INTRAPERITONEAL AND INTRACEREBRAL ROUTES IN SERUM PROTECTION TESTS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

I. A Comparison of the Two Routes in Protection Tests

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The methods most commonly used for the detection of antiviral bodies in the sera of animals immune to the virus of equine encephalomyelitis have consisted of the injection of serum-virus mixtures intracerebrally into mice or guinea pigs. In this way it has been found that the protective power of serum was always of a low order in spite of the fact that a solid resistance was displayed by laboratory animals when tested intracerebrally or by horses convalescent from the disease.

Reports of the demonstration of antiviral substance by intracerebral technique have already been discussed (1). Experiments indicated that guinea pigs later shown to be immune to intracerebral injection of 1,000 minimal infective doses had sera which protected mice only against 1 to 10 doses or not at all. Using a method which was not quantitative, Howitt (2) found that guinea pigs immune to an intracerebral test for immunity could be shown to have protective antibodies in their sera only irregularly. Furthermore, these antibodies disappeared more rapidly than the observed immunity. Horses which have recovered from natural infection are immune to subsequent attacks but the demonstration of protective antibodies in their sera has been difficult and has usually resulted in failure (3–6). TenBroeck and Merrill (7), however, determined that in guinea pig tests antiviral bodies were revealed when serum of convalescent horses was added to low multiples of minimal cerebral infective doses of virus. They have later resorted to another method of testing with more success, namely, guinea pig pad inoculation of serum-virus mixtures.[1]

In the study of certain other viruses, it has been shown that the demonstration of protective antibodies in the sera of immune animals depends to a large extent on the route by which serum-virus mixtures

[1] TenBroeck, C., personal communication.

are inoculated into test animals. Variations in the degree of protection afforded by antiserum in different sites in the same species of animal are of importance in studies of the mechanism of the immune reactions in certain virus diseases. This matter will be discussed later but the findings described in this paper provide, among other results,

TABLE I

Prior Observations in Which the Protective Power of Serum in a Serum-Virus Mixture Varied in the Same Host with the Route of Inoculation

Investigator	Virus used	Animal injected	Route of injection resulting in protection	Route of injection resulting in less or no protection
Manteufel (8)	Fowl pox	Chickens	Subcutaneous	On the comb
Todd (9)	" plague	"	Intramuscular	Intravenous
Hallauer (10)	" "	"	"	"
Andrewes (11)	Vaccinia	Rabbits	Intradermal	Intracerebral, intratesticular, intravenous
Craigie and Tulloch (12)	"	"	"	Intratesticular
Fairbrother (13)	"	"	"	Intracerebral
Sabin (14)	"	"	"	"
Goyal (15)	"	"	Intracerebral	Into the anterior chamber of the eye
Andrewes (11)	Virus III	"	Intradermal	Intratesticular, intravenous
Sabin (14, 16)	B virus	"	"	Intracerebral
Sabin (14)	Pseudorabies	Guinea pigs	Intranasal, subcutaneous	"
Sabin (14)	Herpes	Rabbits	Intradermal	"
Francis and Magill (17)	Rift Valley fever	Mice	Intraperitoneal	Intranasal
Findlay (18)	" "	"	"	"

still another example of such variation. Table I summarizes most of the earlier reports in which differences in protective power of serum depended on the route of inoculation.

The record indicates clearly that with one possible exception, the procedure of intracerebral testing yields poorer results for the demonstration of protective power of serum than other methods.

In experimental equine encephalomyelitis there were definite indications that young mice would be infected by intraperitoneal inoculation of virus and consequently that serum protection tests might be performed by this route.

Mice less than 14 days of age have been found to be susceptible to intraperitoneal inoculation of the Western strain of this virus (19), and more recently it has been shown (20) that this animal, at the age of from 12 to 15 days, is practically invariably susceptible to both Eastern and Western strains, and even with high dilutions of virus. Although experiments by Merrill (21) with mixtures of serum and virus were designed for a purpose other than that under investigation now, and were performed in a manner different from those usually planned for the demonstration of the titre of antibody content of a serum, they leave the impression that a greater degree of protection was afforded when the intraperitoneal route rather than the intracerebral was used for inoculation of mice. In the use of serum tests for an epidemiological study, TenBroeck, Hurst, and Traub (22) stated in a footnote to a table that while most of their tests were done by intracerebral injections, they have since found the intraperitoneal route more satisfactory. No further reference to this finding was made by them.

In view of the fact that the usual intracerebral test for detection of serum antibody yielded little or no antiviral substance in spite of a high degree of resistance to virus injection (1), the question arose as to whether the weak humoral antibody content was to be regarded as absolute or whether the antiviral substance was not readily detectable by means of this method. Furthermore, one of the studies under investigation concerned a comparison of infectivity by intracerebral and intraperitoneal routes simultaneously with relative effect of serum, since prior to the work of Sabin (14), it was thought that the reason it was more difficult to demonstrate antibody by a certain route was because that route was a more sensitive indicator for the presence of virus. The mechanism governing the variations found by the two routes in the relative protective power of immune sera will be discussed, however, in a forthcoming paper.

Methods and Materials

Virus.—The Eastern strain of the virus was used in most of the experiments but the Western strain was also tried. The strains were the same as those employed in previous work in this laboratory (23) and have been maintained by intracerebral passage in mice with storage in 50 per cent buffered glycerol. In

these experiments only fresh virus was used, that is, none but the brains of mice prostrate with the disease or recently succumbed to it. Such brains were frequently kept whole in the refrigerator for several hours but were always used the same day. They were ground with alundum and enough broth to make a 20 per cent suspension; for example, two mouse brains weighing 0.8 gm. were ground with 4 cc. of broth. After centrifugation of about 2,000 R.P.M. for 2 or 3 minutes to deposit the larger particles, serial tenfold dilutions in broth were made from the supernatant. Dilutions were then 2×10^{-1}, 2×10^{-2}, etc. A fresh pipette was used for each dilution.

Sera.—Hyperimmune rabbit serum was obtained from rabbits which had received subcutaneous injections of 10 per cent suspensions of infected mouse brains in doses of 2, 4, 5, and 11 cc. at intervals of 5 to 7 days. They were bled[2] 10 days after the last dose and then at 2 to 4 day intervals. All specimens were pooled. Hyperimmune guinea pig serum consisted of pooled sera. They were derived from guinea pigs immunized with mouse brain virus followed by a test for immunity and further subcutaneous doses of active mouse brain virus.

Hyperimmune mouse serum was obtained from old mice which received intraperitoneal or intramuscular injections of mouse brain virus followed by an intracerebral test for immunity. 13 days later they were bled from the heart and the survivors bled every day or so until all were dead. These specimens were all pooled.

The serum used with the Western strain of the virus was from a rabbit that was given 2.5 cc. of a 2 per cent suspension of mouse brain virus subcutaneously and 5 cc. of a 10 per cent suspension 4 months later. It was bled for serum 9 months after that.

Five horse sera[3] were from animals in areas in New Jersey in which equine encephalomyelitis occurs, but there was no history of disease or inoculations of serum or vaccines in any of them. Eight horse sera[3] were from animals in Virginia and they had either recovered from the disease or had been in contact with known cases. Most of these sera were passed through Seitz filters to insure sterility.

All sera were stored in the refrigerator without preservative.

Serum-Virus Mixtures.—0.5 cc. of the dilution of virus was added to 0.5 cc. of undiluted serum and mixture brought about by shaking. Thus a dilution of 2×10^{-6} of virus added to an equal amount of undiluted serum gave a final dilution of virus of 10^{-6}. Virus was mixed with normal serum first and then with immune serum and the lower dilutions of virus were added to the sera before the higher. The mixtures were injected without incubation except as noted.

Mice.—All of those used were of the Rockefeller Institute albino strain. Mix-

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[2] Operations on animals were performed with the aid of ether anesthesia.

[3] We wish to thank Dr. Carl TenBroeck and Dr. H. C. Givens for their cooperation and generosity in supplying these sera.

tures were injected intraperitoneally into infant mice. Their ages varied from 12 to 15 days and in one instance 16 days, but in the usual experiment all mice born on the same day were used. This particular age of mice was selected because studies of Sabin and Olitsky (20) indicated that some resistance to inoculation by the intraperitoneal route begins to appear even at 21 to 30 days. Regular results in this test depend upon taking into consideration the appearance of resistance at different ages in different mice. As will be noted, in some experiments mice born on 2 or 3 successive days but never more than 3 were employed. These young mice usually averaged in weight between 7 and 9 gm. but larger and smaller ones were encountered. Intracerebral injections were given to adult mice except as noted. Their ages are indicated. The dose by the intraperitoneal route was 0.1 cc. except as noted and the intracerebral dose was 0.03 cc. Intraperitoneal injections were made before intracerebral; immune serum-virus mixtures were injected before normal, and higher dilutions before lower. Both intraperitoneal and intracerebral inoculations of any particular dilution were made from the same tube.

Record and Estimation of Results.—The incubation period in the lower dilutions by either route was usually 2 days. Each day from then on, fewer mice developed the disease and by the 5th day practically all mice still living continued to live. Rarely one would die after that so that all animals were kept for 10 days after inoculation but most at least a week longer. Mice were considered to have developed encephalitis if they were found dead, completely prostrate, or in a state of generalized convulsions. Milder degrees of illness were observed further until such evidence developed. However, no mouse presenting definite signs of the disease has been observed by us to recover. Occasionally when there was doubt as to whether a mouse died of the disease, its brain was ground and injected into other mice to test for virus.

For convenience of designation, it was assumed that in the highest dilution in which more than half the number of the mice developed encephalitis one minimal infective dose of virus was present. In each test separate controls were included with normal serum for each route of inoculation, and results were considered only in comparison with them.

Relative Protection Obtained by Intracerebral and Intraperitoneal Methods

Hyperimmune Serum.—In the first series of experiments the relative protective power of hyperimmune sera derived from guinea pigs, mice, and rabbits was determined by the respective intraperitoneal and intracerebral injection of serum-virus mixtures. Previous intracerebral tests (1) had shown that hyperimmune serum had 10 to 100 times as much antibody, as a rule, as immune serum; that is, it protected against 10 to 100 minimal intracerebral doses of virus. The tests are summarized in Table II.

Examination of Table II shows that all of the hyperimmune sera protected against a very much larger number of minimal infective

TABLE II

*Relative Protective Power of Sera in Serum-Virus Mixtures Inoculated by Intraperitoneal and Intracerebral Routes**

Experiment No.	Strain of virus	Route of injection	Age of mice	Serum	Number of mice developing encephalitis of three injected									Minimal infective doses of virus against which the serum protected	
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
			days												
1	Eastern	ip	16	HGP	2	1	0	0	0	0	–	–	–	10,000	
		"	16	NGP	–	–	3	3	3	2	1	–	–	Control	
		ic	30±	HGP	–	3	3	3	2	1	0	–	–		100
		"	30±	NGP	–	–	–	–	–	3	3	3	1		Control
2	"	ip	14–15	HM	3	0	0	0	0	0	–	–	–	1,000,000	
		"	14–15	NM	–	–	–	–	2	3	3	2	–	Control	
		ic	30±	HM	–	–	–	3	2	0	0	0	–		1,000
		"	30±	NM	–	–	–	–	–	3	3	2	2		Control
3	"	ip	14	HR	2	0	0	0	–	–	–	–	–	100,000	
		"	14	NR	–	–	–	3	2	3	2	–	–	Control	
		ic	30±	HR	–	3	3	2	1	0	–	–	–		100
		"	30±	NR	–	–	–	–	–	–	2	1	0		Control
4	Western	ip	12	HR	3	1	0	0	–	–	–	–	–	10,000	
		"	12	NR	–	–	–	3	3	3	1	–	–	Control	
		ic	14–15	HR	–	–	–	3	2	0	0	0	–		10
		"	14–15	NR	–	–	–	–	–	3	3	1	0		Control

– indicates not tested; HGP, hyperimmune guinea pig serum; NGP, normal guinea pig serum; HM, hyperimmune mouse serum; NM, normal mouse serum; HR, hyperimmune rabbit serum; NR, normal rabbit serum; ip, intraperitoneal; ic, intracerebral.

* Most of the experiments have been done with adult mice for the intracerebral injections and infant mice for the intraperitoneal. The reason for this was that enough young mice for all could usually not be obtained on a single day. The test recorded in Table III shows, however, that the use of adult mice for the intracerebral tests did not account for the results obtained.

doses of virus when the serum-virus mixtures were given by the intraperitoneal route to infant mice than when given by the intra-

cerebral route. Thus the hyperimmune sera protected against 10 to 1,000 minimal cerebral doses as compared with 10,000 to 1,000,000 infective units by the intraperitoneal route. In other words, the protective power by the peritoneal route was from 100 to 1,000 times that by the cerebral. Furthermore, certain serum-virus mixtures which resulted in infection when injected intracerebrally were innocuous intraperitoneally.

As noted in Table I, Findlay (18) confirmed the finding of Francis and Magill (17) that mixtures of immune serum and Rift Valley fever virus produced infection when inoculated intranasally into mice but not when given by the intraperitoneal route. However, Findlay found that this difference between the intranasal and intraperitoneal routes depended on the amount of the inoculum.

Findlay used an intranasal dose of 0.03 cc. and an intraperitoneal dose of 0.4 cc. and found the mice inoculated with the latter remained well. However, when the intranasal dose was kept at 0.03 cc. and the intraperitoneal dose was also 0.03 cc., no difference between the protective power of the serum by the two routes could be detected.

Because of this, the question arose as to whether similar variation in the dose intraperitoneally and intracerebrally in our experiments could account for the difference in the protective power of the serum by the two routes. Accordingly, an experiment was planned to test this. It was done exactly as those in Table II, except that all the mice were 15 days old; intraperitoneal dose was 0.03 cc., and intracerebral dose was 0.03 cc. Table III shows the result.

This experiment indicated that the difference in the protective power of a serum when serum-virus mixtures were given by these two routes did not depend on the amount of the inoculum nor on the age of mice receiving intracerebral injections. In fact, in this series the peripheral inoculation yielded 10,000 times the protective power of the central. Another trial with mice of the same age with similar outcome appears in Experiment 4 (Table II).

The Value of Incubation of Serum-Virus Mixtures before Inoculation.—The purpose of the following experiments was to determine whether incubation had any effect on increasing the amount of virus against which the sera could protect when incubated mixtures were inoculated intraperitoneally. It was also necessary to know whether

incubation would eliminate the difference in protective power observed when unincubated mixtures were given by the two different routes.

There is an extensive record of attempts to disclose the influence of incubation on the action of antisera on viruses, although most workers agree that certain protective power can be secured without incubation being applied to serum-virus mixtures. Yet the question is important from the viewpoint of practical procedure since some viruses deteriorate at incubation temperature. Furthermore, if keeping mixtures at 37°C. could be shown to increase the action of the contained serum beyond the inactivating effect of that temperature on the virus, some evidence for an *in vitro* interaction between it and virus might be supposed to have taken place.

TABLE III

Inoculation of the Same Dose of Serum-Virus Mixtures by Intracerebral and Intraperitoneal Routes

Route of injection	Serum	Number of mice developing encephalitis of three injected									Minimal infective doses of virus against which the serum protected	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
ip	HR	3	0	0	0	0	—	—	—	—	100,000	
"	NR	—	—	—	—	3	3	2	1	—	Control	
ic	HR	—	—	3	3	2	0	—	—	—		10
"	NR	—	—	—	—	—	—	3	1	0		Control

Abbreviations as in Table II. Eastern strain of virus.

Employing a method that was not quantitative, and the virus of equine encephalomyelitis, Howitt (5) studied the effect of incubation for varying periods. The results, however, showed no effect. Cox and Olitsky (1) reported that with the same virus incubation of serum-virus mixtures for 2½ hours at 37°C. increased the number of intracerebral infective units against which a serum could protect. Finally, the work of Merrill (21) with this virus indicated some interaction *in vitro* between the infective agent and the immune serum. Table IV records the results of experiments on the effect of incubation.

The tests revealed that the protective capacity of the serum was not affected by the incubation of serum-virus mixtures when they were done in this way. As a corollary, it is plain that the difference in degree of protective power of unincubated mixtures exhibited by the two routes was not changed by keeping them at 37°C. for 2½ hours.

Sera from Normal Horses Derived from Epizootic Zones.—The results thus far described were obtained entirely with sera of hyperimmunized laboratory animals. Because of the striking difference in the two routes in the demonstration of protective antibody, it was now desired to determine whether the superiority of the intraperitoneal route applied to tests with horse sera and whether such procedures might be of value in epidemiological studies.

There were available for study the sera from five horses which came from districts in New Jersey where cases of equine encephalo-

TABLE IV

Effect of Incubation for 2½ Hours at 37°C. on the Protective Power of Serum When Serum-Virus Mixtures Were Given by the Intraperitoneal Route

Experiment No.	Age of mice	Incubation	Serum	Number of mice developing encephalitis of three injected									Minimal infective intraperitoneal doses of virus against which the serum protected
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
	days												
1	14–16	None	HR	2	1	0	—	—	—	—	—	—	100,000
			NR	—	—	—	—	—	3	2	0	0	Control
		2½ hrs.	HR	1	0	0	0	0	0	—	—	—	100,000
			NR	—	—	—	—	—	2	0	0	—	Control
2	14–16	None	HR	2	1	0	—	—	—	—	—	—	1,000,000
			NR	—	—	—	—	—	3	3	2	—	Control
		2½ hrs.	HR	2	0	0	—	—	—	—	—	—	1,000,000
			NR	—	—	—	—	3	3	2	3	—	Control

Abbreviations as in Table II. Eastern strain of virus.

myelitis have occurred. They had no clinical evidence of the disease and had not received any injections of virus, vaccines, or antiserum.

The five sera had been previously tested for antiviral substance by Dr. TenBroeck and his associates; three were found positive and two, Nos. 0815 and 0806, negative. Because of the possibility that protective capacity might be detected in the latter two by the use of the intraperitoneal technique, additional controls of broth, normal rabbit serum, or normal guinea pig serum were used. Table V shows the results of trials with these horse sera.

From Table V it will be noted that with broth or normal rabbit serum used as a control, serum 0815, previously designated as negative, protected against

10 to 100 minimal intraperitoneal infective doses and against 1 to 10 intracerebral units of virus. This result suggested that specific antibody might be present in small amounts. With normal guinea pig serum as a control, serum 0806, the other "negative" sample, protected against possibly one intraperitoneal or intra-

TABLE V

Protective Power of Horse Sera (New Jersey Series) When Serum-Virus Mixtures Were Inoculated by the Intraperitoneal and Intracerebral Routes

Experiment No.	Route of injection	Age of mice	Serum	Number of mice developing encephalitis of three injected									Minimal infective doses of virus against which the serum protected	
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
		days												
1	ip	14–15	0819	—	3	2	1	0	0	0	—	—	*	
	"	14–15	0815	—	—	—	—	2	1	1	0	—	Control?	
	"	14–15	Broth	—	—	—	—	3	3	2	1	—	Control	
	ic	25+	0819	—	—	—	2†	2	0	0	0	0		*
	"	25+	0815	—	—	—	—	—	2	2	0	0		Control?
2	ip	14–15	0692	3	2	0	1	0	—	—	—	—	*	
	"	14–15	0815	—	—	—	2	2	1	0	0	—	Control?	
	"	14–15	NR	—	—	—	—	3	2	2	2	—	Control	
	ic	21±	0692	—	—	—	3	3	3	1	—	—		*
	"	21±	0815	—	—	—	—	3	3	2	0	—		Control?
3	ip	15	0814	3	1	0	0	0	—	—	—	—	100,000 to 1,000,000	
	"	15	0806	—	—	2	2	3	2	2	0	—	Control	
	"	15	NGP	—	—	—	—	3	3	2	2	—	"	
	ic	23±	0814	—	—	—	3	3	1	0	—	—		10 to 100
	"	23±	0806	—	—	—	—	3	3	3	0	—		Control
	"	23±	NGP	—	—	—	—	—	3	3	2	1		"

Abbreviations as in Table II.

* Amount of virus against which serum protected is explained in the text.

† One died of the inoculation.

cerebral infective dose of virus. Hence serum 0806 was regarded as a more satisfactory control than serum 0815. The data of Table V were therefore evaluated on the basis of serum 0806, broth, normal rabbit and guinea pig sera as controls.

Viewed in this way, the results showed that sera 0819, 0692, and 0814, previously called positive by Dr. TenBroeck, contained protective antibodies, and 0814 protected against a larger number of infective doses of virus when given intraperitoneally than intracerebrally. The latter sample rendered from 10 to 100 units of virus non-infective by the intracerebral test and 100,000 to 1,000,000 by the intraperitoneal method.

The supply of serum 0806 was soon exhausted; a horse serum was therefore sought which showed no protective power by this intraperitoneal technique for use as control in further experiments.

Horse M 33 had been immunized with meningococci and bled for serum on Oct. 7, 1919. This serum was sealed and stored in the refrigerator in this laboratory until Feb. 7, 1938. On the latter date a portion of it was passed through a Seitz filter. An electrometric determination of pH was 7.8 and cultures yielded no growth of bacteria so that it was believed not to have essentially deteriorated.

The serum M 33 was then tested in comparison with normal guinea pig serum as recorded in Experiment 1 of Table VI. The outcome was a difference in titre of only one minimal intraperitoneal infective dose (as with serum 0806) and since this was not significant in respect to the number of animals employed with each dilution, it was decided to use M 33 as a control for further tests with horse sera.

Sera from Horses Recovered from, or Exposed by Contact to Equine Encephalomyelitis.—The next series of tests were performed on sera obtained from four horses that had shown clinical signs and recovered from equine encephalomyelitis, and four others known to have been in contact with one to four horses having signs of the malady. All the animals were from epizootic areas in Virginia and the sera were collected from 6 months to 4 years after recovery from or contact with the disease. Six separate experiments were undertaken and these are recorded in Table VI.

In Table VI it can be seen that sera 1 and 5 protected against a larger amount of virus intraperitoneally than intracerebrally; in the instance of serum 1, 100,000 times as much. Experiments 3, 4, and 5 were not planned to determine the amount of virus against which a serum protected but to show whether the existence of antibody could be detected with a set dose, so as to give a practical aspect to the intraperitoneal test (Experiment 4). In this way, every one of the sera of horses known to have recovered from equine encephalomyelitis showed

TABLE VI

Protective Power of Horse Sera with Horse Serum M 33 as Control

Experiment No.	Route of injection	Age of mice	Serum	Clinical history of horse	Number of mice developing encephalitis of three injected									Minimal infective doses of virus against which the serum protected	
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
		days													
1	ip	15	M 33*	See text	—	—	—	3	3	3	1	0	—	1	
	"	15	NGP	Control	—	—	—	—	1	3	2	0	—	Control	
2	"	13	1*	Recovered Sept., 1933	3	1	0	0	—	—	—	—	—	100,000	
	"	13	M 33*	Control	—	—	—	—	3	3	2	0	—	Control	
	ic	20	1*	Recovered Sept., 1933	—	—	—	3	3	2	0	0	—		1
	"	20	M 33*	Control	—	—	—	—	—	3	3	1	0		Control
3	ip	12–13	2	Exposed to No. 1 Sept., 1933	—	—	—	4†	4†	4†	—	—	—	0	
	"	12–13	3*	Recovered Sept., 1934	—	—	—	0†	0†	0†	—	—	—	At least 100	
	"	12–13	4	Recovered Sept., 1934	—	—	—	0†	0†	0†	—	—	—	At least 100	
	"	12	M 33*	Control	—	—	—	4†	4†	4†	—	—	—	Control	
4 (use of single test dose)	"	12–13	5*	Exposed to No. 4 Sept., 1934	—	—	—	—	2†	—	—	—	—	?	
	"	12–13	6*	Recovered Aug., 1937	—	—	—	—	0†	—	—	—	—	At least 100	
	"	12–13	8*	Exposed to 4 sick horses Aug., 1937	—	—	—	—	4†	—	—	—	—	?	
	"	12–13	9*	Exposed to 2 sick horses Aug., 1937	—	—	—	—	3†	—	—	—	—	?	
	"	12–13	M 33*	Control	—	—	—	—	—	3	2	1	—	Control	

5	"	12	0815	See Table V	—	—	—	—	3	3	1	—	—	10	
	"	12	5*	Exposed to No. 4 Sept., 1934	—	—	—	3	0	0	0	—	—	1,000	
	"	12	8*	Exposed to 4 sick horses Aug., 1937	—	—	—	—	3	3	3	—	—	Less than 10	
	"	12	9*	Exposed to 2 sick horses Aug., 1937	—	—	—	—	3	3	2	—	—	Less than 10	
	"	12	M 33*	Control	—	—	—	—	—	3	3	2	—	Control	
6	"	12	5*	Exposed to No. 4 Sept., 1934	—	—	3	3	1	1	—	—	—	100	
	"	12	M 33*	Control	—	—	—	—	—	3	3	1	—	Control	
	ic	Adult	5*	Exposed to No. 4 Sept., 1934	—	—	—	—	1	2	0	0	—		1
	"		M 33*	Control	—	—	—	—	—	3	3	0	0		Control

* Filtered through a Seitz filter.

† Four mice injected.

power to protect against the virus. Furthermore, the serum of one horse which did not have any illness that was recognized but had been in contact with the disease also gave evidence of specific antibody.

The results of the tests with horse sera show the intraperitoneal route to be more sensitive than the intracerebral (0814, 1, and 5) and also that the intraperitoneal technique would probably be a valuable tool for epidemiological studies since all of the sera of horses known to have had the disease gave strongly positive reactions (1, 3, 4, and 6) and sera of others (four of nine) not known to have shown clinical signs but which have been in contact with the disease also contained measurable, definite antibody (0819, 0692, 0814, and 5).

DISCUSSION

The method ordinarily employed heretofore for the recognition and measurement of humoral antibody in equine encephalomyelitis has consisted of the injection of serum-virus mixtures into the brains of mice. The present experiments show that the intraperitoneal route is more sensitive for this purpose. The basis for this is to be found in the uniform susceptibility of 12 to 15 day old mice to the intraperitoneal injection of the virus (20). In most instances there is only a tenfold or no difference between intracerebral and intraperitoneal titers; 12 to 15 day old mice are approximately equally susceptible to inoculation by the two routes.

The intraperitoneal procedure has been shown to be applicable not only to the sera of laboratory animals immunized with active virus but also to the sera of horses naturally infected, or of those exposed by contact to the disease. It should be of value not only because of its ability to detect antibody to a much higher degree than the intracerebral method, but also, in view of the sensitiveness of the test, because of its capacity to indicate negative findings with greater assurance that antibody is not at all present.

The results of the application of this test to horse sera do not permit general conclusions because of the small number of specimens examined. Nevertheless, they furnish some indication that horses recovered from the disease have serum antibodies regularly and that these may persist for at least 4 years. In addition, antibodies may be found in the sera of horses that have shown no signs of the disease but that

live on farms where the infection has been prevalent, while others from such farms may be negative. That the sera of horses not having clinically apparent disease may contain antiviral substance has already been found by TenBroeck, Hurst, and Traub (22) and confirmed by Giltner and Shahan (6). A more extensive investigation on larger numbers of animals exposed by contact is necessary before one can say whether the intraperitoneal method can disclose a higher percentage of positive reactions for antibody than the intracerebral or other methods.

In prior reports in which animals were described as solidly immune to equine encephalomyelitis, it has been stated that this immunity was associated with a minimal amount of protective antibody in the serum. The present experiments show that perhaps the discrepancy between the amounts of immunity and antibody can be explained by the demonstration of large amounts of antibody by the method of intraperitoneal test.

Certain aspects of the mechanism underlying the phenomenon of the superiority of antibody detection by the intraperitoneal test will be discussed in a forthcoming paper. For the present, some remarks may be made with regard to the reaction of immune serum and virus *in vitro*.

It has been mentioned that the work of Merrill (21) indicated some kind of interaction *in vitro* between this virus and serum. He concluded from his experiments that combination between virus and antibody had occurred *in vitro* probably resulting in aggregation of virus particles. It should be stated that our experiments do not give evidence as to whether there is combination in the test tube. They do demonstrate, however, that in the dilutions which show protection by the intraperitoneal route and not by the intracerebral, the infectious activity has not been abolished *in vitro* by the immune serum; in other words, that the immune serum is not directly virucidal by the intraperitoneal route. This is evident from the fact that material taken from a given tube may not give rise to infection if injected intraperitoneally but will if inoculated intracerebrally. If antibody has combined with virus in such tubes, the combination must be dissociable when in contact with certain tissues, inasmuch as protection may or may not occur, depending on the tissue into which the serum-virus mixture is injected. That variation in protective power of

antiviral serum according to route of inoculation indicates that the consummation of the immune reaction is not based on direct inactivating effect, has been suggested before by several workers among whom may be mentioned Andrewes (11), Sabin (14), and Francis and Magill (17).

Finally to be stressed in this discussion is the point that the behavior of serum-virus mixtures, when injected by different routes, is not the result of the greater capacity of one route to detect unneutralized virus, a fact first demonstrated by similar quantitative, comparative titrations for vaccinia, herpes, B virus, and pseudorabies viruses (Sabin, 14).

SUMMARY AND CONCLUSIONS

Young (12 to 15 day old) mice are approximately as susceptible to the virus of equine encephalomyelitis, Eastern or Western strain, when it is given intraperitoneally as are adult mice when the virus is injected intracerebrally. With this susceptibility by the intraperitoneal route as a basis, the injection of immune serum-virus mixtures intraperitoneally was found to result in protection in dilutions which give rise to infection after intracerebral inoculation.

The difference of protective power by the two indicated routes was shown not to depend on the amount of inoculum nor on the age of the intracerebrally injected mice. Incubation at 37°C. for $2\frac{1}{2}$ hours neither increases nor diminishes the protective action of immune serum when the intraperitoneal method is employed.

The phenomenon of selective protection in different tissues is elicited by the sera of hyperimmunized mice, guinea pigs, and rabbits and by sera derived from horses infected with the disease in nature or exposed to it by contact. Of four horses recovered from the malady, all showed antibody in their sera; of others exposed by contact, four of nine animals revealed antiviral bodies, when the intraperitoneal technique was employed. These tests on horse sera have pointed to the potential value of this procedure for epidemiological studies.

Finally, the reaction itself has significance through its bearing on the mechanism of immunity.

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