and control rats. Two specimens of human liver tissue were fractionated and trace metal distribution determined.

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Patterns of Increase and Spread of Polioviruses in Monkeys after Inoculation into Traumatized Tissue.* (26547)

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If poliomyelitis virus in adequate concentration is inoculated into healing skin wounds of cynomolgus monkeys, an increase of virus in the local site and in other parts of the body follows(1). Data are presented here to show the patterns of increase and spread of 4 different strains of type 1 virus chosen principally for characteristic virulence as measured experimentally or indicated epidemiologically.

Virus strains. Each of the 4 strains of virus used in this study had distinct characteristics of especial interest. Two strains, Mahoney and LSc, had been modified during prolonged passage in tissue culture in other laboratories. The other 2, Chesterfield Inlet and Iberia, were essentially "field" strains; that is, they had undergone only 2 passages in the laboratory. All were identified as type 1 poliovirus by neutralization with homotypic antiserum and by the fact that monkeys infected with each strain developed antibodies to type 1 poliovirus of the Mahoney strain.

The Chesterfield Inlet strain was obtained from feces of a patient who developed poliomyelitis during the epidemic in this arctic community in 1949. This was one of the world's most violent epidemics(2). In a total population of 275 Eskimos living in the village at that time, 18.5% were paralyzed and 5% died. The feces were collected April 27, 1949, and were sent to us by Dr. F. P. Nagler. They were preserved 7 years in the frozen state prior to use. Virus was then freshly isolated in monkey kidney tissue cultures, and second passage material was used

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for inoculation of monkeys in the present experiments.

The Iberia virus was received from Drs. John Fox and Henry Gelfand in the form of feces from a child living in Iberia Parish, Louisiana. The specimen was obtained on June 1, 1955, as a routine collection during a family study of Louisiana communities(3). The child had no illness at that time; clinical poliomyelitis was not prevalent in the community; and the attack rate was low, 2.4/100,000 population, for the entire parish in 1955. Virus of the second passage in tissue cultures of monkey kidney was employed.

The Mahoney strain of virus has been studied extensively and is included in the killed virus vaccine distributed commercially in the United States. For our experiments virus received from the Connaught Laboratories in 1954 was used. This was in fluid from infected cultures of monkey renal tissues and was preserved by freezing.

Dr. Albert B. Sabin provided us with the LSc strain of virus in 1956. It was derived from the Mahoney strain by Drs. Li and Schaeffer in 1954(4) and has been purified by 3 successive single plaque isolations by Dr. Sabin(5). It is notably avirulent for monkeys and man. The virus sent to us was a portion of the culture as seed for Dr. Sabin's type 1 vaccine.

Repeated tests in monkey kidney tissue tube cultures inoculated with 0.1 ml of suitably diluted stock virus showed titers satisfactorily reproducible, expressed as $TCID_{50}$ per ml, at the following levels: Chesterfield Inlet $10^{7.5}$, Iberia $10^{7.2}$, Mahoney $10^{6.8}$, LSc $10^{7.6}$.

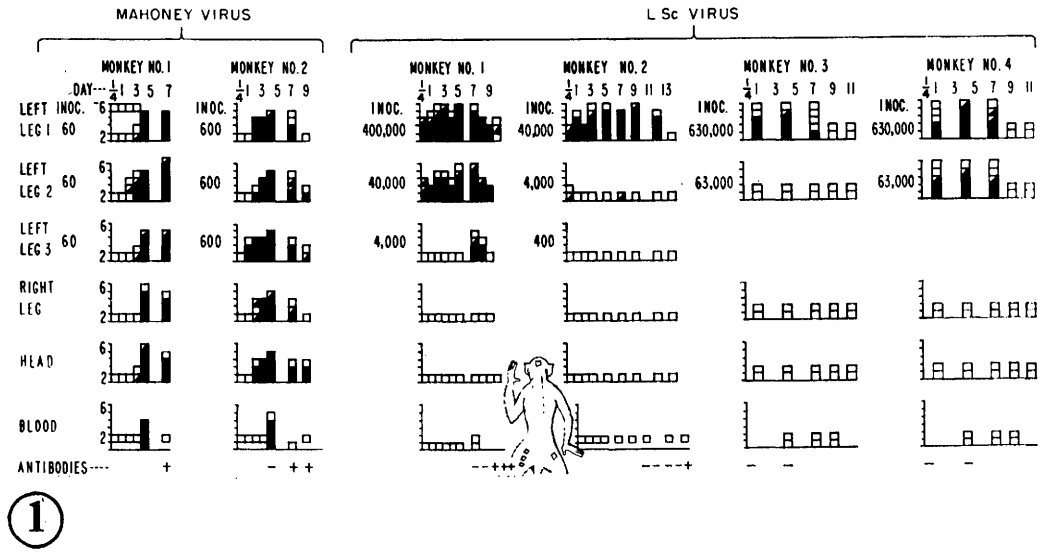
Methods. Procedures employed were essentially similar to those described previously (1). Tissue cultures were prepared from trypsinized monkey (rhesus or cynomolgus) renal tissue. Cells were grown directly on glass in test tubes and were stored for periods of 1 to 6 weeks as previously described. Titrations were based on the inoculation of appropriately diluted specimens into duplicate tube cultures.

Monkeys were obtained from several sources. Excision of 1 square cm of skin was carried out 3 days before inoculation of virus. Procaine penicillin and streptomycin were used to prevent infection, and lesions did not show signs of bacterial invasion. They remained clean and healed at the expected rate. In fact, by the 14th day after inoculation epithelium covered the lesion and had to be abraded to get material for testing.

Five skin lesions were created on each monkey, 1 on the scalp, 1 on the lateral aspect of 1 thigh, usually the right, and 3 in a vertical row on the lateral aspect of the other thigh. Adjacent lesions were separated by 1.5 cm of normal skin (Fig. 1). Three days after excision of skin, the 3 lesions on 1 thigh were inoculated with virus. The lesions on the opposite thigh and the lesion on the scalp were left uninoculated.

After inoculation the monkeys were restrained in a box similar to that described by Ives and Dack(6) or in a "monkey chair" to avoid external transfer of virus from inoculated lesions to those not inoculated. Monkeys were fed by an attendant while restrained. Their food intake was, in many cases, greatly reduced during this time.

In "harvesting" exudate from lesions the monkey was etherized, skin around the lesion was cleansed with anesthetic ether, and the scab was removed with sterile forceps. Then, with a second pair of sterile forceps, a ball of cotton approximately 3 mm in diameter was pressed into all parts of the raw surface to absorb the serous fluid that oozed from the base of the lesion. Little bleeding was ordinarily encountered after the first 3 or 4 days. The cotton was then placed in 1 ml of chilled "medium E" (mixture 199 with 100 units of penicillin, 100 μg of streptomycin, and 25 units mycostatin per ml), to which 0.2 ml of anesthetic ether had been added. The specimen was agitated to mix the ether with the aqueous phase and stored at 4°C. Titration of virus was usually carried out the next day. Specimens collected on Friday (usually the 4th day after inoculation of the monkey) were ordinarily titrated the following Monday. In preliminary experiments biopsy of the base of the lesion was shown to yield no higher concentration of virus than was found in the exudate collected as just described. The amount of serous exudate collected was



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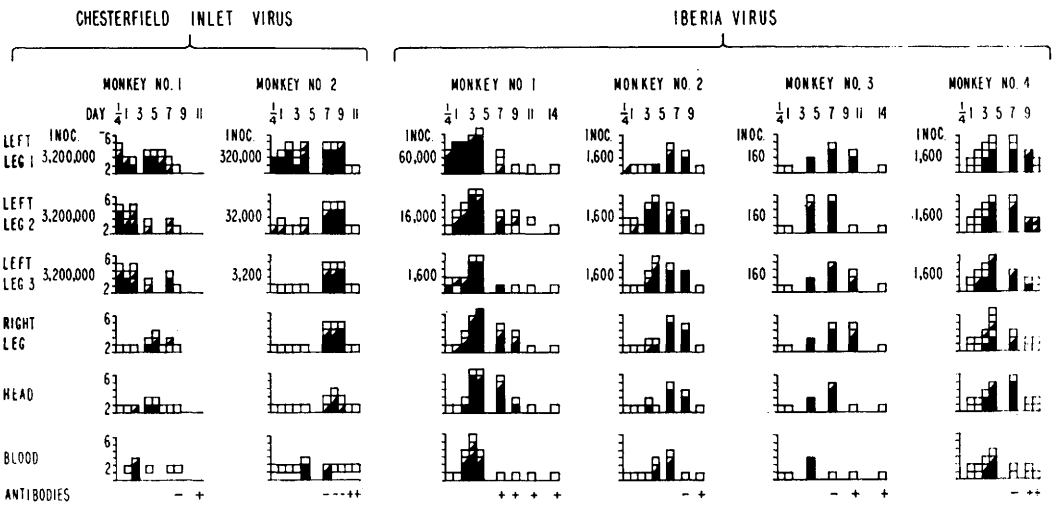

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FIG. 1 and 2. Changes in concentration and distribution of polioviruses after inoculation into skin wounds of cynomolgus monkeys. One cm^2 of skin was removed from sites shown on the diagram in Fig. 1. The 3 lesions on the lateral aspect of 1 thigh, usually the left, are designated as Left Leg 1, 2, and 3 to indicate proximal, middle, and distal lesions. (Monkeys 3 and 4, LSc virus, had only 2 lesions instead of 3.) "Inoc" denotes amount of virus injected, expressed in tissue culture ID_{50}. Numbers on vertical axis represent the negative logarithm of the dilution of exudate or blood tested for virus. Dark areas represent presence of virus as follows: ■ Test for virus was positive in all tissue cultures, usually 2, more if test was repeated. □ Test for virus was negative in all cultures, usually 2, more if test was repeated. ◪ Test for virus was positive in 1 or more cultures and negative in 1 or more cultures. Signs of illness were as follows:

Mahoney Virus: Monkey No. 1—Paralysis first observed on 7th day. Animal died the same day. Monkey No. 2—Tremors at 7 days, paralysis at 9 days. Died on 11th day.

LSc Virus: No illness detected in any of the 4 monkeys.

Chesterfield Inlet Virus: Monkey No. 1—Tremors observed at 8 days. No definite paralysis. Monkey No. 2—Tremors at 11 days, definite paralysis at 13 days.

Iberia Virus: Monkey No. 1, 2, and 3—No illness detected. Monkey No. 4—Paralysis observed at 10 days.

approximately 10 mg. Therefore, the specimens as collected in 1 ml of diluent were regarded as 10^{-2} dilutions of exudate.

Blood was collected from a superficial vein distal to the popliteal area on the leg with the uninoculated skin lesion. One-tenth to 0.2 ml were placed in 1 ml of chilled medium E. The specimen was refrigerated, and a portion was inoculated into tissue cultures at the same time as the other specimens harvested on that day. Tests for antibody were made by removing red cells and mixing 0.27 ml of the supernatant fluid with 0.03 ml of an appropriate dilution of Mahoney virus. After 30 minutes' incubation at 23°C tissue cultures were inoculated with 0.1 ml of this mixture representing approximately 100 ID_{50} of virus and 10% serum.

Histopathologic examination of the spinal cord of monkeys that showed paralysis confirmed the diagnosis of poliomyelitis. Those that showed no paralysis were examined daily in the cage for 1 month.

Results. In Fig. 1 and 2 the results of assays of virus during the course of infections are presented. *Minimal infective dose.* The minimal infective dose of LSc virus appeared to be approximately 4,000 $TCID_{50}$; however, 63,000 $TCID_{50}$ failed to cause infection of 1 lesion. Infection resulted from injection of 160 $TCID_{50}$ of Iberia virus and 60 $TCID_{50}$ of Mahoney virus. In additional experiments not included in the figures, no infection resulted from inoculation of approximately 16 $TCID_{50}$ of Iberia virus into 3 skin lesions of 1 monkey nor from injection of approximately 6 $TCID_{50}$ of Mahoney virus into skin wounds of each of 2 monkeys.

Pattern of viral increase and decrease in inoculated and uninoculated lesion. The LSc virus, unlike the other 3 strains, failed to spread from inoculated skin lesions to uninoculated lesions. In 2 monkeys it even failed to infect a lesion only 1.5 cm from an infected lesion.

In animals inoculated with infecting concentrations of the other 3 strains, virus increased in the injected lesion and invariably appeared in uninoculated lesions on the scalp and opposite thigh. Concentration of virus in exudate was relatively high and approximately equal in inoculated and uninoculated lesions. A pattern of delayed viral increase in obviously infected lesions occurred in several instances (see Mahoney Monkey 1 and Iberia Monkey 2 for example). In left leg lesion 2 of LSc Monkey No. 2, virus was present at 6 hours and 7 days at minimal detectable levels but did not increase.

In some animals virus increased first in inoculated lesions. Chesterfield Inlet Monkey No. 2 is the most striking example of this phenomenon. In most animals rise of virus titer occurred almost simultaneously in all 5 lesions, inoculated and uninoculated.

Decline of virus titer as a rule progressed over a 24- or 48-hour period. However, decrease of virus occurred precipitously in all 5 lesions in one monkey, Chesterfield Inlet Monkey No. 2. Although there was some difference from one lesion to another, the general pattern of viral decline was that of a systemic immunity manifest in all 5 skin sites simultaneously and to an essentially equal extent.

Neuropathogenicity. Paralysis was seen in both monkeys injected with Mahoney virus, in 1 of the 2 injected with Chesterfield Inlet virus, and in 1 of the 4 animals inoculated with Iberia virus. The lack of virulence of the LSc strain is consistent with many other studies of this strain.

In all 4 monkeys that became paralyzed neurological damage appeared and progressed at the same time that virus in skin lesions was declining, presumably in response to antibody as demonstrated in the serum. Monkey No. 2, given Chesterfield Inlet virus, demonstrates this relationship clearly. Virus concentration was maximal in all skin lesions of this animal at 8 and 9 days. At 10 days virus had disappeared from all 5 skin lesions. Yet paralysis was not detected at 10 or 11 days but was definite at 13 days. Antibodies were demonstrated at 10 and 11 days.

Discussion. Wenner and Kamitsuka(7) have stressed the importance of recognizing that sites of implantation of virus and sites of replication are not synonymous. They showed that Brunhilde virus inoculated intramuscularly multiplied first in inoculated muscles(8). Virus in lymph nodes of their ani-

mals did not exceed the amount that could result from implantation. In the present experiments maximal concentration of virus in most lesions far exceeded that found in any specimen of blood. Therefore, it seems clear that virus multiplied in tissues of these areas, whether inoculated directly or infected as a result of spread within the monkey.

The term "infection of skin lesions" in the present work should not be construed to mean infection of skin. The base of the lesion was loose connective tissue altered by the trauma of skin excision and by the healing process. Virus assays were performed on fluid exuding from this tissue. Which cells were infected and whether comparable cells in uninjured tissue of normal animals are susceptible is unknown.

In previous studies a discrepancy has been noted between capacity of poliovirus to grow in testicular and renal tissue *in vitro* and inability to grow in these tissues in the animal (9, 10). The free multiplication of poliovirus in the base of healing skin wounds raises the question of whether the tissues involved are altered by the response to trauma in a manner that makes them physiologically similar to cells in tissue culture. If this is true, it may be a helpful clue in the search for factors that determine the extent of extraneural growth of poliovirus and the likelihood of invasion of the nervous system.

With the Mahoney, Chesterfield Inlet, and Iberia viruses there was no indication that an uninoculated lesion on the opposite thigh was infected earlier or later than one on the scalp. The differences were relatively slight. This finding favors the concept of hematogenous spread, as spread *via* neural pathways might be expected to infect a lesion on the opposite thigh before one on the head.

The procedure used in these studies appears to provide a convenient method of determining the capacity of poliovirus strains to multiply in extraneural sites outside of the alimentary canal and to spread throughout the body by the hematogenous route. This experimental system may be a potentially useful tool in analysis of viruses proposed for use in oral vaccines.

Summary. Four strains of type 1 poliovirus were injected into loose connective tissue at the base of lesions created 3 days previously by excision of 1 cm^2 of skin of one thigh. Viral content of serous exudate from inoculated lesions and from uninoculated skin lesions on the scalp and the opposite thigh was determined. The Chesterfield Inlet and Iberia viruses were chosen as field strains that were epidemiologically virulent and avirulent respectively. The Mahoney and LSc are well known laboratory strains of high and low virulence. Less than 200 $TCID_{50}$ of Mahoney and Iberia viruses were required to infect these lesions. Infection occurred irregularly with LSc virus injected in amounts of 4,000 and 60,000 $TCID_{50}$. The avirulent LSc virus failed to appear in the blood or in uninoculated lesions. The other 3 strains increased in inoculated lesions and in all uninoculated lesions. Although virus appeared to multiply extensively at the lesion site, healing progressed normally; and there was no evidence of local tissue injury. The decrease and disappearance of virus in all lesions of any one animal was essentially parallel. Paralysis occurred in the 2 monkeys infected with Mahoney virus, in 1 of 2 infected with the Chesterfield Inlet virus, in 1 of 4 infected with Iberia virus, and in none of the 4 infected with LSc virus.

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