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ESTIMATION OF TITER OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS PREPARATIONS FROM A SINGLE-DILUTION ASSAY¹

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ABSTRACT

RILEY, JEAN M. (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), WILLIAM C. PATRICK, III, AND WILLIAM E. CAMPBELL, JR. Estimation of titer of Venezuelan equine encephalomyelitis virus preparations from a single-dilution assay. *J. Bacteriol.* 85:1256-1260. 1963.—When suspensions of Venezuelan equine encephalomyelitis (VEE) virus were injected intracerebrally into groups of mice, a nearly linear relationship was observed between the concentration of the virus injected and the mean reciprocal time-to-death of the mice. A total of 91 VEE preparations were assayed in duplicate, and, by plotting the relationship between the reciprocal time-to-death for mice given the 10^{-6} dilution of virus and the MICLD₅₀ (mouse intracerebral challenge, LD₅₀ response) values for the virus preparations, a reference curve was established. Using this reference curve, it was possible to estimate directly the LD₅₀ values of virus suspensions of unknown concentration from the mean reciprocal time-to-death of a group of mice injected with a single dilution. In this work, the number of mice used was reduced by 62.5%, the titrations were complete in 3 to 5 days compared with the usual 10 to 14 days, three to four times as many assays could be done in a day, and no assays had to be repeated since end points were not missed. The precision of the single-dilution assay compared favorably with that of the LD₅₀ titration.

The titration of virus materials by determination of the 50% end point is expensive both in time and in cost of animals used per assay. A less-expensive assay is almost mandatory when many materials must be handled, such as in

¹ In conducting the research reported herein, the investigators adhered to the Principles of Laboratory Animal Care as established by the National Society for Medical Research.

screening programs. However, the LD₅₀ value has for many years been the standard measurement of virulence for virus materials, so any new measurement should be one that would be directly correlated with the LD₅₀ titer. Such an assay based on dose on time-to-response relationship was proposed by Gard (1940), Golub (1948), and Bauer (1960). Gard (1940) reported that there was a rectilinear relationship between the amount of mouse encephalomyelitis virus injected intracerebrally into mice and the length of incubation period (actually time-to-death). This relationship was used as a means of titrating virus suspensions of unknown infectivity by interpolation from a previously established response curve. Golub (1948) demonstrated a similar dose-response relationship with several members of the psittacosis-lymphogranuloma venereum group of viruses and found it possible to estimate the LD₅₀ value of virus suspensions directly from the average day of death of a group of embryonated eggs inoculated with one dilution. Bauer (1960), working with neurovaccinia, ectromelia, dengue I, rabies, and yellow fever viruses, correlated a single-dilution assay to zero mortality (v_0) units that bore a fixed relationship to the LD₅₀ value. These and many other authors have stressed the advantages of the single-dilution assay over the conventional determination of the LD₅₀ value, but the proposed assays have gained little acceptance.

Koprowski and Lennette (1944) recorded average survival times of both mice and chick embryos injected with dilutions (10^{-6} to 10^{-11}) of Venezuelan equine encephalomyelitis (VEE) virus. Their data show that a dose on time-to-death relationship existed, but they did not use this information in any way. The purpose of the present report is to show (i) that a dose-time response relationship exists for VEE infections in mice, (ii) that it is reliable to estimate directly the LD₅₀ value of a suspension from the reciprocal mean time-to-death of a group of mice injected with a single dilution, and (iii) that the precision of the

single-dilution assay compares favorably with that of the LD_{50} titration.

MATERIALS AND METHODS

Virus. VEE virus, strain V-9, was used in this study. A 10^{-2} suspension of embryo select-harvest material was prepared in Heart Infusion Broth (Difco). This suspension was blended for 1 min in a Waring Blendor (semimicro size), and further tenfold dilutions were made in Heart Infusion Broth.

Assay procedure. Groups of ten mice, Swiss Webster strain, weighing between 10 and 14 g were inoculated intracerebrally with 0.03 ml of the diluted virus. To obtain initial dose on time-to-death information, all dilutions from 10^0 to 10^{-10} were used. From this data, the 10^{-6} dilution was selected for the single-dose assay, and the 10^{-7} to 10^{-10} dilutions were used to bracket the 50% end point.

Mice were observed for 10 days, and deaths were recorded at 8:00 AM and 2:00 PM daily. Deaths prior to 24 hr were assumed to be traumatic. Deaths discovered at successive check points were assumed to have occurred at random in the interval between the observation periods, and the midpoint of the interval was taken as the best estimate of time of death. Thus, a mouse alive at one check point and dead at the following was considered to have died at the hour halfway between the two check periods. Mice alive and well at the end of the observation period were considered to have escaped infection.

The mean reciprocal time-to-death (MTD; Brownlee and Hamre, 1951) was calculated for each group of mice given the 10^{-6} dilution. The reciprocal of the time-to-death was multiplied by 100 to provide whole numbers for ease in computation. The LD_{50} value was calculated by the method of Reed and Muench (1938).

RESULTS

Preliminary titrations of six virus suspensions with a mean $MICLD_{50}$ of $10^{-3.9}$ were run to gain initial dose on time-to-death information. As shown in Fig. 1, a nearly linear dose-time relationship was found in the range of concentrations from 10^0 to 10^{-6} ; curvature occurred at the 10^{-7} and 10^{-8} dilutions, in which survivors started to occur. The spread of response among samples was minimal in the range of 10^{-1} to 10^{-4} and maximal in the region of the LD_{50} dose. From

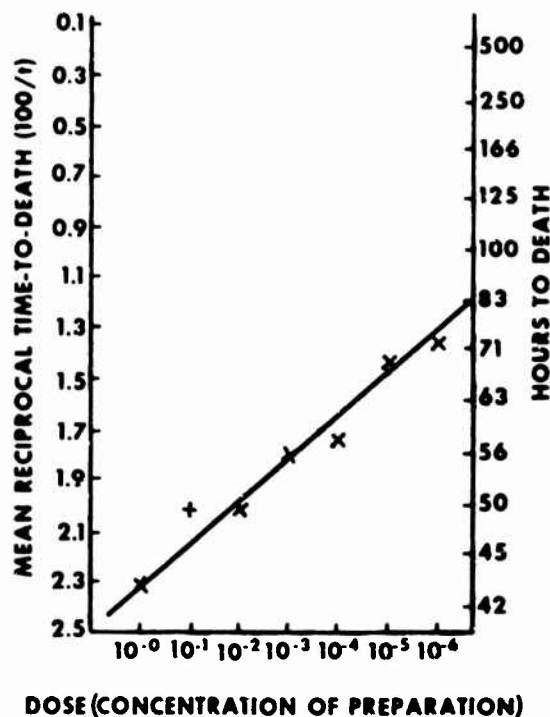


FIG. 1. Dose on time-to-death relationship for mice given Venezuelan equine encephalomyelitis virus. Each point represents the mean of 60 mice. Survivors started to occur at the 10^{-7} and 10^{-8} dilutions, causing curvature.

these data, the 10^{-3} or 10^{-4} dilution was selected as the concentration to be considered in a single-dose assay. A mean time-to-death of 50 to 60 hr was obtained with these dilutions, which (i) assured definition between deaths from trauma and from virus and yet assured 100% mortality and (ii) limited observation of mice to twice daily. However, the viral preparations that were to be used in all future studies had original $MICLD_{50}$ values that were about 2 logs higher than those of the suspensions used in the preliminary work. Thus, it could be expected that the 10^{-5} and 10^{-6} dilutions of the future test materials should give MTD values equal to those obtained with 10^{-3} and 10^{-4} concentrations of the preliminary materials. On this assumption, the 10^{-6} dilution was finally selected for the single-dose assay.

A total of 91 VEE preparations were assayed in duplicate, using the 10^{-6} through the 10^{-10} dilutions. The LD_{50} titers ranged in $MICLD_{50}$ from $10^{-6.0}$ to $10^{-11.2}$. These preparations were grouped by titer in half-log intervals, and response curves were established for each group. Representative response curves for the groups

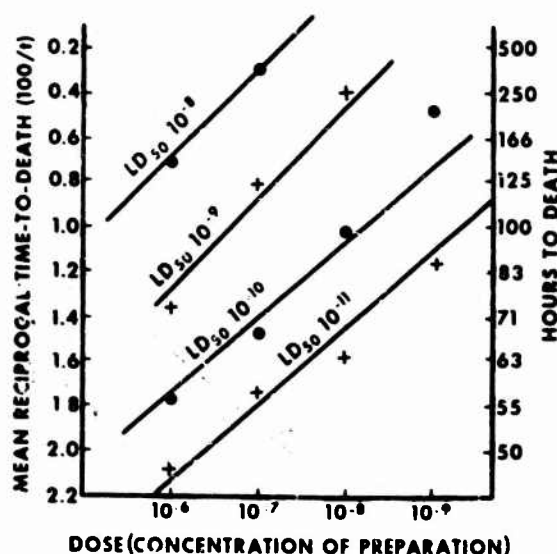


FIG. 2. Dose-response curves for Venezuelan equine encephalomyelitis virus materials having MICLD_{50} values ranging from 10^{-8} to 10^{-11} .

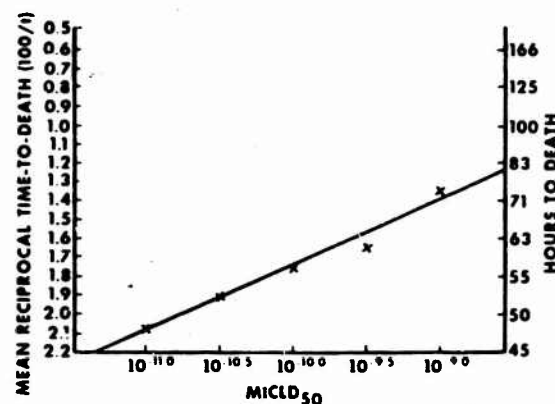


FIG. 3. Reference curve for estimation of MICLD_{50} titer of Venezuelan equine encephalomyelitis virus from mean reciprocal time-to-death for groups of mice injected with a 10^{-8} dilution.

having MICLD_{50} values of 10^{-8} , 10^{-9} , 10^{-10} , and 10^{-11} are shown in Fig. 2. The curves for the intermediate groups were similar, but are omitted from Fig. 2 for clarity of graphic presentation. It can be noted that the slopes of the curves for the groups with MICLD_{50} values of 10^{-8} , 10^{-9} , and 10^{-10} are roughly parallel. The slope of the curve for the group with MICLD_{50} values of 10^{-11} appears to differ from the other three slopes, but this may be due to sampling error.

The relationship between reciprocal time-to-death for the 10^{-8} dilution and the MICLD_{50} value for all 91 assays is shown in Fig. 3. This curve is the reference curve that was used in all future

assays to estimate the MICLD_{50} value from the MTD of groups of mice injected with the 10^{-8} dilution.

An additional 146 preparations were assayed in triplicate using the MTD of groups of mice given the 10^{-8} dilution to estimate the MICLD_{50} titer. In about 20% of these assays, selected at random, a standard LD_{50} titration was also done. The estimated LD_{50} values from the single-dilution assays and the LD_{50} values calculated by the method of Reed and Muench (1938) are shown in Table 1. There was no significant difference between the estimated and calculated values, based on analyses of variance and F tests at the 5% level of probability. In addition, the inherent

TABLE 1. Comparison of LD_{50} values of Venezuelan equine encephalomyelitis preparations obtained by two methods

Prepn	Estimated from reference curve*	Calculated by Reed-Muench method
1	9.4	9.91
2	9.0	9.28
3	8.5	9.16
4	9.0	9.81
5	8.7	10.02
6	8.9	9.89
7	7.3	8.22
8	7.2	8.30
9	7.3	8.03
10	9.5	9.76
11	10.0	10.10
12	9.3	9.85
13	9.6	10.10
14	9.5	10.09
15	9.1	9.90
16	9.3	10.06
17	10.2	10.02
18	10.4	10.24
19	7.4	7.69
20	8.7	8.01
21	7.1	8.02
22	9.7	8.14
23	9.1	8.76
24	8.5	8.12
25	9.9	9.27
26	9.3	10.02
27	9.8	9.19
28	9.8	10.03
29	6.7	7.64
30	7.0	7.98
31	7.6	7.91
$\bar{x}$	8.8	9.14

* See Fig. 3.

variability within each method was not significantly different.

DISCUSSION

The dose-time response relationship shown here for VEE is neither new nor is it limited to viruses. Similar relationships have been used in bacteriology by Hatch et al. (1952), Schewe (1958), and Fernelius et al. (1960); in mycology, by Gale and Devesty (1957) and Rubin (1958); in toxicology and pharmacology, by Ipsen and Toft (1946), Box and Collumbine (1947), and Ipsen (1951). These are only a few examples in various fields of the multitude of reported users of dose-time relationships to simplify and expedite routine assay procedures.

The most frequently discussed advantages of a single-dilution assay over the conventional method of using four to six dilutions and attempting to bracket the 50% end point are: (i) fewer hosts are needed, (ii) the titration is completed in a shorter time, (iii) the time necessary for preparing dilutions and inoculating the hosts is minimized, and (iv) with materials of unknown concentration the end point is not missed by an inaccurate choice of dilutions. In addition, by using a reciprocal transformation, each host contributes a numerical estimate of the amount of agent (virus, bacteria, fungus, or toxin) inoculated into it, and the variance of responses is stabilized over the whole range of doses.

In our work with VEE, the number of mice used was reduced by 62.5%, the titrations were complete in 3 to 5 days compared with the usual 10 to 14 days, three to four times as many assays could be done in 1 day, and no assays had to be repeated since end points were not missed.

Selection of the concentration of the agent to be given is dependent only upon the death pattern of the particular host-agent combination used and upon the investigator's convenience. Thus, Golub (1948) selected the 10^{-2} dilution of psittacosis virus to obtain about the same saving in time as we obtained using the 10^{-6} dilution of VEE. The time needed to complete our assays could be reduced to less than 48 hr by injecting the 10^{-2} dilution, but the mice would have to be observed more often than twice daily, and the cause of death, trauma or virus, would be difficult to define. Of course, with VEE the intraperitoneal route of injection could be used (Lennette and Koprowski, 1944) and thus eliminate

deaths from trauma, but mice would still have to be observed frequently.

The most important factor in using a time-to-death assay is a uniform supply of hosts. This is particularly true with mice which become more resistant with age to many infections. A reference curve established for mice averaging 12 g cannot be used for those which weigh 18 g. The heavier mice live 1 day longer, give smaller mean reciprocals, and give LD_{50} values that are 1 log lower than when 12-g mice are used. On the basis of our experiments, correction factors for the use of mice of various weights should be easily obtained.

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