EXPERIMENTAL STUDY OF A HORSE ANTIPOLIOMYELITIC SERUM

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Up to a recent date two sources of antipoliomyelitic serum have been known: one, human beings who have recovered from attacks of poliomyelitis irrespective of their severity; and the other, monkeys experimentally inoculated with the virus of the disease. Monkeys, like human beings, were found to yield a virus neutralizing serum, not only when they developed symptoms, usually paralytic, of poliomyelitis, but also when they were actively immunized with subclinical doses of the virus itself. There is reason to believe that such subclinical immunization takes place in human beings, perhaps on a large scale, during epidemic prevalences of poliomyelitis.

The knowledge summarized in this brief statement is based on the investigation carried out in 1910 and since then (Flexner and Lewis (1), Roemer and Joseph (2), Landsteiner and Levaditi (3), Zappert, von Wiesner, and Leiner (4), Anderson and Frost (5), Amoss and Chesney (6), and Aycock and Kagan (7)). In 1910, Flexner and Lewis (9) determined definitely, by monkey experiment, that convalescent monkey and human sera possessed therapeutic properties. Netter (8) was the first to apply this knowledge to the treatment of human cases of poliomyelitis. The experiments of Flexner and Lewis (9), carried out with potent virus inoculated intracerebrally, indicated that with a minimal but effective dose of virus, and several intrathecal injections of convalescent serum begun 24 hours after the inoculation, prevention of paralysis or other symptoms of poliomyelitis could be secured. In the years intervening between 1910 and the present time, convalescent human serum has been widely employed in the treatment of early cases of poliomyelitis in man with beneficial results, so far as clinical and statistical indications can be interpreted.

Attempts were early made by several experimenters to produce a virus neutralizing serum in animals other than monkeys.

Flexner (10) in 1910 reported negative results with horse, goat, and several kinds of small laboratory animals. Sheep alone yielded inactivating substances.

But these substances existed in the blood of normal sheep and appeared not to be increased by immunization. The method usually followed was to inject suspensions of the central nervous organs of monkeys succumbing to acute experimental poliomyelitis. One or two exceptions to the common experience were recorded. Neustädter and Banzhaf (11) (1917) believed that they had produced in a horse substances definitely inactivating to the virus when combined *in vitro*. Pettit (12) (1918) produced both in the horse and the sheep, neutralizing sera which have had considerable clinical employment in France. Recently Weyer, Park and Banzhaf (13) and Fairbrother (14) have reported more significant successes with the horse. The former found that the inactivating substances in the serum could be concentrated in the globulin fraction.

Through the kindness of Dr. Park, a quantity of antipoliomyelitic horse serum has been made available to us for study. We have carried through two series of experiments with this serum: inactivating effects in one *in vitro* and in the other *in vivo* have been investigated. It still remains to be determined whether the biological processes in both kinds of inactivation or neutralization are the same; and it is still an open question whether the inactivation which occurs with horse or sheep antipoliomyelitic serum is both qualitatively and quantitatively identical with that produced with convalescent human and monkey serum.

Considerable diversity of view exists with reference to the neutralizing power and process of these alien sera. Rosenow (15) and Nuzum (16) early claimed to have produced antiviral sera in the horse by immunization with streptococci. Amoss and Eberson (17), using serum supplied by the experimenters named, were unable to confirm this work. Stewart and Haselbauer (18) studied Pettit's antipoliomeylitic horse serum and found that it is occasionally virus neutralizing. They also found normal sheep serum to be sometimes inactivating, and reported that sheep yielding this serum when subjected to subsequent immunization not only failed to give a more potent serum, but occasionally lost the power of inactivation originally possessed.

Obviously, therefore, the nature of the inactivating process as exhibited by the antipoliomyelitic horse or sheep serum is worthy of more minute study. It is desirable also to employ a virus of definite and certain activity and preferably, we believe, a virus which possesses these powers when used as a filtrate rather than as suspension or

emulsion. The very active filtrates are more constant in their effects, and the effective dose can be more closely regulated. It is self-evident that the contact between serum and filtrate virus will be more intimate than between serum and emulsion virus, in which relatively large particles of nervous tissues must be penetrated.

Small differences in incubation period in the inoculated monkeys cannot be regarded as significant. Any large series of virus inoculated monkeys will show disparities of this kind, even when intracerebral injection is employed. This is to be expected, since the native resistance of the animals to a fixed inoculum varies within certain limits. Flexner and Lewis (19) (1910) pointed out that with a given virus, four kinds of inoculation effects could be distinguished: no discernible symptoms of disease; abortive poliomyelitis; paralytic poliomyelitis on about the 10th day; and delayed paralytic poliomyelitis in which the first symptoms appeared on about the 15th day. The first two conditions were distinguishable on reinoculation: the abortive animals resisted; the symptomless animals reacted to the second inoculation. In rare instances, paralytic effects appeared as late as the 28th day. Hence 1 month was always permitted to elapse before the experiment was considered concluded.

EXPERIMENTAL

In the experiments to be recorded the highly potent filtrate virus "M.V." has been employed. Injections were made into *Macacus rhesus* alone. The antipoliomyelitic horse sera kindly supplied by Drs. Park and Weyer consisted of unpreserved horse serum, citrated horse plasma, and concentrated globulin fraction. The controls employed consisted of normal horse serum, citrated horse plasma, and the globulin fraction of diphtheria antitoxin. All inoculations which entailed surgical procedures were carried out under ether anesthesia.

Experiment 1.—This test is an exact duplicate of tests frequently made with convalescent human and monkey sera. The virus filtrate, consisting of 0.3 cc., is mixed with serum 0.9 cc., incubated at 37°C. for 2 hours, allowed to stand over night in the icebox, and the whole is injected intracerebrally. Under these circumstances, the immune sera neutralize the virus, while the normal horse and strictly normal human sera do not.

Three monkeys were employed: one received normal, unpreserved horse serum (control), and the other two received the unpreserved antipoliomyelitic horse serum. The inoculations were intracerebral. Table I gives the results. The control animal became paralyzed on the 9th day; the two animals receiving the antipoliomyelitic horse serum remained free of all symptoms.

Comment.—Taken by itself, this *in vitro* experiment has reproduced the usual experience with convalescent human and monkey sera.

Experiment 2.—This test was an orienting one, designed to indicate the limits of effectiveness possessed by the antipoliomyelitic horse plasma. Consequently

TABLE I

Experiment 1. Unpreserved Antipoliomyelitic Horse Serum

No.	Date	Material tested	Amount	Virus amount	Route of inoculation	Results
			cc.	*cc.*		
1	5/20	Unfiltered, unpreserved antipoliomyelitic serum	0.9	0.3	Icer.	No symptoms
2	5/20	"	0.9	0.3	"	"
3	5/20	Normal, unpreserved horse serum	0.9	0.3	"	Typical poliomyelitis on 9th day

TABLE II

Experiment 2. Antipoliomyelitic Horse Plasma

No.	Date	Material tested	Amount	Virus amount filtrate	Result
			cc.	*cc.*	
1	8/23	Normal horse serum. Control	0.1	0.01	Typical poliomyelitis, 20 days
2	8/23	Normal horse plasma. Control	0.1	0.01	No symptoms
3	8/23	Antipoliomyelitic horse plasma	0.1	0.06	Mild symptoms, 12 days
4	8/23	"	0.1	0.12	No symptoms
5	8/23	"	0.1	0.18	Typical poliomyelitis, 4 days

the volume of infecting filtrate was varied widely, and the relations between virus and plasma were so arranged as to comply as closely as possible with the technique used by Park and Weyer. The virus calculated on its usual activity represented

12 to 36 infecting doses. The controls consisted of normal horse plasma and serum.

Comment.—As Table II shows, normal horse plasma is effective in preventing infection under conditions in which normal horse serum has no final effect. On the other hand, in one of three monkeys the antipoliomyelitic horse plasma was protective against at least 12

TABLE III.

Experiment 2. Effect of 1.5 Per Cent Citrate on Poliomyelitis Virus

No.	Date	Material tested	Virus amount	Test material amount	Result
			cc.	cc.	
1	8/23	Citrated horse plasma	0.01	0.1	No symptoms
2	8/23	Uncitrated horse serum	0.01	0.1	Typical poliomyelitis, 19 days
3	10/10	Citrated horse plasma	0.01	0.1	Mild poliomyelitis, 9 days
4	10/10	Uncitrated horse serum	0.01	0.1	Typical poliomyelitis, 5 days
5	10/17	Citrated horse serum	0.01	0.1	No symptoms
6	10/17	Uncitrated horse serum	0.01	0.1	Typical poliomyelitis, 6 days
7	11/1	Citrated horse plasma	0.01	0.1	No symptoms
8	11/1	Control virus alone	0.01	—	Typical poliomyelitis, 7 days

M.L.D. of virus, and ineffective against 6 and 18 M.L.D. The incubation period of the monkey receiving normal horse serum was somewhat prolonged, the reason for which is wholly conjectural. Since the citrate itself may have exerted an inhibitory effect, a set of tests was carried out to determine this point. The result is given in Table III and can be summarized by the simple statement that sodium citrate in concentration of 1.5 per cent does exert an inhibitory chemical effect on the filtered virus.

Experiment 3.—This test was made with the globulin concentrate, and controlled with normal horse plasma and antipoliomyelitic horse plasma, 2 M.L.D. of virus being employed in each test. The results are given in Table IV.

Comment.—Under the conditions of the experiment, all the antipoliomyelitic horse preparations inactivated the virus, while both the normal plasma and serum control animals became paralyzed on the 9th day.

Since the experiments of certain previous investigators and the recent tests of Weyer, Park and Banzhaf all indicate that by the

TABLE IV

Experiment 3. Horse 5 Globulin

No.	Date	Material tested	Amount	Virus amount	Result
			cc.	*cc.*	
1	11/18	Filtered antipoliomyelitic horse globulin	0.1	0.2	No symptoms
2	11/18	Unfiltered antipoliomyelitic horse globulin	0.1	0.2	" "
3	11/18	Antipoliomyelitic horse plasma	0.1	0.2	" "
4	11/18	Normal horse plasma	0.1	0.2	Typical poliomyelitis, 9 days
5	11/18	—	—	0.2	"

injection of poliomyelitic nervous tissues, an occasional horse may be made to develop inactivating substances for the virus effective *in vitro*, the tests which we have made may be regarded as confirmatory. We proceeded, therefore, to our next problems which related first, to the mechanism of the inactivation process, and second, to the *in vivo* effects of the antiserum.

The next experiment deals with the question of the action of adsorbing bodies on the inactivating substances. Andervont and Lewis (20) have shown that colloidal particles, such as aluminium hydroxide, kaolin, and India ink, adsorb vaccine virus and thus render it in-

effective on inoculation. A simple test brought out the fact that the antipoliomyelitic horse serum contained precipitin for normal monkey brain proteins. Hence equal amounts of the antipoliomyelitic horse globulin and 5 per cent saline suspension of normal monkey brain were mixed and shaken for 2 hours, after which they were kept in a water bath at 37°C. for 2 hours and placed in the icebox over night. The clear fluid, separated by centrifugalization, was pipetted off. This fluid failed to give the precipitin reaction.

Experiment 4.—2 M.L.D. of the filtrate virus were mixed with 0.1 cc. each of unfiltered antipoliomyelitic horse globulin absorbed and unabsorbed with normal monkey brain, controlled by a simple virus injection.

TABLE V

Experiment 4. Normal Brain Absorption

No.	Date	Material tested	Amount	Virus amount	Result
			cc.	*cc.*	
1	11/29	Antipoliomyelitic horse unfiltered globulin absorbed by normal brain	0.1	0.2	No symptoms
2	11/29	Antipoliomyelitic horse unfiltered globulin unabsorbed	0.1	0.2	" "
3	11/29	Plain control	—	0.2	Typical poliomyelitis, 7 days

Comment.—As Table V shows, the monkeys injected intracerebrally with the globulin-virus mixtures remained symptomless, while the control animal became paralyzed on the 7th day. In other words, the inactivating substances contained in the antipoliomyelitic horse globulin were not removed by the absorption with normal monkey brain, as carried out in the experiment.

We turn now to what may be termed the crucial experiments, in which the antipoliomyelitic horse serum is compared in its effects with convalescent human and monkey poliomyelitic sera. Weyer, Park and Banzhaf, as well as Neustädter and Banzhaf, have described *in vivo* tests in monkeys in which the antipoliomyelitic horse serum preparations displayed protective and therapeutic properties. The

employment of Pettit's serum for therapeutic purposes in man is postulated on these same properties. Especial interest, therefore, attaches to this class of experiments.

The experience of this laboratory with experiments of this kind is considerable. At the beginning the virus inoculations were made intracerebrally, obviously a severe test for the protective or therapeutic action of an antiserum. Later, intracisternal injection of the virus was employed in order to avoid the traumatic effects of intracerebral injection. More recently, intranasal instillation of virus has superseded the intracisternal method, as involving no trauma whatever and as employing the usual portal of entry of the virus in man.

The observations of Flexner and Stewart (21) on the duration of the protective effects of convalescent sera when injected intraspinally, which observations have been confirmed and extended by ourselves (22), indicate a noteworthy manner of approach towards solving the problem of the nature of the inactivating substances in the antipoliomyelitic horse sera. For instance, it had been shown by Flexner and Stewart and by ourselves that when 2 cc. of convalescent human or monkey serum is injected intrathecally, it is effective for 4 days against a subsequent intracerebral injection of virus; and we found that it is sometimes effective for 6 days against nasal instillation of the virus. In the next experiment, antipoliomyelitic horse globulin was substituted for the convalescent sera.

Experiment 5.—Two series of animals were employed in this experiment. 2 cc. of the antipoliomyelitic horse globulin was injected intraspinally by lumbar puncture, followed 4 days later by intracerebral injection of 1 M.L.D. of virus.

Comment.—As Table VI indicates, under conditions in which convalescent human and monkey sera prevent the development of experimental poliomyelitis, the antipoliomelitic horse globulin solution was without demonstrable effect. The two sets of tests made are consistent. The monkeys given the intraspinal injections of serum exhibited symptoms of experimental poliomyelitis after the injection of virus in average incubation time. Attention should be called to Monkey 2 (Table VI), in which the symptoms are described as being mild. For the purposes of comparison, brief clinical descriptions are appended of the three monkeys employed in this series.

Macacus rhesus 1 received 2 cc. of unfiltered globulin fraction of antipoliomyelitic horse serum, injected into the lumbar subarachnoid space by means of spinal puncture. 48 hours later the animal was inoculated intracerebrally with 0.1 cc. of fresh virus filtrate. After an incubation period of 9 days, the animal presented partial paralysis of one arm and complete paralysis of both legs. On the following day, complete paralysis of the extremities had set in, the respirations were shallow and rapid; the animal was etherized. Autopsy disclosed typical lesions of poliomyelitis.

TABLE VI

Experiment 5. Prophylactic Effect of Antipoliomyelitic Horse Globulin

No.	Date	Intraspinal globulin*		Interval before inoculation	Inoculations			Result
		Type	Amount		Virus filtrate	Route	Amount	
			cc.				cc.	
1	2/18	Antipoliomyelitic horse unfiltered globulin	2	4 days	M.V.	Icer.	0.1	Typical poliomyelitis, 9 days
2	2/18	"	2	4 "	"	"	0.1	Mild poliomyelitis, 7 days
3	2/18	—	Control	—	"	"	0.1	Typical poliomyelitis, 10 days
4	3/24	Antipoliomyelitic horse unfiltered globulin	2	4 days	"	"	0.1	Typical poliomyelitis, 4 days
5	3/24	"	2	4 "	"	"	0.1	Typical poliomyelitis, 3 days
6	3/24	"	2	4 "	"	"	0.1	Typical poliomyelitis, 4 days

* Injected by lumbar puncture.

Macacus rhesus 2. The animal received exactly the same spinal injection of antipoliomyelitic globulin and intracerebral inoculation of fresh filtrate virus as did Monkey 1. 7 days after inoculation, the animal displayed mild symptoms of illness consisting of excitement, fine tremor of the head, and ruffled fur—characteristics of early experimental poliomyelitis. The condition failed to progress further and the animal proved resistant to subsequent inoculation.

Macacus rhesus 3. This test served as a control for the two previously de-

scribed. No globulin was injected, but the animal received the same intracerebral inoculation of active virus as did Monkeys 1 and 2. On the 10th day after inoculation, a marked tremor was present, with wild excitement, very marked ataxia, and weakness of the left arm. The following day, prostration had set in and the monkey was etherized. The lesions found were characteristic of poliomyelitis.

Experiment 6.—The next series of tests may be called therapeutic, in distinction from the immediately preceding tests, which may be called prophylactic. In the therapeutic series, intranasal instillation of the virus was employed for inoculation, and intraspinal injection of the globulin solutions for treatment. The series was controlled not only in the usual way, but with antidiphtheria globulin solution. This experiment can be given adequately in the form of a table (Table VII) which presents all the salient facts.

Comment.—This test is informing. The antipoliomyelitic horse globulin given intraspinally to three monkeys, 3 days after nasal instillation of virus, protected one animal completely, one animal doubtfully (excitement and ruffled fur for 2 days), and one animal not at all. Antidiphtheria globulin administered in the same manner seems to have protected both monkeys to which it was given, as neither developed symptoms of any kind. The precise interpretation of the experiment may be in doubt, and the tests call perhaps for repetition on a larger scale. The markedly inconstant protective effects of the antipoliomyelitic horse globulin are, however, in sharp contrast with the regular effects of convalescent sera (22) under similar conditions of experiment. They do not agree wholly with the reported statement of Weyer, Park, and Banzhaf (13) that the globulin solution injected intraspinally confers immunity of considerable duration against the virus instilled into the nares. We would suggest that the wide differences between Weyer and Park's experimental results and those we have secured are due, not to technical irregularities, but to the potency and certainty of pathogenic action of the strains of virus which were employed by us.

Experiment 7.—A final set of tests was carried out to determine the effect of Berkefeld filtration on the globulin solutions. The technique employed was the usual one of *in vitro* neutralization. The antipoliomyelitic horse globulin was found inactivating before filtration. Antidiphtheria horse globulin was employed for the control test. The results summarized in Table VIII are consistent—Berkefeld filtration deprived the globulin preparations of their inactivating properties.

TABLE VII

Experiment 6. Therapeutic Action of Antipoliomyelitic Horse and Antidiphtheria Globulin

No.	Date	Inoculation					Globulin			Result
		Interval before serum	Route	Virus	Total amount	Method of inoculation	Type	Amount	Route	
					cc.			*cc.*		
1	4/29	3 days	Nasal	M.V. 10% glycerolated	6	1 cc. each nostril daily for 3 days	Antipoliomyelitic horse filtered globulin	2	Spinal*	5/8. Excited
2	4/29	3 "	"	"	6	"	"	2	"	5/8. Typical poliomyelitis, 9 days
3	4/29	3 "	"	"	6	"	"	2	"	No symptoms
4	4/29	3 "	"	"	6	"	Diptheria globulin	2	"	5/9. No symptoms
5	4/29	3 "	"	"	6	"	"	2	"	"
6	4/29	—	"	"	6	"	Control	—	—	5/8. Typical poliomyelitis, 9 days

* Injected by lumbar puncture.

TABLE VIII

Experiment 7. Effect of Filtration

No.	Date	Material tested for neutralizing power	Amount	Virus amount filtrate	Globulin virus ratio	Result
			cc.	*cc.*		
1	4/22	Antipoliomyelitic horse globulin-filtered	0.06	0.4	1:6	Typical poliomyelitis, 5 days
2	4/22	"	0.06	0.1	1:2	"
3	4/22	"	0.24	0.4	1:6	Typical poliomyelitis, 7 days
4	4/22	Control diphtheria horse globulin	0.06	0.1	1:2	"

SUMMARY

Through the kindness of Dr. W. H. Park we have been enabled to study a horse antipoliomyelitic serum. This preparation has been supplied us in three forms: citrated blood plasma, serum, and globulin concentrate.

We have tested these preparations *in vitro* and *in vivo* for inactivating or neutralizing or, to use perhaps a better term, antiviral effects against a constant, potent, filtrate virus of poliomyelitis.

The preparations exhibited these effects when combined *in vitro*. Their action in this respect appears to be greater and more constant than that found by Stewart and Haselbauer for the Pettit antipoliomyelitic horse serum.

On the other hand, *in vivo* tests carried out by us were less successful. In comparison with the constancy of action, under given conditions, of convalescent monkey and human sera, the antipoliomyelitic horse serum displayed striking irregularity, and certain preparations were devoid of protective power.

The precise nature of the inactivating substances in the horse antiserum and their relation to the corresponding substances in convalescent sera have still to be determined. As far as one absorption test carried out by us indicates, precipitin does not play a major rôle in the inactivating process.

When an active globulin concentrate was filtered through Berkefeld candles, it lost its *in vitro* inactivating power. This is not true of convalescent sera in the native state. No tests have, however, been made with globulin concentrates from such sera.

The experiments described in this paper raise the question whether, therapeutically considered, the antipoliomyelitic horse serum should be regarded as an exact equivalent of, and hence employed as a perfect substitute for, convalescent serum. This question can only be answered by further experiment and observation.

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