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

III. Comparison of Antiviral Serum Constituents from Guinea Pigs Immunized with Active or Formolized Inactive Virus

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It has been reported (1, 2) that the protective power of antisera against the virus of equine encephalomyelitis is much greater if serum-virus mixtures are inoculated into 12 to 15 day old mice by the intraperitoneal route, instead of by the intracerebral. The sera tested in the series of experiments referred to were from animals immunized by injections of active virus, or were derived from horses infected naturally.

During the course of these investigations sera obtained from guinea pigs which had been immunized with formolized, inactive virus (3) were submitted to similar comparative intraperitoneal and intracerebral tests for protective potency. The results were unexpected in that they differed from those obtained with sera from animals inoculated with active virus. This was considered important to the problem of the correlation of antibody to immunity and led to further study of the phenomenon. The outcome of this study forms the substance of the present communication.

It may be recalled that previous work (3) demonstrated that a high degree of resistance to experimental equine encephalomyelitis (against 1,000 intracerebral lethal doses of virus) can be induced in guinea pigs by the use of formolized vaccines in which no active virus can be detected. By the application of quantitative methods it was determined that it is necessary to introduce subcutaneously 3,000 to 30,000 mouse intracerebral infective doses of virus three times, at intervals of 7 days, to secure the same degree of protection in guinea pigs by the use of untreated active virus. Thus small amounts of active virus, detectable by animal inocula-

tion, are not sufficient to bring about immunity, while formolized vaccines in which virus is not demonstrable even by elaborate and extensive tests (3) will induce a high degree of resistance.

A continuation of these quantitative studies (4) revealed that guinea pigs immunized either with untreated, active virus or formolized, inactive virus, show no distinctive differences in the antiviral body content of their sera as determined by the mouse intracerebral test. In either event, although guinea pigs are found to resist 1,000 or more intracerebral doses of virus, the antibody content of their sera is, by this test, low and in some instances even absent.

Methods

The method of preparing formolized vaccine used in the experiments was that already described (3). The vaccines were made up of fresh mouse brains infected with the Eastern strain. A concentration of 0.5 per cent formalin was used throughout. Vaccines were kept in the dark at room temperature for 24 hours after the addition of formalin, and thereafter in the refrigerator at 5°C.

The other procedures—animal inoculation, neutralization tests, dosages, etc.—were fully described in the first two papers of this series (1, 2). In Table I will be found the details of immunization of guinea pigs with formolized vaccines and tests for immunity. The sera were obtained by bleeding from the heart[1] at the intervals noted, and this was always performed before the test dose for resistance was given. Equal parts of serum from two to four animals of each group were pooled.

EXPERIMENTAL

The first step in this investigation was the preparation of antisera by immunization of guinea pigs with formolized vaccines. Table I summarizes the results obtained.

The tabulated results indicate that of five groups of guinea pigs one received massive doses of formolized vaccine, *i.e.*, ten times more than the largest amount used in any of the others. The interval between the preparation of the vaccine and its use was from 35 to 100 days,[2] between the last inoculation and the collection of serum was from 14 to 16 days, and between the last inoculation and the later intracerebral test for resistance was about 16 days (in the first group this test was unsatisfactory, hence the repetition). In agree-

[1] All operative procedures on animals were done with the aid of ether anesthesia.

[2] In a prior report (3) it was shown that the vaccines retained their immunizing property for at least 65 days; since then it has been demonstrated after 100 days' storage at about 5°C.

ment with previous experience (3) the vaccines used did not contain any active virus that could be demonstrated by the methods employed. Knowing that small, measurable amounts of active virus are not enough to induce immunity (3) it was believed that these guinea pigs could not have had immunity attributable to any but inactivated

TABLE I

Preparation of Immune Sera by the Immunization of Guinea Pigs with Formolized Vaccines

Guinea pig serum (see Table II)	Formolized vaccine			Immunization			Intracerebral test for immunity	
	Vaccine No.	Mouse infective units per cc. before inactivation	Test for active virus*	Interval between preparation and use	Route and dose	Interval between last injection and bleeding for serum	Minimal infective doses injected	Result†
				days		*days*		
1	X H	3×10^9	Negative	35	1 sc, 1 cc.‡	14, 16 (pooled)	—	—
3	"	3×10^9	"	100	1 sc, 1 cc.	14	1,000	0/2
4	"	3×10^9	"	100	2 sc, 1 cc. each, 7 d. int.‡	14	1,000	0/2
5	"	3×10^9	"	100	3 sc, 1 cc. each, 7 d. int.	14	1,000	0/1
6	XIII H	3×10^9	"	56	3 sc, 10 cc. each, 5–6 d. int.	14	1,000	0/2

* Test for virus included 15 day old mice injected intracerebrally (0.03 cc.) and intraperitoneally (1.0 to 2.0 cc.); eight other vaccines prepared for other purposes in exactly the same way have given negative tests for active virus.

† Figures exclude animals that died from the operations of bleeding from the heart or intracerebral inoculation. The numerator represents the number dead of encephalitis, the denominator, the number of animals given the test dose.

‡ 2 sc, 1 cc. each, 7 d. int. = two subcutaneous injections, 1 cc. each at 7 day interval.

virus. This point is stressed because a comparison is being made between the results of immunization by means of active and of inactive virus. Excluding the animals that died accidentally during bleeding or inoculation, all of those injected with vaccines were found to be resistant to 1,000 guinea pig intracerebral lethal doses of virus.

TABLE II

Sera of Guinea Pigs Immunized with Formolized Vaccine. Comparative Protective Power When Serum-Virus Mixtures Are Inoculated by Intraperitoneal and Intracerebral Routes

Experiment No.	Route of injection	Dose	Age of mice	Guinea pig serum (see Table I)	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which the serum protected	
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
		cc.	*days*												
1	ip	0.1	14–15	1	3	1	3	2	2	0	0	—	—	10	
	"	0.1	14–15	2* (active virus)	2	0	0	0	0	0	0	—	—	100,000*	
	"	0.1	14–15	Normal	—	—	—	—	3	3	2	1	—	Control	
	ic	0.03	25±	1	—	—	—	3	3	3	1	0	—		10
	"	0.03	25±	2* (active virus)	—	—	—	3	3	0	0	0	—		100*
	"	0.03	25±	Normal	—	—	—	—	—	3	2	2	1		Control
2	ip	0.1	15	1	—	—	0	2	2	0	—	—	—	10	
	"	0.1	15	2* (active virus)	3	3	0	—	—	—	—	—	—	10,000*	
	"	0.1	15	Normal	—	—	—	—	3	3	2	0	—	Control	
	ic	0.03	25±	1	—	—	—	—	3	3	1	2	—		0
	"	0.03	25±	2* (active virus)	—	—	—	3	3	1	—	—	—		100*
	"	0.03	25±	Normal	—	—	—	—	—	3	2	2	1		Control
3	ip	0.1	12, 13, 14	3	—	—	1	3	1	3	—	—	—	1†	
	"	0.1	12, 13, 14	4	—	0	1	2	0	—	—	—	—	100	
	"	0.1	12, 13, 14	5	3	2	2	3	—	—	—	—	—	100†	
	"	0.1	12, 13, 14	Normal	—	—	—	—	—	3	2	1	—	Control	
	ic	0.03	26	3	—	—	—	—	3	3	3	0	—		1
	"	0.03	26	4	—	—	—	—	3	2	0	0	—		10
	"	0.03	26	5	—	—	—	—	3	2	1	0	—		10
	"	0.03	26	Normal	—	—	—	—	—	—	3	2	1		Control

ip, intraperitoneal; ic, intracerebral.

* Serum 2 was from a guinea pig that survived an immunizing intramuscular injection of 10^8 mouse infective units of virus and was bled 14, 17, and 25 days later, and the samples so collected were pooled.

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

TABLE II—*Concluded*

Experiment No.	Route of injection	Dose	Age of mice	Guinea pig serum (see Table I)	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which the serum protected	
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
		cc.	*days*												
4	ip	0.03	12	5	—	3	1	3	0	0	0	0	—	1,000	
	"	0.03	12	Normal	—	—	—	—	—	3	3	2	—	Control	
	ic	0.03	30±	5	—	—	—	—	3	1	0	0			10
	"	0.03	30±	Normal	—	—	—	—	—	—	3	1	0		Control
5	ip	0.1	12	4	—	2	1	2	0	0	—	—	—	1,000	
	"	0.1	12	Normal	—	—	—	—	—	3	3	2	—	Control	
	ic	0.03	Adult	4	—	—	—	3	2	2	0	0	—		1
	"	0.03	"	Normal	—	—	—	—	—	3	3	1	0		Control
6	ip	0.1	12	6	3	3	2	3	0‡	1	1	—	—	1,000	
	"	0.1	12	Normal	—	—	—	—	3	3	2	3	—	Control	
	ic	0.03	Adult	6	—	—	—	3	1	0	1	0	0		100
	"	0.03	"	Normal	—	—	—	—	—	3	2	0	0		Control

‡ One died of an accidental cause.

The serum employed in the present experiments could therefore be considered as being derived from guinea pigs highly resistant to artificial infection.

Comparative Intracerebral and Intraperitoneal Inoculations of Serum-Virus Mixtures.—The object of the following tests was to determine whether the same wide variation in protective capacity which follows the inoculation of serum derived from animals immunized with active virus would obtain with serum collected from guinea pigs immunized with inactive formolized virus. The data of the experiments are given in Table II and it is to be noted that in Experiments 1 and 2, as a control, a serum (No. 2) was included which was obtained from a guinea pig immunized with active virus.

From Table II it is evident, first, that the sera of guinea pigs immunized with formolized vaccines protected against only small numbers of minimal infective doses of virus when serum-virus mixtures were introduced intracerebrally in mice, which is in agreement with earlier observations (4); second, that the wide variation observed

previously by two routes of inoculation with sera of animals immunized with active virus did not obtain under present circumstances. For, when the serum and virus were tested by injection intraperitoneally in 12 to 15 day old mice, only a small difference was revealed

TABLE III

Sera of Animals Immunized with Formolized Vaccine or Active Virus

Serum No.*	Animal	Immunization	Minimal infective doses in terms of route, against which the serum protected	
			Intraperitoneal doses	Intracerebral doses
6	Guinea pig	Formolized vaccine	1,000	100
5	" "	" "	1,000	10
4	" "	" "	100	10
5	" "	" "	<100	10
1	" "	" "	10	10
4	" "	" "	1,000	1
3	" "	" "	<1	1
1	" "	" "	10	0
	Mouse	Active virus	1,000,000	1,000
	Rabbit	" "	100,000	100
2	Guinea pig	" "	100,000	100
2	" "	" "	10,000	100
	" "	" "	10,000	100
	Rabbit	" "	100,000	10
0814	Horse	Natural infection	100,000	10
	Rabbit	Active virus†	10,000	10
1	Horse	Natural infection	100,000	1
5	"	" "	100	1

Only those experiments have been included in which control was done with normal serum of the same animal species.

* Numbers of sera in vaccine series taken from Table II; those of active virus series, from Paper I (1), except serum 2, from Table II.

† Western strain.

between the number of minimal infective doses neutralized in this way and the number neutralized intracerebrally. Serum 2, from an animal immunized with active virus, did, on the other hand, reveal the variation, as was to be expected. For example, this latter serum showed 100 cerebral infective units neutralized intracerebrally and

from 10,000 to 100,000 peritoneal units intraperitoneally; whereas the vaccine serum showed 0 to 100 cerebral doses rendered inactive by intracerebral test and from 0 or 1 to 1,000 peritoneal infective units, intraperitoneally.

Comparison of Sera from Animals Immunized with Vaccine or Active Virus.—The next step was to summarize the results of all the experiments with the sera of guinea pigs immunized by means of inactive formolized virus, and of those animals treated with active virus, so as to determine the frequency and regularity with which this different reaction occurred. Table III gives this summary.

It can be seen that sera of guinea pigs immunized with formolized vaccine and neutralizing 100 cerebral doses intracerebrally, protect against 1,000 peritoneal doses intraperitoneally. However, sera of animals immunized with active virus exhibiting the same degree of neutralization intracerebrally, protect against 10,000 or 100,000 doses intraperitoneally. Also, vaccine sera protecting against 10 doses by the cerebral route, neutralize 10 to 1,000 by the peritoneal, while active virus sera giving this same amount of protection cerebrally, inactivate 10,000 to 100,000 peritoneally.

The results, based on average counts, are as follows: The average number of intraperitoneal infective units neutralized by the formolized virus sera was 400 and in the case of active virus sera was about 153,000. This is a striking difference. The average number of intracerebral infective units protected against by the formolized virus sera was about 17 and in the instance of active virus sera was approximately 140.

Even when the amount of vaccine was increased 10 times that ordinarily employed to render guinea pigs firmly resistant, such variation in serum protective capacity by the two routes, as is seen when untreated virus is given as immunizing agent, was not encountered.

DISCUSSION

Guinea pigs immunized either with active virus or with formolized inactive virus have a high degree of resistance to virus injected intracerebrally. While the sera derived from guinea pigs immunized with active virus and those with formolized, inactive virus show the same range of low protective capacity when serum-virus mixtures

are inoculated intracerebrally in mice, a striking difference is revealed when the two sera are injected intraperitoneally. Then serum of animals rendered immune by means of active virus exhibits high protection and that collected from guinea pigs immunized by formolized vaccines reveals only low protective power, approaching the neutralization titers obtained by the intracerebral method. The importance of this phenomenon centers chiefly on the possibility of two antibodies being involved in the action of the two sera.

With respect to the nature of the antibody, the following assumptions may be made.

1. The antiviral body in both sera is a single antibody, the different results obtained by the intracerebral and intraperitoneal methods of testing depending on the quantities of it that are present. This is unlikely, however, although both sera protect against the same low amounts of virus, or do not protect at all, when tested intracerebrally, they are not of equal value in their neutralizing power when injected intraperitoneally.

2. There are two antibodies present; that is, in the serum of guinea pigs immunized with active virus the antibody has properties different in effect from that of animals receiving injections of formolized, inactive virus. (*a*) The difference may be ascribed to the fact that when active virus is employed as immunizing agent, infection is induced and multiplication of virus occurs; when inactive virus is given, no infection or multiplication takes place. The antigenic stimuli in both instances may evoke different antibodies, detectable by intraperitoneal test. (*b*) There is a further suggestion that the "intracerebral" antibody may be present in both sera and the "intraperitoneal" one in greater concentration in serum derived from animals immunized with active virus and to a much lesser extent in that from guinea pigs injected with formolized vaccines. In other words, the reactions of the sera may be conditioned by varying amounts of these substances present.

3. Finally, neither of these possibilities may apply satisfactorily, and the solution of the problem remains still obscure. The present results, however, lend more support to the supposition of the existence and the operation of at least two antibodies, irrespective of their quantitative distribution in the two kinds of sera.

There is still another consideration. Although the protective capacity of sera secured from guinea pigs immunized by means of formolized, inactive virus is low when tested by intraperitoneal inoculation, nevertheless such animals have a high degree of resistance to virus injected intracerebrally. This is consistent with the hypothesis that the content of antiviral antibody is not proportional to the degree of resistance to infection (5).

SUMMARY

Earlier experiments had shown that the sera of animals immunized with active virus have much greater protective potency when serum-virus mixtures are injected intraperitoneally into 12 to 15 day old mice than when given intracerebrally. The present work was concerned with similar tests on sera derived from guinea pigs immunized by vaccines in which the virus had been inactivated by formalin.

In comparing the content of antiviral body by means of intracerebral and by intraperitoneal inoculation, it was found that both sera show about the same low degree of neutralizing capacity by the former method. By intraperitoneal inoculation, on the other hand, serum collected from guinea pigs immunized by means of active virus reveals high protective power, while that from animals receiving formolized, inactive virus exhibits lower neutralization titers which approach those obtained by the intracerebral method. The significance of this unexpected finding is discussed.

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