antigen became detectable when infective virus titer rose up around 10^7 PFU/ml. Then the infectivity rose up to the maximum titer 1−2 days later, followed

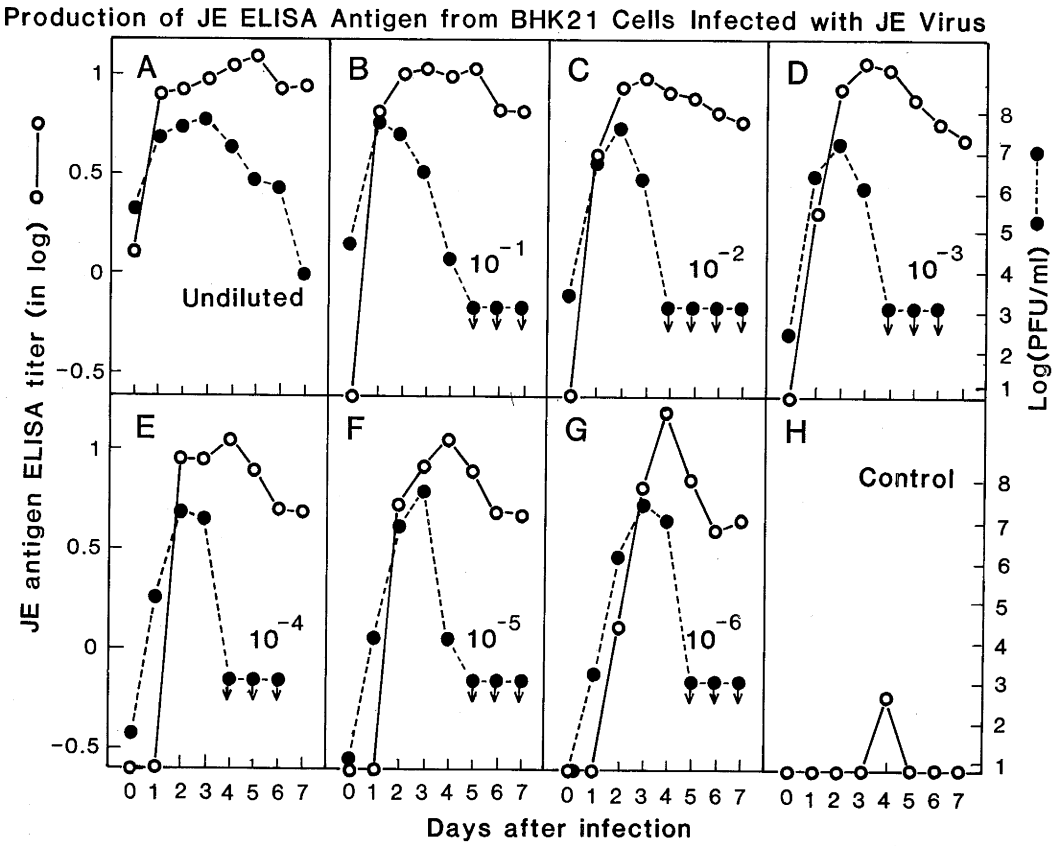

Fig.1. Production of JE virus infectivity and ELISA antigen from BHK21 cells infected with various concentrations of the virus. Replicate cultures of BHK21 cells were inoculated with JE virus without dilution (A), or serially diluted in 10−fold steps up to the 10^{-6} (B−G), and incubated at 37℃. Virus infectivity (●---●) and ELISA antigen titer (○——○) in the culture fluid of infected as well as mock−infected (H) cells were assayed daily.

by rapid decrease to undetectable levels within a few days, probably due to the heat−inactivation. On the other hand, JE−ELISA antigen titer continued to increase or remained at plateau levels even when the infectivity was going down, although some titer decrease was observed. In cells infected with minimal amount of the virus (10^{-6} dilution), JE ELISA antigen definitely became detectable 3 days after infection.

Fig. 2 shows similar experiment as above but performed with C6/36 cells incubated at 28℃. Also, JE−ELISA antigen became detectable when infectivity reached around 10^7 PFU/ml. Quite different from the result with BHK21 cells was the level of infective virus after reaching its plateau, remaining at high titer even for several days. The result is partly due to the lower incubation temperature of 28℃ for C6/36 compared with 37℃ for BHK21 cells. However, another reason may be continuous production of the virus from infected C6/36 cells for several days of duration. In cells infected with the minimal amount of the virus (10^{-6} dilution), JE ELISA antigen became detectable after 5 days of incubation.

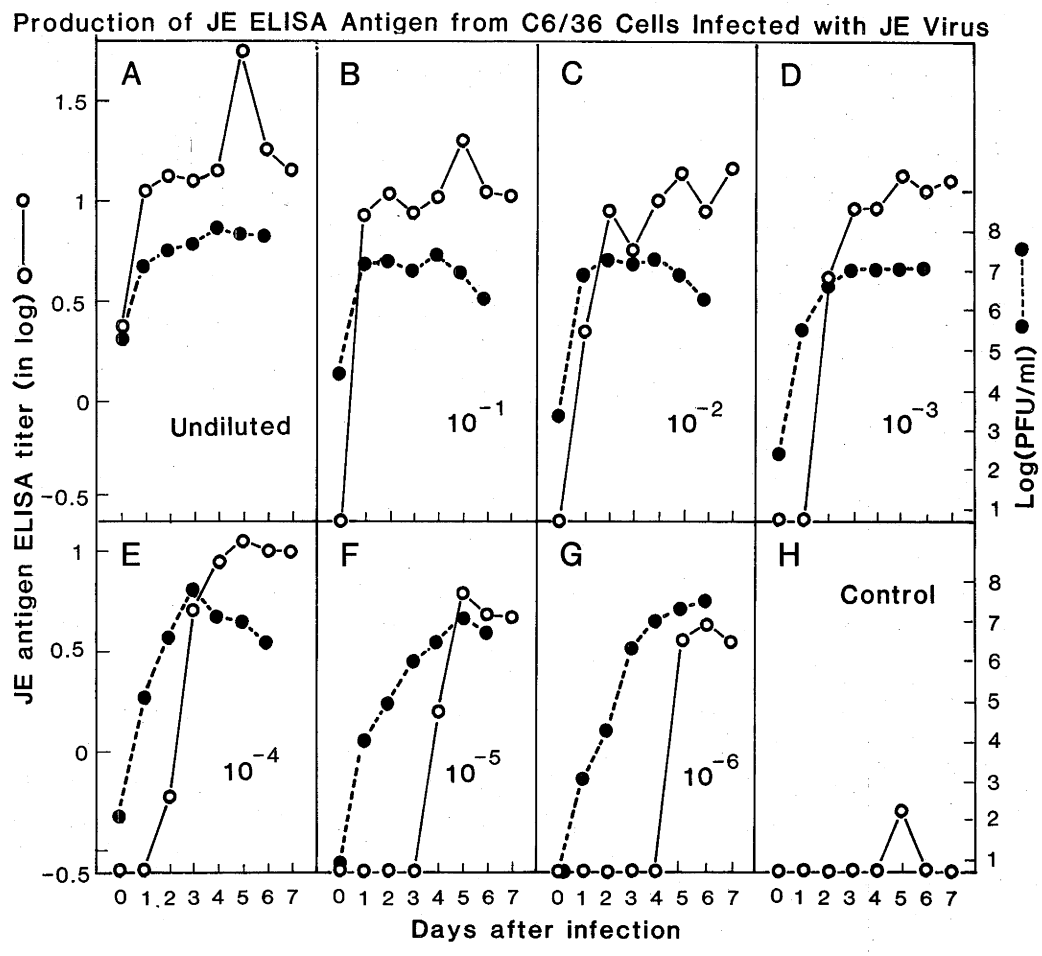

Fig.2. Production of JE virus infectivity and ELISA antigen from C6/36 cells infected with various concentrations of the virus. Replicate cultures of C6/36 cells were inoculated with seed virus without dilution (A), or serially diluted in 10−fold steps up to the 10^{-6} (B−G) and incubated at 28℃. Virus infectivity (●---●) and ELISA antigen titer (○———○) in the culture fluid of infected as well as mock−infected (H) cells were assayed daily.

In order to investigate more accurate time−course of JE virus and virus antigen production, host cells were inoculated with high multiplicity of infection (MOI) around 10 PFU/cell, and BHK21 cells were harvested every 4 hours after incubation at 37℃, and C6/36 cells every 6 hours at 28℃, respectively. The result in Fig. 3 shows that in infected BHK21 cells, virus infectivity, HA, and ELISA titers in the fluid began to increase after 10 hours of infection (Panel A), while HA titer in the cell homogenate rose up after 10 hours of infection, although infectivity and ELISA titer increased from 6 hours (Panel B). In C6/36 cells culture fluid, infective virus and ELISA titer began to increase from 8 hours of infection, while HA increased from 14 hours (Panel C). On the other hand, virus infectivity in C6/36 cell homogenate appeared to increase earlier than 8 hours, while ELISA titer increased from 14 hours, with no HA observed throughout the course of the

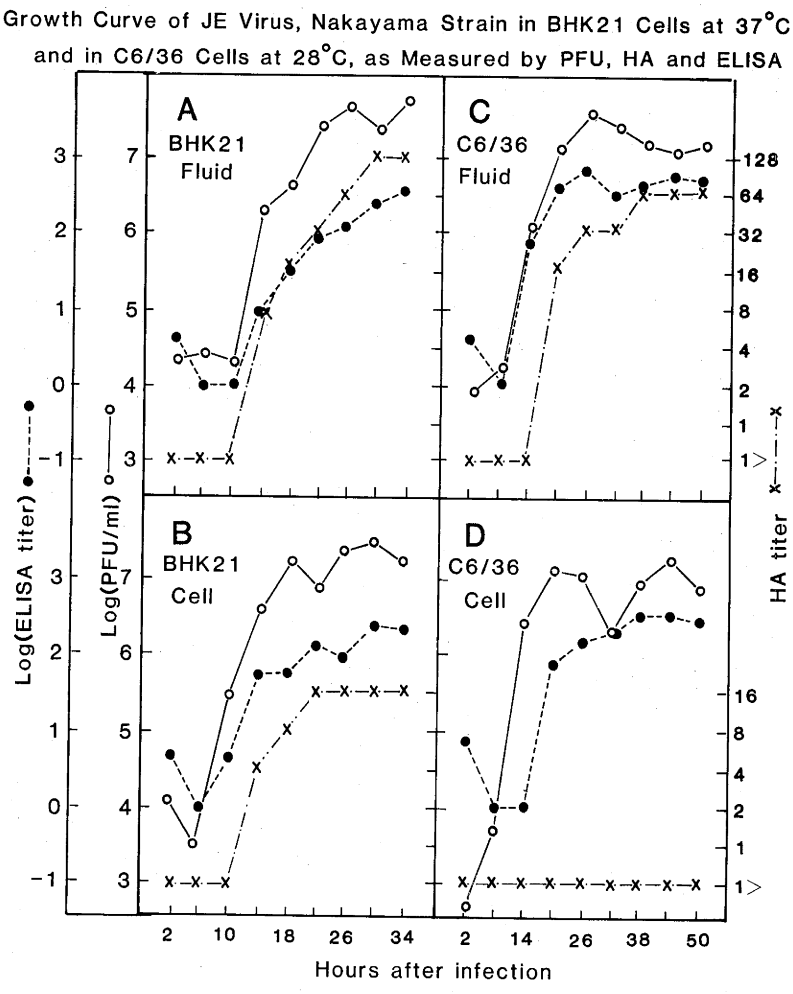

Fig.3. Growth curve of JE virus in BHK21 cells at 37℃ and in C6/36 cells at 28℃. Replicate cultures of BHK21 and C6/36 cells were inoculated with undiluted seed virus. After adsorption, virus inoculum were removed and the cells were covered by the maintenance medium and incubated at 37℃ for BHK21 (A, B) or at 28℃ for C6/36 (C, D) cells, respectively. At various times after the infection, duplicate cultures were harvested from each series and virus infectivity (○———○), HA (×—•—•—×), as well as ELISA titers (●---●) were measured for infected fluid (A, C) and cell homogenate (B, D).

study period (Panel D).

Fractionation of infective JE virus and virus antigens by density gradient sedimentation

Since time course of virus production indicated certain dissociation between infective virus and virus antigen production, specimens harvested after virus growth reached its plateau level were analyzed by sucrose gradient velocity sedimentation. The results in Fig. 4 shows that there are 2 JE antigens with different sedimentation rate, and the bottom component was associated with infective virus with HA and ELISA, while the top component was noninfectious antigen with only HA and ELISA. These 2 components were most

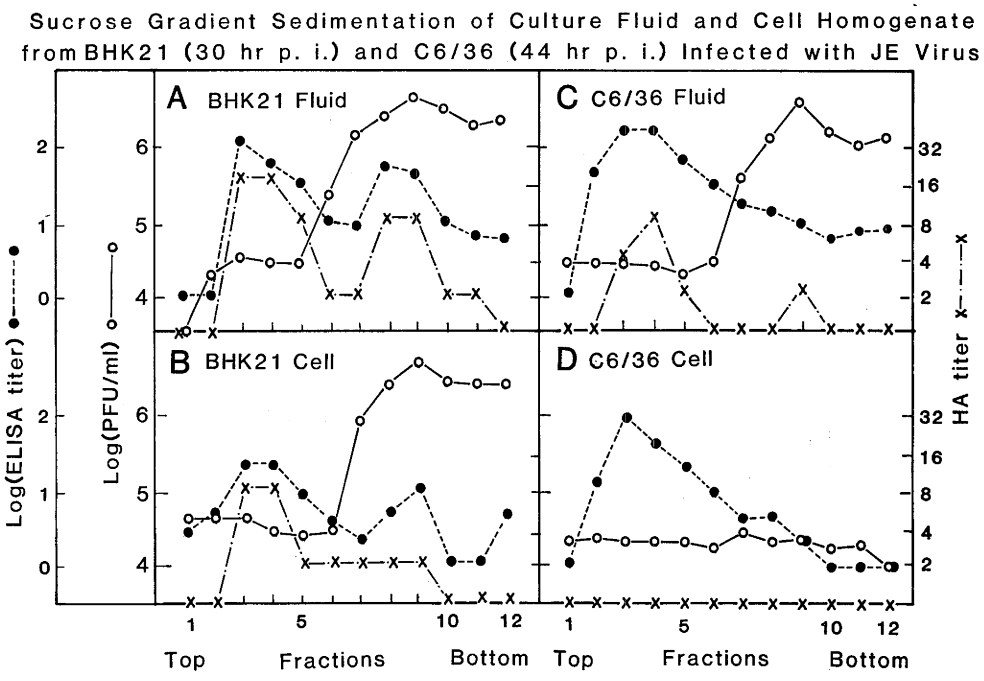

Fig.4. Sucrose gradient velocity sedimentation of culture fluid and cell homogenate from BHK21 and C6/36 cells infected with JE virus. The infected culture fluids (A, C) and cell homogenates (B, D) harvested from BHK21 cells at 30 hours (A, B) and from C6/36 cells at 44 hours (C, D) after infection with JE virus in the experiment in Fig.3 were analyzed by 15−30% sucrose gradient sedimentation. Each fraction was measured for virus infectivity (○———○), HA (×−•−•−×), as well as ELISA titers (●---●).

clearly observed in infected BKH21 cell culture fluid harvested at 30 hours of infection (Panel A), while the HA and ELISA for the bottom component in the infected BHK21 cell homogenate were less marked (Panel B). In the infected C6/36 cell culture fluid harvested at 44 hours of infection, distinction between the top and the bottom components was not clear, perhaps because of the 3rd component in between showing ELISA but no HA (Panel C). In the infected C6/36 cell homogenate, presence of the bottom component was hardly detectable, because of the absence of infectivity and HA peaks, in spite of the prominent top component associated only with ELISA titer. Since infected C6/36 cell homogenate itself possessed virus infectivity (Fig. 3, Panel D), absence of infectivity throughout the gradient (Fig. 4, Panel D) could probably be explained by the association of infective particles with larger membrane fragments which might have sedimented to the bottom of the gradient.

Because JE virus antigens apparently show physical heterogeneity, infected culture fluid of BHK21 cells harvested at 30 hours of infection as well as the top and the bottom components fractionated by the sucrose gradient velocity sedimentation (Fig. 4, Panel A) were subjected to potassium tartrate density gradient sedimentation in order to determine the density of these components. The result in Fig. 5 showed that the infected fluid itself

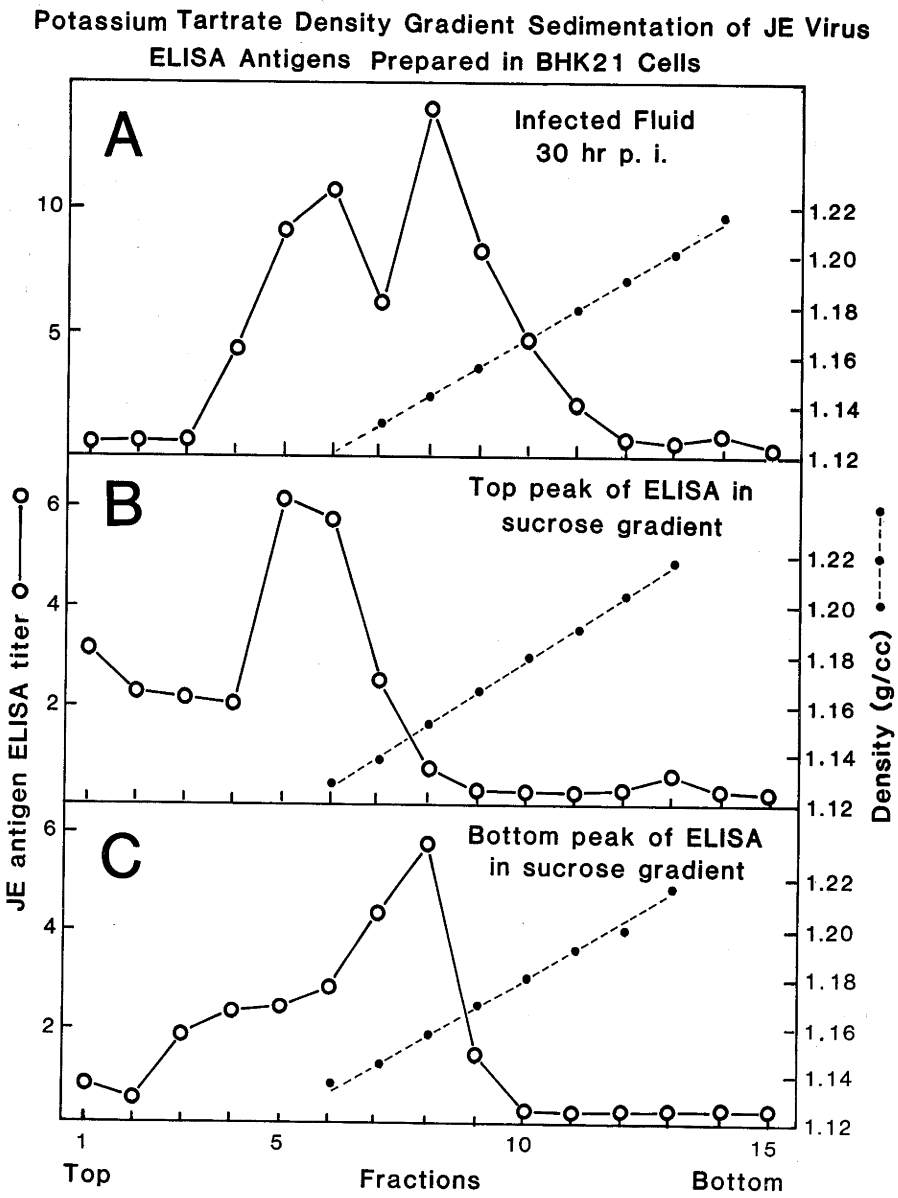

Fig.5. Potassium tartrate density gradient sedimentation of JE virus ELISA antigens. JE virus−infected BHK21 cell culture fluid (A), slowly sedimenting ELISA antigen (B), and rapidly sedimenting ELISA antigen (C) obtained in the experiment in FIg.4A were analyzed by the 15−30% potassium tartrate density gradient sedimentation to determine their densities.

has 2 peaks of ELISA titers at light (1.12 g/cc) and heavy (1.16 g/cc) density (Panel A), while the top cpmponent in the sucrose gradient distributed its ELISA titer at light density (Panel B), and the bottom component at heavy density (Panel C), respectively.

In order to see whether the slowly sedimenting light component and the rapidly

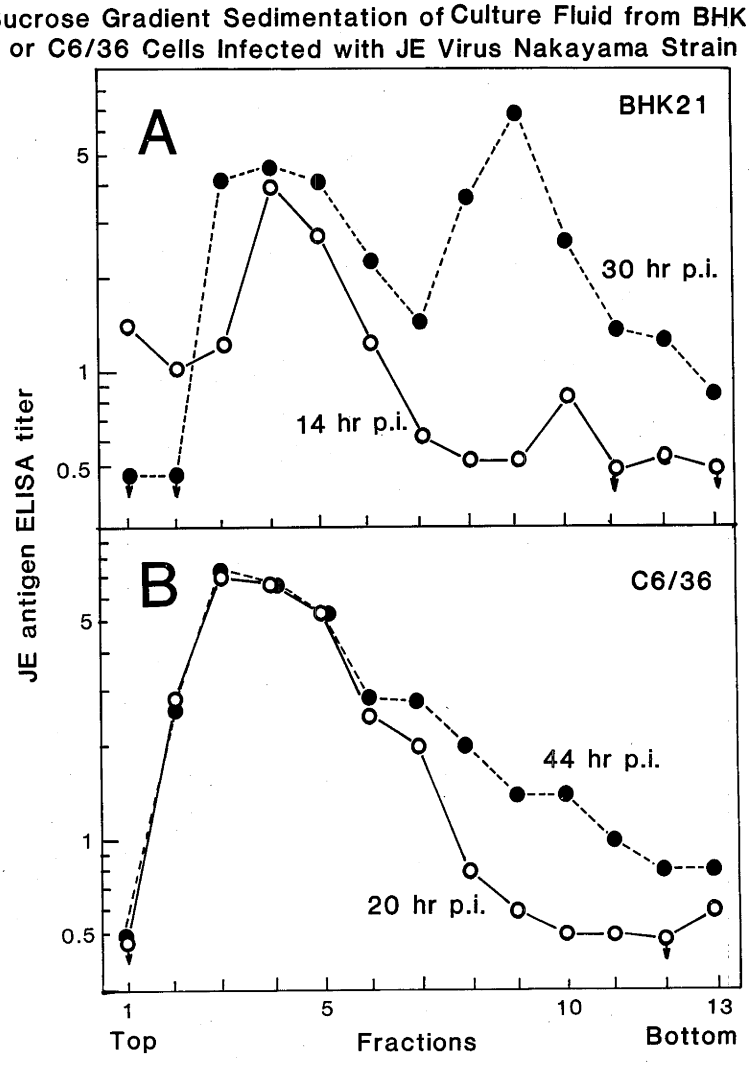

Fig.6. Sucrose gradient velocity sedimentation of culture fluids from BHK21 and C6/36 cells harvested early or late stage of infection with JE virus. Culture fluids harvested from BHK21 cells (A) at 14 hours (○———○) and 30 hours (●---●), and those from C6/36 cells (B) at 20 hours (○———○) and 44 hours (●---●) after infection with JE virus were analysed by 15−30% sucrose gradient sedimentation and each fraction was assayed for JE−ELISA antigen titer.

sedimenting heavy component were produced in parallel during the course of virus growth, infected culture fluid harvested from BHK21 cells at 14 hours and 30 hours of infection as well as culture fluid from C6/36 cells at 20 and 44 hours of infection were analyzed by sucrose gradient velocity sedimentation and fractions were assayed for their ELISA titers. The results in Fig.6 show that the bottom component was less marked for the specimens harvested at earlier phase of virus growth than the specimens harvested after virus growth became plateau levels. This tendency was more pronounced for BHK21 cells (Panel A) than for C6/36 cells (Panel B), in which the bottom componet was less marked.

Application of the ELISA to detect JE virus isolate in the culture fluid of C6/36 cells inoculated with field-caught mosquito homogenates

Virus isolation was performed from 20 pools of *Cx. tritaeniorhynchus* captured in a field by inoculation to C6/36 cells as described in the Materials and Methods, and production of JE-ELISA antigen in the culture fluid was followed daily for 7 days. Result of JE virus isolation was determined by the established method using the 7th day culture fluid. The result in Table 1 shows that for 9 pools which eventually turned out to be positive with JE virus isolation, JE-ELISA antigen became definitely detectable (more than 2 units) between 2 to 5 days and reached its maximum titer between 4 to 7 days after specimen inoculation. On the other hand, for those 11 specimens which were negative with JE virus isolation, JE-ELISA titer remained undetectable throughout the observation period, except a single specimen showing transient and barely detectable level of 1 unit.

Table 1. Production of JE-ELISA antigen in C6/36 cell culture fluid after inoculation of field-caught *Cx. tritaeniorhynchus* homogenate.

Days after inoculation	JE-ELISA antigen titer (unit) for JE virus isolation positive 9 specimens								
1	− *	−	−	−	−	−	−	−	−
2	16	8	8	1	1	−	−	−	−
3	32	32	32	8	8	8	8	1	−
4	64	64	64	64	32	32	16	8	1
5	32	32	64	64	32	32	32	16	8
6	64	64	64	32	64	64	32	32	16
7	64	64	64	64	64	32	32	32	32

Days after inoculation	JE-ELISA antigen titer (unit) for JE virus isolation negative 11 specimens										
1	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−
3	1	−	−	−	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	−

Replicate cultures of C6/C36 cells were inoculated with field-caught *Cx. tritaeniorhynchus* homogenates. A part of infected culture fluid from each specimen was daily assayed for JE ELISA antigen titer. The result was shown with JE virus isolation positive (top panel) as well as negative (bottom panel) specimens. *under 1 unit

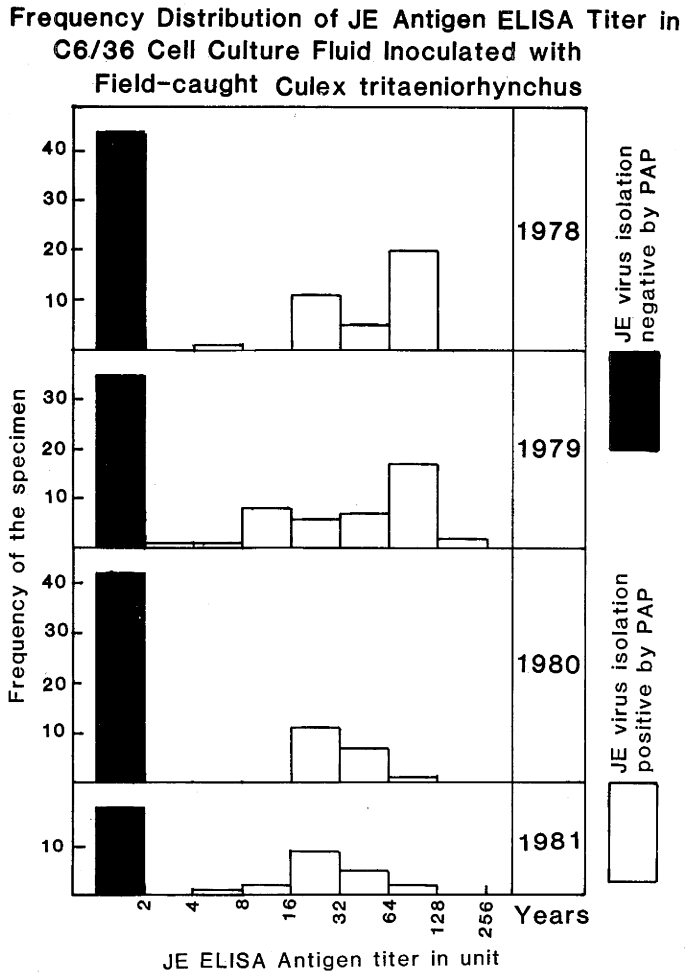

Fig.7. Titer distribution of JE−ELISA antigen in C6/36 cell culture fluids inoculated with field−caught *Cx. tritaeniorhynchus* homogenates. Culture fluids were harvested after 7 days incubation at 28℃ from C6/36 cells inoculated with field−caught *Cx. tritaeniorhynchus* homogenates during the virus isolation studies from 1978 to 1981, and kept frozen at −70℃. ELISA antigen titer was assayed for each fluid with positive JE virus isolation (open column) as well as with negative JE virus isolation (closed column) and frequency of titer distribution was shown in the figure.

A retrospecitive study was performed to examine JE−ELISA antigen titer in a total of 256 culture fluids of C6/36 cells inoculated with field−caught *Cx. tritaeniorhynchus* speciments for virus isolation. All these fluids were harvested after 7 days incubation at 28℃ and kept at −70℃. JE−ELISA antigen titer in 110 specimens which were positive with JE virus isolation were between 4 and 256 units, except a single specimen with titer of 2 units. For 146 specimens with negative JE virus isolation, on the other hand, the titer of JE−ELISA antigen was all under 2 units (Fig. 7).

DISCUSSION

The present data show that JE−ELISA antigen could successfully by detected and measured by using sandwich ELISA method. Although there were some parallelism between the production of infectivity and that of ELISA titer in culture fluids of BHK21 and C6/36 cell infected with various concentration of JE virus, there were also some dissociation as well. Absence of HA in the infected C6/36 homogenate may indicate that the intracellular infective virus can attach to BHK21 cells but the virion may not possess the critical site to attach goose red blood cells or such critical site is masked somehow.

The dissociation between the infectivity and HA or ELISA antigen production in Fig.3 could most probably be explained by the presence of non−infectious ELISA antigen as shown by the sucrose gradient sedimentation study in Fig.4. Presence of slowly sedimenting hemagglutinin (SHA) besides rapidly sedimenting hemagglutinin, which is the complete virion, have been reported for several flaviviruses (Igarashi *et al.*, 1963; Kitaoka and Nishimura, 1963, 1965; Stollar *et al.*, 1966; Smith *et al.*, 1970). Probably, the bottom component with heavier density described in this report corresponds to RHA, and the top component to SHA, respectively, because each component was associated with HA in the infected fluids of BHK21 and C6/36 cells as well as BHK21 cell homogenate, although densities determined in this report were smaller than those previously reported.

Absence of HA in the infected C6/36 cell homogenate (Fig.3, Panel D) could probably be due to the association of infective virions with membranous structures, which might have sedimented to the bottom of the sucrose gradient resulting in the total absence of infectivity throughout the gradient (Fig.4, Panel D). In the case of BHK21 cells infected with chikungunya virus, an alphavirus of Togaviridae, infectivity in the infected cell homogenate sedimented faster than that in the fluid (Igarashi, 1970). Analysis on the specimen harvested during the middle and late stage of virus growth indicated the earlier production of the top component than the bottom component. It may be that each structural component of the virion was first made before its was assembled into complete virion.

JE−ELISA antigen became detectable in C6/36 cell culture fluid as early as 5 days after inoculation even for the specimen with least amount of infective virus both for laboratory and field specimens. Therefore, by assaying the ELISA antigen titer in the infected C6/36 cell culture fluid, the time required for the screening of positive JE virus isolates from field specimens could be reduced to 5 days, significantly shorter than for the previously established method.

The most direct and the shortest way to know the presence of arboviruses in their vector mosquitoes of ticks would, however, be the detection of virus antigen in these vector itself by the ELISA or some appropriate methods. The ELISA has been used for this purpose in the case of dengue virus (Parc *et al.*, 1979), La Crosse virus (Hildreth *et al.*, 1982), eastern equine encephalomyelitis (EEE) virus and Highland J virus (Hildreth and Beaty, 1984, Hildreth *et al.*, 1984), Rift Valley fever virus (Niklasson and Gargan, 1985),

and chikungnya virus (Konishi and Takahashi, 1985). Hildreth *et al.* (1982) reported that their ELISA system for La Cross virus antigen could detect one infected mosquito within a pool of 100 mosquitoes. Hildreth and Beaty (1984) showed that by 3 days post infection after intrathoracic inoculation EEE virus, the ELISA could detect 100% of infected mosquitoes, while indirect fluorescent antibody technique detected only 77%. According to Hildreth *et al.* (1984), the ELISA sensitivity for the specimens containing more than 1500 PFU/ml of the virus was 0.97, while that for the specimens with less than 500 PFU/ml was 0.14. Niklasson and Gargan (1985) showed that plaque assay and the ELISA had similar sensitivity and specificity in detecting mosquitoes capable of transmitting Rift Valley fever virus. Konishi and Takahashi (1985) reported that the ELISA could detect 4×10^6 PFU or more of the purified chikungunya virus and 48.5% of infected mosquitoes with 10^6 to 10^7 PFU of the virus.

Niklasson *et al.* (1983) showed that a double-sandwich ELISA using affinity-purified anti-virus immunoglobulin possessed the sensitivity to detect Rift Valley fever virus of 10^5 PFU/ml for infected Vero cell culture fluid, 10^6 PFU/ml for viremic hamster serum, and 5×10^3 PFU/ml for viremia in Rhesus monkey. Monath and Mystrom (1984) showed that the threshold to detect yellow fever viremia was $10^{3.0-3.6}$ PFU/0.05ml. The lower limit to detect EEE viral antigen in a stock virus was $10^{3.3}$ $TCID_{50}$/ml, while the value was $10^{1.8}$ $TCID_{50}$/0.1ml for experimentally inoculated birds (Scott and Olson, 1986). The sensitivity of our ELISA system appeared to be a little bit less than these reported values, and should be improved further by prolongation of incubation time, using monoclonal antibodies or affinity-purified antibodies, before applied to detect JE virus antigen directly in field-caught mosquitoes.

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