ACTIVE IMMUNIZATION OF GUINEA PIGS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

IV. Effect of Immune Serum on Antigenicity of Active and Inactive Virus

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In one of the preceding papers of this series (1) it was shown that formolization of tissue suspensions containing the active virus of equine encephalomyelitis inactivates the virus. This is reflected by the complete loss of infectivity of even large amounts of material after inoculation by various routes in guinea pigs and mice (1, 2). Yet formolized vaccines induce in these animals a high grade of resistance, as evidenced by development of immunity to as much as 1,000 to 10,000 M.L.D. of virus given intracerebrally. The protection afforded compares favorably, within certain limits, with that evoked by untreated active virus—indeed with the same suspensions of active virus employed in the preparation of formolized vaccines. In another article (3) reference was made to the fact that the antigenic complex existing in active as well as in inactive virus produces practically the same amount of humoral antiviral bodies. The formolization of tissue suspensions of equine encephalomyelitis virus can therefore be regarded as a means of inactivating the virus and at the same time of preserving the antigenicity of the suspension.

In this communication a study is presented on the effects of immune serum on the antigenic capacity of active and of formolized virus.

Methods and Materials

The methods and materials as employed in these experiments, including the Eastern strain of equine encephalomyelitis virus, have already been described in the preceding paper (3). Since guinea pig hyperimmune serum was found to be more potent than ordinary immune serum, the former (Tables I and II, Paper III (3)) was used. This serum, under optimal conditions of time and temperature

(2½ hours at 37°C.), neutralized 1,000 to 10,000 M.L.D. of virus, as manifested by the results of the mouse intracerebral test (3).

The mode of procedure in an experiment was as follows: 1 cc. of hyperimmune serum was injected into subcutaneous tissue of the abdomen of guinea pigs and 1 hour later, 1 cc. of active or formolized virus was inoculated under the skin of the opposite side. The animals received two such courses of treatment at 7 days' interval and were tested for induced immunity by intracerebral inoculation[1] of $> 1{,}000 < 10{,}000$ M.L.D. of virus given 16 days after the last immunizing dose. It will be observed from the protocols to be presented that the amount of serum alone, as given, did not suffice to confer passive immunity.

Immune Serum Followed by Active Virus

In a preliminary experiment, one series of guinea pigs was given active virus alone and another undiluted immune serum followed within an hour by the same virus. The outcome yielded results which prompted us to conduct another experiment with different dilutions of immune serum and with both active and inactive virus.

The results of the preliminary test are summarized in Table I. When active virus alone was used, of guinea pigs receiving two injections of 3×10^5 or more mouse infective units of virus (m.i.u.) (1, 4), seven of seventeen succumbed to virus infection during the period of immunization, the incidence of death being proportional to the amount of virus introduced. Of the survivors, however, all proved to be resistant to an intracerebral test of $>1{,}000$ $<10{,}000$ M.L.D. of virus. On the other hand, those given 3×10^4 or less m.i.u. of virus survived the treatment but only one of eight was found immune to the test for induced resistance. The findings are in agreement with our prior observations (1, 4).

When undiluted hyperimmune serum was injected in advance of the virus, all guinea pigs survived the two courses of inoculation, but none of them proved to be resistant to the described intracerebral test. In other words, in each instance and with concentrations of virus ranging from 3×10^3 to 3×10^8 m.i.u., the relatively small amount of immune serum prevented the antigenic action of the suspensions of active virus.

This blocking effect of undiluted immune serum on the antigenic stimulus of active virus might be considered merely as the result of

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

preventive action of the serum on multiplication of the virus after its introduction in the body, thus producing less antigenic substance and in turn no resistance on the part of the host. That this is not the mode of action can be deduced from the next series of experiments. These were planned to determine the results yielded by smaller

TABLE I

Guinea Pigs Receiving Hyperimmune Guinea Pig Serum and Active Virus or Active Virus Alone

Quantity of undiluted serum in each dose	M.i.u. of virus in each dose	Animals showing fever of 104°F. or above	Animals surviving virus infection during immunization period	Immunity test: Survivors of an intracerebral test of >1,000 <10,000 M.L.D. given 16 days later
1 cc.	3×10^8	3/4	4/4	0/4
None	" "	4/4	1/4	1/1
1 cc.	3×10^7	2/4	4/4	0/4
None	" "	2/4	3/4	3/3
1 cc.	3×10^6	0/4	4/4	0/4
None	" "	3/4	2/4	2/2
1 cc.	3×10^5	2/4	4/4	0/4
None	" "	1/5	4/5	4/4
1 cc.	3×10^4	1/4	4/4	0/4
None	" "	1/4	4/4	1/4
1 cc.	3×10^3	1/4	4/4	0/4
None	" "	0/4	4/4	0/4
1 cc.	None	0/4	4/4	0/4

Animals were given two courses of serum and virus or virus alone at 7 days' interval. In instances in which serum was used, it was followed an hour later by a subcutaneous injection of active virus on the opposite side. Weight of animals = 275 to 325 gm. Denominator represents the number of animals in each test.

amounts of serum and to disclose any blocking effect of both diluted and undiluted immune serum exerted against virus inactivated by formalin. For in the latter material no active virus could be demonstrated (1)—hence no probability of its multiplication *in vivo*—and still a sufficient amount of antigenic substance was present to induce a high degree of immunity (1).

Immune Serum Followed by Active and Inactive Virus

In the following experiment devised to reveal any comparable preventive effect *in vivo* of immune serum on the antigenic capacity of active and of inactive virus, the antigens employed consisted of two materials. The first was active virus as was present in the brain of mice that succumbed to experimental encephalomyelitis. This was employed in doses of 3×10^7 m.i.u., a potent immunizing quantity, and in doses of 3×10^5 m.i.u., the minimal quantity required to immunize guinea pigs against an intracerebral test of 1,000 M.L.D. of virus. The second consisted of formolized vaccine. The last preparation which was shown to be free of demonstrable active virus (1) was made from the same virus tissue suspension employed in the first material just mentioned, and hence contained 3×10^7 m.i.u. before formolization. The data of this experiment are shown in Table II and are summarized as follows:

Untreated, Active Virus (3 × 10⁷ M.i.u.) as Antigen.—With a dose of 3×10^7 m.i.u. of virus given 1 hour after the same undiluted serum as was described in Table I, all animals survived the courses of treatment but none became immune. With 1:4 dilution of serum, again all animals survived; they were, however, proved to be resistant to a later intracerebral test. Finally, with 1:8 or higher dilutions of serum, ten of thirty-one animals succumbed to virus infection during the period of immunization, and all survivors withstood the intracerebral test of >1,000 <10,000 M.L.D. given 20 days after the last immunizing dose.

Untreated, Active Virus in Minimal Immunizing Quantity (3 × 10⁵ M.i.u.) as Antigen.—When 3×10^5 m.i.u. of virus was used, preceded by an injection of either undiluted or 1:4 dilution of immune serum, all animals so inoculated lived through the treatment but only one of eight was found to withstand the intracerebral test for induced immunity. With 1:8 and 1:16 dilutions of serum, all animals survived the subcutaneous injections; three of eight failed, however, to resist the immunity test. With 1:32 or higher dilutions of serum, four of twenty-four guinea pigs died of virus infection during the courses of injections but all survivors except one were successfully immunized to the test dose.

Virus Inactivated by Formalin.—Of eight guinea pigs that received formolized vaccine preceded by the administration of either undiluted or 1:4 dilutions of serum, none succumbed during the period of this treatment and none was found immune to the later intracerebral test. With 1:8 and 1:16 dilutions of serum, all animals withstood the injections and only three of eight the test for immunity. The twenty-four guinea pigs given 1:32 or higher dilutions of serum survived the inoculations, and all but two, the later intracerebral test of >1,000 <10,000 M.L.D. of virus.

TABLE II

Vaccination of Guinea Pigs with Hyperimmune Guinea Pig Serum and Active or Formolized Virus

Dilution of serum	M.i.u. of virus in each dose		Animals surviving virus infection during immunization period	Immunity test
	Active virus	Formolized vaccine		Animals surviving an intracerebral test of >1,000 <10,000 M.L.D. given 20 days later
Undiluted	3×10^7	—	4/4	0/4
	3×10^5	—	"	"
	—	3×10^7	"	"
1:4	3×10^7	—	"	4/4
	3×10^5	—	"	1/4
	—	3×10^7	"	0/4
1:8	3×10^7	—	2/3*	2/2
	3×10^5	—	4/4	2/4
	—	3×10^7	"	"
1:16	3×10^7	—	"	4/4
	3×10^5	—	"	3/4
	—	3×10^7	"	1/4
1:32	3×10^7	—	2/4	2/2
	3×10^5	—	3/4	2/3
	—	3×10^7	4/4	3/4
1:64	3×10^7	—	3/4	3/3
	3×10^5	—	"	"
	—	3×10^7	4/4	4/4
1:128	3×10^7	—	1/4	1/1
	3×10^5	—	4/4	4/4
	—	3×10^7	"	3/4
1:256	3×10^7	—	3/4	3/3
	3×10^5	—	4/4	4/4
	—	3×10^7	"	"
1:512	3×10^7	—	2/4	2/2
	3×10^5	—	3/4	3/3
	—	3×10^7	4/4	4/4
1:1,024	3×10^7	—	"	"
	3×10^5	—	3/4	3/3
	—	3×10^7	4/4	4/4

Animals received two courses of serum and virus at 7 days' interval. Serum injection followed an hour later by an injection of either active or formolized virus on the opposite side. Weight of animals = 275 to 325 gm.

* One of the animals in this group died of an intercurrent, streptococcal infection.

From these experiments it is evident that hyperimmune serum in sufficient quantity injected shortly before the antigen can prevent or block the immunizing action not only of untreated active virus but also of formolized inactive virus. Indeed, the preventive action of 1 cc. of serum is effectively exerted against about 30 million (Table II) to 300 million (Table I) infective units of virus; less serum is needed, however, to block the antigenic capacity of the smaller amount of active agent (300,000 infective units) than of the larger. At this point attention is directed to the fact that with the same serum dilutions, similar results were obtained with formolized vaccine to those with 3×10^5 m.i.u. of active virus—the minimal effective immunizing dose (Table II).

Relation to Immunization with Serum-Virus Combinations.—These experiments, taken together with others carried out by Howitt (5), whose results have been confirmed by our own observations, also have a bearing on the immunization of guinea pigs by means of combinations of immune serum and active virus. It should be stressed that both materials should be introduced into the animal in certain definite proportions if the desired immunity without death from virus infection during the period of immunization is to be attained. Thus with the particular hyperimmune serum studied (Table II) and with 3×10^7 m.i.u. of virus, the optimal conditions for inducing immunity were achieved by employing 1:4 dilution of serum; with 3×10^5 m.i.u., 1:16 to 1:64 dilutions. Furthermore, the proper dilution of each individual immune serum sample should be determined by test with each individual quantity of active virus employed.

Equine encephalomyelitis virus differs in this respect from certain other virus agents, such as yellow fever, in which "when the optimal amount of immune serum has been ascertained, a wide range of virus concentration can be used with success" (6) for immunization. It is clear, nevertheless, that formolized vaccines containing inactivated equine encephalomyelitis virus could replace injections of combined serum and virus for the immunization of guinea pigs with elimination of the necessary, laborious, serum titrations.

It has been assumed that generally antiserum in neutralized and overneutralized suspensions of virus employed for artificial immunization of animals, acts to prevent the development of immunity by

inhibiting the multiplication of virus and thus the production of more antigen in the body. The results of the foregoing experiments show that in so far as the encephalomyelitis virus is concerned, the serum blocks the development of immunity in the instance in which suspensions containing inactivated, nonmultiplying virus are used as immunizing preparations.

SUMMARY AND DISCUSSION

A study was undertaken on the effect *in vivo*, in the guinea pig, of equine encephalomyelitis virus antiserum upon the antigenic response to active, as compared with that to formolized, inactive virus. It was found that when animals were given subcutaneously a proper amount of hyperimmune serum 1 hour before inoculation, in the subcutis, of either active or of inactive virus, no immunity was induced against an intracerebral test of more than 1,000 and less than 10,000 M.L.D. of virus. This preventive power of the serum was lost by its dilution, the loss being proportional to the dilution, and, on the other hand, more serum was needed to obtain the blocking effect as the quantity of virus was increased. When an insufficient amount of serum was introduced into the animals along with the same quantities of active virus or formolized vaccine, a certain number of those receiving the untreated virus succumbed to virus infection in the course of the inoculations, but the survivors were rendered resistant to the intracerebral test; all the guinea pigs treated with higher dilutions of serum and with formolized material were brought safely to an immune state.

The point to be stressed then is that antigenic stimuli present in untreated active virus and in formolized virus tissue suspensions in which no active virus is demonstrable by drastic tests (1) and which are wholly noninfective in animals (1), are completely inhibited from acting by the use of proper amounts of immune serum.

The mechanism underlying this preventive power of adequate amounts of serum may be explained on the basis of facts deduced in preceding papers of this series (1, 3) and in the present article. We have shown that 3×10^7 m.i.u. of active virus contains a sufficient amount of antigen to induce immunity without the necessity of its multiplication in the animal body. This has been fully established by the similar degree of resistance brought about by 3×10^7 m.i.u. of

virus formolized to a degree in which no active virus could be revealed (1). The assumption that the blocking effect of serum in the quantity employed prevents multiplication of the virus which is reflected in the production of inadequate amounts of antigen, is therefore untenable, since this effect was obtained when a sufficient amount of antigen was present in "living" as well as in "killed" virus. On the other hand, with insufficient amounts of immune serum (to be noted in higher dilutions shown in Table II), only the active virus could multiply—the formolized vaccine was not affected in respect to its antigenicity by these quantities of serum—and so produce more antigenic substance. This substance, in turn, brought about greater resistance in the host.

The precise action of proper amounts of serum in preventing development of immunity by both active and inactive virus is not definitely known. However, two hypotheses are offered for consideration: the first implies that the action of the serum is direct, that is, by entering into combination with the antigens to bar antigenic capacity; the second ascribes to the serum an indirect action, on the cells of the body, in such a way as to make them unable to react to the antigenic stimuli present in the inoculated materials.

The identity of these antigenic stimuli in virus suspensions containing the active, infective agent or this agent inactivated by formalin is at the present time undetermined. If virus were obtainable in pure state, free from extraneous material, the answer to this question might be readily given, but it is quite a different matter when the substance called virus is a mixture of the infective agent, of inflammatory tissue products, of tissue, etc. We have, however, shown that induced immunity is not due to the presence of "living" virus, but whether the antigenic action originates from "killed" virus or from another constituent of the suspension is not clear. On the other hand, Sabin (7) suggests the possibility that the virus may not be the direct antigenic stimulus but that some substance on which it acts and which becomes liberated from infected cells may be the factor responsible. While this subject awaits the results of further study, we believe that formalin inactivates the infective agent in virus suspensions and preserves the antigenic component therein, whatever its nature may be.

It would be of interest if this phenomenon of prevention of antigenic

capacity by proper amounts of immune serum might apply to such materials which by their very nature do not multiply in the body of the host, *e.g.*, toxin and antitoxin. Theobald Smith (8) and later Park (9) demonstrated that in mixtures of diphtheria toxin-antitoxin, when smaller amounts of immune serum (antitoxin) are used, the toxicity of the mixture is retained and immunity results; if the serum is increased, toxicity is reduced and immunity occurs irregularly, and if more serum is added, no toxicity nor immunity results. This is supported by the experiments of Hartley (10) on washed precipitates from underneutralized, neutral, and overneutralized mixtures of antitoxin and toxin: those derived from underneutralized material are toxic and powerfully antigenic; those from neutralized, atoxic and of good antigenic action, and from overneutralized, atoxic and of low antigenicity. Hartley states, moreover, that the precipitate reactions of toxicity and antigenicity bear a close relationship to the nature of the mixture from which they are produced.

There is, therefore, a connection between the preventive reactions of the serum on the two forms of virus and of antitoxin on toxin in respect to toxicity and antigenicity. Furthermore, the toxin is rendered atoxic with retention of immunizing capacity by formalin: the production of toxoid or anatoxin (Glenny and Hopkins (11), Ramon (12))—again a condition related to the effects following formolization of the virus. It has, however, been stated that "in an immunizing mixture prepared with modified [formolized, but partially detoxified] toxin the antitoxin present does not within wide limits affect the antigenic power" (Glenny, Hopkins, and Pope (13)). It is not known whether a preliminary injection of antitoxic serum could have prevented the antigenic power of fully detoxified toxin, that is, after the passive immunity induced by the serum disappears. If a preventive action of antitoxic serum could be shown under these circumstances, a remarkable correlation of the reactions of proper amounts of antitoxin to toxoid and of proper amounts of immune serum on the virus would be evident.

Finally, the inhibition of antigenic power of both active and inactive virus by immune serum has been demonstrated to apply to the virus of equine encephalomyelitis in guinea pigs and no generalizations of the application of the phenomenon to other viruses are intended.

CONCLUSION

In active equine encephalomyelitis virus and in the virus inactivated by formalin, there is a sufficient amount of antigen, without necessity of multiplication in the body, to produce immunity in guinea pigs against >1,000 <10,000 intracerebral lethal doses of virus. The antigenic capacity of both materials can be blocked to the same extent by the action of an appropriate amount of hyperimmune serum. The bearing of these findings on the mechanism of immunity induced by virus inactivated by formalin is discussed.

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