An Enveloped Virus Isolated from Leaf Hoppers Captured on East China Sea

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Abstract: By inoculation to *Aedes albopictus*, clone C6/36 cells, an enveloped virus was isolated from a pool of leaf hoppers captured on East China Sea in the year of 1978. The virus structurally and biologically resembled togaviruses, however, did not show cross reactions with known arboviruses. Inability of the virus to grow in some mammalian cell cultures and in suckling mouse brains indicate that the newly isolated virus belong to some insect viruses. The data suggest that certain viruses could be introduced to Japan by insects flying across East China Sea.

INTRODUCTION

It has been established that during summer seasons in Japan, Japanese encephalitis (JE) virus is transmitted between *Culex tritaeniorhynchus*, main vector mosquitoes, and swine, major amplifier vertebrate hosts. However, overwintering mechanisms of the virus during interepidemic season in temperate regions like Japan still remain unsolved. One of the authors, with his colleague, has been studying the ecology of JE virus in Nagasaki and Southern Islands of Japan, and suggested the possibility that the virus might be introduced to Japan by transoceanic insects (Hayashi *et al.*, 1978a). These researches could capture various species of insects including some mosquito species on East China Sea, however, virus isolation turned out to be negative by mouse brain inoculations (Suzuki *et al.*, 1977; Hayashi *et al.*, 1978b; 1979). The senior author developed *A. albopictus*, clone C6/36 cells, and showed that the cells could be used as better hosts to detect mosquito-borne togaviruses, such as JE virus, compared with classical mouse brain inoculations. He and his colleagues showed also that many filtrable and plaque-forming agents could be detected from field-caught mosquitoes by inoculation to C6/36 cell cultures (Igarashi, 1978; 1980; Igarashi *et al.*, 1981). Hoping that some viruses associated with transoceanic insects might be detected by the new method of

Received for publication, December 17, 1982.
Contribution No. 1250 from the Institute for Tropical Medicine, Nagasaki University.

virus isolation, attempts were made to isolate virus from pools of leaf hoppers captured on East China Sea in the year of 1978.

MATERIALS AND METHODS

Insect specimens: Procedures and results of capturing transoceanic insects have been described before (Hayashi *et al.*, 1979). Twenty pools of leaf hoppers (not exceeding 100 insects/pool) were kept frozen with dry ice and sent to Osaka University, Research Institute for Microbial Diseases.

Virus isolation: The method is similar to those described elsewhere (Igarashi, 1980; Igarashi *et al.*, 1981) and was performed in Osaka University. Each pool of insects was homogenized in 2 ml of phosphate-buffered saline (PBS), pH 7.2, containing 0.2 % of bovine plasma albumin fraction V, and was centrifuged at 2,500 rpm for 15 minutes at 4°C. The supernatant was passed through Millipore HA filter (25 mm in diameter) and was inoculated to tube cultures (0.1 ml/tube) and Petri dish cultures (0.2 ml/dish) of C6/36 cells. After 2 hours of adsorption, the cells in the tubes were covered by maintenance medium (2ml/tube) and those in the dishes with agar overlay medium to detect plaque forming agents. The cells in the tubes were observed daily for the appearance of cytopathic effects and infected culture fluid was harvested 7 days after incubation at 28°C. The fluid was examined for the presence of hemagglutinin using goose red blood cells at pH 7.2 (Clarke and Casals, 1958), and also for the presence of infective virus by inoculation to C6/36 cells grown on 8-chamber slides (Miles, I11. USA). The slides were harvested after 3 days of incubation at 28°C and were stained by the immunoperoxidase method using anti-JE and anti-Getah virus rabit sera as the first serum (Okuno et al., 1977).

Cell cultures: Origin and method to grow clone C6/36 of *A. albopictus* cells were as described (Igarashi, 1978). The cells, were grown at 28°C with Eagle's medium supplemented with 10 % heat-inactivated fetal calf serum and 0.2 mM each of nonessential amino acids. Maintenance medium is the same as cell growth medium except that serum concentration was reduced to 2 %. Cells of BHK21, VERO and LLC-MK_2 were grown at 37°C with cell growth medium of 10 % calf serum in Eagle's medium.

Virus growth in C6/36 cells: Medium was removed from replicate cultures of C6/36 in 60 mm Petri dishes and seed virus was inoculated using 0.2 ml/dish. After 2 hours of adsorption, residual virus was removed and cells were washed twice with PBS. The cells were covered by the maintenance medium and were incubated at 28°C in 5 % CO_2-atmosphere. At appropriate time after virus infection, infected culture fluid was taken to measure titer of fluid virus. Cells were scraped into PBS and were homogenized in glass-homogenizer in order to measure cell-associated virus titer.

Assay of virus infectivity: Specimens were serially diluted by 10-fold steps with

0.2 % bovine plasma albumin in PBS and were inoculated to Petri dish cultures (60 mm diameter) using 0.2 ml/dish after removing cell growth medium. After 2 hours of adsorption, the cells were covered by the 1st agar overlay consisting of 1 % Noble Agar (Difco, Mich, USA) and 400 μg/ml of DEAE dextran (Pharmacia, Uppsala, Sweden) in the maintenance medium. After 4 days incubation at 28°C in 5 % CO_2-atmosphere, the cells were stained by the second overlay containing 0.005 % of neutral red. Plaques were observed from the following day and infectivity was shown as plaque-forming units (PFU) per ml.

Extraction and assay of infective RNA: Specimen was mixed with an equal volume of water-saturated phenol and extracted at room temperature for 5 minutes. After centrifugation at 2,500 rpm for 15 minutes, aqueous phase was again extracted with an equal volume of water-saturated phenol and centrifuged. Phenol dissolved in the aqueous phase was removed by extraction with ethyl ether. The specimen was diluted with PBS containing 100 μg/ml of DEAE dextran and was inoculated to C6/36 cells in Petri dishes, which had been washed with PBS containing DEAE dextran (100 μg/ml) After 30 minutes of adsorption at room temperature, the cells were covered by the agar overlay as described above, in order to form plaques.

Purification of the virus and electron microscopy: Cultures of C6/36 cells in 100 mm Petri dishes were inoculated with seed virus. After adsorption the cells were covered by the maintenance medium and incubated at 28°C in 5 % CO_2-atmosphere. When significant cytopathic effect was observed in the cells, infected culture fluid, approximately 250 ml volume, was collected and centrifuged at 2,500 rpm for 15 minutes. Polyethylene glycol 6000 (Nakarai Pure Chemicals, Kyoto) and NaCl were added to the supernatant at final concentration of 6 % and 0.5 M, respectively. The mixture was stirred until solids were completely dissolved, and then centrifuged at 10,000 rpm for 30 minutes. Supernatant was removed and precipitate was dissolved in 1/30 volume of 0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4, and was centrifuged at 2,500 rpm for 15 minutes. The supernatant was layered over 4 ml of 15 % sucrose in the above buffer and was centrifuged at 37,000 rpm for 90 minutes in an SW 41 rotor of a Beckman model L5-50 ultracentrifuge at 4°C. Supernantant was removed and pellet was resuspended in 0.5 ml of 1 % ammonium acetate, pH 7.0, and was dialyzed against the same solution overnight at 4°C. Specimens were mixed with 2 % of uranyl acetate pH 4.0 and were observed under a JEOL model 100B electron microscope at direct magnification of 1 : 30,000.

Arbovirus grouping fluids: The reagents were kindly supplied by Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland, USA.

RESULTS

Isolation of virus from pools of leaf hoppers: Twenty pools of the insects were processed in the year of 1979 as described in the Materials and Methods. A tube culture of C6/36 cells inoculated with one of the specimens showed significant degree of cytopathic effect 5 days after inoculation of the material. Infected culture fluid was passaged to another culture of C6/36 cells and filtrable agent(s) could cause cytopathic effect in the second and the third passages, and also could form plaques on C6/36 cells under agar overlay. Infected culture fluid of the 4th passage level was kept in aliquots at −70°C as seed virus.

Some biological characteristics of the virus isolated: Cultures of C6/36 cells were inoculated with various concentration of the virus and after 2 hours of adsorption, the cells were washed, and incubated under maintenance medium at 28°C. Fig. 1A shows the result of infectivity assay on fluid virus released up to 4 days after infection. Initial growth rate of the virus appeares to be similar irrespective of input multiplicity (MOI), although the time required to reach maximum titer depended on MOI. Replicate cultures of C6/36 cells were inoculated with undiluted seed virus, and specimens were taken every 6 hours after the infection to measure fluid virus as well as cell associated virus titers. The result in Fig. 1B shows that both fluid and cell-associated virus titer

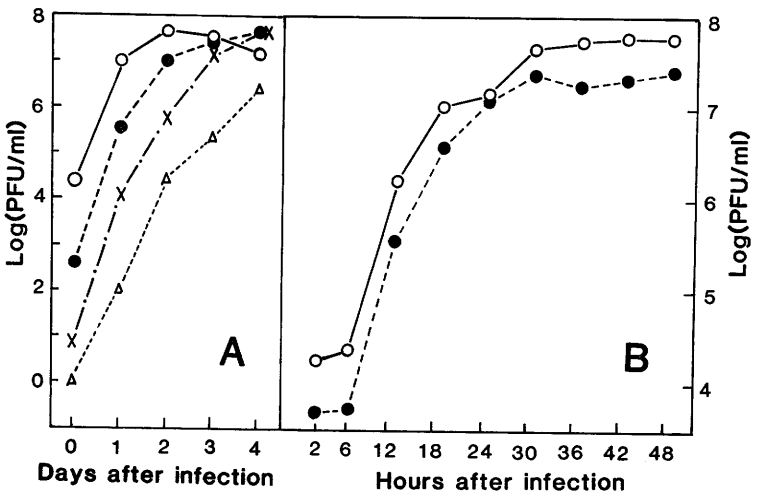

Fig. 1. Growth of the virus in *A. albopictus* clone C6/36 cells. The seed virus was diluted to 10^{-3} (△·····△); 10^{-2} (×—•—•×); 10^{-1} (●·····●); or undiluted (○——○) and then inoculated to C6/36 cells on Petri dishes as described in the Materials and method and titers of infective virus in the fluid was assayed (A). Replicate cultures of C6/36 cells in Petri dishes were inoculated with undiluted seed virus and infective virus titer in the fluid (○——○) and cell homogenate (●·····●) was assayed at various time after the infection (B).

rose up rapidly after 6 hours of latent period until 18 hours, then gradually up to 30 hours reaching plateau level exceeding 10^7 PFU/ml. Titer of infective virus in the fluid appears to be slightly higher than that in the of cell homogenate.

In order to test the presence or absence of virus envelope, the seed virus was treated with ethyl ether or sodium deoxycholate and the remaining infectivity was assayed. Effect of protamine sulfate treatment was also tested in parallel experiment. The result in Table 1 shows that the virus infectivity was sensitive to sodium deoxycholate, although it is resistant to ethylether under the test condition. Infective virus was not precipitated by protamine sulfate treatment.

In order to know the nucleic acid type of the virus genome, effects of some inhibitors of DNA synthesis on the growth of the virus were examined in C6/36 cells infected with the virus. The cells were inoculated with seed virus at high MOI and were incubated under maintenance medium with or without inhibitors for 2 days at 28°C and the yield of infective virus in the fluid was assayed. The result in Table 2 shows that the virus growth was not sensitive to the inhibitors of DNA synthesis, indicating that the virus is an RNA virus. The genome RNA of single-stranded positive polarity is infectious, and demonstration of infectivity of extracted viral RNA will give information on the continuity and polarity of the virus genome. RNA was extracted from seed virus and its infectivity was assayed with and without RNase treatment (1 μg/ml at room temperature for 5 minutes). Although residual infectivity in the RNA after the extraction was significantly low (approximately 10^{-5} of that before extraction), the infectivity in the RNA was completely destroyed by RNase treatment, while the

Table 1. Effect of some chemical treatment on the infectivity of the virus

Treatment	Infectivity Log (PFU/ml)
Control without treatment	5.69
Ethylether, equal volume 4°C overnight	4.19
Sodium deoxycholate, 0.5%, 37°C, 1 hour	<2.69
Protamine sulfate, 1 mg/ml 4°C, 15 min followed by centrifugation at 2,500 rpm, 15 min.	6.01

Table 2. Effects of inhibitors of DNA synthesis on the growth of the virus in C6/36 cells

Inhibitors	Final concentration	Virus yield Log (PFU/ml)
Control without inhibitors		7.62
5-Fluorodeoxyuridine	$10^{-4.5}$M	7.31
5-Iododeoxyuridine	$10^{-4.5}$M	7.59
Hydroxyurea	10^{-2} M	7.28
Cytosine arabinoside	30 μg/ml	7.48

virus infectivity before the extraction was resistant. The result indicate that the genome RNA of the virus is a continuous single-stranded RNA with positive polarity.

These results indicate that the virus isolated from leaf hoppers is an enveloped RNA virus with single-stranded genome of positive polarity, possible belonging to Togaviridae. However, C6/36 cells infected with the virus were not stained either with anti-JE or anti-Getah virus serum by the immunoperoxidase methods. Neither was the infectivity neutralized by any of the 30 grouping fluids for the screening of arboviruses. Thus the virus does not have any relationship with known arboviruses. Preliminary experiment to test the growth potentiality of the virus in some mammalian cell cultures and in suckling mouse brains turned out to be negative. These results indicate that the virus is more likely a member of insect virus carried by leaf hoppers. Several attempts to isolate similar virus(es) from leaf hoppers captured on land in Nagasaki were with negative results.

Morphological observations on the virus isolated: As described above, the newly islated virus appears to have the characteristics of togaviruses. In order to confirm this consideration, the virus was concentrated and partially purified and observed under an electron microscope. Photo 1 shows the morphology of the virus observed by negative staining. Spherical particles of 50 to 60 nm in diameter were observed surrounded by envelope structure with ill-defined outer projections. The size of internal core structure

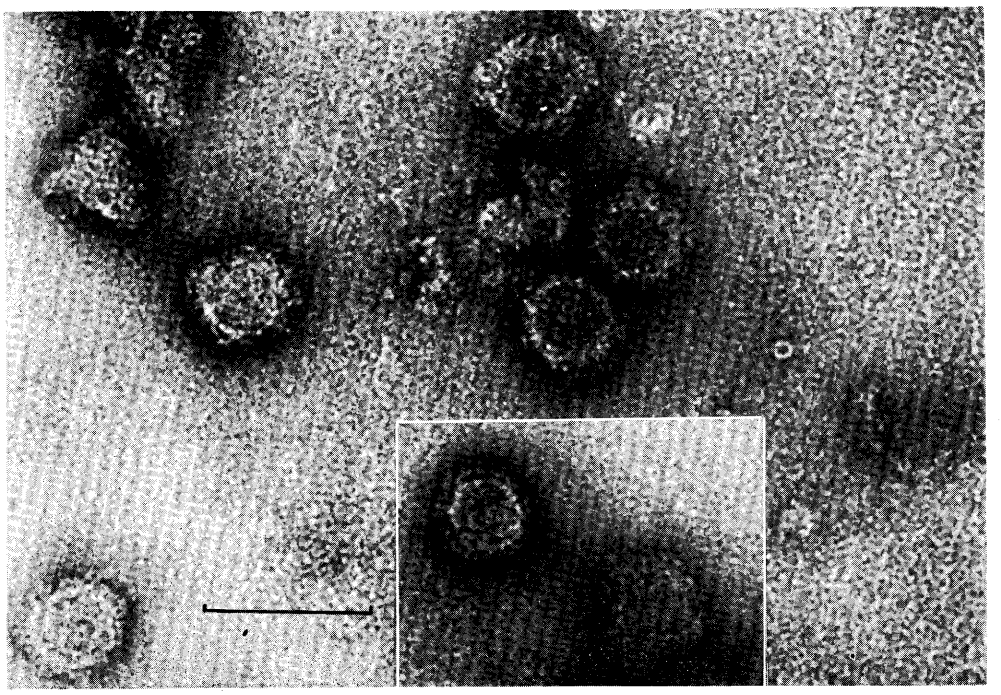

Photo 1. Eletcron micrograph of negatively stained virus.
Magnification: 265,000. Scale 100 nm.

was estimated to be about 35 nm and the thickness of the envelope including 5 nm of projections to be about 10 nm. Sometimes round structure of 12 nm in diameter with central hole was observed along with virions. The structure similar to the small round 12 nm particle was sometimes observed on the surface of the virions, however, exact relationship between these structures is unknown.

DISCUSSION

Present paper describes the isolation of an enveloped virus from leaf hoppers capured on East China Sea. Biological and morphological characteristics of the virus indicate that the virus belong to Togaviridae, however, positive relationship with known arthropod-borne togaviruses could not be demonstrated. Neither did it have any relation with other known arboviruses so far tested. Failure of the growth in some mammalian cell cultures and suckling mouse brains indicate that the virus is a member of insect virus. There has not been any description of such insect virus except cell fusing agent (Stollar and Thomas, 1975; Igarashi *et al.*, 1976) and small-plaque virus isolated from *Culex tritaeniorhynchus* (Okuno *et al.*, 1981). Neutralization test with rabbit antisera against these reported agents gave negative results. Thus the virus described here appears to be a new virus of the insects. We would like to propose a tentative name to the virus, Nagasakimaru virus, according to the name of the University Ship on which leaf hoppers were captured. Further studies will be necessary in order to assign the virus to some family of Togaviridae, such as analyses of virion structural proteins and virus-specific RNA and polypeptides made in the infected cells, as well as the patterns of morphogenesis. Small round particles of 12 nm with central hole appears to be similar to Ht component described for JE virus (Fukai, 1968).

Circumstances to capture the leaf hoppers indicate that the insects from which Nagasakimaru virus was isolated were flying over East China Sea. At present we do not know whether the virus could multiply in leaf hoppers, however, our result shows that certain virus(es) could be brought into Japan along with insects flying across East China Sea. On the other hand, limitted attempts to detect similar virus from leaf hoppers captured on land in Nagasaki were with negative results, indicating that the chance of such association of the virus with transoceanic insects is rather rare phenomenon. The implication of these results still remain to be substantiated in order to draw any reasoning on the possible introduction of JE virus to Japan by transoceanic insects.

ACKNOWLEDGEMENTS

A part of the study, the primary isolation procedure of the virus, was performed in the Department of Preventive Medicine, Research Isntitute for Microbial Diseases of Osaka University. Kind support and encouragement by Prof. K. Fukai is very much appreciated.

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東支那海洋上飛翔ウンカから分離された外被膜を有するウイルス

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1978年東支那海洋上で採集されたウンカ乳剤をヒトスジシマカ培養細胞クローンC6/36に接種する事によって外被膜を有するウイルスが分離された．ウイルスの生物学的並びに形態学的性状はトガウイルスに類似しているが，已知の節足動物媒介ウイルスとの交叉反応は認められず，数種の哺乳類培養細胞および乳呑マウス脳で増殖しない事から，このウイルスは昆虫ウイルスの一つと考えられる．これらの知見は東支那海上を飛翔する昆虫によってある種のウイルスが日本に持ち込まれる可能性を示唆するものと考えられる．

熱帯医学 第24巻 第4号 179−186頁，1982年 12月