INDUCED RESISTANCE OF THE CENTRAL NERVOUS SYSTEM TO EXPERIMENTAL INFECTION WITH EQUINE ENCEPHALOMYELITIS VIRUS

II. Serotherapy in Western Virus Infection*

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From a review of work by many investigators on a variety of virus infections Rivers (1) concludes that immune serum as a therapeutic agent is valueless in most instances if it is given after the onset of signs of disease. His view is that when signs and symptoms of a malady become obvious, the viruses of a number of diseases "have already reached practically all the cells that are likely to be attacked;" the virus then is not reached by antibody so long as the infected cell lives (*cf.* Rous and Jones, (2)) and hence antiserum is not helpful. An exception to this view has recently been taken by Zichis and Shaughnessy (3) who reported successful treatment of experimental infection of mice and guinea pigs with the Western equine encephalomyelitis virus by means of hyperimmune rabbit serum administered at onset of symptoms.

Zichis and Shaughnessy emphasized that their positive results were obtained when large amounts of highly potent serum were injected. The potency required of an antiserum was that 1 cc. of a 1:1000 dilution introduced subcutaneously should protect mice against 10 M.L.D. of virus given intracerebrally 24 hours later. Antiserum of this minimal standard could be produced in rabbits after a course of at least thirty-six inoculations of active virus given over a period of 12 weeks. Single or multiple doses of 1 or 2 cc. sufficed for the treatment of mice, of 5 or 10 cc. for guinea pigs; the largest total amount inoculated into a single 400 gm. guinea pig was recorded as 70 cc.

Howitt (3 *a*) had reported an earlier attempt at experimental serotheraphy in guinea pigs infected with Western virus. The serum was effective when it was administered at the 4th hour but ineffective at the 10th hour after an intracerebral injection of virus. If the latter was given by the nasal route the disease could be prevented by the serum to the 48th hour after virus instillation.

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Wyckoff and Tesar (4) described an attempt to treat Eastern and Western virus infections of monkeys with hyperimmune horse serum.

> No animal survived when serum was administered at the first sign (fever) or later. When it was given early, 3 to 24 hours after nasal instillation of virus, three of six monkeys survived. The survivors, as well as three control monkeys which had received the serum alone, were susceptible to infection by the same route 7 weeks later. This indicated (4) that no active immunity was induced.

The emphasis placed by Zichis and Shaughnessy on the quantitative requirements in effective serotherapy evoked our interest. Previous studies in our laboratory had revealed in actively immunized mice a quantitative relationship between serum antibody titer and the degree of resistance to intracerebral (5) and to peripheral (6) injection of the active equine virus. It was shown later (7) that rabbits actively immunized to such a degree that antibody could be demonstrated in the cerebrospinal fluid or brain tissue resisted an intracerebral test inoculation. The presence of antibody in the spinal fluid was interpreted as an indicator of the availability of antibody to the tissues directly exposed to the virus after intracerebral inoculation.

On this basis the results published by Zichis and Shaughnessy suggested two lines of investigation: (*a*) whether the effectiveness of antiserum in the prevention and treatment of the experimental disease depended on quantitative relations similar to those established in active immunization; (*b*) whether serotherapy, even if it met such requirements, could arrest the course of the infection after onset of signs.

Methods and Materials

Virus.—Two strains of Western equine encephalomyelitis virus were employed, one propagated in this laboratory for 9 years, hereafter designated as the Rockefeller Institute strain, and one which was kindly sent to us by Dr. Joseph Zichis. They are serologically identical but infection with the Zichis virus is characterized by a longer incubation period. Unless otherwise stated the Rockefeller Institute strain was used.

Animals.—Swiss and Rockefeller Institute strains of mice were employed; they were equally susceptible to the virus and equally protected by any given immune serum. Young adult mice, about 1 month old, were used except for neutralization tests by the intraperitoneal method, when 11- to 13-day-old animals were substituted. Rabbits for immunization weighed approximately 3000 gm., and albino and mixed breeds of guinea pigs from 170 to 400 gm. Further details concerning the handling of animals have been described (6, 7).

Immune Sera.—The rabbit antisera have been designated according to the number and the type of injections given; for example, 46 W represents forty-six injections of Western virus. The method of production of antisera in rabbits by means of injec-

tions of active Western virus (Rockefeller Institute strain) is described in the following outline:—

Type of antisera	Virus used for immunization	No. of injections	Dose	Route	Interval between injections
1 W	20 per cent chick embryo in saline solution	1	2 cc.	Subcutaneous	—
3 W	" "	3	2, 4, 5 cc.	"	Weekly
46 W	Mouse brain, then	8	1 to 3 mouse brains, each dose in 5 cc. broth	"	"
	20 per cent chick embryo in saline solution	38	0.5, 1, 2, 4, 5, 5, 5 cc.	Intravenous	3 × weekly
48 W	Same as 46 W, then	46	Same as 46 W		
	20 per cent chick embryo	2	5 cc., 5 cc.	"	—

The Z W serum was kindly sent us by Dr. Zichis at our request for a sample of his potent hyperimmune rabbit serum. According to his records, its titer in mouse protection tests was 1:500 against 100 M.L.D. of virus but in a previous titration had been 1:1000 against 5 to 10 M.L.D. Moreover, he reported that this serum had been used for the treatment of infected guinea pigs with a recovery rate of 60 per cent.

Tests.—Neutralization tests were carried out in mice by the intracerebral (5)[1] or by the intraperitoneal (8) method. Protection tests followed closely the methods of Zichis and Shaughnessy (3): intracerebral injection of active virus 24 hours after subcutaneous inoculation of immune serum. Therapeutic tests were those in which serum was given after the virus regardless of the time of onset of signs. The technique for each test is described in the text.

Tests for Potency of Antisera

The potency of the various antisera was measured by (*a*) protection tests; (*b*) tests for the capacity of sera obtained from passively immunized mice to neutralize the virus; and (*c*) neutralization tests by the intraperitoneal and by the intracerebral methods. The purpose of these tests was to find out whether at least thirty-six injections of active virus (3) were required to produce a rabbit hyperimmune serum of the standard potency.

Protection Tests.—Protection tests were done in order to compare the capacity of various antisera to protect mice against subsequent intracerebral injection of active virus.

[1] All such procedures were carried out with the aid of full ether anesthesia.

1 cc. of undiluted or diluted serum was injected subcutaneously into mice and 24 hours later virus was inoculated intracerebrally. The virus used in these tests was guinea pig brain and the lethal end-point of both the Zichis and the Rockefeller Institute strains of virus in this form was 10^{-5} to 10^{-6}.

A summary of the results obtained is given: In one test undiluted 3 W and 46 W antisera both protected mice against the largest measurable amount of virus, Institute strain, *i.e.*, 10,000 cerebral M.L.D.

In a number of tests employing the Institute strain, no protection against 10 M.L.D. occurred with a 1:1000 dilution of the 46 W antiserum. The Z W antiserum, diluted 1:100, had little or no protective capacity. In 1:500 dilution it was ineffective. On the other hand, when the Zichis strain of virus was used, both the 3 W and the

TABLE I

Neutralizing Capacity of Sera of Mice 24 Hours after Passive Immunization

Rabbit antiserum used for immunization (1 cc. subcutaneously)		Dilution of mouse serum tested with 10^{-8} Western virus*	
Type	Dilution	1:100	1:300
46 W	Undiluted	0/4‡	2/4
	1:100	3/4	3/4
	1:1000	3/4	4/4
3 W	Undiluted	0/4	0/4
	1:100	1/4	3/4
	1:1000	1/4	4/4

* Control mice injected with normal rabbit serum and Rockefeller Institute virus: 10^{-8}, 5/6; 10^{-9}, 2/6.

‡ Number of mice dead/number injected intracerebrally.

46 W sera in dilutions of 1:100 and 1:1000 protected mice against at least 10 cerebral M.L.D. The Z W antiserum was not tested with the Zichis strain of virus because more of the serum was not available.

Neutralizing Capacity of Sera of Passively Immunized Mice.—The purpose of the tests shown in Table I was to compare the antibody titers in sera of mice passively immunized with undiluted or diluted 46 W or 3 W antisera just before the mice were exposed to an intracerebral test dose of the Zichis virus.

The mice were bled 24 hours after the injection of antiserum; the mouse sera were pooled according to type and dilution of antiserum received. Each pool was then diluted 1:100 and 1:1000 and these dilutions were tested for capacity to neutralize virus by intracerebral test in mice, as tabulated.

The results failed to indicate that the injection of 46 W antiserum produced a higher antibody level than that of 3 W antiserum.

Neutralization Tests.—In the following experiments the neutralizing capacities of the rabbit hyperimmune sera were compared.

1. Intraperitoneal Method.—In the neutralization test by this method the different sera were diluted 1:300. At this dilution 46 W serum neutralized 1000 peritoneal lethal doses of virus (on repetition, 1000+ doses); 3 W, between 100 and 1000 (on repetition, 1000+), and 1 W, 10 to 100 doses.

No difference could be detected by the intraperitoneal neutralization test in mice between 46 W and 3 W antisera.

2. Intracerebral Method.—Finally, by means of intracerebral neutralization tests in mice, complete titrations of the neutralizing capacity of serum dilutions were carried out. Antisera 46 W and 3 W were found to be equivalent in their neutralizing capacity over the entire range of serum dilutions; that of 1 W serum was, however, at a lower level. Results obtained in this way will be discussed later (Morgan, 9).

Comment.—The high degree of protection induced in mice by antiserum against infection with the virus was noteworthy. 1 cc. of undiluted serum given subcutaneously 24 hours before virus was introduced directly into the brain protected the animal against as much as 10,000 mouse cerebral lethal doses of virus. When diluted 1:1000, as Zichis and Shaughnessy have already noted (3), it still protected against about 10 lethal doses of one strain of Western virus given intracerebrally.

Mice resisted an intracerebral test inoculation of active virus at a time when the sera of certain ones had a neutralizing titer of only 1:100 or less (*cf.* Table I). It was found previously (7) in actively immunized rabbits that the ratio between serum antibody and antibody in the cerebrospinal fluid was 300:1. The minimal titer at which such rabbits resisted an intracerebral inoculation of 10 to 1000 rabbit cerebral lethal doses was 1:200. This relationship cannot, however, be compared directly with that found in passively immunized mice since: (*a*) The mice were passively, the rabbits actively immunized. (*b*) The mice resisted 10 mouse cerebral lethal doses of the Zichis strain: mice of another series, similarly treated, were fully susceptible to 10 such doses of the Rockefeller Institute virus, which had been employed in the rabbit experiments (7). (*c*) Approximately 1000 times less virus in terms of mouse cerebral lethal doses was injected into mice than into rabbits, and therefore less antibody would be required for its neutralization. (*d*) Intracerebral inoculation in mice causes hemorrhage and consequently virus is exposed to neutralizing action of the blood.

The foregoing tests show that it is not necessary to give a large number of injections of active virus to rabbits in order to produce an antiserum of maximal potency. Indeed, antisera equivalent in all respects were obtained by vaccination of rabbits with from three to as many as forty-eight doses of active virus (*cf.* references 10 and 11).

Therapeutic Use of Antisera

Serotherapy in Mice Injected with Virus by the Intracerebral Route.—Zichis and Shaughnessy (3) stated that serotherapy was successful when antiserum was given intraperitoneally to mice 48 hours after intracerebral injection of

virus. More than one-half the number survived even though certain ones showed visible signs of infection of the central nervous system (prostration and partial paralysis) at the time of treatment.

The results of our experiments on serotherapy in mice after intracerebral introduction of the Rockefeller Institute strain of virus are summarized in Table II.

TABLE II

Effect of Serotherapy in Mice

(1 cc. undiluted antiserum intraperitoneally into mice after intracerebral inoculation of guinea pig brain virus, Rockefeller Institute strain.)

Experiment No.	Interval between virus and serum inoculation	Type of serum injected	Results					Therapeutic effect
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
	hrs.							
1	48*	None	5/5	6/6	3/6	0/8	—	—
		46 W	—	5/6	5/6	0/6	—	0
2	24	Normal rabbit	—	5/6	3/6	0/6	—	—
		46 W	6/6	3/6	0/6	—	—	10 M.L.D.
3	24	Normal rabbit	—	6/6	6/6	3/6	—	—
		46 W	6/6	6/6	6/6	3/6	—	0
		3 W	6/6	5/6	6/6	1/6	—	0
4	24 or 45	Normal rabbit	—	—	6/6	5/8	1/6	—
	24	Z W	—	5/6	2/6	2/6	—	10–100 M.L.D.
	45	Z W	—	6/6	6/6	5/6	—	0
5	24	Normal rabbit	—	—	6/6	1/6	0/6	—
	24	Z W	—	5/6	3/6	2/6	—	<10 M.L.D.?
	45	Z W	—	—	7/8	1/6	—	0

* At 45 hours most of the mice exhibited first signs: ruffled fur, slowness, ataxia.

It will be observed that when any of the different sera was given 45 to 48 hours after the virus, that is to say, at a time when the first signs of the disease became apparent (ruffled fur, slowness, weakness, unsteadiness, and ataxia), it failed to prevent death. Moreover, when the antisera were injected 24 hours after the virus, at a time when no signs were obvious, little or no effect was produced.

The experiment was repeated with the Zichis strain of virus. Mice were injected intracerebrally with 10 M.L.D. of virus. After 3 to 5 days, at the onset of signs, 2 cc. of either 48 W or 3 W, or normal rabbit serum, were administered subcutaneously. In certain cases the dose was repeated after $5\frac{1}{2}$ to 48 hours. In all, twenty-seven

mice received immune serum and thirteen normal serum. All died, the control and test animals within the same period of time.

Thus antiserum even in prodigious amounts given to mice at the first signs of encephalomyelitis induced by intracerebral inoculation of virus was without therapeutic effect. The intracerebral method of inoculating virus, moreover, was believed to be too drastic; the host was overwhelmed at the start and appraisal of a serum under such circumstances would therefore be difficult.

Serotherapy in Guinea Pigs Injected with Virus by the Intralingual Route.—It was reported (3) that guinea pigs were also successfully treated with antiserum given intraperitoneally (in certain instances intracardially or intravenously) after the onset of fever or even of encephalitic signs.

About two-thirds the number of animals receiving such treatment survived (3). Indeed, certain of the survivors treated after onset of signs were resistant to a later intracerebral injection of virus while those treated at an earlier stage were not. This indicated to Zichis and Shaughnessy that infection had proceeded far enough at the time serotherapy was begun to lead to active immunity.

In the present series, twelve 250 to 350 gm. guinea pigs received intralingually 0.3 cc. of 10^{-2} dilution of virus suspension (Zichis strain). 5 to 8 days later, at the first rise of temperature, 10 cc. of antiserum were injected intraperitoneally into six (three of them received an additional 10 cc. from 24 to 48 hours after the first dose) and 10 cc. of normal rabbit serum were inoculated into the remaining six. Two of the six in the control group died and four (including three which received a second dose of immune serum) of the six in the treated group.

There was no indication that serotherapy prevented death although the low incidence of mortality in the control group makes an appraisal difficult. Further experiments are in progress.[2]

Serotherapy in Guinea Pigs Injected with Virus by the Plantar Route.—At this point a paper by Traub (13) became available, in which he reported the results of serum treatment in guinea pigs after pad inoculation of Eastern and Western virus. The regularity with which young guinea pigs could be infected by this peripheral route suggested its use in the present studies.

Traub (13) inoculated horse or guinea pig Eastern or Western virus suspension (0.5 cc. of 10^{-2} dilution) into the pads of guinea pigs. An inoculation was followed by a diphasic febrile course, the afebrile interphase occurring about the 2nd day after injection (13, 14). If serum was given intracardially 24 to 56 hours after Eastern virus, death was prevented, provided the second febrile phase had not as yet begun. If serum was introduced 48 to 105 hours after injection of virus, during the second febrile phase, then it had no effect.

[2] As this paper is being written, a preliminary report by Davison and Fulton (12) has appeared in which it is stated that antiserum is of little value in the treatment of guinea pigs infected by intracerebral injection of Western virus; also that the results of serotherapy of man having the active disease were inconclusive.

The following two experiments were carried out to test the effect of antiserum administered at various intervals after plantar inoculation of active virus.

The Rockefeller Institute strain of virus and 48 W antiserum were employed. The virus was in the form of 20 per cent chick-embryo suspension in broth, 0.125 cc. of which inoculated into each hind pad gave rise to infection of 150 to 200 gm. guinea pigs in all instances and to death in the majority. The serum was administered intracardially and, when repeated, intraperitoneally in doses of 1 cc.

TABLE III

Serotherapy Following Pad Inoculation of Western Virus; One Dose (1 Cc.) of Serum Injected Intracardially

Time of serum treatment Hrs. after virus inoculation	No. of guinea pigs used	Signs at time serotherapy began	Results
No serum (controls)	8	—	6 died of virus effect, average time about 4 d.;* 2 survived but showed 4 and 5 d. febrile reactions
24	4	Fever (104–105.2°F.)	1 died of virus effect 18 d.; 3 survived
48	4	1, afebrile interphase 3, fever (104–105.8°F.)	4 died of virus effect: 1, 4 d.; 1, 9 d.; 1, 13 d.; 1, 24 d.
72	4	2, 2nd febrile phase 2, " " " + CNS signs	3 died of virus effect and 1 survived with posterior limb paralysis since 4 d.
96	4	2 died 4 d. No serum given 2 CNS signs; treated	2 treated found dead next day (5 d.)

* Death due to virus effect established by specific lesions or virus in CNS (by intracerebral passage to 6 to 8 mice) or, as a rule by both; d. refers to day after virus inoculation.

Experiment 1 (Table III).—Of twenty-four guinea pigs injected in the manner just described, eight were not treated. Six of these died of virus effect; the two survivors had fever and as will be shown later, developed active immunity.

Table III shows that hyperimmune serum, given 72 or 96 hours after the virus inoculation during the manifest encephalitic phase, had no influence on the course of the disease. When the serum was administered 24 hours after the injection of virus, three of four animals survived. The fourth died 18 days after virus inoculation of characteristic encephalitis. Of the group receiving the serum 48 hours after the virus, one died after the usual incubation period; the other three 9, 13, and 24 days respectively after virus inoculation.

The guinea pigs which succumbed after such prolonged incubation periods showed no sign of illness until a day or two before death. The course of the disease was as characteristic as that in the control animals which had succumbed 4 or 5 days after inoculation of virus. The lesions found in the central nervous system (Figs. 1 to 3)

were those of acute experimental equine encephalomyelitis (15) with marked degeneration and necrosis of neurons, characteristic vascular changes and hemorrhages, polymorphonuclear cell invasion, and occasionally neuronal intranuclear inclusion bodies.

In an attempt to prevent the fatal infection in serum-treated animals, occurring after an extraordinarily prolonged incubation period (hereafter designated as "delayed reactions") another experiment was undertaken. This time the effect of one, two, and three doses of serum was studied.

Experiment 2 (*Chart 1*).—One dose of antiserum given 24 to 48 hours after pad inoculation of virus failed again to prevent the fatal delayed reaction (*cf.* Table III). On the other hand, even when two or three doses of antiserum were administered at the indicated intervals (Chart 1), one of seven guinea pigs (No. 9-4) developed encephalitic signs and was sacrificed 47 days after inoculation of virus. The brain of this animal was infectious for mice and both brain and cord exhibited lesions characteristic of experimental equine encephalomyelitis (*cf.* Figs. 1 to 3). Another one (8-9) showed fever on the 20th and 21st days and was sacrificed. No virus was isolated from its brain and histological study revealed no lesions indicative of encephalitis.

To sum up the results of the two experiments: Young guinea pigs which received antiserum intracardially 24 to 48 hours following pad inoculation of Western virus either survived or developed delayed reactions terminating in death. When the 48-hour dose of antiserum was repeated once or twice, the animals survived with one exception. This guinea pig developed the disease after 47 days despite three doses of serum. Antiserum given 72 or 96 hours after the injection of virus, during the encephalitic phase, was ineffective.

The conclusions of Traub could thus be confirmed in part. The main point of difference was the frequent occurrence of delayed reaction in the present series of animals.

Traub observed in a group of serum-treated guinea pigs one which developed fatal encephalitis on the 17th day after virus inoculation and another one, transitory myelitis (posterior limb paralysis) on the 14th day. The limited number of such cases reported may be attributable to the fact that Traub tested all survivors within 3 weeks for cerebral resistance, and therefore did not observe them over a sufficiently long period of time.

Earlier observations on the occurrence of such delayed reactions should be recalled: Flexner and Lewis after serotherapy of experimental poliomyelitis in monkeys (16) and Green and coworkers after injection of serum-virus mixtures in experimental fox encephalitis (17).

In the following investigations methods were applied which had been used previously in the analysis of the quantitative relation between neutralizing antibody and resistance in actively immunized animals (7, 18). The antibody titers of sera of the treated and untreated guinea pigs of Experiments 1 and 2

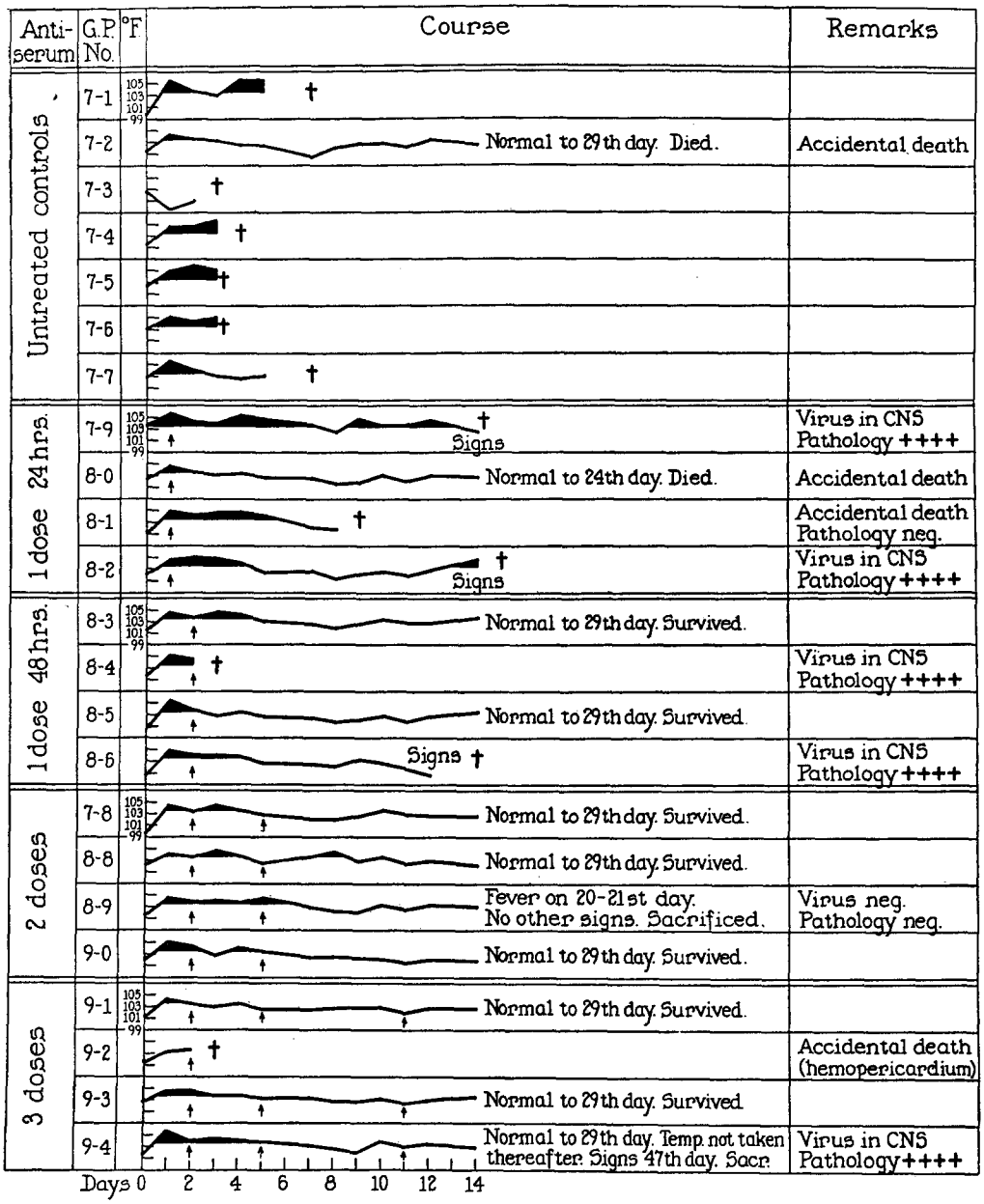

CHART 1. Experiment 2. Effect of single and repeated doses of hyperimmune rabbit serum in guinea pigs inoculated with Western virus (0.125 cc. 20 per cent suspension into each hind pad).

Dosage of antiserum: 1 cc. undiluted serum intracardially for first injection, intraperitoneally for second and third. Injection indicated by arrow-head. Temperatures at 103.6° or above are filled in to show duration and extent of febrile reaction.

were studied to find out (1) whether serum treatment instituted at the specified intervals could prevent the production of antibody (active immunization) in the guinea pig and (2) whether and to what extent the antibody titers in guinea pigs showing delayed reactions differed from the titers in animals which survived.

Neutralizing Antibody and Cerebral Resistance in Untreated and Serum-Treated Guinea Pigs

1. Neutralizing Antibody in Untreated Guinea Pigs.—We have reported (18) previously that in certain adult rabbits after subcutaneous inoculation of active Western virus the virus circulates in the blood for 2 or 3 days. After its disappearance from the circulation neutralizing antibody becomes demonstrable in the serum. On the 4th to 6th day the temperature rises to 106–107°F. and at that time virus can be isolated from the brain. In certain animals permitted to run their natural course the temperature drops critically at the time when the antibody in the serum reaches a titer of 1:300 (tested with at least 10 M.L.D. of virus in mice). This particular level was defined as an indicator of the adequate supply of antibody to the central nervous system. It was also found (18) that young (4-weeks-old) rabbits, after similar exposure to virus, develop typical encephalitis and die before this critical titer of antibody is reached.

The two surviving control guinea pigs from Experiment 1 were bled on the 25th day and No. 7-2 (Experiment 2, Chart 1 and Table IV) was bled on the 14th day after virus inoculation. All three had responded to the virus with fever. The sera, diluted 1:300, of all three neutralized at least 10 mouse cerebral lethal doses.

The reactions of the guinea pig to the inoculated virus patterned themselves therefore after those of the rabbit just described.

2. Neutralizing Antibody in Serum-Treated Guinea Pigs.—Table IV shows that, in contrast to the sera of non-serum-treated guinea pigs which had developed active immunity, those of the treated animals failed to reach an antibody titer of 1:300. Of the sera obtained 12 or 13 days after beginning of serum treatment, only a small number had a titer of 1:100. Even after administration of three doses of antiserum, the last of which was given only 3 days prior to bleeding, the antibody level was no higher.

The serum of guinea pig 1-11 which had received three doses of antiserum alone did reveal a titer of 1:300. The sera of two other animals in the same group did not, however, reach this titer.

The decrease in antibody content in sera obtained a later period of time proceeded at the same rate as that in control guinea pigs (9-8, 1-09, 1-11) which received one, two, and three doses of serum alone.

Tests for Cerebral Resistance of Serum-Treated and Untreated Guinea Pigs.—

TABLE IV

Neutralizing Antibody and Cerebral Resistance Test in Serum-Treated and Untreated Guinea Pigs

Experiment No.	Guinea pig No.	Dosage of serum and time after virus inoculation	Outcome	Bled. Days after virus inoculation	Antibody in serum dilution				Test for cerebral resistance	
					Un.	1:10	1:100	1:300	Time interval	Outcome
1	3-0	None: controls	Survived	25				+	6 mos.	*Survived*
	3-6		"	25				+	6 "	Trauma, died, 1 d.
	1-5	1 dose 1 d.*	"	25	+			−	6 "	Enceph., died
	1-6		"	25				−	6 "	" "
	1-7		"	25	+			−	6 "	" "
	2-0	1 dose 2 d.	Enceph., 24 d. Sacrificed, 25 d.	24	+		−	−		
	2-2	1 dose 3 d.	Post-paralysis, 4 d. Survived	25	+		−	−	6 "	Trauma, died, 1 d.
2	7-2	None: control	Survived. Accidental death 29 d.	14				+		
	7-9	1 dose 1 d.	Enceph., 14 d.	14	+		−	−		
	8-2		Same as No. 7-9	14		+	−	−		
	8-0		Survived. Accidental death 25 d.	14		+	−	−		
	8-3	1 dose 2 d.	Survived	14		+	+	−	10 wks.	Enceph., died
				29	+	−	−			
	8-5		"	14		+	−	−	10 "	" "
				29	+	−	−			
	8-6		Enceph., prostrate 13 d., died 14 d.	14		+	−	−		
	7-8	2 doses 2 and 5 d.	Survived	14		+	+	−	10 "	Trauma, died, 1 d.
				29	+	−				
	8-8		"	14		±	−	−	10 "	Sick; recovered
				29	+	−	−			
	9-1	3 doses 2, 5, and 11 d.	"	14		+	−	−	10 "	Enceph., died
				29	+	−	−			
				47	−	−				
	9-3		"	14		+	+	−	10 "	" "
				29	+	+	−			
				47	+	−				
	9-4		Enceph., 47 d.	14		+	+	−		
				29	+	+	−			
				47	+	−	−	−		

TABLE IV—*Concluded*

Experiment No.	Guinea pig No.	Dosage of serum and time after virus inoculation	Outcome	Bled. Days after virus inoculation	Antibody in serum dilution				Test for cerebral resistance	
					Un.	1:10	1:100	1:300	Time interval	Outcome
	1-19			1‡		+	−	−		
				7		+	−	−		
		1 dose		14	+	−				
	9-8			13		+	−	−	7 wks.‡	Enceph., died
				28	−	−				
Controls (no virus)	1-09	2 doses 2 d. interval		12		+	+	−	6 "	" "
				28	+	+	−			
	1-11	3 doses 3 and 6 d. intervals		12		+	+	+§	6 "	" "
				28	+	+	−			

* 1 d., 2 d., etc., indicates 1, 2, etc. days after virus inoculation.

‡ In the controls the interval after beginning of serum injections is indicated.

§ In two other animals, 1:300 dilution of serum was negative.

Table IV also shows the results of intracerebral test inoculations in certain survivors in Experiments 1 and 2.

All guinea pigs received 0.2 cc. of a 10^{-4} dilution of mouse-brain virus suspension. In addition two normal, control guinea pigs received 10^{-4}, and two others, 10^{-5} virus dilution. All control animals died of encephalitis. Of the two non-serum-treated survivors (3-0 and 3-6), one died of trauma within a few hours after injection. The other one survived without showing signs, even though the test was carried out 6 months after the immunizing pad inoculation. All others showed characteristic signs of encephalomyelitis with fatal outcome in all but one.

It is clear therefore that the guinea pigs surviving after serotherapy of the experimental infection did not become actively immunized since they succumbed to an intracerebral injection of virus, although a control guinea pig receiving virus but no antiserum did resist the test dose. The results of the neutralization and of the resistance tests therefore coincided.

DISCUSSION

The present results indicate that in those guinea pigs in which serum therapy was effective in suppressing or delaying the onset of the fatal disease the antigenicity of the virus was inhibited. The interference with or blocking of the active antibody production occurred even when serum treatment was begun 2 days or, in one instance (2-2), 3 days after the inoculation of virus. Cox and Olitsky (19) had already reported for the guinea pig and later Gochenour (20) for the horse, that immune serum can interfere with the antigenic activity of

the active or formalin-inactivated equine encephalomyelitis virus. Failure of serum-treated guinea pigs to resist intracerebral injection of active virus coincided with failure to develop the high degree of antibody characteristic of active immunity. Similarly Wyckoff and Tesar (4) and Traub (13) found that serum-treated monkeys or guinea pigs were susceptible to an intracerebral test inoculation. The somewhat irregular findings of Traub in this respect might have been due to the fact that he tested guinea pigs after 2 or 3 weeks' observation, *i.e.*, at a time when some of them may still have been passively protected by the injected antiserum. In the present studies, no such protection was found after 10 weeks.

Furthermore, neutralization tests, as summarized in Table IV, failed to reveal any consistent difference between sera of guinea pigs which survived the time of observation (6 months, Experiment 1; 10 weeks, Experiment 2) and the sera of those which developed encephalitis after prolonged incubation period (delayed reactions). Moreover, delayed reaction occurred at a time when antibody had fallen to a titer of 1:10 or lower. Of special interest is the observation that, although the serum of guinea pig 9-4 had an antibody titer of 1:10 on the 29th day, signs of encephalitis in this animal were not manifest until the 47th day, when only undiluted, but not tenfold-diluted serum had a neutralizing capacity. Thus the difference in outcome of serotherapy among individual guinea pigs could not be satisfactorily explained on the basis of antibody levels alone.

Another factor to consider is the persistence of virus in those animals that showed delayed reactions. It is assumed that in those guinea pigs which survived the period of observation all virus was effectively neutralized by the antibody injected but that those which developed delayed reactions might have harbored virus somewhere out of reach of antibody. It has been shown by Francis (21) for the nasopharyngeal mucosa and by us (7, 18) for the central nervous system that the serum antibody titer may be considered as an indicator in characteristic ratio of the antibody content of tissue fluids. Thus it is conceivable that virus might be held in check when fixed by cells and be released to infect other cells as antibody falls to an ineffective level in the surrounding medium. The present investigations did not include attempts to determine in which tissue virus persisted during the long incubation period characterizing delayed reactions. It is difficult, however, to visualize such persistence in extraneural tissues since after its release therefrom the virus would have had to reach the central nervous system by way of the blood stream but, as shown in Table IV, it would then have been exposed to the action of antibody. It is more likely that a small amount of virus reached the central nervous system and there became fixed, before the antiserum was injected. It may be assumed that the spread of such a small amount of virus could have been arrested by a small amount of antibody in the tissue. The titer to which antibody of the serum had fallen at the time of the delayed reaction indicated

that the antibody concentration in the tissue no longer sufficed to hold the virus in check.

In connection with the delayed reactions in serum-treated guinea pigs, the case recently reported by Gold and Hampil (22) is of interest:—

A laboratory helper was accidentally infected with Western virus. On the 6th day after exposure he became ill; on the 8th, his serum contained little or no detectable antibody. Repeated antiserum injections were followed by progressive improvement until 10 days after the beginning of serum treatment, when he suffered a recurrence of signs which were diagnosed as acute Parkinsonism and subsequently recovered with development of a remarkably high titer of antibody.

The question here is whether a similar condition prevailed as in the serum-treated guinea pigs which manifested delayed reactions.

SUMMARY AND CONCLUSIONS

Under none of the experimental conditions here described was treatment of the infection induced by the virus of Western equine encephalomyelitis in mice and guinea pigs with specific hyperimmune rabbit serum effective if begun after the onset of signs of encephalitis. In mice, after intracerebral inoculation of virus, serum was ineffective when given even before that stage. After peripheral introduction of virus in guinea pigs the disease was completely arrested in certain animals by single or multiple doses of antiserum if treatment was begun within 24 to 48 hours after virus inoculation. In others the incubation period was prolonged to 2 or even as long as 7 weeks.

In untreated guinea pigs, injection of virus alone led to active immunity in those which survived. Antiserum blocked the antigenicity of active virus in the serum-treated animals. The decrease in titer in the sera of all antiserum-treated animals proceeded at the same rate as in those of control guinea pigs which received antiserum alone. Thus it was not possible to predict which ones would survive and which would succumb after a prolonged incubation period. Delayed fatal disease occurred at a time when the neutralizing antibody of the treated guinea pigs had fallen to a low titer. It is therefore likely that the virus which persisted throughout this long incubation period had been prevented from passing to and infecting other cells but reached them when antibody fell to an ineffective level in the surrounding medium. The relative frequency of such delayed reactions limits to a further extent the degree to which antiserum can be depended on for effective treatment of infection with the virus of equine encephalomyelitis.

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EXPLANATION OF PLATE 20

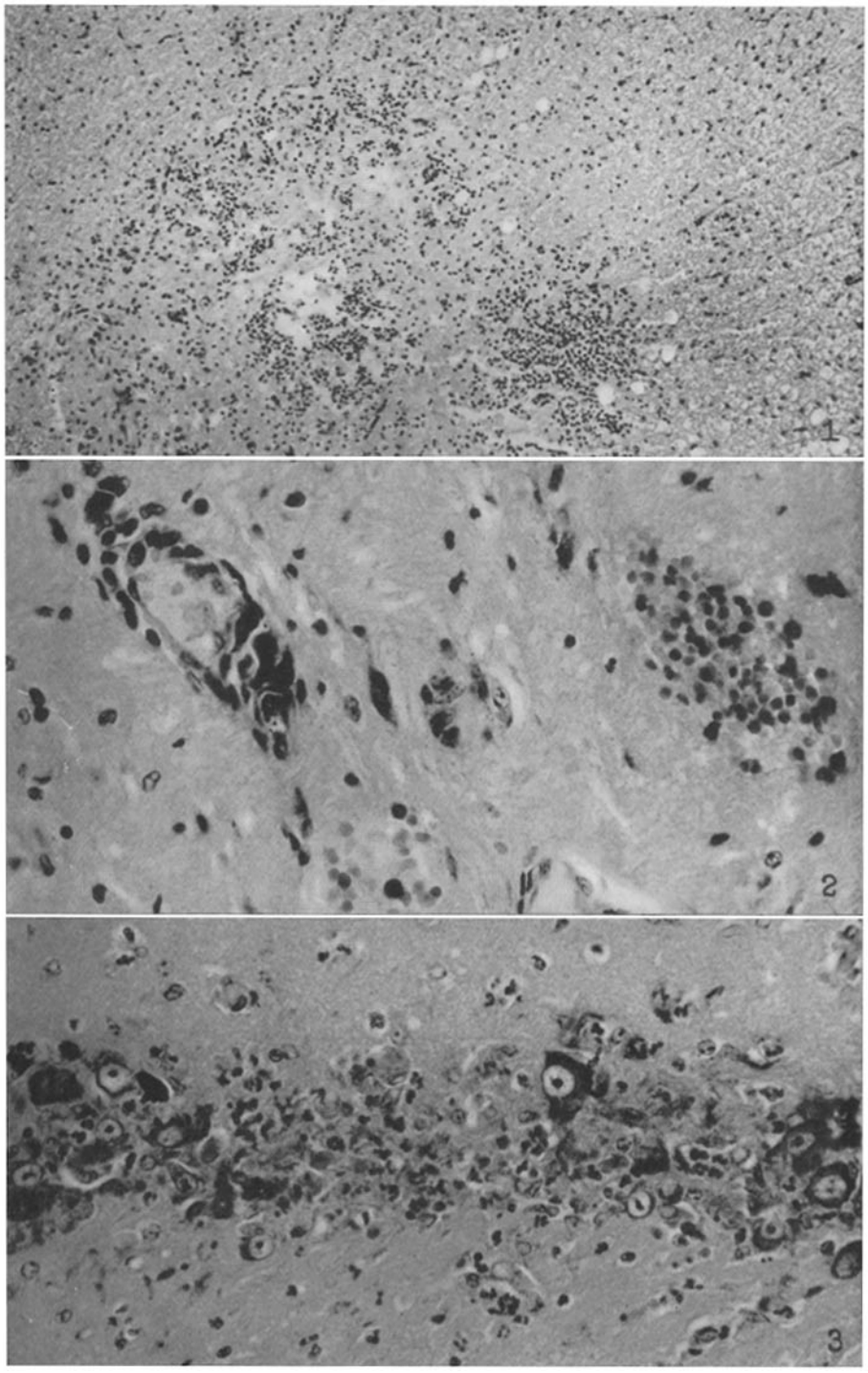
Sections fixed in Zenker's-acetic acid solution and stained with eosin and methylene blue.

The photographs were made by Mr. Joseph B. Haulenbeek.

FIG. 1. Anterior horn of upper cervical cord of guinea pig 8-6 (Chart 1) which died on 14th day after virus inoculation of pads and 12 days after receiving intracardially one dose of hyperimmune serum. To be noted are the absence of normally appearing neurons, the extensive necrosis, and the numbers of polymorphonuclear leucocytes. ×90.

FIG. 2. Medulla of same guinea pig. Hemorrhages and the vascular lesions characteristic of acute equine encephalomyelitis are shown. ×400.

FIG. 3. Hippocampus of same guinea pig. A localized area of necrosis in the layer of large pyramidal cells; off center to right is a neuron showing Nissl type degeneration. ×400.



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