SURVIVAL AND MULTIPLICATION OF THE VIRUS OF POLIOMYELITIS IN VITRO

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The subject of the artificial cultivation of filterable viruses has again brought into prominence the experiments of Flexner and Noguchi (1) and their followers with the so-called globoid bodies of poliomyelitis. The present paper deals with an attempt to repeat the results of the studies of Flexner and Noguchi and it is therefore desirable to present in brief form the results of the earlier work.

The original paper of Flexner and Noguchi (1) described the globoid bodies as minute, formed structures, cultivated by specially devised methods from tissues of the central nervous system from both human and experimental sources. The tissues were either fresh or glycerolated, unfiltered or filtered through Berkefeld V or N candles.

Methods of Cultivation.—The culture medium consisted essentially of human ascitic fluid to which had been added a fragment of sterile fresh tissue, usually rabbit's kidney. The liquid was covered with a deep layer of sterile paraffin oil and of the two sets of cultures, one was placed in an anaerobic jar and the other kept outside. All tubes were incubated at 37°C.

In the tubes kept outside the jar, it was observed after 5 days that about the fragments of tissue an opalescence appeared which could be gradually diffused through the tube by gentle shaking. After another period of 3 to 5 days, the opalescence extended into the upper portion of the medium, but at the expiration of 10 to 12 days the diffuse but slight opacity began to diminish, and irregular particles formed and slowly fell to the bottom of the tube. In control tubes, either no change was observed, or a slight granular precipitate covered the tissue; and, while in tubes in which growth had been noted the opalescence increased in extent, in the control series the precipitate gathered progressively about the tissue fragments. In the corresponding tubes kept in an anaerobic jar, a similar opalescent growth was seen, although it was slighter than that just described and required a somewhat longer time to reach its full development.

For the demonstration of colony formation, a semisolid medium was used which comprised ascitic fluid and sterile rabbit kidney, to which sufficient 2 per cent

agar had been added to produce a solid mixture. This medium was not successful for initial growths, but after about the third generation of growth in fluid medium, transfer could be effected to the solid medium. In this, growth was noted after several days as a diffuse opalescence which first appeared about the tissue fragments and gradually aggregated into visible, minute colonies. Later, the opalescence rose in the medium until it reached about 3 cm. below the surface, the upper limit of growth being sharply demarcated.

In the original paper, mention was made of the fact that initial growth could be secured in a medium from which either rabbit kidney or ascitic fluid had been eliminated, the former being replaced by using a somewhat larger fragment of poliomyelitic brain or the latter by adding brain tissue extract or sheep serum water. Cultures could not be obtained in the absence of both kidney tissue and ascitic fluid, which might each have contained living cells; modified media were inferior generally to a mixture of the above two components.

The results obtained by the use of the substituted media indicate that growth was derived primarily from the inoculum and not from the kidney tissue or ascitic fluid. The original observation supporting this view is that relatively greater success was obtained when fragments rather than emulsions of poliomyelitic tissue were used. For it has been found that bacteria may be introduced from the outside into cultures of brain tissue from herpes-virus encephalitis (2) and from poliomyelitis (3) during the process of grinding the tissue in the preparation of emulsions.

Characteristics of the Globoid Body.—The globoid bodies were originally described as minute, globular structures, measuring from 0.15 to 0.3 micron in diameter, and arranged in pairs, chains, and small masses, according to the nature of the medium. In fluid medium, pairs and chains predominated; within the tissues of poliomyelitic patients or infected monkeys, only pairs or small masses were discernible, never chains. The reaction to Gram's stain was variable, that is, the bodies retained the stain feebly in earlier generations, but more intensely in older cultures. In peptone medium the organisms appeared to be larger and retained the Gram stain more firmly.

In initial cultures, considerable difficulty was encountered in demonstrating the bodies during the first 4 or 5 days. They could be detected more readily on the sixth or seventh day, and maximum growth was obtained from about the eighth to the twelfth day. After 3 or 4 weeks, however, the bodies became enlarged and irregularly stained, although very minute structures, barely visible, were still present. These altered cultures were filterable through Berkefeld filters, just as were the globoid organisms.

It would appear, therefore, from the original description in which the authors stressed the ability of the globoid bodies to form definite colonies and to show a distinctive morphology, that the bodies were unequivocally organisms.

Pathogenicity in Monkeys.—The original paper emphasized the point that only exceptional cultures possessed pathogenicity in the monkey. Furthermore,

the limit beyond which the virus itself was considered not to be infective was that represented by a second transfer of the original virus in the culture medium.

Two sets of inoculation experiments were reported: one comprising fluid and solid cultures of globoid bodies derived from four human cases, and the other, cultures from the M.A. virus highly adapted by prolonged propagation in monkeys.

The mixed cultures of the human strains, in the third generation, were inoculated into a monkey, which developed characteristic experimental poliomyelitis. A second passage from this animal to two normal monkeys, and later, a third passage to a single *rhesus*, proved successful. From three of these four animals, cultures of the microorganisms were recovered.

The monkey strains of globoid bodies were successfully inoculated as follows: One monkey was injected with a mixed culture representing the third and fifth generations, and a second, third and fourth animal, with fifth to sixth generation cultures, all developed typical poliomyelitis. The globoid bodies were recovered in cultures made from the brains of all these animals. Furthermore, from the second and third *Macacus rhesus* successful passage was effected through two successive series of animals. In addition, a fifth monkey was inoculated with a mixed culture representing the eighteenth and twentieth generations. This animal died on the third day from a secondary infection but the inoculation of a filtrate of the site of inoculation in the brain into a normal *cynomolgus* was followed, after 13 days, by characteristic experimental poliomyelitis.

In one instance in the foregoing experiments, mention was made of the fact that characteristic experimental poliomyelitis was induced with a culture derived from an affected monkey which, in turn, had also been inoculated successfully with globoid bodies.

To sum up, the original report offered evidence of specific pathogenicity of globoid bodies even to the eighteenth or twentieth generation and of the successful recovery of a virulent culture from an animal injected with the globoid bodies themselves.

In a later article, Flexner, Noguchi, and Amoss (4) extended the studies on the globoid bodies and found that the microorganisms survived in the semisolid medium kept at room temperature or at 37°C. for over 13 months. A test for virulence showed these cultures to be pathogenic 6 months later, or 19 months after isolation. In this communication the authors also reported the preparation of "mass" culture medium and the employment of the prodigious number of globoid bodies so obtained for pathogenicity tests. The mass cultures were injected three or four times, since the earlier studies had revealed that only exceptional growths were active and that repeated injections of the ordinary virus (5) sometimes induced paralysis. Thus, one *rhesus* monkey was inoculated intraspinally four times at intervals of 6 to 11 days with mass cultures representing the fourth to thirteenth generations. 5 days after the last injection the monkey developed characteristic experimental poliomyelitis. Another monkey was injected intraperitoneally four times with the same material, and at the same intervals as in the preceding

experiment, and typical poliomyelitis was induced. The nervous tissue of these two monkeys, glycerolated for 6 and 70 days respectively, proved infectious. The effect of the repeated injections is cumulative, for it was shown by a control test that the final, or fourth injection, by itself, failed to affect a normal animal.

The experiments described led to the inference that there is a wide fluctuation in pathogenic action of the globoid bodies, and only exceptional strains are infective. In addition, it was emphasized that the recovery of the bodies in culture from affected monkeys is made only with difficulty. Finally, it was pointed out that, by means of mass cultures, the bacterial nature of the globoid bodies is unequivocally established.

The next communication, by Amoss (6), again presented evidence to indicate the fact that the globoid bodies are microorganisms, and that after long cultivation *in vitro* they become saprophytic and grow more readily in a considerable variety of media. Moreover, Amoss reported the recovery of two additional, but nonpathogenic, cultures from monkeys with experimental poliomyelitis induced by human and M.A. virus. He emphasized in this paper a fact that had already been pointed out in the previous articles (1, 4), namely, that highly parasitic cultures are refractory to artificial cultivation.

Smillie (7) contributed a series of twenty-two cultures of globoid bodies in fluid and semisolid media obtained from the tissues of seven monkeys with experimental poliomyelitis. Of these, eight different strains were inoculated into corresponding *rhesus* monkeys. Five animals were unaffected but three, receiving fourth and fifth generation growths, showed some degree of paralysis after intracranial or intraspinal injection. These latter reactions could not, however, be definitely proved as poliomyelitic. Smillie also found that established cultures can survive in the icebox for months; that dye indicators inhibit their growth; that the preferable pH of the medium is at blood neutrality; that strict anaerobiosis is essential and that body fluids, especially ascitic fluid, are required for their proper development. Most noteworthy is the fact, as shown also by Amoss (6), that after having once become established and accustomed to artificial media, the globoid bodies grow more readily and may be more easily transferred.

The observers mentioned in the preceding paragraphs have therefore defined the characteristics of a true culture of globoid bodies. In view of the minute size of the microorganisms, emphasis was laid on the possible confusion which could arise in distinguishing them from granular precipitates in the culture medium. Hence the criteria of a culture should comprise the following principles: (a) characteristic growth in special, anaerobic, fluid medium; (b) definite colony formation; (c) distinctive growth in mass cultures, and (d) agreement with the other properties already mentioned in the foregoing résumé. Finally, (e) in respect to pathogenicity, attention has been called by

the early investigators to the fact that only exceptional cultures show infectiousness and that the saprophytic strains are cultivable with difficulty while parasitic ones are even more refractory. On the other hand, definite experimental poliomyelitis has been induced in monkeys with the first, third, fourth, fifth, sixth, eighth, eighteenth, and twentieth generations of the globoid bodies. The eighteenth generation has been calculated to represent a dilution of $1:24^{17}$ of the original virus—considerably beyond the limits of its inherent infectivity. In one instance, a culture in semisolid medium, in its eighth generation and kept at 37°C. for 13 months, had not lost its infectiousness, whereas ordinary virus in a similar medium survives no longer than 20 to 30 days (4). Furthermore, the microorganism has been recultivated from the tissues of monkeys in which poliomyelitis was induced by the globoid bodies (in one series, from all of four cases) and such strains have, in turn, again produced the experimental disease.

With this definition of a culture of globoid bodies in mind, we can now compare the results of other investigators with those of Flexner and Noguchi (1, 4), of Amoss (6), and of Smillie (7).

In 1918, Heist, Solis-Cohen, and Kolmer (8) reported the isolation from human and monkey poliomyelitic material of four different strains of globoid bodies. These cultures agreed morphologically and culturally, in fluid, semisolid, and mass-culture medium, with the descriptions originally given (1, 6, 7). Furthermore, a definite distinction was made by Heist and Solis-Cohen (9, 10) between globoid bodies and ordinary streptococci—a finding also reported by Smillie (7) and by the prior investigators. Although the cultures of Heist and his co-workers were carried successfully through at least ten generations, no inoculation tests were made.

On the other hand, the results of the cultivation tests reported by Tsen (11) are not so clearcut. After "many trials" he succeeded in finding organisms which he believed to be similar to globoid bodies. But they could not be maintained for more than three generations, and hence were not inoculated into animals. Since only fluid medium was used, and the "organisms" therein disappeared rapidly, it is likely that Tsen may have confused granular precipitate with the globoid microorganisms.

Into this category of granulations may also be placed the so-called globoid body cultures obtained by Foster (12) from the nasopharyngeal secretions of common cold cases, and those of Bradford, Bashford, and Wilson (13). Olitsky and McCartney (14), in discussing the significance of Foster's cultures, pointed out how the error of confusing precipitates with minute organisms may arise and, further-

more, attempted to indicate the methods by which such misinterpretations may be eliminated. In the case of Bradford and his co-workers, their cultures of so-called "globoid bodies," obtained from patients with influenza, trench fever, war nephritis, and a number of other diseases, were later shown to consist of artefacts (Arkwright—15)—a view which the investigators themselves finally accepted.

In a study concerning the etiology of epidemic encephalitis lethargica, Loewe and Strauss (16), using the fluid and semisolid culture media already described (1), recovered what was believed to be a minute, globular organism from the brain, from nasopharyngeal mucous membrane tissue and washings, from spinal fluid, and from blood of cases of the affection. These investigators stated that their filterable organism resembled in morphology and cultural characteristics the globoid bodies, but that it possessed distinctly different pathogenic effects. They have not, however, reported the distinctive bacteria-like growth, as described by Amoss (6), in mass-culture medium. The fact, however, that colonies were obtained indicates the occurrence of bodies of microorganismal nature in their cultures, which, the authors believe, should be placed in the globoid body group.

EXPERIMENTAL

As indicated in the introductory paragraph, we undertook to repeat the earlier work on the cultivation of the globoid bodies and, especially, to secure cultures which possessed pathogenic properties.

For this purpose, we had access to abundant material taken from monkeys which had developed experimental poliomyelitis after the inoculation[1] of a highly active mixture of two virus strains—M.A. and K—(17, 18) which had been propagated for many years in monkeys. The animals, as soon as definitely paralyzed and before becoming moribund, were etherized and the brain and spinal cord were removed with sterile precautions. The tissues were generally free from secondary bacterial contamination, or bacteria derived, during exposure, from the air. Inoculation of media was immediately carried out, using fragments of brain or spinal cord, or a Berkefeld V filtrate of a 5 per cent physiological saline suspension of these tissues.

Cultivation

The culture medium was that of Noguchi, consisting of ascitic fluid and a fragment of sterile, fresh, rabbit kidney.

In the preparation of initial cultures, we attempted to repeat precisely the original method described by Flexner and Noguchi (1). The fragment of rabbit kidney

[1] All inoculations in the monkey were carried out under ether anesthesia.

was placed in a test tube measuring 1.5 x 20 cm. The inoculum, consisting of either a small fragment of brain or of spinal cord, or of 0.5 cc. of the filtrate of these tissues, was next added and then about 15 cc. of sterile ascitic fluid. Finally, about 2 cc. of sterile liquid albolene was added in such a way as to form a layer over the surface of the medium. One set of tubes of each series was placed in the Boëz anaerobic jar (19), and a duplicate set kept outside. The incubation was at 37°C.

A modified technique was also used: sufficient 1 per cent neutral cysteine hydrochloride solution was introduced into the ascitic fluid to give a final concentration of 1:2000. The cysteine medium was sealed with solid petrolatum instead of liquid albolene. We found, however, that this modification did not lead to an increase in the number of positive "cultures."

As a rule, about 50 tubes of medium were inoculated during each original attempt at cultivation: two-thirds of them were set up as first described, and one-third with the cysteine.

After 10 to 14 days, the tubes were removed from the thermostat and a small amount of the medium was withdrawn from the bottom of each tube with a sterile capillary pipette, for film preparations. These preparations were allowed to dry in the air and, after being fixed by heat, were stained for 3 minutes with well-ripened, alkaline, methylene blue.

All tubes found by microscopic examination to be contaminated with ordinary, familiar bacteria were discarded. On the other hand, each culture thought to contain material resembling globoid bodies was subplanted to six or eight tubes of Noguchi's fluid medium and upon two rabbit-blood, dextrose-agar plates. In the case of each subplant, one-half the number of tubes and plates was placed in an anaerobic jar and the other half kept outside. In addition numerous uninoculated control tubes of the medium, and inoculated control tubes of dextrose broth were included in each series of transfers.

No characteristic macroscopic changes were visible in the inoculated tubes at any time. The opalescence described by Flexner and Noguchi (1) as appearing around the kidney at the end of the fifth day was not detected but a distinct clouding, possibly due to autolysis, was noted at the end of 24 hours.

With regard to the formation of colonies, which other workers (Flexner and Noguchi—1; Flexner, Noguchi, and Amoss—4; Amoss—6; Smillie—7, and Heist, Solis-Cohen, and Kolmer—8) agree in emphasizing as characteristic of the globoid bodies, and as indicative of their microorganismal nature, we were unable to secure any evidence. This may perhaps have been due to the fact that we did not employ the semisolid medium of Noguchi. However, no colonies could be detected on the plates, incubated anaerobically, which were made from tubes showing suspected growth. Nor were we successful in obtaining growth in the special mass-culture medium (4), although no less than twelve attempts were made by this method.

In view of our having used different media in this part of the work, any comparison to be made between our results and those of the original investigators should be based on findings in fluid cultures.

To recapitulate: Efforts at cultivations were made with fragments of the brain or cord or with Berkefeld V filtrates of the nervous tissues of seven monkeys inoculated with poliomyelitic virus and suffering from experimental poliomyelitis. With these materials, 315 tubes were inoculated, thirty-six of which showed minute morphological particles or "bodies" suggesting microorganisms. The subplanting from these "positive" tubes was disappointing, owing to the failure to obtain secondary growths in later subplants or to failure due to contamination with ordinary bacteria. Thus, while all seven monkeys yielded minute particles suggesting growths in initial cultures, the "growths" derived from three animals failed after the first subplant, from one after the third, from one after the fourth, from one after the eighth, and from the last monkey after the eleventh subplant.

Morphology

The microscopic appearances to be described relate wholly to the fluid subplants and it should be recognized that because of the presence of the autolyzing kidney fragment and the resulting granular precipitate, it is just such cultures that are most subject to misinterpretations. Indeed, the distinction in early subplants between minute microorganisms and precipitate is so indefinite that we were led to apply the method of cataphoresis in order to attempt possible differentiation. We already knew that the poliomyelitic virus in nervous tissue suspensions migrates to the anode (20). Applying the same experimental procedures (21) to washed "cultures" in twenty-three tubes derived from six different "strains" in five separate tests, no conclusive evidence could be obtained, by this method, of differentiation of "microorganisms" from precipitate. For whatever picture was detected at the anode could be seen also at the cathode. On the other hand, control experiments with *Bacterium pneumosintes* and *Bacterium coli* were clearcut; there was active migration of both to the anode.*

Notwithstanding these facts, we were able to pick out thirty-six from 315 tubes in which the distinctions were sufficiently wide to suggest the possibility of microorganisms being present. The main points of

* In carrying out the cataphoresis experiments, we were aided by the effective cooperation of Dr. D. C. Hoffman of The Rockefeller Institute.

such distinctions are as follows: In early subplants, the minute and "globoid bodies" were single, in pairs, or in short chains, the individual bodies being of a fairly uniform appearance. In older subplants, the bodies were more numerous and in part formed agglomerated masses. In remote subplants, they were somewhat larger. The bodies stained well with well-ripened alkaline methylene blue and, as a rule, did not retain Gram's stain. In stained specimens the "bodies" appeared to be raised above the background of amorphous material, cellular detritus, and indefinite particles and were not refractile. It should be mentioned that in practically all film preparations we observed well-preserved kidney tissue cells and leucocytes, the latter probably derived from the ascitic fluid.

Power to Infect Monkeys

We pass now to the most significant part of our study, namely, the power of the "cultures" to infect and induce experimental poliomyelitis in monkeys. Indeed, in one series of subplants, derived from the brain and cord of a monkey experimentally infected with the virus described in Experiment 1, we were successful in producing experimental poliomyelitis in monkeys with the materials taken from "cultures" in the seventh, eighth, ninth, and tenth transfers. It should be noted that the seventh transfer represents a dilution of the original material cultivated of about 1.5×10^{-12}, and the tenth, of about 1.3×10^{-18}. The history of the monkey yielding the active material is as follows:

Experiment 1.—Macacus rhesus inoculated intracerebrally on February 8, 1929, with 1 cc. of the suspension of anodic material obtained by cataphoresis of brain and cord tissues from a monkey in the paralytic stage of the experimental disease (20). February 19, excitement, tremor, right facial paralysis, and ptosis were noted. February 20, ataxia, tremors, weakness of right arm and shoulder and both legs were observed. February 21, the animal was prostrate and unable to move its arms and legs. Etherized. The gross and histopathological examinations revealed characteristic lesions of experimental poliomyelitis.

Sixty-nine tubes were inoculated with the infected nervous tissue of this animal.

Of the sixty-nine tubes, eight showed what appeared to be a minute, "globoid microorganism." These were subplanted to sixteen tubes of which, after 12 days' incubation, five were "positive." A third subplant into thirty tubes yielded sixteen; a fourth into forty-two tubes yielded nine; a fifth of forty-five, eleven; a sixth of sixty-six, twenty; a seventh of sixty, thirty; an eighth of forty-eight,

thirty-seven; a ninth of thirty-two, four; and a tenth of thirty-two, five "positives." All the forty tubes of the eleventh subplant were contaminated by moulds and diphtheroid organisms. By the time the sixth subplant was reached, all the "positive" tubes had a common origin from one tube of the first transfer.

The medium is notably irregular and uncertain in its composition because of the variations in quality of the ascitic fluid and possibly of the kidney tissue. Noguchi (22) originally pointed out that certain samples of ascitic fluid were more suitable than others for cultivating spirochetes and other investigators have emphasized this factor as influencing cultures of globoid bodies (4, 6, 7). That uncontrolled fluctuating factors played a part in our results is indicated by the discrepancies arising in the several subplants. The irregularity in positive tubes is correlated with the number of "bodies" visible under the microscope: In the first three subplants they were few, from the fourth to the eighth subplant, many; with the seventh subplant they predominated, but in the ninth to eleventh they became very few and difficult to find.

Monkeys were inoculated with materials from the seventh, eighth, ninth, and tenth subplants of these "cultures." The material inoculated consisted of centrifuged sediment of the tubes, washed, as a rule, five times with physiological salt solution. In one instance (Experiment 2) to the washed sediment of the seventh subplant was added the unwashed sediment of the sixth and fourth subplants.

Experiment 2.—Macacus rhesus A. April 18, 1929, pooled material from the bottom of the positive tube in the sixth transfer of the culture obtained in Experiment 1 (hereafter designated as Bodies 1) was prepared and of this mixture 2 cc. were injected intracerebrally, 10 cc. were injected into the peritoneal cavity and 2 cc. into the spinal canal.

April 23, pooled material from the bottom of the positive tube in the fourth transfer of Bodies 1 was prepared, and of this mixture 2 cc. were injected intracerebrally, 9 cc. intraperitoneally, and 2 cc. intraspinally.

May 1, the material from the bottom of the positive tube of the seventh transfer was centrifuged at high speed for 15 minutes; the sediment was suspended in 3 cc. of physiological saline solution, and then centrifuged again. This was repeated three times. 1.75 cc. of the final suspension were injected into the left cerebral hemisphere.

May 11, tremor; right facial paralysis; ataxia; complete paralysis of left arm, and legs very weak.

May 12, prostrate. Etherized.

Autopsy.—Meninges normal. A very small area of softening was visible in the border of the right internal capsule, involving the lenticular nucleus. This site contained old blood and yellow pigment. The midbrain showed spotty translucent areas about 1 to 2 mm. in diameter. The cervical cord contained pink, translucent, soft areas in the region of the anterior horn cells.

Microscopical Examination.—Forebrain: A moderate mononuclear cellular reaction was present. Lateral to and partly involving the lenticular nucleus was a small area of softening containing red blood cells in various stages of degeneration. Around this area were many phagocytic microglia. Certain of the nerve cells showed varying degrees of degeneration and some of them were phagocytized.

Medulla: A slight mononuclear infiltration of the pia-arachnoid membrane was seen. Moderate perivascular infiltration was present. A certain amount of nerve cell degeneration with and without accompanying cellular reaction occurred.

Cord: A slight lymphocytic reaction was observed in the pia-arachnoid membrane, together with a marked perivascular mononuclear infiltration. In the cervical region there was almost complete destruction of both the anterior and posterior horn cells, with accompanying phagocytosis of the débris by microglia. The same reaction was present but was progressively less marked in the lower levels of the cord.

A 5 per cent saline Berkefeld N filtrate was prepared from portions of the cord and of this 1 cc. was injected intracerebrally into a monkey. This monkey developed typical experimental poliomyelitis within 6 days.

Cultivation experiments in Smith-Noguchi medium were carried out with fresh central nervous tissue from Monkey A. Seventy-one tubes were inoculated and after 14 days' incubation, five tubes contained bodies resembling those with which Monkey A had been inoculated.

Experiment 3.—Macacus rhesus B. May 1, 1929, 2.5 cc. of a saline suspension of washed materials from the bottom of the positive tube of the seventh transfer of Bodies 1 were injected into the left cerebral hemisphere.

May 6, slight tremor; ataxia; marked left facial paralysis; both arms practically completely paralyzed.

May 7, prostrate; facial paralysis increased. Etherized.

Autopsy.—At a site of inoculation there was a soft area 2 x 2 mm. containing a small amount of golden yellow pigment. No cyst formation or frank hemorrhage was observed. The region of the anterior horn cells in the spinal cord showed injection and slight hemorrhage. This was most marked in the cervical region.

Microscopical Examination.—Brain: Slight perivascular lymphocytic reaction in the region of the thalamus. Irregular nerve cell degeneration. The choroid

plexus was infiltrated with lymphocytes. In the sub-arachnoid space was a diffuse lymphocytic and mononuclear cellular reaction.

Medulla: Foci of mononuclear cells, some surrounding degenerated nerve cells, were present in the region of the fourth ventricle. A marked perivascular mononuclear infiltration occurred throughout the medulla. All stages of nerve cell degeneration were seen.

Cervical cord: A moderate meningeal and perivascular reaction was observed. The neurons showed marked destruction and active neuronophagia.

Thoracic cord: The meninges were thickly infiltrated with lymphocytes. The cells of the anterior and posterior horns showed marked acidophilic degeneration, but their outline was preserved. The nerve cells were surrounded by a large number of polymorphonuclear and mononuclear leucocytes. The same changes were observed in the lumbar cord.

Intervertebral ganglia: There was a marked interstitial lymphocytic reaction and a moderate acidophilic degeneration of the nerve cells.

Five monkeys were inoculated with suspensions and filtrates prepared from the cord of Monkey B. Three of these control monkeys developed typical experimental poliomyelitis within 7 days; the remaining two were unaffected.

Cultivation experiments in Smith-Noguchi medium were made with fresh material from the brain and spinal cord of Animal B. In the second transfer bodies closely resembling those inoculated were isolated.

Experiment 4.—Macacus rhesus C. May 15, 1929, 1.5 cc. of a saline suspension of washed material from the bottom of the positive tubes of the eighth transfer of Bodies 1 were inoculated into the left cerebral hemisphere and 1.5 cc. of the same suspension were injected into the spinal canal.

May 21, tremors; slight ptosis of both upper eyelids; ataxia; partial paralysis of both arms and shoulders; legs very weak. Etherized.

Autopsy.—Meninges normal. Site of inoculation used for touch preparations. A definite small hemorrhage was seen in the striate body. The cervical cord revealed definite areas of congestion and hemorrhage in the region of the anterior horn cells. These changes were less marked in the lumbar and sacral cord.

Microscopical Examination.—Site of inoculation: No bodies were seen in touch preparations. Lateral to the anterior part of the striate body was a narrow zone of softening containing red blood cells, granular débris, and a moderate amount of pigment. About the periphery of this area was a large number of glial nuclei.

Cord: The anterior horn cells showed varying degrees of degeneration, some being slightly involved while others were completely destroyed. A diffuse cellular infiltration of polymorphonuclear and mononuclear leucocytes was present. This reaction became less marked in the lumbar and sacral regions.

Intervertebral ganglia: A diffuse interstitial lymphocytic infiltration was present and a moderate number of nerve cells showed partial or complete degeneration.

A 5 per cent saline Berkefeld N filtrate was prepared from the brain and cord of Monkey C. 1 cc. of this filtrate was injected into the left cerebral hemisphere of a normal control monkey. Within 8 days the animal developed typical experimental poliomyelitis.

Cultivation experiments in Smith-Noguchi medium were made with fresh tissue of the central nervous system from Monkey C. After 14 days' incubation, one of forty-seven inoculated tubes contained bodies similar to those with which the monkey had been inoculated.

Experiment 5.—*Macacus rhesus* D. May 31, 1929, 1.25 cc. of a washed saline suspension of the material from the bottom of the positive tubes of the ninth transfer of Bodies 1 were injected into the right and left cerebral hemispheres.

June 6, tremor; ataxia; both arms paralyzed; legs weak.

June 7, prostrate. Etherized.

Autopsy.—The meninges were slightly injected. The left site of inoculation showed an old hemorrhage with some yellow pigment and an area of softening extending into the internal capsule and the caudate nucleus. A similar picture was present in the right site of inoculation. Typical translucent, congested and hemorrhagic areas were visible in the medulla and in the region of the anterior horn in the cervical cord. No organisms were seen in preparations made from the sites of inoculation.

Microscopical Examination.—The site of inoculation showed a hemorrhage with hemosiderin pigment and was surrounded by phagocytic mononuclear cells. A slight diffuse increase in glial nuclei was present. Nearly all of the anterior horn cells in the cervical region had disappeared. Many foci of phagocytic mononuclear cells were present. A moderate perivascular lymphocytic reaction was observed.

A 5 per cent saline suspension was made of the cord of Monkey D. 0.5 cc. was inoculated into the left cerebral hemisphere of a normal, control monkey, which in the course of 7 days developed typical experimental poliomyelitis.

Cultivation experiments in Smith-Noguchi medium were made with the fresh central nervous tissue from Monkey D. None of the forty-eight tubes showed bodies similar to those with which Monkey D had been inoculated.

Experiment 6.—*Macacus rhesus* E. May 31, 1929, 1.25 cc. of a washed saline suspension of the material from four negative tubes of the ninth transfer of Bodies

1 were inoculated into the right and left cerebral hemispheres. This material contained no demonstrable bodies.

The monkey remained well and was discarded after 1 month's observation.

Experiment 7.—Macacus rhesus F. June 14, 1929, 1.5 cc. of washed saline suspension of material from the bottom of five positive tubes of the tenth transfer of Bodies 1 were injected into the right and left cerebral hemispheres.

June 20, excited; tremors; right deltoid weak.

June 21, prostrate. Etherized.

Autopsy.—There was slight meningeal congestion. An area on the left side, greenish gray to red in color, was filled with tenacious, soft, elastic material which contained staphylococci. The area extended from the superior surface of the frontal lobe to a region near the internal capsule, 1 to 2 mm. wide, and spread out at the base to a pyramidal area about 4 mm. in diameter. On the right side a similar condition prevailed, although the area was not as large but more hemorrhagic and extended almost to the internal capsule. The fourth ventricle revealed the usual picture of poliomyelitis, but its edges were bulging and the glistening surface showed pink, turgid, tiny, translucent, somewhat hemorrhagic areas. The cervical cord exhibited the characteristic injection and translucence of the anterior horns, but in the dorsal and lumbar cord the pinkish tinge was not as prominent.

Microscopical Examination.—Forebrain: At the site of inoculation an elongated area was seen extending from near the cortex to the external surface of the lenticular nucleus. This area contained erythrocytes, polymorphonuclear and endothelial cells, and granular débris. A small amount of golden yellow pigment was present. The tissue forming the border of this area comprised a mass of phagocytic mononuclear cells and proliferated neuroglia; there was also perivascular infiltration by lymphocytes. Lymphocytes infiltrated the pia-arachnoid. In the medulla there were present occasional nerve cell degeneration and neuronophagia with a moderate perivascular infiltration.

Cord: Cervical region. Moderate lymphocytic infiltration of the meninges. Almost all neurons showed acidophilic degeneration and some were in the process of neuronophagia. The gray matter was diffusely infiltrated with lymphocytes and with occasional polymorphonuclear cells.

Thoracic region: Here the cord was the seat of an intense reaction. No normal appearing nerve cells were present, all being degenerated and fragmented, the subject of active neuronophagia. Very marked extra-adventitial infiltration was noted.

Lumbar region: Lesions of meningeal mononuclear infiltration; nerve cell degeneration; neuronophagia; perivascular infiltration with some diffuse lymphocytic and mononuclear cellular infiltration throughout the gray matter. In general, the reaction was less marked here than in the cervical or thoracic cord.

Intervertebral ganglia: Moderate diffuse lymphocytic and mononuclear cell infiltration; a few necrotic nerve cells in various stages of degeneration with some showing neuronophagia.

A 5 per cent saline suspension was made of the cord of Monkey F; 0.5 cc. was inoculated into the left cerebral hemisphere of a normal monkey, which developed typical experimental poliomyelitis after an incubation period of 7 days.

Cultivation experiments in Smith-Noguchi medium were made with fresh tissue of the central nervous sytem of Monkey F. Two of twenty-eight tubes showed bodies similar to those employed in the inoculation of Monkey F.

Experimental 8.—Macacus rhesus G. June 14, 1929, 1.5 cc. of a washed saline suspension of the material from five negative tubes, that is, free from "globoid bodies," of the tenth subplant of Bodies 1 were inoculated into the right and left cerebral hemispheres. The monkey remained well throughout the period of observation.

One observes from the preceding experiments that the inoculation in monkeys of a saline suspension of the washed sediment from Smith-Noguchi cultures of poliomyelitic tissue induced, in the seventh, eighth, ninth, and tenth transfers, the clinical syndrome and the pathological effects characteristic of experimental poliomyelitis. It is noteworthy that in the successful experiments, the washed material contained small bodies resembling the globoid bodies described by Flexner and Noguchi. Equally important is the fact that in parallel experiments with material derived from the ninth and tenth transfers, the washed sediment of tubes showing similar bodies was active in monkeys while that of tubes not containing the bodies was inactive.

SUMMARY AND DISCUSSION

The study here reported concerns attempts at bacteriological cultivations with fragments of brain or cord, or with Berkefeld V filtrates of the nervous tissues, from seven monkeys successfully inoculated with poliomyelitic virus. With these materials, 315 tubes were inoculated, of which thirty-six showed minute bodies resembling the globoid bodies described by Flexner and Noguchi. However, a study of subplants from these minute, morphological particles did not convince us that we had in hand actual cultures of the globoid bodies, or indeed of any living microorganism.

Nevertheless, when washed sediments from subplants of one of the

strains, representing the seventh, eighth, ninth, and tenth transfers, were inoculated into monkeys, the clinical signs and pathological effects characteristic of experimental poliomyelitis could be induced. The virulence of the "cultures" could not be ascribed to carrying over the original material into these remote subplants since the seventh transfer represented a dilution of the original cultivated material to about 1.5×10^{-12}, and the tenth, to about 1.3×10^{-18} if one assume, as the transfer technic justifies, a thorough mixing of the contents of each tube. On the contrary, it appears as if the poliomyelitic virus had multiplied *in vitro*, and had increased as a consequence of being in a medium of a modified living tissue-cell culture. For in practically all specimens we observed many well-preserved kidney tissue cells and leucocytes, the latter probably derived from human ascitic fluid, a component of the Smith-Noguchi medium. In this connection, it should be mentioned that the several lots of ascitic fluid used in the cultivation tests were recently obtained from patients and employed from a week to a month after their collection.

There remains for consideration the problem of the selective pathogenicity of the "cultures:" only the material of those tubes of the ninth and tenth transfers which showed the "globoid bodies" proved pathogenic; those respective tubes of the same transfers which were free from the minute bodies but apparently identical in all other respects, were avirulent. It may be that the virus was adsorbed to the particular bodies which we have found in the "cultures" and which resemble closely the globoid bodies of Flexner and Noguchi. Further elaboration of this study would be necessary, however, before such an inference could be regarded as a definite hypothesis.

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