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Interference with Eastern Equine Encephalomyelitis (EEE) Virus in the Brains of Mice Immune to Lymphocytic Choriomeningitis (LCM)

By

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It has long been suspected that neutralizing antibodies are not the sole immunity factor in mice immunized against LCM as mature animals (5). Although such mice are not tolerant immunologically, they form neutralizing antibodies rather poorly (4, 10). Since many of them do not carry demonstrable amounts of infectious virus in their brains and other organs (5, 4), there was at first no reason to suppose that their immunity might be correlated with the interference phenomenon, which appears to be responsible for the strong immunity of tolerant mice (virus carriers) infected congenitally with LCM virus (7, 11).

It is the purpose of this communication to show that slight but definite interference with EEE virus occurs in the brains of both categories of LCM-immune mice and that the persistence of detectable quantities of infectious LCM virus is not a prerequisite for such interference in non-tolerant animals.

Materials and Methods

Viruses: As in previous experiments reported in this journal (10, 11), strain W of LCM virus and strain S 18888 of EEE virus were used.

Mice: The tolerant animals came from our infected stock mentioned previously (11) in which all mice, young or old, are carriers of strain W of LCM virus. The mice immunized as mature animals and the controls were obtained from stock III of the Institute (11), from which the ancestors of the infected colony originated.

Immunization of mature mice with LCM virus: The attribute "mature" is used here for animals beyond the age at which the "carrier state", based on immunological tolerance, frequently develops upon artificial infection

with LCM virus (7, 9). Female mice were used exclusively. The animals were immunized at the age of about 5 weeks either by a single subcutaneous (s. c.) inoculation with 0.2 ml. of a 2 per cent mouse brain suspension from the second or third intracerebral (i. c.) passage of strain W or, in the majority of the experiments, by a similar injection followed by i. c. challenge with 0,04 ml. of a suspension of the same strength 14 to 184 days later (see tables). Many of the mice challenged 184 days after s. c. infection showed an accelerated, nonfatal reaction (7, 4).

Titration of EEE virus: Twenty per cent suspensions from the second or third i. c. passage of strain S 18888, kept under vaseline seal in the refrigerator, were diluted serially in phosphate-buffered saline (pH 7.4). The 10^{-5} to 10^{-9} dilutions were tested i. c. using 6 to 11 LCM-treated mice (see tables) and

Table 1. Titration of EEE virus in the brains of congenitally infected (tolerant) mice and normal controls

Experiment No.	No. of mice per decimal virus dilution	LD_{50} of EEE virus when titrated in the brains of		Average incubation period (hours)		Average survival period (hours)	
		LCM-infected mice	normal mice	LCM-infected mice	normal mice	LCM-infected mice	normal mice
1	6	7.1*	7.9*	61	52		
2	8	6.9	7.4	63	54		
3	8	7.0	7.7	60	50		
4	10	7.4	8.4	62	51		
5	6	7.3	8.4	69	55	94	70
6	8	7.1	7.3	61	52	97	69
Mean values		7.1	7.7	63	52	95	69

* Negative $\log_{10}$ of dilution of infectious 20 per cent mouse brain suspension.

equal numbers of normal controls of the same age per decimal dilution. The inoculated animals were observed 3 times daily and the incubation and survival periods recorded as accurately as possible. The LD_{50} was computed according to the method of *Reed* and *Muench* (3).

Tests for LCM virus: Simultaneously with some titrations of EEE virus (see Tables 2, 3, and 4) the brains of parallel mice not infected with EEE were tested individually for LCM virus by i. c. inoculation of 4 week-old mice with 20 per cent suspensions.

In the experiment presented in Table 4 heparinized blood obtained by cardiac puncture was tested for infectivity also. Before removing the brains and upper parts of the cervical cords the heads of the animals were perfused through the left ventricle each with at least 12 ml. saline.

Since i. c. infection with very small amounts of LCM virus often fails to cause definite symptoms but produces cerebral immunity, surviving test mice were challenged i. c. with 2 per cent mouse brain suspension 2 weeks after the first inoculation.

Interference with EEE virus in the brains of congenitally infected (tolerant) mice

In a preceding experiment (11) in which EEE virus was titrated intraperitoneally in young tolerant mice and normal controls, the infectivity titer of the virus was by 1.1 log lower in the former animals. The results of the present tests, in which about 4 week-old mice were used and the i. c. route of inoculation chosen, are recorded in Table 1.

As can be seen from the table, the LCM-infected mice were consistently slightly less susceptible to EEE infection than normal controls of the same age. The mean difference in the LD_{50} of EEE virus, in combination with prolonged average incubation and survival periods in the LCM-infected mice, indicates a low degree of interference. In Experiments 1—4 the average survival periods were not determined. Infected animals were chloroformed when they presented definite encephalitic symptoms because we anticipated cannibalism and accidental oral infections (13) resulting from it. Future experiments, however, showed this fear to be unwarranted.

Interference with EEE virus in the brains of non-tolerant mice infected s. c. and challenged i. c. with LCM virus

It has been reported (1) that cerebral challenge with LCM virus in mice immunized by s. c. inoculation of active virus if followed by considerable viral multiplication in the brain in spite of the fact that the animals do not show signs of disease. The virus, however, is eliminated more rapidly than in non-immune controls (1, 4). When challenged mice are given a second i. c. injection of LCM virus there is no evidence of viral growth and the challenge virus disappears from their brains within 48 to 72 hours (4, 10). Local antibody formation has been suspected to cause the relatively rapid inactivation of the virus in such cases, but it has not been possible to demonstrate neutralizing antibodies in brain extracts or suspensions (4, 10).

In the following experiments we investigated the fate of EEE virus in the brains of mice possessing a solid cerebral immunity towards LCM virus. Since the two viruses are unrelated serologically, antibodies were not expected to complicate the picture.

Table 2 gives the results of 6 titrations. It shows that the interference observed was similar in degree to that in tolerant mice in spite of the fact that the brains of the latter animals as a rule contain considerable amounts of active LCM virus. Judging from the average incubation and survival periods, there still was some interference on the 37th day after i. c. challenge with LCM virus. The length of the interval between the primary s. c. infection and the i. c. challenge did not appear to influence the degree of interference to any great extent.

Table 2. Titration of EEE virus in the brains of LCM-immune (non-tolerant) mice and normal controls

(Experiments 1—6)

Experiment No.	Days between s.c. and i.c. inoculation of LCM virus	Days between i.c. challenge with LCM and i.c. titration of EEE virus	No. of mice per decimal dilution of virus	LD_{50} of EEE virus when titrated in the brains of		Average incubation period (hours)		Average survival period (hours)	
				LCM-immune mice	normal mice	LCM-immune mice	normal mice	LCM-immune mice	normal mice
1	14	12	10	7.0	7.8	71	53	89	82
2*	148	12	11	6.7	7.3	93	56	130	87
3	30	12	10	6.7	7.1	79	61	112	91
4**	16	15	10	7.1	7.7	65	58	94	77
5	148	35	10	6.8	7.1	73	66	111	94
6	16	37	8	7.6	7.4	61	45	105	75
Mean values (Experiments 1—4)				6.9	7.5	77	57	106	84

* Of 14 parallel mice tested 4 carried traces of LCM virus in their brains.

** The brain of 1 out of 10 parallel mice contained a trace of LCM virus.

Table 3. Titration of EEE virus in the brains of LCM-immune (non-tolerant) mice various periods of time after i.c. challenge with LCM virus

(Experiment 7)

Days after i.c. challenge with LCM*	LD_{50} of EEE virus when titrated** in the brains of		Average incubation period (hours)		Average survival period (hours)		Tests for LCM virus in the brains of LCM-immune parallel mice	
	LCM-immune mice	normal mice	LCM-immune mice	normal mice	LCM-immune mice	normal mice	No. tested	No. positive
3	6.2	7.9	97	51	132	85	4	3†
7	6.7	7.6	78	61	114	83	4	0
14	7.2	8.1	64	53	91	79	4	1†
21	7.4	7.7	66	56	91	81	10	1†
28	7.0	7.0	63	57	91	83	10	0
35	7.3	7.6	62	53	90	80	10	0
42	7.4	7.8	56	53	92	80	10	0

* 31 days after s. c. infection with LCM virus.

** Using 8 mice per decimal dilution of virus.

† Only traces of virus detected.

Infectivity tests carried out with brain suspensions from parallel LCM-immune mice in Experiments 2 and 4 showed that, by the 12th and 15th day, only a small percentage of the animals tested still carried trace amounts (4) of active LCM virus in their brains.

The diminution of the interference with time was studied more systematically in Experiment 7 presented in Table 3. There was a moderate antagonistic effect towards EEE virus on the third day after i. c. challenge with LCM virus, when 3 of 4 parallel mice still harbored very small quantities of LCM virus in their brains. The effect decreased gradually in the following two weeks, but a low degree of interference was still evident

Table 4. Titration of EEE virus in the brains of mice various periods of time after s.c. inoculation with LCM virus

Days after s.c. inoculation with LCM virus	LD_{50} of EEE virus when titrated* in the brains of		Average incubation period (hours)		Average survival period (hours)		Tests for LCM virus in the blood and brains of parallel mice	
	LCM-treated mice	normal mice	LCM-treated mice	normal mice	LCM-treated mice	normal mice	LCM-treated mice	normal mice
3	7.1	7.7	71	51	101	69	2/10	0/10
7	5.9	7.2	66	47	100	70	5/10	1/10**
14	7.6	7.9	50	51	78	74	0/10	0/10
21	6.9	6.8	55	50	84	86		
26	7.7	7.8	53	51	80	82		

* Using 8 mice per decimal dilution of virus.

** The brain of 1 out of 10 mice tested contained a trace of active virus which failed to cause symptoms but produced cerebral immunity in 1 of 3 test animals.

on the 42nd day, when the experiment was discontinued. The results of infectivity tests of the brains of parallel mice indicate that persisting infectious virus was not responsible for the phenomenon.

In these animals, the virus content of the brains was unexpectedly low on the third day after i. c. challenge with LCM virus. There was no definite evidence of virus multiplication after challenge, which is in disagreement with the observations referred to above (1). The explanation may be that mice from different stocks react differently towards LCM virus (6).

Interference with EEE virus in the brains of non-tolerant mice infected s. c. with LCM virus

Since s. c. infection with LCM virus usually takes a mild course in mature mice, it appeared unlikely that the virus would affect the central nervous system in a high percentage of such animals. This has been con-

firmed by the infectivity tests recorded in Table 4, in which the perfused brains, with one exception, were non-infectious 3, 7, and 14 days after inoculation.

In spite of this fact, there was slight interference with EEE virus on the 3rd day and a stronger effect on the 7th day, which rapidly decreased thereafter. This is in contrast to the results obtained with mice challenged i. c. with LCM some time before the titration of EEE virus (cf. Tables 2 and 3).

Table 5. Duration of cerebral immunity in mature mice infected s. c. with LCM virus

Experiment No.	Result of i. c. inoculation with 2 per cent mouse brain suspension							
	Days after s. c. inoculation with LCM virus	immunized mice				normal controls		
		No. tested	accelerated reaction	death in convulsion	no symptoms	No.	accelerated reaction	death or killed in convulsion
1	14	9	0	0	9	9	0	9
	46	9	2	0	7	9	0	9
	88	9	9	0	0	9	0	9
	131	10	10	0	0	10	0	10
	203	10	0	0	10	8	0	8
2	19	10	0	0	10	10	0	10
	61	9	9*	0	0	9	0	9
	118	9	0	0	9	9	0	9
	148	54	majority	0	0	9	0	9

* Slightly ill with ruffled fur on 2nd and 3rd day, no tremors or convulsions, rapid recovery; clear difference between immunized mice and controls.

Duration of cerebral immunity in mature mice infected s. c. with LCM virus

In order to facilitate a correlation of the interference phenomenon with the duration of the specific immunity resulting from s. c. infection with LCM virus, we present in Table 5 the results of two experiments in which groups of mice were tested for cerebral immunity different periods of time after s. c. injection of 0.2 ml. of a 2 per cent infectious mouse brain suspension.

As the table shows, the immunity was strong at first but waning rather rapidly. This result is in line with previous ones (7). Numerous mice with a decreasing immunity showed a non-fatal accelerated reaction after a shortened incubation period (7, 4). There were indications in both experi-

ments that, after a period of decline, the immunity gained strength again. This observation requires confirmation by further tests. It may be connected with differences in the virus content of the suspensions injected i. c., although this is not a very convincing explanation.

To test the degree of antiviral immunity under standard conditions is a difficult task. As Tables 3 and 4 show, the titer of the same suspension of EEE virus may vary by about 1 log when tested at different times under identical conditions. The variation is somewhat less with suspensions of LCM virus, which have much lower infectivity titers. Experience has shown that, in the case of these two viruses, the use of deep-frozen or lyophilized brain suspensions does not eliminate the fluctuations in titer.

Discussion

There is no evidence suggesting that some unknown latent virus was causing the interference with EEE virus reported above, although one can never be quite certain that such an agent is not present. The search for another virus in the virus suspensions used and in the test animals has been unsuccessful.

Even though the degree of the interference was of a low order, there can be little doubt about its significance if one considers the data as a whole.

In evaluating the role of the interference phenomenon as an immunity factor in non-tolerant mice the possibility should be taken into account that the interfering effect may be much greater towards the homologous virus than towards a heterologous agent like EEE virus. This is indicated by the finding (11) that in tolerant mice there was no evidence of multiplication of the challenge virus following i. c. inoculation of about one million ID_{50} of an LCM strain distinguishable from the carried strain on account of its pathogenicity for new-born mice. It is not feasible to perform such an experiment with non-tolerant animals because in them specific antibodies impede the interpretation of the results. One may further assume that the duration of the effect against the homologous virus is longer than that towards an unrelated one. It is therefore likely that the interference phenomenon plays some part in the immunity of non-tolerant mice also, especially in its early stage. The results of recent studies on the immunity of mice towards EEE virus (12) suggest that a similar mechanism is prevailing there in spite of the fact that antibody production is much more marked than in LCM-immune mice.

The degree of interference with EEE virus was about the same in tolerant mice, whose brains always contain active LCM virus, as in non-tolerant animals, the brains of which were either not infectious or contained only traces of virus. It appears therefore that infectious LCM virus is not required to bring about the state of relative refractoriness. We have thus far been unable, however, to demonstrate an interferon (2) by the use of tissue culture methods. Despite this failure we believe that a sub-

stance of this sort is involved. The data given in Tables 3 and 4 can be interpreted in this way. In the first case (Table 3) an interferon was possibly produced in the brain securing there an effect of relatively long duration. In the second case (Table 4), the substance may have arisen in other organs and reached the brain indirectly at the time of maximum production. This may account for the shorter period of effectiveness in the central nervous system. The rapid subsidence of the interference during the second week after s. c. inoculation of LCM virus, when the antibody titer no doubt was still rising, indicates that LCM antibodies did not cause the antagonistic effect towards EEE virus.

The interference factor was able to make mice refractory to infection with small amounts of EEE virus and to retard the progress of the disease in animals infected, but it could not stop the encephalitis at a certain point. Among the many mice tested we have not seen a single animal recover and there were no inapparent infections followed by specific immunity. The phenomenon described here differs in this respect from the mechanism prevailing in mice infected with EEE virus and treated with specific immune serum of medium potency (8).

Summary and Conclusions

EEE virus was titrated in the brains of mice infected or immunized with LCM virus and in normal controls. There was slight but definite interference in the former animals.

The interfering effect was about the same in tolerant mice infected congenitally with LCM virus, whose brains contain considerable amounts of active virus, as in non-tolerant mature animals infected experimentally, the great majority of which did not carry detectable quantities of LCM virus in their brains. The interference thus appeared to be independent of the amount of infectious LCM virus present in the central nervous system. It lasted longer in mature mice infected subcutaneously and challenged intracerebrally with LCM virus than in those inoculated subcutaneously only.

It has not been possible by the use of tissue culture methods to demonstrate an interferon in brains in which interference occurred. In spite of this negative result a substance of this kind appears to be involved.

The possible role of the interference phenomenon as an immunity factor in non-tolerant mice is discussed.

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