ENDURING IMMUNITY FOLLOWING VACCINATION OF MICE WITH FORMALIN-INACTIVATED VIRUS OF RUSSIAN SPRING-SUMMER (FAR EASTERN, TICK-BORNE) ENCEPHALITIS

Correlation with Serum-Neutralizing and Complement-Fixing Antibodies*

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The present paper is concerned with the working out of a laboratory test, other than actual infection, which would indicate the efficacy of vaccination with the inactivated virus of Russian spring-summer encephalitis through the determination of the titer of circulating antibody. If a direct correlation exists between the antibody and immunity, the test should provide an indicator of the degree of immunity. In our laboratory such a correlation has been demonstrated with the virus of equine encephalomyelitis (1–3), but the results were based on a relatively short period, after immunization, in the life-span of the animal. Now, a study is presented of the immune response of mice to vaccination with formalin-inactivated virus that includes (*a*) immunity on intracerebral and on peripheral inoculation of test doses of active virus; (*b*) complement-fixing substance, and (*c*) neutralizing antibody determined by intraperitoneal and intracerebral methods, over a period covering almost the entire life of the animal. This paper will describe, furthermore, an enduring immunity of high degree that develops following a single course of two intraperitoneal injections of formolized, inactivated virus. Finally, the possible application of the results to vaccination of man will be discussed.

The virus of Russian spring-summer encephalitis (4) is notably well adapted to investigations of the sort outlined, since it has a peripheral portal of entry, under natural conditions being transmitted to man by ticks, while furthermore it is pathogenic for mice (among other animals) by several routes of inoculation, including the subcutaneous and the intraperitoneal. Subcutaneous injection as employed experimentally approximates the natural route of infection in man; and since the pathogenicity of the virus when thus introduced is not affected by the age of the mice employed, a challenge subcutaneous injection is possible at any time during their life-span.

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Kagan (5) reported that mice could be immunized by means of formolized virus of Russian spring-summer encephalitis. Immunity to infection by intracerebral or subcutaneous routes was thereby achieved, but serum-neutralizing antibody, as determined by the intracerebral method, could not be detected until late; no correlation was made out between immunity and circulating antibody. Smorodintsev (6), on the other hand, stated that there is a close correlation between immunity to infection by the subcutaneous route and circulating neutralizing antibody as determined by the intracerebral method, but his protocols were limited and his observations carried out for no longer than 2 months.

It should be mentioned here that formolized virus of Russian spring-summer encephalitis has been used in epidemic areas as a vaccine in man apparently with good results (6).

Materials and Methods

Virus.—The strain of Russian spring-summer encephalitis virus used in this work was transported in ticks (7) in 1941, from the laboratory of Dr. M. P. Chumakov in Moscow, U.S.S.R., to that of Dr. R. R. Parker, Director of the Rocky Mountain Laboratory of the United States Public Health Service, at Hamilton, Montana. This strain was made available to us by Dr. Parker and Dr. H. R. Cox in the fall of 1942, through the Commission on Neurotropic Virus Diseases, in the form of mouse brain in 50 per cent glycerol. From this material, successive mouse-to-mouse passages by the intracerebral route were carried out. The virus has been kept in our laboratory with a minimum number of mouse passages (four or five), by storing a suspension of infected mouse brain in buffered distilled water in a carbon-dioxide refrigerator at −76° C. As virus was needed, mice were inoculated intracerebrally with the stock suspension and the required virus was obtained from their brains. As diluent for the virus, except for the preparation of vaccine, fresh normal rabbit serum in physiological saline solution in a proportion of 1 to 9 was used.

Animals.—Albino mice of the W-Swiss strain (8) were used for this study. All animals were purchased from one dealer and their age and average weights determined; uniform ones were selected for each experiment. Following injection with serial dilutions of virus, the mice were housed in metal boxes, each containing no more than four or five animals which had received the same dilution of virus. This was done because mouse-to-mouse cage infection had been noted, probably the result of cannibalism, when large numbers of mice inoculated with different dilutions of virus were kept together. By segregation according to dilutions used, transmission of infection from one animal to another in the same cage was avoided, and it was assumed that when this did happen, the error could be limited within one dilution.

Vaccine.—Suspensions of formalin-inactivated virus were prepared as vaccines as follows: From the stock suspension of virus in the dry-ice refrigerator, a 10^{-2} suspension was made and groups of 40 to 50 mice, about 3 weeks old, were inoculated intracerebrally. When sick or prostrate, usually on the 4th day after injection, they were sacrificed, their brains removed and emulsified in physiological saline solution in a Waring blendor to a concentration of 10 per cent mouse brain, and 0.5 per cent formalin was added (final concentration). The suspension was stored in the refrigerator at 4° C. in a glass-stoppered flask and left there long enough for inactivation, usually 10 to 12 days. At the end of that time, the vaccine was tested for virulence by intracerebral injection into ten mice, 20 to 22 days old. They were observed for 21 days and if any of them died the test was repeated until no virus could be recovered from the vaccine. Since no preparation could be considered avirulent until the end of this test, vaccines were not used until 35 and 45 days after preparation, which was more than three times the number of days required to inactivate the virus under the conditions described. No attempt was made to determine how long the vaccine might be stored and still be effective.

Vaccination.—Mice were vaccinated by means of two intraperitoneal injections of 0.2 cc. each of the 10 per cent vaccine at 2 days' interval, making a total of 0.4 cc. of vaccine for each mouse. Although solid immunity can be achieved with less vaccine, no attempt was made in these experiments to find out the smallest amount.

Challenge Test.—At definite intervals, before or after vaccination was begun, mice were tested for immunity to the virus. Five mice, 22 to 25 days of age, received intracerebrally a 10^{-3} dilution of frozen virus. When the animals were prostrate or sick, usually on the 4th day, their brains were removed, ground in a mortar, and suspended in saline-serum (10 per cent rabbit serum in 0.9 per cent saline solution) diluent to a concentration of 10^{-1}. This suspension was then centrifuged at 2000 R.P.M. for 10 minutes, the supernate removed and diluted with saline-serum in serial tenfold dilutions from 10^{-2} to 10^{-11}. Vaccinated mice as well as controls were inoculated with these suspensions, five animals usually being employed for each dilution; 0.5 cc. of virus suspension was introduced into the subcutaneous tissue of the groin. When the intracerebral route was used, the inoculum consisted of 0.03 cc.

Neutralizing Antibody.—For the investigation of circulating antibody, mice were bled from the heart under ether anesthesia,[1] the blood from individual animals was pooled and, after standing at room temperature for 1 hour, centrifuged. The sera were either tested immediately after or were kept at −76° C. until studied. Thus at the same definite intervals after vaccination when mice were tested for immunity, other mice from the vaccinated and control groups were bled and the material was kept frozen.

Neutralizing antibody was detected by mixing undiluted serum with increasing dilutions of virus. To this end, a 10^{-1} fresh suspension of virus was prepared as described under Challenge test. After centrifugation at 2000 R.P.M. for 10 minutes, the supernate was further diluted in serial tenfold dilutions beginning with 2×10^{-2} and ending with 2×10^{-10}. 0.4 cc. of each of these dilutions was added to 0.4 cc. of undiluted serum deriving from control or from vaccinated mice. The mixtures were incubated at 37° C. for 2 hours in a water bath. Two different routes of inoculation, intracerebrally with 0.03 cc. and intraperitoneally with 0.1 cc., were used in mice 25 to 35 days old. With each dilution five mice were injected for the intracerebral test and four for the intraperitoneal.

Complement-Fixing Antibody.—All samples of sera were tested for complement-fixing antibody against the virus. Mouse brain antigens were used; the method of procedure and of preparation of antigen has been described (9). Antigen and serum controls were included, and in all cases the sera were tested against another unrelated brain antigen, *e.g.* of Western equine encephalomyelitis or St. Louis encephalitis viruses, in order to rule out non-specific reactions. The highest dilutions of serum giving a 2+ or higher reaction determined the titer of the serum. Sera from both vaccinated and from control mice were always tested together.

Estimation of End-Points, Neutralization and Immunity Index.—The mice used in this work were uniform in their response to inoculation of the virus regardless of their age. Hence the detailed protocols of neutralization or immunity tests presented no difficulty for evaluation of the titer of the virus. The $L.D._{50}$ titer was calculated according to the formula of Reed and Muench (10). From this the immunity or neutralization index was obtained as the ratio between $L.D._{50}$ of virus in vaccinated mice and in control mice (the index of the latter being taken as 1). For simplification, neutralization or immunity indices of 1 to 50 were expressed as the nearest whole number, of 50 to 100 to the nearest 10, 101 to 1000 to the nearest 100, 1001 to 10,000 to the nearest 1000, and so on.

EXPERIMENTAL

Correlation of Serum-Antibody with Immunity on Peripheral Inoculation of Virus

The type and degree of correlation between circulating antibody and immunity on peripheral inoculation of a test dose of virus in a vaccinated mouse were determined from the results of the two following experiments.

[1] All operations on animals were performed with the aid of full ether anesthesia.

TABLE I

Immunity on Challenge Tests, Virus-Neutralizing, and Complement-Fixing Antibodies in Mice, Following Vaccination with Formalin-Inactivated Virus of Russian Spring–Summer Encephalitis

Day of test	Treatment of mice	Immunity of mice on subcutaneous injection of virus		Neutralization test with sera of mice				Complement-fixing titer of sera
				Intracerebral route		Intraperitoneal route		
		L.D.$_{50}$	Immunity index	L.D.$_{50}$	Neutralization index	L.D.$_{50}$	Neutralization index	
−4	V	8.7	−1					
	C	8.6						
−2	V	7.5	−2					
	C	7.3						
2	V	<2.0	>4,000,000	7.8	4	7.2	6	0
	C	8.6		8.4		8.0		0
3	V	<3.5	>30,000	7.4	2			0
	C	8.0		7.7				0
6	V	<1.5	>12,000,000	7.6	4	6.8	25	1:2
	C	8.6		8.2		8.2		0
7	V	<2.5	>300,000	7.2	1			1:8
	C	8.0		7.3				0
13	V	<1.5	>63,000,000	6.6	8	<1.5	>2,000,000	1:16
	C	9.3		7.5		7.8		0
14	V	<1.5	>16,000,000	6.7	6			1:16
	C	8.7		7.5				0
21	V	<1.5	>50,000,000	6.8	5	<2.0	>160,000	1:16
	C	9.2		7.5		7.2		0
29	V	<1.5	>50,000,000	7.0	6	<2.0	>500,000	1:16
	C	9.2		7.8		7.7		0
31	V	<1.5	>200,000	7.3	1	2.0	2,000,000	1:16
	C	6.8		7.4		8.3		0
61	V	<1.5	>10,000,000	7.4	2	3.9	3,000	1:2
	C	8.5		7.6		7.4		0
120	V	<1.5	>3,000,000	7.0	6	2.0	1,600,000	1:8
	C	8.0		7.8		8.2		0

TABLE I—*Concluded*

Day of test	Treatment of mice	Immunity of mice on subcutaneous injection of virus		Neutralization test with sera of mice				Complement-fixing titer of sera
				Intracerebral route		Intraperitoneal route		
		L.D.$_{50}$	Immunity index	L.D.$_{50}$	Neutralization index	L.D.$_{50}$	Neutralization index	
224	V	<1.5	>16,000,000	7.5	8	3.3	10,000	1:2
	C	8.7		8.4		7.3		0
348	V	<2.5	>300,000	6.7	6	2.4	20,000	1:2
	C	8.0		7.5		6.7		0
437	V	<1.5	>8,000,000	5.6	8	<2.0	>30,000	1:2
	C	8.4		6.5		6.5		0

L.D.$_{50}$ = log of dilution giving the 50 per cent end-point.

Immunity index = ratio between L.D.$_{50}$ of virus in vaccinated mice and in control mice.

Neutralization index = ratio between L.D.$_{50}$ of virus in presence of serum from vaccinated mice and that in presence of serum from control mice.

Complement-fixing titer = highest dilution of serum giving a 2+ or better reaction. First dilution is 1:2.

V = vaccinated, C = control, not vaccinated. Neutralization and immunity index of control is 1.

Experiment 1.—700 mice, 60 to 90 days of age and weighing from 20 to 26 gm. at the beginning of the experiment, were selected. 350 were set aside as untreated controls; the other 350 were vaccinated with formalin-inactivated, mouse brain vaccine which had been prepared 45 days before its use. Two injections of 0.2 cc. each were given intraperitoneally at 2 days' interval. Then at intervals of 3, 7, 14, 21, 29, 61, 120, 224, and 348 days, tests for immunity on subcutaneous injection of virus and for neutralizing and complement-fixing antibody were performed in the groups of vaccinated mice and corresponding controls.

Experiment 2.—This experiment was a repetition of the first; here the tests for immunity and for the presence of antibody were carried out at other intervals. In addition, two groups of mice were injected with virus first and then vaccinated, in order to ascertain whether vaccination following exposure would be effective.

680 mice from 40 to 70 days old at the beginning of the test were selected and of these 300 were kept untreated as controls. The others were immunized in the same way as in Experiment 1, that is, 0.2 cc. of vaccine was given twice, the vaccine having been prepared 35 days before. Two groups of the latter mice were injected subcutaneously with virus 4 and 2 days before they were given the first dose of vaccine. The corresponding controls were injected with virus alone; *i.e.*, no vaccine was given them. The remaining animals, vaccinated in the usual way, and untreated, control mice, were tested in groups for peripheral resistance and antibodies at the following intervals from the date of the first dose of vaccine: 2, 6, 13 31, and 437 days. Besides, tests for circulating antibody, although not for immunity, were carried out 203 and 206 days after vaccination. Finally, on the 437th day, a group of mice were tested for immunity on intracerebral, as well as on subcutaneous inoculation of virus.

Immunity to Virus Given Subcutaneously.—Since the results obtained in both experiments were similar, they were combined and are shown in Tables

I and II and graphically in Text-fig. 1. It is apparent that a single course of two doses of vaccine induced a solid protection against virus given subcutaneously at varying intervals from 2 days to 15 months. It was not possible in any of the tests to bring down a vaccinated mouse (with the only exception of two on the 2nd day test), even when a 10^{-2} dilution of virus (*i.e.*, from 100 thousand to 10 million lethal doses) was given. Therefore the values of the immunity index as stated are only an indication of the minimal amount of virus resisted, since no end-point reading was obtained in the vaccinated mice. The fluctuations in the values of the immunity index are due to the fact that the titer of the virus in the controls varied from test to test from a minimum of $10^{-6.8}$ to $10^{-9.3}$. Vaccination carried out 4 and 2 days after injection of virus did not modify the degree of the induced immunity.

Immunity to Virus Given Intracerebrally.—On the 437th day after vaccination, mice were challenged by active virus introduced into the brain (Table III). Vaccinated mice revealed scarcely any immunity to the test dose of virus as compared with controls: the L.D.$_{50}$ was $10^{-7.3}$ in the vaccinated, and $10^{-7.5}$ in the unvaccinated. On the other hand, mice similarly vaccinated but tested subcutaneously had, on the same (the 437th) day, a solid immunity (Table II) to 8 million, at least, M. L. D. Other instances were observed which added to the evidence that it is difficult to induce immunity to virus given intracerebrally, by means of similar dosages of formalin-inactivated virus of Russian spring-summer encephalitis. Thus a wide difference exists in the degree of protection afforded by a given preparation of inactivated virus, according to the route (intracerebral or peripheral) used for the challenge test.

Serum-Neutralizing Antibody.—A striking contrast was found in the results of neutralization tests performed by intraperitoneal and by intracerebral routes. As shown in Tables I and II and Text-fig. 1, mixtures of virus and antiserum introduced intracerebrally gave neutralization indices in vaccinated series between 1 (*i.e.*, the same as for the controls) and 8. According to accepted practice these low values might be taken to indicate that no circulating antibody was present in the vaccinated mice. The same serum-virus mixtures, however, when injected intraperitoneally, yielded an entirely different result: Neutralizing indices of the sera from vaccinated mice were 6 on the 2nd day, 25 on the 6th, 2,000,000 or more on the 13th and 31st days, and high thereafter to the 437th day after vaccination. Again here, as with the index of immunity to infection, an end-point of the higher values was not often reached. The neutralization index (Table I) represents only the minimal number of M.L.D. against which sera protected. To conclude, a correlation was observed between immunity on peripheral injection of virus and circulating neutralizing antibody as determined by the intraperitoneal route. Only on the earliest days, namely, the 2nd and 6th, was solid immunity after injection of virus unaccompanied by as high a titer of neutralizing antibody as in the more remote periods after vaccination.

Complement-Fixing Antibody.—Complement-fixing antibody revealed itself on the 6th day following vaccination with a 1:2 titer which became 1:16 in about 2 weeks and remained there or somewhat lower, 1:8, to the 4th month.

TABLE II

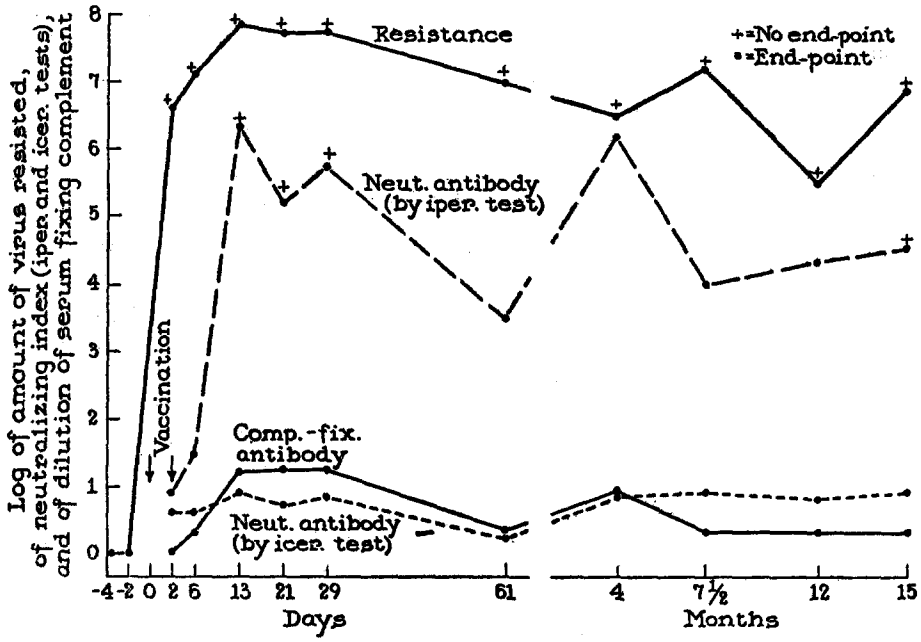
Results of Immunity and Neutralization Tests in Mice Vaccinated with Formalin-Inactivated Virus of Russian Spring-Summer Encephalitis

Day of test	Treatment of mice	Immunity test: Fate of mice given virus in dilution (log)										Intracerebral neutralization test: Fate of mice given virus in dilution (log)							Intraperitoneal neutralization test: Fate of mice given virus in dilution (log)								
		−2	−3	−4	−5	−6	−7	−8	−9	−10	−11	−4	−5	−6	−7	−8	−9	−10	−11	−2	−3	−4	−5	−6	−7	−8	−9	−10
−4	V		5	5	5	5	5	4	2*																			
	C						5	5	1	0	0																	
−2	V		5	5	4	4	4	2	2																			
	C					5	3	1	0	0	0																	
2	V	1	1	0	0	0	0	0	0				5	5	5	2	0	0		4	3	3	4	4	3	1*		
	C					5	5	5	1	0					5	4	0	0	0				4	4	4	2	0	0
6	V	0	0	0	0	0	0	0						5	5	1	0	0		3	3	4	4	3	2	2		
	C					5	5	4	2	0				5	5	3	0	0						4	4	2	1	0
13	V	0	0	0	0	0	0	0					5	5	1	0	0	0		0	0	0	0	0	0	0		
	C					5	5	5	3	1				5	4	1	0	0						4	3	2	0	0
21	V	0	0	0	0	0	0	0	0			5	5	4	2	1	0	0		1	0	0	0	0	0	0		
	C			5	5	5	5	5	3	0				5	4	1	0	0	0		4	4	4	4	2	1	0	
29	V	0	0	0	0	0	0	0				5	5	5	2	1	0	0		0	1	1	0	0	0	0		
	C			5	5	5	5	5	3	0				5	5	2	0	0	0		4	4	4	4	4	1	0	
61	V	0	0	0	0	0	0	0				5	5	5	4	0	0	0		4	3	0	2	0	2	0		
	C				5	5	5	4	1	1	0			5	5	1	0	0					3	4	4	0	0	
120	V	0	0	0	0	0	0	0					5	5	2	1	0			1	1	0	1	0	0	0		
	C					5	4	3	0	0				5	5	2	0	0					4	4	3	3	0	0
224	V	0	0	0	0	0	0						5	5	4	1	0			3	1	1	2	1	0	0		
	C				4/4	4/4	4/4	4/4	1/4	0/4‡				5	4	4	1	0					4	4	3	0	0	0
348	V		0/3	0/3	0/3	0/3	0/3						5	5	1	0	0			0	1	2	0	1	1	0		
	C				4/4	4/4	3/4	2/4	0/4	1/4				5	5	0	0	0					4	4	1	0	0	0
437	V	0	0	0	0	0	0						5	1	0	0	0			0	2	1	0	0	0	0		
	C			4/4	3/4	4/4	4/4	4/4	1/4	0/4				5	0	0	0	0					4	4	0	0	0	

*Mice dead of 5 inoculated (in intraperitoneal test, of 4 inoculated).

‡ 4/4 = 4 mice dead of virus infection of 4 used in test.

Thereafter the titer was low, 1:2, or negative. In view of this low reading at stated intervals after the 4th month, one might assume that the complement-fixation test is of dubious value as an indicator of immunity. However, when the test was positive, the immunity on peripheral inoculation of virus was of high degree.



TEXT-FIG. 1. Immunization by intraperitoneal injection of formalin-inactivated virus of Russian spring-summer encephalitis.

TABLE III

Immunity on Intracerebral Injection of Russian Spring-Summer Encephalitis Virus in Mice Vaccinated with Formalin-Inactivated Vaccine

437th Day from Vaccination

Treatment of mice	Fate of mice given virus in dilution (log)							L.D.$_{50}$	Immunity index
	-4	-5	-6	-7	-8	-9	-10		
V	4/4	4/4	4/4	3/4	0/4	0/4		7.3	2?
C		3/3	3/3	2/4	1/4	1/4	0/4	7.5	

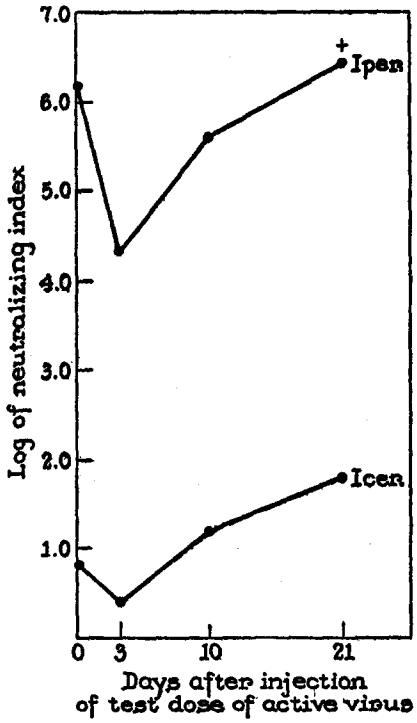
See other tables for abbreviations.

Neutralizing Antibody Following Virus Inoculation during a Late Stage of Active Immunity

The correlation of immunity, on subcutaneous infection, with high titer of antibody, as determined by the intraperitoneal method, together with the absence of appreciable immunity on intracerebral introduction of virus, suggested that the circulating antibody prevented the spread of virus from the site of inoculation to the central nervous system, as happens in the case of

equine encephalomyelitis virus (1, 11). Additional evidence for this was found by following the titers of neutralizing antibody in mice vaccinated as described and then, at a much later date, exposed to the virus by the subcutaneous route.

Experiment 3.—One group of vaccinated mice from Experiment 1 and another of corresponding controls were bled on the 120th day after vaccination. Ten additional vaccinated mice were injected subcutaneously with 0.5 cc. of virus in a 10^{-5} dilution (approximately 1000 lethal doses). 3, 10, and 21 days after inoculation of virus, these mice were bled from the heart



TEXT-FIG. 2. Neutralization tests by intracerebral and intraperitoneal methods following vaccination and injection of test doses of active virus given on the 120th day after vaccination.

and the sera tested for neutralizing antibody both by intracerebral and intraperitoneal routes. Between the interval of bleeding and the test, the sera were kept frozen. Although the blood had been derived from mice inoculated with active virus, it was not infective. The sera were tested simultaneously with those derived from mice not given test doses of virus, on the 120th day after vaccination. The results, shown graphically in Text-fig. 2, revealed a definite drop in the amount of virus neutralized by serum by the intraperitoneal test, especially on the 3rd day after injection of virus. The neutralization index of the serum increased on the 10th day and was at the same level, or even higher than it was originally, on the 21st day. The results of neutralization by the intracerebral test paralleled those obtained by the intraperitoneal method but at a much lower level.

It would seem from this test that the introduction of active virus into animals already having neutralizing antibody brings about within a few days a drop in the protective power of their serum, as though the antibody were being used for the neutralization of the virus introduced into the immune animals (*cf.* Schlesinger, Olitsky, and Morgan, 12). Eventually, within about 3 weeks, the serum either regains the original high titer or shows a still higher titer than ever before.

DISCUSSION

The immunity of albino mice after a single course of two injections of 0.2 cc. of formalin-inactivated virus of Russian spring-summer encephalitis has been observed, with tests for immunity given just before vaccination, and at varying periods from 2 days to 15 months after vaccination. At the final period, the oldest mice were about 1½ years of age, and all of them were showing signs of old age. The vaccines employed were free from active virus in so far as the present tests could show: (1) Vaccines were injected 35 and 45 days after preparation and in no instance has active virus been recovered after 12 days' formalinization. (2) Virus was not recovered from the blood deriving from vaccinated animals and collected 1 hour, or later, after treatment. (3) Mice exhibited no visible signs of illness after vaccines were introduced intracerebrally or intraperitoneally.

After vaccination, an immunity of such pronounced character prevails in mice during almost a lifelong period that they can resist many thousands of lethal doses of virus injected peripherally. This finding is in line with the encouraging results of vaccination of man against the Russian spring-summer virus observed by Smorodintsev and his colleagues (6). The fact that a strong and enduring immunity results from subcutaneous injection of inactivated virus is a point of practical interest. Such an immunity, following a single course of injections, is highly unusual, and is ordinarily called forth only by infection with virus, or through the persistence of the latter within the body of the host (so called "persistence immunity").

It should be pointed out, however, that the total amount of formalin-inactivated virus given in the single course of vaccination (0.4 cc.) is an extraordinarily large amount. The dosage was selected at random, no attempt having been made to find the minimal amount of vaccine which would be needed to induce so great an immunity. The amount required in mice may give no index to the quantity needed for effective immunization of other species including man, as was shown by Cox and Olitsky (13) in connection with the relative dosages of equine virus vaccines needed for the protection of the mouse and the horse, as well as by Beard *et al.* (14) for the production of neutralizing antibody against the latter virus in the mouse and in man.

A wide difference was noted between the immunity on subcutaneous exposure

to the virus and that on intracerebral. For example, mice tested about 15 months after vaccination exhibited resistance to at least 8 million lethal doses of virus introduced subcutaneously but not to more than two doses of virus injected into the brain. It is probable that the immune reaction is consummated chiefly in the tissues and blood at some place between the site of introduction of the virus and its entrance into the central nervous system. However this may be, the intracerebral method of testing for immunity is not sufficiently delicate to indicate the degree of immunity developed. The challenge test for efficacy of preparations of vaccines should therefore be carried out by peripheral routes which offer a more delicate test, as shown by Cox and Olitsky (13) for equine encephalomyelitis virus, and by Webster (15) for rabies virus. The results, furthermore, can be better correlated with a natural disease in which the portal of entry of the virus is peripheral, as by an insect or tick bite.

A noteworthy disparity in result is encountered when the neutralization test is performed by the intracerebral and by the intraperitoneal routes respectively, intraperitoneal injection being far the more efficacious. The difference has already been pointed out for several viruses (16, 17). In mice vaccinated with the Russian spring-summer encephalitis virus the neutralization index by the intracerebral method was 8 or less; by the intraperitoneal, later than the 6th day after vaccination to the 15th month, several thousand to more than 2 million. Indeed, in one instance the intracerebral test showed a neutralization index of 1—the value for the control—while the intraperitoneal exhibited an index of 2 million. However, with one or two exceptions in a score of tests, the readings of the intracerebral neutralization test were higher than 1, that is, 2 to 8, numbers generally considered hitherto as not significant in indicating neutralization of neurotropic viruses (18). But such low indices have meaning since (*a*) they can be obtained on repetition; (*b*) they are correlated with a large amount of antibody—never with an absence—as determined by the intraperitoneal test and (*c*) neutralizing antibody revealed by the latter test exhibits a close correlation with immunity to peripheral challenge doses of Russian spring-summer virus.

With respect to correlation of immunity to circulating antibody, it is of interest that the index of immunity on intracerebral injection of virus on the 437th day after vaccination was 2, whereas on the subcutaneous challenge dose it was greater than 8 million. The intracerebral immunity index at 2 was of the same magnitude as the intracerebral neutralization index; the high index of immunity on subcutaneous test with active virus was in effect associated with the high index of neutralization shown by the intraperitoneal method. This seems to be the interrelation, in general, of the four factors involved, and may apply to other neurotropic viruses as well (16, 17). The practical point is that tests for potency of vaccines should, when possible, depend on the use

of peripheral routes both for the challenge test for immunity and for the neutralization test for antibody. It should be stated here that the low indices of neutralization obtained by the intraperitoneal route on the 2nd and 6th days after vaccination do not disturb the concept of a correlation of immunity with neutralizing antibody; for the incubation period of the Russian spring-summer virus is 8 to 12 days following its subcutaneous introduction. In the earliest phase following vaccination the production of antibody proceeds apace and it is apparent that a sufficient amount is available to block the progression of virus from the site of injection to the central nervous system.

Attention should be drawn to the fact that the possible presence of virus in the test serum in early days after the challenge exposure of vaccinated mice to active virus may serve to reduce the amount of detectable antibody (Text-fig. 2) (12).

Finally, the relation of complement-fixing antibody to immunity in vaccinated mice is not as close as that of neutralizing antibody: the former is slower in development and persists for a shorter time—after 4 months the titer is low or practically negative. Its presence is, however, accompanied by solid immunity whereas the converse does not hold.

The findings here reported on the production in mice of an enduring, staunch immunity developed through the use of formalin-inactivated virus; the correlation of immunity with serum-neutralizing antibody, and the demonstration of effective immunization by titration of such antibody by means of appropriate tests, may have application to vaccination of man with the Russian spring-summer encephalitis virus. How far these applications go can be determined only by trials in the field.

CONCLUSIONS

A single course of two intraperitoneal injections of formalin-inactivated virus of Russian spring-summer encephalitis induced in albino mice a solidly immune state which endured almost throughout life. Active virus is therefore not essential for the production of a high degree of lasting immunity. The immune response to vaccination consists of resistance to peripherally introduced active virus and development of circulating antibody.

A correlation has been found to exist throughout the long period of the immune state between the titer of neutralizing antibody, as determined by the intraperitoneal method described, and the degree of immunity to peripherally introduced active virus. Thus laboratory tests for the immunizing power of a vaccine suggest themselves, to be carried out by an estimation in vaccinated mice of (*a*) immunity to peripherally inoculated active virus, and (*b*) serum virus-neutralizing antibody determined by the intraperitoneal method.

The rôles as indicators of immunity in vaccinated mice of complement-fixing antibody in the serum, of the intracerebral challenge dose of virus, and of the intracerebral method for testing neutralizing antibody are discussed.

Finally, if the immune response of man to vaccination with formalin-inactivated virus of Russian spring-summer encephalitis follows the pattern of the response of mice as here described, and if the correlation of neutralizing antibody with immunity to peripherally introduced virus applies to man as to mice, then possibly the degree of immunity in human beings following vaccination can be appraised by a peripheral test for neutralizing antibody in the serum.

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