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Autor: Hotta, Susumu
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Propagation of Dengue Virus in Tissue Culture.[1]

By SUSUMU HOTTA.[2]

I. Introduction.

Since STEINHARDT et al. (1913) and LEVADITI (1913 a, b) reported the first successful maintenance of viruses in tissue cultures, many viral agents pathogenic for man and animals have been propagated *in vitro* by tissue culture methods. Detailed consideration of tissue culture techniques and their biological implications is found in monographs by FISCHER (1925, 1946), PARKER (1950), CAMERON (1950) and KIMURA (1953). General aspects on the application of tissue culture in virological field were discussed in chapters by HALLAUER (1938) and ENDERS (1952). A critical review of the literature on the cultivation of viruses in tissue cultures was presented by SANDERS et al. (1953). Recently an extensive bibliography on tissue culture research has been published by MURRAY & KOPECH (1953), which includes complete lists of viruses cultivated in tissue cultures and must be consulted by any worker interested in this subject.

Although the study of viruses in tissue culture had advanced steadily since 1913, a revival occurred around 1950 when the multiplication of polioviruses in cultures of extra-neural tissues was unequivocally demonstrated (ENDERS et al., 1949; WELLER et al., 1949; SMITH et al., 1950, 1951; MILZER et al., 1950; SYVERTON et al., 1951; LEDINKO et al., 1951). During the succeeding years a large number of developments, of both fundamental and practical significance, have been made in this field. Some of them are the following:

(1) Particular attention has been paid to the search for tissues suitable for the propagation of viruses. Theoretically speaking, a hypothetical tissue most appropriate for virus study would be able (a) to support the proliferation of many kinds of viruses quickly and abundantly; (b) to show signs attributable to the infection, by which the viral growth could be clearly detected; and (c) to be procured easily by an average laboratory. Although no perfect tissue has been discovered, it has recently been found that, besides the widely used chick embryo tissues, the monkey testicular or kidney cells and the HeLa cells (GEY et al., 1952) fulfill these requisites fairly well. These cells, in most instances, exhibit degeneration that is directly associated with viral infection and is suppressed by the specific antiviral antiserum. The degeneration, usually termed the cytopathogenic effect of virus, is of great advantage to animal virology.

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[2] Present address: Department of Microbiology, Kobe Medical College, Kobe, Japan.

Detection of virus is facilitated wherever the virus under investigation produces this effect. Viral neutralization tests in tissue cultures can be performed by taking advantage of this phenomenon. Of particular interest is a technique, by which plaques can be produced on a monolayer of cultured cells as a result of viral infection, in a manner comparable to plaque formation by bacteriophage (DULBECCO, 1952; DULBECCO & VOGT, 1954).

(2) Certain newer methods of cultivation of tissues have proved useful in virus research. Included in these developments are: Use of improved culture media; cultivation of cells in a roller apparatus, and treatment of tissue fragments with trypsin.

(a) Although serum or tissue extracts prove universally useful as nutrient fluids for tissue culture systems, they contain complex unidentified substances which constitute a serious disadvantage in certain kinds of experiments. Attempts have been made to develop chemically defined media for biochemical studies on virus growth in tissue culture. "Synthetic Mixture 199" of MORGAN et al. (1950) has been employed most frequently in recent years. This solution is not only advantageous because of its chemical purity, but also, when used with relatively small amounts of native protein products such as lactalbumin hydrolysate or serum, is more favorable for growth of cells or propagation of viruses than is plain serum or tissue extract. In this connection, interesting results have recently been obtained as to the basic nutrient requirements of cultured cells (EAGLE, 1955 a, b, c, d). Another material helpful in tissue culture work is Simm's serum-ultrafiltrate (SIMMS & SANDERS, 1942). It is believed that the ultrafiltrate is capable of maintaining culture cells for long periods of time without formation of intracellular fat granules. Recently it has been recorded that omission of ox-serum-ultrafiltrate from a culture medium resulted in apparent failure of multiplication of polioviruses (WELLER et al., 1952).

(b) There is agreement that roller tube cultures can support growth of virus more abundantly than the flask cultures previously used (ROBBINS et al., 1952; EVANS et al., 1954 b). Although no cause for such difference has been clearly established, it is possible that the abundant multiplication of virus in roller tube cultures may result from better growth of cells or from more effective contact of cells and virus. It may likewise be true that cells treated with trypsin and grown directly on the surface of glass without the conventional plasma clot are more completely exposed to infection with virus. Trypsin removes extracellular substances that might interfere with the cell-virus affinity. Based on the original studies on action of trypsin on tissue (SIMMS & STILLMAN, 1937), newer methods have been developed, by which monolayer cell cultures can be prepared (DULBECCO & VOGT, 1954; YOUNGNER, 1954). Trypsin-treated cell cultures have a definite advantage in the preparation of replicate cultures in large numbers. Trypsinized cells grown as a monolayer on glass are particularly favorable for microscopic study.

Recent reviews of these methods are found in papers by POMERAT et al. (1954), ENDERS (1954), and HABEL et al. (1955).

After ASHBURN & CRAIG (1907) established the viral etiology of dengue, many attempts to isolate the virus were made (CLELAND, 1930; DOERR, 1930; VAN ROOYEN & RHODES, 1948). Successful isolation of dengue virus by infection of mice was first achieved during the Second World War. The results obtained by American investigators are summarized by SABIN (1950, 1955), and those by Japanese investigators are reviewed by HOTTA (1947, 1951). However, no attempt to cultivate dengue virus in tissue culture prior to the present study, had been unequivocally successful. The reported exceptions are contained in short discussions by YAOI & ARAKAWA (1948), SCHLESINGER (1950), and SABIN (1955). The former two groups reported experiments in which dengue virus

was propagated in chick embryo tissues, and the latter presented results stating that dengue virus multiplied in monkey kidney tissue cultures without producing a cytopathogenic effect. The present work [3] was designed to cultivate *in vitro* the dengue viruses that have been adapted to mice. Particular consideration was given to the utilization of the newer tissue culture developments that were briefly reviewed in the preceding paragraphs.

II. Materials and Methods.

A. Strains of Dengue Virus.

Three mouse-adapted strains representing two known antigenic types were used.

(a) *Mochizuki strain (Type 1 dengue):*

This was isolated by KIMURA & HOTTA during a dengue epidemic in Japan in 1943 by injecting white mice intracerebrally with heparinized blood from a patient in the febrile stage of the disease (HOTTA, 1952). Since then it has been maintained continuously by intracerebral passage in mice. A homogenate was prepared containing approximately 10% glycerinated infected mouse brain in Ringer's solution with 20% inactivated normal horse serum.

(b) *Hawaiian strain (Type 1 dengue):*

This was originally isolated by SABIN & SCHLESINGER (1945) in 1944 from serum of a patient. After several passages in human volunteers, it was passed serially in brains of white mice. The virus used in the present experiments, 114th mouse passage, was obtained on November 29, 1954, from Dr. A. B. Sabin, as a form of lyophilized mouse brain homogenate in heated rabbit serum. After storage at 4°C for 5 days in this laboratory, it was dissolved in 2 ml. of medium E of Youngner (mixture 199 with 1.25 g. sodium bicarbonate per liter).

(c) *New Guinea C strain (Type 2 dengue):*

Isolated from a patient's serum, this strain was adapted to unweaned mice by MEIKLEJOHN et al. (1952) after serial intracerebral passages. The virus is now adapted to growth in the brain of 2 to 3-week-old mice. A lot of the 19th mouse brain passage was received by the author on January 25, 1955, from Dr. Edwin H. Lennette, State of California Department of Public Health, in the form of 20% brain homogenate in 30% normal rabbit serum frozen by dry ice. After storage for 4 days in a dry ice chest, the frozen material was thawed at room temperature and diluted 10-fold with Hanks' balanced salt solution.

In every case, the homogenates were centrifuged at 3,000 r.p.m. for 15 minutes. The supernatant fluid was diluted appropriately and used as the initial virus suspension. Unless otherwise indicated, Ringer's solution with 20% inactivated horse serum was employed for dilution of viral materials.

B. Glassware and its Washing.

Glassware routinely employed in the present study included the following items: 1 ml. and 10 ml. graduated pipettes; capillary pipettes with a bent tip of about 1 mm. in diameter; 16 × 150 mm. size tubes, either screw-capped or rubber-stoppered; 30 ml. centrifuge tubes; 50 ml. and 200 ml. dilution bottles,

[3] Preliminary accounts for part of the work have already been published by HOTTA & EVANS (1955, 1956 a, b).

with a screw cap; 250 ml. centrifuge bottles. Leighton tubes and Porter flasks were also used for special purposes.

The glassware was cleaned in Calgon-Metasilicate solution, and rinsed several times with tap water and distilled water, and once with diluted hydrochloric acid. For details of the cleaning procedures, refer to SCHERER (1955).

C. Basic Nutrient Fluids for Tissue Culture and Other Liquid Materials.

(a) *Chick embryo extract:*

Chick embryos 10 days old were minced in a Waring blender for a few minutes, with an equal volume of Hanks' balanced salt solution. After centrifugation at 2,500 r.p.m. for 20 minutes, the supernatant fluid was removed and 100 units of penicillin and 100 micrograms of streptomycin were added per ml. Just prior to use, 1 part of chick embryo extract and 2 parts of Hanks' balanced salt solution were mixed.

(b) *Chicken plasma:*

From an adult male chicken previously starved for 2 days, blood was drawn by puncture of the wing vessel. Heparin was added at a concentration of 0.01 mg. per ml. of the blood. After centrifugation the plasma was removed.

(c) *Normal horse serum:*

From a horse previously starved overnight, blood was collected by puncture of the jugular vein. The serum was separated after allowing the clot to stand for about 24 hours. Erythrocytes were removed by centrifugation. The serum was inactivated by heating at 56°C for 30 minutes.

(d) *Lactalbumin hydrolysate solution:*

Lactalbumin hydrolysate (Nutritional Biochemicals Corporation) was dissolved in glass-distilled water at desired concentrations.

(e) *Phosphate buffered saline (PBS):*

This was prepared according to the formula adopted by DULBECCO & VOGT (1954):

Solution (A):	NaCl	8.0 g.
	KCl	0.2 g.
	Na_2HPO_4	1.15 g.
	KH_2PO_4	0.2 g.
	Distilled water	800 ml.
Solution (B):	$CaCl_2$	0.1 g.
	Distilled water	100 ml.
Solution (C):	$MgCl_2 \cdot 6H_2O$	0.1 g.
	Distilled water	100 ml.

Each solution was prepared separately, and all three were mixed after the complete dissolution took place.

(f) *Trypsin solution:*

Bacto-Trypsin 1 : 250 (Difco Laboratories) was dissolved in PBS in a concentration of 0.25%.

(g) *Commercial nutrient fluids:*

Simms' ox-serum-ultrafiltrate was purchased from the Microbiological Associates. Synthetic mixture 199, Hanks' balanced salt solution, and 2.8% $NaHCO_3$ solution were obtained from Microbiological Associates and from the Virus Research Laboratory of the University of Pittsburgh or were prepared in our laboratory. Phenol red was present in these fluids as a pH indicator.

(h) *Sterilization and storage of the fluid materials:*

Chick embryo extract and chicken plasma were prepared using sterile precautions. Horse serum, PBS and trypsin solution were sterilized by filtration through a Selas filter candle under positive pressure with carbon dioxide. Lactalbumin hydrolysate solution was autoclaved at a pressure of 15 pounds for 20 minutes. The commercial nutrient fluids were used without any sterilizing treatment in this laboratory. The materials, except for chick embryo extract, were stored at 4°C for periods not exceeding 4 months. Chick embryo extract was stored at — 10°C.

D. Culture Media.

One of the following media was chosen for each experiment:

(a) Medium D of Youngner:

Synthetic mixture 199	95 parts
2.8% $NaHCO_3$	3 parts
Inactivated normal horse serum	2 parts

(b) 0.5% lactalbumin hydrolysate medium:

5% lactalbumin hydrolysate in distilled water	10 parts
Ox-serum-ultrafiltrate	22.5 parts
Hanks' balanced salt solution (10-fold concentrated)	10 parts
2.8% $NaHCO_3$	2.5 parts
Distilled water	55 parts

(c) 0.1% lactalbumin hydrolysate medium:

The concentration of lactalbumin hydrolysate in the second medium was reduced to 0.1%.

In all media penicillin and streptomycin were added to concentrations per ml. of 200 units and 200 micrograms, respectively. Phenol red was included as a pH indicator. In the case of roller-tube cultures embedded in chicken plasma clot, 5% carbon dioxide in air was used to reduce the pH to approximately 6.8.

For the initiation of cellular growth in tissue cultures, either medium D or 0.5% lactalbumin hydrolysate medium was used. The culture medium used after inoculation of virus was exclusively the 0.1% lactalbumin hydrolysate medium. This medium appeared to be more satisfactory for prolonged maintenance of culture cells than either of the other two media or Synthetic Mixture 199 alone.

E. Tissue Culture Systems.

Tissues used for culture were mainly from the rhesus monkey. The animals were anesthetized by an intraperitoneal injection of sodium pentobarbital solution [4]. Testes and kidneys were removed under sterile precautions. Two major systems of tissue culture were investigated. One was tissue culture em-

[4] Five grains of crystaline sodium pentobarbital were dissolved in 25 ml. distilled water. Of this solution 1 ml. per pound was injected.

bedded in chicken plasma clot and incubated in a roller apparatus (SMITH & EVANS, 1954). The second consisted of trypsin-treated cells grown directly on a glass surface and incubated in the stationary state (YOUNGNER, 1954). An additional experiment was carried out with human kidney tissue treated with trypsin.

(a) *Rhesus testicular tissue culture embedded in chicken plasma clot:*

After the tunica albuginea was stripped from each testis, the remaining tissue was minced into fragments less than 1 mm^3 in size with a curved steel scissors. During the mincing the tissue was dipped in Hanks' balanced salt solution containing 100 units of penicillin and 100 micrograms of streptomycin per ml. The tissue fragments were washed several times by changing and decanting the Hanks' solution. The final tissue suspension contained approximately 2 to 10 fragments per drop [5].

Two drops of fresh heparinized chicken plasma were spread in a thin layer on the lower half of a tube, 16 × 150 mm. in size. One drop of the tissue suspension was introduced into the tube, and the tissue fragments were placed linearly at a distance of 3 to 5 mm. apart. Each tube contained approximately 6 fragments. Four drops of chick embryo extract were then distributed as evenly as possible throughout the plasma layer. After clotting took place, 2 ml. of culture medium was added to each tube.

The tissue cultures prepared were incubated in a roller apparatus at 37°C. The slant of the rotary drum was adjusted so that the nutrient fluid covered the tissue fragments sufficiently without coming into contact with the stoppers of the tubes. The drum was rotated at about 12 revolutions per hour.

(b) *Rhesus kidney tissue culture embedded in chicken plasma clot:*

After the renal capsule was stripped, the kidney was cut in half and the connective tissue of the pelvis and medulla was removed as completely as possible. The remaining tissue was treated by the method described above with the testicular tissue.

(c) *Rhesus and human kidney tissue cultures treated with trypsin:*

Trypsin-treated kidney tissue cultures were prepared by a method slightly modified from those described by DULBECCO & VOGT (1954) and YOUNGNER (1954). The kidney, from which the connective tissue of the pelvis and medulla were removed, was minced with a curved steel scissors into fragments of approximately 2 mm^3 in size. The minced tissue was then treated by the following procedures:

1) The fragments were washed three times in a 250 ml. centrifuge bottle with PBS. Fluid was drawn off with a capillary pipette attached to a suction device.

2) The fragments were suspended in prewarmed trypsin solution [6] (20 ml. per kidney) and incubated at 37°C for 10 minutes.

3) Trypsin was discarded and replaced with fresh trypsin (20 ml. per kidney) and the tissue suspension was agitated with a stirrer [7] for 10 minutes. The speed of stirring was controlled to prevent foaming.

[5] A drop delivered from the capillary pipette. This designation will be applied to later paragraphs of this section.

[6] Prior to use, the trypsin solution was warmed to 37°C and kept in an incubator until ready to use.

[7] Stainless steel screw-propeller, each wing of which is about 15 mm. in length and 5 mm. in the widest width and attached to a stainless steel shaft of about 20 cm. in length.

4) The suspension was centrifuged at 600 r.m.p. for 5 minutes, and the supernatant fluid was decanted into a 250 ml. centrifuge bottle through three layers of sterile cheesecloth. The filtrate was collected in a bottle in an iced bath in order to stop the trypsin activity.

5) The extraction with trypsin was repeated 7 more times.

6) The turbid trypsinized cell suspension was centrifuged at 1,000 r.p.m. for 5 minutes, and the supernatant fluid was discarded.

7) The sediment was resuspended in medium D of Youngner.

8) This centrifugation-and-resuspending was repeated 3 times.

9) The final sediment was resuspended in a small amount of medium D, and transferred to a 30 ml. centrifuge tube in which it was centrifuged at 600 r.p.m. for 2 minutes.

10) The packed cell sediment was diluted with medium D to make a 1 : 50 dilution based on the volume of cell sediment.

11) Appropriate dilution of the 1 : 50 suspension was prepared in medium D so as to provide 600,000 to 700,000 kidney cells per ml.

Cell counts were performed with a hemocytometer and a white blood cell pipette. The living cells were recognized as bright, round particles under a microscope and thus distinguished fairly easily from masses of debris which looked dark and irregular. Total cell numbers per ml. were calculated by the formula:

$$4N/4 \times 10{,}000$$

where N is the number of cells seen in a white blood cell counting square.

One-half ml. of the suspension was put into each of the tubes which were then incubated at 37°C in the stationary state at a tilt of approximately 5 degrees from the horizontal. For uniform dispensing, a combination of a magnetic stirrer and an automatic pipette was used. Porter flask cultures were prepared by dispensing 2 ml. of the cell suspension per flask, and Leighton tube cultures were prepared with 1 ml. suspension per tube.

Procedures for preparation of human kidney tissue cultures were the same as described above with the rhesus kidney tissue.

F. Inoculation of Virus into Tissue Cultures.

Following initial incubation at 37°C for 4 or 5 days, cultures showing good cellular growth were selected for virus inoculation. Occasionally, the cultures were stored prior to use at 10 or 20°C for a period not exceeding 1 week.

Viral inoculation was carried out as follows in the initial cultivation and in the serial subcultures. Just prior to inoculation, the nutrient fluid was discarded. Two-tenth ml. of the appropriately diluted virus material, either mouse brain homogenate or tissue culture fluid, was put into each culture tube, and allowed to cover the cellular explants for about 10 minutes. Then 1.8 ml. of the 0.1% lactalbumin hydrolysate medium was added. The tissue cultures embedded in chicken plasma clot were incubated in a roller apparatus, and the trypsin-dispersed cell cultures were held in the stationary state. The temperature for incubation was 35°C.

The medium was renewed at intervals of 2 to 7 days in order to keep the pH of the fluid phase within the range of 7.2 to 7.6. The changes of fluid involved replacement of either 50% or 90% of the volume. The pH values were determined by comparison with a series of standard tubes containing phosphate buffer solutions and phenol red.

G. Assay of Viral Activity.

Titration of virus in tissue culture fluid was performed, in most instances, by intracerebral injection of mice. At suitable periods after the inoculation, portions of the culture fluid were taken from each tube and pooled with fluids from other tubes similarly inoculated. Serial 10-fold dilutions of the pools were then made, and 0.02 ml. of each dilution was injected into mice. Swiss albino mice, 2 to 3 weeks old, raised in the University of Washington Animal Quarters, were used throughout. A minimum of 3 mice were inoculated with each dilution. All mice were checked daily for the characteristic signs of dengue infection such as tremor and flaccid paralysis. Mice which died within 3 days after the injection, and those which died without showing definite signs of infection were not included in the results. The observation period was at least 3 weeks. LD_{50} titers per 0.02 ml. were calculated by the method of REED & MUENCH (1938).

In some cases the titration was carried out in tissue cultures. Two-tenth ml. from each of the 10-fold dilutions was inoculated into each tissue culture tube with the subsequent addition of 1.8 ml. of medium. Three to 5 tubes were used with each dilution. The fifty per cent infective dose (ID_{50}) for tissue cultures was calculated on the basis of appearance of definite degeneration at 2 weeks after the virus inoculation.

The titrations were usually done immediately after harvesting the fluid. In a few instances the materials were stored before titrating; they were sealed in pyrex glass tubes and held in an iced thermos overnight or in a dry ice chest for periods not exceeding 4 months.

H. Morphological Examination.

Following the inoculation of virus, the cultures were checked daily for morphological changes visible under a microscope at magnifications of 32 × and 100 ×. In the case of trypsinized cells, two other types of culture were also examined:

(a) *Cultures in Porter flasks:*

Two ml. of the kidney cell suspension prepared as indicated previously was dispensed into Porter flasks with a bottom 3 cm. in diameter. Following incubation at 37°C for 4 or 5 days, a continuous sheet of cells was usually formed. The cells were then washed with Hanks' solution; the 0.1% lactalbumin hydrolysate medium was added and the cultures were incubated again at 37°C for a day or two. Then the cultures were inoculated with virus in the manner described previously, except that the volumes of the viral inoculum and of the culture medium per flask were 0.3 ml. and 2.7 ml., respectively.

(b) *Cultures in Leighton tubes:*

Test tubes, 18 × 150 mm., with a flattened area of about 15 × 30 mm. near the bottom, were used. A cover slip, approximately 10 × 22 mm. in size, was placed on the flattened area. On this 2 ml. of the kidney cell suspension was deposited. After 4 or 5 days incubation at 37°C in the stationary state, a cellular sheet had grown on the cover slip. The cultures were infected with virus in the same way as the cultures in test tubes.

The ordinary test tube cultures and the Porter flask cultures were observed usually *in situ* with the microscope. In order to take photomicrographs of the cells, the tubes or flasks were fixed with adhesive tape on the platform of a microscope under a Selge-Huhne's Orthophot apparatus. A green filter was used in most instances.

The cultures in Leighton tubes were examined as follows: The cover slips were removed from the tubes by using a metal bar approximately 20 cm. long, the end of which was bent at a right angle. Then they were subjected either to staining or to phase-contrast microscopy:

1) *Staining:*

The coverslip preparations were placed vertically for 3 minutes in a Columbia jar with Hanks' balanced salt solution previously warmed at 37°C. Then they were fixed with Zenker-formol solution (formula described by PARKER, 1950) for 30 minutes. After being washed in running water for 6 hours, the preparations were stained by the following procedure, which represents a slight modification from that devised by Jacobson (HANKS et al., 1955).

1. Stain for 10 minutes in May-Grünwald stain.
2. Wash in distilled water once.
3. Stain for 5 minutes in Giemsa stain diluted 1 : 20 with distilled water.
4. Dehydrate rapidly in two changes of acetone.
5. Place in xylol for a few minutes.
6. Mount in clarite

2) *Phase-contrast microscopy* [8]:

The technique of making the phase-contrast microscopic preparations was similar to that devised by Gey and described by PARKER (1950). The small coverslip removed from a Leighton tube or Porter flask was placed on a drop of medium on a large coverslip (43 × 50 mm. and 0.1 mm. thickness). The wet coverslip remained attached to the large coverslip, and the cells were immersed in the thin layer of fluid between the two coverslips. This preparation was mounted on a metal slide with a central opening to accommodate the small coverslip. A second large coverslip was used to seal the opposite side of the hole in the metal slide. The metal slide and the large coverslips were sealed with paraffin.

In some instances, entire test tube cultures were fixed with Zenker-formol solution and stained with May-Grünwald-Giemsa stain.

J. Anti-Dengue Immune Serum.

Anti-type 1 dengue immune serum was obtained from a male rabbit immunized with the Mochizuki strain of virus that had been passed only in mouse brain and never in tissue culture. The immunizing materials were supernatant fluids from 10% infected mouse brain homogenates in Ringer's solution, centrifuged at 3,000 r.p.m. for 15 minutes. Mouse-intracerebral LD_{50}'s of the suspensions were presumed to be near 10^6 per 0.02 ml. on the basis of occasional titrations. The immunizing procedures consisted of injections as follows: 3 times intravenously (0.5 ml. to 0.7 ml., each), 5 times subcutaneously (1.0 ml. to 2.0 ml., each), 4 times intraperitoneally (2.5 ml. to 3.0 ml., each), and again 2 times intravenously (2.0 ml., each), at intervals of 5 to 10 days. One week after the last injection, the rabbit was exsanguinated, and the serum was separated. Serum collected from this rabbit prior to immunization served as a control non-immune serum. The serums were inactivated by heating at 56°C for 30 minutes, and stored at — 10°C until used.

[8] A phase-contrast microscopic apparatus assembled by the late Dr. P. H. Ralph, Department of Anatomy of the University of Washington, was used throughout. Assistance given by Dr. Eichi Yamada is gratefully acknowledged.

Anti-type 2 dengue immune serum was obtained with the New Guinea C strain of virus by the procedure similar to that used to produce the anti-type 1 serum. In this case, mouse-intracerebral LD_{50}'s of the immunizing materials were not determined because of irregularity in mortality ratios of inoculated mice (MEIKLEJOHN et al., 1952). However, since the infected brain homogenates used for immunization were prepared freshly every time, it was probable that reasonably large amounts of the active virus were introduced into the immunized rabbit.

Viral neutralization tests were performed in the following manner: Virus at a given concentration was mixed in equal volume with serum diluted appropriately in lactalbumin hydrolysate medium or in 20% heat-inactivated normal horse serum in Ringer's solution. The mixture was put into a pyrex glass tube which was then stoppered tightly with a rubber stopper. The tube was held at 37°C for 1 hour, and at 4°C for 1 more hour. Then 0.02 ml. of the mixture was either injected into mice intracerebrally, or 0.2 ml. was inoculated into tissue cultures with the subsequent addition of 1.8 ml. of medium. In the latter case, the culture fluid used for changing the medium included 1% of immune or control serum.

III. Results.

A. Control Experiments.

Dengue virus was incubated at 35°C in tubes containing culture medium minus cells. Some tubes were placed in a roller apparatus; others were held stationary. Portions of the nutrient fluid were removed at intervals and tested for viral activity. The data indicated that dengue virus disappeared within a short period of time under such conditions. One example is shown in Table 1.

TABLE 1.

Survival of dengue virus of the Mochizuki strain in culture medium lacking cells, incubated in a roller apparatus at 35°C.

Dilution of culture fluid	Mortality ratio of mice inoculated with culture fluid removed at					
	1 hour	24 hours	72 hours	7 days **	10 days	14 days
10^{0}	3/3 *	3/4	0/3	0/3	0/3	0/3
10^{-1}	3/3	0/4	0/3	0/3	0/3	0/3
10^{-2}	3/3	0/4	0/3	0/3	0/3	0/3
10^{-3}	3/3	0/4	0/3	0/3	0/3	0/3
10^{-4}	2/3	0/4	0/3	0/3	0/3	0/3

Infected mouse brain homogenate from the 133rd passage was inoculated. Two-tenth ml. of a 10-fold dilution was introduced into each of 6 tubes, with the subsequent addition of 1.8 ml. of culture medium. The inoculum contained $10^{6.5}$ mouse LD_{50}'s. At the given intervals, 0.2 ml. of the fluid was removed from each tube. Pooled material from the 6 tubes was diluted 10-fold, and 0.02 ml. of each dilution was inoculated into mice intracerebrally.

* Numerator indicates number of mice which died showing typical signs of infection; denominator indicates number of mice inoculated.

** Eight days after the beginning of incubation, 90% of the medium was replaced with fresh culture fluid.

In every experiment 3 to 7 cultures with no virus were included. They were incubated and subjected to change of medium exactly the same as infected cultures. They exhibited only slight degenerative changes after incubation lasting 3 to 10 weeks.

It was confirmed repeatedly that homogenate of normal mouse brain diluted 1,000-fold or more had no apparent effect on the culture cells.

Specimens of undiluted culture fluid from various control experiments were injected intracerebrally into 5 or more 2-week-old white mice. Except for a few isolated mice which died presumably from the effect of trauma, deaths were not observed.

Ten serial blind passages were carried on through normal rhesus kidney tissue cultures by transferring 0.2 ml. of the culture fluid to new cultures with subsequent addition of 1.8 ml. of medium. No mouse-infective or cytopathogenic agent, however, was found in this experiment. A cytopathogenic agent isolated from a control uninoculated rhesus kidney tissue culture will be discussed in a later section of this article.

All specimens were inoculated onto bacteriological culture media and checked for bacterial and fungal growth. Contaminated tubes which were infrequent were discarded.

B. Potency of Anti-Dengue Immune Rabbit Serum.

Preliminary measurement of the potency of the immune rabbit serum was accomplished by neutralization tests in mice. The results tabulated in Tables 2a and 2b indicate that the undiluted

TABLE 2.

Neutralization tests to determine the potency of anti-dengue immune rabbit serum.

(a) Tests with serially diluted virus and a fixed concentration of serum.

Dilution of virus	Mixed with		
	Immune serum	Control serum	Normal horse serum
10^{-2}	0/3	3/3	3/3
10^{-3}	0/3	3/3	3/3
10^{-4}	0/3	3/3	3/3
10^{-5}	0/3	0/3	1/3
10^{-6}	0/3	0/3	0/3
10^{-7}	0/3	0/3	0/3

Virus: Mochizuki strain; ten-fold dilutions of a mouse brain homogenate from the 135th passage were employed.

Serum: Anti-Mochizuki; undiluted.

The virus-serum mixture was held at 37°C for 1 hour and at 4°C for 1 more hour. Then 0.02 ml. of the mixture was inoculated into mice intracerebrally.

TABLE 2 (continued).

(b) Tests with serially diluted serum and a fixed concentration of virus.

Dilution of serum	Mixture containing	
	Immune serum	Control serum
$10^{-1.0}$	0/5	4/5
$10^{-1.5}$	0/5	
$10^{-2.0}$	0/5	4/5
$10^{-2.5}$	0/5	
$10^{-3.0}$	2/5	

Virus: Hawaiian strain; a 10^{-4} dilution of a mouse brain homogenate from the 116th passage was employed. It contained approximately 5 mouse LD_{50}'s.

Serum: Anti-Mochizuki; diluted with 20% inactivated normal horse serum in Ringer's solution.

The virus-serum mixture was subjected to the same treatment as indicated in Table 2 a.

anti-type 1 immune serum neutralized 1,000 times more virus of the Mochizuki strain than the control serum did, and that approximately 5 LD_{50}'s of the Hawaiian strain virus were neutralized completely by the immune serum diluted 320-fold. The control serum was found to have no neutralizing activity against dengue at all.

Anti-type 2 immune serum, diluted 10-fold, was shown to neutralize the New Guinea C strain virus in a 10^{-2} dilution of infected mouse brain homogenate from the 20th passage. No quantitative tests were performed with this virus because of irregularity in its infectivity for mice.

C. Dengue Virus in Rhesus Testicular Tissue Culture Embedded in Chicken Plasma Clot.

1) *Initial inoculation:*

Two series of experiments were carried out, the results of which are summarized in Table 3.

The data indicate that dengue virus remained active in rhesus testicular tissue cultures for relatively long periods of time. This finding appeared to be significant when compared with the results of control experiments in which the virus disappeared within a short period of time in medium devoid of living cells. However, the data do not provide evidence of viral multiplication.

2) *Serial transmission:*

Results of serial passages through testicular tissue cultures are shown in Tables 4a and 4b. The virus was detected only in the

TABLE 3.

Mortality ratios of mice injected intracerebrally with culture fluid from dengue-infected rhesus testicular tissue cultures.

Expt. No.	Dilution of culture fluid	* Tissue culture incubation period in days										
		1/12	1	3	5	7	8	10		14		17
								Pooled	From a single tube	Pooled	From a single tube	
I	10°	3/3	1/3	2/4	7/8	9/9	5/10	7/8	6/7	4/6	6/6	6/6
	10^{-1}	3/3	0/3	1/4						3/5	2/6	3/6
	10^{-2}	1/3	0/3	0/4	1/8	2/9	0/9	2/8	4/7	1/5	0/6	1/5
	10^{-3}	0/3	0/4	0/3								0/5
	10^{-4}	0/3	0/3	0/3	0/8	0/8	0/8	0/7	0/7			0/5
		* Calculated dilution of original inoculum in tissue culture fluid.										
		$10^{-3.0}$	$10^{-3.0}$	$10^{-3.0}$	$10^{-3.0}$	$10^{-3.0}$	$10^{-4.0}$	$10^{-4.0}$		$10^{-4.0}$		$10^{-4.3}$

		* Tissue culture incubation periodi n days						
		14	28	42	52	63	77	108
II	10°	5/6	5/5	6/6	2/5**	4/5	4/6	0/6
	10^{-1}	1/6	3/5	5/6	0/5 **	1/5	0/6	0/6
	10^{-2}	0/6	3/5		0/5 **			
	10^{-3}				0/5 **			
		* Calculated dilution of original inoculum in tissue culture fluid.						
		$10^{-3.3}$	$10^{-5.3}$	$10^{-6.6}$	$10^{-7.5}$	$10^{-8.4}$	$10^{-8.7}$	$10^{-8.7}$

Fluid from replicate cultures were pooled except on the 10th and 14th day in experiment I, when tests were made of single tubes as well as from pools.

Seed virus: In experiment I, a 10^{-2} dilution of a homogenate from infected mouse brain of the Mochizuki strain, 132nd passage, was used; its mouse-intracerebral LD_{50} was approximately $10^{7.0}$ per 0.02 ml. In experiment II, a 10^{-2} dilution of a mouse brain homogenate from the Mochizuki strain, 135th passage, was employed; mouse-intracerebral LD_{50} of this material was $10^{6.5}$ per 0.02 ml.

* The volume of medium removed and replaced varied; a 90% change was made on the 7th day, and a 50% change on the 14th day in experiment I. Changes of fluid in experiment II involved replacement of half volume or 90% of fluid at different times.

** The surviving mice were rechallenged intracerebrally at the end of observation period (4 weeks) with the mouse-passaged type 1 Mochizuki strain virus containing approximately 10^2 mouse LD_{50}'s. All of them died showing typical signs of infection within 2 weeks after the rechallenge.

(Reproduced, with slight modification, from the Journal of Infectious Diseases, vol. 98. 1956.)

TABLE 4.

Summary of results on the serial passage of dengue virus of the Mochizuki strain in rhesus testicular tissue culture.

(a) Experiment I.

Passage	Incubation period of each passage, in days	Cumulative dilution of initial inoculum [1]	Dilution of culture fluid	Mortality ratio of inoculated mice
Second [2]	9	$10^{-5.0}$	10^{0}	4/6
			10^{-1}	1/6
			10^{-2}	0/5
			10^{-3}	0/5
			10^{-4}	0/5
	14	$10^{-6.0}$	10^{0}	4/5
			10^{-1}	1/5
			10^{-2}	0/5
			10^{-3}	0/5
			10^{-4}	0/5
Third [3]	7	$10^{-6.0}$	10^{0}	0/4
			10^{-1}	0/4
			10^{-2}	0/4
			10^{-3}	0/4
			10^{-4}	0/4
	14	$10^{-7.0}$	10^{0}	0/5
			10^{-1}	0/5
			10^{-2}	0/5
			10^{-3}	0/5
			10^{-4}	0/5

1. On the basis of passage and medium-change.

2. Two-tenth ml. of culture fluid harvested 8 days after the beginning of the first passage was transferred to each culture tube of the second passage, with the subsequent addition of 1.8 ml. medium.

3. Nine-day-specimen from the second passage was transmitted to the third passage, in the same way as in the transfer from the first to the second passages.

TABLE 4 (continued).

(b) Experiment II.

Passage	Incubation period of each passage, in days	Cumulative dilution of initial inoculum [1]	Dilution of culture fluid	Mortality ratio of inoculated mice
Second [2]	14	$10^{-4.6}$	10° 10^{-1}	4/5 3/5
	28	$10^{-5.9}$	10° 10^{-1}	2/6 * 0/6 *
	42	$10^{-6.8}$	10° 10^{-1}	0/6 0/6
Third [3]	14	$10^{-5.9}$	10° 10^{-1}	2/7 * 0/7 *
	28	$10^{-6.8}$	10° 10^{-1}	0/6 * 0/6 *
Fourth [4]	14	$10^{-7.5}$	10° 10^{-1}	0/6 * 0/6
	28	$10^{-8.1}$	10° 10^{-1}	0/6 0/6

1. On the basis of passage and medium-change.

2. Two-tenth ml. of culture fluid harvested 14 days after the beginning of the first passage was transferred to the second passage, and 1.8 ml. of culture medium was added in each tube.

3. Fourteen-day-specimen from the second passage was transmitted to the third passage.

4. Twelve-day-specimen from the third passage was used for the transfer to the fourth passage.

* At the end of a 4-week observation period, the surviving mice were re-challenged intracerebrally with the Mochizuki strain mouse-passaged virus. Inoculum for the rechallenge contained approximately 10^2 times LD_{50}'s. All of the rechallenged mice died showing characteristic signs of dengue infection.

first two passages in Experiment I, and the first three passages in Experiment II. Again evidence for multiplication was negative.

3) *Morphological findings:*

Very few changes were noted in the appearance of fibroblastic cells in inoculated tissue cultures. In some cases certain degenerative alterations, such as cytoplasmic granulation and separation of cells, were seen, but they were hardly distinguishable from those found in the control tubes subjected to equally prolonged incubation.

4) *Neutralization tests:*

Virus harvested from culture fluid of the inoculated tissue cultures was submitted to neutralization tests with anti-dengue immune serum. The results obtained are summarized in Table 5. It is evident that the virus in culture fluid was neutralized by the anti-dengue immune rabbit serum.

TABLE 5.

Neutralization tests with dengue virus harvested from rhesus testicular tissue cultures.

Experiment	Culture fluid[1] harvested at	Mortality ratio of mice inoculated with mixture containing	
		Immune serum[2]	Control serum[2]
I	17 days	0/5	5/5
II	42 days	0/5	5/5
	63 days	0/5	4/5

1. Undiluted. See Table 3 for further data.
2. Diluted 10-fold with 20% inactivated normal horse serum in Ringer's solution.

D. Dengue Virus in Rhesus Kidney Tissue Culture Embedded in Chicken Plasma Clot.

Each of 7 tissue cultures received 0.2 ml. of a 10^{-6} dilution from an infected mouse brain homogenate of the Mochizuki strain, 136th passage. Then 1.8 ml. of 0.1% lactalbumin hydrolysate medium was added to each tube. The cultures were incubated in a roller apparatus at 35°C.

Infectivity of the initial virus inoculum was determined by injecting mice intracerebrally with 0.02 ml. of the 10-fold dilutions. Mortality ratios of the injected mice were: 2/5 for the 10^{-6} dilution, and 0/5 each for dilution of 10^{-7} and 10^{-8}.

Following the inoculation of virus, half of the volume of culture fluid in each tube was replaced every 4 or 5 days. At given periods, portions of the fluids were pooled and tested for mouse-infectivity. The results obtained are shown in Table 6. Virus was detected in 0.02 ml. of a 10^{-2} dilution of the 16-day specimen, a 10^{-3} dilution of the 24-day specimen, and undiluted fluid of the 32-day specimen. These data suggested an increase of virus in the culture fluid, because the amount of virus contained in the original inoculum was relatively small and the inoculum was subsequently diluted by the frequent replacements of culture medium.

TABLE 6.

Mortality ratios of mice inoculated intracerebrally with culture fluid removed from dengue-infected rhesus kidney tissue cultures embedded in chicken plasma clot.

Dilution of culture fluid	Tissue culture incubation period in days			
	16	24	32	56
10^{0}	5/5 *	5/6 *	2/7 *	0/7
10^{-1}	5/5	2/6	0/7	0/7
10^{-2}	5/5	1/5		
10^{-3}		1/5		

* Neutralization tests were performed in mice, and the virus was identified as dengue.

Degeneration of the culture cells was found under the microscope 2 weeks after the virus inoculation, although its extent was relatively slight. Two weeks later, however, the degeneration became marked. Eight weeks after the inoculation, almost the entire population of cells that had initially grown out from explants had disappeared.

Neutralization tests using the culture fluid from infected cultures were not performed. However, brains of mice that died in the groups marked with asterisks in Table 6 were stored in a 50% glycerin saline solution and used later for neutralization tests. It was shown that the mouse-infectivity of a 10^{-3} dilution from the homogenates of these brains was completely neutralized when mixed with the anti-dengue immune rabbit serum. The same suspension mixed with control serum killed all 5 mice inoculated. It is evident that the agent derived from the tissue culture fluids was dengue virus.

E. Dengue Virus in Cultures of Trypsinized Rhesus Kidney Cells.

1) *Preliminary experiment:*

The results of an experiment with the Mochizuki strain virus, 133rd mouse brain passage, were strongly suggestive of an increase of the virus in the fluid phase of cultures of trypsinized rhesus kidney tissue. The inoculum contained less than 10 mouse LD_{50}'s of the virus. The medium was changed by half volumes every 2 to 4 days. Undiluted culture fluid removed 17 days after the virus inoculation, and its 10-fold dilution killed all of 6 injected mice. Similarly the undiluted fluid from the 24-day specimen and its

10-fold dilution killed 5 out of 6 mice, and 2 out of 6 mice, respectively. Moreover, a distinct cellular degeneration was noticed two weeks following the virus inoculation, and two weeks later the majority of the culture cells had disappeared. In view of these encouraging results, the following more comprehensive studies were carried out.

2) *Multiplication of virus:*

Definite evidence for the multiplication of dengue virus in trypsinized rhesus kidney tissue cultures was provided by (a) determining the growth curve of virus in cultures inoculated with a relatively small amount of virus, and (b) serial transmission of virus through a number of subcultures.

(a) *Growth curve experiment:*—A growth curve of the virus was determined in a single series of cultures. Seven tubes were used for the infected group, and 3 for the control. The initial virus inoculum was 0.2 ml. of a 10^{-6} dilution from an infected mouse brain homogenate of the Mochizuki strain, 136th passage.

One mouse LD_{50} of this material was contained in 0.02 ml. of a $10^{-6.5}$ dilution. One and eight-tenth ml. of culture medium was added to each tube. At given periods following the inoculation, portions of the culture fluid were removed, pooled and tested for virus concentration. The control tubes received a normal mouse brain homogenate and were treated the same as the infected cultures. The results obtained are shown in Table 7. The virus increase was unequivocal. The maximum titer of the fluid phase was approximately one-hundredth of that of infected mouse brain. The dengue virus multiplied in this system of tissue culture more slowly than certain other viruses such as poliovirus.

(b) *Serial transmission:*—Initial inoculum was the same as that used in the experiment stated in the preceding paragraph. Two one-tenth ml. of undiluted culture fluid taken 17 days after the beginning of the first passage was inoculated into each of 5 trypsinized rhesus kidney tissue cultures, with subsequent addition of 1.8 ml. of culture medium. In the similar manner serial transmission of virus through tissue cultures was carried out. For each passage at least 3 tissue culture tubes were used. Virus content of each inoculum was determined by intracerebral injection of mice. Results obtained with the Mochizuki strain virus are summarized in Table 8, which includes the mouse LD_{50} titers in each passage, the cumulative dilution of initial virus inoculum calculated on the basis of passages in successive tissue cultures, and the cumulative dilutions based on both passages and changes of medium, as well

TABLE 7.

Growth of dengue virus of the Mochizuki strain in cultures of trypsinized rhesus kidney cells.

Period of incubation in days	Mouse-intracerebral LD_{50} per 0.02 ml
1/12	No virus detected
1	No virus detected
4	$10^{1.5}$
7	$10^{2.5}$
10	$10^{4.67}$
13	$10^{4.25}$
17	$10^{4.0}$
21	$10^{4.0}$
28	$10^{2.5}$
35	$10^{1.5}$
43	No virus detected

Seven cultures were inoculated with 0.2 ml. of a 10^{-6} dilution from a homogenate of infected mouse brain, 136th passage, with a mouse LD_{50} titer of $10^{6.5}$ per 0.02 ml.; 1.8 ml. of culture medium was added to each tube. Pooled culture fluid removed at the times indicated was titrated for virus content by intracerebral injection of mice.

TABLE 8.

Summary of results of the serial passage of dengue virus of the Mochizuki strain in trypsinized rhesus kidney tissue culture.

Passage	Mouse LD_{50} per 0.02 ml of each passage	Dilution of initial inoculum by passage	Dilution of initial inoculum by passage and medium-change	Cumulative time of cultivation in days
1	$10^{4.0}$	10^{-7}	10^{-9}	17
2	$10^{5.33}$	10^{-8}	10^{-11}	35
3	$10^{1.75}$	10^{-9}	10^{-12}	43
4	$10^{2.75}$	10^{-10}	10^{-14}	62
5	$10^{3.46}$	10^{-11}	10^{-16}	75
6	$10^{5.75}$	10^{-12}	10^{-17}	85
7	$10^{5.25}$	10^{-13}	10^{-19}	93
8	$10^{4.0}$	10^{-14}	10^{-21}	103
9	$10^{4.63}$	10^{-15}	10^{-22}	113
10	$10^{4.50}$	10^{-16}	10^{-23}	120
15	$10^{4.75}$	10^{-21}	10^{-31}	162
20	$10^{3.0}$	10^{-26}	10^{-37}	200
25	$10^{5.25}$	10^{-31}	10^{-44}	236
27	$10^{4.50}$	10^{-35}	10^{-47}	251
30	$10^{4.33}$	10^{-41}	10^{-53}	272
31	$10^{5.50}$	10^{-43}	10^{-55}	279
40	$10^{4.75}$	10^{-52}	10^{-69}	352

Initial inoculum was the same as that used in the experiment stated in Table 7.

TABLE 9.

Summary of results of the serial passage of dengue virus of the Hawaiian strain in trypsinized rhesus kidney tissue culture.

Passage	Mouse LD_{50} per 0.02 ml of each passage	Dilution of initial inoculum by passage	Dilution of initial inoculum by passage and medium-change	Cumulative time of cultivation in days
1	$10^{5.0}$	10^{-4}	10^{-5}	10
2	$10^{4.5}$	10^{-5}	10^{-7}	19
3	$10^{4.5}$	10^{-6}	10^{-9}	29
4	$10^{4.25}$	10^{-7}	10^{-10}	38
5	$10^{3.5}$	10^{-8}	10^{-12}	48
6	$10^{4.5}$	10^{-9}	10^{-13}	56
7	$10^{4.5}$	10^{-10}	10^{-15}	66
8	$10^{5.25}$	10^{-11}	10^{-17}	78
9	$10^{5.5}$	10^{-12}	10^{-18}	88
10	$10^{4.25}$	10^{-13}	10^{-20}	102
15	$10^{4.5}$	10^{-18}	10^{-27}	139
20	$10^{4.75}$	10^{-23}	10^{-35}	179
26	$10^{4.0}$	10^{-29}	10^{-47}	232

The initial inoculum was a mouse brain homogenate from the 114th passage. The lyophilized material, delivered from Dr. A. B. Sabin, was dissolved with medium E of Youngner (synthetic mixture 199 containing 1.25 g. sodium bicarbonate per liter) into a 10-fold volume, and diluted further with 20% inactivated normal horse serum in Ringer's solution. Two-tenth ml. of a 10^{-3} dilution was inoculated into each tube, with the subsequent addition of 1.8 ml. culture medium.

as cumulative time of cultivation in days. Forty passages were carried out during 352 days, and 1 LD_{50} at the 40th passage represented a 10^{-74} dilution of the original virus inoculum. Essentially similar results were obtained with the Hawaiian strain virus (see Table 9).

Results with the New Guinea C strain virus are summarized in Table 10. In this case, however, mouse-infective titers of culture fluid are represented by the highest dilutions causing death of mice, instead of LD_{50}'s. Significant LD_{50} values could not be determined because of the previously mentioned irregularity in mortality of inoculated mice.

3) *Morphological findings:*

(a) *Uninoculated control cultures:*—In uninoculated cultures usually a continuous sheet of cells was formed. Each cell observed microscopically in the living state had a homogeneous cytoplasm and a round and homogeneous nucleus with one or two dark

TABLE 10.

Summary of results of the serial passage of dengue virus of the New Guinea C strain in trypsinized rhesus kidney tissue culture.

Passage	Minimum infective titer * in mouse (intracerebral)	Dilution of initial inoculum by passage	Dilution of initial inoculum by passage and medium-change	Cumulative time of cultivation in days
1	10^{-4}	10^{-4}	10^{-5}	11
2	10^{-4}	10^{-5}	10^{-7}	25
3	— **	10^{-6}	10^{-9}	38
4	10^{-4}	10^{-7}	10^{-12}	50
5	10^{-4}	10^{-8}	10^{-13}	65
6	10^{-3}	10^{-9}	10^{-15}	74
7	10^{-3}	10^{-10}	10^{-17}	81
8	10^{-3}	10^{-11}	10^{-18}	92
9	10^{-3}	10^{-12}	10^{-19}	100
10	10^{-4}	10^{-13}	10^{-21}	110
18	10^{-5}	10^{-21}	10^{-37}	176

The initial inoculum was a mouse brain homogenate from the 19th passage. The material frozen in dry ice was received from Dr. E. H. Lennette. It was thawed at room temperature, and diluted with Hanks' balanced salt solution. Two-tenth ml. of a 10^{-3} dilution was inoculated, and 1.8 ml. of culture medium was added in each tube.

* Highest dilution causing death of mice. Mortality ratio in mice infected was characteristically erratic.

** No mice inoculated died.

(Reproduced, with partial modification, from the Proceedings of the Society for Experimental Biology and Medicine, vol. 93, 1956.)

nucleoli. The stained preparations exhibited a similar appearance. Under the phase-contrast microscope mitochondria and round granules were seen. After incubation at 35°C for 2 weeks or more certain abnormal appearances developed in some cells of the uninoculated cultures. These cells were dark and round, and showed irregular granulation of the cytoplasm. The majority of them eventually became detached from the glass surface. However, the number of such cells was so small that an almost unbroken sheet of cells with normal appearance remained throughout the incubation.

(b) *Infected cultures:*—The culture cells infected with dengue virus exhibited characteristic degeneration visible under the microscope. Early changes were clearly detectable at 5 days to 1 week after the inoculation of virus. At this time a portion of the cells assumed a spherical shape and a dark appearance. Soon after the cells acquired these features, they lost their firm attachment to the glass surface. At 10 days it was common to see a culture in which the population of normal-looking cells was reduced by about half

TABLE 11.

Parallel titration of dengue virus in mice and in trypsinized rhesus kidney tissue cultures.

Virus			Inoculated into *	Dilution of inoculated fluid						LD_{50}	ID_{50}
Type	Strain	Passage in tissue culture		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
1	Mochizuki	4	Mice	3/3 **	3/3	3/3	1/3	0/3	0/3	$10^{3.75}$	—
			Tissue cultures	3/3 **	3/3	3/3	2/3	0/3	0/3	—	$10^{4.25}$
		30	Mice	6/6	6/6	5/5	2/5	1/5	0/5	$10^{4.0}$	—
			Tissue cultures	4/4	4/4	4/4	3/4	0/4	0/4	—	$10^{4.33}$
2	New Guinea C	10	Mice	1/3	2/3	0/3	1/3	0/3	0/3	***	—
			Tissue cultures	3/3	3/3	0/3	0/3	0/3	0/3	—	$10^{2.5}$
		18	Mice	3/3	2/3	0/3	2/3	1/3	0/3	***	—
			Tissue cultures	3/3	3/3	2/3	0/3	0/3	0/3	—	$10^{3.25}$

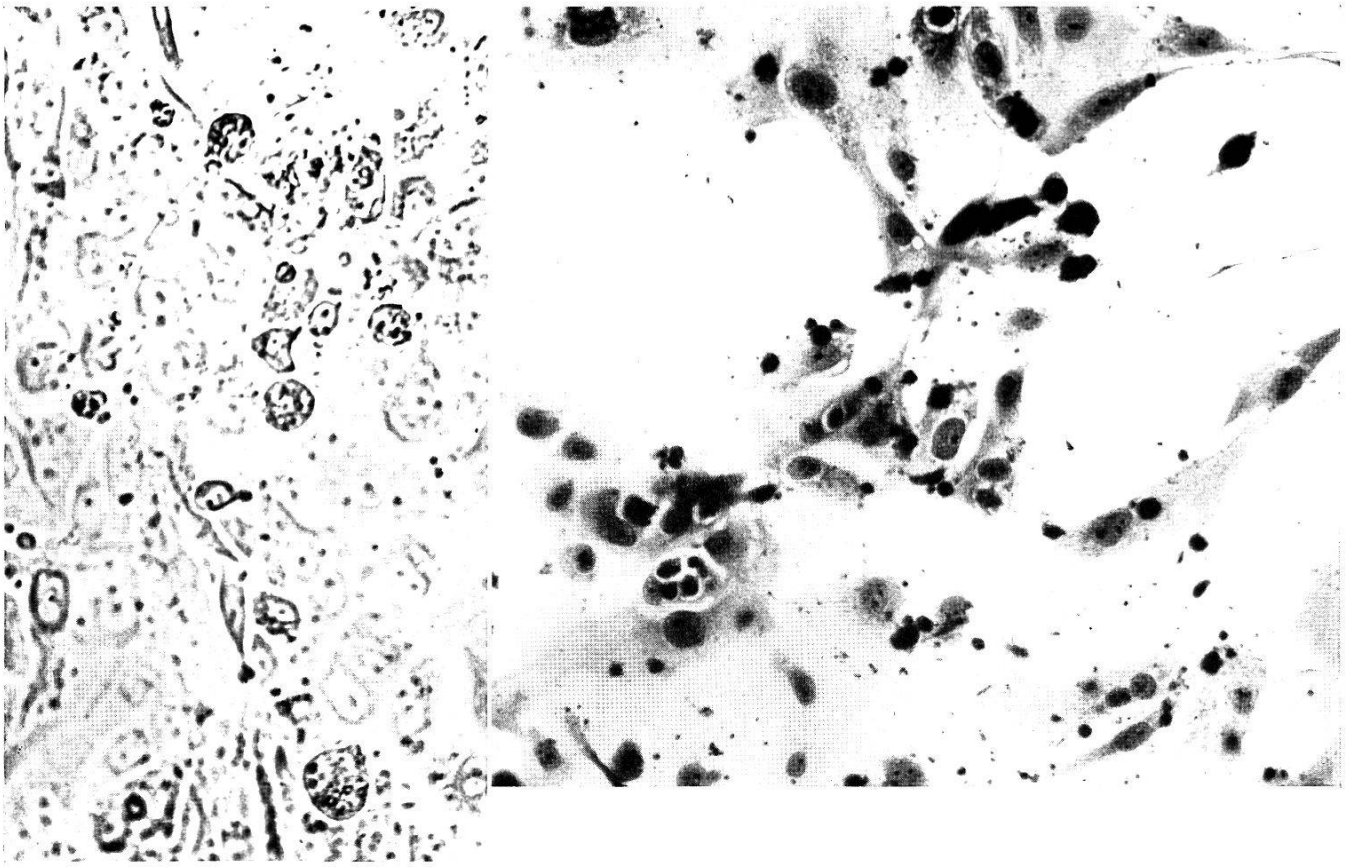
* Inoculum: 0.02 ml. in mice intracerebrally, and 0.2 ml. in tissue cultures with 1.8 ml. of medium.

** Numerator indicates number of mice that died showing typical signs of infection, or tissue culture tubes showing definite cellular degeneration; denominator indicates number of mice or tissue culture tubes inoculated.

*** No LD_{50} titer calculated, because of irregularity in mortalities.

(Reproduced in part from the Journal of Infectious Diseases, vol. 98, 1956, and in part from the Proceedings of the Society for Experimental Biology and Medicine, vol. 93, 1956.)

and fine masses of debris were floating in the fluid phase. Presumably the affected cells had been washed off the glass in handling the tubes and some cells had disintegrated. At 2 weeks only a relatively sparse population of cells usually remained. Some of these cells exhibited long, slender strands or dense cytoplasmic masses, that were also regarded as signs of degeneration. It was noted that even at 2 weeks after the virus inoculation normal-looking cells remained in the cellular population. Practically all cells disappeared after 3 to 4 weeks. This pattern of degeneration appeared to be compatible with the relatively slow multiplication of dengue virus as shown in the growth curve experiment. Figures 1 to 14 inclusive depict the typical findings observed in control, as well as infected tissue cultures.



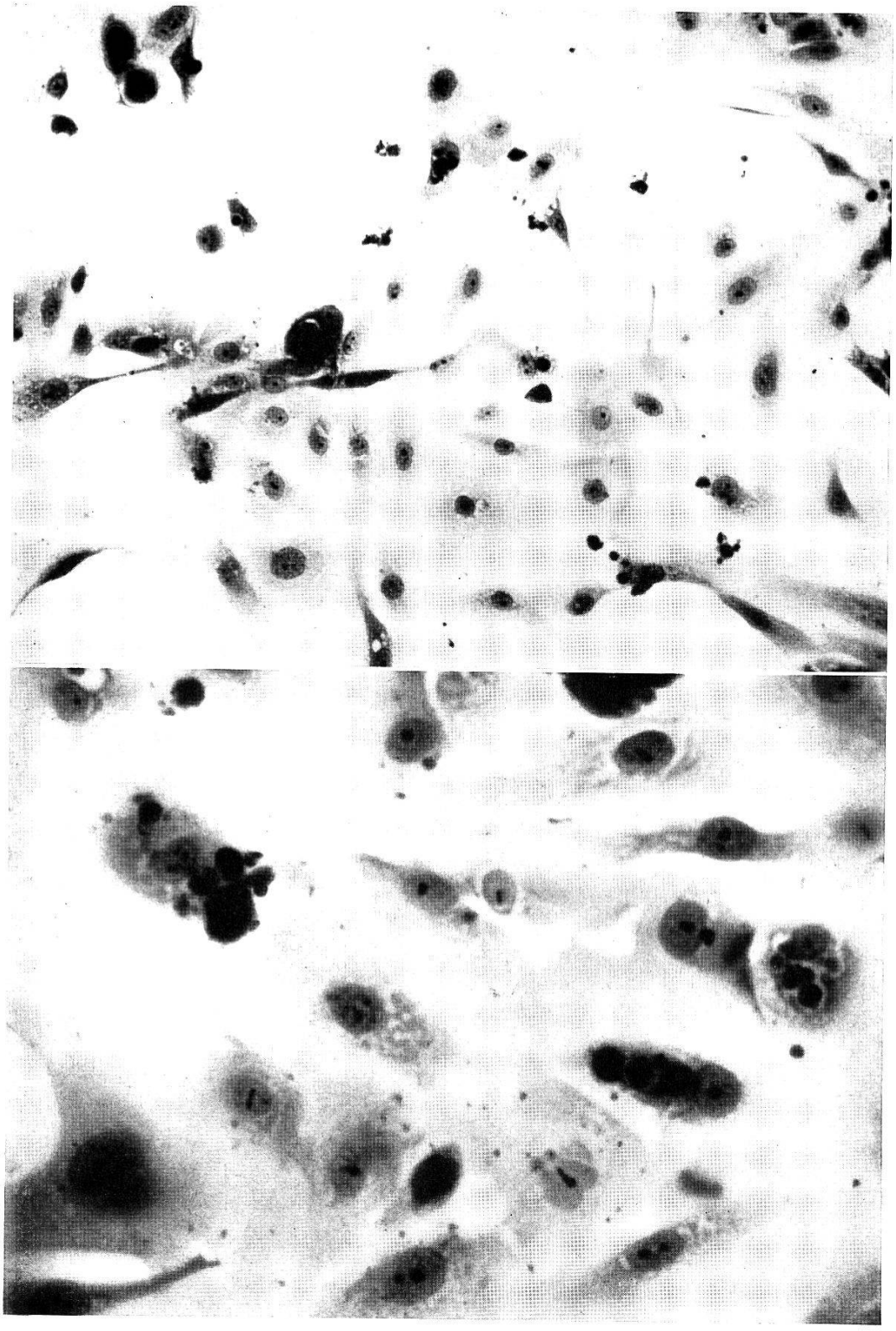
1 2

Fig. 1. Dengue-infected rhesus kidney cells in a Porter flask. Ten days after inoculation with Mochizuki strain virus. Twenty-third tissue culture passage. ×150. Some cells are dark and rounded. (Reproduced from the Journal of Infectious Diseases, vol. 98, 1956.)

Fig. 2. Dengue-infected rhesus kidney cells on a cover slip. Twelve days after inoculation with Mochizuki strain virus. Fourth tissue culture passage. Zenker-formol fixation, May-Grünwald-Giemsa stain. ×150.—About half of the cellular population is gone. Some of the remaining cells exhibit long, slender strands, and others include dense cytoplasmic masses. A small number of normal-looking cells is seen.—Increase of the virus in this culture was confirmed as follows: Two-tenth ml. of undiluted fluid from the third tissue culture passage was inoculated into this culture with the subsequent addition of 1.8 ml. of lactalbumin medium. Mouse LD_{50} of the inoculum was $10^{3.5}$ per 0.02 ml. The medium was changed every 2 to 4 days, and the resultant dilution of the original inoculum was $10^{2.2}$ fold by the time of photography. The culture fluid removed just before the photography showed a mouse LD_{50} titer of $10^{5.25}$ per 0.02 ml. (Reproduced from the Journal of Infectious Diseases, vol. 98, 1956.)

4) *Parallel titration in mice and in tissue cultures:*

The titers of cultivated virus were compared in mice and in tissue cultures. The inoculum was 0.02 ml. for mice by the intracerebral route, and 0.2 ml. for tissue cultures containing 1.8 ml. of medium. Examples of the results obtained are shown in Table 11. In the case of type 1 dengue virus, practically no difference was



3a

3b

Fig. 3a. Dengue-infected rhesus kidney cells on a cover slip. Fourteen days after inoculation with Hawaiian strain virus. Ninth tissue culture passage. Zenker-formol fixation, May-Grünwald-Giemsa stain. ×150.—Cellular degeneration, essentially similar to that shown in Fig. 2, is seen.

Fig. 3b. Dengue-infected rhesus kidney cells on a cover slip. Fourteen days after inoculation with Mochizuki strain virus. Twenty-sixth tissue culture passage. Zenker-formol fixation, May-Grünwald-Giemsa stain. ×230.—Dense cytoplasmic masses are included in some of the cells.

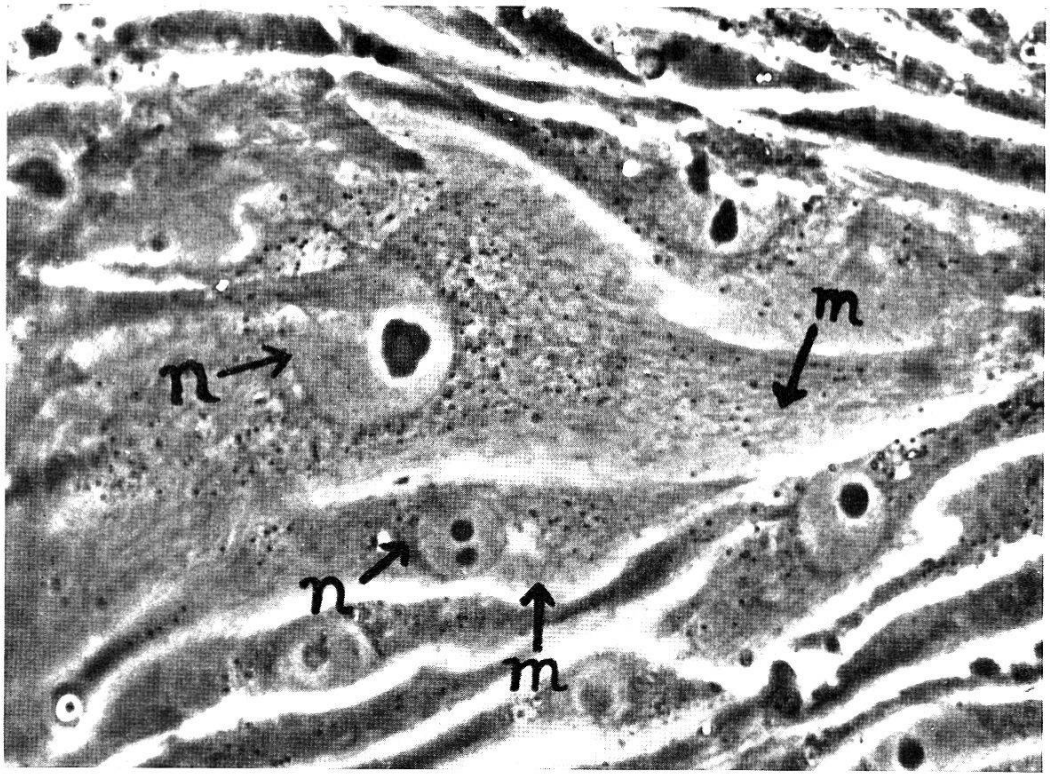


Fig. 4. Uninoculated rhesus kidney cells. Twelve-day culture on a cover slip, observed with a phase contrast microscope. ×450.—Each cell has a smooth contour. The nucleus is round or oval, and has one or two nucleoli. The cytoplasm contains a number of mitochondria and granules which are of regular shape and are arranged regularly (n: nucleus; m: mitochondria).

noted between the titers in mice and in tissue cultures. In the limited experiments with the type 2 dengue virus, a discrepancy in the titers was found: the highest dilution of culture fluid, that caused death of mice, was higher than that producing definite degeneration of tissue culture cells. However, the infection of tissue cultures by the type 2 dengue virus appeared to be regular.

5) *Neutralization tests:*

The cultivated viruses were neutralized by the anti-dengue immune rabbit serum. The neutralization was demonstrated in parallel tests with mice and tissue cultures, as tabulated in Table 12. Figure 15 illustrates a culture to which virus and immune serum were added. No degeneration is seen. Figure 16 shows the control culture to which virus and non-immune serum was added. A marked cellular degeneration developed. These results were considered to present clear evidence for the identification of the tissue-cultured virus as dengue virus.

Positive neutralization was observed in the combination of virus and its homotypic serum, but not in the combination with hetero-

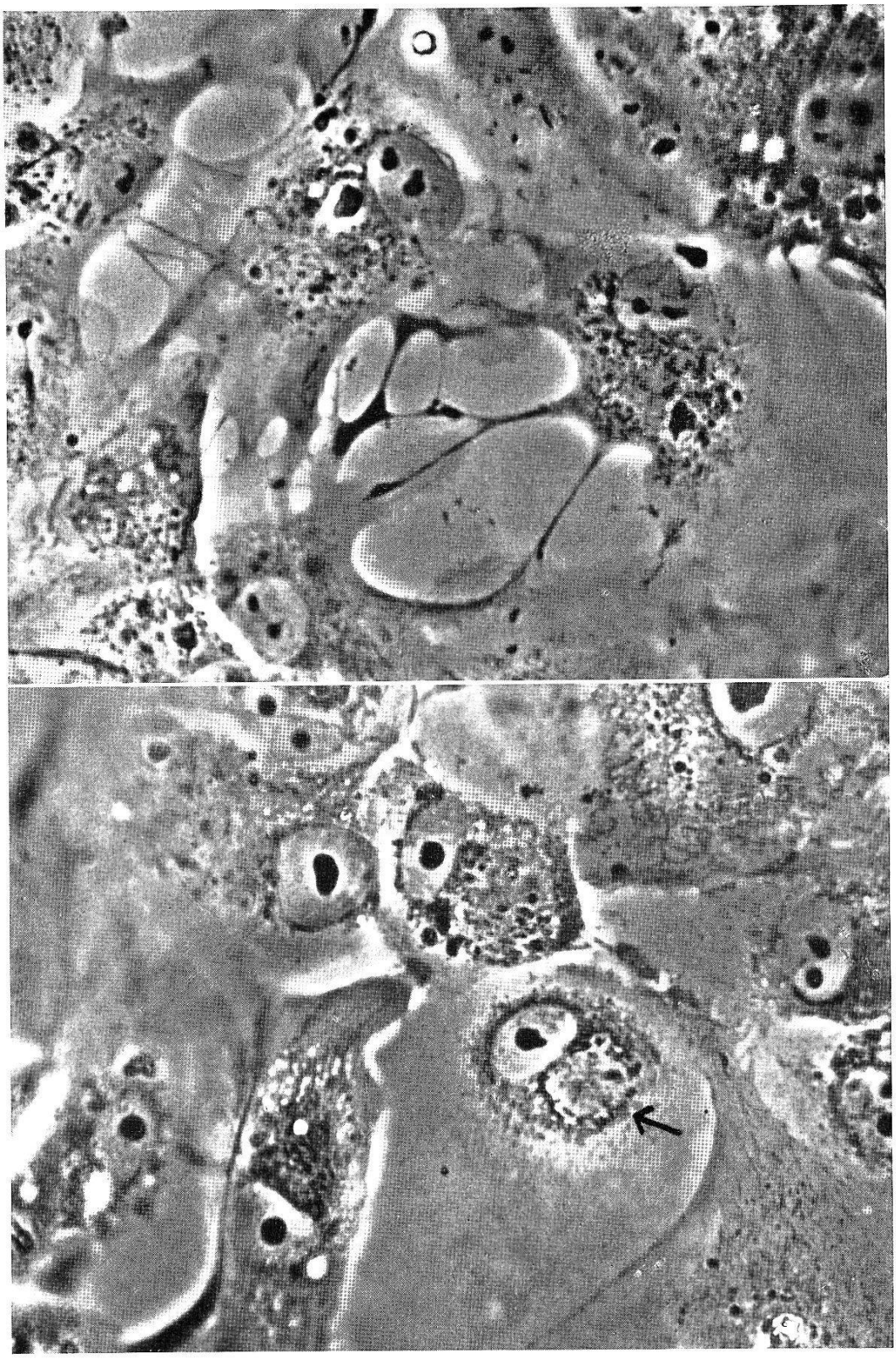


Fig. 5. Dengue-infected rhesus kidney cells on a cover slip, observed with a phase contrast microscope.—Twelve days after inoculation with Mochizuki strain virus. Twenty-second tissue culture passage. ×450.—The contour of cell is irregular, and slender strands are projected. The cytoplasm contains a number of irregular granules.

Fig. 6. Dengue-infected rhesus kidney cells on a cover slip, observed with a phase contrast microscope.—Fourteen days after inoculation with Mochizuki strain virus. Twenty-second tissue culture passage. ×450.—The cytoplasm contains granules of irregular shape and arrangement. Juxtanuclear inclusion-like structures (indicated by an arrow) are seen.

TABLE 12.

Neutralization of type 1 dengue virus cultivated in rhesus kidney tissue culture: Parallel tests in mice and in tissue cultures.

Virus				Immune serum[1] (Anti-Mochizuki)		Control serum[1] (Before immunization)	
Strain	Passage in tissue culture	Mouse IC LD_{50} (per 0.02 ml)	Dilution	Mouse	Tissue culture[2]	Mouse	Tissue culture[2]
Mochizuki	6	$10^{5.0}$	Undiluted	0/10	0/8	10/10	6/8 *
	8	$10^{4.0}$	Undiluted	0/5	0/8	5/5	8/8
	28	$10^{4.5}$	1000-fold	0/7	0/4	6/7	5/5
Hawaiian	2	$10^{4.5}$	Undiluted	0/5	0/6	5/5	7/7
	8	$10^{5.25}$	100-fold	0/7	0/5	7/7	5/5

1. Diluted 5 times with 0.1% lactalbumin hydrolysate medium.

2. The culture fluid used for changing medium included 1% of immune or control serum.

* Two tubes showed nonspecific degeneration.

(Reproduced from the Journal of Infectious Diseases, vol. 98, 1956.)

TABLE 13.

Neutralization of type 1 and type 2 dengue viruses cultivated in rhesus kidney tissue culture: Cross tests in tissue cultures.

Virus					Serum[1]		
Type	Strain	Passage in tissue culture	ID_{50} for tissue culture (per 0.2 ml)	Dilution of infected tissue culture fluid	Anti-Mochizuki immune	Anti-New Guinea C immune	Control[2]
1	Mochizuki	43	$10^{5.0}$	100-fold	0/5 *	5/5	5/5
	Hawaiian	25	$10^{5.25}$	100-fold	0/5	5/5	5/5
2	New Guinea C	18	$10^{3.25}$	Undiluted	5/5 **	0/5	5/5

1. Diluted 1 : 5 with 0.1% lactalbumin hydrolysate medium. Aliquots of this were mixed with equal volumes of virus suspension.

2. Taken before immunization from a rabbit used for immunization against New Guinea C strain virus.

* Numerator indicates number of tissue culture tubes showing definite degeneration; denominator indicates number of tissue culture tubes inoculated. The culture fluid used for changing medium included 1% of immune or control serum.

** Degeneration of tissue cultures inoculated with a mixture of type 2 virus and anti-type 1 serum was milder than that seen in the control cultures exposed to type 2 virus and non-immune serum.

(Reproduced from the Proceedings of the Society for Experimental Biology and Medicine, vol. 93, 1956.)

typic serum. Examples are shown in Table 13. In these limited observations it appeared that the cellular degeneration produced by type 2 dengue virus mixed with anti-type 1 immune serum was a little milder than that produced by the same virus exposed to the control non-immune serum.

F. Dengue Virus in Culture of Trypsinized Human Kidney Cells.

A single experiment was carried out, in which cultures of trypsinized human kidney cells were inoculated with dengue virus.

The kidney was excised from an adult diagnosed as suffering from a kidney tumor. Unaffected parts of the kidney were employed. The preparation and incubation of cultures, and constituents of the culture medium, etc., were the same as described previously with rhesus kidney tissue.

Three of the cultures were inoculated with 0.2 ml. of undiluted fluid from trypsinized rhesus kidney tissue cultures infected with the Mochizuki strain of dengue virus, 27th tissue culture passage. One and eight-tenths ml. of 0.1% lactalbumin hydrolysate medium was added to each tube. The mouse-intracerebral LD_{50} of the inoculum was $10^{4.5}$ per 0.02 ml. The cultures were incubated at 35°C in the stationary state, and half of the fluid phase was replaced every 2 or 3 days. Controls consisted of 2 cultures treated the same as the infected ones, but not exposed to any inoculum. As another control, 7 tubes of rhesus kidney tissue cultures were inoculated with the same batch of virus.

At given periods following the inoculation of virus, portions of the culture fluid were harvested and assayed for viral content. The results obtained are shown in Table 14. There was no definite evidence that dengue virus multiplied in the trypsinized human kidney tissue cultures, although the virus remained active in the culture medium for at least 15 days.

Culture fluid harvested 12 days after inoculation was used for neutralization test. It was shown from the results that, of 7 mice injected intracerebrally with a mixture of the undiluted culture fluid and the anti-dengue immune rabbit serum, all survived, while 6 mice died in the control group consisting of 7 mice injected with a mixture of the same fluid and the control non-immune serum.

Cells in the inoculated human kidney tissue cultures exhibited no significant changes during 15 days after the virus inoculation. It was noted that separation of cells in the control uninoculated cultures subjected to the relatively prolonged incubation was more

distinct in the human kidney tissue cultures than in the rhesus kidney tissue cultures. It was not known whether this finding was due to an effect of the lactalbumin medium on the human kidney cells or to the fact that the cells originated from a tumor-bearing kidney, or was unrelated to either of these factors.

TABLE 14.

Mortality ratios of mice inoculated intracerebrally with culture fluid from dengue-inoculated human kidney tissue cultures.

Dilution of culture fluid	Tissue culture incubation period in days					
	2	4	7	10	12 *	15
10^{-1}	3/3	3/3	3/3	3/3	3/3	3/3
10^{-2}	3/3	3/3	3/3	3/3	2/3	2/3
10^{-3}	3/3	3/3	1/3	0/3	1/3	0/3
10^{-4}	3/3	3/3	0/3	0/3	0/3	0/3
10^{-5}	0/3	0/3	0/3	0/3	0/3	0/3
LD_{50} (per 0.02 ml.)	$10^{4.5}$	$10^{4.5}$	$10^{2.75}$	$10^{2.5}$	$10^{2.5}$	$10^{2.25}$

Three of the cultures were inoculated with 0.2 ml. of undiluted fluid from trypsinized rhesus kidney tissue cultures infected with the Mochizuki strain virus, 27th tissue culture passage. One and eight-tenth ml. of culture medium was added to each culture tube. Mouse-intracerebral LD_{50} of the inoculum was $10^{4.5}$ per 0.02 ml.

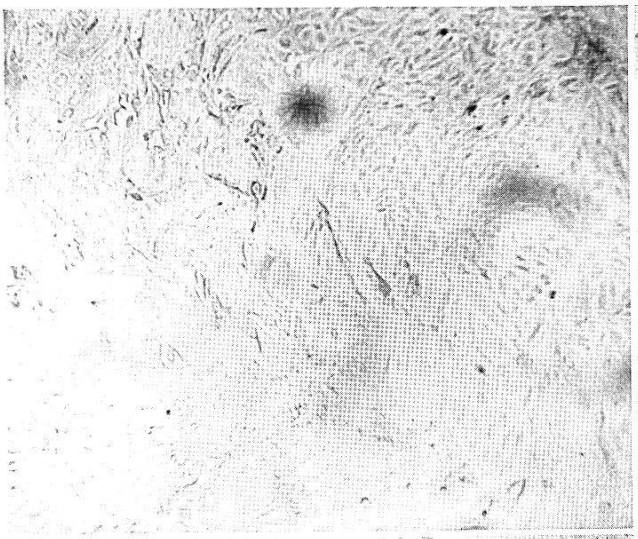
* Neutralization tests were performed in mice, and the virus was identified as dengue.

Figs. 7-10 inclusive. Serial pictures showing a sequence of changes in one uninoculated rhesus kidney tissue culture.—The culture was incubated in the stationary state at 35°C. and the fluid phase was replaced by half volume every 24 hours. Observation *in situ*. ×32.

Fig. 7. Two days after the beginning of observation. No degeneration is seen.
Fig. 8. Four days after the beginning of observation. No degeneration is seen.
Fig. 9. Nine days after the beginning of observation. No degeneration is seen.
Fig. 10. Fourteen days after the beginning of observation. The cellular sheet has a few circular holes, where some cells have detached.

Figs. 11-14 inclusive. Serial pictures showing a sequence of changes in one dengue-infected rhesus kidney tissue culture.—Two-tenth ml. of culture fluid from tissue cultures infected with Mochizuki strain virus, 44th tissue culture passage, was inoculated into tube, and 1.8 ml. of 0.1% lactalbumin hydrolysate medium was added. The culture was incubated in the stationary state at 35°C. and the fluid phase was replaced by half volume every 24 hours. Observation *in situ*. ×32.

Fig. 11. Two days after the virus inoculation. No apparent degeneration is seen.
Fig. 12. Four days after the virus inoculation. The culture cells show no definite change attributable to the infection.
Fig. 13. Nine days after the virus inoculation. Some cells are dark and rounded; some have detached from the cellular sheet.
Fig. 14. Fourteen days after the virus inoculation. An almost complete degeneration has taken place. Still a small number of cells looks apparently normal.



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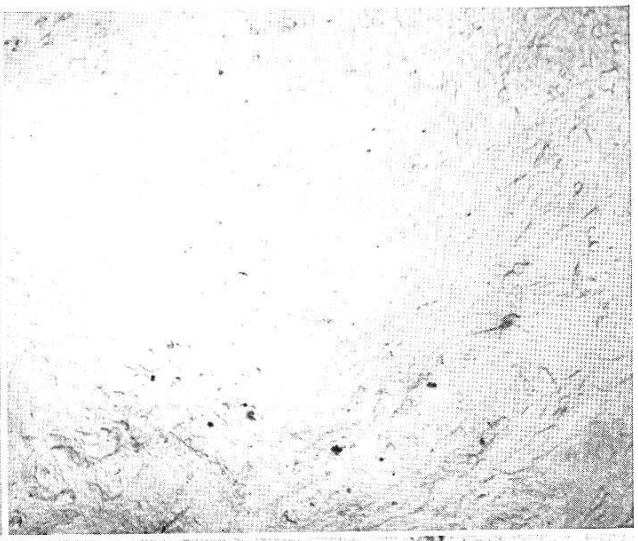

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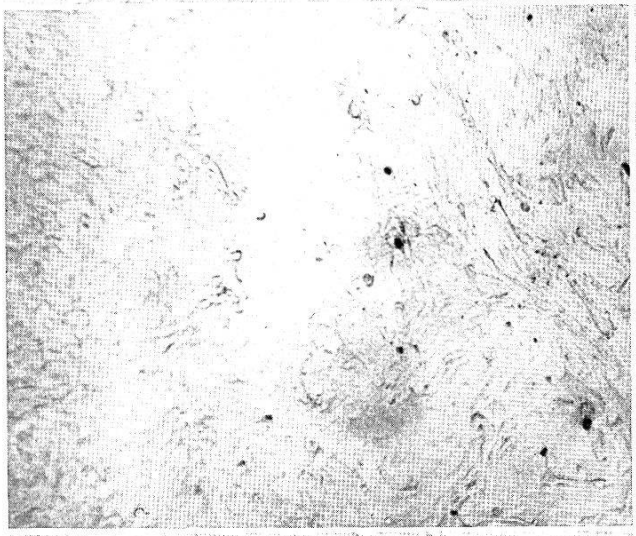

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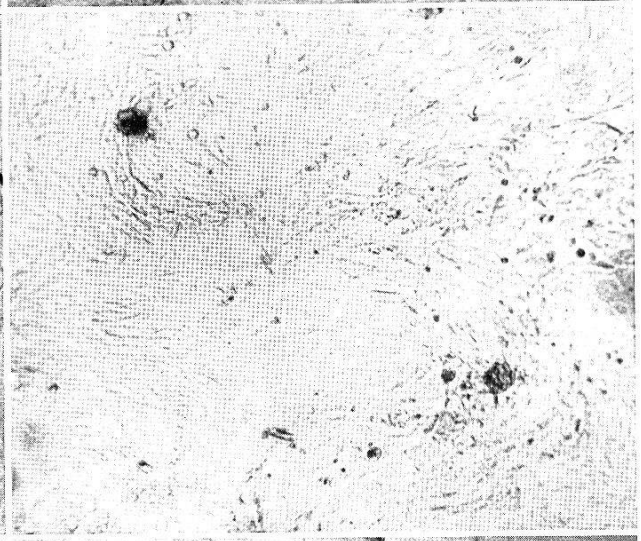

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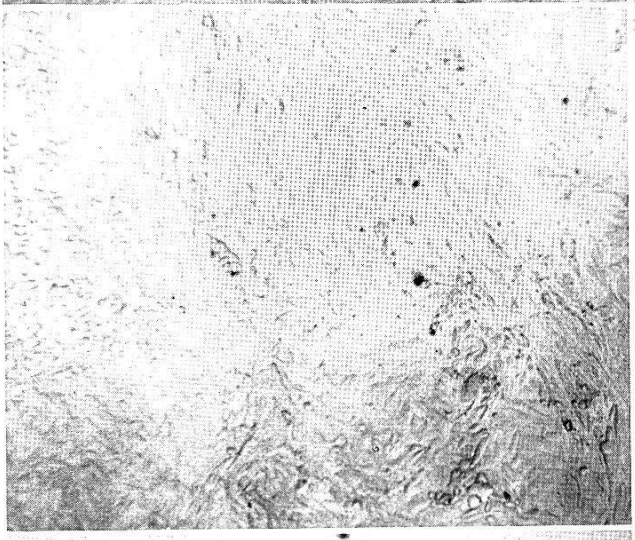

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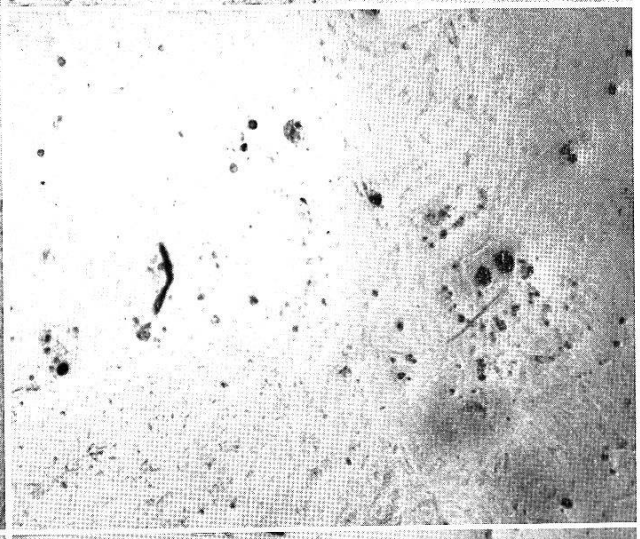

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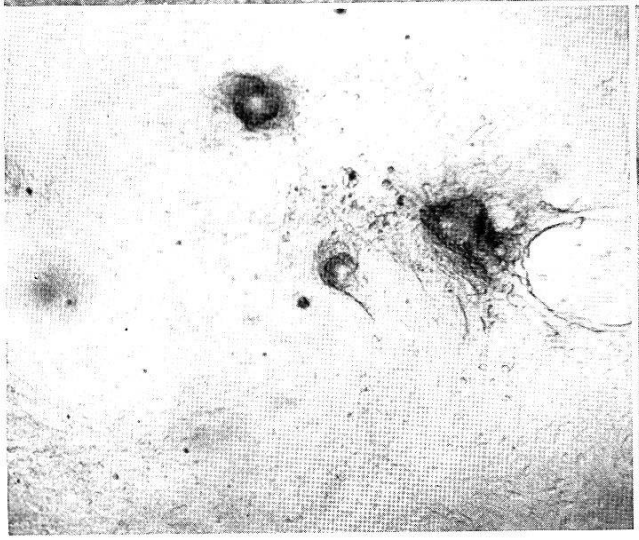

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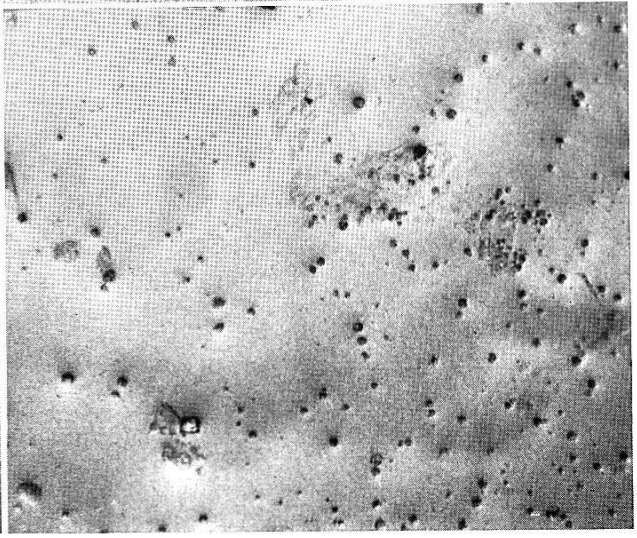

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In contrast to these observations, the inoculated rhesus kidney tissue cultures showed marked degeneration 9 days after virus inoculation. Culture fluid harvested at that date was found to contain $10^{5.25}$ mouse-LD_{50}'s of virus per 0.02 ml. Six days later the majority of the cell population had disappeared.

G. A Cytopathogenic Agent Recovered from an Uninoculated Rhesus Kidney Tissue Culture.

During the experiments with trypsinized rhesus kidney tissue cultures, a cytopathogenic agent of unknown origin was encountered. It was originally found in one of 4 uninoculated tubes used as controls. The first sign, which was noted 7 days after the beginning of incubation, was a small circular hole devoid of cells in a continuous cellular layer. Surrounding this clear area was a zone of abnormal cells with numerous cytoplasmic vacuoles of various sizes. On the 8th day the discrete area had increased in size and became more distinct. Some of the surrounding cells appeared to have fused and formed a syncitial multinuclear structure. In the others the cytoplasm had been replaced with huge vacuoles. Thus the general view of the area looked like a collection of foam.

A subculture was made from the tube by transferring 0.2 ml. of the undiluted culture fluid to 5 new tubes with the subsequent addition of 1.8 ml. of medium to each tube. Eight passages were carried out in the same manner at intervals of 6 to 10 days, using 3 to 5 tubes for each passage. Inoculation of culture cells grown in

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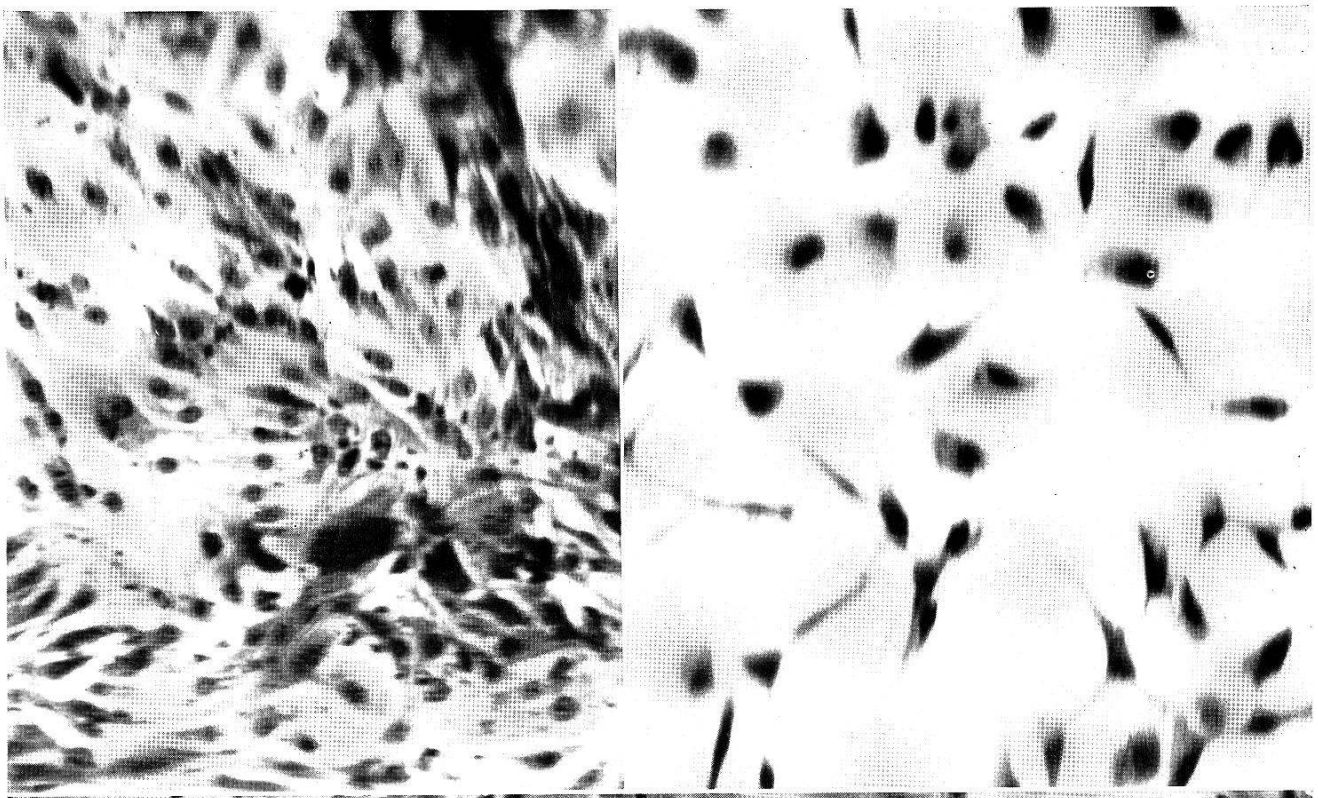
Fig. 15. Rhesus kidney tissue culture, inoculated with a mixture of Mochizuki strain dengue virus, 8th tissue culture passage, and immune rabbit serum. Eleven days after the inoculation of virus-serum mixture. Zenker-formol fixation, May-Grünwald-Giemsa stain. ×130.—No apparent degeneration is seen.—The culture fluid was changed by half volume every 2 or 3 days with medium containing 1% of the immune serum.—The picture is not well-focused, because this is taken of a stained culture in a test tube filled with xylol. However, the normal cell shapes and fully intact sheet of cells are shown.

Fig. 16. Rhesus kidney tissue culture, inoculated with a mixture of dengue virus, the same batch used for Figure 15, and control non-immune serum. Eleven days after the inoculation of virus-serum mixture. Zenker-formol fixation, May-Grünwald-Giemsa stain. ×130.—A marked cellular degeneration has taken place.—The culture fluid was changed by half volume every 2 or 3 days with medium including 1% of the control serum.—The photograph is not in clear focus for the same reason stated for Figure 15.

Fig. 17. Rhesus kidney cells grown on a cover slip and infected by an unidentified cytopathogenic agent obtained from an uninoculated culture of rhesus kidney cells. Ten days after inoculation. Second passage. Zenker-formol fixation, May-Grünwald-Giemsa stain. ×200.—Some cells are multinuclear, or contain vacuoles of various sizes. The remaining cells are apparently normal.—(Reproduced from the Journal of Infectious Diseases, vol. 98, 1956.)



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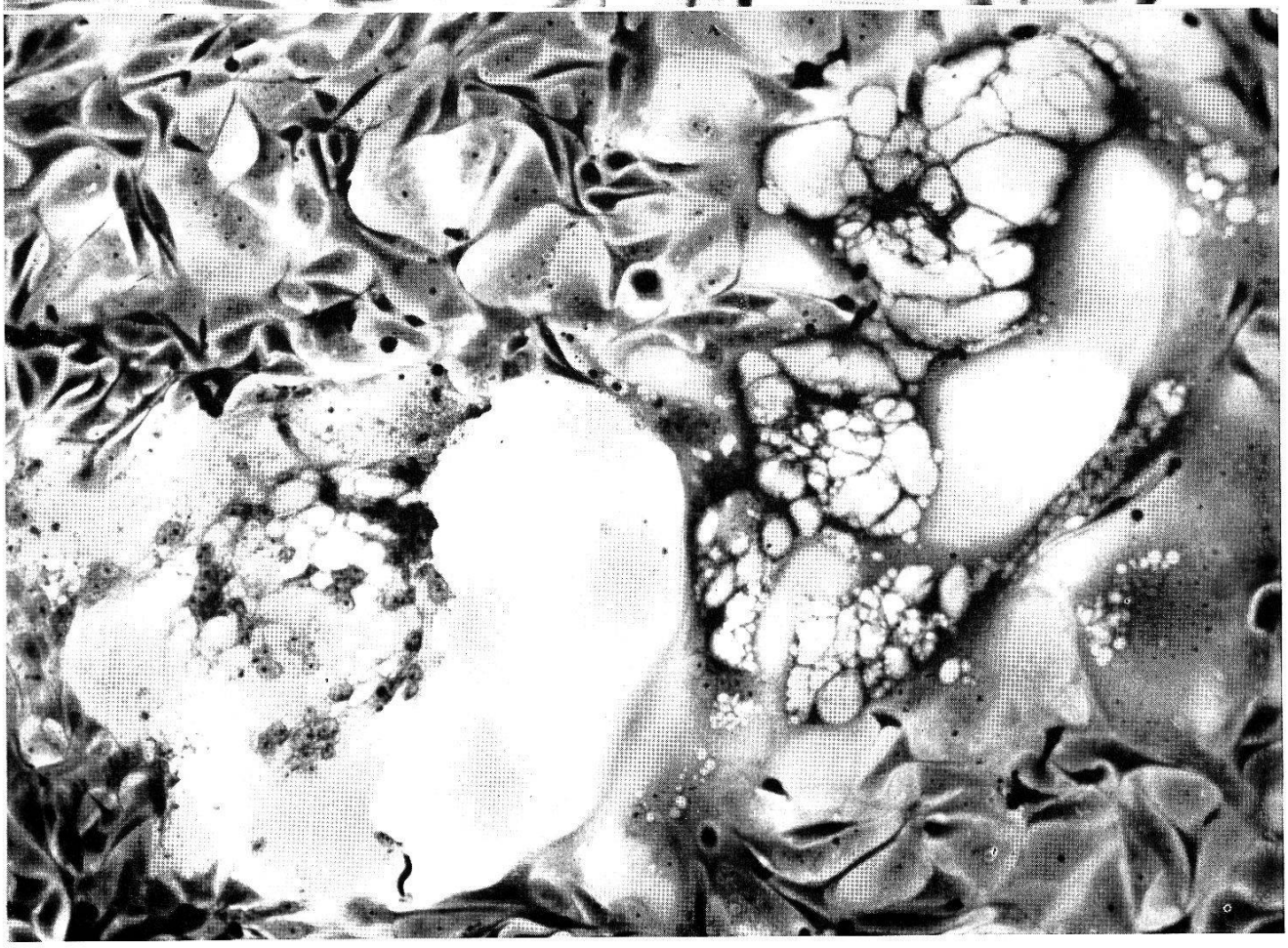

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Porter flasks or on cover slips in Leighton tubes were also performed. In every inoculated culture, the same morphological changes were found. The clear areas usually became 1 to 3 mm. in diameter 7 to 10 days after the inoculation, and were easily observed with the naked eye. In some cases they merged to form a larger hole. Cells not immediately adjacent to such areas were apparently normal at that time. However, the whole cellular layer underwent degeneration after 2 to 3 weeks. Figure 17 shows an example of the typical findings.

Neither bacterial nor fungal growth were found in bacteriological culture media inoculated with the culture fluids from these tubes.

Undiluted culture fluid from the 7th passage, injected intracerebrally into 2-week-old white mice, did not cause death or specific signs of illness during a 4-week observation period.

Results of a single experiment indicated that at the 7th passage there were $10^{2.75}$ ID_{50} for tissue culture of this agent per 0.2 ml. on the basis of cellular degeneration observed at 1 week after the inoculation.

The cytopathogenicity of this agent was not affected by being mixed with the anti-dengue immune rabbit serum, as shown in Table 15. No antigenic relationship was detected, therefore, between this agent and dengue virus.

It is curious that, so far as the present observations are concerned, this agent has been encountered much more frequently in uninoculated control cultures than in the dengue-inoculated cultures.

TABLE 15.

Action of dengue-immune and control serums upon an unidentified cytopathic agent recovered from rhesus kidney tissue culture.

Inoculum[1]	Degeneration in tissue culture[2]
Agent *plus* Dengue-immune serum [3]	5/5
Agent *plus* Control serum	5/5
Agent only	5/5
Control (without agent nor serum)	0/3

[1] Unidentified cytopathic agent: 7th tissue culture passage, diluted 10-fold with lactalbumin hydrolysate medium. Serum: diluted 50-fold with lactalbumin hydrolysate medium. Procedures of inoculation were the same as those of neutralization tests with dengue virus.

[2] Numerator indicates number of tissue culture tubes showing characteristic degeneration; denominator indicates number of tissue culture tubes inoculated. The judgement was based on observations made 1 week after the inoculation.

[3] Anti-type 1 dengue rabbit serum.

From one tube which showed signs of concomitant infection with this agent and dengue virus, the culture fluid was transferred to new cultures, and serial transmissions were carried out. From the second to fourth subcultures the degeneration pattern exhibited both the large vacuolated multinuclear cells induced by this agent and the small round dark cells associated with dengue infection. After the fifth subculture, however, the affected cells showed only changes similar to the original dengue infection. Mouse-infectivity tests with the undiluted culture fluid from these tubes indicated that dengue virus was retained throughout the subcultures. It appeared that this agent might have been excluded by dengue virus.

No agent similar to this was found by the present author in the rhesus testicular tissue cultures or in the human kidney tissue cultures.

IV. *Discussion.*

Testicular and renal tissues from rhesus monkeys were found to differ in their capacity to support the *in vitro* growth of mouse-adapted dengue virus.

It was shown that dengue virus of the Mochizuki strain survived in the testicular tissue cultures for a relatively long period of time, whereas it disappeared completely within a few days from culture medium lacking cells. In experiment II described in Section C of Chapter III, the successive changes of culture fluid resulted in a dilution of the original virus inoculum beyond the original LD_{50} titer by the 42nd day. Virus was still present 3 weeks later in spite of further medium-changes and resultant dilution of the original inoculum. Nevertheless, it was uncertain as to whether the rhesus testicular tissue cultures supported multiplication of dengue virus. No definite morphological change was noticed in explants of the fibroblastic cells after the inoculation of virus. Active virus could not be detected after a few passages through tissue cultures. It remains uncertain whether this reflected the actual disappearance of virus or was the result of a change of the virus to reduced virulence for mice. It may be pointed out in this connection that mice which survived after inoculation of the culture fluid from which no active virus was detected, were shown to be as susceptible to dengue virus as untreated mice of the same age (see Table 3 and Table 4b). Although no quantitatively controlled data were collected in these observations, it was probable that the mice surviving after the inoculation of culture fluid had no immunity to dengue virus. These results suggest that during the serial transmissions through rhesus testicular tissue cultures dengue virus

actually disappeared. It is difficult to understand the cell-virus relationship that would result in survival of virus without multiplication for weeks in the presence of cells in an environment in which it would become noninfective in a few days in the absence of cells. This seems to have been the case.

In contrast to the results with testicular tissue, it was evident that dengue virus propagated in tissue cultures consisting of trypsinized rhesus kidney cells. The evidence was unequivocal from the results of the growth curve experiment, as well as of the serial subcultures. Similar results were obtained with representative virus strains of the two known antigenic types of dengue. Reasons for the difference between testicular and kidney cells in their capacity of supporting viral growth remains unsolved. The problem as to whether the difference was due to a difference of culture methods, or resulted from intrinsic properties of the testicular and kidney cells, warrants further studies. It was noted that dengue virus multiplication produced a cytopathogenic effect in rhesus kidney tissue cultures embedded in chicken plasma clot.

In view of the apparent suitability of the epithelial cells from rhesus kidney for propagating dengue virus, it appeared of interest to investigate the possibility of using human kidney tissues for dengue research. In a single experiment, however, the evidence that dengue virus multiplies in tissue cultures of trypsinized human kidney cells was equivocal. According to observations by many investigators (VAN ROOYEN and RHODES, 1948; SABIN, 1952), dengue infection of rhesus monkeys is usually mild (so-called inapparent infection) while clinical signs of human dengue, including renal involvement, are more severe. With polioviruses a discrepancy between *in vitro* and *in vivo* multiplication has been shown (EVANS et al., 1954a; KAPLAN, 1955). It has also been demonstrated, that, although cultures of kidney tissue of the primates generally can support the growth of poliovirus well, kidney cells from a particular species of ape lack such capacity (BARSKI et al., 1954; JEZIERSKI, 1955; KAPLAN, 1955). The results with dengue virus may be correlated essentially with these findings with polioviruses, and would appear to suggest specific cellular affinity for a particular kind of virus.

The dengue infection of rhesus kidney tissue cultures was unique in its slowness of progress. It was necessary to continue cultivation at least 3 weeks in order to observe the full effect of dengue virus upon cultured cells. A medium suitable for prolonged maintenance of cultures was required, and frequent replacement of the medium was needed. It is likely that many unsuccessful attempts to cultivate dengue virus in tissue culture systems in the

past (including the present author's own experiences) might have been due to situation where the cultured tissues had undergone non-specific degeneration before the multiplication of virus could be effectively initiated.

The tissue-cultured rhesus kidney cells exhibited degeneration following exposure to virus. Association of the cellular degeneration with the infection of dengue virus was indicated by several lines of evidence. Parallel titrations in mice and in tissue cultures showed that in general the minimal infective dose of type 1 virus for mice was approximately the minimal amount that would cause degeneration of inoculated tissue cultures. The suppression of cytopathic effect by the antiserum paralleled its capacity to protect mice from infection. With type 2 virus some discrepancy was noted between the titers in mice and those in tissue cultures. However, this was due to the well known irregularity of infection in mice with certain strains of dengue virus such as the type 2 strain used in these studies. Suppression of cellular degeneration by the specific antiserum was clearly demonstrated in the case of both type 1 and type 2 viruses.

The morphological changes visible under a microscope with the lower magnifications were first clear at 5 to 7 days after the inoculation of virus. It took 3 to 4 weeks until whole cellular populations were virtually destroyed. In the growth curve experiment it was noted that maximal titers of virus were found in the tissue culture fluid at about 7 to 10 days when apparent degeneration was beginning to occur. Therefore, the possibility that much virus was released from infected culture cells prior to development of obvious degeneration must be considered. It should be stated in this connection that some uninoculated control cultures showed a certain abnormal appearance after a prolonged incubation lasting 3 weeks or more. However, the changes seen in the control cultures were relatively limited and never equaled the widespread destruction of cells observed invariably in the infected cultures.

It is important to realize that, in the experimental system used, degeneration of fairly large numbers of cells may go unnoticed. As long as there is an essentially continuous sheet of cells on the glass surface, an observer may consider the population undiminished. Reproduction of cells might compensate for loss of a substantial number of infected cells, and the culture might still be regarded as showing no definite signs of damage. It is not possible at the present time to state whether the virus detected in the early stage of infection resulted from release of virus from infected, but morphologically normal, cells, or from virus liberated by degenerating cells.

Morphological studies were based on three techniques. Besides the routine direct microscopic observations of test tube cultures, fixation and staining and phase contrast microscopy were performed with the coverslip cultures. Cytological changes, including granulation or constriction of the cytoplasm, formation of long, slender strands or dense, cytoplasmic masses, etc., were observed in some detail. Although these observations were not made with a single, particular cell by a time-lapse cinematographic method (BARSKI et al., 1955), nor in correlation with the release of virus from single cells (LWOFF et al., 1955), the changes observed by the present author appear to be of similar nature to those described by other investigators with either monkey kidney or HeLa cells infected by polioviruses (KLÖNE, 1955; HARDING et al., 1956; DUNNEBACKE, 1956; REISSIG et al., 1956). This suggests that these cytological alterations may represent a pattern common to infections of tissue cultures by certain viruses.

In the neutralization tests it appeared that a partial neutralization by heterologous antibody may have taken place, since the cellular degeneration in rhesus kidney cell cultures exposed to the mixture of type 2 virus and anti-type 1 immune serum was milder than the degeneration produced by the same virus mixed with control non-immune serum. The neutralization was complete only when performed with homologous serum. It has been found by means of hemagglutinin-inhibition tests that type 1 and type 2 dengue viruses show a cross-reaction, and also are related to certain other kinds of viruses (SWEET and SABIN, 1954; CASALS and BROWN, 1954; CASALS, 1957). These results warrant further study regarding the antigenic relationships of prototypes of dengue.

The cytopathogenic agent recovered from cultures of kidney cells from an apparently normal rhesus monkey caused no confusion, as it was distinguished clearly from dengue virus. This agent was not infective for 2-week-old white mice by the intracerebral route, and it was not neutralized by the specific anti-dengue immune rabbit serum. Moreover, the appearance of the cells affected by this agent was quite different from the changes induced by dengue virus. Recovery of agents of cytopathogenic character from uninoculated tissue cultures was reported (ENDERS and PEEBLES, 1954; RUSTIGIAN et al., 1955). Recent data showed that cytopathic "simian" agents are encountered not infrequently (HULL et al., 1956). The agent recovered by the present author is probably in the same category as the agents reported by previous investigators.

Strains of dengue virus used in the present study were mouse-adapted. In view of the difficulty of isolating dengue virus from

natural sources by inoculation of mice, trials to isolate it with tissue cultures should be undertaken. Recovery of new strains, or even of new type(s) of dengue virus may be expected.

Cultivation of virus in tissue culture also may make possible the development of sublines that presumably represent mutants from the parent virus. Such mutants are of fundamental interest, and, in addition, may be of practical value in developing measures for preventive immunization.

References.

ASHBURN, P. M. & CRAIG, C. F. (1907). Experimental investigations regarding the etiology of dengue fever. — J. infect. Dis. *4:* 440-475.

BARSKI, G., JEZIERSKI, A. & LÉPINE, P. (1954). Sensibilité au virus de la poliomyélite *in vitro* des tissus de différentes espèces de singes d'Afrique centrale. Non réceptivité des tissus de certains mammifères. — Ann. Inst. Pasteur *86:* 243-247.

BARSKI, G., ROBINEAUX, R. & ENDO, M. (1955). Phase contrast cinematography of cellular lesion produced by poliomyelitis virus *in vitro.* — Proc. Soc. exp. Biol. *88:* 57-59.

CAMERON, G. (1950). Tissue Culture Technique. New York: Academic Press.

CASALS, J. (1957). The arthropod-borne group of viruses. — Trans. N.Y. Acad. Sci., Ser. II, *19:* 219-235.

CASALS, J. & BROWN, L. V. (1954). Hemagglutination with arthropod-borne viruses. — J. exp. Med. *99:* 429-449.

CLELAND, J. B. (1930). Dengue Fever. *in* A System of Bacteriology. — London: HM Stationary Office *7:* 376-385.

DOERR, R. (1930). Pappatacifieber und Dengue. *in* Handbuch d. pathogen. Mikroorgan., ed. KOLLE, W., KRAUS, R. & UHLENHUTH, P. — Jena: Fischer, VIII, 501-546.

DULBECCO, R. (1952). Plaques produced on a monolayer tissue culture by single particles of an animal virus. — Science *115:* 481-482.

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. — J. exp. Med. *99:* 167-182.

DUNNEBACKE, T. H. (1956). Correlation of the stage of cytopathic change with the release of poliomyelitis virus. — Virology *2:* 399-410.

EAGLE, H. (1955a). Nutritional needs of mammalian cells in tissue culture. — Science *122:* 501-504.

— (1955b). The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. — J. exp. Med. *102:* 37-48.

— (1955c). The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. — J. biol. Chem. *214:* 839-852.

— (1955d). The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure. — J. exp. Med. *102:* 595-600.

ENDERS, J. F. (1952). Propagation of viruses and rickettsiae in tissue cultures. *in* Viral and Rickettsial Infections of Man, ed. RIVERS, T. M. Philadelphia: Lippincott, pp. 126-140.

— (1954). Cytopathology of virus infections: Particular reference to tissue culture studies. — Ann. Rev. Microbiol. *8:* 473-502.

ENDERS, J. F., WELLER, T. H. & ROBBINS, F. C. (1949). Cultivation of the Lansing

strain of poliomyelitis virus in cultures of various human embyronic tissues. — Science *109:* 85-87.

EVANS, C. A., BYATT, P. H., CHAMBERS, V. C. & SMITH, W. M. (1954a). Growth of neurotropic viruses in extraneural tissues. VI. Absence of *in vivo* multiplication of poliomyelitis virus, types I and II, after intratesticular inoculation of monkeys and other laboratory animals. — J. Immunol. *72:* 348-352.

EVANS, C. A., CHAMBERS, V. C., SMITH, W. M. & BYATT, P. H. (1954b). Growth of neurotropic viruses in extraneural tissues. V. Survey of tissues of various animal species for capacity to support multiplication of poliomyelitis virus *in vitro.* — J. infect. Dis. *94:* 273-283.

FISCHER, A. (1925). Tissue Culture. — London: William Heinemann.

— (1946). Biology of Tissue Cells. — New York: Cambridge Univ. Press.

GEY, G. O., COFFMAN, W. D. & KUBICEK, M. T. (1952). Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. — Cancer Res. *12:* 264-265.

HABEL, K., Editor. (1955). Biology of Poliomyelitis. — Ann. N.Y. Acad. Sci. *61:* 737-1064.

HALLAUER, C. (1938). Die Züchtung der Virusarten außerhalb ihrer Wirte. A. Die Viruszüchtung im Gewebsexplantat. *in* Handbuch d. Virusforschung, ed. DOERR, R. & HALLAUER, C. — Wien: Springer, I, 369-419.

HANKS, J. H. *et al.* (1955). Staining reagents and procedures. *in* An Introduction to Cell and Tissue Culture. — Minneapolis: Burges, pp. 120-122.

HARDING, C. V., HARDING, D., MCLIMANS, W. F. & RAKE, G. (1956). Cytological changes accompanying the growth of poliomyelitis virus in cells of human origin (strain HeLa). — Virology *2:* 109-125.

HOTTA, S. (1947). Recent advance in the research of dengue. — Nisshin Igaku *34:* 252-259 (in Japanese).

— (1951). Isolation and identification of the etiologic virus of dengue. — Virus *1:* 187-197 (in Japanese).

— (1952). Experimental studies on dengue. I. Isolation, identification and modification of the virus. — J. infect. Dis. *90:* 1-9.

HOTTA, S. & EVANS, C. A. (1955). Cultivation of mouse-adapted dengue virus in tissue culture. — Fed. Proc. *14:* 466.

— (1956a). Cultivation of mouse-adapted dengue virus (type 1) in rhesus monkey tissue culture. — J. infect. Dis. *98:* 88-97.

— (1956b). Cultivation of type 2 dengue virus in rhesus kidney tissue culture. — Proc. Soc. exp. Biol. *93:* 153-155.

HULL, R. N., MINNER, J. R. & SMITH, J. W. (1956). New viral agents recovered from tissue cultures of monkey kidney cells. I. Origin and properties of cytopathogenic agents S.V.$_1$, S.V.$_2$, S.V.$_4$, S.V.$_5$, S.V.$_6$, S.V.$_{11}$, S.V.$_{12}$ and S.V.$_{15}$. — Amer. J. Hyg. *63:* 204-215.

JEZIERSKI, A. (1955). Action cytopathogène des trois prototypes de virus de la poliomyélite *in vitro* sur les tissus de différentes espèces de singes d'Afrique centrale. Non réceptivité des tissus de certains animaux. — Ann. Inst. Pasteur *89:* 78-82.

KAPLAN, A. S. (1955). Comparison of susceptible and resistant cells to infection with poliomyelitis virus. — Ann. N.Y. Acad. Sci. *61:* 830-838.

KIMURA, R. (1953). Tissue Culture: As Applied Especially Within Bacteriology and Immunology. — Copenhagen: Ejnar Munksgaard.

KLÖNE, W. (1955). Untersuchungen zur Cytopathogenität des Poliomyelitis-Virus (Typ Leon). — Arch. ges. Virusforsch. *6:* 36-44.

LEDINKO, N., RIORDAN, J. T. & MELNICK, J. L. (1951). Differences in cellular pathogenicity of two immunologically related poliomyelitis viruses as revealed in tissue culture. — Proc. Soc. exp. Biol. *78:* 83-88.

LEVADITI, C. (1913a). Virus de la poliomyélite et culture des cellules *in vitro*. C. R. Soc. Biol. *75:* 202-205.

— (1913b). Virus rabique et culture des cellules *in vitro*. — C. R. Soc. Biol. *75:* 505.

LWOFF, A., DULBECCO, R., VOGT, M. & LWOFF, M. (1955). Kinetics of the release of poliomyelitis virus from single cells. — Virology *1:* 128-139.

MEIKLEJOHN, G., ENGLAND, B. & LENNETTE, E. H. (1952). Propagation of dengue virus strains in unweaned mice. — Amer. J. trop. Med. Hyg. *1:* 51-58.

MILZER, A., LEVINSON, S. O., VANDERBOOM, K. & ADELMAN, P.: (1950). Cultivation of poliomyelitis virus (Lansing strain) in human embryonic and placental tissues. — Proc. Soc. exp. Biol. *74:* 136-138.

MORGAN, J. F., MORTON, H. J. & PARKER, R. C. (1950). Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. — Proc. Soc. exp. Biol. *73:* 1-8.

MURRAY, M. R. & KOPECH, G. (1953). A Bibliography of the Research in Tissue Culture. — New York: Academic Press.

PARKER, R. C. (1950). Methods of Tissue Culture, 2nd ed. — New York: Hoeber.

POMERAT, C. M., Editor. (1954). Tissue Culture Technique in Pharmacology. — Ann. N.Y. Acad. Sci. *58:* 971-1326.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent endpoints. — Amer. J. Hyg. *27:* 493-497.

REISSIG, M., HOWES, D. W. & MELNICK, J. L. (1956). Sequence of morphological changes in epithelial cell cultures infected with poliovirus. — J. exp. Med. *104:* 289-304.

ROBBINS, F. C., WELLER, T. H. & ENDERS, J. F. (1952). Studies on the cultivation of poliomyelitis viruses in tissue culture. II. The propagation of the poliomyelitis viruses in roller-tube cultures of various human tissues. — J. Immunol. *69:* 673-694.

VAN ROOYEN, C. E. & RHODES, A. J.: (1948). Dengue fever, *in* Virus Diseases of Man. — New York: Thomas Nelson, pp. 437-454.

RUSTIGIAN, R., JOHNSTON, P. & REIHART, H. (1955). Infection of monkey kidney tissue cultures with virus-like agents. — Proc. Soc. exp. Biol. *88:* 8-16.

SABIN, A. B. (1950). The dengue group of viruses and its family relationships. — Bact. Rev. *14:* 225-232.

— (1952). Dengue. *in* Viral and Rickettsial Infections of Man, ed. RIVERS, T. M. — Philadelphia: Lippincott, pp. 556-568.

— (1955). Recent advances in our knowledge of dengue and sandfly fever. — Amer. J. trop. Med. Hyg. *4:* 198-207.

SABIN, A. B. & SCHLESINGER, R. W. (1945). Production of immunity to dengue with virus modified by propagation in mice. — Science *101:* 640-642.

SANDERS, M., KIEM, I. & LAGUNOFF, D. (1953). Cultivation of viruses: A critical review. — Arch. Path. *56:* 148-225.

SCHERER, W. F. (1955). Glassware and its cleaning. *in* An Introduction to Cell and Tissue Culture. — Minneapolis: Burges, pp. 2-4.

SCHLESINGER, R. W. (1950). Propagation in chick embryos of the Hawaiian strain of dengue virus. I. Sustained serial passage in eggs after one hundred and one intracerebral passages in mice. — Amer. J. Hyg. *51:* 248-254.

SIMMS, H. S. & SANDERS, M. (1942). Use of serum ultrafiltrate in tissue cultures for studying deposition of fat and for propagation of viruses. — Arch. Path. *33:* 619-635.

SIMMS, H. S. & STILLMAN, N. P. (1937). Substances affecting adult tissue *in vitro*. I. The stimulating action of trypsin on fresh adult tissue. — J. gen. Physiol. *20:* 603-619.

SMITH, W. M., CHAMBERS, V. C. & EVANS, C. A. (1950). Growth of neurotropic

viruses in extraneural tissues: Preliminary report on the propagation of poliomyelitis virus (Lansing and Hof. strains) in cultures of human testicular tissue. — Northw. Med. *49:* 368-372.

— (1951). Growth of neurotropic viruses in extraneural tissues. IV. Poliomyelitis virus in human testicular tissue *in vitro*. — Proc. Soc. exp. Biol. *76:* 696-700.

SMITH, W. M. & EVANS, C. A. (1954). Growth of neurotropic viruses in extraneural tissues. VII. Cultivation of MM and Western equine encephalitis viruses in cultures of rhesus testicular tissue. — J. Immunol. *72:* 353-359.

STEINHARDT, E., ISRAELI, C. & LAMBERT, R. A. (1913). Studies on the cultivation of the virus of vaccinia. — J. infect. Dis. *13:* 294-300.

SWEET, B. H. & SABIN, A. B. (1954). Properties and antigenic relationships of hemagglutinins associated with the dengue viruses. — J. Immunol. *73:* 363 to 373.

SYVERTON, J. T., SCHERER, W. F. & BUTORAC, G. (1951). Propagation of poliomyelitis virus in cultures of monkey and human testicular tissues. — Proc. Soc. exp. Biol. *77:* 23-28.

WELLER, T. H., ENDERS, J. F., ROBBINS, F. C. & STODDARD, M. B. (1952). Studies on the cultivation of poliomyelitis viruses in tissue culture. I. The propagation of poliomyelitis viruses in suspended cell cultures of various human tissues. — J. Immunol. *69:* 645-671.

WELLER, T. H., ROBBINS, F. C. & ENDERS, J. F. (1949). Cultivation of poliomyelitis virus in cultures of human foreskin and embryonic tissues. — Proc. Soc. exp. Biol. *72:* 153-155.

YAOI, H. & ARAKAWA, S. (1948). Studies on dengue (Résumé). — Jap. med. J. *1:* 4-12.

YOUNGNER, J. S. (1954). Monolayer tissue cultures. I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. — Proc. Soc. exp. Biol. *85:* 202-205.

Résumé.

Des recherches ont été faites pour savoir si du virus de la Dengue adapté à la souris est capable de se multiplier dans des cultures de tissu du singe Rhesus. Dans une autre expérience on a étudié le comportement de ce virus inoculé à des cultures de cellules provenant d'un rein humain contenant une tumeur.

Le type 1 de la souche Mochizuki du virus de la Dengue peut être maintenu à l'état actif pendant au moins 11 semaines dans des cultures de tissu testiculaire de Rhesus inclus dans du plasma de poulet, incubé à 35° C. dans un dispositif à « roller-tubes ». La méthode appliquée ne permet pas de déterminer si l'inoculat initial persiste simplement ou si une certaine multiplication du virus intervient.

Dans des cultures de cellules rénales de Rhesus, traitées à la trypsine, s'accroissant sur une surface de verre et incubées à l'état immobile à 35° C., le virus de la Dengue type 1 (souche Mochizuki et hawaïenne), ainsi que celui du type 2 (souche Nouvelle Guinée C) se multiplient. La multiplication observée dans ces conditions de culture est relativement lente, comparée avec celle d'autres virus. On a pu maintenir la souche Mochizuki à travers 40 passages de cultures de tissu pendant 352 jours, la souche hawaïenne à travers 26 passages pendant 232 jours et la souche Nouvelle Guinée C à travers 18 passages pendant 176 jours. La contagiosité pour la souris a été démontrée à l'aide de solutions de cultures représentant les dilutions suivantes : 10^{-74} pour l'inoculat de la souche Mochizuki, 10^{-51} pour celui de la souche hawaïenne et 10^{-42} pour

celui de la souche Nouvelle Guinée C. Le virus a conservé sa virulence pour la souris à travers tous les passages de cultures de tissu.

Les cellules rénales Rhesus infectées ont montré à l'examen microscopique in situ, ou après coloration, des dégénérescences caractéristiques. Dans le cas du virus du type 1, on note un parallélisme évident entre la dégénérescence et la contagiosité pour la souris. On peut démontrer aussi que la dégénérescence et la contagiosité sont inhibées simultanément par des antiséra de lapins immunisés avec du virus de la Dengue, provenant de passages sur souris. La suppression de la dégénérescence est complète après application du sérum homologue, tandis que le sérum hétérologue ne produit qu'une neutralisation partielle.

Il semble établi que le virus de la Dengue, souche Mochizuki, se multiplie et produit des dégénérescences cellulaires typiques, lorsqu'il est cultivé à 37° C. sur du tissus rénal Rhesus dans du plasma de poule dans un dispositif à « roller-tubes ».

Dans une seule expérience, comprenant l'inoculation du virus de la Dengue à des suspensions de cellules rénales humaines (suspensions effectuées à l'aide de trypsine), l'auteur n'a pas obtenu de multiplication certaine du virus. Le pouvoir infectieux du liquide de cultures diminue progressivement au cours des 15 premiers jours de culture.

On a réussi à isoler un agent cytopathogène à partir de tissue rénal Rhesus non inoculé. Cet agent peut être distingué du virus de la Dengue du point de vue histologique et immunologique.

Zusammenfassung.

Es sind Versuche durchgeführt worden, um die Vermehrungsfähigkeit von mäuse-adaptiertem Dengue-Virus in Gewebekulturen von Rhesusaffen zu untersuchen. Zusätzliche Versuche betrafen die Züchtung dieses Virus auf Gewebe-Explantaten aus einer tumorhaltigen menschlichen Niere.

In Rhesus-Hodengewebekulturen, die in Hühnerplasmagerinnsel eingeschlossen in einem «Rollertube»-Apparat bis 35° C bebrütet wurden, ließ sich der Dengue-Virus Typus 1 des Mochizuki-Stammes während mindestens 11 Wochen in aktivem Zustand erhalten. Es war bei der verwendeten Methode nicht zu entscheiden, ob lediglich das Original-Inokulat persistierte, oder ob eine beschränkte Vermehrung des Virus stattgefunden hatte.

In Trypsinbehandelten Rhesus-Nierenzellkulturen, welche direkt auf Glasplatten gezüchtet und stabil in 35° C bebrütet wurden, vermehrten sich sowohl das Dengue-Virus Typ 1 (Mochizuki und hawaiianischer Stamm) als auch Typ 2 (Neuguinea C Stamm). Unter den gewählten Bedingungen war die Vermehrung relativ langsam im Vergleich zu anderen Viren. Es konnten gehalten werden der Mochizuki-Stamm über 40 Gewebekulturpassagen während 352 Tagen, der hawaiianische Stamm über 26 Passagen während 232 Tagen und der Neuguinea C Stamm über 18 Passagen während 176 Tagen. Die Infektivität für Mäuse wurde geprüft mittels Gewebekulturaufschwemmungen in folgenden Verdünnungen: 10^{-74} für das Inokulat des Mochizuki-Stammes, 10^{-51} für dasjenige des hawaiianischen und 10^{-42} für dasjenige des Neuguinea C Stammes. Es zeigte sich, daß das Virus seine Infektivität durch alle Gewebekulturpassagen hindurch beibehielt.

Infizierte Rhesus-Nierenzellen zeigten bei mikroskopischer Prüfung in situ oder nach Färbung charakteristische Degenerationserscheinungen. Im Fall des Typus 1 zeigte sich ein klarer Parallelismus zwischen dieser zellulären Degeneration und der Infektivität gegenüber Mäusen. Degeneration und Infektivität

konnten gleichzeitig verhindert werden durch Antisera von Kaninchen, die mit Dengue-Virus aus Mäusepassagen immunisiert worden waren. Die Unterdrükkung der Degeneration gelang vollständig mit homologem Serum, mit heterologem war nur eine partielle Neutralisierung möglich.

Es darf als wahrscheinlich angenommen werden, daß sich das Dengue-Virus des Mochizuki-Stammes vermehrte und Zelldegeneration hervorrief, wenn es auf Rhesus-Nierengewebe unter Hühnchenplasmagerinnsel bei 35° C in einem «Rollertube»-Apparat gezüchtet wurde.

In einem einzigen Versuch, bei welchem Dengue-Virus auf mit Trypsin aufgeschwemmte menschliche Nierenzellen inokuliert wurde, konnte eine Vermehrung des Virus nicht einwandfrei festgestellt werden. Der infektiöse Titer der Kulturlösung verminderte sich zunehmend während 15 Tagen nach der Inokulation.

Aus nicht inokulierter Rhesus-Nierengewebekultur konnte ein cytopathogenes Agens isoliert werden, welches biologisch und immunologisch vom Dengue-Virus unterschieden werden konnte.