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# Experimental Attempts to Transmit Phlebotomus (Sandfly, Pappataci) and Dengue Fevers to Chimpanzees.*

J. R. PAUL, J. L. MELNICK, AND A. B. SABIN.

*From the Section of Preventive Medicine, Yale University School of Medicine, New Haven, and the Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati College of Medicine.*

As part of a study designed to test the susceptibility of chimpanzees *(Pan satyrus)* to certain human viruses, the infectivity of phlebotomus (pappataci, sandfly) fever virus and of dengue virus was tested in this species of animals. The primary object of these experiments was to determine whether clinical or subclinical infection could be established in this species of primates and, if so, whether any recognizable symptoms or signs of these diseases could be produced.

*A. Phlebotomus (Sandfly, Pappataci) Fever. History of Experimental Infections.* To our knowledge this virus has never been proved to infect any laboratory animal.[1]

*Strain of Virus Used.* The strain of virus used was isolated in Cairo, Egypt, in 1943, from blood samples taken from a series of patients in the acute phase of sandfly fever. It has been termed the *Middle East strain.* Its properties have been previously described and it has been used extensively to produce the experimental disease in human volunteers both in this country and abroad.[1] Previous attempts had been made to infect animals with this strain by the inoculation of serum or blood of proven infectivity for human beings, by the intracerebral, intracutaneous, subcutaneous, intratesticular, intranasal or intraperitoneal routes, in the following animals; young baboons *(Papio hamadryas);* several varieties of Cercopithecus and rhesus monkeys, and various rodents, including white mice, wild gray mice, Syrian hamsters, Egyptian desert rats (jerboas), rabbits, guinea pigs and cotton rats. No evidence of pathogenicity was found in these animals. The virus could not be demonstrated in the serum of 3 rhesus monkeys 3 to 4 days after inoculation in attempts made by one of us (A.B.S.) by subinoculation in human volunteers of proved susceptibility. Material used for inoculating the chimpanzees in this experiment represented a pooled lot, totalling 7.4 ml of human serum collected from natural cases, and which had been kept frozen in 5 different ampoules (with occasional thawing in some) for about 3 years. Inoculations were given both subcutaneously (0.5-0.9 ml for each) and intracutaneously (0.5-0.9 ml for each). It should be pointed out that one of us (A.B.S.) has found human serum to be infective after 4 years of storage on dry ice.

*Number of Animals.* Six chimpanzees were used†—Webb, Flora, Jent, Nina, Maria and Pinta. All of these animals had been previously used for experimental work in poliomyelitis,[2] but none were considered to be "infective" as far as poliomyelitis was concerned, at the time the sandfly fever experiments were begun. Only 2 of the animals were sufficiently tractable to allow temperatures to be taken. These temperatures were taken daily when possible.

*Results.* The period of observation lasted

* This investigation was conducted by the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of The Surgeon General, U. S. Army, Washington, D. C.

1 Sabin, A. B., Philip, C. B., and Paul, J. R., *J.A.M.A.*, 1944, **125**, 603.

† Several of the animals used in this and the experiment on dengue were generously made available to us by the Yerkes Laboratories of Primate Biology, Orange Park, Florida. We are indebted to Dr. K. S. Lashley for his assistance in providing these animals.

2 Melnick, J. L., and Horstmann, D. M., *J. Exp. Med.*, 1947, **85**, 287.

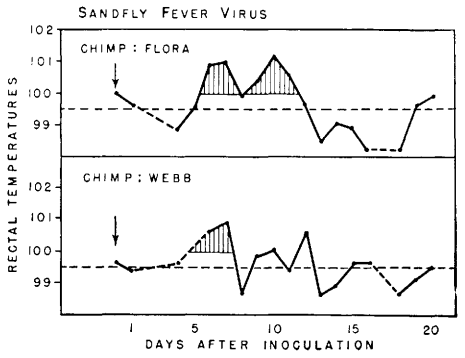


FIG. 1.

20 days. None of the 4 inoculated animals on which temperatures were not taken, showed any noticeable symptoms nor was there evident malaise or loss of appetite.

On the 2 animals in which temperatures were taken, the story is as follows:

*Webb*—During a 15-day-period prior to inoculation, the rectal temperature had maintained a fairly flat level ranging between 99° and 100.1°, which is normal for the chimpanzee. Following inoculation on 24 October 1946, the temperature continued normal for about 5 days to be followed by a rise of 0.5° to 1°. This slight fever was irregular with 2 humps, spaced about 4 days apart (Fig. 1).

*Flora*—The rectal temperature prior to inoculation had also ranged between 98.6° and 100.1° and beginning 5 days after inoculation, there was a rise reaching 1.2° of fever, and lasting in all about 6 days. During this period, the animals seemed quiet and somewhat dull on two occasions; during the end of the febrile period the eyes had a slightly glazed appearance.

The slightly elevated temperature observed in the 2 animals appeared after an incubation period of 5 days and although irregular was protracted over a period of 6 to 7 days. We did not attempt to demonstrate viremia in these chimpanzees during their febrile period by inoculating human volunteers with chimpanzee blood. Furthermore, a test for antibodies for sandfly fever virus is not yet available and so this determination was not done. Therefore, we cannot be sure that the fever exhibited by these two animals was due to the inoculation of virus. While the course of this fever and the length of the incubation period in the 2 chimpanzees might be comparable with that of human sandfly fever,‡ it is also possible that the fever may have been due to serum disease. One of us (A.B.S.) observed fever of 2 to 3 days' duration in human volunteers who were inoculated with serum of rhesus monkeys used in tests on sandfly fever, but the usual changes in the leukocytes seen in natural and experimental sandfly fever were absent, passage to other human volunteers was negative, and the original volunteers were subsequently found to be susceptible to the virus. No conclusion is possible, therefore, concerning the susceptibility of chimpanzees to the virus of sandfly fever.

*B. Dengue Fever. History of Experimental Dengue Infections.* In laboratory animals experimental infections with dengue virus apparently have never been very dramatic except after adaptation in mice[3] and in certain rhesus monkeys inoculated intracerebrally with mouse-adapted virus.[4] Simmons, *et al.*[5] found that it was possible to produce inapparent infection in *Macacus philippinensis* monkeys caught at elevations above 4000 feet in the dengue-free mountains of the Philippine Islands, and in *M. fuscatus* monkeys imported from Japan. Blanc and his coworkers[6] and Findlay[7] have reported similar results in Asiatic and African monkeys. By subinoculation tests in human volunteers, one of us (A.B.S.) in association with Dr. Max Theiler (unpublished experiments) demonstrated inapparent infection in rhesus monkeys inoculated intracerebrally or intraperi-

‡ In humans the incubation period ranges in experimental cases from 2½ to 7 days. The febrile course of the disease in the great majority of cases ranges from 2 to 4 days.[1]

[3] Sabin, A. B., and Schlesinger, R. W., *Science*, 1947, **101**, 640.

[4] Sabin, A. B., Paper presented at meeting of Am. Soc. of Trop. Med., December 3, 1947.

[5] Simmons, J. S., St. John, J. H., and Reynolds, F. H. K., *Philippine J. Sc.*, 1931, **44**, 1.

[6] Blanc, G., Caminopetros, J., Dumas, J., and Saenz, A., *C.R. Acad. Sci., Paris*, 1929, **188**, 468.

[7] Findlay, G. M., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1932, **26**, 157.

toneally with the strain of human dengue virus used in the present experiments. Subsequently one of us (A.B.S.) also found that rhesus monkeys inoculated with human dengue virus developed neutralizing antibodies for the mouse-adapted virus.

*Strain of Virus Used.* The Hawaii strain of dengue virus recovered by one of us (A.B.S.) in 1944 from natural cases of the disease in Hawaii was used in the present tests. Serum collected within less than 24 hours after onset of fever from the first group of human volunteers inoculated with the virus from the natural cases, and stored in the lyophilized state in an ordinary refrigerator since 17 April 1944 was used in the first experiment on 11 December 1946. Serum obtained within 24 hours after onset of fever in other human volunteers, inoculated with subsequent passages of this virus, and stored in a box containing solid $CO_2$ since 1944, was used 3 years later in the second experiment on 31 July 1947. Recent tests carried out by one of us (A.B.S.) on human volunteers showed that dengue virus in human serum stored for more than 3 years either in the frozen state in a box containing solid $CO_2$ or in the lyophilized state in an ordinary refrigerator was still infectious.

*Number of Animals.* Nine chimpanzees from 2 to 5 years of age were used in these experiments. Several of them had been used previously for experimental work in poliomyelitis,[2] Fort Bragg (pretibial) fever,[8] and sandfly (phlebotomus) fever. Five of these animals were tractable enough to have their temperatures recorded daily. However, all animals were observed daily for signs of illness and evidence of a rash for a period of 4 to 5 weeks.

*Technique of Neutralization Tests.* All of the neutralization tests were carried out by one of us (A.B.S.). Through them it was possible to confirm the presence of subclinical infection in the inoculated chimpanzees, by the use of the neutralization test developed by Sabin and Schlesinger following their adaptation of the Hawaii strain of dengue virus in mice.[3] The technique of the test§ and the extensive observations made by these investigators on the development of antibodies in human volunteers, natural cases and experimental animals have not as yet been published. The essentials of the test are: 1) that the sera to be tested be maintained in the frozen state; 2) that the original suspension of dengue-infected mouse brain as well as the dilutions be prepared in undiluted rabbit serum which had been heated at 56° C for 30 minutes; and 3) that the serum-virus mixtures be incubated in a water bath at 37° C for 2 hours prior to intracerebral inoculation in mice. In the present study the pre-inoculation and post-inoculation serum specimens were kept frozen in a box containing solid $CO_2$, and tested simultaneously.

*Results. Exp. 1*—Dec. 11, 1946. Four animals (Webb, Jent, Flora and Pinta) were each inoculated subcutaneously with 0.5 ml of human dengue serum. This was from a lyophilized sample which had been collected on 17 April 1944. It was redissolved on the day of the chimpanzee inoculations. Daily temperatures are recorded for 2 of the animals, Webb and Flora, and one of them, Flora, had a slight fever on the 9th day (Fig. 2). There were no other signs of disease.

Neutralization tests on matched specimens of preinoculation and postinoculation serum were carried out in one animal, Webb.

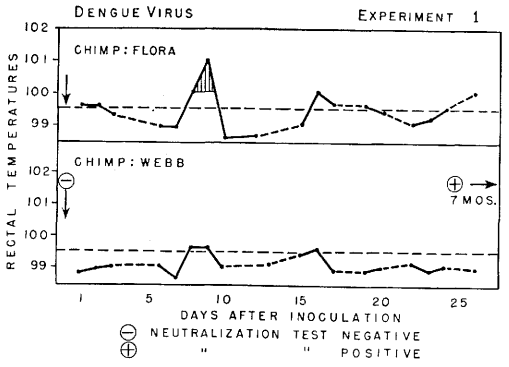


FIG. 2.

8 Melnick, J. L., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 263.

§ See Sabin, A. B., in *Procedures for Virus and Rickettsial Diseases*, 1948, p. 289, Am Pub. Health Assn., New York City.

TABLE I.
Development of Neutralizing Antibodies in Chimpanzees Inoculated with Human Dengue Virus (Hawaii Strain)
Virus Used—59th mouse brain passage of Hawaii Dengue.

| Serum tested | Mortality at indicated dilutions of virus | | | | | $LD_{50}$ Log of Dilution | Neutralization Index* |
|---|---|---|---|---|---|---|---|
| | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | | |
| Control—undiluted rabbit | — | 5/5 | 3/5 | 3/5 | 1/5 | 5.0 | — |
| Webb (11-2-46)—before dengue | 5/5 | 5/5 | 5/5 | 2/5 | — | 4.8+? | 2—? |
| " (7-19-47)—7 mos. post dengue | 0/5 | 0/5 | 0/5 | 0/5 | — | 1.5—? | 3,200+? |
| Rosebud (7-31-37)—before dengue | 5/5 | 5/5 | 5/5 | 4/5 | — | 5.4+? | —3 |
| " (9-23-47)—54 days post dengue | 0/4 | 1/5 | 0/5 | 0/5 | — | 1.6—? | 2,500+? |
| Hickory (7-31-47)—before dengue | 5/5 | 5/5 | 4/4 | 1/5 | — | 4.6+? | 3—? |
| " (9-23-47)—54 days post dengue | 1/5 | 0/5 | 0/5 | 0/5 | — | 1.6—? | 2,500+? |
| Becky (7-31-47)—before dengue | 5/5 | 5/5 | 5/5 | 2/5 | — | 4.8+? | 2—? |
| " (9-23-47)—54 days post dengue | 3/5 | 0/4 | 0/5 | 0/5 | — | 2.2—? | 630+? |
| Catawba (7-31-47)—before dengue | 5/5 | 5/5 | 5/5 | 5/5 | — | 5.5+? | —3 |
| " (9-23-47)—54 days post dengue | 1/5 | 0/2 | 0/5 | 0/5 | — | 1.6—? | 2,500+? |
| Mary Lou (7-31-47)—before dengue | 5/5 | 5/5 | 5/5 | 5/5 | — | 5.5+? | —3 |
| " " (9-23-47)—54 days post dengue | 0/2 | 1/4 | 0/2 | 0/4 | — | 1.7—? | 2,000+? |

* The neutralization indexes were calculated on the basis of the control titer ($10^{-5.0}$) obtained with heated undiluted rabbit serum.

Neutralizing antibodies, absent before inoculation, were present in the convalescent specimen. See Table I.

*Experiment 2*—July 31, 1947. Tests with ultracentrifuged fractions. 100 ml of human serum, frozen since 1944, were thawed and spun at 15,000 r.p.m. for 30 minutes in the cold in the RR-1 International centrifuge. The fatty cake was discarded and the aqueous phase spun in 12 tubes in the ultracentrifuge (10° angle rotor) at 33,000 r.p.m. for 90 minutes with a force of 60,000 x gravity at the middle of the tubes.

No visible sediment was obtained. The top 7.2 ml of each tube was drawn off and pooled. The bottom 0.6 ml of each tube was then rubbed against the sides and bottom of the tube with a rubber tipped glass rod. The bottom fractions were then pooled. Nitrogen determinations (micro-Kjeldahl) were carried out in duplicate as an indication of the amount of serum proteins sedimented under these conditions, and these were as follows:

| | N mg/ml |
|---|---|
| Serum before ultracentrifugation | 12.3 |
| Top fraction (86.4 ml) | 11.6 |
| Bottom fraction (7.2 ml) | 17.8 |

The top fraction was inoculated subcutaneously (1 ml) and intracutaneously (1 ml) into each of 2 chimpanzees (Catawba and Mary Lou) and the bottom fraction by the same routes and in the same volumes into each of 3 (Rosebud, Becky and Hickory). Daily temperatures were taken on the latter 3 animals over a period of 5 weeks. (Fig. 3). No clinical signs of illness were manifest in any of the 5 animals other than a slight fever, but in examining the temperature charts carefully (Fig. 1 and 2) one can perhaps see that in all inoculated animals there is a consistent, postinoculation trend with a slight elevation of temperature at some time between the 5th and 10th days after inoculation, to be followed by a secondary slight elevation from the 12th to the 17th days. This temperature pattern does not resemble the diphasic temperature occasionally seen in human dengue.

Neutralization tests were carried out simultaneously on the chimpanzee sera collected on 31 July, prior to the virus inoculations, and 54 days later on 23 September. All 5 animals responded to the inoculation with the development of neutralizing antibodies in high titre (Table I).

*Discussion.* Six chimpanzees inoculated with human serum, believed to contain phlebotomus fever virus, exhibited no signs of illness, other than fever which was measured in but 2 of them and the very slight temperature elevation which occurred in the 2 animals whose temperature was recorded, cannot be interpreted with certainty. In the absence of

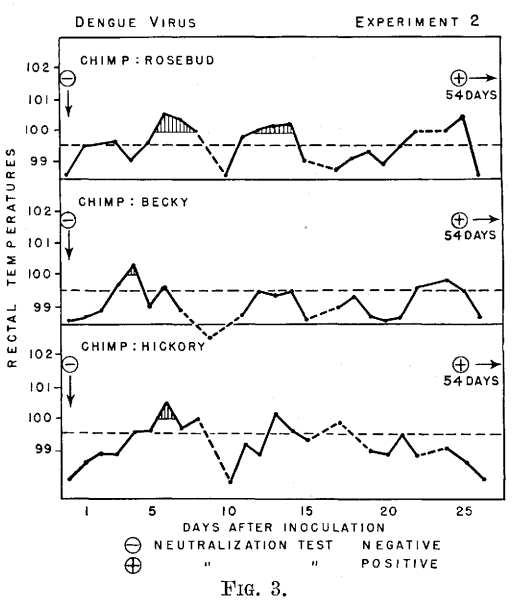


FIG. 3.

leucocyte studies and subinoculation tests in human volunteers, there is no evidence to indicate one way or another, whether chimpanzees are susceptible to this virus.

Among 9 chimpanzees inoculated with human dengue virus, there was again no clinical evidence of infection or striking elevation in temperature. However, by means of the neutralization test developed by Sabin and Schlesinger it was found that all those tested developed neutralizing antibodies for the homologous mouse-adapted dengue virus, and this may be interpreted as indicating that an inapparent infection followed the inoculation of human dengue virus in chimpanzees. Sabin and Schlesinger (unpublished experiments) found that in unsusceptible animals such as hamsters, cotton rats, guinea pigs and rabbits, a single inoculation of a large dose of dengue virus is not followed by the development of neutralizing antibodies, while inapparent infections resulting from single inoculations of mouse-adapted virus in rhesus monkeys or human beings invariably give rise to these antibodies. It is of interest that none of the 6 chimpanzees tested had any antibody for the Hawaii strain of dengue virus used in these tests prior to inoculation and that all developed antibodies of high titre after inoculation. The persistence of the neutralizing antibody in high titre for at least 7 months is in keeping with observations one of us (A.B.S.) has made on the long persistence of dengue antibody in human beings.

Previous experiments, not yet reported in detail, carried out by one of us (A.B.S.) in association with Drs. R. W. Schlesinger and Wendell M. Stanley, on the ultracentrifugation of human dengue virus,[8] indicated that the virus can be concentrated in the sediment obtained by centrifugation at 24,000 r.p.m. (50,000 x gravity at middle of tubes) for 90 minutes in a 33° angle, 8-inch rotor. In the present tests it is of interest that centrifugation at 33,000 r.p.m. (60,000 x gravity) for 90 minutes in a 10° angle 6½-inch rotor did not yield any visible sediment although under these conditions an appreciable fraction of the serum proteins sedimented into the bottom part of the tubes. It should be recalled that before being subjected to ultracentrifugation, these sera had been frozen and thawed, and then spun at 15,000 r.p.m. for 30 minutes. The fact that after ultracentrifugation the top fraction produced neutralizing antibodies in the 2 chimpanzees inoculated with it, as well as did the bottom fraction in three other chimpanzees, need only mean that a certain amount of virus remained unsedimented. Other viruses centrifuged in such angle rotors have also been found to remain in small amounts in the supernatant liquids.[9,10]

*Summary.* 1. Six chimpanzees inoculated with human serum believed to contain phlebotomus (sandfly, pappataci) fever virus, exhibited no clinical signs of infection. A slight febrile response in the 2 chimpanzees, whose temperatures were being recorded, could not be interpreted with certainty, and no evidence, pro or con, was adduced regarding the susceptibility of chimpanzees to this virus.

2. Nine chimpanzees inoculated with human dengue virus (Hawaii strain) also exhibited no clinical signs of infection, but

[9] Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1936, **64**, 503.

[10] Melnick, J. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 553.

evidence of inapparent infection was obtained by neutralization tests with the homologous mouse-adapted virus. None of 6 chimpanzees, whose serum was tested, had any antibodies for the dengue virus before inoculation and all developed them in high titre after inoculation.

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## Accidental Laboratory Infection with Human Dengue Virus.*

J. L. MELNICK, E. C. CURNEN, AND A. B. SABIN.

*From the Section of Preventive Medicine, Yale University School of Medicine, New Haven, and the Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati College of Medicine.*

Accidental laboratory infections with a wide variety of different viruses including viscerotropic yellow fever virus and Rift Valley fever[1] virus have frequently occurred. No such infection with dengue virus, however has previously been recorded. It is the purpose of this paper to present observations upon a patient with dengue who acquired this disease by accident in the laboratory.

The patient, a 23-year-old male negro laboratory assistant was exposed to infection under the following circumstances. In July, 1947 he participated in one of the experiments (Experiment No. 2) which were described in the previous paper on the transmission of dengue to chimpanzees.[2] It will be recalled that a pooled sample of infected human serum obtained in 1944 in Hawaii from patients acutely ill with dengue and preserved in the frozen state was separated into two fractions designated "top" and "bottom" following ultracentrifugation at 33,000 R.P.M. for 90 minutes. On 31 July each fraction was injected into chimpanzees and both fractions were subsequently found to contain dengue virus. During the efforts to restrain and inoculate the chimpanzees a syringe containing the "bottom" fraction suddenly separated from the needle, and some of the serum which squirted out struck the patient in the eye. His eye was immediately washed out with water, and he resumed his work. The subsequent developments to be described are charted in Fig. 1.

The patient remained well during the next week. On 7 August he noted a macular rash distributed over the bearded area of his face. On 9 August after a good breakfast he became tired and weak on his way to work. During the morning he developed aches in the calf muscles and behind his knees as well as throbbing frontal headache. He returned home at noon, ate an egg and vomited promptly. His temperature at noon was 101°F, at 3:30 P.M., 103.6°, at 8:00 P.M., 102.6°. During the afternoon he had repeated chills. On 10 August he complained of headache, anorexia, nausea and vomiting. His eyes burned and the muscles of his legs ached. He had no symptoms referable to the respiratory tract. His wife and two children were well and had not recently been ill. On physical examination he appeared sluggish but not particularly ill. His temperature was 102.2°. The pulse and respiratory rates were not accelerated. A macular rash was present on the face as well as a few indistinct macules on the right shoulder and back. Tenderness over the eyeballs and slight injection of the conjunctivae and pharynx were noted. Small cervical and axillary lymph nodes were palp-

*This observation occurred in the course of investigations conducted by the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of The Surgeon General, U. S. Army, Washington, D. C.

1 Sabin, A. B., and Blumberg, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 385.

2 Paul, J. R., Melnick, J. L., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 193.