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STUDIES ON CHIKUNGUNYA VIRUS.
II. PURIFICATION OF MOUSE BRAIN VIRUS[1]

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SUMMARY A method of purification of Chikungunya virus grown in suckling mouse brain is described. The infective virus was purified 40–50 times and concentrated 20–30 times over the starting material with a recovery rate of 20–40%. Only a single component of virus activity was found by sucrose density gradient centrifugation of the purified sample, and the infective virus seemed to be associated with hemagglutinating activity.

INTRODUCTION

Chikungunya virus was first isolated in Tanganyka, East Africa in 1953 (ROSS, 1956). Later it was also found in Thailand during an epidemic of Thai hemorrhagic fever and was suspected to be one of the etiological agents of this disease (OYA, 1959, HAMMON *et al.*, 1960). Although several clinical and epidemiological studies have been made on this virus disease (HAMMON *et al.*, 1960, DASANEYAVAJA *et al.*, 1963, HALSTEAD *et al.*, 1963, NELSON *et al.*, 1964 and HALSTEAD, 1965, 1966), almost nothing is known about the virus particle itself except from an electron microscopic study of tissue culture cells infected with the virus (HIGASHI, 1966). To obtain more information on the properties of this virus, which is a member of group A arthropod-borne viruses, attempts were made to purify the mouse-adapted virus. The purification of this virus will also be valuable for production of purified vaccine for human use.

MATERIALS AND METHODS

1. *Virus*

Chikungunya virus, prototype African strain at the 174th passage level in suckling mouse brain (SMB), and the BaH 306 strain, isolated in Bangkok (OYA, 1959), at the 7th SMB passage level were supplied by Dr. Sompop Ahandrik of this Institute in Bangkok. All the virus materials were prepared after 2 more passages of these strains in SMB.

2. *Preparation of infected SMB*

Volumes of 0.02 ml of virus solutions containing about 10^5 LD_{50} of virus were inoculated intracerebrally into 1–3 day old suckling mice. After 40

1 This work was performed by cooperation between Thailand and Japan under the Colombo plan.

hours for the African strain and 80 hours for the BaH 306 strain, infected mice were bled by cutting the axillary artery, and the brains were harvested and stored at −70°C.

3. *Tissue culture*

An established cell line of African green monkey kidney cells (VERO) was originarry supplied by Dr. Oya's laboratory in the National Institute of Health of Japan, Tokyo, at the 184th passage level. The cell line was transferred every 3–4 days at a density of 10^5 cells/ml in YLE medium (0.1% yeast extract, 0.5% lactalbumin hydrolyzate and 0.55% glucose in Earle's balanced salt solution with antibiotics and phenol red) supplemented with 5% bovine serum. The cell line was used by the 270th passage level.

4. *Assay of infectivity*

The infectivity of Chikungunya virus was assayed by the plaque technique using VERO cell monolayers as described elsewhere (IGARASHI and TUCHINDA, 1967), with overlay medium containing 400 μg/ml of protamine sulfate. The virus titer was expressed as plaque forming units (PFU) per ml.

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

The HA activity of the virus and HI titer of the immune sera were determined by the method of CLARKE and CASALS (1958), using VAD 6.2 for the African strain and VAD 6.0 for the BaH 306 strain at their optimal pH values for HA.

6. *Immune serum*

Hyperimmune mouse serum against the African strain obtained by 4 successive immunization and that against the BaH 306 strain obtained by 5 successive immunization were also kindly supplied by Dr. Sompop Ahandrik of this Institute in Bangkok. The 50% plaque reduction test to measure the neutralizing antibody titer was carried out according to the method of HASHIMOTO and PRINCE (1963) except that the diluent used was 0.2% gelatin in YLE.

7. *Sucrose density gradient centrifugation*

Columns of 10–35% sucrose in BS (0.12 M NaCl in 0.05 M borate buffer, pH 9.0) were made in lusteroid tubes (1.2 cm × 5.0 cm) by layering 35, 30, 25, 20, 15 and 10% (w/w) sucrose solutions in BS. Layered columns were kept overnight in a refrigerator. Virus samples of 0.2–0.5 ml were layered onto the top of the gradient columns and centrifuged in a SW-39 rotor of a Beckman model L ultracentrifuge with refrigeration at 30,000 rpm for 90 min. The rotor was stopped without braking and fractions were collected by the droplet method from the bottom of the centrifuge tube.

8. *Protein determination*

Protein contents were determined by the method of LOWRY *et al.* (1951), using bovine plasma albumin fraction V (Armour) as a standard.

9. *Chemical reagents*

a) Protamine sulfate

Protamine sulfate was a product of Nutritional Biochemical Cooperation U.S.A. It was dissolved in BS at a cencentration of 10 mg/ml, and the pH was adjusted to 9.0 with NaOH. Then the mixture was sterilized by autoclaving.

b) Active carbon

Active carbon, Shirasagi mark, was purchased from Takeda Pharmaceutical Company, Japan. It was suspended in, and thoroughly washed with double distilled water, then rinsed 3 times with BS and finally suspended in BS at 100 mg/ml for use as stock solution, and sterilized by autoclaving.

c) Fluorocarbon

Fluorocarbon, Daiflon S_3, was a product of Daikin Industrial Company, Japan.

RESULTS

1. *Selection of the strain and starting material for purification*

With regard to vaccine production. The newly isolated strain (in this case BaH 306) would seem preferable to an older one (African). However, the BaH 306 strain gives a slightly lower titer in SMB than the African strain as described previously (IGARASHI and TUCHINDA, 1967) and is less stable at 37°C. Also the immunologic al difference between these strains is not so great as judged by the HI and 50% plaque reduction test (Table 1). Viruses in tissue culture were also considered as possible starting materials because of their low content of non-viral materials; but the final yield of virus in tissue culture is one to half a log lower than that in a 10% homogenate of

TABLE 1 *Immunological Difference between African and BaH 306 Strain*

Virus \ Antiserum	African (4 immunizations)		BaH 306 (5 immunizations)	
	$\log_{10}$ of 50% plaque reduction	HI	$\log_{10}$ of 50% plaque reduction	HI
African	3.4	1600	3.3	3200
BaH 306	3.0	800	3.8	6400

infected SMB. Accordingly the African strain grown in SMB was adopted as the starting material for virus purification.

2. *Purification procedure*

The procedure used to obtain the purified product from the 10% homogenate (H) is illustrated in Fig. 1. All the steps were performed at 4–10°C. The P_4 fraction was dissolved in one-fifth the volume of the H fraction, and the P_6 fraction in one-hundredth the volume of the H fraction. The virus activity and protein content of each fraction were measured. The results of three experiments starting with about 10 gm of SMB are summarized in Table 2. The virus titer in the H fraction was $1.4–2.7\times10^9$ PFU/ml and that in the P_6 fraction was $2.7–6.5\times10^{10}$ PFU/ml. About 20–40% of the infective virus was recovered in the P_6 fraction, and it was 40–50 times purer and 20–30 times more concentrated than in the H fraction. Sometimes better

TABLE 2 *Summary of Purification of Chikungunya Virus*

Experiment	Fraction	Virus activity (10^9 PFU/ml)	Protein content (mg/ml)	Volume of fraction (ml)	Specific activity (10^6 PFU/mg protein)	Purifiication factor*	Total virus yield (10^{10} PFU)	Recovery of virus PFU (%)
I	H	1.35	4.78	106	2.83	1.00	14.3	100
	S_1	1.30	3.00	93	4.33	1.53	12.1	84.7
	S_2	1.00	1.98	100	5.05	1.79	10.0	70.0
	S_3	1.15	1.19	103	96.7	3.42	11.9	83.3
	P_4	4.30	0.83	21	51.9	18.3	9.03	63.1
	S_5A	3.30	0.26	19	127	44.9	6.28	43.9
	P_6	26.5	3.26	1.1	81.4	28.8	2.91	20.4
II	H	2.70	4.10	98	6.59	1.00	26.5	100
	S_1	2.15	3.12	83	6.89	1.05	17.8	67.3
	S_2	1.45	1.98	86	7.89	1.10	12.5	47.2
	S_3	1.75	0.77	87	22.8	4.08	15.2	57.4
	P_4	4.80	0.65	18	73.9	11.2	8.65	37.2
	S_5A	6.50	0.21	16	310	47.1	10.4	39.3
	P_6	58.8	2.27	1.0	248	37.6	5.88	22.2
III	H	2.10	5.15	102	4.08	1.00	21.4	100
	S_1	2.00	4.06	89	4.93	1.21	17.8	83.3
	S_2	1.85	2.71	91	6.83	1.68	16.8	78.6
	S_3	2.75	1.72	91	16.0	3.92	25.0	117
	P_4	10.5	1.50	18	70.0	17.1	18.9	88.4
	S_5A	8.0	0.59	17	135	33.1	13.6	63.6
	P_6	65.0	3.33	1.3	195	47.8	8.45	39.5

* Ratio of specific viral activity of the fraction to that of the H fraction.

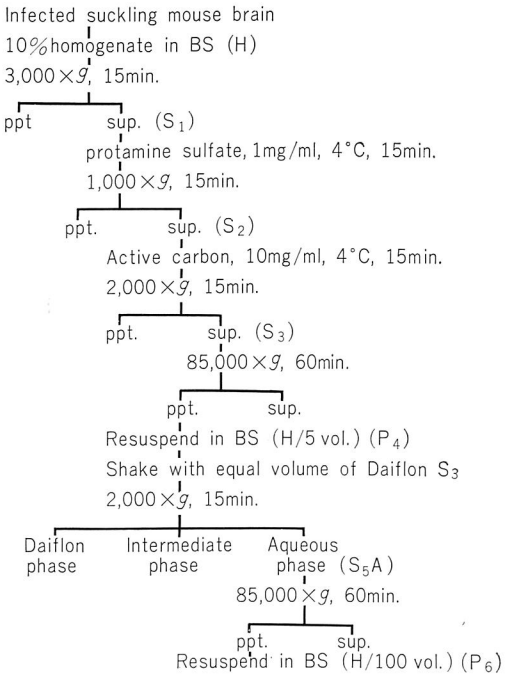

FIGURE 1 Purification of Chikungunya Virus.

specific activity was obtained with the S_5A fraction rather than the P_6 fraction because the virus was inactivated during the final procedure. The ratio of PFU to HA of the P_6 fraction was in the order of 10^7.

3. *Homogeneity of the virus activity*

The diluted P_6 fraction was analyzed by sucrose density gradient centrifugation and the result is shown in Fig. 2. The recoveries of PFU and HA were 58% and 100% respectively. The single peak of PFU was found to coincide with the single peak of HA. Similar single peaks of virus activity were found in the S_5A and P_4 fractions and there were no other peaks of virus activity. When concentrated fraction P_6 was centrifuged in a sucrose gradient column, a single light-scattering band was observed (Fig. 3), and the position of this band corresponded to the peak of virus activity in the previous experiment in which the diluted

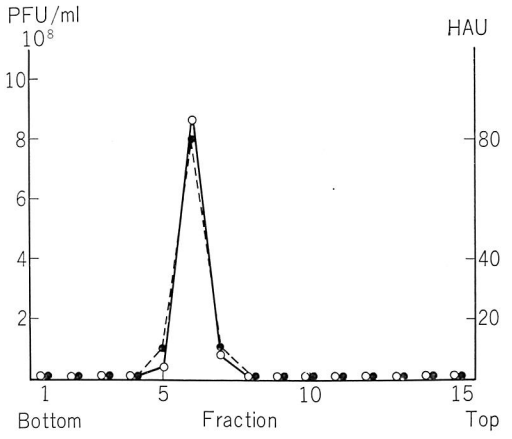

FIGURE 2 Sucrose Density Gradient Centrifugation of Purified Chikungunya Virus.
○———○ PFU
●------● HA
sample: 0.2 ml of P_6(6×10^8 PFU, 32 HA)
Sucrose column: 10–35% in BS, 5 ml
Centrifugation: 30,000 rpm, 90 min.

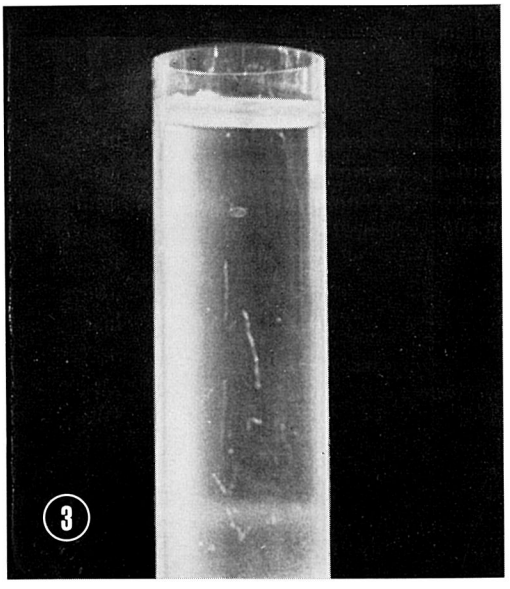

FIGURE 3 Virus Band of Chikungunya Virus in a Sucrose density Gradient Column
Sample: 0.5 ml of P_6(2×10^{10} PFU)
Sucrose column: 10–35% in BS, 4.5 ml
Centrifugation: 30,000 rpm, 90 min.

P_6 fraction was used. Almost 100% of the HA activity was recovered at this band. No similar band was found using fraction P_6 prepared from normal SMB.

DISCUSSION

Since its first application (WARNER *et al.*, 1949), protamine sulfate has often been used in the purification of arthropod-borne viruses. In this study the concentration and duration of protamine treatment were less than those used originally, as shown in Fig. 1, to obtain better and more rapid recovery of the virus PFU. Adsorption by active carbon was found to be effective for removing non-viral protein and it caused a slight increase of PFU. This might be due to removal of some virus-inhibiting substances. Ultracentrifugation is the most popular method for purification of animal viruses, but repeated differential centrifugation in the absence of a certain amount of protein resulted in a loss of virus PFU. The observed loss of virus PFU during the second ultracentrifugation may be attributed partly to poor resuspension in a small amount of medium. Fluorocarbon treatment is effective in removing non-viral proteins and a significant amount of flocculating material was removed in the intermediate phase after centrifugation. Though the nature of this material is unkown, this step is effective in increasing the specific activity of the virus.

In the purified virus preparation only a single component of virus HA was found to be associated with a single component of virus PFU, by sucrose density gradient analysis. Also by density gradient centrifugation through potassium tartrate columns (20–36%) only a single peak of HA associated with a single PFU peak was observed at a density of about 1.2 gm /ml after 3 hours' centrifugation at 35,000 rpm, with a quantitative recovery of virus activities. CHENG (1961b) observed a single componet of virus activity in a purified sample of Semliki Forest virus. Later PFEFFERKORN and HUNTER (1963) obtained a purified sample of Sindbis virus material with a single HA component associated with PFU. On the other hand SMITH and HOLT (1961) reported two separable hemagglutinins for arthropod-borne viruses by column chromatography. MUSSGAY and ROTT (1964) observed two hemagglutinin components with different densities in Sindbis virus by cesium chloride density gradient centrifugation. With Chikungunya virus it was found that concentrated cesium chloride exerts a very deleterious effect on the virus PFU and almost no virus PFU was recovered after equilibrium density gradient centrifugation in it. However, two peaks of HA were observed at densities of 1.27 and 1.22 gm/ml even using the band of purified virus obtained by sucrose density gradient centrifugation. Thus in cesium chloride one HA component may result from virus disintegration. The lability of the virus particles is also observed by electron microscopy of the virus band from sucrose density gradient column. After overnight dialysis against 1% ammonium acetate at 4°C, the shadowed specimen showed spherical viruslike particles with a diameter of 38–40 mμ together with smaller particles with a diameter of 11 mμ. The latter were considered to be disintegrated virus hemagglutinin. The details of this morphological study will be published by the Virus Research Institute in Bangkok.

CHENG (1961a) observed a considerable increase in HA titer during purification of Semliki Forest virus. With Chikungunya however, this was not observed. The specific activity of HA was almost parallel to that of PFU in fractions S_2 to P_6.

Chikungunya virus can be concentrated and purified with relative ease and reproducibility by the method described in this paper. This is essentially the same as in the case of Japanese encephalitis virus (YAMASHITA *et. al.*, 1965).

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