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HEMAGGLUTININ PREPARED FROM CELLS OF *AEDES ALBOPICTUS*, CLONE C6/36, INFECTED WITH TYPE 1 DENGUE VIRUS

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SUMMARY A high titer of hemagglutinin (HAnin) was found in culture fluids of cells of *Aedes albopictus*, clone C6/36, infected with type 1 dengue virus. The HAnin (TC antigen) was associated with complete virions and no appreciable small-sized HAnin was produced, in contrast to the case in an infected suckling mouse brain (SMB) homogenate, in which most of the HAnin is smaller than complete virions. Extraction of the TC antigen with Tween 80-ether disrupted the HAnin into smaller-sized particles, resulting in complete loss of infectivity (TE-TC antigen), but it did not affect the titer or reactivity of the HAnin. Comparative hemagglutination-inhibition (HI) assay of human sera using TC antigen, TE-TC antigen or standard antigen extracted from infected SMB (SMB antigen) showed that TC or TE-TC antigen could be used for routine diagnostic or epidemiological HI tests instead of SMB antigen, which is rather hard to prepare.

INTRODUCTION

Dengue hemorrhagic fever is a very common and often serious disease of children in Southeast Asia (Hammon et al., 1960; Halstead, 1966). In serodiagnosis and seroepidemiological studies on dengue infections (Nimmanitya et al., 1969; Halstead et al., 1969; Fukunaga et al., 1974), the hemagglutination-inhibition (HI) test has most often been used, because it is simple and rapid (Hammon and Work, 1964). Antigens for this test have been prepared from infected suckling mouse brain (SMB antigen) as described by Clarke and Casals (1958). However, in many laboratories, especially in developing countries, maintenance of mouse colonies and preparation of diagnostic antigens by sucrose-acetone or acetone-ether extraction is not easy, due to

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the limited facilities available and high running cost involved. Buckley and Srihongse (1963) reported the production of dengue hemagglutinin (HAnin) in infected HeLa cells, but this HAnin has not been used instead of standard SMB antigen in routine tests. Recently, LLC-MK_2 cell cultures infected with dengue viruses were found to produce sufficiently high titers of HAnin in the culture fluid for use as diagnostic antigens under specified conditions (Rosen, personal communication; Inoue, personal communication). In this laboratory, a clone of *A. albopictus* cells was isolated that was sensitive to all 4 types of dengue virus, as well as to chikungunya virus (Igarashi, 1978), and the cells have much higher growth rate and are easier to cultivate than ordinary vertebrate cells. Therefore, we examined the production of dengue HAnin in infected *A. albopictus* cells to see if the concentration of HAnin in the culture fluid was high enough for use of the fluid in routine diagnostic or epidemiological HI tests.

MATERIALS AND METHODS

1. *Viruses*

Dengue virus, type 1 (DEN-1) Hawaiian strain, type 2 (DEN-2) New Guinea B strain, type 3 (DEN-3) H-87 strain, and type 4 (DEN-4) H-241 strain were used. The origins and passage histories of these viruses as well as the preparation of stock viruses from infected suckling mouse brain (SMB) have been described (Igarashi, 1978). Infectivities were assayed by focus assay (Igarashi and Mantani, 1974; Okuno et al., 1977; 1978) and are shown as focus forming units (FFU) per ml.

2. *Cells*

The isolation of a virus-sensitive clone C6/36 from Singh's *A. albopictus* cells (Singh, 1967) and the method of cell culture have been described (Igarashi, 1978). BHK21 cells were grown at 37 C with 10% calf serum in Eagle's minimal essential medium (MEM, Eagle, 1959). These cells were used for infectivity assay of viruses.

3. *Virus inoculation and sampling*

Cells of *A. albopictus*, clone C6/36, were grown in 2-ounce rubber-stoppered prescription bottles by seeding 5×10^5 cells into 5 ml of cell growth medium (10% fetal calf serum in MEM supplemented with 0.2 mM each of nonessential amino acids) per bottle. After 3-days incubation at 28 C, the culture medium was removed and stock virus was inoculated (0.2 ml/bottle). Adsorption was carried out at 28 C for 2 hr, and then residual virus was removed and the cells were washed twice with 4 ml-volumes of phosphate buffered saline (PBS) and incubated at 28 C under maintenance medium (cell growth medium in which fetal calf serum was reduced to 2%). Portions of infected culture fluid were taken every day for assay of virus titers.

4. *Human serum specimens and hemagglutination-inhibition (HI) test*

Thirty-five specimens of human serum were collected from individuals staying at Osaka International Training Center (OITC), Ibaraki City, Osaka. These subjects (Quina et al., 1978) were visitors from abroad participating in training courses operated by Japan International Cooperation Agency. The sera were treated with kaolin and goose red blood cells before being assayed by the HI test (Clarke and Casals, 1958), modified for a microtiter system (Sever, 1962).

5. *Radioisotopic labeling and sucrose gradient sedimentation*

Cells of *A. albopictus*, clone C6/36, infected with DEN-1 were incubated at 28 C for one day under maintenance medium. Then ^{3}H-uridine (New England Nuclear, 27 Ci/mmole) was added to a final concentration of 10 μCi/ml. The culture fluid was collected after further incubation at 28 C for 6 days and was centrifuged at 3,000 rpm for 15 min. A sample (0.5 ml) of the supernatant was layered on a 15–30% sucrose gradient (4.5 ml volume) in STE buffer [0.1 M NaCl, 0.01 M tris (hydroxymethyl) amino methane (Tris), 0.001 M EDTA, pH 7.6] containing 0.2% bovine plasma albumin fraction V (Armour) and was centrifuged in an SW 50.1 rotor of a Beckman model L2-65B ultracentrifuge at 35,000 rpm for 90 min at 4 C. Fractions were collected from the bottom of the gradient and part of each fraction was used for assay of HA and FFU titers. The remainder of each fraction was mixed with an equal volume of 10% trichloroacetic acid (TCA), and stood at 4 C for 30 min. The precipitate was collected on a

glass fiber filter (Whatman, GF/A), washed three times with 5% TCA, dried and immersed in 5 ml of toluene-based scintillator (4 g of diphenyloxazole/liter). Radioactivity was measured in a Beckman model LS-150 scintillation spectrometer

RESULTS

1. *Production of HAnin in dengue infected A. albopictus cells*

Cells of *A. albopictus*, C6/36, were infected with DEN-1 stock virus and viral HA and FFU in the medium were assayed every day after infection. The results in Fig. 1 show that the infectivity increased from 2 to 4 days after infection and then remained at a plateau, while the HA increased from 4 to 7 days after infection to a titer of 128 units and then remained steady. These curves for HA and FFU suggest that non-infectious HAnin was produced at a late stage of infection after the FFU had reached a maximum. This possibility is discussed in the following section. Lower production of HAnin was observed in similar experiments on other types of dengue viruses: the maximum HA titers up to 7 days after infection were 16, 4 and less than 2 units for DEN-2, DEN-3 and DEN-4 viruses, respectively, while their maximum infectious titers were around 10^8, 10^6 and 10^6 FFU/ml, respectively.

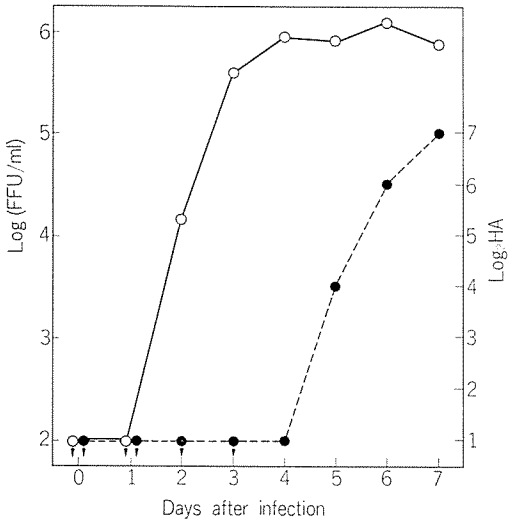

FIGURE 1. Production of infectivity and HA in culture fluid of *A. albopictus*, C6/36, cells infected with DEN-1 virus.

A. albopictus, C6/36, cells were prepared in prescription bottles by seeding 5×10^5 cells into 5 ml medium per bottle. After 3-days incubation at 28 C, cells were infected with DEN-1 virus at an input multiplicity of 0.1 FFU/cell. Production of infective virus (○——○) and HAnin (●- - -●) were followed by assaying the titers in the culture fluids every day after infection for 7 days as described in the Materials and Methods.

There was no marked increase in production of HAnin on increasing the magnesium ion concentration in the medium, unlike the case with Vero cells (Matsumura et al., 1972). Moreover, a high titer of HAnin could not be extracted from infected cells by alkaline treatment, unlike the case with rubella virus (Halonen et al., 1967). Furthermore, production of HAnin was not improved by using fetal calf serum pretreated with kaolin in the medium (Stewart et al., 1967), or by using bovine plasma albumin in place of serum to avoid the presence of non-specific serum inhibitors (Kundin and Diercks, 1960; Salminen, 1962).

2. *Size of DEN-1 TC-HAnin and its disruption by Tween 80-ether extraction*

DEN-1 HAnin was produced in infected *A. albopictus* cells labeled with ^{3}H-uridine and was analyzed by sucrose gradient sedimentation. The results in Fig. 2A show that most of the HAnin was associated with particles containing radioactivity of incorporated uridine and sedimenting at a similar speed to infectious particles from DEN-1 infected SMB homogenate (Fig. 2C). This result is not consistent with the idea that the difference in the curves for HA and FFU shown in Fig. 1 was due to production of small-sized noninfectious HAnin. The difference can be explained better by supposing that the high HA titer observed on the 7th day after infection is due to accumulation of complete virions, some of which may

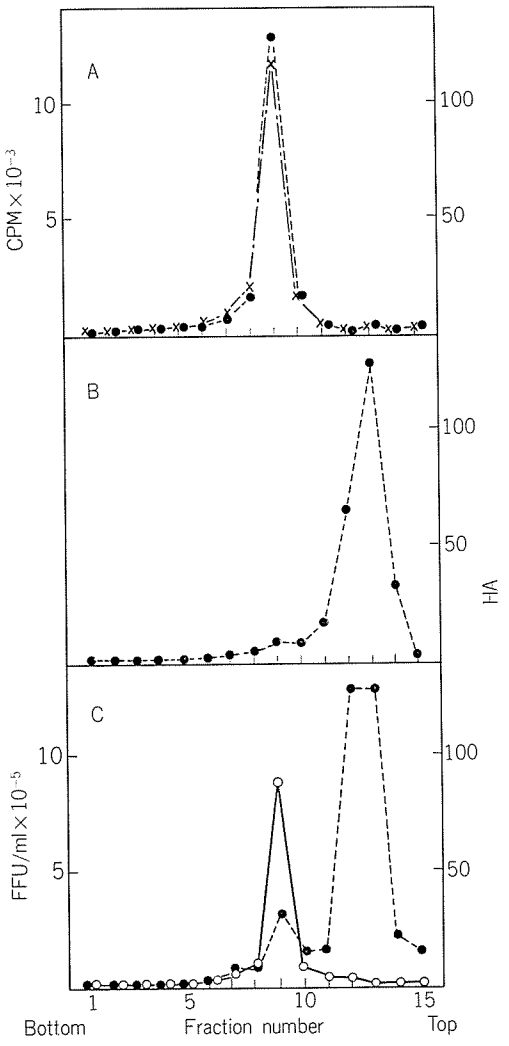

FIGURE 2. Sucrose gradient sedimentation of DEN-1 specimens. (A) Culture fluid was collected from DEN-1 infected *A. albopictus* cells labeled with ^{3}H-uridine (10 μCi/ml) from 1 to 7 days after infection. After centrifugation at 3,000 rpm for 15 min, the supernatant was applied to a 15–30% sucrose gradient in STE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6) containing 0.2% bovine plasma albumin. The specimen was centrifuged in an SW 50.1 rotor of a Beckman model L2-65B ultracentrifuge at 35,000 rpm for 90 min at 4 C. Fractions were collected and assayed for HA (●- - -●) and acid-insoluble radioactivity (×-·-·-·×). (B) A similar specimen to that in (A), but not labeled with ^{3}H-uridine, was extracted with Tween 80-ether and fractionated as described above. (C) Stock virus of DEN-1 infected SMB homogenate was centrifuged through a sucrose gradient as described above, and each fraction was assayed for HA (●- - -●) and infectivity (○——○).

TABLE 1. *Tween 80-ether extraction of DEN-1 TC antigen*

Experiment No.		1	2	3
Before extraction	HA	128	128	128
	FFU	8.2×10^5	7.4×10^5	8.6×10^5
After extraction	HA	128	128	128
	FFU	$<10^2$	$<10^2$	$<10^2$

have lost their infectivity due to thermal inactivation. On the other hand, when the supernatant of DEN-1 infected SMB homogenate was analyzed by sucrose gradient sedimentation, most of the HAnin was found near the top of the gradient and only a small amount associated with infective virions (Fig. 2C).

Culture fluid of C6/36 cells infected with DEN-1 could itself be used as a diagnostic antigen, because it has a high titer. However, it would be better to use antigen without infectivity. This infectivity could be completely removed without affecting HA by Tween 80-ether extraction (Norrby, 1962) of DEN-1 TC antigen (Table 1). Sucrose gradient sedimentation analysis of the material extracted with Tween 80-ether (TE-TC antigen) showed that the HA was associated with smaller particles remaining near the top of the gradient (Fig. 2B). Thus, Tween 80-ether extraction seems to disrupt HAnin associated with particles of the size of complete virions into smaller particles, resulting in loss

of infectivity, as in the case of Sindbis virus (Mussgay and Rott, 1964). However, there was no increase in the titer of HAnin on Tween 80-ether extraction of DEN-1 TC antigen.

3. *Comparative HA titration of human DEN-1 antibody using standard SMB antigen and antigens from infected A. albopictus cells*

As described above, high titers of HAnin were easily obtained from DEN-1 infected cells of *A. albopictus*, C6/36, into their culture fluid (TC antigen), or as preparations after Tween 80-ether extraction (TE-TC antigen). Therefore, we examined the possibility of using these preparations to assay the HI antibody titers of human sera by comparing their effectiveness with that of standard SMB antigen. Some of the human sera tested were obtained from healthy adults coming from Southeast Asia, and all of them had previously been exposed to dengue viruses, as revealed by antibody assay (Quina et al., 1978). The results in Fig. 3A indicate that the titer of each serum against DEN-1 SMB antigen was almost the same as that against DEN-1 TC antigen. Although there were some differences between the titers assayed with these 2 antigens, the differences were within the limits of technical error, that is less than 2-fold. Moreover no false-positive or false-negative results were obtained on assay with TC antigen. There was also no significant difference between the HI titers assayed with TE-TC antigen and with TC antigen (Fig. 3B), indicating that Tween 80-ether extraction did not alter the antigenicity of DEN-1 TC HAnin significantly.

DISCUSSION

The HI test is probably the most widely used serological test for flavivirus infections including dengue, and SMB antigens have been

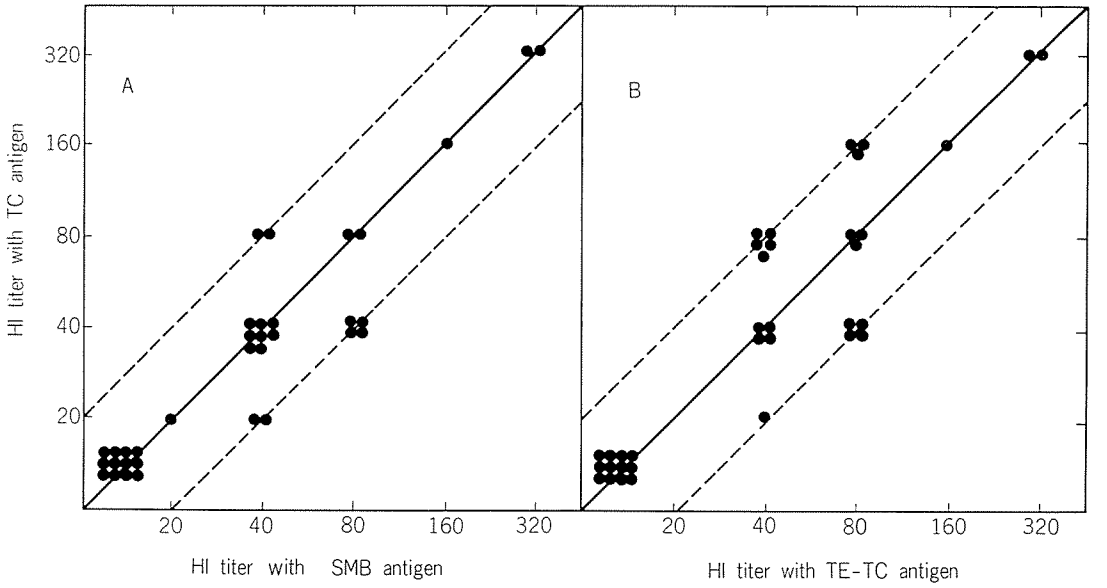

FIGURE 3. Comparative HI titration of DEN-1 antibody of human sera using standard SMB antigen, TC antigen, or TE-TC antigen. The DEN-1 HI antibody titers of 35 individual human sera were assayed using SMB antigen and TC antigen (A), or using TC antigen and TE-TC antigen (B). Dots represent values for individual sera. Dots on oblique solid lines indicate that the same titer was recorded with two different antigens. Dots on oblique dotted lines indicate that a 2-fold difference was observed between the HI titers obtained with 2 kinds of antigens.

used as standard antigens (Clarke and Casals, 1958; Hammon and Work, 1964). There have been many reports on the production of high titered HAnin in flavivirus infected cell culture fluids (Diercks et al., 1961; Salminen, 1962; Darwish and Hammon, 1966; Lee et al., 1967) including dengue (Buckley and Srihongse, 1963). However, TC antigens of dengue viruses have not been widely used until recently. According to Rosen (personal communication) a high titer of HAnin was found in LLC-MK_2 cell culture fluid infected with a certain plaque isolate of DEN-1 virus, and this HAnin was used for serological tests. Moreover, Inoue (personal communication) found that HAnin was produced when serum in the medium was replaced by bovine plasma albumin and he also used the resulting dengue infected LLC-MK_2 cell culture fluids as diagnostic antigens. Since TC antigens can be obtained without any tedious extraction procedures, their practical value is obvious. Our results indicate that culture fluid from *A. albopictus*, C6/36, cells infected with DEN-1 can itself be used as diagnostic antigen without the necessity for any complex procedure. Moreover, *A. albopictus* cells can grow faster than ordinary vertebrate cells and are easy to cultivate and so have a good reason to be used as a source of HAnin production.

Most of the HAnin in DEN-1 infected *A. albopictus* cell culture fluid was associated with complete virions, in contrast with that in preparations from infected SMB, which is mostly of a smaller size. This finding is reminiscent of the findings of Sinarachatanant and Olson (1973) that only complete virions, RHA, of DEN-2 virus are produced in *A. albopictus* cells, while both RHA and SHA are produced in LLC-MK_2 cells. Although Tween 80-ether extraction seems to disrupt the virion-associated HAnin into smaller size, the HA titer was not enhanced by this procedure. It may be conceivable that the HA of dengue virus may depend on total mass of virion glycoprotein rather than the number of particles carrying the glycoprotein. Or else, envelope of the virions was stripped and condensed into smaller size without increasing the number of particles.

In spite of the high infectious titer of virus produced in DEN-2 infected cells, the HA titer was rather low. With DEN-3 and DEN-4 also much less HAnin was produced. Thus, crude culture fluid of cells infected with other types of dengue virus could not be used as diagnostic antigen unless it was concentrated in some way before use (Horzinek, 1969; Della-Porta and Westaway, 1972).

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