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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Investigations of the thermostability of the virus of lymphocytic choriomeningitis.

by F. Lehmann-Grube.

Archiv f. d. Gesamte Virusforschung, 9: 56-63 (1959).

Introduction.

Attempts to establish titers and titer progressions of neutralizing antibodies in human sera after infection with the virus of lymphocytic choriomeningitis (LCM) in tests with mice led to initial contradictory results, necessitating an improvement of this method. Within the framework of these endeavors, the stability of virus suspensions had to be tested under all conditions of subsequently planned experiments.

Method.

Repeated attempts to induce a cytopathogenic effect in tissue culture with LCM virus have failed also in the laboratory of the Cologne University Nerve Clinic. We were forced to resort to the mouse as a system of evaluating our tests.

Virus: We utilized strain WE3 which had been sent to us in 1952 by Dr. K.F. Meyer, San Francisco, Calif. The virus has since undergone 50 guinea pig passages by subcutaneous inoculation and was adapted to the mouse prior to the commencement of testing by intracerebral transmission. We used the material of the 8th to the 14th mouse-to-mouse passage.

The strain suspensions were prepared as follows: 30-40 mice received 0.04 ml (later 0.03 ml) of a 10% mouse brain suspension intracerebrally (i.c.). The brains of animals dying on the 6th day were stored at -70° C. All remaining mice were sacrificed by bleeding on the 7th day -- if they had not succumbed sooner --, the brains were extracted aseptically and also stored at -70° C. The material usually was treated on the same day, at times a few days later.

Upon addition of 100 units of penicillin and 20 γ streptomycin per ml of prepared suspension, the organ material was reduced in size to a 10% or 20% suspension with physiological saline or with inactivated rabbit serum in the homogenizer (Fa. Buehler)(about 5 min.). After centrifugation -- first 10 min. at 2,500 RPM, later twice 10 min. at 5,000 RPM in a customary laboratory centrifuge -- the supernatant was placed in ampullae in quantities of 1-2 ml and stored at -70°C. All steps followed each other in rapid succession. Glass equipment and solutions were pre-cooled.

Sera: Control sera came from healthy individuals whose anamneses presented no suspicion of a previously overcome LCM infection. A large

amount of blood taken from the arm vein was exposed to room temperature for about 2 hours and then centrifuged. Subsequently the serum was placed in ampullae in lots of 1,0 ml without further treatment and stored at -20°C. The required amount was defrosted just prior to use and, unless otherwise indicated, inactivated at 56°C for 30 minutes.

We obtained the mice from a major breeder in Hamburg whose animals had proved reliable for almost 10 years. There were never any indications of LCM, such as experienced by E. Traub (1939). However, an increase in non-specific deaths among the controls was noted in the course of the investigation. Although significant dimensions were not reached -- in a major test in which 180 control mice were inoculated intracerebrally with non-LCM mouse brain suspension, only 8 died between the 6th and 25th day ---, we investigated the cause, which was first sought in bacterial infections. In this connection we isolated ectromelia virus. Later it became impossible to establish the time at which the mouse colony had been infected. A repetition of the entire previously conducted work did not seem justified. However, all tests requiring a greater precision were repeated with unobjectionable animals (*). The titers were in some cases more easily reproduced, as a comparison of Table 1 and Table 2 will show. Otherwise the results were principally unchanged.

The weights of the mice within the individual shipments were highly uniform. Thus, an average weight of 11.9 g with a standard deviation of only 1.2 g was found among 100 mice received on 27 Feb. 56. The average weights revealed only small fluctuations from one shipment to the other. They lay between 11 and 14 g. As a rule, the animals were utilized a few days after their arrival.

Measurement of effectiveness (activity): Departing from 10% brain suspensions, series with the factor 10 (= 1 $\log_{10}$) were prepared with different dilution agents. Initially we used 4 or 6 mice per dilution. Later 6 or more animals were inoculated. We dispensed 0.04 ml intracerebrally according to E. Traub's method. It was discovered, however, that the mice tolerate 0.03 ml more easily, causing us to use the smaller dose exclusively in later runs. LD_{50} was computed by the method of B. Behrens (1929) and L.J. Reed and H. Muench (1938). In accordance with the literature and our own experiences, we included in our computations all animals that had died between the 6th and the 25th day.

In order to reduce the method's error, we endeavored to bracket LD_{50} symmetrically from both sides (M. Pissi - 1950).

Penicillin (100 units/1 ml)and streptomycin (20 γ/1 ml) were added to all diluting agents.

Results.

Even extended storage of the virus suspension at -70°C does not influence the titer noticeably, as shown by Tables 1 and 2. A systematic drop in LD_{50} undoubtedly does not exist. Table 3 reveals the loss of

activity of a mouse brain suspension prepared with physiological saline and exposed to freezer temperatures. A distinct drop is noted only after more than 20 hours. Table 4 contains parallel analyses of an identical suspension (columns 4 and 5) in comparison to a suspension produced with pure, inactivated rabbit serum in place of physiological saline. In accordance with a method to be described later, titration was accomplished with physiological saline + 10% inactivated rabbit serum. No drop in activity is indicated in either case even after 50 hours. The conditions are similar when the diluted series instead of the concentrated suspension was subjected to the same treatment prior to inoculation. Repeated experiments tested the effect of freezer temperatures on a brain suspension diluted with physiol. saline. Table 5 shows that the loss in activity was small. Although an important inactivation during preparations leading to inoculation with physiol. saline was not indicated after these tests, we nevertheless decided not to work with physiol. saline in the future, but to use a liquid which promised sufficiently stabilizing properties.

Table 6 contains the results of a test series which established the effect of different diluting fluids on virus activity at a temperature of + 2°C, partly in parallel tests (cf. the dates). It is noticeable that saline + 10% rabbit serum, phosphate buffer and phosphate buffer + 10% rabbit serum are equally stabilizing. Physiological saline alone causes a distinct decrease in virus activity after 50 hours. Moreover, the initial titers were lower; an observation that was made repeatedly in the course of the investigation.

Finally, tests were made to determine whether the starting suspension prepared with pure, inactivated rabbit serum would show deviations after dilution with 10% rabbit serum. A glance at Table 7 reveals that here again there was no uniform attenuation after 50 hours.

The effect of the incubation temperature of 37°C seemed to be significant particularly for the choice of incubation time. Earlier tests with the method described above had indicated that the drop in titer was very low even after 6 hours in the presence of an inactivated control serum. The results of the following test (see Table 8) confirm these observations. The starting suspension consisted of a mouse brain suspension produced with pure, inactivated rabbit serum. Dilution was accomplished with 10% rabbit serum and — for comparison — with pure physiol. saline. The ultimate concentration was obtained by the addition of 1. human control serum, 2. 10% rabbit serum and 3. physiol. saline in equal parts. It is clear that in the presence of serum the temperature of 37°C does not cause an appreciable titer drop even after 6 hours. The fact that the matter is different in the case of saline is not surprising. It is noteworthy, however, that here again the initial value of the non-serous mixture is relatively low without the influence of temperature.

Discussion.

In order to improve the analytical methods for neutralizing serum antibodies against the virus of lymphocytic choriomeningitis, it seemed

indicated to test the stability of the virus extensively under different circumstances. It may be concluded from the test results disclosed here that the LCM virus is considerably more labile than, for instance, the polio types (J.S. Younger, 1957) or the ECHO viruses (F. Lehmann-Grube, 1958). Our practical investigations reveal, however, that temperatures unavoidably induced in the course of preliminary steps leading to inoculation are not important. Our observations therefore do not agree with those of other authors who reported considerable titer losses under identical conditions, as, for instance, J.T. Heyl and others (1948). They noted a considerable loss of activity after 60 minutes in the ice water bath. --- Another question seeks to establish whether the use of unbuffered saline might not exert a disturbing influence -- perhaps by shifts in pH --- directly and independently of time. Repeatedly reported observations made in parallel tests to the effect that virus suspensions containing serum yield considerably higher titers than those without serum (see Tables 7 and 8), speak for a prejudicial influence of unbuffered saline.

We were forced thereby to work with a more stable system. Based on the frequently used standard method of the U.S. Army, described by J.S. Simmons and C.J. Gentzkow (1944), we chose for our additional tests a dilution fluid composed of physiological saline + 10% rabbit serum inactivated for 30 minutes at 60°C. The same reasons prompted us to adopt a method described there for the preparation of virus suspensions in pure inactivated rabbit serum. This measure also improved the effect of centrifugation. The sediment was solidly packed and the supernatant could be withdrawn easily, an action that was possible only with losses in the case of physiol. saline. A large number of sera was used in the course of further tests for the production of brain suspensions and dilution series. There were never any indications of virucidal properties of inactivated rabbit sera.

Summary.

The stability of LCM virus was tested under conditions that seemed significant for the future development of a method of neutralization. Neither 10% mouse brain suspensions nor dilution series made with various liquids showed a noteworthy titer loss after 24 hours at freezer temperatures. [illegible] this interval saline was distinctly inferior to other dil[illegible] — addition of 10% rabbit serum, phosphate buffer with or without admixture of serum. Moreover, the titers were usually lower, when dilutions were made with saline. At 37°C a titer drop was evident already after a few hours, if this dilution agent was used. Here, too, the stability was considerably improved by admixture of serum.

(*) Footnote on p. 2: We are indebted to Prof. Traub (Tuebingen) for mice free of ectromelia.

Table 1. Influence of storage at -70°C on a brain suspension prepared with physiological saline. Average log LD_{50}: -5.44.

Date of inoculation	Storage at -70°	Number of animals per dilution	Log LD_{50}	Deviation from the average
18 Apr 56	29 days	4	-5.5	∕0.06
3 May 56	44 days	6	-5.24	-0.20
16 Jul 56	118 days	6	-5.53	∕0.09
5 Sep 56	169 days	6	-6.37	∕0.93
5 Sep 56	169 days	4	-6.03	∕0.59
21 Dec 56	276 days	4	-4.53	-0.91
15 Jan 57	301 days	4	-4.96	-0.48
19 Feb 57	336 days	6	-5.31	-0.13

Table 2. Influence of storage at -70°C on a brain suspension prepared with 10% rabbit serum. Average log LD_{50}: -6.28.

11 Feb 58	0 days	6	-6.40	∕0.12
4 Mar 58	21 days	6	-6.00	-0.28
9 Apr 58	57 days	6	-6.40	∕0.12
22 Apr 58	70 days	7	-6.19	-0.09
12 May 58	90 days	6	-6.39	∕0.11

Table 3. Influence of a temp. of ∕2°C on an undiluted mouse brain suspension emulsified with physiological NaCl. Titrated with saline. 6 mice per dilution. 0.04 ml i.c.

Time in hours	Log LD_{50}
0	-5.12
	-5.12
20	-4.72
47	-3.0
72	-3.0
96	-2.24
120	-1.64
168	< -1.0
192	< -1.0

Table 4. Influence of freezer temperatures on 2 different mouse brain suspensions. Two analyses. Titrated with physiol. saline ∕ 10% Rabbit serum. 4 animals per dilution. 0.04 ml i.c.

Time in hours	Suspension with rabbit serum		Susn. with physiol. NaCl	
	29 Aug 56	13 Feb 57	29 Aug 56	13 Feb 57
0	$10^{-5.46}$	$10^{-5.7}$	$10^{-5.87}$	$10^{-4.93}$
6	$10^{-6.03}$	$10^{-5.45}$	$10^{-5.96}$	$10^{-6.7}$
24	$10^{-5.2}$	$10^{-5.2}$	$10^{-6.47}$	$10^{-5.2}$
50		$10^{-5.93}$		$10^{-5.2}$

Table 5. Influence of a temperature of +2°C on the dilution series of a virus-mouse brain suspension prepared with physiological saline. 4 identical tests. 4 animals per dilution. 0.04 ml i.c.

Date	Time in hours			
	0	8	24	50
22 Aug 56		$10^{-4.95}$	$10^{-5.2}$	$10^{-4.95}$
5 Sep 56	$10^{-6.03}$		$10^{-5.34}$	
21 Dec 56	$10^{-4.53}$	$10^{-4.47}$		$10^{-3.7}$
15 Jan 57	$10^{-4.96}$	$10^{-4.54}$		$10^{-3.87}$

Table 6. Influence of the refrigerator temperature (about +2°C) on the dilution series of a mouse brain suspension emulsified with physiological saline in the presence of different diluting agents. Two analyses. 4 animals per dilution. 0.04 ml i.c.

Time in hours	Physiol NaCl		NaCl+10%rab.serum		Phosphate buf.	Ph.buf+10%rab.ser.		
	29 Aug 56	11 Jan 57	29Aug56	13Feb57	20Sep56	26Sep56	20Sep56	26Sep56
0	$10^{-4.7}$	$10^{-4.2}$	$10^{-5.87}$	$10^{-4.93}$	$10^{-5.2}$	$10^{-5.54}$	$10^{-5.7}$	$10^{-5.53}$
6	$10^{-5.2}$	$10^{-4.37}$	$10^{-5.7}$	$10^{-5.92}$	$10^{-5.34}$	$10^{-5.2}$	$10^{-5.87}$	$10^{-5.37}$
24	$10^{-5.08}$	$10^{-4.03}$	$10^{-6.2}$	$10^{-5.03}$	$10^{-5.4}$	$10^{-4.86}$	$10^{-6.47}$	$10^{-6.09}$
50		$10^{-2.7}$		$10^{-5.48}$		$10^{-4.45}$		$10^{-5.13}$

Table 7. Suspension prepared with rabbit serum in a dilution series (physiol. saline + 10% rabbit serum) at refrigerator temperature. Two analyses. 4 animals per dilution. 0.04 ml i.c.

Time in hours	Physiol. NaCl and 10% rabbit serum	
	29 Aug 56	11 Jan 57
0	$10^{-5.48}$	$10^{-5.26}$
6	$10^{-5.83}$	$10^{-5.67}$
24	$10^{-5.93}$	$10^{-5.95}$
50		$10^{-4.7}$

Table 8. Mouse brain suspension prepared with rabbit serum in dilution series in the presence of different diluting agents, exposed to 37°C. 6 mice per dilution. 0.03 ml i.c.

Time in hours	Diluted w/phys. NaCl+10%rab.serum Control serum	Diluted w/phys. NaCl+10% rab. serum. No control serum	Diluted w/ physiol. NaCl without rab. ser.
0	$10^{-0.0}$	$10^{-5.96}$ (5.66?)	$10^{-4.85}$
2	$10^{-5.64}$	$10^{-6.16}$	$10^{-3.76}$
4	$10^{-5.16}$	$10^{-5.06}$ (5.66?)	$10^{-3.33}$
6	$10^{-5.12}$	$10^{-5.42}$	$10^{-3.12}$