toxin. The spleen esterase is not associated with a lipoprotein and no lipoprotein was detected in the isolated fraction. The spleen esterase also differs from the plasma esterases in chromatographic behavior and in heat stability.

1. Rutenburg, S., Rutenburg, A., Smith, E., and Fine, J., Ann. N. Y. Acad. Sci. 133, 663 (1966).

2. Rutenburg, S., Skarnes, R., Palmerio, C. and Fine, J., Proc. Soc. Exptl. Biol. Med. 125, 155 (1967).

3. Rutenburg, S., Smith, E., Rutenburg, A. and Fine, J., Antimicrobial Agents Chemotherapy 142 (1961).

4. Skarnes, R., Ann. N. Y. Acad. Sci. 133, 644 (1966).

5. Skarnes, R., Bacteriol. Proc. 131 (1961).

6. Skarnes, R. and Chedid, L. in "Bacterial Endotoxins," Landy, M. and Braun, W., eds., p.575. Rutgers Univ. Press, New Brunswick, New Jersey, 1964.

7. Freeman G. G., Biochem. J. 36, 340 (1942).

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Cross Plaque Neutralization of Two Antigenically Closely Related Dengue Viruses (Type 2 New Guinea C and TH-36)* (32947)

ADLY N. IBRAHIM, WILLIAM MCD. HAMMON, AND BOSKO POSTIC
Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

In 1958 an epidemic of hemorrhagic fever occurred in and near Bangkok, Thailand during the rainy season. The disease there was called Thai hemorrhagic fever. Over 2500 patients were hospitalized with about 10% case fatality rate. During the epidemic, Hammon *et al.* (1) isolated several viruses both from human sera and from *Aedes aegypti*. Among these isolates a prototype strain named TH-36 (representing a number of apparently identical isolates) was found to be antigenically closely related to dengue type 2 (D-2). However, further studies by the same investigators revealed some antigenic differences between these two viruses (2). In an attempt to explore further such possible differences, cross plaque neutralization tests were undertaken.

Materials and Methods. Virus stocks were 20% infected suckling mouse brain suspensions of D-2, New Guinea "C" strain, and of TH-36, both at the twenty-third mouse passage level. Each virus stock was diluted to contain approximately 300 plaque forming units (pfu) per 0.1 ml. The diluent was: Tris (hydroxymethyl) aminomethane, buffered salt solution with 0.4% bovine albumin, adjusted to pH 8.4 with 0.1 N HCl.

The D-2 and TH-36 hyperimmune mouse sera were prepared in 2-fold serial dilutions in the aforementioned diluent. The dilutions used, based on earlier trials were: 1:320, 1:640, 1:1280, and 1:2560. Serum–virus mixtures were prepared by mixing 1 ml of antiserum dilution with an equal volume of virus suspension. Controls consisted of (a) normal mouse serum (NMS) diluted 1:320, and (b) diluent, each mixed with an equal volume of virus suspension. All of the above sets of virus–serum and virus–control mixtures were incubated at 30°C for 1 hour, and then transferred to an ice bath.

Bottles (3-oz prescription type) used for plaque assays were seeded with approximately 3×10^5 versinized LLC-MK$_2$ cells, a continuous line of rhesus monkey kidney cells, at the thirtieth to forty-eighth serial passage level. The medium was Eagle's basal medium supplemented with 10% heat inactivated calf serum. This medium was changed on the second day, and the cells were inocu-

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TABLE I. Cross Plaque Neutralization Tests with TH-36 Hyperimmune Mouse Serum Versus TH-36 and Dengue 2 Viruses.

Reciprocal antiserum dilution	No. of TH-36 plaques[a]		No. of D-2 plaques	
	Expt. I	Expt. II	Expt. I	Expt. II
320	24, 28, 27 (26)	14, 12, 12 (13)	70, 72, 75 (72)	65, 71, 63 (66)
640	50, 46, 53 (50)	34, 29, 34 (32)	85, 79, 86 (83)	79, 79, 88 (82)
1280	61, 66, 63 (63)	56, 52, 50 (53)	100, 102, 108 (103)	98, 93, 96 (95)
2560	89, 90, 95 (91)	62, 69, 67 (66)	128, 119, 134 (127)	109, 118, 110 (112)
Controls with diluent	146, 154, 151 (150)	109, 104, 106 (106)	144, 138, 140 (140)	117, 125, 123 (122)

[a] Refers to individual bottles, the means are in parentheses.

lated on the third day when a heavy cell layer had developed. After decanting the medium, 0.1 ml of each virus–serum or control mixture was inoculated onto the cell sheet of three bottles. The inoculum was evenly distributed over the cell sheet during a 1.5-hour period of absorption at 37°C by rocking the bottle at the time of inoculation and every 30 min thereafter. Each bottle, without decanting received 8.0 ml of agarose–overlay medium, consisting of Eagle's basal medium supplemented with 10% heat-inactivated calf serum, 1% agarose (Sea Kem, Marine Colloid, Inc.), and 5% of a 1:1000 filtered neutral red. Bottles were incubated in the dark at 37°C, and examined daily for the appearance of plaques. Plaques were counted when they reached maximum size and number. Dengue 2 plaques started to appear on the fifth and sixth day of incubation, increased gradually to reach maximum in size and number (2–4 mm) on the ninth to tenth day. The TH-36 plaques became visible and countable on the ninth day and reached maximum size (1–2 mm) and number on the twelfth day. For confirmation, the experiment was repeated at a subsequent date using the same antisera and the same viruses.

Results. Results of two separate cross plaque neutralization tests are presented in Tables I and II and in Figs. 1 and 2. Details of plaque numbers in each bottle with calculated means, together with those of the controls with medium only are presented in the tables. Controls with NMS gave plaque numbers which did not vary significantly from those with medium. Percentage of plaques neutralized was calculated by using the mean of the medium controls and the various antiserum–virus means shown in the parentheses in the Tables I and II. The four sets of points shown in each figure appeared to fit straight, parallel lines. Arrows indicate the 50% end points for each dose response curve, i.e., the 50% serum dilution end points. These end points and the calculated relative potencies of each antiserum for the duplicate experiments are presented in Table III.

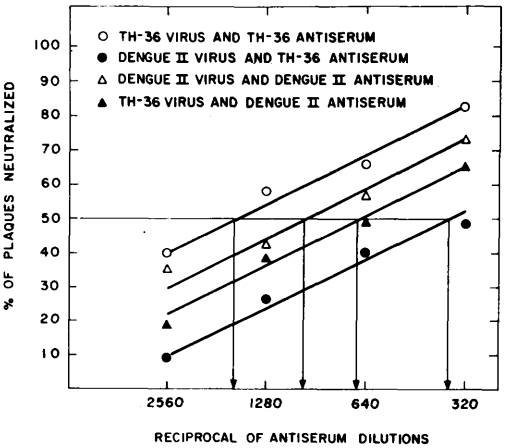

FIG. 1. Cross plaque neutralization with dengue 2 and TH-36 viruses and their antisera. (Exp. 1).

TABLE II. Cross Plaque Neutralization Tests with Dengue 2 Hyperimmune Rabbit Serum Versus Dengue 2 and TH-36 Viruses.

Reciprocal antiserum dilution	No. of D-2 plaques[a]		No. of TH-36 plaques	
	Expt. I	Expt. II	Expt. I	Expt. II
320	36, 41, 38 (38)	27, 22, 23 (24)	54, 49, 55 (52)	45, 48, 40 (44)
640	61, 56, 64 (60)	40, 41, 46 (42)	82, 74, 71 (76)	53, 58, 53 (55)
1280	80, 80, 82 (80)	76, 72, 70 (73)	98, 89, 90 (92)	82, 76, 80 (79)
2560	95, 84, 87 (89)	90, 85, 97 (91)	117, 125, 124 (122)	96, 89, 88 (91)
Controls with diluent	144, 138, 140 (140)	117, 125, 123 (122)	146, 154, 151 (150)	109, 104, 106 (106)

[a] Refers to individual bottles, the means are in parentheses.

Comparison of the results of the two experiments show very close agreement as may be expected from plaque neutralization tests in a suitable system. They show clearly that antigenic differences (relative potencies of 4.6 or 4.3) were detected between D-2 and TH-36 when using TH-36 antiserum. However, D-2 antiserum did not show the same degree of difference (relative potencies of 1.5 or 1.9) and at first glance one might question the significance of the difference in this direction.

Discussion. At the same time that the tests reported here were carried out we made comparative tests with these same two closely related dengue viruses using immunodiffusion and immunoelectrophoresis (3,4). Both viruses, as well as several other types of dengue viruses, and some other group B arboviruses were shown to contain one or more common antigens, but TH-36 contained unique antigens as did also D-2, establishing substantial evidence of their antigenic differences as well as their relatedness. With present data it is difficult to relate neutralizing antibodies with precipitins, but based on other current studies in our laboratories they appear to be different. In any case it is obvious that in related group B arboviruses antigenic differences demonstrated by any of the many types of more discriminating neutralization tests show differences only by quantitative rather than by qualitative procedures. The present study using the plaque neutralization test to at-

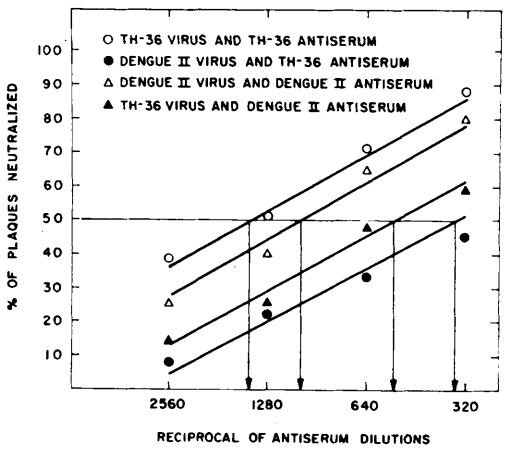

FIG. 2. Cross plaque neutralization with dengue 2 and TH-36 viruses and their antisera (Exp. 2).

TABLE III. Relative Potencies of TH-36 and Dengue 2 Hyperimmune Mouse Sera Versus the Homologous and Heterologous Virus.

System		Expt. I		Expt. II	
Antiserum	Virus	SDE[a]	RP[b]	SDE	RP
TH-36	TH-36	1650	4.6	1500	4.3
TH-36	D-2	370		350	
D-2	D-2	980	1.5	1040	1.9
D-2	TH-36	670		540	

[a] Serum dilution end point = the antiserum dilution neutralizing 50% of the plaques as determined by the dose response curve.

[b] Relative potency = SDE versus homologous virus /SDE versus heterologous virus.

tempt to differentiate between these two dengue viruses simply confirms that as previously suggested by complement fixation tests, specially with human sera from convalescents of primary infections (2), then definitely by immunodiffusion and immunoelectrophoresis (3,4), a significant difference can be shown by use of a quantitative plaque neutralization test. The differences shown by the TH-36 antiserum are quite substantial. Using D-2 serum the differences are less, but considered significant, since in eight different serum dilution–virus combinations (2 experiments, 4 serum dilutions each) a difference was shown and the direction of difference was consistently the same, evidence of excellent reproducibility.

The question again rises as to whether these differences demonstrated by several types of immunological tests constitute a basis for calling TH-36 a distinct type, type 5 having been suggested earlier (2), or whether this constitutes simply a strain difference among type 2 dengue viruses. As pointed out previously (3,4), many strains will have to be compared before such a decision can be made with any degree of reasonable certainty. Present classification of arboviruses by the extensive work of Casals and his associates utilizes only immunologic criteria (5). The original suggestion that TH-36 might represent another type was based in part on the suggestion that this and other agents apparently like it came from patients with hemorrhagic fever acquired in Bangkok and not from a case of classical clinical dengue fever, the source of D-2. Presently, there is no laboratory or other experimental basis to support a difference in biological activity between TH-36 and D-2, nor on the other hand, can such be ruled out. Thus, it appears wise to limit comparisons to antigenic analyses and to study them by quantitative and qualitative means. The experiments reported here add precision to previous quantitative comparisons and support the qualitative differences (3, 4).

Summary. Dengue type 2, New Guinea "C" virus, from a case of classical dengue fever was compared with dengue TH-36, an agent antigenically closely related to it but isolated from a case of hemorrhagic fever. Cross neutralization tests by the plaque method were performed in duplicate with essentially duplicate results. These were plotted and the 50% serum end points were determined. Relative potencies for homologous and heterologous serum–virus combination were calculated. Significant differences in degrees of neutralization of the two viruses were obtained, the homologous serum and virus resulting in the highest percentage plaque reduction at every serum dilution in each experiment. It is concluded that these two viruses differ antigenically as demonstrated in relatively precise quantitative tests, confirming previous differences shown by complement fixation, immunodiffusion, and immunoelectrophoresis.

1. Hammon, W. McD., Rudnick, A., Sather, G., Rogers, K. D., and Morse, L. J., Trans. Assoc. Am. Physicians **73**, 140 (1960).

2. Hammon, W. McD., Sather, G. E., and Rudnick, A., SEATO Med. Res. Lab. Monograph No. **2**, 30 (1961).

3. Ibrahim, A. N. and Hammon, W. McD. J. Immunol. **100**, 86 (1968).

4. Ibrahim, A. N. and Hammon, W. McD. J. Immunol. **100**, 93 (1968).

5. Casals, J., Trans. N. Y. Acad. Sci. **19**, 219 (1957).

Received Jan. 9, 1968. P.S.E.B.M., 1968, Vol. 128.