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

II. Mechanism Underlying the Difference in Protective Power by the Two Routes

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In the first paper of this series (1) it was shown that when mixtures of the virus of equine encephalomyelitis, Eastern or Western strain, and its antiserum are inoculated intraperitoneally in 12 to 15 day old mice, protection is obtained against much larger amounts of virus than when the mixtures are given intracerebrally. That is, with the same material, 1 to 1,000 cerebral infective doses of virus are neutralized by the intracerebral method as compared with 10,000 to 1,000,000 peritoneal doses by the intraperitoneal.

The purpose of the present study was to determine the mechanism underlying the more potent action exerted by the antiviral serum by one route than by another. At the outset it should be mentioned that it has not proved possible thus far to elucidate this completely; the experimental results have served to eliminate, however, divers theories hitherto offered to account for the phenomenon, to delimit more closely the place where the immune mechanism may be consummated and, what is more important, to correlate the mechanism of immunity with the pathways taken by the virus from the point of inoculation at various sites to the central nervous system.

In the first communication (1), mention was made of the work with other viruses in which it was shown that the protective power of serum-virus mixtures depends to a large extent on the route of inoculation. A summary follows of some of the interpretations of this reaction presented by earlier investigators, together with the bearing of our previous work on the question as it applies to equine encephalomyelitis in the mouse.

Thus Craigie and Tulloch (2) considered the possibility that differences in protection according to route might be explained, in the case of vaccine virus and its antiserum, on the ground of greater susceptibility of one organ (*e.g.*, testis) to the virus than another (skin). On the other hand, Sabin (3) studied the problem of greater sensitivity of certain tissues to the action of vaccinia, herpes, B virus, and pseudorabies viruses, to note whether smaller amounts of these infective agents could be detected that way and hence more serum required for protection. With strictly quantitative methods in which the minimal infective doses were the same by two different routes, and by varying the amounts of serum and virus, Sabin found that the difference in protective capacity of mixtures could not be ascribed to the fact that one route may be more sensitive than another in detecting small amounts of virus. The results with equine encephalomyelitis virus, as already reported (1), confirmed the latter findings. With this virus, the lesser degree of protection by a given route (intracerebral) did not depend on the greater sensitivity of that tissue for detection of the infective agent. It was shown that one minimal infective dose of virus by the intraperitoneal route in 12 to 15 day old mice was approximately the same as by the intracerebral, yet the same amount of serum protected against many more doses by the former than by the latter route of inoculation.

Andrewes (4) obtained greater protection with antivaccinial serum by intradermal inoculation than by other routes and he suggested that this might be due to less ready diffusion of antibody in that site. Again the experiments of Sabin (3) indicated that this might not be the explanation: when the virus was used as a suspension of testicular tissue, marked diffusion took place from the action of the Duran-Reynals spreading factor of testicular tissue but the protective effect of the serum was, nevertheless, apparent.

That the difference in protective power depends on unknown factors peculiar to the tissue itself was indicated by the phenomenon described by Shope (5) in which mixtures of immune serum and pseudorabies virus which are innocuous subcutaneously in the guinea pig, produced fatal infection when given by the same route to the rabbit. This problem was also studied quantitatively by Sabin (3) and it was disclosed that the varying protective capacity of anti-pseudorabies serum in rabbits and guinea pigs was not due to the greater sensitivity of the subcutaneous tissue of rabbits in revealing smaller amounts of virus but rather to other conditions in this tissue of both species that may be only indirectly related to their susceptibility. Thus the results depended more on species involved than on route of inoculation. In this connection the investigator also studied the possibility of pseudorabies virus being fixed or entering the susceptible cells more rapidly or in greater quantity than the immune serum when the two are injected subcutaneously in rabbits. One must restrict oneself to measured quantities of materials in such trials: when such quantitative relationships were brought into consideration (3), and when subeffective amounts of serum were injected at intervals before virus in the same cutaneous sites, the writer concluded that no protection was gained. It is therefore probable that the poor protection by this route did not depend on the more rapid fixation of the virus by the dermal cells.

Findlay (6) investigated anew the finding of Francis and Magill (7) that antiserum of the virus of Rift Valley fever protected against more virus when the serum-virus mixtures were given mice intraperitoneally rather than intranasally. Findlay pointed out that in this instance the difference in protective capacity depended on the amount of inoculum, for when the dose was equal no variation occurred. In the preceding paper (1), it was shown, however, that with equine encephalomyelitis virus, variation in the protective capacity of antiserum by intraperitoneal and intracerebral methods of injections persisted when the amount of inoculum given was the same for both routes. In addition, the variation was still evident not only when the ages of the mice employed in the two methods were equal but also when the serum-virus mixtures were administered either freshly prepared or incubated for 2½ hours at 37°C.

It was brought out previously (1) and this is in agreement with the work of Sabin on other viruses (3), that the variation in protective power by the two routes, intracerebral and intraperitoneal, was in itself evidence that the action of the antiserum against equine encephalomyelitis virus was not an *in vitro* effect but was contingent upon the tissues into which it was injected.

The foregoing summary brings to light the fact that while the precise mechanism underlying the variation in the protective potency of antiserum-virus mixtures, when administered by different routes, is still unknown, certain theories advanced to explain this variation are not consistent with experimental data subsequently obtained. The virus now being studied was found to resemble in a general way several other viruses in that the effect of its antiserum is not consummated *in vitro* and that the variation in protective capacity is not primarily the result of the dose of inoculum or incubation of serum-virus mixtures but is influenced by the tissues into which such mixtures are injected (1). In this latter connection, no definite proof could be offered that the variation is dependent on the greater sensitivity of the tissues of one route to detect small amounts of virus over that of another.

For the purposes of the present investigation, namely, the elucidation of the mechanism involved in the variation of protective capacity by two routes of inoculation, the approach to the problem consisted of inoculation of serum intraperitoneally followed by the injection of virus by various routes. The results were then correlated with the pathways known to be taken by the virus according to the route of inoculation. In so doing, use was made of the knowledge at hand of the pathogenesis of the Eastern strain of the virus, that is, of the pathways pursued by it after its inoculation into mice at various sites.

Sabin (8) and Sabin and Olitsky (9, 10) have employed a method of partial serial tissue section of mouse (and guinea pig) central nervous system combined with tests for detection of virus by inoculation of animals, and have been able thereby to indicate the localization of lesions and virus, thus delineating the probable pathways of the infective agent from the periphery to the central nervous system. After intranasal instillation of Eastern equine encephalomyelitis virus into young or old mice, the central nervous system is invaded along the olfactory pathway. After intraperitoneal or intramuscular injection of this virus into 15 day old mice, it becomes demonstrable in the blood, and in the greater number of the animals, it migrates from the blood onto the nasal mucosa, whence it invades the central nervous system by the olfactory pathway. In some of these mice, however, invasion of the central nervous system occurs along the local peripheral nerves or along the auditory nerve pathway and possibly along the seventh nerve fibers. It has also been shown that while the virus enters and persists or multiplies in the circulating blood, no evidence was found of a direct passage of virus across the blood vessels of the brain (8–10).

Methods

These were essentially the same as those previously employed (1). The serum studied was the hyperimmune rabbit serum—the same sample was used throughout this work; the virus, the Eastern strain of equine encephalomyelitis, and the mice, the Rockefeller Institute albino strain of 12 to 15 days of age (unless otherwise mentioned). The mode of procedure, preparation of materials, and dosages were described in the first paper (1) and need not be repeated here.

Experiments on Passive Immunity

It was planned to inject mice with hyperimmune serum intraperitoneally in varying amounts and to follow this at certain intervals by administration of virus in different sites,—brain, nose, peritoneal cavity, and leg muscles. In other tests antiserum-virus mixtures were inoculated into the tissues mentioned; the results of the latter trials would serve as a check on those in which the serum was injected in advance of the virus. It was believed that by these means the virus could be placed either in the brain itself or in peripheral sites from which regions it would invade the central nervous system *via* the pathways already designated; the virus and tissues would then be under the influence of antiviral serum given either along with or before the infective agent. From the outcome of such experiments indications might be derived as to the relationship of pathways traversed to the effectiveness of serum neutralization.

Passive immunization experiments with this virus have already been reported. Howitt (11) introduced serum into the muscles of guinea pigs and found them resistant to intracerebral inoculation of virus; also Rottgardt and Riglos (12) gave serum intraperitoneally to guinea pigs and found them refractory either to an intracerebral or to a combined intranasal and subcutaneous test dose. These experiments were not performed on a quantitative basis, thus making it impossible or difficult to apply these data to the elucidation of the problem under consideration here.

In the following experiment an effort was made to determine quantitatively the protective capacity of a certain amount of serum, given in the peritoneal cavity, against virus introduced directly into the brain.

Serum Given Intraperitoneally, Virus Intracerebrally.[1]—In the first test 15 day old mice were injected intraperitoneally with the rabbit hyperimmune serum in the amounts indicated in Table I, and Eastern equine encephalomyelitis virus intracerebrally in varying doses and at the intervals as noted in the table in Experiments 1 to 3.

The results recorded indicate that even with as much as 2 cc. of hyperimmune serum given intraperitoneally—the largest amount that can be given safely in this way to 15 day old mice—either very little protection or only a questionable one could be secured against virus introduced intracerebrally 24 hours later. The result is similar to that achieved when serum-virus mixtures were used (1). There is striking contrast between this effect and that resulting from the inoculation of 1/133 of this amount (0.015 cc.) of serum mixed with virus and administered intraperitoneally whereby protection was afforded against 10,000 to 1,000,000 infective doses (1). From these tests it is apparent that a low degree of protection, or none at all, results when the virus is given into the brain which is the main seat of viral attack. The next step was to disclose any difference in reaction when the virus was inoculated peripherally rather than centrally; the nasal route was the first to be tried.

Serum Given Intraperitoneally, Virus Intranasally.—Sabin (13) has already shown that the intraperitoneal injection of 1 cc. of immune serum, or the equivalent of 50 cc. per kilo, given 4 hours before infection, protected mice against a lethal amount of this virus instilled

[1] All such operations were performed with the aid of ether anesthesia.

TABLE I

Passive Immunity to Virus Given by Intracerebral, Intranasal, Intraperitoneal, and Intramuscular Routes

Experiment No.	Amount of serum intraperitoneally		Interval between serum and test with virus	Route of injection of virus	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which there was protection			
	Immune	Normal			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intracerebral doses	Intranasal doses	Intramuscular doses	Intraperitoneal doses
	cc.	*cc.*	*hrs.*														
1	0.05		24	ic	—	—	3	—	—	3	—	—	—	10+			
	2.0		24	"	—	—	3	—	—	3	—	—	—	10+			
				"	—	—	—	—	—	—	3	3	0	Control			
2	2.0		24	"	—	—	—	—	2*	3	2*	—	—	10+			
				"	—	—	—	—	—	—	3	3	3	Control			
3	2.0		24	"	—	—	—	—	—	4†	3†	2†	—	1+			
				"	—	—	—	—	—	—	3	3	2	Control			
4	0.015		4	in	—	3	3	3	2	—	—	—	—		0		
		0.015	4	"	—	3	3	3	0	—	—	—	—		Control		
	0.015		4	ic	—	—	—	—	—	3	2	0	0	1			
		0.015	4	"	—	—	—	—	—	—	3	3	0	Control			
5	0.015		4	im	—	—	—	0	0	0	0	0	—			10,000 or more	
		0.015	4	"	—	—	—	—	2	3	3	3	—			Control	
	0.015		4	ip	—	0	0	0	0	0	0	—	—				100,000 or more
		0.015	4	"	—	—	—	—	3	3	3	0	—				Control

Dose of virus dilution by any route was the same, namely, 0.03 cc.

ip, indicates intraperitoneal; ic, intracerebral; in, intranasal; im, intramuscular; —, no test made.

\+ Undetermined but equal to or less than the amount indicated.

* One died of the inoculation of serum.

† Four mice inoculated.

intranasally. It was now desirable to know whether the small amount of serum used in our experiments for demonstration of protective antibody by the intraperitoneal route (0.015 cc.) might have the same or a different effect. As a control, and furthermore to check the results obtained in the previous passive protection test in which the intracerebral route for inoculation of virus and large amounts of serum were used, this latter test was repeated with the small amount of antiserum.

For this purpose, 15 day old mice received rabbit serum intraperitoneally—one group, normal serum and the other, hyperimmune—in a dose of 0.015 cc. each. The sera were diluted 1:1 in saline solution so as to make for greater accuracy in measurement of the amounts needed as inocula. 4 hours later they were given either intranasal or intracerebral test doses of various dilutions of virus, the dose by both routes being 0.03 cc. The results are shown in Experiment 4, Table I.

The data reveal that in the mice receiving virus peripherally, no protective influence of the serum could be discerned. As was to be expected, the control group of animals having been given virus centrally, again showed a low degree of protection, that is, against only one infective unit. A similar outcome was found on repetition, as recorded in Table II. It would appear, therefore, that by intranasal and intracerebral methods the immune serum exhibited in both instances the same low or ineffective neutralization.

The results are now reported of passive immunity tests in which virus was given by other peripheral routes, namely, the intramuscular and intraperitoneal.

Serum Given Intraperitoneally, Virus Intraperitoneally or Intramuscularly.—The plan of this experiment was to introduce immune serum intraperitoneally and to follow it later by the simultaneous injection of virus, intramuscularly in one group of 15 day old mice and intraperitoneally in another.

As will be noted in Experiment 5, Table I, hyperimmune rabbit serum was given intraperitoneally in doses of 0.015 cc. (as in Experiment 4) followed 4 hours later by an intramuscular inoculation (muscles of a posterior extremity) of 0.03 cc. of each dilution of virus. (A preliminary titration of virus activity by this route exhibited the limiting titer of infectivity in the 10^{-8} dilution.) Intraperitoneal inoculations were made at the same time in another group of serum-treated 15 day old mice, employing the same amount of inoculum, 0.03 cc.

The results reveal that the immunity to intramuscularly or intraperitoneally injected virus after serum has been given intraperitoneally is of a remarkably high degree. The serum induced protection against at least 10,000 minimal intramuscular infective doses introduced into the muscles, and at least 100,000 peritoneal units inoculated into the peritoneal cavity. (A still higher degree of resistance to intramuscularly introduced virus is shown in Table II.) It is therefore clear that on the basis of effective protection as revealed by the methods employed, the administrations of virus by the intramuscular and

TABLE II

Passive Immunity to Virus Introduced Intracerebrally, Intranasally, and Intramuscularly

Amount of serum		Interval between serum and test with virus	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which there was protection		
Immune	Normal		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intracerebral doses	Intranasal doses	Intramuscular doses
cc.	*cc.*	*hrs.*												
0.015		5*	—	—	—	—	3	3	1	0	0	1		
	0.015	5*	—	—	—	—	—	—	3	1	0	Control		
0.015		4½*	—	—	3	3	0	0	—	—	—		1	
	0.015	4½*	—	—	3	2	2	1	—	—	—		Control	
0.015		4	1	0	0	0	—	—	—	—	—			10,000,000
	0.015	4	—	—	—	—	—	3	3	2	0			Control

Abbreviations as in Table I.

Mice were a mixture of the ages of 12, 13, and 14 days.

* Intervals due to the time used in inoculation.

intraperitoneal routes (peripheral) align themselves in one class of high protective capacity, the nasal (peripheral) and the cerebral (central) in another of low protective power. However, all the experiments on which this classification is based were not performed at the same time; this factor was therefore taken into consideration in the next test in which was studied the simultaneous use of intracerebral, intranasal, and intramuscular methods.

Comparison of Results with Serum Given Intraperitoneally and Virus Intracerebrally, Intranasally, and Intramuscularly.—The procedures followed in this experiment are outlined in Table II. From the

results it is apparent that again hyperimmune serum injected intraperitoneally yielded protection against only one cerebral or nasal infective unit when virus was administered intracerebrally or intranasally, and against as many as 10,000,000 intramuscular units when the virus was inoculated intramuscularly. The remarkable fact brought to light is that such a relatively minute quantity of antiserum (0.015 cc.) can be capable of exerting so high a degree of protective effect, even though the serum is introduced into the abdominal cavity and the virus into the muscle. Another interesting observation is revealed in these experiments as well as in those recorded in Table III. Less virus is required to induce encephalitis after its intramuscular rather than intranasal introduction. This may be taken as evidence that the virus may multiply before arriving at the central nervous system. The larger amounts of virus needed to produce encephalitis after its intranasal instillation may be explained by the fact that a great deal of it is washed away. It should be stressed also that the intramuscular minimal infective dose of virus is the same as the intracerebral and often the same as the intraperitoneal.

Up to this point experiments were made on the basis of passive immunity with the antiserum given prior to the virus. The results, however, are in accord with those already reported (1), which were derived from tests with serum-virus mixtures injected intracerebrally and intraperitoneally. In the following a comparison was made of the effects secured from the inoculation of serum-virus mixtures intranasally as an example of a route by which only low protective potency is demonstrable, and intramuscularly, where high protective capacity is discerned.

Comparison of Infectivity of Serum-Virus Mixtures by Intranasal and Intramuscular Routes

Serum-virus mixtures, without incubation, were inoculated intramuscularly or intranasally into groups of mice 14 or 15 days old. The amount of inoculum by both routes was 0.03 cc. prepared as previously described (1) and contained 0.015 cc. of serum. The results are given in Table III.

The outcome of this experiment is plain: the antiserum in the mixtures protected against 10 infective nasal units of virus by the intranasal, as against 1,000,000 intramuscular doses by the intramuscular route. There is very little difference in effect when immune

serum is mixed with virus just before animal inoculation or when the serum is given separately and prior to the virus. Here again occurs the wide variation in protective capacity elicited by two routes of inoculation.

The data can be related to the pathways traversed by the virus after inoculation by these various routes. With the intranasal and intracerebral methods, the virus enters nervous tissue immediately since in the former it progresses along the olfactory pathway to the brain, and in the latter it is placed within the brain itself, the cells of which it attacks directly. But after inoculation into the peritoneal cavity or into the muscles, virus circulates in the blood and is demon-

TABLE III

Comparison of Infectivity by Intranasal and Intramuscular Routes

Route of inoculation of serum-virus mixtures	Serum	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which there was protection	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intranasal doses	Intramuscular doses
in	HR	—	1	3	0	0	—	—	—	—	10	
"	NR	—	—	3	3	2	—	—	—	—	Control	
im	HR	1	0	0	0	—	—	—	—	—		1,000,000
"	NR	—	—	—	—	—	—	3	1	0		Control

Abbreviations as in Table I.

HR, hyperimmune rabbit serum; NR, normal rabbit serum.

strable there (9, 10). Thus it appears that the large amounts of protection may in some way be related to an action occurring at the peripheral site or during the passage of the virus through the blood on its way to the central nervous system. Attention was then turned to a study of the relation of the circulating blood to the mechanism of the immune reaction.

Virus in Blood Following Intraperitoneal Injections of Serum-Virus Mixtures

It has been shown (9, 10) that virus may be detected in the blood of young mice inoculated intraperitoneally or intramuscularly. In a preliminary test it was found that virus could be recovered from

the blood at 1, 2, 4, and 24 hours (longer periods of time were not studied) after intraperitoneal introduction of virus, which is in general agreement with the prior observations. The plan of the following tests was to inject serum-virus mixtures by this route and to observe whether virus could be detected later in the blood stream.

Mixtures of 10^{-3} dilution of virus (1,000 cerebral infective units) and hyperimmune rabbit serum were injected intraperitoneally (dose 0.03 cc.) in a group of 14 day old mice. At the same time 10^{-6}, 10^{-7}, and 10^{-8} dilutions of virus mixed with normal serum were similarly inoculated into another group. 24 hours later subgroups of each were bled to death by cardiac puncture by means of syringes washed with sterile 1:500 solution of heparin. The blood secured was directly transferred to other mice intracerebrally (dose 0.03 cc.). The remainder of the animals which were not bled were observed for signs of infection with the virus, as controls. The results are summarized in Table IV.

It is clear from Table IV that by the methods used, virus was detected in the blood 24 hours after intraperitoneal injection of normal serum-virus mixtures but not in the animals receiving the immune serum.

The work of Smith (14), Long and Olitsky (15), and Sabin (16) has shown that after intravenous injection of vaccine virus into normal rabbits, the virus can be demonstrated in the whole blood. However, following similar injections of immune serum-virus mixtures, or of virus into immune rabbits, the infective agent can be recovered from leucocytes but not from whole blood. An attempt was therefore made to search for virus in the leucocyte suspensions in mice given normal and immune serum-virus mixtures intraperitoneally.

Groups of mice were inoculated intraperitoneally with normal and immune serum-virus mixtures as in the last experiment. The same samples of serum were used; dilution of virus was 10^{-3}; dose, 0.03 cc. Mice were 14 and 15 days old. 24 hours later they were bled from the heart as before and the heparinized blood from each of the two groups of animals was pooled. The washed buffy coat of the centrifuged blood was secured and injections of these leucocyte-containing suspensions were made into adult mice in groups of six. The result was that all the mice receiving leucocyte suspensions derived from mice given normal serum-virus mixtures died and all those injected with immune-serum preparations survived.

By means of the methods here employed virus was found to be present constantly in the whole blood or in its leucocyte layer after

normal serum-virus mixtures were given intraperitoneally. By the same methods, no virus was apparently detectable in these materials

TABLE IV

Test for the Presence of Virus in the Whole Blood of Mice 24 Hours after Intraperitoneal Inoculation with Normal and Immune-Serum-Virus Mixtures, Respectively

Mouse No.	Dilution of virus in mixture given intraperitoneally	Serum	Fate of ones not bled	Results of test for virus in the whole blood*
1	10^{-3}	HR		0/3
2	10^{-3}	"		0/3
3	10^{-3}	"		0/3
4	10^{-3}	"		†
5	10^{-3}	"	S	
6	10^{-3}	"	"	
7	10^{-3}	NR		3/3
8	10^{-3}	"		3/3
9	10^{-3}	"		3/3
10	10^{-3}	"	E	
11	10^{-3}	"	"	
12	10^{-3}	"	"	
13	10^{-6}	"	"	
14	10^{-6}	"	"	
15	10^{-6}	"	"	
16	10^{-7}	"	"	
17	10^{-7}	"	"	
18	10^{-7}	"	"	
19	10^{-8}	"	"	
20	10^{-8}	"	S	
21	10^{-8}	"	"	

S, survived; E, encephalitis and death.

Other abbreviations as in Tables I and III.

* Numerator indicates the number of mice developing encephalitis; denominator indicates the number injected.

† No blood obtained.

after immune serum-virus preparations were similarly introduced into mice.

In Vitro Effect of Normal Mouse Blood on Serum-Virus Mixtures.—While these tests for virus in the blood could not be considered conclusive, they were taken as evidence that in some manner the immune

serum prevents the circulation of virus in the blood. The precise mechanism by which this is done is not apparent. Although it is known that serum does not have an inactivating effect in the test tube, there was a possibility that the whole blood of the normal animal might enhance the action of antiviral bodies. The following experiment was performed to test this point.

Hyperimmune rabbit serum plus normal heparinized blood obtained by pooling whole blood from 18 normal, 15 day old mice, plus dilutions of virus in broth, were mixed, using 0.3 cc. of each ingredient. The final dilutions of virus were 10^{-1} to 10^{-9}.

TABLE V

Effect of Heparinized Blood on the Protective Power of Serum When Mixtures of Blood, Serum, and Virus Are Injected Intracerebrally

Blood, or broth control	Serum	Number of mice developing encephalitis of three injected									Minimal cerebral infective doses of virus against which the serum protected with	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Blood	Broth
Blood	HR	3	3	3	3	3	—	—	—	—	100*	
	NR	—	—	—	—	—	3	3	3	0	Control	
Broth	HR	—	—	3	3	2	0	—	—	—		1,000
(control)	NR	—	—	—	—	—	—	3	3	2		Control

Abbreviations as in Table I.

* Undetermined but equal to or less than the amount indicated.

Another mixture was prepared in precisely the same way except that normal rabbit serum was substituted for the immune serum; other controls are also shown in Table V. Of each of these materials, 0.03 cc. was withdrawn and injected intracerebrally in mice. These components were in contact in the test tube during the period of animal inoculations, which contact lasted from about 10 minutes before the first injection to about 30 before the last.

It will be noted from the table that normal mouse blood *in vitro* probably contained no factor enhancing the protective power of immune serum, nor was the immune reaction found to be completed therein. It is natural to conclude from all these tests with blood that under the influence of immune serum after intraperitoneal injection of serum-virus mixtures, virus is not found in the blood.

Résumé

In the first paper of this series (1) it was shown that antiserum against equine encephalomyelitis virus protected against many more minimal infective doses when serum-virus mixtures were given intraperitoneally, instead of intracerebrally. It was indicated that this variation in protective capacity by two routes of inoculation (*a*) was not due to inactivation of virus by serum *in vitro*, (*b*) that it did not depend on the greater sensitivity of tissues to detect virus, nor (*c*) on the amount of inoculum, nor (*d*) on incubation of the mixtures.

The purpose of the present investigation was to throw more light on the mechanism underlying the phenomenon. Our procedure consisted of passive immunization of 12 to 15 day old mice by intraperitoneal inoculation of hyperimmune serum followed by virus given in the brain, nose, muscles or peritoneal cavity. When intracerebral or intranasal inoculations of virus were given, only minimal protection or none at all was demonstrated; when intramuscular or intraperitoneal injections were made, a marked protection was revealed. Further experiments exhibited an agreement in effect when antiserum and virus were mixed and then without incubation were introduced into animals. The results bring out the fact that a relatively minute amount of antiserum (0.015 cc.) has the capacity to protect against a remarkably high amount of virus given intramuscularly or intraperitoneally, even against as many as 1,000,000 to 10,000,000 infective doses.

The experimental findings on the variation in the protective capacity by different routes of inoculation appear to be correlated with the pathways traversed by the virus from the periphery to the central nervous system. Since after intraperitoneal or intramuscular inoculation the pathway includes the circulating blood, studies were made on the blood. After introduction of serum-virus mixtures intraperitoneally, virus was not found in the circulation 24 hours later.

DISCUSSION

An interpretation of the results will be proposed on the basis of existing knowledge as to the pathways traversed by the virus of equine encephalomyelitis after inoculation by various routes in young mice.

It is known (8) that virus given intranasally in mice reaches the brain by the olfactory chain of neurons and this pathway is thus entirely within nervous tissue. Also, virus placed within the brain comes into direct contact with the nerve cells. After intramuscular and intraperitoneal inoculation, however, virus, in the greater number of mice, reaches the central nervous system from the periphery through the mediation of the blood stream. That is, the infective agent is deposited from the blood onto the nasal mucosa whence the invasion of the brain is by way of the olfactory pathway (9, 10). The experiments have shown that when the pathway of virus is only in nervous tissue there occurs little or no protection by antiserum; when the pathway includes the circulating blood, on the other hand, the protective capacity is great, even though relatively minute amounts of antiserum are used.

Interest centers on the point along the pathways taken by the virus where the immune effect is consummated. It is not likely to be in the brain because after the intraperitoneal injection of antiserum there is often neutralization of only one minimal intracerebral infective dose of virus or even none at all. Because of the fact that after intramuscular or intraperitoneal injection of virus it migrates in most cases from the blood stream to the nasal mucosa, there was the possibility that the immune reaction took place on the nasal mucosa. However, it was found that when serum is given intraperitoneally and followed by virus intranasally, little or no protection occurred, so that this possibility seemed unlikely. By elimination, then, the most important part of the immune reaction is probably effected somewhere in non-nervous tissue. But the experiments leave undetermined the exact tissue or site of the reaction.

In the latter connection, antiserum injected intraperitoneally followed by virus intramuscularly results in a high degree of protection. If the action of the serum is to protect the muscle cells at the local site of inoculation, antibody must have entered the blood stream before reaching the muscle. Plainly, great diffusion is possible in the blood and therefore the suggestion alluded to earlier that antivaccinal serum is more effective in one tissue than in another because of less diffusion (4) may not apply here. Although by the methods used virus was not shown to be present in the blood 24 hours after

intraperitoneal inoculation of serum-virus mixtures, no conclusive proof is adduced from this fact and from the *in vitro* experiments as reported, that the reaction takes place in the blood stream. Sabin (13) had previously demonstrated that with the virus now under investigation the prior nasal instillation of antiserum produces resistance against infection by the same route, 2 to 4 hours later, provided the dose of virus is not too great, by virtue of the local specific protective action on the cells by the serum.

A corollary may be offered to strengthen the general proposition that the protective capacity varies, depending on whether nervous or non-nervous tissues are included in the pathways of virus progression to the central nervous system from the point of inoculation. Sabin (3, 13, 17, 18) has shown that, following injection of pseudorabies (or B virus) intramuscularly, the virus multiplies locally and then progresses to the central nervous system by way of the peripheral nerves; furthermore, injection of serum-virus mixtures intramuscularly results in protection, while intracerebrally there is none. It is possible that the difference may be ascribed (18) to the fact that the antiserum prevents the local multiplication of virus and hence no progression along peripheral nerves occurs. Pseudorabies antiserum-virus mixtures are infective intracerebrally even when minimal doses of virus are used, but do prevent infection when administered by the intranasal (or subcutaneous) route. The question is whether the antiserum here acts in the same way to prevent local multiplication of virus and so no further progression can take place. By way of contrast, pseudorabies virus does not take the olfactory pathway as equine encephalomyelitis does (8), therefore the intranasal route is one by which pseudorabies virus progresses first through non-nervous tissue; consequently one should expect a higher protective capacity by this route than by the intracerebral.

One final point, perhaps of practical bearing, remains for discussion,—if one assumes that in the horse as in the mouse immunity which results from circulating antibody depends similarly on the route of inoculation of virus. The most probable hypothesis as to the mode of transmission in nature of equine encephalomyelitis is that the virus is carried by mosquitoes. If this is so, the portal of entry is such in natural infection as to correspond with intraperitoneal

or intramuscular inoculation in the mouse, and the immunity from circulating antibodies might be expected to be maximal. Such a state of affairs would explain the effective use of antiserum in the field as a prophylactic agent (19, 20).

CONCLUSIONS

Minute amounts of antiserum injected intraperitoneally protect against large doses of equine encephalomyelitis virus given intramuscularly or intraperitoneally in 12 to 15 day old mice. Antiserum given intraperitoneally with virus intracerebrally or intranasally results in little or no protection. These phenomena occur as well when serum-virus mixtures are injected at the different sites. The marked variation of the protective capacity of antiserum as thus displayed would appear to be dependent upon the differing pathways of progression of the virus from the site of injection to the central nervous system.

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