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NEUTRALIZATION OF DENGUE VIRUS BY IgM AND IgG ANTIBODIES

BY

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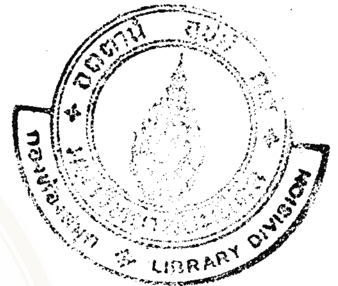

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I, Miss Chinda Charupat hereby submit this thesis of "NEUTRALIZATION OF DENGUE VIRUS BY IgG AND IgM ANTIBODIES" for the Master Degree to the Faculty of Post Graduate Studies, University of Medical Sciences.

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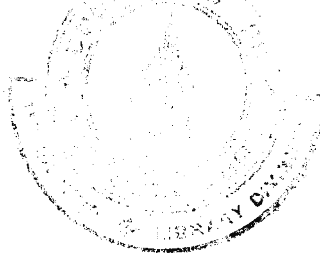

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Introduction

Dengue viruses are members of Casal's group B arboviruses (1) and are the etiological agents of undifferentiated fevers, classical dengue fever, dengue hemorrhagic fever and dengue shock syndrome. Four distinct serotypes of dengue viruses are recognized. The prototype strains are Dengue-1 (Hawaii), Dengue-2 (New Guinea "C"), Dengue-3 (H-87) and Dengue-4 (H-241) (2,3). The four type of dengue viruses are antigenically differentiable but are very closely related. Antigenic relationship between the dengue serotypes are sufficiently different that infection with one dengue type does not confer lasting resistance to infection by the other serotypes. (2,4).

All four dengue serotypes have been etiologically associated with dengue hemorrhagic fever as well as milder illnesses. The reasons why dengue viruses in some instances produce epidemics of severe illness and in other instances epidemics of benign disease are as yet unknown. Several hypothesis have been proposed to explain this observation. One hypothesis proposes that differences in virulence among viruses within the same serotype explains the phenomenon (4). However, the hypothesis which is best supported by epidemiologic observations proposes that the severe forms of disease with manifestations of dengue hemorrhagic fever are prodiced by secondary dengue infections in an individual who has previously experienced a primary dengue infection with a different serotype (4). This hypothesis implies that an immunologic mechanism is involved in

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the pathogenesis of the disease. Further explanation of this phenomenon will require detailed knowledge of the interactions between dengue viruses and immunoglobulins.

Three types of serological tests are useful for measuring anti-dengue antibodies. They are Hemagglutination Inhibition (HI) test, Complement Fixation (CF) test and Neutralization (N) test (5). The HI test is a sensitive method but its usefulness is limited by entensive cross reactivity within the dengue group and with other group B arboviruses. The CF test is more specific but much less sensitive. Neutralization tests have a higher degree of specificity than either the HI or CF tests and, when carried out by a quantitative method using a plague reduction technique, are also highly sensitive (6).

In man, three major types of immunoglobulins exist which have antibody activity (7). The recent studies of Mellors et al indicate that plasma cell, primitive reticular cells and other cells of the germinal centers of the lymphoid tissue are capable of synthesizing all three immunoglobulins (7). Immunoglobulins are not restricted to the serum, but may be found in the other body fluids or tissues, such as urine spinal fluid, lymph node, spleen etc. The major immunoglobulins in serum are designated IgG, IgA, IgM. They can be differentiated by their sedimentation rates as 19S (IgM) and 7S (IgA and IgG). The classification of immunoglobulin is based on the fact that these molecules differ from one another in size and electrophoretic mobility. The molecular weights of the 19S and 7S

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immunoglobulins are approximately 1,000,000 and 160,000 respectively. Each of the immunoglobulin classes has a base molecule consisting of two pairs of symmetric peptides joined by disulfide bonds into an ovoid macromolecule. The base molecules are composed of two general type of peptide units "heavy" chain and "light" chain. IgM is inactived by reduction with 2-mercaptoethanol but IgG is resistant to reduction by 2-mercapto ethanol (8). IgA is less sensitive to reduction than IgM.

The antiviral activities of the immunoglobulins have been studied in several host-virus systems. Immunization of human infants with live attenuated poliomyelitis vaccine resulted in IgM (19S) antibody in first two weeks. By eight weeks the 19S had disappeared and replaced by 7S (IgG)(9). IgA antibodies appear during the interval between the appearance of IgM and IgG. Immunization rabbits with poliovirus results in 19S antibody alone in the first 2-3 days sera, on day 3 and 4 7S antibody was also detectable (10).

Studies with inactivated measles virus in guinea pigs shows that the early response to primary infection was production of 19S antibody alone. A 7S antibody component was detected in the late phases of primary response. The secondary response to the same antigen was characterized by a rapid increase in 7S antibody production and by the absence of 19S antibody (11).

Experimental infection of guinea pigs with Japanese encephalitis and Russian-Spring summer encephalitis virus showed that the earliest detectable HI and N antibodies at 7-10 days were associated with 19S

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globulin. After 14 days antiviral activity was found in 7S globulin (12). The complement fixing antibodies were associated with 7S globulin at various times over a 4 month and there was no evidence of early 19S complement fixing antibody (12).

Thus in all of the systems studies a primary virus infection resulted in a similar response i.e. are early 19S (IgM) response followed by a 7S (IgG) response.

Dengue virus can produce inapparent infection in certain species of monkeys (2). The immunologic response of monkeys to experimental dengue-2 Virus infection was studied to determine the time of appearance and specificity of 7S and 19S antibodies fraction for 2 month after infection. Monkeys with primary infection had no detectable antibodies for 6 days post infection by showing peak titers. At day 14 a large 19S components was presented and by day 60 all of antibodies were 7S type. HI antibody titers of 19S and 7S antibodies were approximately equal at 14 days. In the monkeys with a secondary infection, the HI and N antibody in day 6 were 7S globulins. Subsequently a rapid rise in 7S antibody to high levels was observed with little or no 19S antibody detected (13).

6

Serum from human cases of dengue hemorrhagic fever have been studied by density gradient centrifugation and gel filtration methods to determine the type and time of appearance of the immunoglobulins produced by primary and secondary infection. Thai children with primary dengue infection also have an early 19S antibody response followed by 7S antibody response (14). Patients with dengue shock

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syndrome have secondary type antibody response with a very rapid rise of 7S antibodies activity and little or no 19S antibody activity (14).

19S immunoglobulins were found to have HI antibody activity but no CF antibody activity. The HI antibody of 19S fractions was destroyed by reduction with 2-mercaptoethanol (15), 7S globulin which are resistant to reduction by 2-mercaptoethanol had both HI and CF antibody. Under optimum in vitro conditions it was shown that 19S antibody was capable of blocking the CF reaction between homologous antigens and antisera. This blocking action was shown to be specific for the homologous system (16).

The studies referred to above indicate major differences in anti-viral activity between IgM and IgG antibody. Perhaps the most important aspect of anti-viral action of immunoglobulins is their virus neutralizing activity. This, however, has not yet been investigated with respect to anti-dengue IgM and IgG immunoglobulins. Since understanding of the immunue mechanisms and the pathogenesis of dengue hemorrhagic fever requires a thorough knowledge of these antibody activities such investigations appear necessary.

The purpose of the experimental work presented in this thesis was to study the anti-dengue neutralizing properties of IgG and IgM antibodies, using experimentally infected monkeys as a source of anti-dengue immunoglobulins.

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Materials and Methods

Virus

Dengue-1 virus (strain 12900) used in the following experiments was isolated from the sera of a dengue fever patient in Thailand in 1964. The virus was isolated in both BS-C-1 cellculture and suckling mice and proven to be a strain of dengue-1 by utilizing reference monkey antiserum and a plague reduction neutralization test for the identification (17) in the virology Department, SEATO Medical Research Laboratory and Department of Microbiology, Faculty of Public Health University of Medical Sciences, Bangkok, Thailand.

Monkeys

Five 8-10 month old *Macaca irus* were used in these experiments. All were tested and found to be free from HI antibody and N antibodies to dengue viruses. These monkeys were designated V129, V130, V132, V133, V152.

Mice

One to two day old suckling albino Swiss mice were used for preparation of CF and HI antigens. These mice were free of known enzootic mouse diseases.

Tissue culture

The LLC-MK_2 strain of rhesus monkey cells used was originally obtained from Dr. Robert N. Hull, Biological Research Division, Eli Lilly corporation (18). The growth media were modified in Virology Department, SEATO Medical Research Laboratory and Department

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of microbiology, Faculty of Public Health University of Medical Sciences, Bangkok, Thailand (19). Stock LLC-MK_2 cell cultures were propagated in the growth-media (Table 1) in one litre Blake bottles. The 4-7 days old cell cultures were removed from glass surface with trypsin versene mixture containing 0.2% and 0.002% versene, and were then suspended in four times of the orinigal volume of growth media and distributed into 4 Blake Bottles. One ounce prescription bottles seeded with 4ml of cell suspension containing 10^5 cells/ml and used after incubation at 37°c for 4-5 days.

Preparation of virus seeds.

Four day old culture of LLC MK_2 cell in one ounce bottle were inoculated with 0.3 ml of 3rd BSC-1 passage dengue-1 (12900). After a two hours adsorption period, fur ml of maintenance media (Table 8) were added to each bottle and inocubated at 37°C. Cell sheets were removed on the 10th day by trypsin - versene treatment, and suspended in 4 ml of M199 + 50% inactivated fetal bovine serum, frozen and thawed 3 times, and centrifused at 12,000 rpm for 30 min. at 4°C. One ml aliquots of virus seeds were distributed into screw capped vials, quickly frozen and stored at - 70°C.

Virus assay methods

Infectivity titrations were performed by making ten-fold dilutions of seed viruses (10^{-1} to 10^{-7}) in virus diluent solution (Table 8). 0.3 ml of each dilution was inoculated into four-day old cell cultures in one ounce bottles and allowed to adsorb at 37°C for six days, a second overlay containing neutral red stain (Table 4) was added.

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The number of plaques were counted on the following day.

Determination of level of viremia in infected monkey serum was similarly done.

Plaque Reduction Neutralization Test (PRNT)

Serum or serum fractions were inactivated at 56°C for 30 minutes in water bath. Serial two-fold dilutions from 1:10 to 1:640 of serum or serum fraction were made on virus diluent solution and mixed with equal volume of virus suspension containing an estimated 50 to 75 plaque forming units (pfu)/0.15 ml. Virus-serum mixtures were incubated at 37°C in the water bath for 30 minutes. Growth medium was aspirated from cell cultures and the cell sheets washed with 2 ml. of Hank's Balance Salt Solution (Table 7). The washing fluids were aspirated and 0.3 ml. of each dilution of the virus-serum mixture was inoculated into three replicate bottles. Three bottles were used as control: each was inoculated with 0.3 ml. of the test virus dose in normal monkey serum diluted 1:10. Inoculated bottles were rocked well on a rocking machine at 37°C for 1 1/2 hours. The inoculum was removed the first overlay media added, and the cultures incubated at 37°C for six days. On the sixth day the 2nd agar overlay containing neutral red was added.

The culture were incubated at 37°C for 18 hours and the number of plaques counted. Fifty percent plaque reduction end points were estimated by method of Cutchins (20). The arithmetic means of the three plaque counts at each serum dilution and control bottles were

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calculated. The percent of control of each dilution of serum or serum fraction were plotted on the log-probit paper. The best fitting straight line was determined by visual inspection and the fifty percent plaque reduction end point read from the graph.

Kinetic neutralization test by plaque technique

IgM or IgG containing fractions were diluted to four times the concentration of percent plaque reduction titer. Dengue virus strain 12900 P-5 was diluted to contain 50 to 75 pfu/0.15 ml. The virus and antibody was mixed in equal volumes incubated at 37°C water bath. Controls without antibody were similarly treated. At intervals of 1, 5, 10, 15, 20, 25, and 30 minutes, aliquots from the experimental and control tubes were pipetted into three replicate bottles each of washed LLC-MK_2 cells. Each bottle was inoculated with 0.3 ml of virus-antibody mixture or virus control. Virus was allowed to adsorb at 37°C for 1 1/2 hours. The inoculum was aspirated by vacuum pump and the first and second overlay media were added as describe in plaque reduction neutralization test. The arithmetic mean of the number of plaque counts of the control bottles were calculated, and percent of control were calculated.

Preparation of seed viruses for CF and HI antigens

1-2 day old mice were inoculated intracerebrally with 0.02 ml of 10^{-1} dilution of dengue virus strain 12900 SM6 infected sucking mouse brain. The brains of surviving paralized mice were harvested and hemagglutination and complement-fixing antigens were prepared by the

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sucrose-acetone extraction method of Clake and Casals (21). The infected mouse brain was weighed and added with four volumes of a chilled 8% solution of sucrose in a Waring Blendor. The homoginate was added dropwise into 20 volumes of chilled acetone under agitation and then centrifuged at 1,800 rpm for 15 minutes. The sediment was collected and reextracted with chilled acetone. After centrifuging the sediment was spread thinly on the surface of centrifuged tube, dried under vacuum and rehydrated with two times the volume of the original brain tissue in normal saline solution, allowed to stand overnight at 4°C and centrifuged at 10,000 rpm for 1 hour. The supernatant was dispensed into 1 ml. steriles ampoules, lyophilized and stored at 4°C.

Hemagglutination Inhibition (HI) test

The HI tests were performed by using a modification of Clarke and Casals (21). Sera were inactivated at 56°C for 30 minutes. 0.1ml of serum fraction was added with 0.1 ml of 4% bovine plasma albumin as protein carrier. The aliquots of serum or serum fractions were extracted twice with 10 ml of chilled acetone. The precipitate dried overnight at 37°C and rehydrated in 1 ml of borate saline pH 9. The rehydrated sera were adsorbed with 0.02 ml of packed goose red cells. The supernatant fluid was diluted in microtiter plates. Eight units of D-1 12900 antigen were added and the mixture incubated overnight at 4°C. An 0.33% suspension of goose erythrocytes was added, pH was controlled at 6.2, and incubated at 24°C for one hour. End points

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were taken as the highest dilution showing complete inhibition of hemagglutination.

2-Mercaptoethanol treatment

One part of 1:10 dilution of 2-mercaptoethanol (2ME) in borate saline pH9 was added into nine parts of each acetone treated fraction (22,23). The mixture was incubate at 37°C, 30 minutes, and then placed at 4°C for 30 minutes. 2ME-treated and untreated fraction were diluted simutaneously as described in HI test.

The complement fixation (CF) test

To determine CF titers of heat-inactivated whole sera and serum fraction, serial two fold dilution of serum or serum fraction were made in veronal buffer saline (VBS) at pH 7.2 (Table 12) CF test was done in disposable microtiter plates. Four united of denque antigen in 0.025 ml and two exact units of complement in0.05 ml were added. The plates were covered and shaken with vertex mixer and incubated overnight at 4°C . The plates were removed and held at 24°C for 20 minutes while the sheep red cells were sensitized. To each well 0.05 ml of sensitized sheep red cells were added, the plates were covered shaken, and incubated at 37°C for 1 hr. The plates were centrifuged at 1500 rpm for 30 minutes. The highest serum dilution showing no hemolysin was read as the end point.

Immunoglobulin Fractionation Methods

DEAE-cellulose chromatography was performed in this experiment

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to fractionate sera into three major fractions containing the immunoglobulin, IgG, IgA, IgM. Inactivated sera were packed in sterile cellophane bags and dialyzed against 0.03 M phosphate buffer at pH 6.4 on magnetic stirror at 4°C for 72 hours with 6 changes of buffer. DEAE-cellulose was packed in a column 2.5 by 50 cm in 0.03 M phosphate-buffer pH 6.4. Flow rate was adjusted to about 5 ml/min. Dialyzed sera were applied into the column. Stepwise elution was effected by the sequential application of 0.03M, 0.01M and 0.3M phosphate buffer at pH 6.4. The eluted fractions (Fractions I, II and III) were collected by observing the protein concentration as measured by optical density at 280 mu in a direct recording spectrophotometer.

<u>Gel filtration on Sephadex G-200</u>

Gel filtration was performed by a modification of the method of Folin and Killander. Sephadex G-200 Lot No. 5822 with particle size 40-120 u were soaked with distilled water for 2 days, washed several time with distilled water and washed several time with 0.2M phosphate buffered saline(PBS) at pH 7.4 (Table 13) A3 by 100 cm column was packed with sephadex G-200 in PBS under gravity flow. Flow rate was adjusted approximately 45 ml per hour. One ml of inactivated serum was carefully layered on the top of G-200 by capillary pipette. After the serum entered the Gel. The column was filled with PBS at pH 7.4. Fractions were collected in 3 ml portions by using a fraction collector. The protein concentration of the eluted fraction was measured as above.

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Ultrafiltration for concentration of fractions

The first, and third eluted fractions (I and II) from DEAE-cellulose chromatography and the first eluted fraction from gel-filtration were concentrated by ultrafiltration in a pressure dialysis cell under 100 pounds per square inch pressure. Flow rate of the dialysate phosphate buffer saline at pH 7.4 was 180 ml/hr. The fractions were concentrated to the original volume of sera (approximate 50 to 80 X) and steriled by dispensing from hypodermic syringe with millipore Swinnex Filter Cat. no. SXHA 0130S, Type HA0.45 u pore size. The concentrated fractions were checked the purity of IgM of IgG immunoglobulin by immunoelectrophoresis and single radial diffusion in immunophate.

Immunoelectrophoresis and Single radial diffusion

Immunoelectrophoresis was carried out by a modification of the microtechnique of Scheideggar (24). Rabbit antimonkey globulins were prepared by Dr. Phanu Sitthisomwong, Goat anti-monkey serum and goat anti-human IgG, IgM was obtained from Hyland Laboratories, Los Angeles, U.S.A. The 1% agarose was heat in water bath until agar was melt in barbital buffer at pH8.2 (Table 14). 3 ml of agar was pipetted to a clean 2.5 x 7.5 cm microscope slide. The agar was allowed to solidify at room temperature. A central round well was cut 4 ml in diameter and the fractions added. Electrophoresis was carried out for two hours in barbital buffer at pH8.2 with a constant potential of 75 volts and a current range of 29-35 millampares. After electrophoresis, lateral through of 2 x 36 mm were cut in the agar and 0.15 ml

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of antisera added (Rabbit anti-monkey globulin, goat anti-monkey globulin and goat anti-human IgM of IgG). Precipitin bands were observed after incubation for 20-24 hours at 24°C. The slides were soaked in 0.9% normal saline solution for 3 days, washed in distilled water and dried. The dried slides were immersed in 1:1000 Buffalo Black stain in acetate buffer (Table 15) for 1-2 minute, and transfered to 'decolorizing solution(20ml)acetic acid and 150 ml glycerol/L) for one minute and rinsed in distilled water and dried.

Single radial diffusion

Two drop samples of each fraction were placed into Immunoplates (Hyland) containing anti-human IgM globulin that kept in moist gauge-lined box, and then incubated at 24°C for 18 hrs. Antihuman IgG globulin plates were incubated 37°C minute and added with sample and the incubated at 37°C for 4 hrs. The diameter of the precipitating was measured using a calibrated viewer (Hyland Laboratories), compared with the standard, and the concentration of protein computed.

Experimental infection of monkeys for preparation of dengue immune partially purified immunoglobulin fraction.

Five 8-10 month old *Macaca irus* were tested of HI antibody and neutralizing antibody to dengue viruses, Monkey V129, V130, V132,V133 and V152 were bled 2 ml each for using as normal monkey serum in immunoelectrophoresis. The monkeys were infected with 3000 pfu of dengue virus strain 12900 P-5 ($2x10^4$ pfu/0.3ml) by single subcutaneous infection. Virus inocula were titrated on the same day.

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Each monkey was bled 2ml on day 2 through day 10 for viremia test. The clotted blood was cooled and centrifuged at 2000 rpm for 30 minutes. Sera were quickly frozen in screw-capped vial at $-70^{\circ}C$ until tested for infectivity by direct plaque technique. 10-12 ml of blood from each monkey was obtained on day 14 and day 60 by femoral venipuncture. Blood was allowed to clot at room temperature and then centrifuged with 2000 rpm for 30 min. at $4^{\circ}C$.

Sera on day 14 and day 60 were tested in HI, CF and PRNT to measure the titers of whole sera. Sera were fractionated by DEAE-cellulose chromatography and Gel filtration chromatography. The concentrated fraction containing IgG and IgM were tested to measure the HI , CF and N titers by Hemagglutination-Inhibition test, Complement fixation test and plaque reduction neutralization test as described above. In order to confirm the purity of IgM or IgG fraction, immunoelectrophoresis and single radial diffusion were performed.

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Results

Viremia:

Viremia occurred after single peripheral inoculation of 3000 pfu of dengue virus strain 12900 in all five monkeys. Viremia occurred on day 2 through day 6 in monkey V 129. On day 2 and 3 in monkey V132 on day 4 through day 7 in monkey V133 and day 2 through day 7 in V152. Plaque counts were in range of 1 to 7 pfu/ 0.3 ml when undiluted sera was tested (Table 16).

Antibody in whole sera:

HI, CF and N antibody titers in whole sera on day 14 and 60 were shown in Table 17. HI antibody titer of monkey V129, V130, V132, V133 remained in the range of 1:80 to 1:160, CF titers were about 1:8 to 1:16, and N titers remained in the range 1:23 to 1:80 except V129 that had N titer on day 60 was 1:625. Serum on day 11 of monkey V152, HI, CF and N titer were 1:40, 1:2 and 1:220 respectively. Serum on day 13, 15, 17 H I titers were 1:160, but the CF titer was rising from 1:8 to 1:64, N titers were rising from 1:180, 1:700 and 1:800 respectively. V152, sera on day 60:HI titer was 1:320, CF titer was 1:8 and N titer declined to 1:340.

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Relative purity and concentration of IgM and IgG in fractions from DEAE cellulose chromatography and gel-filtration:

Preliminary experiments indicated that goat antisera prepared against human IgG and human IgM reacted in immunoelectrophoresis with monkey serum, giving specific precipitation bands. Antisera prepared in rabbits and goats against whole monkey serum produced readily identifiable precipitation bands in the IgG, IgM and IgA regions when tested against monkey serum by immunoelectrophoresis. Concentrated fractions from DEAE cellulose chromatography were tested by immunoelectrophoresis with rabbit and goat anti-monkey sera. Fraction I (eluted with .03M buffer) from DEAE cellulose chromatography in each case produced only an IgG band, the IgM content being the low detectable levels. Fraction III (eluted with 0.3M buffer) produced IgM bands and albumin bands on immunoelectrophoresis, the IgG being the low detectable levels in each case. Similarly, fraction I from gel filtration experiments produced only IgM bands on immunoelectrophoresis.

Concentrated fractions obtained from DEAE cellulose chromatography and gel filtration were also tested by single radial diffusion using "Immunoplates" containing anti-human IgG and IgM. Results are given in table 18. Fraction I from the DEAE cellulose chromatography contained IgG in concentrations between 10 and 1500 mgs%. IgM concentrations in fraction I were below detectable levels in every instance. Fraction III when tested by immunoplate contained

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concentrations of IgM between 17 and 80 mgs%. In each case, fraction III also contained residual IgG in concentrations between 7 and 45 mgs%.

Fraction I from the gel filtration preparations contained IgM as the only detectable immunoglobulin in concentrations of 10-25 mgs%.

Immunologic activity of globulin fractions:

The HI, CF and neutralizing antibody of the fractions from DEAE cellulose chromatography and gel filtration were measured. The results are summarized in Tables 20 and 21. The day 60 serum from monkey V129 showed HI, CF and N activity in the IgG fraction, the HI activity being resistant to 2-mercaptoethanol. The IgM fraction, in contrast, showed 2-mercaptoethanol sensitive HI activity, absence of CF activity, and presence of N activity.

Monkey V130 at day 14 had only low level CF and N activity in the IgG fraction, and mercaptoethanol-sensitive HI activity in the IgM fraction with a significant N titer in the IgM fraction. On day 60 the majority of the HI, CF and N activity was in the IgG fraction.

The sera from 4 monkeys collected 14 days after inoculation and fractionated by gel filtration each had mercaptoethnol

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sensitive HI activity, no CF activity and low titers of N activity in the IgM fraction.

On day 11 serum from monkey 152 showed antibody activity only in the IgM fraction. On day 13 antibody activity was present in both IgG and IgM containing fractions. Again, in each instance, HI activity of the IgM fraction was sensitive to mercaptoethanol, and complement fixing activity was found only in the IgG containing fractions.

<u>Results of Kinetic Neutralization Tests using partially purified immunoglobulins</u>.

Figure 1 shows the results of neutralization of dengue-1 by a 1:7.3 dilution of DEAE fraction III (IgM) from 14 day sera of monkey V130. The 50% plaque reduction titer of the fraction was 1:35. Plaque counts were very rapidly reduced at first resulting in reduction to 35% of control counts within one minute, and subsequently decreasing slowly in a linear fashion to 12% of control at 30 minutes. This fraction had only mercaptoethanol sensitive HI activity and only 7 mg% IgG content compared to 35mg% IgM content.

Figure 2 shows the results obtained with DEAE fractions I (IgG) from the 60 day sera of the same animal, V130. In this

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case a 1:10 dilution was used; the 50% plaque reduction titer of the sera being 1:40. A similar curve of neutralization was obtained with rapid reduction within the first minute to 45% of control and subsequent show reduction to 15% at 30 minutes. This fraction contained only IgG (325 mg%) with no detectable IgM and had mercaptoethanol resistant HI activity and CF activity.

DEAE Fraction III (IgM) from 11, 13 and 17 day sera of monkey V152 reacted similarly in the kinetic neutralization test as shown in figures 3, 4, and 5 . These fractions contained residual IgG but had no CF activity and the HI activity was mercaptoethanol sensitive. The neutralization curves were very similar to those obtained with the fractions from the V130 sera with most of the neutralization occurring within the first minute.

Figure 6 shows the results of the DEAE fraction III (IgG) from 17 day sera of monkey V152. The curve appears similar to the curves obtained with IgM fractions from the same monkey on days 11, 13 and 17 (Figures 3, 4 and 5).

Figure 7 and 8 were obtained from gel filtration fraction I (IgM) of day 14 and DEAE fraction I (IgG) of day 60 sera of monkey V133. Again a similarity in the kinetics of neutralization is seen.

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The kinetic neutralization tests presented in figure 1 through 8 are representative of 16 tests, 9 with IgM fractions and 6 with IgG fraction. Three of the 9 tests using IgM fractions were carried out with fractions prepared from gel filtration. No differences in kinetic of neutralization were seen between IgM fractions prepared by DEAE chromatography and those prepared by gel filtration.

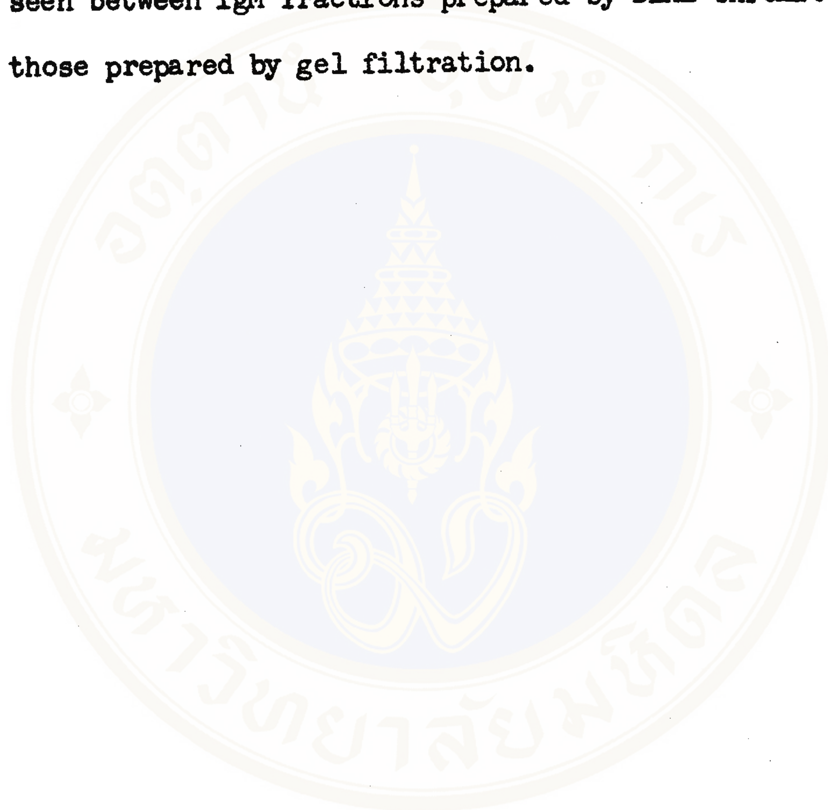

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Table 1

Growth medium for MK_2-cells

Calf serum	200.0	ml.
5% T.C. glutamine	6.0	ml.
Kanamycin	0.5	ml.
7% $NaHCO_3$	7.0	ml.
10% yeastolate	10.0	ml.
P & S*	2.0	ml.

Medium 199 (W/O $NaHCO_3$ or serum) added to 1000 ml.

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*Penicillin (100,000 units/ml) and Streptomycin (0.1 gm/ml)

Table 2

Maintenance medium for MK_2-cells

Calf serum (inactivated 56°C. 30 min.)	5.0	ml.
P & S*	0.5	ml.

7% $NaHCO_3$ to adjust at pH 8.3

1 x medium 199 was added to 100 ml.

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Table 3

Medium for first Agar Overlayer (2 X solution)

Calf serum (inactivated at 56°C, 30 min.)	10.0	ml.
100 x T.C. vitamins, Eagle, Difco	2.0	ml.
100 x T.C. amino acid, Hela, Difco	2.0	ml.
5% T.C. glutamine	1.2	ml.
20 x Hank's stock A	10.0	ml.
20 x Hank's stock B	10.0	ml.
7% $NaHCO_3$	4.5	ml.
P & S*	1.2	ml.

Dilute to 100 ml with demineralized water.

Equal parts of the first overlay medium (Table 3) which is warmed to 37°C. on water bath was added to DEAE dextran treated 2% Noble agar which is heated to 42°C.

Table 4

Medium for second Agar Overlayer (2 x solution)

Somes as first overlayer medium without calf serum and $NaHCO_3$. Add millipored filtered neutral red (1:100 solution) 1 ml. before mixed with DEAE dextran treated 2% agar in equal part.

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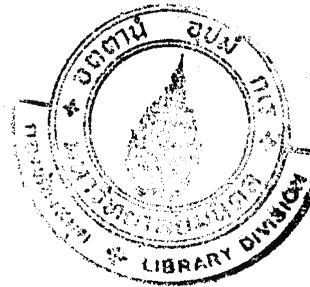

Table 5

Hank's Stock A

NaCl	160.0	gm.
KCl	8.0	gm.
$MgSO_4.7H_2O$	2.0	gm.
$MgCl_2.6H_2O$	2.0	gm.
$CaCl_2$	2.8	gm.

Demineralized water was added to 1000 ml.

(Sterile 10 minutes, 15 lb/inch^2)

Table 6

Hank's Stock B

Na_2HPO_4	1.2	gm.
KH_2PO_4	1.2	gm.
Glucose	20.0	gm.

Demineralized water was added to 1000 ml.

(Sterile 7 minutes, 15 lb/inch^2)

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Table 7

Hank's balanced salt solution (HBSS)

X20 stock A.	50	ml.
X20 stock B.	50	ml.
P & S*	5	ml.
7% $NaHCO_3$	11	ml.

Demineralized water was added to 1000 ml.

Table 8

Virus diluent

Calf serum (inactivated 56°C. 30 min.)	50.0	ml.
5% T.C. glutamine, Difco	6.0	ml.
P & S*	3.0	ml.
7% $NaHCO_3$	45.0	ml.

1 x medium 199 was added to 1000 ml.

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Table 9

Borate Saline pH 9.0

1.5 M NaCl	80.0 ml.
0.5 M H_3PO_3	100.0 ml.
1.0 M NaOH	24.0 ml.

Demineralized water to make 1000 ml.

Adjusted pH 9.0 with 1.0 M NaOH

Table 10

0.4% Bovine albumin in borate saline pH 9.0

Bovine albumin (Armous fraction V) 4.0 gm.

Borate saline pH 9.0 was added to 1000 ml.

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Table 11

Phosphate buffer pH 6.2

Solution A

1.5 M NaCl	100 ml.
0.5 M Na_2HPO_4	400 ml.

Demineralized water was added to 1000 ml.

Solution B

1.5 M NaCl	100 ml.
0.5 M NaH_2PO_4	200 ml.

Demineralized water was added to 1000 ml.

Phosphate buffer pH 6.2

Solution A	22 ml.
Solution B	78 ml.

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Table 12

Stock Veronal Buffered Saline (VBS) pH 7.2

5,5 diethyl barbituric acid (barbital)	5.75	gm.
Na-5,5 diethyl barbituric acid (Sod.babital)	3.75	gm.
NaCl	85.0	gm.
$Mg\ Cl_2.6H_2O$	1.68	gm.
$CaCl_2$	0.28	gm.

Demineralized water was added to 2000 ml.

5.75 gm. of 5,5 diethylbarbituric acid was dissolved in 500 ml. of hot demineralized water. 3.75 gm. of Na-5,5 diethylbabituric acid and 85 gm. of NaCl were added. 1.68 gm. of $MgCl_2.6H_2O$ and 0.28 gm. of $CaCl_2$ were added to the mixture. Adjusted the volume to 2000 ml. with demineralized water. The mixture was autoclaved at 15 lbs. for 20 min. The final pH., was 7.2. The stock solution was stored at $4^{o}C$. and diluted to 1:5 with demineralized water when used.

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Table 13

Phosphate Buffered Saline (PBS) pH 7.4

Stock A

NaCl	8.0 gm.
KCl	0.2 gm.
Na_2HPO_4	1.15 gm.
KH_2PO_4	0.2 gm.
Demineralized water was added to	800 ml.

Stock B

$CaCl_2$	0.1 gm.
Demineralized water was added to	100 ml.

Stock C

$MgCl_2.6H_2O$	0.1 gm.
Demineralized water was added to	100 ml.

Autoclave each separately. The cooled stocks were combined after autoclaving.

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Table 14

Barbital Buffer pH 8.2

0.05 M Sodium barbital (20.618 gm/2L)	1540	ml.
0.1 N Hydrochloric acid.	230	ml.

Table 15

Buffalo Black Stain

Buffalo Black	2.0	gm.
12% Acetic acid	900	ml.
1.6% Sodium acetate	900	ml.
qs. ad to 2 L.		

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Table 16

Determination of Viremia by Direct Plaque Technique

Day after Inoculation	Plaque count/0.3ml of sera from indicated monkey				
	V129	V130	V132	V133	V152
2	1	7	2	0	3
3	0	1	1	0	1
4	2	0	0	1	0
5	2	0	0	1	2
6	1	0	0	0	1
7	0	0	0	1	1
8	0	0	0	0	NT(1)
9	0	0	0	0	0
10	0	0	0	0	NT(1)

(1) = Not Tested

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Table 17

Hemagglutination Inhibition, Complement Fixation

and Neutralizing Antibody Titers of whole sera.

Monkey	Day after Inoculation	HI titer (1)	CF titer (2)	N titer (3)
V129	14	160	16	29
	60	160	8	625
V130	14	80	16	40
	60	80	8	80
V132	14	80	8	40
	60	160	8	30
V133	14	80	8	23
	60	160	16	30
V152	11	40	2	220
	13	160	8	180
	15	160	32	100
	17	160	64	800
	60	320	8	340

(1) = Reciprocal of titer VS 8 units of antigens.

(2) = Reciprocal of titer VS 4 units of antigens.

(3) = Reciprocal of 50% plaque reduction titers.

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<u>Table 18</u>

Relative Purity and Concentration of IgM and IgG in Concentrated serum Fraction from DEAE cellulose chromatography.

Monkey	Day after Inoculation	Globulin Fraction	Immuno plate reaction VS	
			anti IgG	anti IgM
V129	14	FI(IgG)	NT	NT
		FIII(IgM)	NT	NT
	60	FI(IgG)	350[1]	0
		FIII(IgM)	10	80
V130	14	FI(IgG)	1560	0
		FIII(IgM)	7	35
	60	FI(IgG)	325	0
		FIII(IgM)	10	17
V132	14	FI(IgG)	1560	0
		FIII(IgM)	13	52
	60	FI(IgG)	1000	0
		FIII(IgM)	25	80
V133	14	FI(IgG)	1560	0
		FIII(IgM)	10	50
	60	FI(IgG)	1000	0
		FIII(IgM)	15	50

(1) = mg % Protein

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Table 18 (Continued)

Relative Purity and Concentration of IgM and IgG in Concentrated serum Fraction from DEAE cellulose chromatography.

Monkey	Day after Ineculation	Globulin Fraction	Immuno plate reaction VS	
			anti IgG	anti IgM
	11	FI(IgG) FIII(IgM)	300[1] 35	0 45
	13	FI(IgG) FIII(IgM)	300 35	0 30
	15	FI(IgG) FIII(IgM)	500 40	0 35
	17	FI(IgG) FIII(IgM)	500 10	0 70
	60	FI(IgG) FIII(IgM)	100 45	0 50

(1) = mg% Protein

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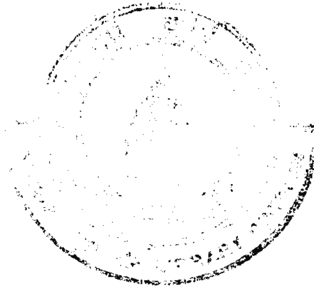

Table 19

Relative Purity and Concentration of IgM in Concentrated serum Fractions from gel filtration

Monkey	Day after Inoculation	Globulin Fraction	Immunoplate reaction VS	
			anti IgM	anti IgG
V129	14	FI(IgM)	15[1]	0
V132	14	FI(IgM)	25	0
V133	14	FI(IgM)	25	0
V152	15	FI(IgM)	10	0

(1) = mg% Protein

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Table 20

HI,CF and N antibody titers of Concentration Serum Fractions from DEAE cellulose chromatography

Monkey	Day after Inoculation	Globulin Fraction	HI titer(1)		CF titer(2)	N-titer(3)
			untreated	treated -ME		
V129	14	FI(IgG)	NT	NT	NT	NT
		FIII(IgM)	NT	NT	NT	NT
	60	FI(IgG)	20	20	32	480
		FIII(IgM)	20	0	0	100
V130	14	FI(IgG)	0	0	4	5
		FIII(IgM)	20	0	0	35
	60	FI(IgG)	20	20	8	40
		FIII(IgM)	0	0	0	17
V132	14	FI(IgG)	0	0	8	4
		FIII(IgM)	40	0	0	30
	60	FI(IgG)	40	40	8	250
		FIII(IgM)	20	20	0	35
V133	14	FI(IgG)	0	0	8	0
		FIII(IgM)	40	0	0	0
	60	FI(IgG)	40	40	16	90
		FIII(IgM)	0	0	0	18

(1) = Reciprocal of titer VS 8 units of antigens

(2) = Reciprocal of titer VS 4 units of antigens

(3) = Reciprocal of 50% plaque reduction titer

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Table 20 (continued)

HI, CF and N antibody titers of Concentrated Serum

Fractions from DEAE cellulose chromatography

Monkey	Day after Inoculation	Globulin Fraction	HI titer(1)		CF titer(2)	N titer(3)
			untreated	treated -2ME		
V152	11	FI(IgG)	0	0	ac(4)	0
		FIII(IgM)	20	0	0	45
	13	FI(IgG)	20	20	ac	10
		FIII(IgM)	80	0	0	55
	15	FI(IgG)	40	40	32	70
		FIII(IgM)	80	0	0	250
	17	FI(IgG)	80	80	64	150
		FIII(IgM)	80	0	0	230
	60	FI(IgG)	80	80	64	320
		FIII(IgM)	20	0	0	24

(1) = Reciprocal of titer VS 8 units of antigens

(2) = Reciprocal of titer VS 4 units of antigens

(3) = Reciprocal of 50% plaque reduction titers

(4) = Anticomplementary effect.

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Table 21

HI, CF and N antibody titers of IgM in Concentrated Serum Fractions from Sephadex G - 200 for gel filtration.

Monkey	Day after Inoculation	Globulin Fraction	HI titer (1)		CF titer (2)	N titer (3)
			untreated	treated -2ME		
V129	14	FI(IgM)	20	0	0	10
V132	14	FI(IgM)	40	0	0	13
V133	14	FI(IgM)	40	0	0	12
V152	15	FI(IgM)	20	0	0	25

(1) = Reciprocal of titers VS 8 units of antigens

(2) = Reciprocal of titer VS 4 units of antigens

(3) = Reciprocal of 50% plaque reduction titers

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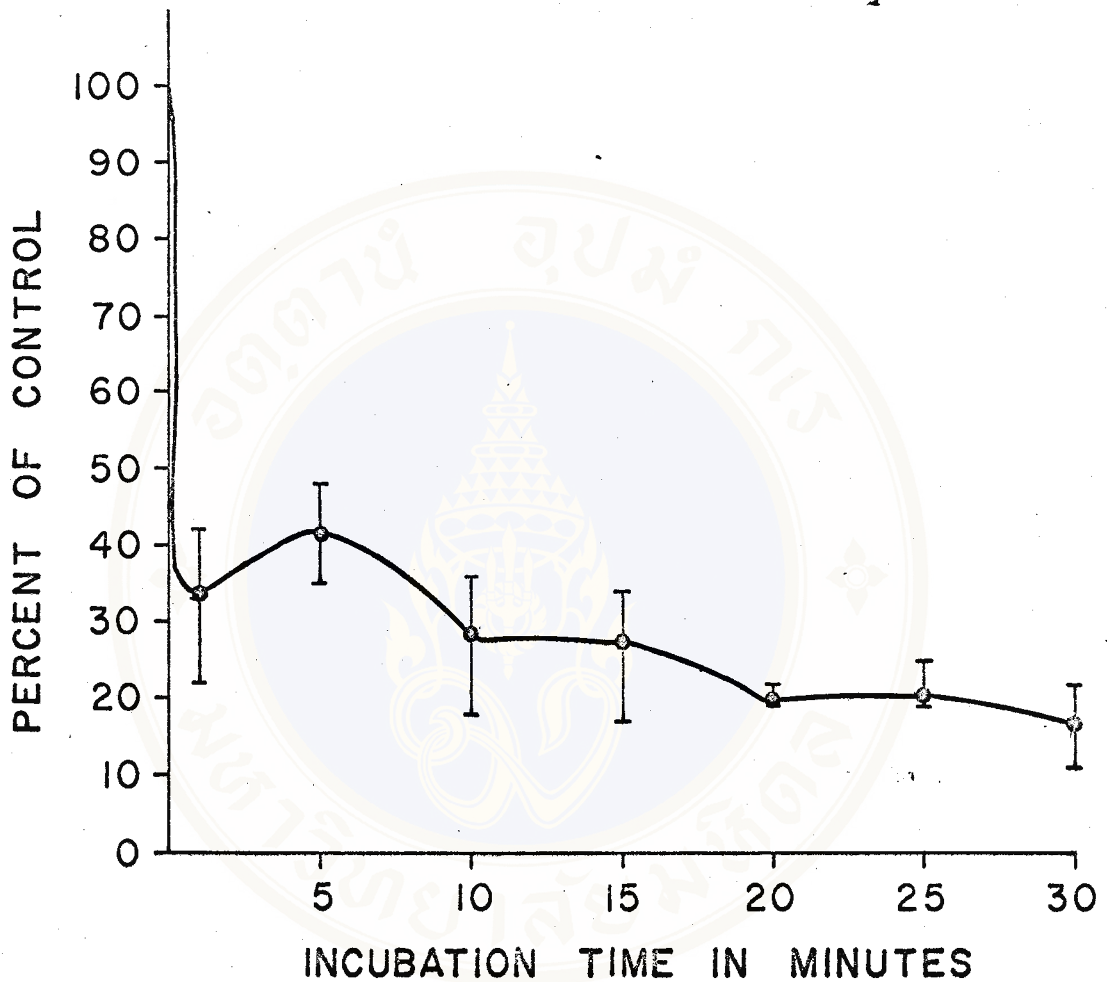

Figure I Kinetic neutralization test with dengue - I virus (77 pfu) and DEAE fraction III (IgM) from day 14 sera, monkey V130. Fraction III used at 1:8.75; PRNT titer = 1: 35.

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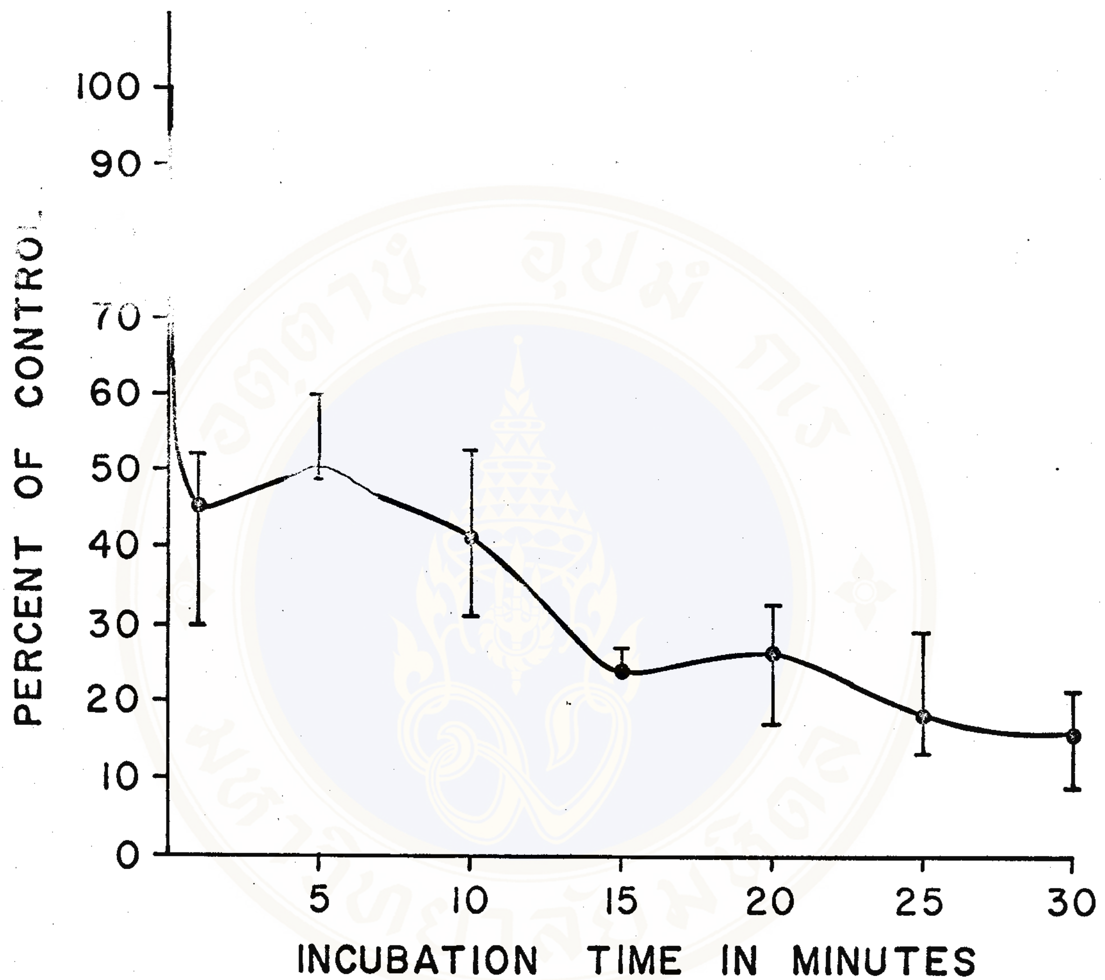

Figure 2 Kinetic neutralization test with dengue -1 virus (88 pfu) and DEAE fraction 1 (IgG) from day 60 sera, monkey V130. Fraction 1 used at 1:10; PRNT titer = 1:40.

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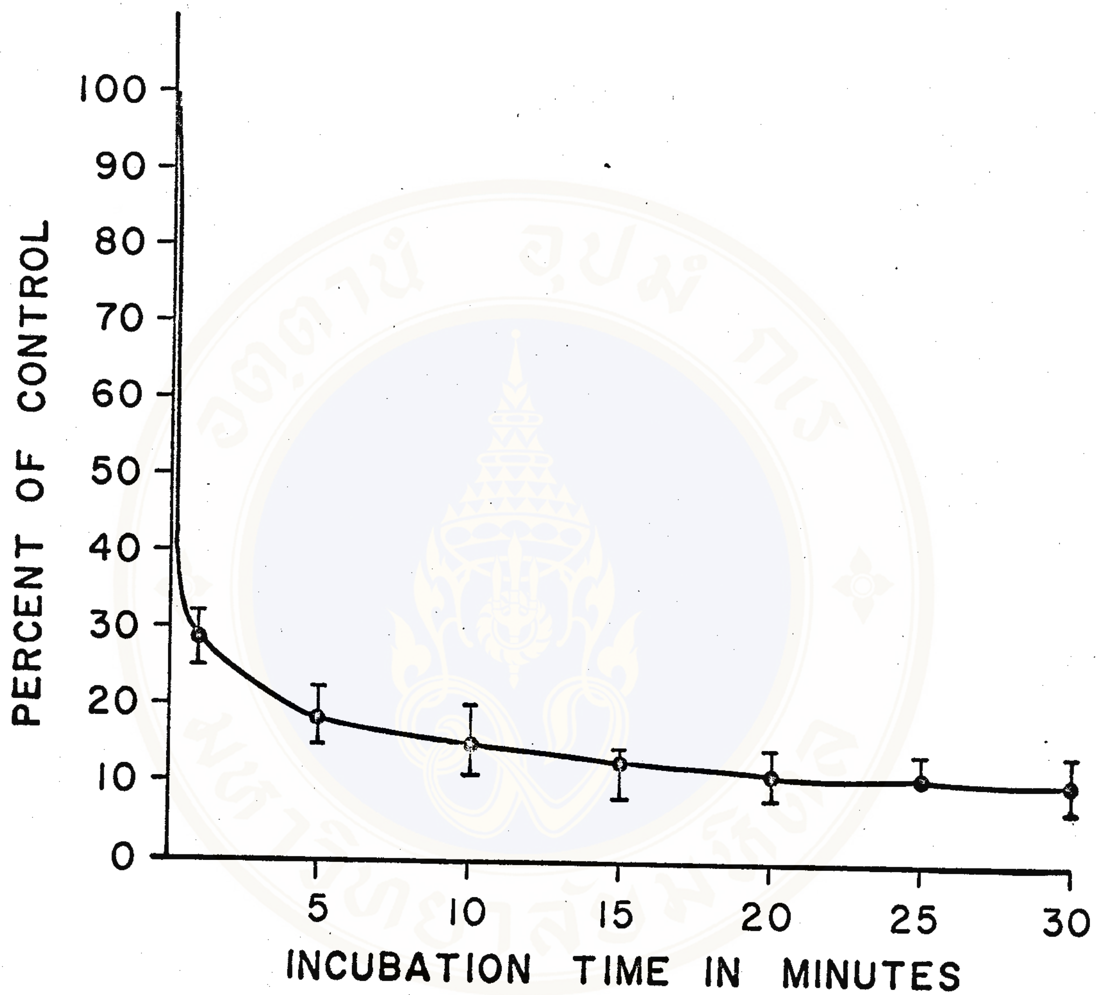

Figure 3 Kinetic neutralization test with dengue-1 virus (65 pfu) and DEAE fraction III (IgM) from day 11 sera, monkey V152. Fraction III used at 1:11.25; PRNT titer 1:45.

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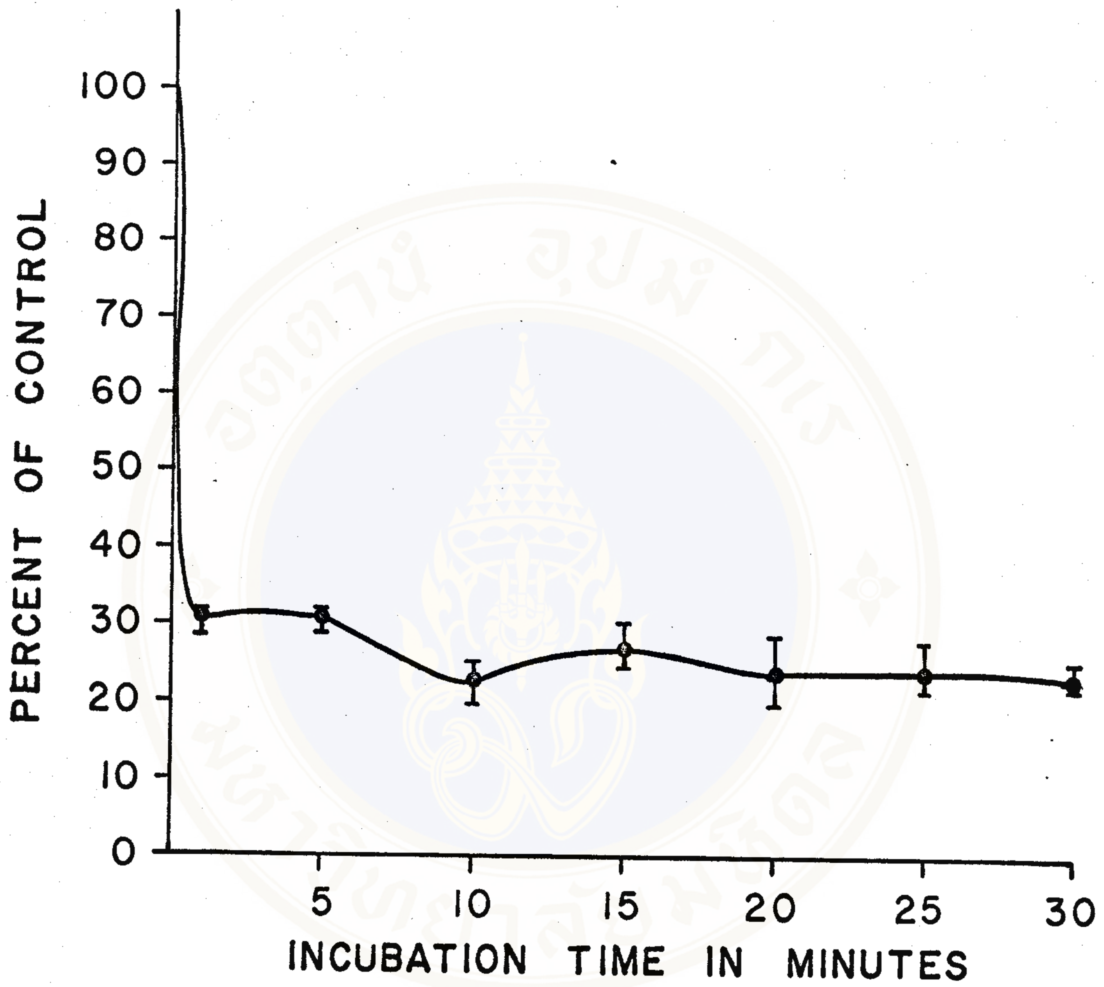

Figure 4 Kinetic neutralization test with dengue - I virus (78 pfu) and DEAE fraction III (IgM) from day 13 sera, monkey V 152. Fraction III used at 1:13.75; PRNT titer 1:55.

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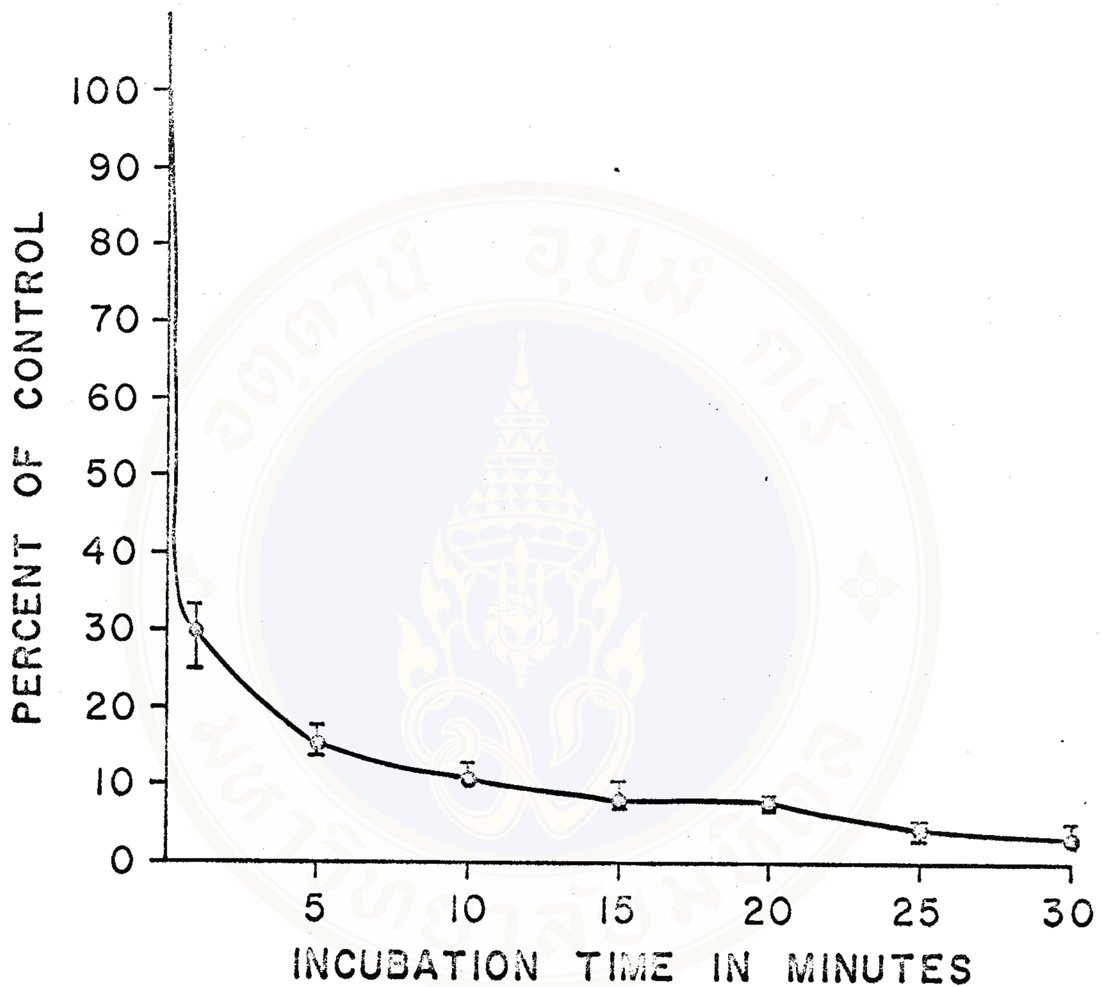

Figure 5 Kinetec neutralization test with dengue - I virus (67 pfu) and DEAE fraction III (IgM) from day 17 sera, monkey V 152 Fraction III used at 1:57.5; PRNT titer 1:230.

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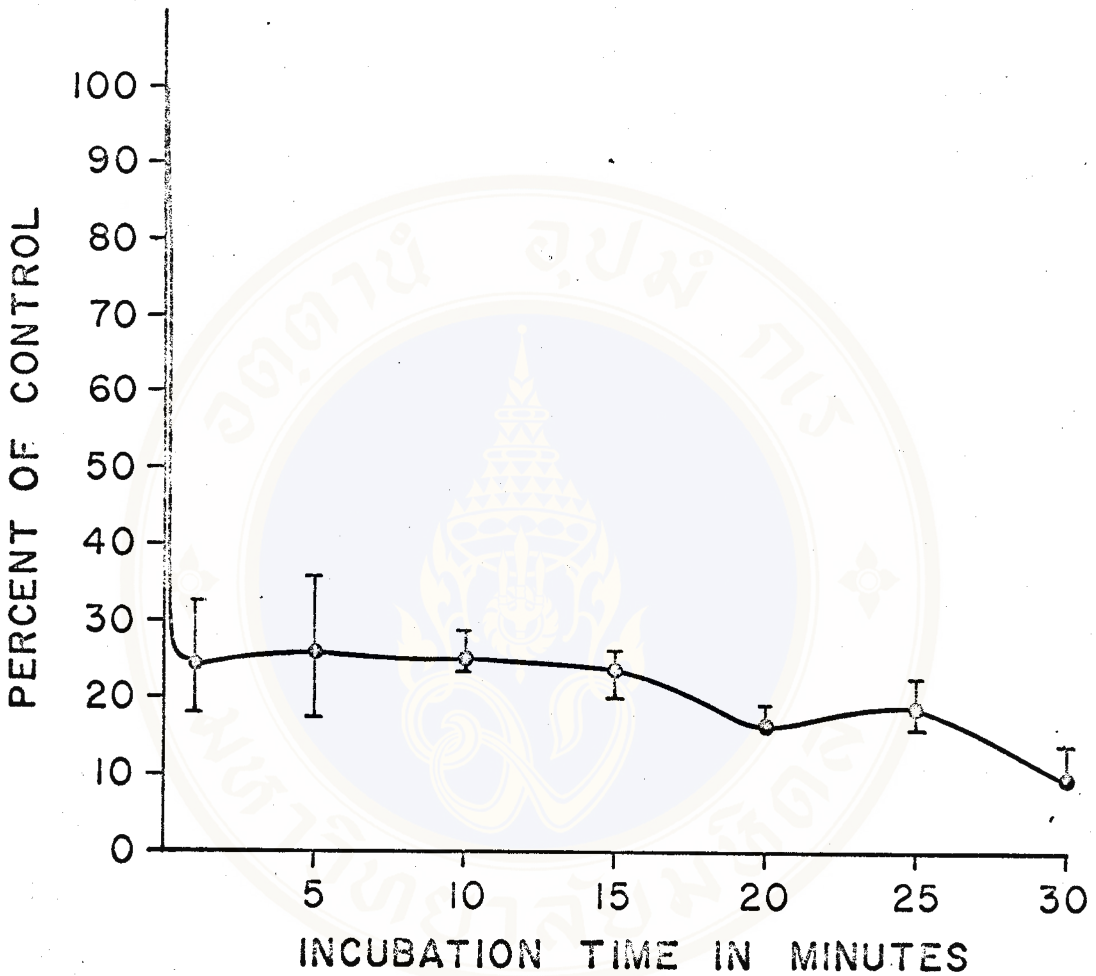

Figure 6 Kinetic neutralization test with dengue-1 virus (65 pfu) and DEAE fraction 1(IgG) from day 17 sera, monkey V152. Fraction 1 used at 1:37.5; PRNT = 1:150.

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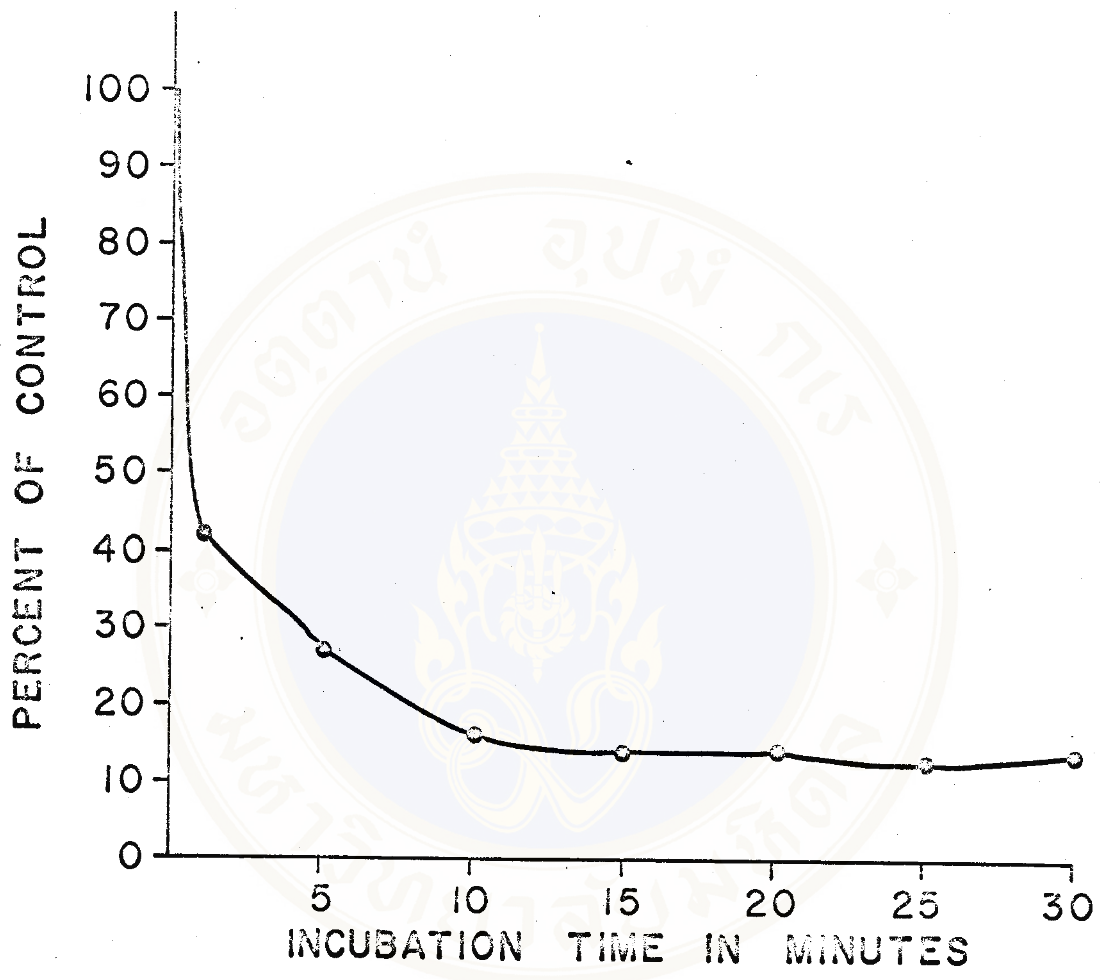

Figure 7 Kinetic neutralization test with dengue - I virus (80 pfu) and get filtration fraction I (Igm) from day 14 sera, monkey V133. Fraction I used at 1:3, PRNT titer = 1:12.

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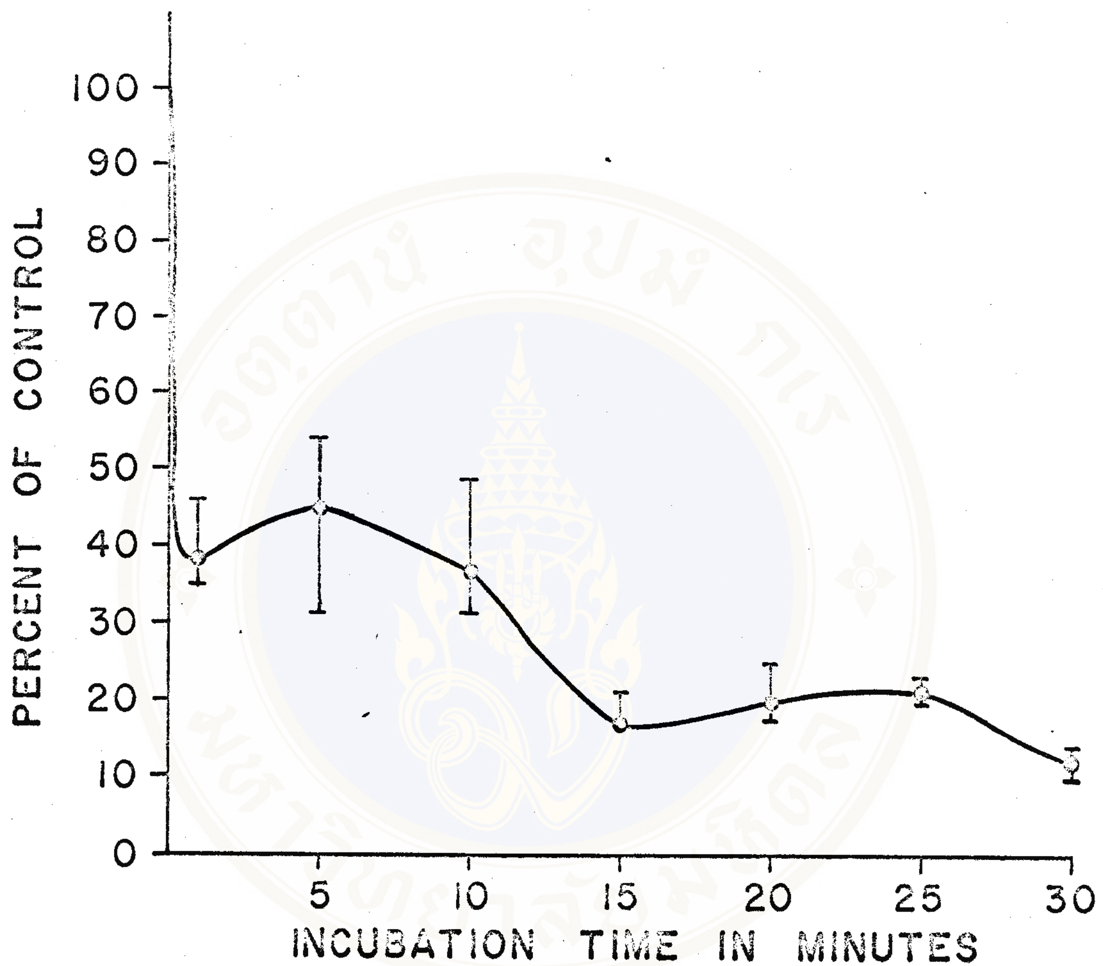

Figure 8 Kinetic neutralization test with dengue-1 virus (90 pfu) and DEAE fraction I (IgG) from day 60 sera, monkey V133. Fraction I used at 1:22.5, PRNT titer = 1:90.

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Discussion

As has been previously observed, (13, 25) dengue-1 virus produced viremia in *Macaca irus* monkeys when injected subcutaneously. Measurement of the viremia levels by direct plaque method, however indicated very small amounts of circulating virus. The observation was probably strongly influenced by normal inhibitors in sera which reduced plaque counts giving very low counts when undiluted sera was used. Therefore the level of viremia may have been significantly higher than the data indicate.

The antibody response as measured by HI, CF and N antibody levels in whole sera were those expected from primary dengue infection in monkeys. This is confirmed by the relatively large amounts of IgM antibody activity found in the sera obtained on days 11 to 17.

A very significant observation was the demonstration of the cross reaction in immunoelectrophoresis of monkey IgG immunoglobulins with antisera prepared against human IgG and IgM. This has not previously been recorded. This cross reaction allowed the use of immunoplates containing anti-human IgG and anti-human IgM antisera for quantification of monkey immunoglobulins by single radial diffusion. The use of immunoplates showed the contamination of DEAE fraction III (IgM) with some residual IgG;this

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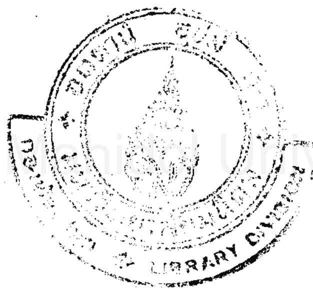
residual IgG; this contamination was not detectable by immuno-electrophoresis.

Fractionation of sera to obtain purified immunoglobulins was only partially successful. Highly purified IgG was obtained in Fraction I from DEAE chromatography in each ease. Fraction III from DEAE chromatography however, was in each instance contaminated with some IgG. The fel filtration method gave purer preparations of IgM but with lower concentration of IgM and low antibody activity.

The purity of the fractions III obtained from DEAE was sufficient to allow the conclusion, based on lack of anti-dengue CF activity and the mercaptoethanol sensitivity of HI activity, that most of the antibody activity of DEAE Fraction III was due to the IgM content in each case.

These experiments confirmed the lack of CF activity of IgM anti-dengue antibody previously observed (16).

The results of the kinetic neutralization tests indicated that, in this test system, the speed of the neutralization reaction observed with DEAE and gel filtration preparations of IgM, and DEAE preparations of IgG was similiar. Both immunoglobulin reacted rapidly resulting in marked reduction of infectivity with in one minute. The results indicate a high avidity for virus by both IgM and IgG antibodies and also rapid neutralization in the absence of complement or heat labile assessary factors since all sera were heat inactivated.



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The results obtained do not conclusively prove, however, the two immunoglobulins IgM and IgG neutralize dengue virus at the same rate or have the same avidity. The above experiments have two major failings. The IgM preparations were not completely pure and probably of more importance, the neutralization reaction in the kinetic neutralization test was not actually stopped at the times indicated but continued to an unknown extent during the adsorption period. The rates of neutralization actually could be different but were undetectable by the method used. Further investigation is indicated.

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Summary

Anti-dengue neutralizing properties of IgG and IgM antibody were studied by using infected monkeys as a source of anti-dengue immunoglobulin. Macaca irus monkeys were experimentally infected with dengue-1 virus. HI, CF and N tests were performed to detect antibody titers of sera following infection. Neutralizing antibody was measured by plaque reduction neutralization test. Sera were fractionate by DEAE cellulose chromatrography and gel filtration. Fraction I from DEAE cellulose chromatography contained highly purified IgG immunoglobulin as shown by immunoelectrophoresis and by single radial diffusion, fraction III from DEAE chromatography were contaminated with some IgG immunoglobulin. IgM fractions from gel filtration were free of IgG but with low concentrations and low antibody titers. HI tests and PRNT indicated HI and N antibody activities were associated primarily with IgM immunoglobulin on day 14, HI and N titer were associated with IgG immunoglobulin on day 60. Only IgG immunoglobulin had CF activity; IgM fractions had no CF titer. The results from kinetic neutralization test indicate that anti-dengue IgG and IgM antibodies have similiar rates of neutralization, and both have a relatively high degree of avidity.

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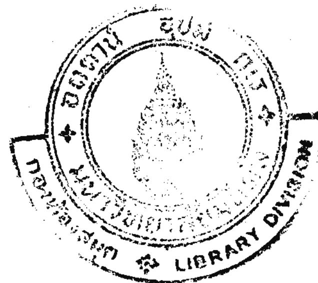

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