Growth of Neurotropic Viruses in Extraneural Tissues IV. Poliomyelitis Virus in Human Testicular Tissue *In vitro*.* (18598)

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Multiplication of the Lansing strain of poliomyelitis virus has been demonstrated in tissue cultures of human embryonic tissue (1,3) and of human foreskin(2). Evidence suggesting limited multiplication in human placenta in 2 of 13 tests has also been reported (3). The Brunhilde strain has been maintained in human embryonic skin and muscle cultures for 73 days and in human embryonic brain cultures for 39 days(2).

In this paper it will be shown that poliomyelitis virus of the Lansing and Hof. strains multiplied extensively in human testicular tissue *in vitro*. In experiments with other human tissues our results have been largely, but not completely, negative. In a preliminary report of this work, a more extensive review of pertinent literature will be found (4).

Materials and methods. Source of tissue. The testes used in these experiments were from men 57 to 93 years old, most of whom were suffering from carcinoma of the prostate gland. Some had received estrogen therapy prior to operation. Histologically the testes varied considerably. In most of them, spermatogenesis was reduced and in some cases it was apparently absent.

Viruses employed. The Lansing virus used in these experiments was received from Dr. John F. Enders in December, 1948. It had been passed in mice 2 times in our laboratory prior to use in these experiments. For preservation of the virus, suspensions of infected CNS of mice were stored in the dry ice chest.

The Hof. virus, which we received from Dr. A. B. Sabin in May, 1949, was isolated from stools and throat swabs from a patient in Cincinnati in 1947 and has since been found to belong to the Brunhilde group of poliomyelitis viruses. The virus had been passed through rhesus monkeys 3 times before we received it. Suspensions of the infected spinal cords from the cynomolgus monkeys representing the 1st and 2nd passages in our laboratory were preserved in the dry ice chest until used in these experiments.

Tissue culture methods. Promptly after the testes were removed from the patient, small fragments were cut from the central portion of the organ, placed in warm tissue culture medium and minced to approximately 1 mm to 3 mm in size. About 1 g of the testicular material in 30 cc of medium was transported to the laboratory in a portable incubator where the fragments were minced again with a Bard Parker scalpel until they were 1 mm or less in diameter. The tissue was washed with medium several times during the mincing process in an attempt to reduce the amount of antibody carried over with the tissue. Approximately 0.02 to 0.04 ml of a heavy suspension of minced tissue was transferred by means of a graduated pipette to the center of a perforated cellophane disc in each of 3 50-ml Erlenmeyer flasks. Virus was promptly added as 0.4 ml of a centrifuged 10% suspension of infected tissue in tissue culture medium. A period of 5 minutes was allowed to elapse after which 3.6 ml of medium were added, producing a tissue-to-fluid ratio of approximately 1:100. The tissue culture medium consisted of bovine serum ultrafiltrate† diluted with 3 volumes Hank's bal-

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† The serum ultrafiltrate and Hank's solution were obtained from Microbiological Associates, Coral Gables, Fla.

anced salt solution. It contained phenol red and 100 units of penicillin and 100 μg of streptomycin per ml. The atmosphere in the flasks was flushed with 5% CO_2 and 95% air. The flasks were closed with rubber stoppers and loose aluminum caps were put over the top to minimize contamination of the lip of the flask. The cultures were placed in the incubator at 36°C. The medium in the tissue cultures was changed regularly at intervals of 3 or 4 days. At this time 3.6 ml of fluid were removed and replaced by an equal volume of fresh medium. Tests for virus were made by injecting the harvested fluid (pooled from 3 flasks) intracranially in amounts of 0.03 ml into mice or 0.5 to 1.0 ml into monkeys. The inoculum contained a minimum of cells and no attempt was made to free virus from cells. Transfer of virus to new tissue was made by adding 0.4 ml of pooled tissue culture fluid from flasks of previous cultures to the tissue of the new culture. Frequently the fluid was frozen in a sealed glass tube in the dry ice chest for some time prior to being used for transfer or for virus titrations. There was no evidence of fall in pH values (change in color of phenol red) of the tissue culture fluid during the periods of incubation. Only new pyrex glass flasks were used. The flasks were washed with Ivory soap and rinsed 5 times with tap water and 5 times with distilled water. The cellophane, perforated with the Beckley OC design, was obtained from the Tissue Culture Association and cut into discs slightly smaller than the bottoms of the flasks. These were washed with 3 changes each (10 minutes per "change") in ether, absolute alcohol, acetone, distilled water, and glass distilled water. They were then placed in clean flasks which were autoclaved and stored until used.

Multiplication of Lansing virus in human testicular tissue. Lansing poliomyelitis virus was inoculated onto human testicular tissue in our 1st experiment on November 29, 1949. As shown in Fig. 1, it was passed through 8 successive tissues. The calculated dilution of the original inoculum was 10^{-23}. In no instance did loss of virus necessitate going back to an earlier specimen to resume the passage of virus. It is estimated that each time fluid was changed, a 10-fold dilution of virus occurred. Evidence for the validity of this method of calculation is found in Fig. 1. With a few exceptions, every specimen of fluid removed in the periodic change of medium was tested for infectivity. Results showed that the virus content gradually increased throughout the period of incubation up to the time transfer was made to new tissue. In all cases, the amount of virus removed was at least equal to that expected on the basis of simple dilution, and in most cases it was 10 times that amount. Daily tests of virus in the period beginning November 29 provide further evidence for the continuing presence of virus in fluid and support this method of calculation.

Identity of the virus. Observations were made periodically in order to check the identity of the virus during the course of these experiments. Cultures for bacterial contamination were consistently negative. The wide range in incubation period in test mice, from 3 to 30 days, is characteristic of Lansing virus. Mice that were observed while showing signs of illness almost invariably had paralysis of the front legs, the pattern of paralysis known to be characteristic of Lansing virus. Periodic checks made for ether resistance of the virus were always positive. Intramuscular injection of mice failed to produce visible infection. The titers of virus in the CNS of mice succumbing in tests of tissue culture fluid were all in the range expected with Lansing virus. No infectious agent was detected by intracranial injection of mice with suspensions of spleens from mice infected with tissue culture fluid. Extensive tests were made to check the identity of virus in the 8th serial passage representing estimated dilutions of 10^{-23} of the original inoculum. Evidence of the sort described above was obtained. In addition pathogenicity for monkeys was checked. The undiluted tissue culture fluid, treated with ether overnight, induced typical poliomyelitis in 2 injected monkeys. A 10^{-1} dilution of the same fluid produced poliomyelitis in 2 injected monkeys; neither of the 2 monkeys injected with a 10^{-2} dilution was infected. In 3 other experiments, Lansing virus has been maintained

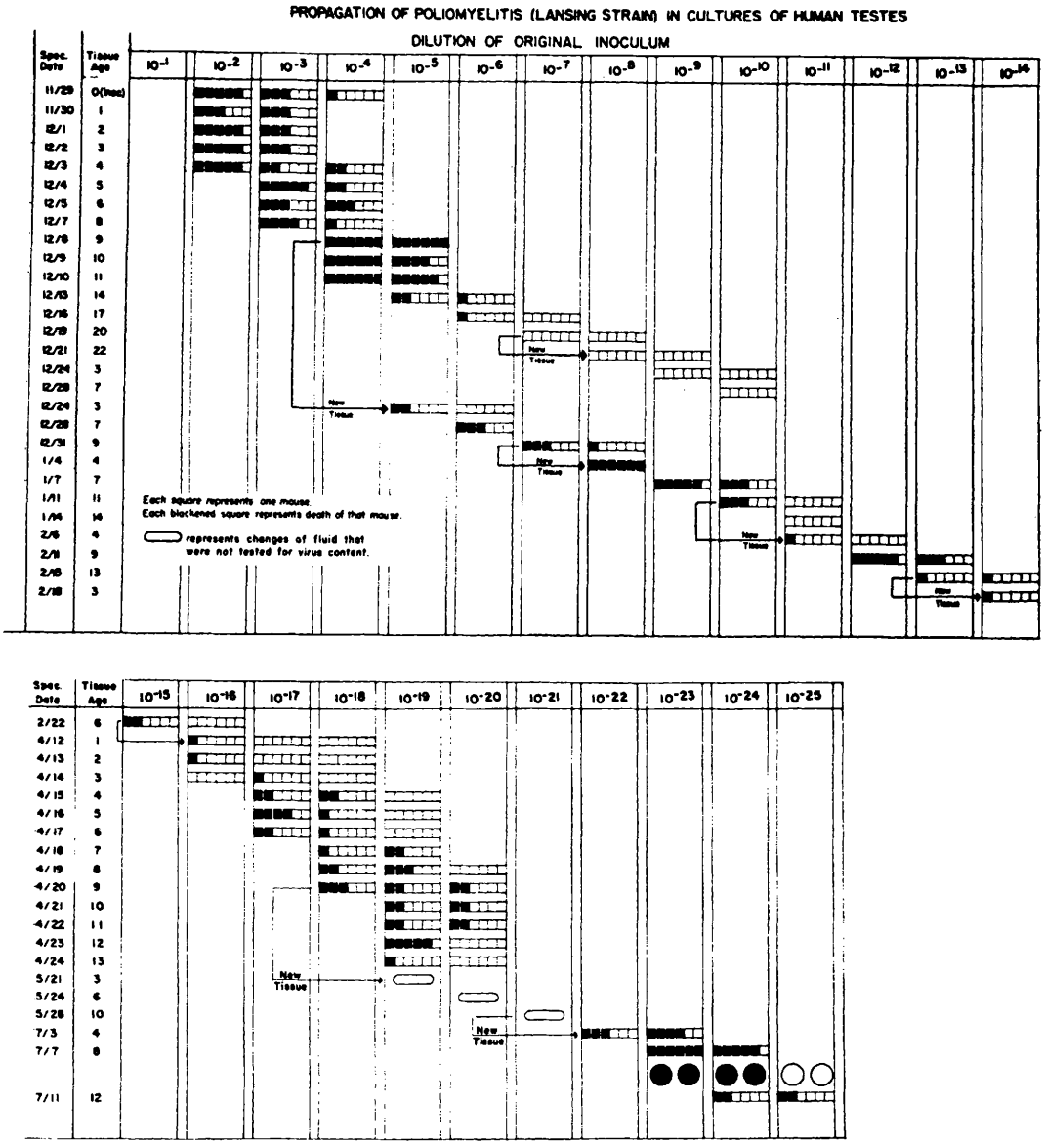

FIG. 1.

Tests for virus were made by intracranial injection of mice in groups of 6. Undiluted tissue culture fluid, fluid diluted 10-fold and in some cases fluid diluted 100-fold were used. The first horizontal line represents the titration of the virus used to inoculate tissue cultures on 11/29. At intervals of 3 or 4 days, 90% of the fluid was removed from each culture and replaced by fresh culture fluid. A 10-fold dilution of the original inoculum resulted each time fluid was changed and each time a transfer was made to a new culture.

Spec. date = date specimen was removed from tissue culture.

Tissue age = number of days the tissue in this culture had been maintained *in vitro*. In each case, transfer of infected fluid was made to new tissue on the day the new tissue was first placed in tissue culture medium.

Circles refer to monkeys inoculated. If infection occurred, the area inside the circle is black.

through 3, 4, and 5 successive tissue cultures of human testes with calculated dilutions of 10^{-8}, 10^{-13}, and 10^{-10} of the original inoculum. In each of these experiments the virus was present in the last passage tested. That is, in none of them was the virus lost due to

failure to multiply. In a 5th experiment, Lansing virus was inoculated into a testicular tissue culture and was carried through a 2nd tissue with tests at 3- or 4-day intervals. Virus decreased markedly in the first few days and in all tests after this time only 1 or 2 mice succumbed in each group of 6 injected with tissue culture fluid. However, subinoculations from mice succumbing in these tests proved they were infected with virus having the characteristics of Lansing virus (ether resistance, incubation period, initial paralysis of front legs). This culture has been carried to a calculated dilution of 10^{-7} of original inoculum. The virus evidently maintained itself at a level insufficient to give a titer of 1 LD_{50} in the supernatant fluid. The same appeared to be true at some stages in the experiment represented in Fig. 1.

Multiplication of Hof. Virus in human testicular tissue. The Hof. strain of poliomyelitis virus was first inoculated into tissue cultures of human testicular tissue on December 31, 1949. Since that time, it has been passed through 7 successive tissue cultures. The calculated dilution of original inoculum was 10^{-21} in the 7th tissue culture. Since this virus will not infect mice, tests for virus have been much less frequent than in the experiments with Lansing virus. Four specimens representing the 1st, 2nd, 3rd, 5th and 7th passages were tested. Of the 13 monkeys injected with various specimens of undiluted tissue culture fluid, 1 escaped infection. Of the 19 monkeys injected with 1:100 dilutions of tissue culture fluid, 15 were infected. Of 9 monkeys injected with 1:1000 dilutions of tissue culture fluids, 3 were infected. Since the tissue-to-fluid ratio was 1:100 in these cultures, the titer of virus, in terms of tissue present, was between 10^{-4} and 10^{-5}. In other words, it equalled the amounts of virus obtained in equivalent amounts of infected monkey spinal cords. A pool of specimens removed at 6 or 7 days from cultures of 3 different tissues in this series infected 4 of 4 monkeys injected with a 10^{-2} dilution and 1 of 4 injected with a 10^{-3} dilution. A similar pool of specimens removed at 13 or 14 days from 3 cultures infected 2 of 4 animals injected with the 10^{-2} dilution and 1 of 4 injected with the 10^{-3} dilution.

Identification of this virus after passage in tissue cultures was based on the following evidence, all demonstrated with virus in the 7th passage in tissue culture:

1. The infective agent was ether resistant.
2. Intracranial inoculation of mice with material infectious for monkeys failed to cause illness of the mice.
3. Nearly complete flaccid paralysis of voluntary muscles of all 4 limbs developed in most infected monkeys; less severe involvement occurred in a small proportion of animals.
4. Typical histopathology of poliomyelitis was regularly found in spinal cords of infected monkeys.
5. Surviving monkeys were resistant to intracranial challenge with Hof. stock virus suspension representing more than 100 LD_{50}.

In 2 other experiments Hof. virus was maintained through 2 and 4 serial passages and virus was demonstrated in tissue culture representing 10^{-7} and 10^{-13} dilutions of the original inoculum. In no case did this virus fail to multiply in a tissue culture of human testicular tissue.

Discussion. It is evident that poliomyelitis virus of the Lansing and Hof. strains has multiplied extensively in tissue cultures of human testicular tissues. Our largely negative results obtained with other human tissues have been briefly summarized elsewhere(4).

Attempts to cultivate the viruses of herpes simplex and western equine encephalitis in tissue cultures of the same tissues used in experiments on poliomyelitis were largely unsuccessful except in testicular tissue. With the latter herpes virus grew; western equine encephalitis was not tested in this tissue. It seems probable that when suitable tissue culture conditions are defined, these 2 viruses and possibly poliomyelitis virus will multiply in some of the human tissues with which we have had negative results up to this time.

The kind or kinds of cells which support the growth of virus in testicular tissue cultures have not as yet been determined in spite of considerable effort to do so. Germinal cells

degenerated rapidly in tissue cultures whether infected or not. Some of the testes in our series had markedly depressed germinal cell activity *in vivo*. Therefore it was probably not the germinal cells that were infected. The Sertoli cells, the interstitial cells of Leydig, and the fibroblasts remain to be considered. The susceptibility of interstitial cells to certain other viruses injected into the intact testis has been well demonstrated.

The capacity of testicular tissue from monkeys, bulls, hamsters, guinea pigs and mice to support multiplication of Lansing virus *in vitro* has been explored to a limited extent. At the present time it appears that Lansing virus has not multiplied in any of these experiments except possibly those in which tissue of cynomolgus monkeys' testes were employed.‡

Summary. The Lansing and Hof. strains of poliomyelitis virus multiplied extensively in tissue cultures of testes from men 57 to 93 years old, most of whom had carcinoma of the prostate gland. Lansing virus was carried through 8 successive tissue cultures and Hof. virus was carried through 7 cultures. Several less extensive series were completed with each virus. The amount of Hof. virus in these tissue cultures per milligram of tissue was approximately the same as was obtained in infected monkey spinal cords.

‡ In experiments completed after this paper was submitted for publication poliomyelitis virus multiplied readily in tissue cultures of testicular tissue from both rhesus and cynomolgus monkeys.

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A Metabolic Intermediate of Pamaquine from Chickens. (18599)

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(Introduced by N. B. Eddy)

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Berliner(1) observed that pamaquine (8-(4 - diethylamino - 1 - methylbutylamino)-6-methoxyquinoline), though markedly plasmodicidal *in vivo*, was relatively inactive when added to cultures of *Plasmodium falciparum in vitro*. Geiman(2) made essentially the same observation for the related 8-aminoquinolines, pentaquine and isopentaquine, using *in vitro* cultures of *P. knowlesi*. Berliner (1) also found that a solution of pamaquine, which had aged for several years, was highly active *in vitro*. Zubrod, Kennedy and Shannon(3) found that pamaquine was so extensively metabolized in the vertebrate body that extremely little of the drug was recovered in the excretory products of treated patients. Brodie and Udenfriend(4) and Hughes and Schmidt(5) have described metabolic intermediates of pamaquine and related 8-aminoquinolines, but none of these have been fully characterized and their antimalarial activities have not been determined. In an earlier report by three of us(6) it was shown that pamaquine, when added to *in vitro* cultures of *P. gallinaceum*, did not possess the marked antimalarial activity associated with the drug *in vivo*. In further trials (unpublished data) it was found that concentrates from the blood, tissues and droppings of chickens treated with pamaquine possessed antimalarial activity *in*

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