ACTIVE IMMUNIZATION OF GUINEA PIGS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

III. Quantitative Studies of Serum Antiviral Bodies in Animals Immunized with Active and Inactive Virus

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In the first paper of this series (1) a basis was constructed for determining the minimal amount of untreated active virus of equine encephalomyelitis needed for the successful production of resistance in guinea pigs to 10^3 or 10^4 intracerebral lethal doses of the virus. In the second article (2) we showed that formolized material induced, within certain limits, as high a degree of resistance as did untreated active virus, and this refractory state was shown not to be due to the action of "living" virus as such in the formolized immunizing preparation.

The results of a study on humoral antibodies in guinea pigs treated with known quantities of active virus and of virus inactivated by formalin (2) will be described in the present communication. It was necessary first to determine the optimal conditions by which the presence of serum antiviral bodies could be brought to light; and having found the best means for their demonstration, to note any difference in content of antibody occurring in the serum of animals immunized with active and with inactive virus. The findings served to explain certain phases of the mechanism of the resistance developed by guinea pigs after injections of formolized inactive virus.

In early work on equine encephalomyelitis virus, Meyer, Haring, and Howitt (3) reported that sera of spontaneously recovered or resistant horses failed to neutralize homologous virus, while those of recovered rabbits, guinea pigs, and monkeys neutralized only irregularly. It was later shown that antiviral bodies were demonstrable in convalescent animals only when serum was added to low multiples of M.L.D. of virus (4, 5, 6). Howitt (4, 7) recorded more constant pres-

ence of serum antibodies in recovered animals, especially horses, after hyperimmunization by means of reinjection with active virus. In guinea pigs, moreover, tissue immunity conferred by vaccination was found to last longer than humoral: resistance to an intracerebral test was therefore present without demonstrable neutralizing bodies in the blood, but the converse could not be proved. Hurst (8) found antiviral substance in the blood of surviving monkeys as early as the 4th day after peripheral inoculation of virus. Neutralization of 1 to 10 M.I.D. of virus as determined by the mouse intracerebral test could "almost invariably" be effected—in some instances even 100 to 1,000 M.I.D. Monkeys that withstood a later intracerebral or intramuscular injection of large amounts of virus did not reveal an appreciable increase of the neutralizing titer of their sera.

Viewing the recorded experiments on the production of serum antiviral bodies in animals recovered from infection or immunized by virus inoculations, one observes that the antibodies occur irregularly or in low concentration.

Materials and Methods

Virus.—The Eastern strain of equine encephalomyelitis virus was employed as fresh or glycerolated guinea pig or mouse brain tissue. Its activity was measured by the results of intracerebral injection of mice (1).

Animal Inoculation.—Guinea pigs were immunized with active or formolized virus, as already described (1, 2). In most instances mouse brain passage virus was used as immunizing preparation and in all cases wherein guinea pigs were tested for induced immunity, the homologous passage virus was injected intracerebrally.[1]

Serum.—Pooled immune serum was employed, being derived from groups of animals, all of which were treated alike with either active or inactive virus. Sera were collected, as a rule, from 4 to 8 days before tests were made. Hyperimmune guinea pig serum was obtained from animals surviving intracerebral inoculation of virus and which, in addition, had resisted three later subcutaneous injections of 3×10^8 to 6×10^8 mouse intracerebral infective units (m.i.u.) of virus (1, 2), given at weekly intervals. These animals were bled for collection of serum 8 to 10 days after the last subcutaneous dose.

Neutralization Tests.—A preliminary test was always made to determine the infectivity of a stock virus suspension which consisted of infective mouse brain ground in hormone broth pH 7.4 and spun in an angle centrifuge at 3,000 R.P.M. for 20 minutes. This suspension, when stored at 5°C., was found to retain its original infectivity for at least 14 days. In conducting the neutralization test,

[1] Operative procedures on animals were performed with the aid of ether anesthesia.

TABLE I

Comparison of the Antiviral Content of Sera of Guinea Pigs Treated with Active and with Formolized Virus. Serum-Virus Mixtures Held at 20 to 22°C. for 15 Minutes before Injection

Experiment No.	No. of sera pooled	Immunized with	No. of weekly injections	M.i.u. of virus in each dose	Time between 1st inoculation and serum collection	Survivors in treated group after intracerebral test of >1,000 M.L.D. given 24 to 36 days after 1st inoculation	Mice survivors after intracerebral injection of serum-virus mixtures — Virus dilutions (absolute)					
							10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
					days							
	4	A V	1	3×10^2	29	0/4	0/3	0/3	0/3	0/3	0/3	3/3
	4	"	3	3×10^2	22	0/4	0/3	0/3	0/3	0/3	2/3	3/3
	5	"	1	3×10^3	29	3/5	0/3	0/3	0/3	0/3	2/3	3/3
	5	"	3	3×10^3	22	5/5	0/3	0/3	0/3	2/3	2/3	3/3
	5	"	1	3×10^4	28	2/5	0/3	0/3	0/3	0/3	2/3	2/3
	5	"	1	3×10^5	30	5/5	0/3	0/3	0/3	3/3	2/3	3/3
	6	"	2	3×10^5	28	6/6	0/3	0/3	0/3	1/3	1/3	3/3
1	5	"	1	3×10^7	30	5/5	0/3	0/3	0/3	2/3	3/3	3/3
	3	F V II	2	N T	21	3/3	0/3	0/3	0/3	0/3	1/3	3/3
	4	F V IX	2	"	28	8/8	0/3	0/3	0/3	0/3	2/3	2/3
	4	F V XIV	1	3×10^7*	31	s c test 4/4	0/3	0/3	0/3	0/3	3/3	3/3
	4	" "	2	"	32	5/5	0/3	0/3	0/3	1/3	3/3	3/3
	4	F V XV	2	"	19	5/5	0/3	0/3	0/3	1/3	1/3	3/3
	4	" " (conc. 5-fold)	2	1.5×10^8	19	5/5	0/3	0/3	0/3	1/3	3/3	2/3
	4	Normal guinea pig sera				0/4	0/3	0/3	0/3	0/3	0/3	3/3
	5	A V	3	3×10^3	22	5/5	—	0/3	0/3	1/3	1/3	3/3
	5	"	1	3×10^7	30	5/5	—	0/3	0/3	2/3	3/3	3/3
2	4	F V XIV	2	3×10^7	32	5/5	—	0/3	0/3	0/3	3/3	3/3
	4	F V XV (conc. 5-fold)	2	1.5×10^8	19	5/5	—	0/3	0/3	2/3	2/3	3/3
	10	Hyperimmune guinea pig sera				10/10	—	1/3	3/3	3/3	3/3	3/3
	4	Normal guinea pig sera				0/4	—	0/3	0/3	0/3	0/3	3/3

In this and the succeeding table the numerator indicates the number of animals surviving; the denominator, the number of animals injected.

A V = active virus; F V = formolized vaccine, the number following representing the particular vaccine used; i c = intracerebral; s c = subcutaneous; N T = not titrated for virus content prior to formolization.

* Titration prior to formolization revealed 3×10^7 m.i.u. per cc.

the stock virus was diluted decimally in broth to 1:500,000,000 and to each dilution was added an equal volume of undiluted serum. The resulting serum-virus mixtures were tested soon after mixing (kept up to 20 minutes at room temperature) or after retention for 2 or 3 hours at 37°C. Each of three mice was injected intracerebrally with 0.03 cc. of a serum-virus preparation.

EXPERIMENTAL

In Table I is shown a comparison of the antiviral body content of the sera of guinea pigs treated with active virus and with formolized vaccine. The different serum-virus mixtures were tested at the same

TABLE II

Comparative Effect of Incubation in Revealing Antiviral Bodies in Serum-Virus Mixtures

Sera	Serum-virus mixtures injected													
	up to 20 minutes after mixing							after 2½ hours at 37°C.						
	Virus dilutions							Virus dilutions						
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Normal guinea pig	0/3	0/3	0/3	0/3	0/3	1/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3
Guinea pigs injected twice at 7 days' interval with F V XV. Bled 19 days later	0/3	0/3	0/3	0/3	2/3	3/3	3/3	0/3	0/3	0/3	2/3	3/3	3/3	3/3
Guinea pigs injected once with 3×10^5 m.i.u. A V. Bled 30 days later	0/3	0/3	0/3	0/3	3/3	3/3	3/3	0/3	0/3	0/3	2/3	3/3	3/3	3/3
Hyperimmune guinea pig	0/3	0/3	1/3	2/3	3/3	3/3	3/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3

Abbreviations as in Table I.

time and in each case were held at room temperature not longer than 20 minutes before being injected intracerebrally in mice. It is apparent that under these conditions only the hyperimmune guinea pig serum revealed an appreciable quantity of virus antibodies, *i.e.*, neutralizing 100 to 1,000 M.I.D. The sera derived from guinea pigs which resisted the intracerebral test for induced immunity exhibited partial or complete protection against only 1 to 10 M.I.D. of virus. Guinea pigs which had received an amount of virus inadequate to

bring about immunity against the intracerebral test for resistance possessed no significant humoral protective properties. Finally, no distinct difference could be seen in antibody content of serum collected from guinea pigs treated with active or inactive virus.

Table II summarizes the experiments on the relation of incubation to titration of humoral antiviral bodies. Incubation clearly enhanced the protective substance in equine encephalomyelitis antisera. Here a tenfold increase was demonstrable in all of the immune sera tested. Hence to reveal small amounts of antiviral bodies it is best to incubate serum-virus mixtures before animal inoculation.

SUMMARY AND DISCUSSION

An analysis of the preceding experiments discloses that antiviral bodies are demonstrable not at all or in small amounts in the sera of guinea pigs injected with a quantity of active virus not sufficient to induce immunity against the described intracerebral test for induced resistance. However, neutralizing bodies are found in immune animals, although in low concentration, and are regularly manifested when serum is added to low multiples of infective doses of virus under optimal conditions of time and temperature. Hyperimmune serum, on the other hand, reveals a distinct increase in the amount of antiviral bodies present.

Irrespective of the mode of procedure for revealing neutralizing bodies, there does not appear to be any notable difference in the content of such bodies in the serum of animals immunized with active virus or with formolized vaccine in which active virus could not be demonstrated. In other words, the antigenic complexes in active as well as in inactive virus produce similar degrees of antibody reaction. The formolization of virus tissue suspensions, therefore, can be considered as a process whereby the virus is inactivated but the antigenicity of the suspensions is preserved, as is also shown in the preceding paper of this series in tests on tissue immunity. In that article is described the remarkably high degree of tissue immunity which results from injections of inactive virus; now we demonstrate that this resistance is associated with a minimal degree of serum antibody.

Finally, the question may well be asked, if practically no antiviral bodies are demonstrable immediately or soon after mixing immune

serum and virus, and are recognizable in a tenfold increase when functions of time and temperature are brought into play, whether the bodies are "neutralizing" or the phenomenon is due merely to aggregation of virus particles by the serum. From the recent work on the same virus and immune serum (9) by Merrill, there appears to be warrant for the belief in aggregation of virus particles which in turn diminishes the virus activity to the indicated degree.

CONCLUSIONS

Guinea pigs injected with amounts of active equine encephalomyelitis virus inadequate to induce protection against an intracerebral test of 1,000 or more M.L.D. of virus show no significant humoral antiviral bodies. The latter are, however, regularly present in immune animals and are best demonstrated by adding serum to low multiples of infective doses of virus under optimal conditions of time and temperature ($2\frac{1}{2}$ hours at 37°C.). Guinea pigs immunized either with active or with inactive (formolized) virus reveal no distinctive differences in the antiviral body content of their sera.

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