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Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1978, 21(4), p. 137-147
Version Type	VoR
URL	https://doi.org/10.18910/82563
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NEUTRALIZATION TESTS FOR DENGUE AND JAPANESE ENCEPHALITIS VIRUSES BY THE FOCUS REDUCTION METHOD USING PEROXIDASE-ANTI-PEROXIDASE STAINING¹

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(Received June 2, 1978)

SUMMARY Neutralization tests were made on 4 types of dengue (DEN) virus and Japanese encephalitis (JE) virus by incubation of serially diluted antisera and constant amounts of the viruses and then focus assay of surviving virus infectivity with peroxidase-anti-peroxidase (PAP) staining. Neutralization reactions were virtually completed in 2 hr on incubation of serum-virus mixtures at 28 C. A straight regression line was obtained on a probit chart by plotting the focus reduction rates at various dilutions of a given serum against the logarithm of the serum dilution used in the test. The slopes of the probit regression lines for the neutralization for DEN types 1 and 3 were similar, but differed somewhat from those for DEN type 2 and type 4. The slope of the line for JE virus was quite different from those for DEN viruses. Using these relations, the fifty percent focus reduction titer (FR₅₀) of neutralizing antibodies of a given serum could be estimated from the focus reduction rates at several dilutions of the test serum when the latter was between 25-75% of the value of the control.

INTRODUCTION

Dengue hemorrhagic fever is a very common and serious epidemic virus disease in South-east Asia caused by 4 different serotypes of dengue (DEN) virus (Hammon et al., 1960; Halstead, 1966). Japanese encephalitis (JE) virus is also endemic in this area (Miles, 1960) sometimes causing epidemics (Yamada et al.,

1971; Grossman et al., 1973). The hemagglutination-inhibition (HI) test is usually used routinely for the serodiagnosis and sero-epidemiology of these virus diseases. However, because of the high cross-reactivity between the 4 types of DEN viruses and JE virus, which are all group B arboviruses (Casals, 1957; Clarke and Casals, 1965), it is usually difficult, and sometimes impossible, to determine type-specific antibodies against these viruses in areas where multiple group B arbo-

¹ A part of this work was presented at the 25th Annual Meeting of Society of Japanese Virologists held in Osaka, October 1977.

viruses, the flavivirus of *Togaviridae* (Melnick, 1974), coexist. Although the neutralization (N) test is considered to be the most type-specific of all serological reactions (Hammon and Work, 1964), the standard N test, involving inoculation of suckling mice, requires a relatively long incubation time before results can be obtained and furthermore the number of the specimens tested is limited by the supply of test animals and the space available for animal experiments. Even the more recent applications of tissue culture techniques to the N test in the challenge virus resistance (Halstead et al., 1964) and plaque reduction (Russell et al., 1967) methods require a fairly long incubation time especially for DEN viruses.

Previously we reported a rapid method for titration of DEN virus type 4 infectivity by counting foci of infected BHK21 cells stained by the indirect fluorescent antibody (FA) or peroxidase-anti-peroxidase (PAP) technique (Igarashi and Mantani, 1974; Okuno et al., 1977). Since the PAP technique was found to be more useful than FA especially for testing many specimens at the same time, we tried to adapt this method for use in N tests on all 4 types of DEN viruses and JE virus in routine serodiagnosis or epidemiological survey of type-specific antibodies against these viruses in such areas as Southeast Asia, where multiple flavivirus infections are prevalent.

MATERIALS AND METHODS

1. *Viruses*

Dengue viruses, 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 have been described (Igarashi, 1978). The viruses were propagated in suckling mouse brain. Japanese encephalitis (JE) virus, Nakayama strain, was obtained from Kanonji Institute, the Research Foundation for Microbial Diseases, Osaka University, and was propagated in adult mouse brain. Infected mouse brains were stored at -70°C until use. Seed viruses

were prepared from 10% homogenates of infected brains in Eagle's minimal essential medium (MEM, Eagle, 1959) containing 0.25% bovine plasma albumin fraction V (Armour, Ill. USA). The homogenates were clarified by centrifugation at 10,000 rpm for 30 min and the supernatants were divided into aliquots and stored in sealed ampoules at -70°C .

2. *Cells*

The BHK21 cells used for the focus assay of virus infectivities were grown with MEM containing 10% calf serum. The cells were seeded at 10^5 cells/0.4 ml/chamber on Lab-Tek 8-chamber slides (Miles, Ill. USA). Monolayers were ready for use after one day's incubation in a 5% CO_2 atmosphere at 37°C .

3. *Antisera*

Antisera against dengue viruses were prepared in rabbits by two intramuscular inoculations with one month interval between inoculations, using Freund's complete adjuvant and partially purified virus prepared from infected suckling mouse brain by protamine sulfate clarification and ultracentrifugation (Smith et al., 1970). Rabbits were bled 10 days after the second immunization.

Anti-JE rabbit serum and anti-rabbit IgG sheep serum were kindly supplied from Kanonji Institute, the Research Foundation for Microbial Diseases, Osaka University.

Anti-peroxidase (PO) rabbit serum was prepared as described before (Okuno et al., 1977) using horseradish peroxidase (PO), type VI, obtained from Sigma Chemicals, Mo. USA.

Fluorescein isothiocyanate-conjugated anti-rabbit IgG goat serum was obtained from Hyland Division, Traverol Co. Calif. USA.

Twenty nine individual human serum specimens were collected from participants in various training courses operated by the Japan International Cooperation Agency (JICA). They were taken from subjects of between 24 to 44 years old who were staying at Osaka International Training Center, Ibaraki City, Osaka.

4. *Preparation of soluble peroxidase-anti-peroxidase (PAP) complex*

Soluble PAP complex was prepared by the method of Sternberger et al. (1970) with slight modifications. The antibody titer of anti-PO rabbit

serum was determined by the quantitative precipitation reaction (Dougherty et al., 1972). Five milliliters of anti-PO rabbit serum was mixed with an equal volume of PO solution in saline containing an equivalent amount of PO (1.25 mg). After one hour's incubation at room temperature, the antigen-antibody complex was precipitated (10,000 rpm, 20 min, Sorvall SS-34 rotor), washed 3 times with 10–15 ml volumes of saline by centrifugation and suspended in 2 ml of saline. Five milligrams of PO in 5 ml of saline was added and the pH was adjusted to 2.3 with 0.1 N HCl to dissolve the precipitate. Then 2 ml of PBS was added and the solution was adjusted to pH 7.0–7.4 with 0.1 N NaOH to obtain the PAP complex. An equal volume of saturated ammonium sulfate was added to the solution, and after standing the mixture for one hour at room temperature, the PAP complex was precipitated by centrifugation (15,000 rpm, 15 min) leaving uncombined PO in the supernatant. Precipitation with ammonium sulfate was repeated once more to remove uncombined PO completely, and the resulting precipitate was dissolved in 10 ml of saline and dialyzed against 3–5 liters of PBS for 2 days at 4 C. The small amount of insoluble material formed during dialysis was removed by centrifugation (2,700 rpm, 10 min), and the concentrations of total protein and PO in the supernatant were determined by measuring E_{280} and E_{403} , respectively. (The values were usually about 0.8 and 0.3 respectively.) Then the solution was adjusted to $E_{280}=0.2$ with PBS and stored at 4 C as the stock solution of PAP complex.

5. Infectivity assay of the viruses by focus counting after staining by the PAP technique

The virus specimen was inoculated onto BHK21 cells in 8-chamber slides (0.1 ml/well). After virus adsorption for 2 hr. the cells were incubated in MEM at 37 C under 5% CO_2 for various periods. Then the cells were washed with PBS and fixed with acetone (4 C, 20 min). Fixed cells were stained successively at room temperature for 40 min each with the first serum (anti-DEN or JE rabbit serum diluted 1:1000), the second serum (anti-rabbit IgG sheep serum diluted 1:50) and the third serum (PAP complex diluted 1:20). After each serum treatment, the specimens were washed with 3 changes of PBS for 5 min each. Then virus antigens in infected cells were stained by the method of Graham and Karnovsky (1966) using

3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemicals, Mo. USA) and H_2O_2 . After the reaction, the cells were washed 3 times with PBS, sealed with glycerin and examined under an ordinary light microscope. Fluorescent antibody staining was carried out by the indirect method.

6. Kinetic neutralization

Virus and serum samples were diluted with MEM containing 2% calf serum. For neutralization tests, sera were inactivated by heating at 56 C for 30 min. Virus specimens containing about 10^5 focus forming units (FFU)/0.1 ml were mixed with an equal volume of suitably diluted antiserum and incubated at various temperatures. Virus controls were set up by mixing the virus specimen with diluent only and were run in parallel with the test specimens. After various times of incubation, samples of 20 μ liter of the reaction mixture were diluted with 2 ml of diluent in an ice bath to stop the neutralization reaction, and the specimens were further diluted 10 times. Samples of 0.1 ml of the diluted specimens were inoculated into each well of 8-chamber slides containing BHK21 cell monolayers to assay the surviving virus infectivity as described above. The survival ratio of the infective virus was plotted on a semi-logarithmic scale against the incubation time of the serum-virus mixture.

7. Determination of neutralization (N) antibody titers

Virus specimens containing about 200 FFU/0.1 ml were mixed with equal volumes of serially diluted test sera, which had been heat-inactivated, and the mixtures were incubated at 28 C for 2 hr. Virus controls were incubated in parallel. Then the serum-virus mixtures were cooled in an ice bath and the remaining virus infectivity was assayed as described above. The focus reduction rate was calculated by dividing the difference between the numbers of foci in the control and the test sample by the number of foci in the control. The percentage focus reduction rate was transformed into its probit (Y) using Bliss' Table in the book of Fisher and Yates (1953). Fitting of a linear probit regression line ($Y=a+bX$) to the observed probit (Y) of the focus reduction rate observed at various serum dilutions (where X is the logarithm of the serum dilution) was performed as described by Goulden (1952).

8. Hemagglutination-inhibition (HI) test

The method of Clarke and Casals (1958) adapted to microtiter systems (Sever, 1962) was followed using antigens extracted from infected mouse brains with sucrose-acetone. Test sera were extracted with kaolin (acid-washed American standard, Fisher Scientific Co. N. J., USA) and were absorbed with goose red blood cells.

RESULTS

1. Determination of the optimum incubation time for focus assay of virus infectivity

First the optimum incubation time of BHK 21 cells for focus assay of virus infectivity was examined. Replicate cultures of BHK21 cells on 8-chamber slides were inoculated with diluted stock DEN-4 virus. Specimens were harvested after various times of incubation at 37 C and the foci of infected cells were stained by the PAP and FA techniques. Infected cells were stained as paired cells 16 hr after inoculation by either technique. The increase in the number of foci is shown in Fig. 1. In this experiment groups of more than 4 stained cells were recorded as foci. Foci appeared 24 hr after inoculation, and increased in number to a plateau after 36 hr, subsequently increasing only in size for up to 48 hr after inoculation. Then the enlargement of existing foci and the appearance of secondary foci made it impossible to count the actual number of primary foci. Thus 2 days (44-48 hr) of incubation was an appropriate time for focus assay of DEN-4 virus infectivity. Similar results were obtained by the PAP and FA technique, confirming previous results that essentially the same dose-response relationships between virus concentration and the number of foci were obtained by these two methods (Okuno et al., 1977). The appropriate incubation times for focus assay of other types of DEN viruses and JE virus were determined similarly: suitable times were one day (20-24 hr) for JE, 2 days (36-40 hr) for DEN-2, and 3 days (66-72 hr) for DEN-1 and DEN-3 viruses.

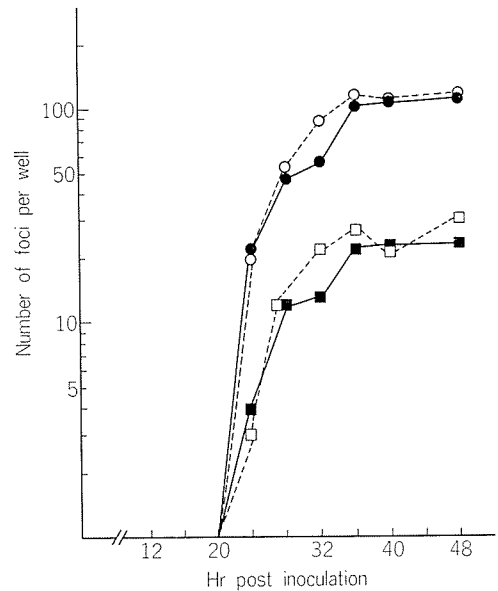


FIGURE 1 Development of foci of BHK21 cells infected with DEN-4 virus as measured by the PAP and FA techniques. Replicate cultures of BHK21 cells prepared in 8-chamber slides were inoculated with stock DEN-4 virus diluted 5×10^4 -fold (■—■, □.....□), or 10^4 -fold (●—●, ○.....○). After various times of incubation at 37 C, specimens were harvested and stained with either PAP (■—■, ●—●), or FA (□.....□, ○.....○) to detect foci of infected cells. Groups of more than 4 stained cells were recorded as foci. Values are averages for 4 replicate wells.

2. Neutralization kinetics

The optimum temperature and time of incubation of serum-virus mixtures for assay of neutralization (N) antibody titers were determined by kinetic studies. When virus controls of DEN-2 virus without any antiserum were incubated at 37, 32, and 28 C for 120 min, virus infectivity was reduced to less than 20% 30% and 70-80%, respectively, of the titer before incubation (Fig. 2). Therefore, an incubation temperature of 28 C was used in further N tests. Results on the kinetics of neutralization of DEN-2 virus (Fig. 2) indicate that the neutralization reaction was almost completed in 120 min in serum-virus mixtures

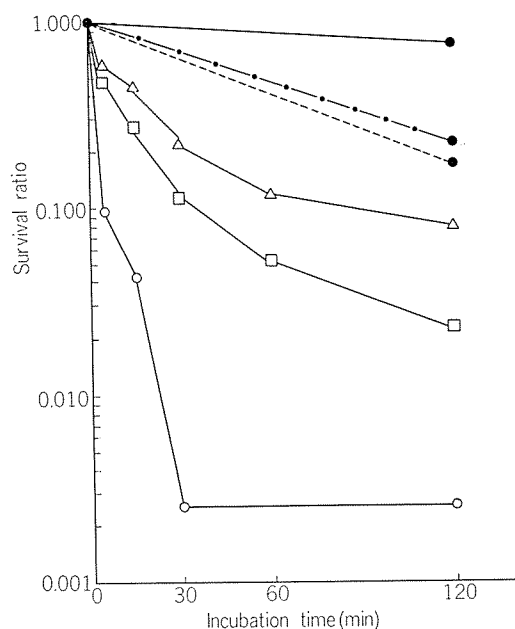


FIGURE 2 Kinetics of neutralization of DEN-2 virus. Stock DEN-2 virus containing about 10^5 FFU/0.1 ml was mixed with an equal volume of anti-DEN-2 rabbit serum diluted 1:25 (○—○), 1:50 (□—□), or 1:100 (△—△), and incubated at 28 C. Control specimens were incubated with virus diluent only (●—●). After various times of incubation, 20 μ liter samples of the mixture were diluted 1:100 and 1:1,000 with the diluent in an ice bath. The infectivity of the surviving virus was assayed by the focus counting method as described in the Materials and Methods. Survivals of the control specimen at 32 C (●—●) and at 37 C (●—●) are also shown.

at 28 C. The kinetics of neutralization of other types of DEN viruses and JE virus was studied similarly, and their neutralizations followed almost the same course as that of DEN-2 virus. From these results, incubation of the serum-virus mixtures at 28 C and for 2 hr were selected as the standard conditions for determination of N antibody titers of test sera.

3. Determination of neutralization (N) antibody titers of the sera

Serially diluted human and rabbit sera were

incubated with a constant amount of DEN-2 virus at 28 C for 2 hr and then the residual virus infectivity was assayed. When the percent focus reductions observed at various dilutions of a given serum were plotted on a probit chart against the logarithm of the serum dilutions, the points fell approximately on a straight line. A probit regression line ($Y = a + bX$) was fitted to these points as described in the Materials and Methods, where Y is the probit focus reduction, and X is the logarithm of the serum dilution. The slopes (b) of the probit regression lines for human and rabbit sera were almost the same ($b = -2.381$ and $b = -2.595$, respectively) as shown in Fig. 3. The fifty percent focus reduction titer of N antibody (FR_{50}) was defined as the serum

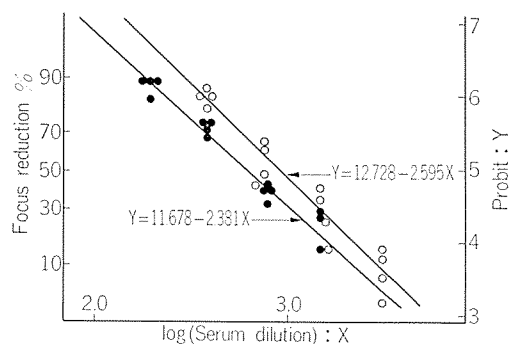


FIGURE 3 Neutralization of DEN-2 virus with human and rabbit antisera. Stock DEN-2 virus was diluted to about 200 FFU/0.1 ml and mixed with an equal volume of human (●—●) or rabbit (○—○) antiserum diluted serially 2-fold. The control virus specimen was mixed with diluent only. After 2 hours' incubation at 28 C, infectivity of the surviving virus in each specimen was assayed in 4 replicate wells on 8-chamber slides. The focus reduction rate was calculated by dividing the difference between the numbers of foci in the control specimen and the test specimen by the number of foci in the control. The resulting values were plotted on a probit chart against the logarithm of the serum dilution (X). Linear regression lines ($Y = a + bX$) were fitted by the method of Goulden (1952) after transforming the focus reduction rates into their probits (Y).

dilution that reduces the focus number to 50% of the control without any antiserum. To estimate the FR₅₀ titer of a given serum, we have to find the point of 50% focus reduction on the probit regression line (or 5.0 of the probit shown on the ordinate) and the X value of the point on the abscissa will give the logarithm of FR₅₀, which can then be transformed into its antilogarithm (643 for human and 951 for rabbit serum in Fig. 3), which is the titer of FR₅₀.

Similar probit regression lines were obtained for human sera from persons coming from Southeast Asia, when N tests were performed against DEN-1, DEN-3, DEN-4, and JE viruses (Fig. 4).

The probit regression lines were obtained by this procedure for 8 individual human sera collected from persons coming from Southeast Asia. The lines obtained for neutralization of DEN-2 virus are shown in Fig. 5. The slopes (b values) of these lines were rather similar,

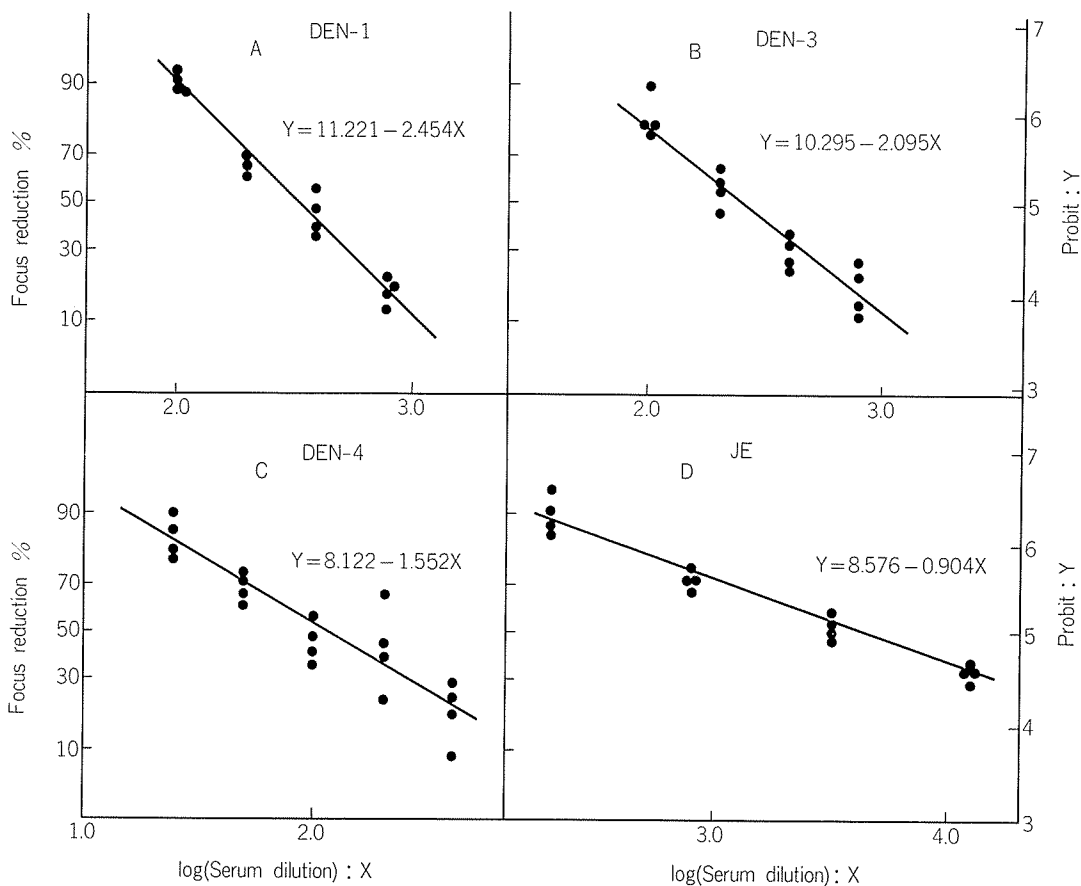


FIGURE 4 Neutralizations of DEN-1, DEN-3, DEN-4, and JE virus by human serum preexposed to dengue and JE virus. Stock DEN-1 (A), DEN-3 (B), DEN-4 (C), and JE (D) viruses, each containing approximately 200 FFU/0.1 ml were mixed with equal volumes of serially diluted human sera obtained from persons coming from Southeast Asia. Control virus specimens were mixed with diluent only. Specimens were incubated at 28 C for 2 hr and the focus reduction rates were plotted as described in the Materials and Methods and in the legend to Fig. 3.

ranging from -2.075 to -3.000 . The average of these 8 regression lines was calculated and is shown as a broken line with a slope of $b = -2.536$ in Fig. 5. Probit regression lines were obtained for other types of DEN viruses and JE virus and the averages of these lines for each virus were calculated similarly. The slopes (b value) of these average regression lines were compared with the arbitrary value of "a" (Fig. 6). The slopes for DEN-1 (-2.285) and DEN-3 (-2.280) were almost the same, but differed somewhat from those

for DEN-2 (-2.536) and DEN-4 (-1.737). The slope for JE (-0.823) was quite different from those for DEN viruses. On the other hand, the slope of the regression lines did not vary significantly for a given virus with various human or rabbit sera (data not shown).

Assuming that the results of all N tests of human sera against DEN and JE viruses fall on such probit regression lines having the same, or very similar, slopes as the b-value shown in Fig. 6, the FR_{50} titer of a given human serum could be estimated from the

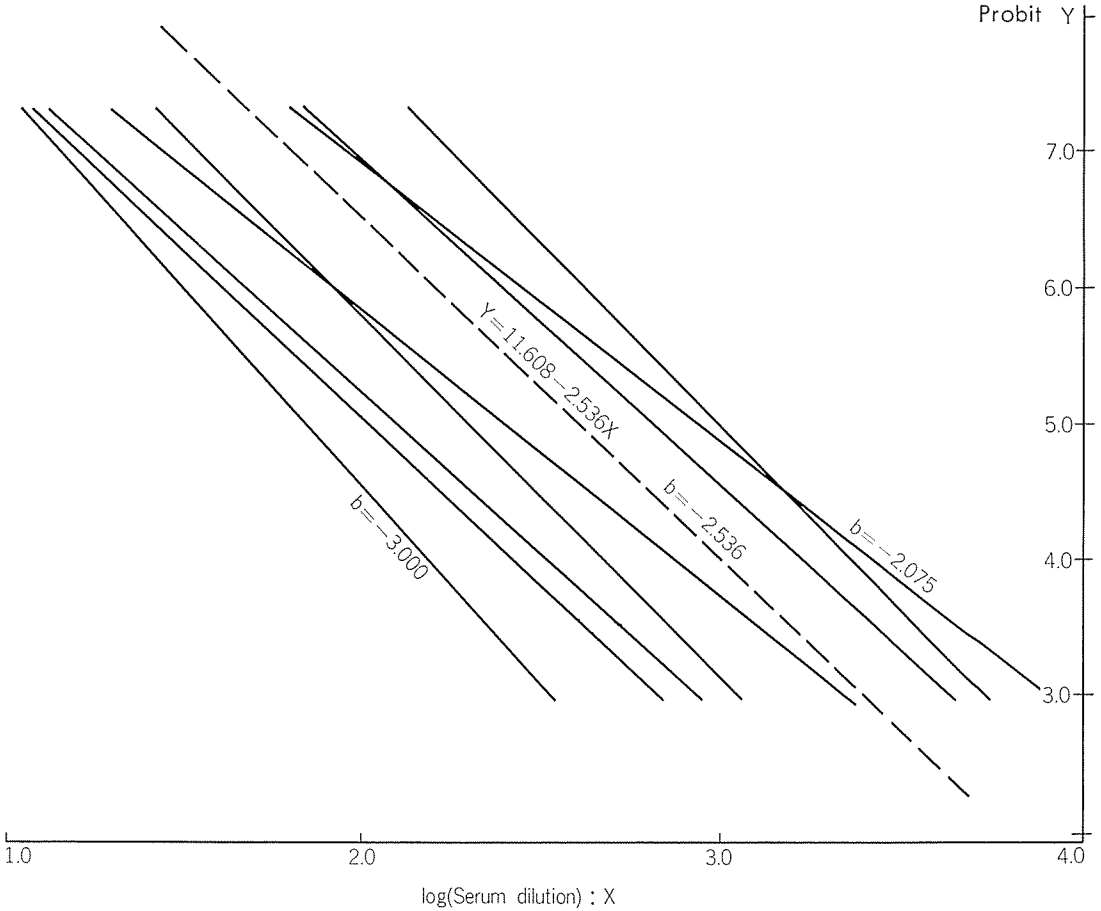


FIGURE 5 Slopes of linear probit regression lines of several human sera against DEN-2 virus. N-tests against DEN-2 virus were performed on 8 individual human sera from persons coming from Southeast Asia, as described in the Materials and Methods and in the legend to Fig. 3. The broken line shows the average of the 8 regression lines.

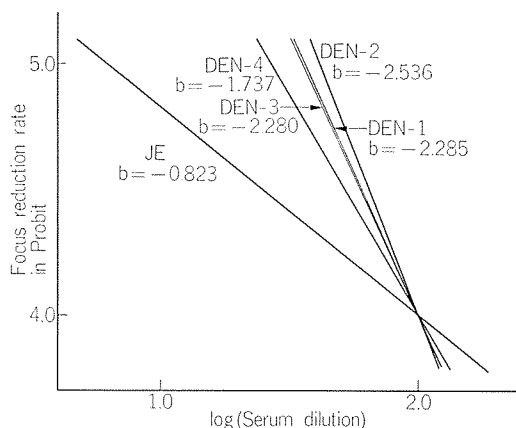


FIGURE 6 Slope variation of the probit regression lines for DEN and JE viruses obtained with human sera. N-tests were carried out against each of the 4 types of DEN viruses and JE virus using several of the human sera used in the experiments shown in Fig. 5. Probit regression lines were calculated for each virus and serum. Average regression lines were calculated for each virus as shown in the legends to Figs. 3 and 5, and they were represented in arbitrary values of "a" to show the variation of their slopes (b-value).

focus reduction rates at several dilutions of the serum using the following equations:

$$m = \frac{5-Y}{-2.285} + X \text{ for DEN-1;}$$

$$m = \frac{5-Y}{-2.536} + X \text{ for DEN-2;}$$

$$m = \frac{5-Y}{-2.280} + X \text{ for DEN-3}$$

$$m = \frac{5-Y}{-1.737} + X \text{ for DEN-4;}$$

$$m = \frac{5-Y}{-0.823} + X \text{ for JE}$$

where m is the logarithm of the serum dilution giving 50% reduction, Y is the probit corresponding to the reduction % obtained in the experiment, and X is the logarithm of the serum dilution used in the experiment. The FR_{50} titer is obtained by transforming the m into its antilogarithm 10^m . These equations are valid when the focus reduction rate observed is between 25 to 75% of the control.

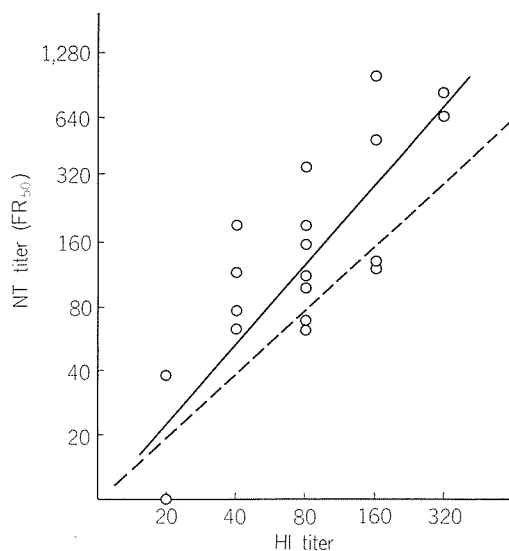


FIGURE 7 DEN-2 HI and N antibody titers of human serum specimens. The DEN-2 antibody titers of 29 individual human serum specimens were tested by the HI and N tests. The latter test was performed using the method described in the text with several dilutions of the sera. Each point represents the value for an individual. The straight broken line is the line expected if there is a 1:1 relationship between the HI and N antibody titers. The straight solid line was obtained by the least square method.

When the focus reduction rate is higher or lower than this range, another N test must be made with a higher or lower dilution of the test serum giving a reduction rate of 25–75%. To estimate the FR_{50} titer easily without calculations, we have prepared tables and graphs for each of the 4 types of DEN viruses and JE virus for use in later N tests. These tables and graphs are available on request.

4. An example of FR_{50} assay of human N antibody titers against DEN-2 virus

Human sera were obtained from persons coming from various countries, as shown in the Materials and Methods, and their DEN-2 antibody were assayed by the HI and N tests. In general, when a given serum showed a high

HI titer, its N titer was also high (Fig. 7), but considerable differences were observed among the N antibody titers of several sera with the same HI titer. Thus the HI titer does not always reflect the N titer, that is the type specific antibody titer, against DEN-2 virus. All persons coming from Southeast Asia had DEN-2 HI and N antibodies, while 10 people from Africa, the Far East and South America did not. Although the number of test specimens was too small to draw any definitive conclusion, these results may reflect the high prevalence of dengue viruses in Southeast Asia, and also indicate the value of the N test for serological diagnosis and epidemiological survey in this area.

DISCUSSION

The principle of plaque titration of dengue virus (Schulze and Schlesinger, 1963) has been applied in the neutralization test for assay of anti-dengue antibodies by the plaque reduction method (Russell, et al., 1967). This method has been useful in studies on the biology, epidemiology and laboratory diagnosis of dengue virus. However, dengue viruses are among the most difficult viruses to assay for infectivity and the usual plaque titration requires a fairly long incubation time. Thus the results are often affected by accidental microbial contamination or nonspecific cellular damage. Microadaptation of the plaque assay (Sukhavachana, Yuill and Russell, 1969; Fujita et al., 1975) reduces the incubation time slightly, but since only a few plaques can be counted in a single well on a microtray, test specimens must be diluted carefully at many dilutions to obtain appropriate numbers of plaques.

In the focus counting methods by the FA (Igarashi and Mantani, 1974) and PAP techniques (Okuno et al., 1977), 200 foci can be counted in each well on a 8-chamber slide with reasonable accuracy and there is a linear relationship between the virus concentration and the number of foci. Thus on 10-fold serial

dilution of the virus specimen, 20 to 200 foci are usually observed per well, which allows a fairly good estimation of virus infectivity. As mentioned previously (Igarashi and Mantani, 1974), this technique has several advantages: (1) Foci of cells containing virus antigens can be detected sooner than plaques, which involve drastic damage of infected cells; an incubation period of at most 3 days is required for focus assay, and thus the chance of microbial contamination is greatly reduced. (2) All 4 types of dengue viruses can be assayed after an appropriate incubation time. (3) Since this method is a microtechnique and amounts of cells, medium and incubator space required are only less than one-tenth of those used in the ordinary plaque assay.

However, the technique has some drawbacks. One disadvantage is that antiserum against each virus with good staining properties must be prepared in advance. However, our preliminary results showed rather strong cross reactions between the staining titers of homologous and heterologous DEN viruses: for example, anti-DEN-1 rabbit serum could stain the foci of cells infected with other types of DEN viruses. Moreover, anti-dengue rabbit sera prepared in our laboratory could be diluted more than 1,000-fold for use as the first serum in PAP staining, and thus very potent antiserum, once prepared, could be used for a very long time. Furthermore, the PAP complex is easy to prepare and its antibody titer and enzymatic activity do not decrease on storage at 4°C for more than one year. A second difficulty in the focus counting method is observation of the specimen under a microscope to record the numbers of foci. However, the reaction product of the peroxidase is stable and the stained specimens can be kept at room temperature until there is time to count them. Moreover, infected cells on 8-chamber slides can be stored at -20°C after fixation with acetone, and so several stored specimens harvested on different days could be stained at the same time. A third drawback of the method is plaque isolation of

some virus clones. Some other device such as limiting dilution of the specimen should be considered for this purpose (Lubinski et al., 1973).

Using the linear relationship between the focus reduction rate and the logarithm of the serum dilution (X) on a probit chart, or the probit (Y) of the focus reduction rate and X on an arithmetic scale, convenient method was presented for estimating the FR_{50} titer of N antibodies for each of the 4 types of DEN viruses and JE virus from the focus reduction rates at several dilutions of the serum. Provided that the same virus and host cell system are used, these relationship are valid for estimation of FR_{50} titers, based on the assumptions mentioned in the text. Estimation of the N antibody titers of JE virus from the plaque reduction rate at a certain dilution of the serum has been used (Oya et al., 1967). With JE, however, values of the plaque reduction rate were used rather than its probit. When our results on focus reduction rate were plotted on an arithmetic scale against the logarithm of the serum dilution, the regression line tended to be sigmoidal at high and low percentages of focus reduction, and the results fitted a straight line better when the latter was transformed into its probit or when it was plotted on a probit chart. Both methods are

valid provided the linear portion of the sigmoid dose-response curve is used. However, the slope of the regression line reported for the plaque reduction method of JE virus appears to differ significantly from the b-value shown in Fig. 6. The difference may be mainly attributable to the difference in the assay systems.

The focus reduction method using the PAP staining technique and its application for FR_{50} estimation as described in this paper will be valuable in serodiagnosis and seroepidemiological studies of dengue and JE virus infections. The principle of this method can also be applied to other virus systems in which it is difficult to titrate virus infectivity or N-antibody (Smith, et al., 1973) by ordinary methods and this method should be useful when rapid results are required (Katzenelson, 1976).

ACKNOWLEDGMENTS

We are very grateful to members of the Research Foundation for Microbial Diseases for generous supply of antisera, and to the people in Osaka International Training Center who provided blood specimens. Mr. T. Masuyama and Mr. T. Yamamoto participated in this work as medical students.

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