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Experiments on Heterologous and Homologous Interference in LCM-infected Cultures of Murine Lymph Node Cells

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In mice infected congenitally or experimentally with the virus of lymphocytic choriomeningitis (LCM) there is slight but definite interference with a subsequent infection with eastern equine encephalomyelitis (EEE) virus (5, 6). The effect is of relatively short duration in mature mice infected experimentally. In the brain, it appears to be independent of the presence of infectious LCM virus, but it has not been possible to demonstrate interferon as the causative or mediating factor. Similar observations were reported more recently by *Wagner* and *Snyder* (11).

Results obtained with congenitally infected (tolerant) mice (5, 6) indicate that the interfering effect is much stronger towards the homologous virus than towards a heterologous agent like EEE virus. While the solid immunity shown by tolerant mice (virus carriers) is probably due exclusively to the interference phenomenon, the latter appears to be a factor of some importance in the immunity of non-tolerant animals as well, at least in its initial stage (6).

In the experiments to be reported in this paper, heterologous and homologous interference were studied in cultures of lymph node cells (LNC) from tolerant and normal (or leukemic) mice, and new attempts were made to demonstrate interferon in such cultures.

Materials and Methods

Strains of virus: The strains of LCM virus used (W and WCC) and the behavior of the unmodified strain W in murine LNC were described in earlier papers (5, 7). Strain WCC is highly pathogenic when injected intracerebrally (i. c.) in newborn mice, while the pathogenicity of strain W for such animals is low. This marker was used to differentiate the two strains in tests for homologous interference *in vivo* (5).

Strain 18,888 of EEE virus and type "Indiana" of vesicular stomatitis (VS) virus were chosen for tests for heterologous interference. Both viruses are known to be susceptible to the effect of interferon. The EEE virus had undergone 1 passage in chick embryos and 2 i. c. passages in mice in this laboratory, the VS virus 2 passages in chick embryos.

Mice: In addition to animals from stock III, normal or congenitally infected (5), inbred mice from a small colony of the AKR strain were used, in which lymphatic leukemia is very frequent. Leukemic mice, whose LNC usually grow better than normal LNC, served as sources of tissue in some tests (see tables).

Preparation of tissue cultures: The method was described previously (7) and slightly modified with regard to fractionated trypsination later (8). To obtain optimal growth, the cultures of normal LNC were usually prepared from a mixture of trypsination fractions III and IV, those of leukemic LNC from fractions II and III.

Infectivity titers: These are expressed in the tables as the negative $\log_{10}$ of the ID_{50} computed as before (7).

Further technical details will be given in the following section.

Experimental

Tests for heterologous interference with EEE and VS viruses in cultures of LNC from tolerant mice and of normal LNC infected with LCM in vitro

It should be recalled in this connection, in LNC from tolerant mice, the multiplication of LCM virus continues *in vitro* at about the same level as *in vivo* for weeks (7), the viral growth curve resembling a horizontal straight line. There is no definite lag phase in these cultures (8). In view of such a curve, periodical production of interferon is unlikely to begin with. There is hardly more evidence for continuous production of small amounts of interferon, since the infectivity titers of such cultures are relatively high.

The odds were different with LNC from normal (or leukemic) mice infected *in vitro*. The viral growth curve obtained from such cells resembles a sinus line during the first six weeks of cultivation (7, 8). In other words, viral multiplication occurs in waves, and this may well be due to periodical formation of virus and interferon. To test this hypothesis, interference tests with heterologous viruses were carried out different periods of time after infection with LCM virus.

In the experiments with EEE virus recorded in Table 1, test tube cultures of normal or leukemic LNC were infected with LCM virus (strain W) using a Berkefeld N filtrate of infectious culture fluid from tolerant cells. The filtrate was diluted 1:2 with standard medium (7) and added to the cell layers in amounts of 2 ml. A number of cultures remained uninfected to serve as controls in the titration of EEE virus. Media were changed every 3 or 4 days.

For superinfection with EEE virus, decimal dilutions of infectious 20 per cent mouse brain extract were made in standard medium and the dilutions 10^{-6} to 10^{-10} added to the cells instead of regular medium using 3 (experiments 1 and 2) or 6 (experiments 3) tubes per dilution. Hereafter, the cultures

were incubated at 36,5° C for 66 hours. Since murine LNC infected with EEE virus do not regularly show conclusive cytopathogenic changes, the cultures were individually tested for infectivity by i. c. mouse inoculation. The rate of thermal inactivation of the seed virus was checked by including control tubes without cells. In view of the results of this test and the short incubation

Table 1. Tests for interference with EEE virus in LCM-infected cultures of murine LNC

Experiment No.	Origin of LNC	Infection with LCM virus	Titration of EEE virus (20 per cent mouse brain suspension)	
			Time	ID_{50} for cells
1	Congenitally infected (tolerant) mice	in vivo	34th day of cultivation	7.5
	dtto.	dtto.	19th day of cultivation	7.2
	Normal mice (III)	—	20th day of cultivation	7.2
2	Congenitally infected (tolerant) mice	in vivo	16th day of cultivation	8.0
	Normal mice (III)	3rd day of cultivation	14 days after LCM	7.5
	dtto.	13th day of cultivation	4 days after LCM	7.5
	dtto.	—	17th day of cultivation	7.5
3	Leukemic AKR mice	7th day of cultivation	7 days after LCM	7.6
		—	14th day of cultivation	7.5

periods in the mice injected with culture fluid, it is certain that the EEE virus demonstrated in the cultures had actually multiplied there. Cultures previously infected with LCM, which did not contain infective amounts of EEE virus, without exception produced typical LCM in the injected mice.

The EEE titers recorded in Table 1 show that infection with LCM virus failed to reduce the susceptibility of the cells for EEE virus. In other tests (not included in the table) made with a number of cultures infected with endpoint dilutions of EEE virus, previous infection with LCM virus did not significantly change the rate of multiplication of the superinfecting virus.

As a preliminary to *in vitro* experiments with VS virus, this agent was titrated i. c. in tolerant and normal mice using 8 animals per decimal dilution of virus. The titers obtained were 7.6 and 7.4, respectively. They indicate that the chronic LCM infection did not reduce the susceptibility of the mice for VS virus. However, there was definite retardation of its multiplication as shown by prolonged average incubation (84 vs. 56 hours) and survival periods (102 vs. 79 hours) in the tolerant animals.

Apart from minor modifications, the interference tests made with VS virus *in vitro* were carried out in the same way as those with EEE virus.

Table 2. Tests for interference with VS virus in LCM-infected cultures of murine LNC

Experiment No.	Origin of LNC	Infection with LCM virus	Titration of VS virus			
			Time	Dilution factor	No. of cultures per dil.	ID_{50} for cells
1	Leukemic AKR	5th day of cultivation	7 days after LCM	10	4	8.0
		—	10th day of cultivation			8.7
2	Normal mice (III)	5th day of cultivation	4 days after LCM	10	6	8.3
		—	9th day of cultivation			8.0
3	Mice (III) infected with LCM 14 days before preparation of cultures	*in vivo* (cells still infectious when harvested)	6th day of cultivation	5	7	8.3
	Normal controls (III)	—	dtto.			8.2

A deep-frozen suspension of ground embryos and membranes in allantoic-amniotic fluid (second egg passage) was titrated (10^{-6} to 10^{-10}) in test-tube cultures previously infected with LCM virus and in uninfected controls. In spite of the fact that VS virus has a very distinct cytopathogenic effect in murine LNC, the cultures infected with endpoint dilutions of this virus were tested for infectivity i. c. in mice. These tests were made after incubation for 47 hours in experiment 1, 71 hours in experiment 2, and 69 hours in experiment 3. There was close agreement between their results and those of the microscopic examination. Tests made in mice also showed that LCM virus had infected the cells with absolute regularity.

Except in experiment 1, in which the VS titers in LCM-infected and uninfected cells differed by 0.7 log, there was no indication of a reduction

of the susceptibility of the cells for VS virus as a consequence of the persisting LCM infection. The significance of the difference noted in experiment 1 appears doubtful in view of the negative results obtained in the other tests.

Experiments on homologous interference between strains W and WCC in vitro

Supplementing earlier *in vivo* experiments in tolerant mice (5), interference tests with strains W and WCC have been carried out *in vitro* using LNC from tolerant and normal mice. Other cultures of normal cells were infected with strain W 4—14 days prior to the test with strain WCC (see Table 3).

It should be mentioned here that newborn mice injected i. c. with strain WCC show a decreased growth rate which, as a rule, is noticeable by the 7th day after inoculation. The great majority of the animals dies from emaciation 2 to 6 days later. Surviving mice usually have the appearance of runts. They will have scanty, rough hair and usually remain exceptionally small for several weeks. Strain W produces such symptoms very rarely. The few animals which die from neonatal i. c. infection with this strain will grow hair normally but show severe diarrhea 13 to 20 days after infection. In addition, signs of involvement of the central nervous system are not rare in such cases.

Newborn mice infected i. c. with either strain will regularly infect their mothers by contact. While contact infection with strain WCC, modified by 367 i. c. passages in adult mice, is usually harmless for the mothers, contact infection with strain W sometimes causes severe disease. The animals may even die in typical convulsions. In such cases it is necessary to provide foster mothers to save the young from starvation.

In the experiment presented in Table 3 there was not a single fatal case among 75 suckling mice infected with strain W, while all of 18 controls receiving strain WCC died within 9 to 13 days.

Of 56 newborn mice infected with culture fluids from tolerant LNC exposed to strain WCC, 5 died presenting a picture as just described for strain W. It is therefore very unlikely that strain WCC had gained a foothold in any of these cultures.

Culture fluids from normal LNC infected with strain W *in vitro* and exposed to strain WCC 9 or 14 days later failed to cause fatal disease in 32 newborn mice indicating that strain WCC had not multiplied in those cultures. There is definite evidence, however, that this strain persisted for 4 days, but not for 6 days, in cells exposed to strain WCC 4 days after *in vitro* infection with strain W. In the subsequent titration of the respective (deep-frozen) culture fluid in newborn litters a clear distinction between mice infected with strains W and WCC, respectively, was possible. The result suggests that only a small amount of WCC virus was present in that culture.

All surviving infantile mice were immune to i. c. inoculation of virus at the age of 4 weeks.

In the second experiment on homologous interference recorded in Table 4, LNC from normal and immune mice were exposed to strain WCC 0 to 7 days after *in vitro* infection with strain W.

Table 3. Homologous interference between strains W and WCC in cultures of LNC from tolerant and normal mice

Origin of cells	Infection with W	Addition of WCC	Mortality in newborn mice injected i. c. with culture fluids harvested		
			Before WCC	4 days after WCC	6 days after WCC
Tolerant mice	*in vivo*	45th day of cultivation	0/9	0/8	1/9 (18 days)*
		30th day of cultivation	0/8	0/9	0/9
		16th day of cultivation	0/7	0/12	4/9 (18 days, D)*
Normal mice	—	WCC only		9/9 (11 days)*	9/9 (10 days)*
	in vitro	4 days after W	0/8	5/8† WCC titer: 0.5 W titer: 4.1	0/14
		9 days after W	0/8	0/7	0/9
		14 days after W	0/9	0/8	0/8
		—**	7 days after W: 0/10	13 days after W: 0/9	18 days after W: 0/7

D = Diarrhea.
* Average survival periods in mice which died.
** Additional tests to confirm the low pathogenicity of strain W after different periods of growth in cultures.
† 3 survivors were "runts".

The cultures of LNC from non-tolerant immune mice were made 77 days after s. c. infection of the cell donors with serum from tolerant mice of the infected stock (strain W). The cells proved to be virus-free to begin with. They and the normal LNC were infected with strain W on the 14th day of cultivation, followed by strain WCC 0 to 168 hours later as indicated in the table. Hereafter, the cultures were incubated for 4 days and then tested, i. c. in newborn litters consisting of 8 or 9 animals each. In dubious cases, the inoculations were repeated using the deep-frozen cultures. Moreover, passages to new litters were made from the first ones using the brain of one suckling mouse per litter. The animals were killed when sick or else on the 14th day after inoculation. All survivors acquired a solid cerebral immunity.

The interpretation of the results given in Table 4 is somewhat difficult due to the fact that interference is not always an "all or none" effect, but varies considerably in degree. Besides the mortality, the percentage of runts and the average survival period in mice which died may serve as criteria in identifying or guessing the strain or strains of virus present in the cultures.

Table 4. Homologous interference between strains W and WCC in cultures of LNC from normal and immune mice

No.	Interval between W and WCC	Infectivity tests of culture fluids in newborn mice (i. c.)							
		Cultures of LNC from normal mice				Cultures of LNC from immune mice			
		a	b	a + b	Mean	c	d	c + d	Mean
		Mortality rate	Surviving "runts"		Survival period*	Mortality rate	Surviving "runts"		Survival period*
		per cent	per cent	per cent	days	per cent	per cent	per cent	days
1	1 min.	12	82	94	11	47	35	82	15
2	1 hour	37	17	54	14	53	47	100	13
3	6 hs.	12	0	12	14	82	12	94	12
4	24 hs.	0	0	0		29	25	54	15
5	48 hs.	0	0	0		29	18	47	14
6	72 hs.	18	0	18	15	47	6	53	12
7	96 hs.	0	0	0		6	0	6	19
8	120 hs.	31D†	6	37	17	0	0	0	
9	144 hs.	21D	0	21	19	0	0	0	
10	168 hs.	0	0	0		29 D	0	29	18
11	W only	14D	0	14	17	0	0	0	
12	WCC only	89	11	100	10	86	14	100	13

* In mice which died. D† = Diarrhea.

In cultures of LNC from normal mice, the presence of strain WCC (possibly besides W) appears certain in No. 1, highly probable in No. 2, and possible in No. 8, while the cultures of LNC from immune mice may be judged as follows: certain Nos. 1, 2, and 3; highly probable Nos. 4, 5, and 6. This result indicates a difference between LNC from immune and normal mice, but requires confirmation by further tests. On the whole, there can be little doubt that homologous interference between distinguishable strains of LCM virus does occur *in vitro*.

Tests for interferon

In principle, these tests were carried out as follows: culture fluids removed from non-tolerant LNC infected *in vitro* or from tolerant LNC infected *in vivo* were subjected to gentle thermal inactivation followed by high-speed centrifugation. Normal cells were treated with the superna-

Table 5. Tests for interferon in LCM-infected cultures of LNC from non-tolerant and tolerant mice

Experiment No.	Culture fluids tested for interferon: Origin	Days after *in vitro* infection	Infectivity titer at time of harvest	Titration of LCM virus* in normal cells treated with sterilized culture fluid: Cells	ID_{50} for cells	Virus production in treated cultures: i. c. titers of cultures infected with virus* dilution 10^{-1} and incubated for: 24 hours	48 hours
1	Cultures of LNC from normal (non-tolerant) mice infected with LCM virus *in vitro*	2	4.3	Mouse embryo cells	2.5	1.2	2.7
		5	3.1		2.5	1.2	2.7
		9	1.5		2.5	1.5	3.5
		13	1.2		2.5	1.2	2.5
		17	1.3		2.2	1.2	2.5
		21	1.2		2.2	0.7	2.5
		normal control			1.7	0.7	2.2
		9—21 mixture	1.2	LNC	1.5	0.7	2.2
		normal control			1.7	0.5	3.2
2	dtto.	4	4.2	LNC	2.5	1.0	3.7
		14	2.5		2.0	1.2	4.0
		normal control			2.5	0.7	4.2
3 a	Tolerant LNC		3.7	Mouse embryo cells	2.5	0.7	3.5
	Normal control		—		2.7	0.0	2.5
3 b	Tolerant LNC		3.7	LNC	3.5	1.2	3.2
	Normal control		—		2.5	1.5	4.2

* Berkefeld N filtrates of culture fluids from LNC of tolerant mice.

tants *in vitro* and their susceptibility to LCM virus and suitability for viral reproduction tested subsequently.

In experiment 1 (Table 5) the culture media to be tested for interferon were obtained from cultures of normal LNC infected *in vitro* with LCM virus on the 14th day of cultivation. The media collected 2—21 days after infection were deep-frozen and titrated simultaneously i. c. in mice. To inactivate the virus, they were incubated at 36.5° C for 90 hours and then centrifuged at 30,000 r. p. m. for 90 minutes using the medium rotor of a Spinco Model L

preparate ultracentrifuge. As controls, media from uninfected cultures were subjected to the same treatment.

The supernatants were mixed with equal quantities of standard medium and the mixtures added in 2 ml. amounts to monolayers of well-grown normal embryo or lymph node cells in test tubes. It was not feasible to use the plaque technique, since murine LNC do frequently not form a dense cell sheet in cultures. After incubation for 24 hours, undiluted infectious culture filtrate and decimal dilutions (10^{-1}—10^{-5}) of it in standard medium were added to the cultures in amounts of 0.5 ml. using 3 tubes per dilution. Of the triplicate cultures, one was incubated for 24 hours, one for 48 hours, and the third for 72 hours after addition of virus and then tested for infectivity i. c. in mice. The titers recorded in column 6 of Table 5 were computed from the results of these infectivity tests.

To get an idea of the amounts of virus formed in treated and untreated cells in the early phase of viral reproduction, the deep-frozen cultures infected with virus dilution 10^{-1} and incubated for 24 and 48 hours, respectively, were titrated i. c. in mice (7). The titers are given in columns 7 and 8 of Table 5.

The same method was used in experiments 2 and 3.

The culture fluids tested in experiment 1 were suitable for tests for interferon inasmuch as the cells in the cultures of origin behaved in a typical fashion. The infectivity titer dropped from 4.3 on the 4th day to 1.2 on the 13th day (see column 4) and rose again to 3.5 between the 25th and 30th day (the culture fluids harvested after the 21st day were not tested for interferon in view of the negative result obtained with the fluids collected earlier).

The titration results presented in columns 6—8 give no clear indication of the presence of interferon in any of the culture fluids examined. This applies also to the mixture of fluids tested in normal LNC. The difference of 1 log (48 hours, column 8) cannot be regarded as significant in view of the equal titers obtained at 24 hours (column 7) and the rather crude method of titration used. The results of experiments 2 and 3 must be regarded as negative also.

Discussion

Observations reported from different laboratories concerning the interference phenomenon in murine LCM may be summarized as follows: (a) Congenitally infected mice (tolerant virus carriers) are already immune at birth and maintain a solid immunity for many months (3) in spite of the fact that they do not form antibodies (9, 1, 4). This observation has been correlated with the interference phenomenon (3). (b) Small amounts of LCM virus of one strain can interfere with the infection by another, distinguishable, strain in mature (2). (c) In tolerant mice, there is strong homologous interference of strain W with strain WCC (5), but a much lower degree of heterologous interference with EEE or VS viruses (6, 11). (d) An interfering effect towards EEE virus of relatively short duration is demonstrable in LCM-infected and in cerebrally immune (non-tolerant) mature mice (6, 11). In the brain, this effect does not seem to depend

strictly upon the presence of infectious LCM virus (6). (e) A moderate degree of heterologous interference with VS virus has been observed in LCM-infected cultures of murine L-cells (11). (f) In murine LNC infected *in vivo* or *in vitro* with LCM virus, homologous interference can be readily demonstrated, but not heterologous interference with the viruses of EEE or VS (see above). (g) It has not been possible to detect interferon as the cause of the interference either *in vivo* (6, 11) or *in vitro* (11, see above).

One may conclude from these observations that there is a mechanism responsible for heterologous interference *in vivo*, but missing or of very low efficacy in tissue cultures. Since it is often very difficult to demonstrate interferon in the animal (10), the failure to detect it in the present case is not convincing proof for its absence. Stronger evidence is the failure in different laboratories to find it in tissue cultures. However, the possibility still cannot be ruled out that interferon is present in a concentration too low to be detected by the methods employed.

The mechanism of the homologous interference demonstrated in murine LNC thus remains a problem. The same applies to the cause of the remissions in viral growth curves obtained from non-tolerant LNC infected *in vitro*.

Summary

Heterologous interference with the viruses of EEE and VS was not demonstrable *in vitro* in LCM-infected lymph node cells from tolerant or non-tolerant mice in spite of the fact that slight interference with these viruses occurs in the LCM-infected mouse.

However, there was marked homologous interference in such cultures with strain WCC of LCM virus, which can be distinguished from the infecting strain W by its higher pathogenicity for newborn mice.

It has not been possible to detect interferon in cultures in which homologous interference occurs and to identify such a substance as the cause of the fluctuations of the viral growth curve characteristic for lymph node cells from non-tolerant mice.

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