THE MECHANISM OF ACTIVE CEREBRAL IMMUNITY TO EQUINE ENCEPHALOMYELITIS VIRUS

II. The Local Antigenic Booster Effect of the Challenge Inoculum

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In earlier studies on the mechanism of immunity to the viruses of Eastern (E.E.E.) or Western equine encephalomyelitis (W.E.E.), it was possible, under certain experimental conditions, to demonstrate a quantitative correlation betwee degree of cerebral resistance of vaccinated mice and titer of neutralizing antibody in their serum (1). The high serum titer generally required to insure resistance to intracerebral challenge was believed to be due to penetration of only a small fraction of serum antibody into the central nervous system since the serum/ brain tissue/ spinal fluid ratio for antibody titers in vaccinated rabbits was of the order of 300/3/1 (2).

However, when the response of immunized rabbits or guinea pigs to an intracerebral inoculum was studied in some detail (3), it was found that the serum/ brain antibody ratio was not unalterably fixed. The inoculation was followed first by a febrile response closely resembling that of non-vaccinated animals. In some instances, there were also transitory signs of damage to the central nervous system. It was possible to isolate the virus from the brains of a few guinea pigs during this abortive attack, but attempts to demonstrate viral multiplication failed repeatedly. In one experiment, the rise and fall in neutralizing titers of sera and brains was followed after challenge inoculation, and it was found that at 1 week the brain titer had risen so high that the serum/brain ratio had been reduced from about 100/1 to 1/1 and 10/1, respectively, in the two animals tested.

It seemed possible that such high neutralizing titers of brain tissue, if reproducible, might be of significance in relation to the survival of immune animals. This was suggested, in particular, by the finding (4) that the degree of resistance of vaccinated mice to W.E.E. virus depended on the rate of multiplication of the strain used as challenge inoculum. This difference in response indicated that the concentration of antibody present *at the time of challenge* was certainly not the sole factor determining the fate of the immune animal. It was likely that the intracerebral inoculum served as an antigenic booster and that the effectiveness of the resulting antibody response depended on the rate of viral multiplication. Experiments in this direction were carried out in mice because it was desirable to use large numbers of animals, and because the growth rates

of viral variants were being studied in that host (4). This paper will deal with the following aspects: (*a*) the fate of a "fast" and a "slow" virus strain in the immune host; (*b*) the antigenic booster effect of the challenge inoculum; (*c*) the nature of the neutralizing substance found in brain tissue.

Materials and Methods

The R.I. and the Kelser strains of W.E.E. virus were used as representing "fast" and "slow" variants of the virus. Their characteristics and most of the other materials and methods used have already been described (4).

Serological Tests.—Test material for serological study was obtained as follows: Mice were exsanguinated from the heart, and then their heads were perfused slowly with at least 10 ml. of saline. The efficacy of perfusion was tested by suspending homogenized perfused normal brain tissue in 2.2 parts of distilled water (1:3.2) and then clarifying the suspension in the cold for 30 minutes at about 13,000 R.P.M. in an angle centrifuge.[1] The supernatant fluid was water-clear and colorless indicating the absence of detectable hemolysis.

Brains to be tested for antibody were kept frozen in lusteroid tubes. For test, they were ground up in 2.2 ml. per gm. of tissue of either saline containing 2 per cent of normal guinea pig serum previously heated at 56°C. for 30 minutes, or plain saline. The resulting suspensions were used either uncentrifuged or centrifuged for 30 minutes at 13,000 R.P.M. Neutralization tests with crude suspensions and supernatant fluids yielded identical results.

(*a*) *Technique and Interpretation of Neutralization Tests.*—Serial 0.5 log-fold (actually 3.2-fold) dilutions of serum or brain extract were mixed with equal parts of 2×10^{-8} diluted R.I. stock virus. The mixtures were held for 2 hours in the 37°C. water bath, and then 0.03 ml. was inoculated intracerebrally into groups of 4 to 6 mice per dilution. Control mixtures with normal mouse serum or brain extracts were always injected last.

The final concentration of virus in the mixtures (10^{-8}) represented $10^{1.2}$ LD_{50}/0.03 ml., since, as shown in the earlier paper (4), the mean titer of this virus was $10^{9.2}$ LD_{50}/0.03 gm. of brain tissue. In the period during which neutralization tests were carried out, a total of 36 control titrations were performed in which the 10^{-8} dilution was included. The number of mice used per test ranged from 4 to 10. Of 169 mice inoculated, only 4 survived; in 36 tests, there were only 3 in which 20 to 25 per cent of the mice survived (1 of 4, 1 of 5, 2 of 10, respectively). The odds, therefore, of 1 of 4, 1 of 5, or 1 of 6 mice surviving may be considered as 1 in 10, 1 in 8, 1 in 7 tests, respectively. The chance of 2 surviving in such groups would have been less than 1 in 100 or 1 in 1,000. Therefore, in neutralization tests the occurrence of 2 or more survivors in a group of mice may be considered as due to specific neutralization. However, deaths may be scattered among mice inoculated with "neutral" mixtures containing antibody dilutions over the entire effective range, the mortality rate not necessarily increasing with decreasing concentration of antibody. This variation in response of individual mice to a given inoculum makes difficult the determination of the actual limiting neutralizing dilution. If an adequate range of dilutions and enough animals are used, consistent and reproducible titers are obtained by applying the method of Reed and Muench (5) to calculate the cumulative 50 per cent survival end-point, or the estimated highest (final) dilution which, when injected together with $10^{1.2}$ LD_{50} of virus, would protect 50 per cent of the mice (ND_{50}).

(*b*) *The Serum/Brain Ratio of Neutralizing Antibody in Immunized Mice.*—The methods just described can best be illustrated by an example which, at the same time, will show the "normal" serum/brain ratio in mice immunized by intraperitoneal inoculation of W.E.E. vaccine.

[1] A Sorval angle centrifuge with a 9 inch head was used.

Mice were immunized with a total of 0.17 ml. of vaccine, a relatively large dose. Two weeks after the first dose, they were exsanguinated and perfused, and their brains and sera were tested for neutralizing antibody.

TABLE I

Comparative Neutralizing Titers of Serum and Brain Tissue of Hyperimmunized Mice against $10^{1.2}$ LD_{50} of W.E.E. Virus

Mouse No.	Test material	Rate of survival of mice: Log final dilution of serum or brain in mixture 0.8	1.3	1.8	2.3	2.8	3.3	3.8	Log ND_{50}*	Ratio Serum/Brain: Log	Antilog
1	Serum	—	4/4‡	3/3	3/4	4/4	2/4	—	>3.20§	>2.40	>252/1
	Brain	0/4	1/4	1/4	—	—	—	—	<0.80‖		
2	Serum	—	2/4	4/4	3/3	2/4	3/4	—	>2.95	>2.15	>142/1
	Brain	2/4	0/4	0/4	—	—	—	—	0.80		
3	Serum	—	3/4	4/4	4/4	3/4	2/4	—	>3.07	>2.12	>132/1
	Brain	3/4	0/4	0/4	—	—	—	—	0.95		
4	Serum	—	—	4/6	5/6	4/6	3/6	5/6	>3.30	>2.45	>282/1
	Brain	3/6	1/6	—	—	—	—	—	0.85		
5	Serum	—	—	3/6	5/6	6/6	3/6	1/6	3.10	2.25	178/1
	Brain	3/6	1/6	—	—	—	—	—	0.85		
Mean 1, 2, 3, 4, 5	Serum								>3.12	>2.27	>186/1
	Brain								<0.85		
1, 2, 3, 4, 5 pooled	Serum	—	—	—	5/5	5/5	4/5	0/5	3.49	2.29	195/1
	Brain	3/5	3/5	0/5	—	—	—	—	1.20		

Total dosage of vaccine (7 doses) = 0.17 ml. intraperitoneally.

* ND_{50} = estimated highest dilution of test material which would protect 50 per cent of the mice.

‡ Fractions indicate number of mice surviving in numerator, number inoculated in denominator.

§ >3.20, no end-point; ND_{50} = 3.20 or higher.

‖ <0.80, no end-point; estimate of ND_{50} based on assumption that with lower dilutions of antibody all mice would survive.

The results are given in Table I. While end-points were not obtained in all titrations of individual sera, the approximate mean serum/brain ratio for the 5 mice studied was similar to that obtained in a test with pooled materials from all 5 animals. A ratio of the order of 200/1 is in close accord with that described earlier (2) for rabbits vaccinated with W.E.E. virus. Similar values have also been found by Freund (6) for bacterial antibody in rabbits and by Fox (7) for yellow fever antibody in mice vaccinated by extraneural routes.[2] Since

[2] For the purpose of comparison, the ratios given by Fox (7) should be multiplied by 10, since 10 per cent brain suspensions served as starting materials in his titrations.

Freund found that this ratio is established shortly after either intravenous (6) or intracisternal (8) injection of antibody, one may assume that it expresses an equilibrium between plasma and brain tissue under normal physiological conditions. Accordingly, there were only a few exceptional tests in which the lowest dilutions of brain tissue from immunized mice neutralized even though the corresponding serum titer was less than 1:1,000.

(*c*) *Technique of Complement Fixation Tests.*—Brain suspensions serving as source of complement-fixing antibody were prepared as described above. They were heated for 20 minutes in the 60°C. water bath and then centrifuged for 30 minutes at 13,000 R.P.M. The resulting supernatant was water-clear and colorless. Sera were diluted 1:4 or higher in saline, heated at 60°C. for 20 minutes, and then also centrifuged for 30 minutes at 13,000 R.P.M.

Eastern and Western E.E. antigens were obtained from the Lederle Laboratories, Inc.

Tests were set up with 0.1 ml. of antigen, 0.1 ml. of serum or brain dilution, 0.2 ml. containing 2 hemolytic units of complement, and 0.2 ml. of sensitized sheep erythrocytes, and the technique used was that recommended by Casals (9). Titers have been expressed in terms of highest original dilutions of test material giving 2 plus or greater fixation.

EXPERIMENTAL

Fate of W.E.E. Virus after Intracerebral Inoculation in Immune Mice

For a study on the fate of W.E.E. virus in the brains of immunized animals, it was desirable to contrast the response to a "fast" variant with that to a "slow" one. Therefore, mice were vaccinated to a degree at which they were expected to resist the "slow" but not the "fast" inoculum. The dependence of demonstrability of this strain difference on the level of immunity has been described elsewhere (4).

Accordingly, mice received intraperitoneally a total of 0.018 ml. of vaccine. Four weeks after the first dose, 33 received intracerebrally $10^{5.7}$ LD_{50} of the "fast" R.I. strain, and 39 $10^{5.5}$ LD_{50} of the "slow" Kelser strain. Non-vaccinated mice of the same age served as controls. Of the mice challenged with the R.I. strain, 9 were sacrificed at various intervals up to the 3rd day. The remaining 24 died from 2 to 11 days (average 3.7 days) after inoculation. Of those challenged with the Kelser strain, 35 were sacrificed at various intervals up to 114 days after inoculation. None showed any signs of illness. Mice from the control groups were sacrificed at corresponding intervals as long as survivors were left.

Thus, vaccinated mice were resistant to $10^{5.5}$ LD_{50} of the slow strain but not to $10^{5.7}$ LD_{50} of the fast one.

All mice were perfused except those sacrificed before the 3rd day after challenge inoculation. The brains were used in tests for virus or antibody content.

The fate of the virus in the brain was followed in the manner already described (4) for experiments on viral multiplication. Brains of individual mice were titrated. The range of titers is given for the R.I. strain in Fig. 1, for the Kelser strain in Fig. 2. The curves for the 2 strains in the non-vaccinated groups were comparable with those described in detail elsewhere (4). In vaccinated mice, the rate of multiplication of the "fast" variant paralleled that in non-vaccinated controls but at a lower level (Fig. 1). Inasmuch as the brains at 3 hours and at 1 and 2 days were not perfused, it is possible that the

difference between normal and vaccinated mice may have been largely the result of *in vitro* neutralization.

On the other hand, in the vaccinated group challenged with the "slow" strain, there was a much more marked depression in the titer of demonstrable virus (Fig. 2). Nevertheless, multiplication apparently occurred, and in some

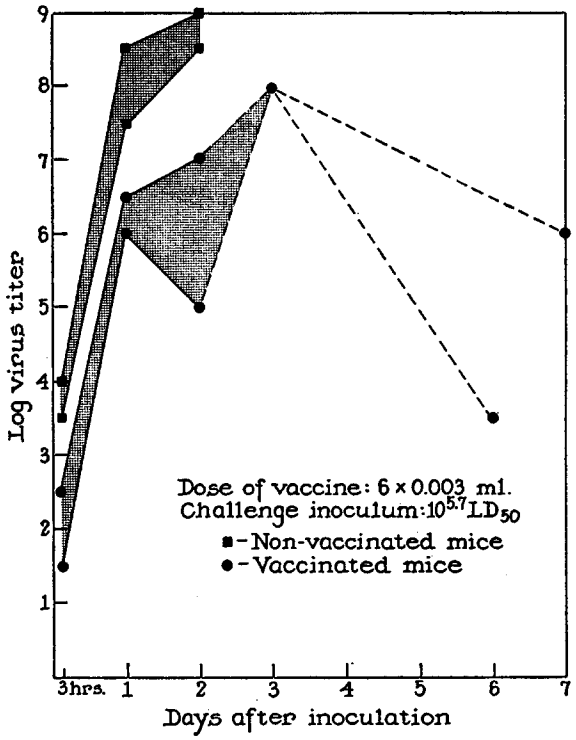

FIG. 1. Multiplication of W.E.E. virus, "fast" R.I. strain, in brains of normal and of vaccinated mice.

animals the virus maintained itself for 4 days at a level 100-fold higher than that found at 3 hours.

Because of the small number of specimens available in this test and because of the importance attached to the unequivocal demonstration of viral multiplication in immune animals, another experiment was done in which mice immunized with a total of 0.015 ml. of vaccine were challenged with $10^{6.5}$ LD_{50} of the "slow" strain. Brains were harvested at 3 hours and at 2 days from 9 mice each. Of 30 additional mice, only one succumbed, and the group as a whole may be considered as resistant. At each interval, 3 pools of 3 brains each were titrated for virus. The results of these titrations are presented in Table II. It will be seen again that there was about a 100-fold increase in virus at 2 days in the brains of vaccinated, resistant mice.

Thus, immunity of a degree high enough to insure resistance to challenge did not preclude transitory multiplication of the challenge virus. However, while some factor arrested further multiplication of the "slow" strain, this factor failed to operate effectively when the challenge virus multiplied rapidly. The following sections will deal with the experimental evidence identifying this factor as neutralizing antibody believed to be produced locally in response to the challenge inoculum.

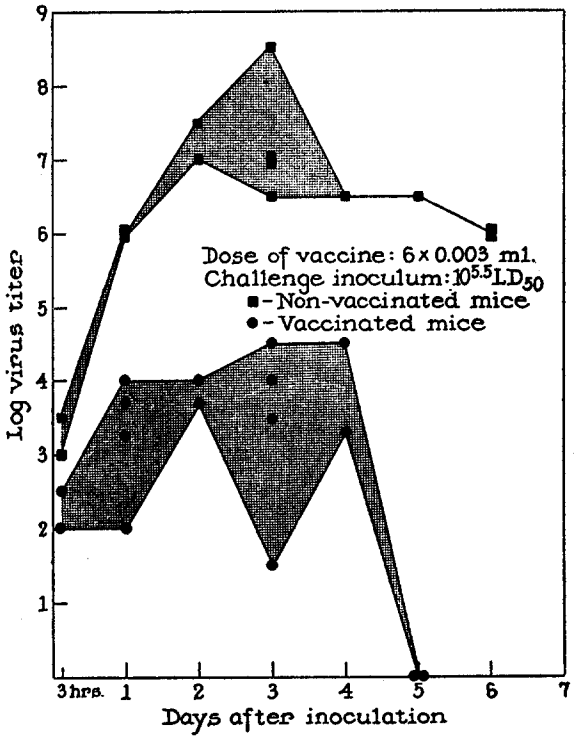

FIG. 2. Multiplication of W.E.E. virus, "slow" Kelser strain, in brains of normal and of vaccinated mice.

Changes in the Serum/Brain Antibody Ratio in Vaccinated Mice after Intracerebral Challenge Inoculation

In continuation of the first experiment described above, the sera and brains obtained from mice at various intervals after challenge inoculation with the "slow" Kelser strain were tested for neutralizing antibody. The outcome of these tests is presented in Table III and Fig. 3.

A pool of 9 sera collected from vaccinated but unchallenged mice at the time of challenge had a neutralizing titer of 1.8 log (1:64). As expected, with a serum titer of such low order,

no significant amounts of antibody could be measured in the corresponding brains even though they were not perfused. Only 3 of them, when tested individually, neutralized in the lowest dilution tested (1:6.4). Beginning on the 5th day after challenge, the brain titer increased out of proportion to the serum titer with a resulting marked shift in the serum/brain ratio. The brain titer was maintained on a high level from the 7th to at least the 114th day, the ratio decreasing from the "normal" value of about 200/1 to 16/1 on the 6th day to less than 10/1 from the 13th day on. It is particularly noteworthy that the neutralizing titer of brain tissue was relatively high on the 5th day in view of the fact that brains harvested on the 4th day had contained considerable amounts of virus (see Fig. 2).

TABLE II

Multiplication of W.E.E. Virus, "Slow" Strain, in Brains of Immune and of Normal Mice

Dose of vaccine	Time sacrificed after intracerebral inoculation	Lot	No. of brains	Log LD_{50} recovered		Mean increase 3 to 48 hrs.
				Single lots	Mean	
	hrs.					
0.015 ml.	3	A	3	3.16	3.18	1.80
		B	3	3.25		
		C	3	3.12		
	48	D	3	5.16	4.98	
		E	3	4.62		
		F	3	5.08		
None	3	G	3	3.61	3.41	2.91
		H	3	3.25		
		I	3	3.36		
	48	J	3	6.50	6.32	
		K	4	6.14		

Challenge inoculum, $10^{5.5}$ LD_{50}.

Thus, in animals with a relatively low degree of initial immunity (as indicated by a serum titer of 1:64 just before challenge with virus) the multiplication of a large inoculum of the "slow" strain was arrested, and neutralizing antibody appeared in the brain in excess before the virus had spread to an extent incompatible with the animal's recovery. A causal relationship between the accumulation of antibody in the brain and survival of the animal is suggested by the fact that other animals in the same experiment failed to resist a comparable dose of the "fast" variant which attained lethal concentrations well before the 5th day (see Fig. 1).

As a corollary, in an attempt to show whether or not the neutralizing substance found in brain tissue was antibody, some of the brain suspensions and

TABLE III

Titers of Neutralizing Antibody in Sera and Brains of Vaccinated Mice before and after Intracerebral Challenge Inoculation

Time after challenge inoculation	Mouse No.	Log ND_{50}				Mean ratio $\frac{\text{Serum*}}{\text{Brain}}$	
		Serum		Brain			
		Single or repeated tests	Mean	Single or repeated tests	Mean	Log	Antilog
days							
5	B 15	—	—	1.42	1.42	—	—
	B 16	—	—	1.42			
6	B 17	2.60, 2.55‡	2.58	0.92	<1.36	>1.22	$\frac{>16.6}{1}$
	B 18			<1.17			
	B 19			1.87			
	B 20			1.49			
7	B 21	—	—	1.72, 1.65	1.69	—	—
10	B 22	—	—	2.82	2.52	—	—
	B 23	—	—	2.22			
13	B 24	3.10, 2.60	2.85	2.17, 1.77	1.89	0.96	$\frac{9.1}{1}$
	B 25			1.82			
24	B 26	3.02, 2.30	2.66	1.82, 2.07 (1.59)	1.77	0.89	$\frac{7.8}{1}$
	B 27						
55	B 28	2.90, 2.80, 2.80 (2.70)	2.80	1.42, 2.17, 2.42 (2.02)	2.01	0.79	$\frac{6.2}{1}$
	B 29						
	B 30						
114	B 31	2.55	2.63	1.80	1.98	0.65	$\frac{4.5}{1}$
	B 32						
	B 33	2.70		2.15			
	B 34						
	B 35						

Total dosage of vaccine, 0.018 ml. Challenge dose, $10^{5.5}$ LD_{50} of "slow" variant.

* A pool of 9 sera obtained before challenge inoculation gave a mean ND_{50} of 1.8 (1:64). The corresponding brains were not perfused, and only 3 of the 9 showed some evidence of neutralization in the lowest dilution tested (1:6.4). The prechallenge ratio therefore could not be determined and is assumed to be of the usual order; *i.e.*, about 200/1.

‡ Braces indicate that pools were tested.

For further explanation see Table I.

sera were tested for complement-fixing antibody. The results are shown in Table IV. Complement-fixing antibody was first demonstrated in the brains of mice sacrificed on the 10th day after intracerebral inoculation. On the 13th

day both serum and brain titers were at maximum, and the ratio was 8/1. Thereafter, both titers fell, but the ratio remained at 8/1 on the 24th day and at 4/1 on the 55th day.

Assuming that a "physiological" serum/brain ratio of the order of 200/1 applies to complement-fixing antibody as it does to other types of antibody, the shift in neutralizing titers was paralleled by a similar shift in complement-fixing titers.

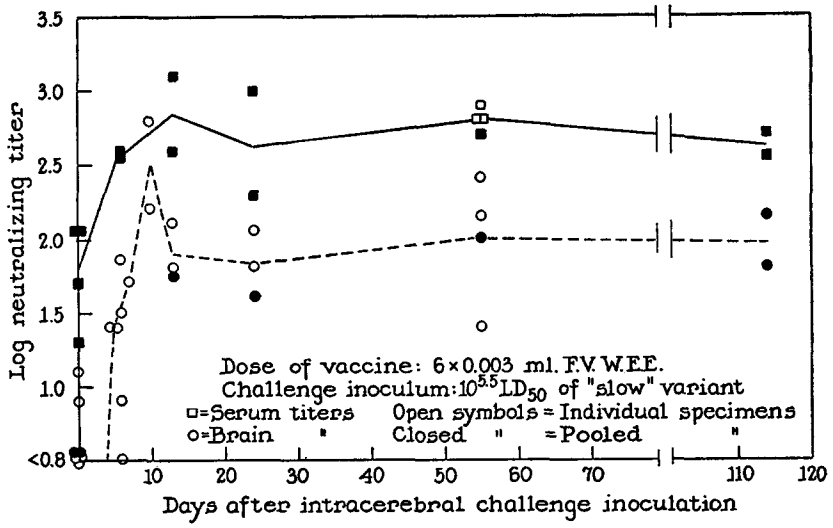

FIG. 3. Titers of neutralizing antibody in sera and brains of vaccinated mice before and after intracerebral challenge inoculation.

Further Evidence that the Neutralizing Substance in Brain Tissue Is Antibody

(*a*) *The Specificity of the Antibody in Brain Tissue.*—Equal parts of Eastern and Western E.E. vaccines were mixed, and 36 mice were given intraperitoneally a total of 0.15 ml. of the mixture. Two weeks after the first dose of vaccine, 6 mice were sacrificed, 15 received intracerebrally $10^{4.5}$ LD_{50} of E.E.E. stock virus and the remaining 15 by the same route $10^{5.2}$ LD_{50} of the "fast" R.I. strain of W.E.E. virus. At 2 weeks after challenge inoculation, there were 8 survivors in the E.E.E. group, but only 3 in the W.E.E. group. All these were bled from the heart, perfused, and their brains were harvested. Sera and brains were pooled in lots as indicated in Tables V and VI and titrated for neutralizing antibody against W.E.E. virus and for complement-fixing antibody against both the Eastern and Western types.

The result of the neutralization test is summarized in Table V. The outstanding finding is that after challenge with W.E.E. virus, the homologous titer of brain tissue rose to at least the same level as that of the serum, while after challenge with E.E.E. virus, the Western antibody remained unchanged in both serum and brain tissue. This finding, as will be discussed later on,

confirms the belief that the accumulation of antibody in the brain is the result of local production.

TABLE IV

The Rise and Fall of Complement-Fixing Antibody in Serum and Brain Tissue of Immune Mice before and after Intracerebral Challenge with $10^{5.5}$ LD_{50} of the "Slow" Strain of W.E.E. Virus

Interval after intracerebral challenge	No. of mice	Test material	Antigen											
			W.E.E.								E.E.			
			Dilution of test material 1:											
			4	8	16	32	64	128	256	512	4	8	16	32
0 (before challenge)	9	Serum	—	**3**	±	0	—	—	—	—	—	0	0	0
		Brain	0	0	—	—	—	—	—	—	±	0	—	—
6	4	Serum	—	4	4	4	4	**3**	0	—	—	0	0	0
		Brain	—	0	0	0	0	—	—	—	—	0	0	0
7	1	Brain	—	0*	0	0	0	0	0	—	—	0*	0	0
10	2	Brain	—	**3***	1	0	0	0	0	—	—	0*	0	0
13	2	Serum	—	4	4	4	4	4	**3–4**‡	0	—	0	0	0
		Brain	—	4*	4	**3–4**	**0–2**	0	0	0	—	0*	0	0
24	2	Serum	—	4	4	4	**3–4**	0	0	0	—	0	0	0
		Brain	—	**2–3**	±	0	0	0	0	—	—	0	0	0
55	3	Serum	—	—	**4**	1	0	0	0	—	—	—	0	0
		Brain	**3**	1	0	±	0	—	—	—	0	0	—	—
114	5	Serum	—	—	0	0	0	0	0	—	—	—	0	0
		Brain	0	0	0	0	0	0	—	—	0	0	—	—

The results are presented as, 4 = complete fixation; 0 = complete hemolysis with intermediate degrees of fixation expressed as 3, 2, and 1. The highest dilution giving a reading of at least 2 is considered as end-point (bold-faced type).

* Indicates that the lowest dilution tested was 1:10 instead of 1:8.

‡ Two figures indicate results of duplicate tests.

In Table VI, the outcome of the complement fixation test with the same materials is shown. Here again, the serum/brain ratio for antibody reacting with the virus used for the intracerebral test was reduced to 2/1 to 8/1. In these doubly immunized mice, the antibody response to challenge was just as specific in the brain as in the serum.

(*b*) *The Percentage Law.*—Table VII illustrates one of several tests in which constant amounts of serum or brain extract of vaccinated and challenged mice

were mixed with graded amounts of virus and in which the resulting mixtures after 2 hours' incubation at 37°C. were diluted serially so that the highest dilution contained 10^{-8} virus. It may be seen that in both cases the percentage of virus neutralized was independent of the amount of virus originally added to the test material. In other words, the "percentage law" as described by Mer-

TABLE V

W.E.E. Neutralizing Antibody in Serum and Brain Tissue of Mice Immunized against W.E.E. and E.E.E. Viruses before and 2 Weeks after Intracerebral Challenge Inoculation of Either Virus

Specificity of the Local Response

Challenge virus	Lot	No. of mice	Test material	Log ND_{50}	$\frac{\text{Serum}}{\text{Brain}}$ log ratio
None	A	3	Serum	2.15	>1.45
			Brain	<0.70	
	B	3	Serum	1.80	0.78
			Brain	1.02	
W.E.E.	C	3	Serum	2.80	<0.02
			Brain	>2.78	
E.E.E.	D	4	Serum	1.80	>1.15
			Brain	<0.65	
	E	3	Serum	2.21	>1.31
			Brain	<0.90	
	F	1	Serum	2.07	>0.80
			Brain	<1.27	

The lowest dilution of brain tissue tested was $10^{-0.9}$ for lots A to E, and $10^{-1.4}$ for lot F. In some instances, there was a suggestion of neutralization by these dilutions. The estimated ND_{50} titers are unduly high because they are based on the assumption that with lower dilutions all mice would have survived.

For explanation of symbols see Table I.

rill (10) for Eastern E.E. virus and its antibody and as observed for other antigen-antibody mixtures (11) applied equally to the neutralizing substance in the brain and that in the serum.

The Effect of Varying Degrees of Immunization on the Shift in Serum Antibody/Brain Antibody Ratio after Intracerebral Challenge

Mice which had been immunized with graded amounts of W.E.E. vaccine and had survived after intracerebral challenge inoculation of varying amounts of the "slow" Kelser

TABLE VI

Complement-Fixing Antibody in Serum and Brain Tissue of Mice Immunized against W.E.E. and E.E.E. Viruses before and 2 Weeks after Intracerebral Challenge Inoculation of Either Virus

Specificity of the Local Response

Challenge inoculum	Lot	No. of mice	Test material	Antigen																			
				W.E.E.									E.E.E.								Saline		
				Dilution of test material 1:																			
				4	8	16	32	64	128	256	512	1024	4	8	16	32	64	128	256	512	1024	4	8	16
None	A	3	Serum	4	3	1	0	—	—	—	—	—	4	4	4	2	—	—	—	—	—	0	—	—
			Brain	0	0	—	—	—	—	—	—	—	0	0	—	—	—	—	—	—	—	0	—	—
	B	3	Serum	4	4	1	±	—	—	—	—	—	4	4	4	3	—	—	—	—	—	0	—	—
			Brain	0	0	—	—	—	—	—	—	—	0	0	—	—	—	—	—	—	—	0	—	—
W.E.E.	A	3	Serum	—	4	4	4	4	4	2	0	0	—	4	±	0	—	—	—	—	—	—	0	—
			Brain	4	4	4	4	4	0	0	0	0	0	0	0	0	—	—	—	—	—	0	—	—
E.E.E.	A	4	Serum	—	3	2	±	—	—	—	—	—	—	4	4	4	4	3	0	0	0	—	±	—
			Brain	0	0	0	0	—	—	—	—	—	4	4	4	4	1	0	0	0	0	0	—	—
	B	3	Serum	—	0	0	0	—	—	—	—	—	—	4	4	4	4	3	0	0	0	—	0	—
			Brain	0	0	0	0	—	—	—	—	—	4	4	4	4	2	0	0	0	0	0	—	—
	C	1*	Serum	—	—	0	0	—	—	—	—	—	—	—	4	4	4	4	4	3	0	—	—	0
			Brain	—	0	0	0	—	—	—	—	—	—	4	4	4	4	1	0	0	0	—	0	—

For explanation of figures see Table IV.

* The single mouse of lot C was of special interest because it was sacrificed after a course of encephalitis with gradually improving paresis of the extremities lasting 10 days.

strain, were sacrificed at various intervals. These mice were survivors from the experiment described in Fig. 6 of the preceding paper (4). The sera and perfused brains were each pooled according to the amount of vaccine received. Each pool contained specimens from mice challenged with 10^5, 10^3, and 10^1 LD_{50} of virus.

The results of a neutralization test with these pools are summarized in Table VIII. It is shown that the relative concentration of antibody in the brain increased with decreasing dosage of vaccine used for immunization. As a result, while all the serum titers were of the same order of magnitude, there was a

TABLE VII

The "Percentage Law" in the Neutralization of W.E.E. Virus by Immune Mouse Serum and Brain Tissue

Test material	Original mixture: Final dilution of		Survival rate in mice inoculated with						
	Serum or brain	Virus	Original mixture	Dilution of mixture to final virus concentration equivalent to: 10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Immune serum	$10^{-1.3}$	10^{-3}	0/4	0/4	0/4	0/3	**1/3**	**4/4**	—
		10^{-4}	0/4	—	0/4	0/4	**2/4**	**3/4**	—
		10^{-5}	0/4	—	—	0/4	**2/4**	**3/4**	—
		10^{-6}	0/4	—	—	—	**0/4**	**4/4**	—
		10^{-7}	**2/4**	—	—	—	—	**4/4**	—
		10^{-8}	**3/4**	—	—	—	—	—	—
Immune brain	$10^{-0.8}$	10^{-4}	0/4	—	0/4	0/4	0/4	**4/4**	—
		10^{-5}	0/4	—	—	0/4	1/4	**3/4**	—
		10^{-6}	0/4	—	—	—	0/4	**3/4**	—
		10^{-7}	1/4	—	—	—	—	**3/4**	—
		10^{-8}	**2/4**	—	—	—	—	—	—
Normal mouse serum	$10^{-1.3}$	10^{-3}	0/4	—	—	—	0/4	**0/4**	3/4
		10^{-7}	0/4	—	—	—	—	**0/4**	2/4
		10^{-8}	**0/4**	—	—	—	—	—	—
		10^{-9}	3/4	—	—	—	—	—	—
		10^{-10}	3/4	—	—	—	—	—	—

tenfold difference between the serum/brain ratios in mice vaccinated with 1.8 ml. of vaccine (ratio 24.6/1) and in those vaccinated with 0.0018 or 0.0057 ml. (ratio 2.3/1 or 2.8/1), with intermediate values for those vaccinated with 0.018 or 0.057 ml. A single complement fixation test was carried out with pooled sera and brains from the latter 4 groups. As shown in Table VIII, the ratio was 4/1 which is again in good agreement with that for the neutralizing titers. It is noteworthy that the time interval which elapsed after challenge inoculation did not influence the consistency of the pattern. This is in agreement with the experiment described earlier in which the ratio remained more or less constant from about the 13th to the 114th day after inoculation.

One may assume that in animals immunized with large amounts of vaccine a considerable proportion of the challenge inoculum would be neutralized and thereby rendered incapable of multiplication and also less effective as antigen. Conversely, after vaccination with small doses, the primary antibody response may be minimal but the animal would be sufficiently sensitized to respond to the challenge virus with a large and rapid local output of antibody. By the same token, in highly immunized animals, survival may be largely due to the suppressive effect of antibody initially present which would be effective against either "fast" or "slow" variants of the virus (see Fig. 6 in reference 4). In less

TABLE VIII

Effect of Degree of Prechallenge Immunity upon Local Antibody Response to Intracerebral Challenge Inoculation

Total dosage of vaccine	Sacrificed after challenge	No. of mice in pool	Material tested for neutralizing antibody	Log ND_{50}	Ratio Serum/Brain		Titer of complement-fixing antibody
					Log	Antilog	
ml.	*days*						
1.8	15	10	Serum Brain	2.80 1.41	1.39	24.6/1	n.t.
0.057	22	8	Serum Brain	2.91 1.98	0.93	8.5/1	
0.018	33	12	Serum Brain	2.38 1.55	0.83	6.7/1	Pooled sera 1:32 Pooled brains 1:8
0.0057	26	9	Serum Brain	2.38 1.93	0.45	2.8/1	Ratio 4
0.0018	127	10	Serum Brain	2.41 2.05	0.36	2.3/1	

For explanation of figures see Table I.
n.t., not tested.

thoroughly immunized animals, survival would then depend more and more predominantly on their potential ability to respond to the challenge inoculum with local antibody production, and this in turn would be effective only if the rate of viral multiplication were not too rapid. This complex mechanism in which viral growth competes with local antibody production probably accounts for individual variations often seen among animals vaccinated and challenged in the same manner.

The "Paradoxical" Response of Lightly Immunized Mice to Small Intracerebral Challenge Doses

Another observation in support of the concept just discussed is that of a "paradoxical" response of lightly immunized mice to large or small intracerebral

challenge doses of active virus. Table IX and Fig. 4 illustrate two examples of this. In both experiments, graded doses of the "slow" Kelser strain were injected intracerebrally into mice immunized with very small amounts of vaccine. Table IX shows that mice vaccinated with 0.0057 or 0.0018 ml. resisted 10^5 or 10^3 LD_{50} but that 4 of 8 succumbed after inoculation of 10^1 LD_{50}. Similarly, Fig. 4 shows the response of mice immunized with 0.0006 ml. of vaccine. While 10 of 24 mice survived after inoculation of 10^3 to 10^6 LD_{50} of virus, there was no sign of protection among mice challenged with smaller amounts (to the right of the broken line in Fig. 4) when compared with non-vaccinated controls.

It is reasonable to assume that the degree of the local immune response should depend on the amount of antigen injected. While in mice with a higher degree of immunity small doses of challenge virus may be expected to be effec-

TABLE IX

"Paradoxical" Response to Challenge Doses of W.E.E. Virus, Slow Strain, in Mice with Low Grade Immunity

Total dose of vaccine	Challenge dose LD_{50} of W.E.E.-Kelser virus		
	$10^{5.0}$	$10^{3.0}$	$10^{1.0}$
ml.			
1.8	1/4*	0/4	0/4
0.18	0/4	0/4	0/4
0.057	0/4	0/4	0/4
0.018	0/4	0/4	0/4
0.0057	0/4	0/4	**2/4**
0.0018	0/3	0/4	**2/4**

* Numerator, number of deaths, denominator, number of mice inoculated.

tively blocked by the antibody initially present in the brain tissue, in those with a very low degree of immunity this amount of antibody must be infinitesimal. In the preceding paper (4) the differences in viral growth rate after inoculation of various amounts of W.E.E. virus were described. It was shown that with large seed inocula the rates tended to converge while, in the case of the R.I. strain, with decreasing inocula there was a tendency for the rates to parallel each other. It was assumed that with increasing amounts seeded, a correspondingly larger proportion was in excess of that utilized immediately to initiate infection. This excess could then, in the sensitized animal, act as free antigen. A very small inoculum, on the other hand, if taken up quantitatively by susceptible cells, would be ineffective as antigen, with the result that viral multiplication could proceed unchecked.

How delicate this balance between virus increase and immune response may be is indicated by the consistently high incidence of transitory encephalitic signs among mice with borderline degrees of immunity. This has been graphi-

cally illustrated by shading individual squares in Fig. 4. The mice so symbolized all had definite convulsive seizures and were expected to die. Instead, they recovered completely after a few days or continued to live, sometimes for months, with gradually improving partial paralysis of the extremities.

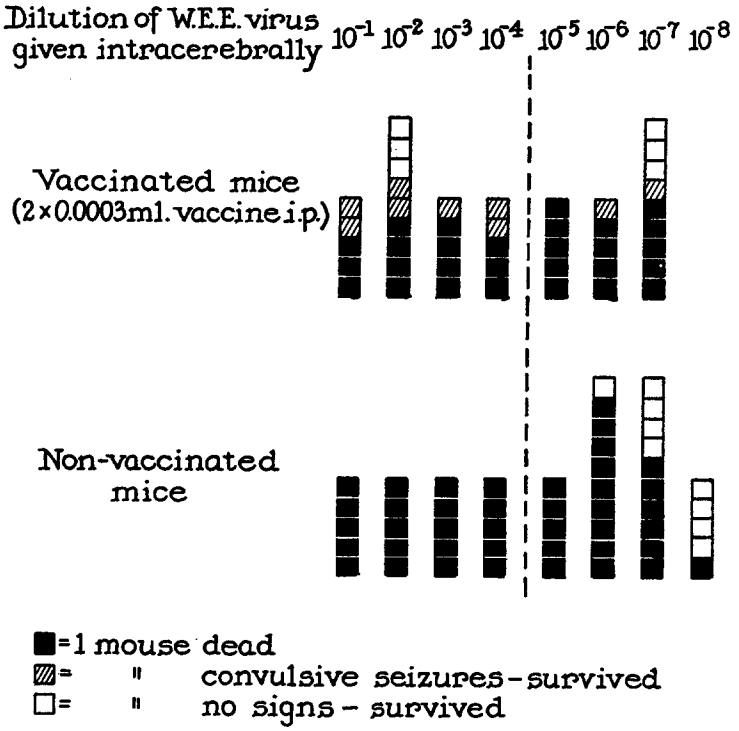

FIG. 4. "Paradoxical" response to graded challenge doses of W.E.E. virus in mice with low grade immunity.

RECAPITULATION AND DISCUSSION

While the virus-inactivating and protective action of neutralizing antibody is readily evident both *in vitro* and in passively immunized animals, its rôle in actively immunized hosts has been a subject of doubt and speculation. This has been due to the inability, especially in the case of neurotropic virus infections, to consistently correlate varying titers of neutralizing antibody acquired as a result of vaccination with corresponding variations in the degree of resistance to infection. This has been especially true for those studies in which the degree of resistance was measured by intracerebral challenge inoculation of virus. Thus, conflicting opinions have been expressed concerning the possible significance of neutralizing antibody in relation to cerebral resistance to the

viruses of poliomyelitis (12), equine encephalomyelitis (1, 13), St. Louis encephalitis (13), and rabies (14). Morgan, Schlesinger, and Olitsky (2) showed that vaccinated rabbits were able to resist large intracerebral challenge doses of E.E. virus provided their serum contained neutralizing antibody of high titer. In such animals demonstrable amounts of antibody penetrated into the central nervous system, the serum/brain tissue/spinal fluid ratio being of the order of 300/3/1. It was thought that adequate amounts of antibody available to the exposed tissue at the time of challenge were necessary to insure resistance. Subsequent finding of a shift in this ratio through an increase in the neutralizing titer of brain tissue after challenge inoculation (3) first suggested that local production of antibody might be stimulated by the challenge inoculum itself.

The work reported in this and the preceding paper (4) has revealed a relationship between local antibody accumulation and survival of immune animals. It has led to the following conclusions regarding the mechanism of cerebral immunity to W.E.E. virus:—

(*a*) The degree of resistance of vaccinated mice to intracerebral challenge inoculation is in part determined by the rate of multiplication of the challenge virus.

(*b*) In mice immunized with relatively large doses of vaccine, the difference between fast and slow variants of W.E.E. virus is masked, because the high initial concentration of antibody in their serum and central nervous system can effectively block or retard the spread of the virus regardless of its rate of multiplication.

(*c*) In animals with lower degrees of immunity, the amount of antibody initially present may be inadequate to retard significantly the rate of growth of a "fast" variant of the virus. Because of the rapid rate at which it multiplies there may be no chance for local antibody production to be effective in preventing fatal infection. The "slow" variant, on the other hand, not only affords the immediately available antibody a better chance to react with it, but it also gives time for sufficient additional antibody to be produced locally. Hence even though the virus may multiply and persist in the brain at a relatively high level for several days the animal survives.

(*d*) As a result of this successful competition of local antibody production with viral multiplication, the serum antibody/brain antibody ratio is considerably reduced.

(*e*) The degree of this shift in serum/brain ratio varies inversely with the initial degree of immunity: the more antibody available initially the greater the proportion of the inoculum which is rendered inert as antigen.

(*f*) The intensity of the local immune response is also proportional to the amount of antigen injected as challenge. In animals with extremely low grade immunity, this may lead to a "paradoxical" response to large and small intra-

cerebral challenge doses: while such animals are sufficiently sensitized to give a rapid anamnestic immune response to excessive amounts of virus, small amounts are not sufficiently antigenic and are therefore more apt to cause fatal infection.

The latter finding, in particular, suggests that the antigenic booster effect is exerted chiefly by the virus contained in the inoculum itself. For, as has already been pointed out, the growth rate studies (4) lead one to believe that with increasing amounts of virus inoculated, correspondingly larger proportions of the inoculum exist in excess of the amount utilized to initiate infection. Presumably this excess virus does not attack susceptible cells and is free to stimulate antibody formation. Similarly, after inoculation of small infectious doses, some of the virus liberated by infected cells after multiplication would be free to act as antigen but probably too late to affect the outcome; it would be ineffective in arresting the course of the infection, especially if, as is probable, the virus progressed along the neuronal pathway (15), for in that case virus may already have reached distant neurons at the time when antibody becomes available. It has been shown by Morgan (12) that non-fatal infection of the brain and cord of the monkey with poliomyelitis virus leaves in the wake of its neuronal pathway a trail of high neutralizing titers.

The concept of the mechanism of cerebral immunity just outlined may appear complex, but it is in accord with generally accepted principles of immunology (16). Local accumulation of specific antibody at the site where antigen is deposited has been described by Walsh and Cannon (17) and DeGara and Angevine (18) for bacterial agglutinins and by Oerskov and Andersen (19) and Hartley (20) for antivaccinial antibody. Similarly, local concentration of neutralizing substances, presumably antibody, in the central nervous system after infection with viruses has been reported by Fox (7) for yellow fever in mice and by Morgan (12) for poliomyelitis in monkeys. The present study leaves no doubt that the neutralizing substance present in high concentration in the brain tissue of mice immune to and challenged with W.E.E. virus is antibody: (*a*) it reacts specifically with homologous antigen, (*b*) it follows the "percentage law" on dilution of underneutralized mixtures, (*c*) its concentration in relation to that in the serum is paralleled by that of complement-fixing antibody.

The finding of complement-fixing antibody of high titer in blood-free brain tissue is a novel observation in the field of virus diseases. It is reminiscent of the presence of complement-fixing antibody in the cerebrospinal fluid of neurosyphilitic patients. Kabat, Moore, and Landow, on the basis of electrophoretic comparison of serum and cerebrospinal fluid proteins, came to the conclusion that this antibody is produced within the central nervous system (21). This interpretation has been favored also by Morgan (12) and Fox (7) for the systems studied by them, and evidence presented in this paper lends significant support to it: that is the observation that in mice vaccinated with a mixture of

inactive Western *and* Eastern E.E. viruses and challenged intracerebrally with active virus of either type, the relative concentration of the homologous, but not of the heterologous antibody in brain tissue rises. Such specificity could not be expected in doubly immunized animals if the local accumulation of antibody were assumed to be due to increased permeability of capillary walls or to some non-specific disturbance in the protein balance between blood and brain tissue.

An interesting problem for further investigation is the reason for the long persistence of local antibody in high concentration. In the present report, 127 days was the longest interval after intracerebral inoculation of W.E.E. virus at which the ratio serum titer/brain titer was examined, and in that particular experiment (see Table VIII) it had remained at 2.3/1. Similarly, Morgan (12) found the neutralizing titer against the Lansing strain very high in monkey cord as long as 5 months after onset of paralysis. Earlier observations on the markedly delayed onset of encephalitis in W.E.E.-infected guinea pigs treated with large doses of hyperimmune serum (22) suggested that in passively immune animals the virus sometimes persisted in the central nervous system masked by antibody which blocked the development of active immunity. In contrast, persistence of antigen in actively immune animals may be responsible for continued presence of local antibody in high titer.

The "paradoxical" type of response of mice with low degree of immunity to various amounts of active virus, *i.e.* their failure to survive minimal challenge doses although they can resist large ones, is not without parallel. Casals (23) and Habel and Wright (24) have noted the same phenomenon in mice vaccinated and challenged with rabies virus, and the explanation given here for the findings with W.E.E. virus may apply equally for rabies.

In general, there is no reason to believe that the principles found to govern the mechanism of cerebral immunity to W.E.E. virus should not be applicable to the wider field of other virus infections of the central nervous system and of other organ systems. The findings reported in the preceding paper (4) and in this one reaffirm that the actively immunized animal differs from the normal one not in principle but only in its greater ability to respond to an antigenic stimulus. The fate of the immune host after challenge—even that of the mouse inoculated intracerebrally with W.E.E. virus—is not either death or intact survival. There may be any intermediate degree of involvement. What happens is determined by a delicate quantitative balance with relation to (*a*) degree of initial immunity or sensitization, (*b*) amount of the challenge virus, (*c*) rate of its multiplication, (*d*) ability of the host to respond with local antibody production.

SUMMARY

The fate of W.E.E. virus has been followed in the brains of mice vaccinated to such an extent that they failed to resist a large intracerebral challenge dose

of a viral variant with a rapid rate of multiplication but were fully protected against a similar amount of a "slow" strain.

The growth rate of the "fast" variant in vaccinated animals paralleled that in non-vaccinated ones at a slightly lower level. The "slow" strain also multiplied, but its rate of growth was depressed. Nevertheless, it persisted for 4 days at a level 100-fold higher than its initial titer.

After the 4th day the virus was no longer demonstrable and was replaced by neutralizing antibody which rose so high that the serum antibody/brain antibody ratio was reduced from a "physiological" value of about 200/1 to less than 10/1. Antibody persisted in brain tissue in high titer until at least 127 days after challenge inoculation.

The shift in the serum/brain ratio of neutralizing antibody was paralleled by a similar shift in the ratio of complement-fixing antibody.

The neutralizing antibody in brain tissue, like that in serum, followed the "percentage law" on dilution of underneutralized mixtures.

In mice immunized with small doses of vaccine, the intracerebral challenge inoculum induced a significantly greater local immune response than in those immunized to a higher degree.

Mice with very low grade immunity were found more resistant to large amounts of virus than to small amounts. This "paradoxical" response to challenge was explained as due to the antigenic booster effect exerted by amounts of virus in excess of that utilized to initiate infection which were present in large inocula but absent in small doses.

The broader relation of these findings to the problem of antiviral immunity has been discussed.

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