13402

Effect of Dilution of Neutral Mixtures of Eastern Equine Encephalomyelitis Virus and Antiserum.*

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Neutral mixtures of a number of viruses and their homologous antisera have been found to be reactivated by simple dilution with 0.85% saline solution.[1-10] The same phenomenon holds true for bacteriophage-antibacteriophage serum,[11, 12] toxin-antitoxin,[13-16] as well as a neutral mixture of the pneumococcus type III.[17]

The work of Merrill[18] suggested an exception to the above general findings. In his report, a mixture of eastern equine encephalomyelitis virus and serum composed of a 10% suspension of guinea pig brain and undiluted immune horse serum when serially diluted 10^{-1} to 10^{-5} did not produce infection when injected intraperitoneally into mice. The question as to whether or not this particular virus-serum mixture becomes active on dilution has an important bearing on neutralization tests which are so frequently used in research with this agent of disease and seemed worthy of reinvestigation.

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In carrying out the studies, advantage was taken of the various contributions of Olitsky and colleagues[19-21] which emphasized the importance of the intraperitoneal route of inoculation in mice. These workers showed this avenue to be the most effective method for demonstrating the protective power of the serum but called attention to the necessity of using 15-day-old albino mice which are highly susceptible to the virus.

Methods. The virus used was a strain of eastern equine encephalomyelitis obtained from Dr. Jerome T. Syverton in 1938. It has been maintained by intracerebral passage in mice with frequent storage of the infected brain tissue in 0.85% saline solution at 10°C. Before each experiment the virus was given several passages in mice to insure a high virulence, and the mld of each preparation was determined.

Immune serum was obtained from a rabbit which had received an intravenous injection of a 10% suspension of virus, 2 injections of Lederle vaccine by the same route and one intraocular injection of virus. Several rabbits had been immunized in this manner for other studies and the one showing the highest antiviral titer was selected for the present work. The rabbit was bled at intervals by cardiac puncture, and the serum without preservative was stored in the refrigerator (10°C).

To prepare the virus for the virus-serum mixture, a 20% suspension of infected brain tissue made by grinding with sand and diluting with 0.85% saline solution was centrifugated at 1600 rpm for 20 minutes in the angle head centrifuge. The supernatant solution was diluted with 0.85% saline solution to the desired concentration and added in equal amounts to undiluted immune serum and after thorough shaking was allowed to stand at 26°C (room temperature) for 15 minutes. The neutrality of each mixture was determined by mouse injection. To determine the influence of dilution on reactivation, the desired amount of 0.85% saline solution was added and incubation carried out at room temperature for 15 minutes. Mice were then injected, each mouse receiving 0.1 cc of each dilution intraperitoneally. The animals used were either *P. maniculatus* under 90 days of age, which have been found in this laboratory to be highly susceptible to the virus when introduced by the intraperitoneal route, or 15-20-day-old albinos. Two controls

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were employed: (1) the determination of the potency of the virus when mixed with an equal volume of undiluted normal rabbit serum, and (2) tests for the neutralization of the virus by the immune serum as mentioned above. The titer of the virus varied between 10^{-6} to 10^{-7} cc. Over-neutralized, neutral, and under-neutralized mixtures were obtained by the use of concentrations of virus as follows: 1:1,000, 1:100, and 1:10 in the presence of an equal volume of undiluted immune serum. The data for 7 experiments have been combined and are presented in Table I.

Discussion. It is strikingly evident from the data in Table I that the virus in the neutral mixture was reactivated following dilution of 1:100 with 0.85% saline solution. Nineteen of the 27 mice injected with the diluted material died with typical signs of eastern equine encephalomyelitis. In contrast only 3 of the 26 animals injected with the undiluted material succumbed. Confirmation of the cause of death of the animals injected with the diluted material was obtained in two ways: (1) by mouse passage of bacteria-free brain tissue with the production of characteristic signs of this infection, (2) protection tests against this passage virus with the specific antiserum.

Now, turning to the mixtures in which the dilution of the virus was 1:1,000, further dilution evoked no detectable reactivation. If one calculates the titer of virus in the final dilution, the quantity injected should still be adequate to kill the mice if dissociation of virus and antivirus were complete. The controls on this dilution show an ex-

TABLE I.
Reactivation of Mixtures of Eastern Equine Encephalomyelitis Virus and Antiserum by Dilution with Saline Solution.

Conc. of virus in original virus-serum mixture at time of dilution	Dilution of virus-serum mixture tested for reactivation	Results of test for reactivation
1:1000	1:10	1/21*
"	1:100	3/27
"	1:200	0/5
"	undiluted (control)	1/15
1:100	1:10	6/18
"	1:100	19/27
"	1:1000	0/3
"	undiluted "	3/26
1:10	1:10	9/12
"	1:100	11/12
"	undiluted "	8/13

*The denominator indicates the total number of animals injected and the numerator the number of deaths.

cess of antiserum. Under these conditions, as might be expected, reactivation rarely occurs. In the mixture containing 1:10 virus we have the other extreme, namely, virus in excess. The injection of this material produced death even when diluted, merely confirming the potency of the virus.

The importance of having balanced mixtures of virus and antiserum in order to demonstrate reactivation by dilution is clearly brought out in the table. If antiserum is in excess, there is no detectable liberation of virus, and when virus is in excess the mixture is naturally potent. These findings agree with the work of Goyal[22] on vaccinia virus and possibly account for the negative results of Merrill. The extent of dilution is also a factor since the mixture containing virus in a concentration of 1:100 was not reactivated when further diluted 1:10 and 1:1000 but was reactivated in a dilution of 1:100.

Conclusions. 1. A neutral mixture of eastern equine encephalomyelitis virus and homologous antiserum was reactivated when a 1:100 concentration of virus with antiserum was diluted a hundredfold with 0.85% saline solution and was allowed to stand at 26°C (room temperature). 2. It appears that reactivation will occur only in a balanced mixture of virus and serum and furthermore, the extent of dilution is important.

13403

Effects of Repeated Small Doses of Roentgen Rays on Canine Blood and Bone Marrow.

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Since the human and experimental data on the effect of roentgen rays on the blood and the bone marrow deal with large doses and their sequelae over relatively short periods of time, 4 dogs were exposed to doses of 75 r or 100 r units administered at intervals of a week or longer over several months.

Before irradiation, dogs 11, 21 and 24 were given a course of subcutaneous benzol injections (preceded by 4% novocaine local

22 Goyal, G. K., *J. Immunol.*, 1935, **29**, 111.