STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

IV. Infection in the Mouse with Fresh and Fixed Virus

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(Received for publication, October 31, 1939)

In previous papers on Eastern equine encephalomyelitis (1), some of the general features of infection in guinea pigs, as well as some differences in behavior between fresh and fixed strains of virus, were described. The widespread use of the mouse as an experimental subject indicated the need of comparable studies on this animal.

Material and Methods

Two strains of virus have been employed. One was freshly isolated from a horse in the summer of 1938, and immediately passaged once in guinea pigs. The brains of these animals were preserved in glycerine, and for supply of virus subinoculations were made into mice as needed. Mouse brain emulsion was used for all experiments involving the fresh virus. When a particular supply was exhausted, the original guinea pig material was freshly inoculated intracerebrally into more mice. The guinea pig brain used as source has remained virulent for almost a year, and fresh subinoculations into mice yield a constant titer of virus. The fresh virus is designated as M_1, indicating the first mouse passage of virus, removed by only one guinea pig transfer from its equine host.

The fixed virus is the same as that used in previous papers, namely, the strain modified by Traub and TenBroeck (2) through intracerebral passage in pigeons. The 115th to 126th serial passages in pigeons have been utilized in these experiments. One part of mouse or pigeon brain was emulsified in 9 parts of diluent (saline or 50 per cent glycerine). This is called a 10^0 dilution. The titration end-point, by intracerebral inoculation, was quite constant, 0.03 cc. of a 10^{-6} or 10^{-7} dilution of the stock suspension causing death. Usually the volume of the inoculum was 0.05 cc. Accordingly, animals succumbed to 3 (or 5) $\times 10^{-9}$ to 3 (or 5) $\times 10^{-10}$ gm. of infective tissue injected intracerebrally.

From the strain of white mice inbred at this laboratory for over 20 years young adults, 8 and 9 weeks of age, were used. Animals are sexually mature at this age. Males generally weighed 25 to 27 gm., females, 2 to 3 gm. less. Unless otherwise stated, in any single experiment only animals of the same sex and the same age (in weeks) were used. Where infant mice were employed, only those animals with a well developed coat of hair but with eyes as yet not open were used. Such animals are 10 to 12 days old. In one set of experiments, females between 6 and 8 months of age were used.

Susceptibility to Inoculation by Different Routes

Previous work with guinea pigs has shown that a fresh strain of virus is highly infectious with all modes of inoculation, even in high dilutions. The fixed strain given subcutaneously is of extremely low virulence, but is more active if given in the nose (3) or the eye (1 *c*). It was first deemed desirable to find out if these relationships held for mice.

Groups of animals, 3 to a dilution, were simultaneously injected by a variety of routes. Intracerebral inoculation (0.05 cc.) was used to show the titer of the virus. Subcutaneous injection was done into the ear. A maximum of 0.02 cc. was injected, but there was usually some leakage from the needle track. The effective dose varied from 0.01 to 0.02 cc. For intranasal instillation, about 0.08 cc. was dropped onto the nares of etherized animals. The inoculum was inhaled and much of it was swallowed. The effective amount acting on the nasal mucosa was probably only a small proportion of the total volume instilled. Virus was also injected into the vitreous of the right eye. 0.01 to 0.02 cc. was expressed from the syringe, but most of this leaked from the eyeball into the conjunctival sac. Because of the lachrymo-nasal duct, and the possibility that virus might leak from the eye into the conjunctival sac and thence into the nose, a control group was utilized in which virus was placed directly in contact with the surface of the eye and conjunctiva, without trauma, and allowed to run down by whatever pathway might be utilized.

TABLE I

Susceptibility of Adult Mice to Different Routes of Inoculation with Fresh and Fixed Virus

Dilution	Fresh virus					Fixed virus				
	Intracerebral (0.05 cc.)	Subcutaneous (0.02 cc.)	Intranasal	Intraocular (±0.01 cc.)	Conjunctival instillation	Intracerebral (0.05 cc.)	Subcutaneous (0.02 cc.)	Intranasal	Intraocular (±0.01 cc.)	Conjunctival instillation
10^{-8}	5, 0, 0					0, 0, 0				
10^{-7}	1, 2, 0					3, 0, 0				
10^{-6}	2, 2, 2		0, 0, 0	0, 0, 0		2, 2, 3				
10^{-5}	1, 1, 1	6, 7, 7	0, 0, 0	7, 0, 0		1, 2, 3		0, 0, 0	5, 0, 0	
10^{-4}		5, 6, 8	5, 5, 6	0, 0, 0	0, 0, 0			0, 0, 0	0, 0, 0	
10^{-3}		4, 5, 5	5, 5, 6	5, 5, 10	0, 0, 0		0, 0, 0	0, 0, 0	3, 4, 0	0, 0, 0
10^{-2}		7, 7, 0	3, 4, 5	4, 4, 0	0, 0, 0		0, 0, 0	2, 3, 3	2, 3, 3	0, 0, 0
10^{-1}		4, 7, 0					0, 0, 0	2, 3, 4	2, 2, 3	0, 0, 0
10^{0}							0, 0, 0			

0 = mouse survived.

1 = mouse died of encephalitis after 1 day, *i.e.*, between 24 and 48 hours following inoculation.

2 = mouse died of encephalitis after 2 days, *i.e.*, between 48 and 72 hours following inoculation, etc.

Blank spaces = not tested.

The results of this experiment are shown in Table I. Several features are worthy of note. The intracerebral titer for the fresh virus is 0.05 $\times 10^{-7}$, for the fixed strain, 0.05 $\times 10^{-6}$. This difference is not considered of significance. The same tube of glycerolated virus might titrate out to 10^{-6} on one occasion, 10^{-7} on another.

The fresh virus proved highly infectious by subcutaneous inoculation, even with the minute doses injected into the ear. Slightly larger doses, injected into the foot-pads, were even more virulent, as shown in Table II. With fixed virus, larger doses (0.2 cc.) of undiluted 10 per cent suspension injected into the pads might cause death in about one-third of the cases. With diluted suspensions, however, fatality never resulted.

The fresh virus administered by the intranasal and intraocular routes was highly infectious, but the results are somewhat more irregular than with subcutaneous inoculation. Fixed virus injected into the eye or nose

TABLE II

Subcutaneous Inoculation of Fresh Virus into the Ear and the Foot-Pad of Young Adult Mice

Dilution	Ear (0.02 cc.)	Pad (0.10 cc.)
10^{-6}	0, 0, 0	10, 10, 0
10^{-5}	9, 0, 0	6, 7, 0
10^{-4}	4, 8, 10	5, 6, 0
10^{-3}	5, 6, 0	5, 5, 5
10^{-2}	5, 5, 6	5, 5, 6

is considerably more effective than into the subcutaneous tissue. The ocular or nasal routes, however, are less favorable than intracerebral.

It is further noteworthy that where the fixed virus is effective, by any mode of peripheral inoculation, the elapsed time until death is somewhat less than with the fresh virus. Shortened incubation period is a recognized feature of a fixed virus. With many viruses this has been especially true in relation to direct intracerebral inoculation. Here, on the contrary, difference of incubation period occurs only with peripheral and not with intracerebral routes of inoculation.

Intraperitoneal Inoculation, and Susceptibility of Infant Mice

The researches of Theiler (4) and of Andervont (5), on yellow fever and herpes viruses respectively, demonstrated that infant mice may be more susceptible to peripheral inoculation of some viruses than are adults. This phenomenon in relation to vesicular stomatitis viruses has recently been studied by Sabin and Olitsky (6). The same authors have also reported

(7) similar observations for their strains of equine encephalomyelitis. It seemed desirable to obtain quantitative data in respect to the two distinct strains available. Consequently infant and adult mice (as described under Methods) were injected intraperitoneally, adults receiving 0.25 cc. and infants 0.10 cc.

TABLE III

Inoculation of Infant and Adult Mice with Fresh and Fixed Virus

Dilution	Fresh virus				Fixed virus			
	Intracerebral		Intraperitoneal		Intracerebral		Intraperitoneal	
	Adult (0.05 cc.)	Infant (0.30 cc.)	Adult (0.25 cc.)	Infant (0.10 cc.)	Adult (0.05 cc.)	Infant (0.03 cc.)	Adult (0.25 cc.)	Infant (0.10 cc.)
10^{-8}	4, 0, 0	2, 0, 0			0, 0, 0	0, 0, 0		
10^{-7}	0, 0, 0	2, 2, 0			0, 0, 0	2, 2, 0		
10^{-6}	2, 3, 3	1, 1, 2	10, 0, 0	2, 2, 2	2, 2, 0	2, 2, 0		0, 0, 0
10^{-5}	2, 2, 2	1, 1, 2	7, 9, 0	4, 6, 7	2, 2, 2	1, 1, 2		0, 0, 0
10^{-4}			6, 0, 0	2, 2, 3		1, 1, 1		0, 0, 0
10^{-3}			5, 6, 7	2, 2, 2			0, 0, 0	2, 7, 0
10^{-2}			5, 8, 0	2, 2, 2			0, 0, 0	1, 2, 2
10^{-1}							4, 0, 0	1, 1, 1
10^{0}							3, 3, 3	

TABLE IV

Fresh Virus Inoculated Intraperitoneally into Mice 6 to 8 Months in Age

Dilution	Experiment 1	Experiment 2
10^{-5}	9, 12, 0*	6, 7, 8, 0, 0
10^{-4}	5, 5, 0	7, 8, 12, 0, 0
10^{-3}	9, 0, 0	7, 8, 0, 0, 0
10^{-2}	10, 0, 0	7, 0*, 0*, 0, 0
10^{-1}	10, 0*, 0	6, 5, 0, 0, 0
10^{0}	4, 5, 5	4, 4, 5, 5, 0

* These animals showed signs of encephalitis at the end of the arbitrary 14 day observation period, but had not yet succumbed.

The results of one pair of experiments are presented in Table III. Adult mice are susceptible to intraperitoneal inoculation of fresh virus in high dilutions, although there is some irregularity. (See, as well, Table VI, controls.) Infants, on the other hand, succumb much more quickly and with complete regularity. Fixed virus, however, acts somewhat differently. Adults, inoculated intraperitoneally, succumb regularly to undiluted virus suspension, only occasionally to a dilution of 10^{-1}. Infants are 100 to 1000 times more susceptible to the same route of inoculation

than are adults. But with the fixed virus, from 1000 to 10,000 minimal cerebral lethal doses are required to cause death in infants from intraperitoneal injection, while with fresh virus from 1 to 10 cerebral lethal doses are adequate. Thus, with both strains of virus, infants are more susceptible to peripheral administration than are adults. But infants succumb far more readily to a fresh strain of virus given intraperitoneally than to a strain that is fixed by pigeon passage.

Because of the statement appearing in the literature, presumably with the use of undiluted suspensions (7), that mice over the age of 3 months are 95 per cent resistant to equine encephalomyelitis virus, the possibility arises that the animals 8 or 9 weeks of age are not adequately representative of adult mice. Consequently titrations were carried out in mice 6 to 8 months old. Two experiments are presented in Table IV. A few of the survivors at the end of the arbitrary 2 week observation period showed marked signs of encephalitis, and are designated by asterisks in the table. It is clear that even 6 to 8 month old mice are quite susceptible to high dilutions of fresh virus.

Intrasciatic Inoculation of Fixed Virus

In the guinea pig it has been demonstrated that virus, especially the fixed strain, may travel along nerves quite readily when the cell body is infected first. This was particularly shown in the case of the retina (1 *c*). In the studies on mice it is of interest to determine the ease with which virus may travel in the reverse direction, that is, following direct inoculation into the axone, whereby virus, if it is to travel along the nerve, must go towards the cell body.

Inoculation into the sciatic nerve in a small animal is attended by the risk that the inoculum may spread mechanically along the sheath of the nerve, and by rupture of anatomical boundaries, be forced into the subarachnoid space of the spinal cord. Preliminary experiments were therefore undertaken wherein, after the nerve had been surgically exposed, the needle was inserted in a distal direction and the inoculum forced downwards, away from the spinal cord. The needle was pushed back and forth to rupture the fiber sheaths and facilitate contact of virus and axis cylinder. It was possible by this means to produce infection characterized by paralysis of the injected extremity as the first sign of disease. This is interpreted as presumptive evidence that the virus spread along the nerve fibers upwards into the spinal cord.

The effective quantity of inoculum is extremely small. Injection of even 0.01 to 0.02 cc. is attended by considerable leakage into the surrounding muscle tissue. For controls, therefore, the sciatic nerve was exposed as usual, and about 0.05 cc. of virus suspension dropped on the nerve and into the operative wound, but without any trauma to the nerve. This is considered adequate to control leakage from the injected nerve and contact between escaped virus and muscle tissue.

Once the possibility of disease following direct nerve injection was established, other experiments were performed with the injection made towards the spinal cord, in order to allow all possible chance for infection after direct nerve inoculation. In this way, largely negative results are especially significant.

An example of such an experiment is presented in Table V. The controls were treated by simple dropping of virus on to the exposed sciatic nerve. Other animals received drops of virus suspension in the nostrils. Virus so administered readily attains the nerve cell bodies of the olfactory nerve. Even though complicating factors related to intranasal injection cannot be neglected, a rough comparison between the attack of fixed virus on nerve cell bodies and on nerve fibers or axones may thus be made.

It is evident that fixed virus placed in direct contact with nerve fibers has but little power to invade the nervous system. On the other hand virus

TABLE V

Comparison of Intrasciatic Injection of Fixed Virus with Intranasal Instillation

Dilution	Intrasciatic injection	Sciatic controls*	Intranasal instillation
10^{-5}	N. T.		0, 0, 0, 0
10^{-4}	0, 0, 0, 0, 0		0, 0, 0, 0
10^{-3}	7, 0, 0, 0, 0		2, 3, 3, 3
10^{-2}	0, 0, 0, 0, 0		3, 3, 3, 3
10^{-1}	8, 9, 0, 0, 0	0, 0, 0, 0, 0	2, 3, 3, 3
10^{0}	7, 0, 0, 0, 0	0, 0, 0, 0, 0	N. T.

* Sciatic controls: Exposed sciatic nerve and operative wound bathed in virus, without trauma to the nerve.

placed in contact with olfactory or retinal (Table I) nerve cells does so invade with much greater ease. The axone is, of course, a part of a cell, but a part with a much lower metabolic rate than the cell body. It seems probable that the differing abilities of cell invasion in the cell body as compared with the axone may be correlated with the metabolic rate.

Brain Trauma in Relation to Infection

Working with mice, Sawyer and Lloyd (9) showed that intraperitoneal inoculations of yellow fever virus, without pathological effect in the intact adult animal, produced encephalitis if the brain was injured. Later Webster and Clow (10) indicated a similar facilitating effect of brain injury with the virus of St. Louis encephalitis. Burnet and Lush (11) found that brain injury enhanced the effect of louping ill virus. On the other hand, Olitsky, Cox, and Syverton (12) were unable to induce infection with vesicular stomatitis virus injected intraperitoneally, even though the brain was simultaneously traumatized.

The fresh strain of Eastern equine encephalomyelitis virus is infectious in high dilutions when injected intraperitoneally. The fixed strain has only a very limited invasiveness by this route. It was of interest to determine whether brain injury would increase the susceptibility to intraperitoneal injection.

Young adult mice, as described under Methods, were divided into groups of 10. Each group was composed of 5 males and 5 females. A hot needle stab was made into the brains of the test groups. The next day the traumatized animals, together with an equal number of controls, were given intraperitoneal injections of virus (0.25 cc.) at different dilutions. This was done for both fresh and fixed virus. Ether anesthesia was used for all operations.

TABLE VI

Relation of Brain Trauma to Intraperitoneal Administration of Virus in Young Adult Mice

Dilution	Fresh virus (0.25 cc.)		Fixed virus (0.25 cc.)	
	Trauma	Control	Trauma	Control
10^{-6}	5, 6, 6, 0, 0, 0, 0, 0, 0, 0	8, 9, 12, 0, 0, 0, 0, 0, 0, 0		
10^{-5}	5, 5, 5, 5, 6, 7, 7, 8, 0, 0	6, 6, 6, 7, 7, 10, 0, 0, 0, 0		
10^{-4}	3, 4, 4, 4, 5, 5, 5, 5, 7, 9	5, 6, 6, 6, 6, 6, 6, 7, 7, 0		
10^{-3}				
10^{-2}			0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
10^{-1}			5, 5, 0, 0, 0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
10^{0}			2, 3, 3, 4, 4, 4, 5, 5, 0, X*	2, 3, 3, 3, 3, 3, 4, 4, 0, 0

* X = Mouse died within a few hours after inoculation. Death not due to virus action.

The results are shown in Table VI. One fact is not indicated in the table, namely, that the males and females reacted entirely similarly. It may be seen from the table that a few more of the traumatized animals died than did the controls. This difference does not seem to be of significance, for the effective titer of the virus is not increased by the brain injury. Other experiments in which intracerebral injections of starch were the traumatizing agent, with virus injected intravenously, gave similar results.

One fact should be noted in Table VI. Of the animals receiving fresh virus, the traumatized mice succumbed after an incubation period that was almost constantly less than for the controls. This is not the case with the fixed virus. The mechanism of the reaction requires further study.

Intravenous and Intramuscular Inoculations of Fixed Virus

Numerous experiments have shown that undiluted suspensions of fixed virus are quite regularly infective when injected intraperitoneally. When the same quantity of undiluted virus is injected intramuscularly, encepha-

litis results only infrequently. At the same time, fixed virus (after centrifugation to remove coarse particles) is constantly more infectious when given intravenously than intraperitoneally. A 10^{-1} suspension given intravenously produces encephalitis about as regularly as 10 times that amount given intraperitoneally. And a 10^{-2} dilution, intravenously, may produce encephalitis in as many as half the cases, being thus comparable to a 10^{-1} dilution intraperitoneally. With considerable regularity, the intravenous route is 10 times more effective than the intraperitoneal. The intramuscular route, in turn, is approximately 5 to 10 times less effective than the intraperitoneal.

Facilitation of Infection by Intraperitoneal Glycerine

When it first became apparent that intraperitoneal inoculation was more effective than the same dose given intramuscularly, it was thought that the use of glycerine in the inoculum might have had some effect. In many experiments the pigeon brain used as virus source was emulsified in 50 per cent glycerine which was kept as stock solution and used diluted or undiluted as needed. It is readily demonstrable, however, that virus suspended in saline solution is as infectious by the intraperitoneal route as the same virus suspended in 50 per cent glycerine.

It was found, however, that if an undiluted saline suspension of virus is injected intramuscularly, and at the same time 50 per cent glycerine is injected into the peritoneum, there is a much increased rate of infection. Thus, in a total of 81 mice so treated, 64 died. At the same time, of 81 controls, receiving the same quantity of virus intramuscularly, but without intraperitoneal glycerine, only 28 died.

Not every irritant injected intraperitoneally produces the facilitating effect. A 5 per cent starch solution, infusion broth, horse serum, and glycerine were injected into different groups of mice. Only the glycerine exerted an effect. The dose of glycerine is of considerable importance. At least 0.20 cc. of 50 per cent glycerine is required to produce a significant effect, and 0.25 cc. is even more effective. Experience has shown that more than this amount is not well tolerated by mice. These experiments are summarized in Table VII.

This facilitating effect of intraperitoneal glycerine is exerted only when the glycerine is injected approximately simultaneously with the intramuscular virus. Thus, in a series of animals with glycerine injected into the peritoneum at various intervals before and after the intramuscular virus, a significant effect is produced only when the two injections occur within a few minutes of each other. This experiment is summarized in Table VIII.

After intramuscular injections of virus, mice which die 2 and 3 days (that is, less than 96 hours) after inoculation invariably show encephalitic symptoms as the first clinical signs of disorder. On the other hand, animals which die 4 or more days after inoculation show in about two-thirds of the

TABLE VII

Effect of Type and Dosage of Intraperitoneal Irritant on Facilitation of Infection in Young Adult Mice

Experiment No.	Intraperitoneal irritant	Dose of irritant intraperitoneally	Virus intramuscularly (0.25 cc. undiluted suspensions)
		cc.	
1	5 per cent starch	0.25	6, 0, 0, 0, 0, 0
	Broth	0.25	3, 4, 0, 0, 0, 0
	Horse serum	0.25	4, 5, 0, 0, 0, 0
	50 per cent glycerine	0.20	2, 2, 2, 3, 3, 3
	None	Control	5, 0, 0, 0, 0, 0
2	50 per cent glycerine	0.05	3, 0, 0, 0, 0, 0, 0, 0
	" " " "	0.15	3, 4, 0*, 0, 0, 0, 0, 0
	" " " "	0.20	2, 2, 3, 3, 3, 3, 5, 6
	" " " "	0.25	2, 2, 2, 3, 3, 3, 3, 3
	None	Control	3, 4, 6, 0, 0, 0, 0, 0

* Signs of encephalomyelitis at end of 10 day observation period.

TABLE VIII

Effect of Time Intervals on the Facilitating Action of Intraperitoneal Glycerine in Young Adult Mice

Glycerine injected intraperitoneally	Intramuscular virus (0.25 cc., undiluted suspension)
24 hrs. before virus	3, 3, 3, 0, 0, 0, 0, 0
15 min. " "	2, 3, 3, 3, 3, 4, 6, 0
12 hrs. after "	4, 5, 5, 0, 0, 0, 0, 0
36 " " "	3, 0, 0, 0, 0, 0, 0, 0
Controls—no glycerine	3, 4, 0, 0, 0, 0, 0, 0, 0, 0

cases a flaccid paralysis of the hind quarters as the first sign of disease. The latter instances may reasonably be interpreted as passage of virus up the peripheral nerves of the leg. The action of glycerine intraperitoneally appears to facilitate the production of primary encephalitis, with death 2 to 4 days after inoculation. There is no evidence with this virus that the passage up the peripheral nerves is equally facilitated.

DISCUSSION

A rigorous definition of a fixed virus cannot readily be given, nor will any attempt be made here. It is known, however, that many viruses, after a prolonged series of brain to brain passages, may alter their properties.

Whereas fresh equine encephalomyelitis virus is detectable in the blood in high titer after peripheral inoculation of even minimal doses, the passage virus was shown by Traub (3) to have lost this property and to be practically indetectable in the blood stream. Traub worked with guinea pigs. In the mouse, massive doses of virus injected, say intraperitoneally, may be reflected by the presence of virus in the blood stream. But as already shown by Sabin and Olitsky (7) for their strain, such circulating virus need not lead to infection. This has been confirmed for the fixed strain used herein, and virus has been repeatedly found in the circulating blood of animals that do not develop disease. Sawyer and Lloyd (9) note similar findings with yellow fever virus in the mouse, and quote literature on other viruses.

As is well recognized, by repeated brain passages the virus becomes adapted to the nervous system and has suffered corresponding loss of power to multiply in non-nervous tissue. At the same time, fixed virus which does circulate finds much greater difficulty in infecting the brain from the blood stream than does fresh virus.

Fixed equine encephalomyelitis virus is much less infectious by peripheral inoculation than the fresh strain. But there is considerable difference attending the route chosen for inoculation. When placed in fairly direct contact with nerve cell bodies, as in intranasal or intraocular injection, considerable virulence is present. However, there is only a very limited power of invasion along the axones, even with direct injection into a nerve trunk.

Infant mice are more susceptible to intraperitoneal inoculations than are adults. This is true of both fresh and fixed virus. But the fixed virus is less invasive than the fresh, not only in adults, but also in infants.

The reason for the relatively greater susceptibility of infants as compared with adults has never been satisfactorily explained. The assumption of a hypothetical change in the quality of the blood vessels appears to be purely gratuitous. Far more probable is a change in the brain tissue itself. That there are such changes as infants grow older, as, for example, in myelination, cell maturity, and water content, as well as in reaction to specific or non-specific injury, is well known. It is reasonable that some of these

changes may be correlated with the phenomenon of greater susceptibility. But the subject is obviously in need of detailed study.

It is of some interest that brain trauma does not increase the effective titer of either fresh or fixed virus, yet may (with fresh virus) shorten the incubation period. On the other hand, a non-specific irritation, by injection of glycerine into the peritoneum, does produce facilitation of infection. In this connection Marie (13) reported a similar facilitation with rabies virus after intraperitoneal injection of India ink. German and Trask (14), working with poliomyelitis virus, found increased susceptibility to peripheral inoculations after various operative procedures which did not directly involve the central nervous system. The subject, in so far as it involves the use of glycerine, is still under investigation.

SUMMARY

A fresh strain of equine encephalomyelitis virus is infectious for adult mice in high dilutions by all modes of peripheral inoculation. A fixed strain has very limited invasive power when injected peripherally unless virus is placed in fairly close contact with nerve cell bodies, as in the intranasal or intraocular routes. For fixed virus the effectiveness of the mode of inoculation may be graded in the following descending order: intracerebral, intraocular and intranasal, intravenous, intraperitoneal, intramuscular, subcutaneous.

Fixed virus has a very limited power of invading the central nervous system along the axones of peripheral nerves even when injected directly into the nerve.

Infants are more susceptible to infection than are adults. But even for infants, intraperitoneal inoculation with fixed virus is significantly less effective than similar inoculations with fresh virus.

Brain trauma does not increase the effective titer of fresh or fixed viruses but may shorten the incubation period for fresh virus. With fixed virus injected intramuscularly, a pronounced facilitating effect may be produced by the simultaneous intraperitoneal injection of 0.20 to 0.25 cc. of 50 per cent glycerine. Other irritants tried are without effect.

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