COMPARISON OF GRADED AND QUANTAL VIRULENCE TESTS FOR *BACILLUS ANTHRACIS SPORES*[1]

ALBERT L. FERNELIUS, IRA A. DEARMON, JR., FREDERICK KLEIN, AND RALPH E. LINCOLN

U. S. Army Chemical Corps, Fort Detrick, Frederick, Maryland

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A comparison of the intraperitoneal virulence of two strains of *Bacillus anthracis* for mice was made by an LD_{50} method and a survival time method in an earlier report (Roth *et al.*, 1956). This median survival time method was referred to as an ST_{50} method, but it will be called more appropriately in the present paper, the median-time-to-death method.

In the present study, the virulence of four highly virulent and four relatively avirulent strains or variants of *B. anthracis* were compared by these two methods employing both intraperitoneal and subcutaneous routes of injection in mice.

MATERIALS AND METHODS

Test organisms. Strains or variants of *B. anthracis* employed in these tests are given in table 1. The term "variant" is not used in a genetic sense, but connotes suspensions with either strain or cultural differences. Cultures of relatively low virulence were predominantly of the rough colonial type, whereas those of high virulence were smooth or mucoid and encapsulated when cultured on serum agar under CO_2 tension (Sterne, 1937).

Method of preparing spores. For each of the three replicate tests in this experiment, cultures were grown in shake flasks containing a casein acid digest medium (Roth *et al.*, 1955), and inocula were prepared by dilution of spores in a gelatin-phosphate buffer by the method of Roth *et al.*, (1956). Viable spore counts for each culture determined by a surface plate-count method are given by replicates in table 1.

Experimental animals. Albino Swiss mice from one pure-bred stock were held under conditions described in an earlier report (Roth *et al.*, 1956).

Method of determining mouse median-time-to-death values. Freshly grown heat-shocked spores were injected into the test mice either subcutaneously or intraperitoneally. The size of the injected dose was 0.2 ml to determine median-time-to-death values; twelve mice per dose and two doses, one undiluted and one diluted 100-fold with gelatin-phosphate buffer were employed. Times-to-death were recorded to the nearest half hour and the median times-to-death calculated (Roth *et al.*, 1956).

Mean reciprocal death times were computed from

$$1/n \sum_{i=1}^{n} 100/t_i$$

where n was the number of mice in a group and t_i was the time-to-death of the ith mouse with t equal to ∞ for a survivor (Finney, 1952*a*). The mean reciprocal death time was converted to time-to-death in hours and was approximately equal to the median-time-to-death values.

Method of determining mouse LD_{50} values. Logarithmic dilutions of the variants described in table 1 were made in gelatin-phosphate buffer and doses were injected in 0.2-ml amounts. Ten mice per dose with four doses were employed and point estimate LD_{50} determinations calculated by the maximum likelihood method of Finney (1952*b*).

RESULTS

Comparison of intraperitoneal and subcutaneous routes for the LD_{50} method. Point estimate LD_{50} values determined by the maximum likelihood method for each of the variants administered to mice by each of two injection routes are presented in table 2. The mean LD_{50} for the four strains of high virulence by the intraperitoneal route was approximately 13 times greater than the mean LD_{50} by the subcutaneous route with a 95 per cent confidence interval of 4 to 43 times,[2] whereas

[1] A preliminary report of this work was presented at the 58th General Meeting of the Society of American Bacteriologists, Chicago, Illinois, April 29, 1958.

[2] The variance determined from an analysis of variance using log LD_{50} of 48 observations.

TABLE 1

Source and viable spore counts of eight strains or variants of Bacillus anthracis

Relative Virulence	Designation	Original Source or Treatment Condition	Viable Spore Count (10^8/ml) for Replicate Test:		
			I	II	III
Low	298-R	Wool worker-Pakistan	7.6	16.4	17.0
	30-R	Ft. Detrick variant '54	15.5	0.9	4.8
	21-R	Vollum variant '54	14.7	0.2	0.02
	32-R	Vollum B variant (Rough)	4.9	10.4	4.9
High	32-S	Vollum B variant (smooth)	7.5	11.2	4.6
	Vol-3	Vollum stored at 3 C for 180 weeks	200.0	154.5	202.0
	Vol-3-Re	The above culture regrown	13.7	0.4	0.007
	Vol-39-Re	Vollum stored at 39 C for 150 weeks, then regrown	21.1	0.06	8.3

TABLE 2

Mean LD_{50} values of spores from eight variants of Bacillus anthracis injected intraperitoneally and subcutaneously

(Values are means of three replications)

Relative Virulence	Strain Designation	No. of Spores for an LD_{50}	
		Intraperitoneal route	Subcutaneous route
Low	298-R	50.0×10^5	79.0×10^5
	30-R	13.7×10^5	23.4×10^5
	21-R	5.7×10^5	10.4×10^5
	32-R	36.0×10^4	9.8×10^4
Geometric mean of "low" variants..		10.9×10^5	11.7×10^5
High	32-S	537	28
	Vol-3	132	9
	Vol-3-Re	132	32
	Vol-39-Re	740	29
Geometric mean of "high" variants..		287	22

for the strains of low virulence, the LD_{50} values did not differ significantly with the injection route.

Individual log LD_{50} values by replicates are plotted in pairs in figure 1. The log LD_{50} by the subcutaneous route is shown in relation to the log LD_{50} by the intraperitoneal route. If the two routes of injection gave the same approximate LD_{50} values, all the points would fall on or near the diagonal (45°) line. Most of the points for the high-virulence group fell well below this line which indicates that inoculation by the intraperitoneal route required more spores for a response than did inoculation by the subcutaneous route. For the low-virulence group, the points fell equally on both sides of the diagonal line which indicates the LD_{50} values by both routes of injection are approximately equal.

Comparison of intraperitoneal and subcutaneous routes by the median-time-to-death method. The median-time-to-death values of mice injected by both routes are given in table 3 for both the undiluted and 1:100 dilutions of *B. anthracis* spores. The mean median-time-to-death values for the undiluted spores injected by the intraperitoneal route were shorter than the response times of mice injected by the subcutaneous route. This was true for spores of both low and high virulence. In a comparison of mean median-time-to-death values given by mice injected with spores diluted 100-fold, approximately equal response times were obtained for both routes of challenge with spores of high virulence; however, spores of low virulence caused shorter median-time-to-death values when injected by the subcutaneous route. These apparent reversals at the 1:100 dilutions are discussed later at which time a conversion of all the data (both LD_{50} and median-time-to-death results) to reciprocal death times is made.

Comparison of LD_{50} and median-time-to-death values. Comparisons of LD_{50} and median-time-to-death values can be made, if these comparisons

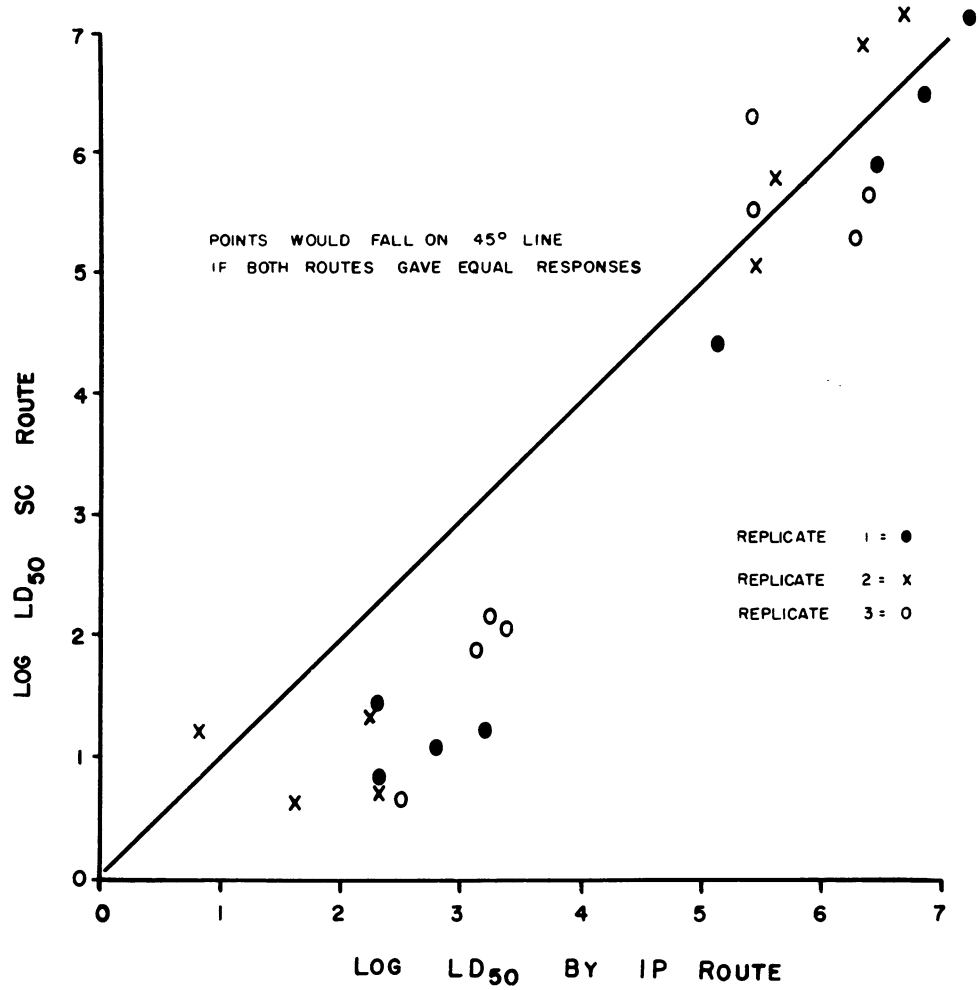

Figure 1. Log LD_{50} by intraperitoneal (*IP*) route plotted against log LD_{50} by subcutaneous (*SC*) route in mice for spores of eight variants of *Bacillus anthracis*.

are restricted to the same route of injection. By the intraperitoneal route of injection, either test will distinguish between strains of high and low virulence since the LD_{50} values differ by some 3800-fold and median-time-to-death responses for undiluted culture differ by approximately 30 hr. By the subcutaneous route of injection, again either test will distinguish between strains of *B. anthracis* of low and high virulence, since the LD_{50} values differ by some 50,000-fold and median-time-to-death values for undiluted culture differ by about 60 hr.

DISCUSSION

It has been shown by the data presented that the time-to-death of mice is related to the concentration of *B. anthracis* spores injected. It seems reasonable to believe that the time-to-death of an animal is an accumulation of the time periods involved in: (a) germination and conditioning of spores to initiate vegetative growth; (b) rate of cell division or generation time; and (c) the number of cell divisions necessary to produce a critical number of vegetative organisms to cause death of the host.

Although death is associated with some number of infectious organisms in the host, it could be attributable to production of toxin as described by Smith *et al.* (1955), or to an inflammatory factor and to the presence of bacterial capsular material as hypothesized by Watson *et al.* (1947). The functional relation of time-to-

TABLE 3

Observed median-time-to-death values of mice injected intraperitoneally and subcutaneously with two dilutions of Bacillus anthracis spores

(Each value is the mean of three replicate tests)

Relative Virulence	Strain Designation	Median-Time-to-Death			
		Intraperitoneal route		Subcutaneous route	
		Undiluted	1:100 Dilution	Undiluted	1:100 Dilution
		hr	*hr*	*hr*	*hr*
Low	298-R	50	225	88	150
	30-R	49	253	77	176
	21-R	44	493	93	171
	32-R	34	147	64	178
Mean..		43.4	232	78.9	168
High	32-S	15	28	20	28
	Vol-3	13	17	18	19
	Vol-3-Re	17	36	23	32
	Vol-39-Re	13	22	17	25
Mean..		14.6	23.6	19.4	25.0

death (T), the three stages of reactions in animals, and the initial spore dose (D) can be formulated as follows:

$$T = I + \frac{\log (V_c/V_0)}{k} = f(D) \qquad (1)$$

where T is time-to-death of an animal injected with D spores, I is the average time involved in germination and conditioning of spores (D) for vegetative growth and $\log \frac{(V_c/V_0)}{k}$ is the time necessary for vegetative cells to reach a critical level V_c starting at a level V_0 and multiplying at the rate k where k is a function of the number of multiplications per unit time.

Each of the elements I, V_c, and k may be equal or different according to route of challenge, virulence of the organism, and the resistance of the host. It has been pointed out by Dutton (1955) that the virulence of microorganisms is influenced by the operation of localization as a defense mechanism by the host, i. e., there is a high defensive value of the phagocytes of the reticuloendothelial system bordering the blood stream, thus the route of injection could influence the death rate of experimentally infected animals.

If the organism strain and host are held constant and the route of challenge is varied between, say, intraperitoneal and subcutaneous, then the values of I, V_c, and k for these two routes will be equal or different and eight combinations of these elements are possible. For example, for a given spore inoculant, I could be a longer time by intraperitoneal than by subcutaneous route; and V_c and k could be the same for either route, therefore the resultant T values for each route would differ. It follows also that if the routes of challenge and the host are held constant but the organism strains are varied, say, low or high virulence, then again I, V_c, and k will each be equal or different according to organism strains and again eight combinations are possible.

In the study carried out in this paper, it was first noted that in the comparison of intraperitoneal and subcutaneous routes of injection of *B. anthracis* spores, the median-time-to-death values elicited by a large number of spores (undiluted culture) were shorter for the intraperitoneal route than for the subcutaneous route; however, a smaller number of spores (1:100 dilutions of the culture) caused the median-time-to-death values by the two routes to become closer or even reverse themselves in the case of the less virulent spores. To investigate this observation further, animal responses (median-time-to-death values) were combined over the entire range of doses by utilizing observations made from the median-time-to-death and LD_{50} sets of data. This combination of data was made possible by using the mean reciprocal time-to-death ($100/t$) as a function of $\log_{10}$ dose, since for the LD_{50} data, mice were tallied as to day of death. Those mice surviving any given dose were assigned a time-to-death of infinity which, on the reciprocal scale, becomes zero.

The combinations and plots of all data are shown in figure 2 along with continuous curves fitted by the least squares method. Each point, weighted according to the number of mice observed, represents at least 80 mice. The mathematical form of the curves relating reciprocal time to dose of spores injected is:

$$100/t = AD^b - 1.0 \qquad (2)$$

where t is death time in hours, D is the dose, and A and b are intercept and slope, respectively.

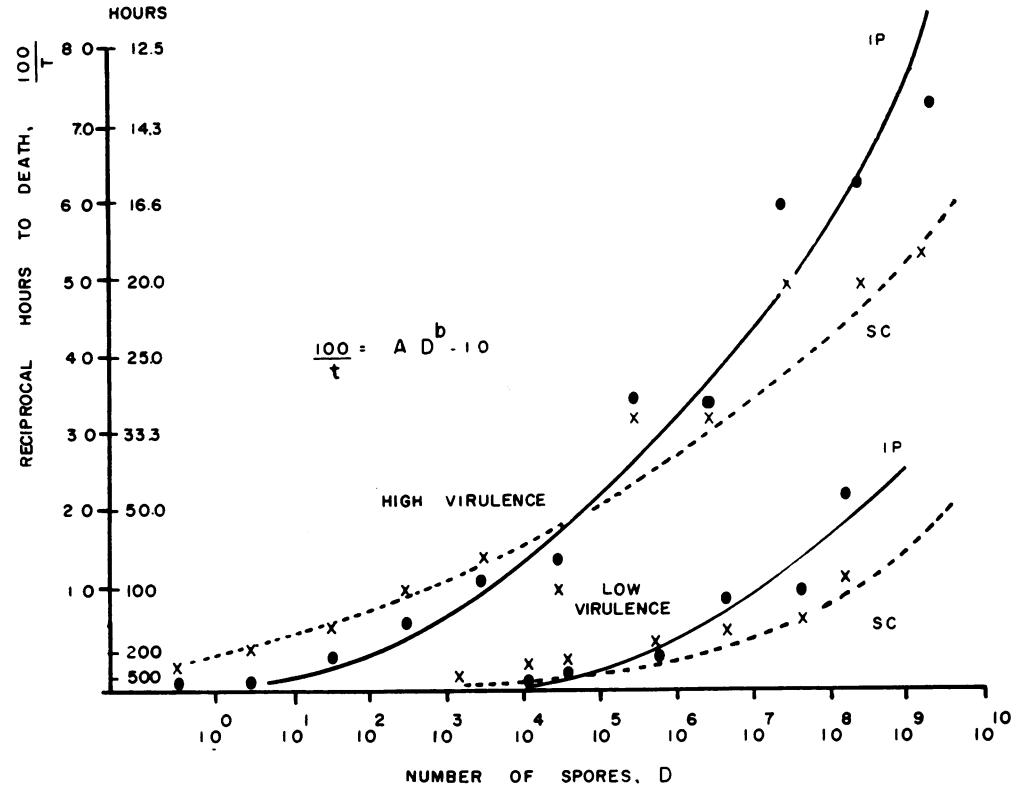

Figure 2. Relation between reciprocal of mean time-to-death of mice and the number of *Bacillus anthracis* spores injected by intraperitoneal (*IP*) and subcutaneous (*SC*) routes.

The values of A and b by routes and degrees of virulence of the spores are as follows:

Routes	Spores of high virulence		Spores of low virulence	
	A	b	A	b
Intraperitoneal	1.06	0.10	0.43	0.10
Subcutaneous	1.48	0.07	0.57	0.07

A study of the curves in figure 2 and the constants for the mathematical form indicate that the curves associated with *B. anthracis* strains of high or low virulence are parallel whereas the intraperitoneal and subcutaneous curves are not parallel and intersect each other at a spore concentration of approximately 10^5. The times-to-death associated with selected spore doses by both subcutaneous and intraperitoneal routes of infection have been extracted from the curves of figure 2 and are shown in table 4. These values are synonymous with the functional relation of death time and spore dose as given by equation (1). Although equation (2) is not sufficiently complex to explain the entire function related in equation (1), it is suggested by the data that at a dosage of 10^5 spores the time periods (I), i. e., times associated with germination and conditioning of spores, by either subcutaneous or intraperitoneal routes of challenge appear to be the same.

TABLE 4

Times-to-death of mice incurred by inocula of varying sizes administered by two routes

Inoculum Size (No. of Cells)	High Virulence		Low Virulence	
	Intraperitoneal route	Subcutaneous route	Intraperitoneal route	Subcutaneous route
	hr	*hr*	*hr*	*hr*
10^9	13	19	40	70
10^5	43	43	500	500
$10^{3.5}$	73	62	∞	2500

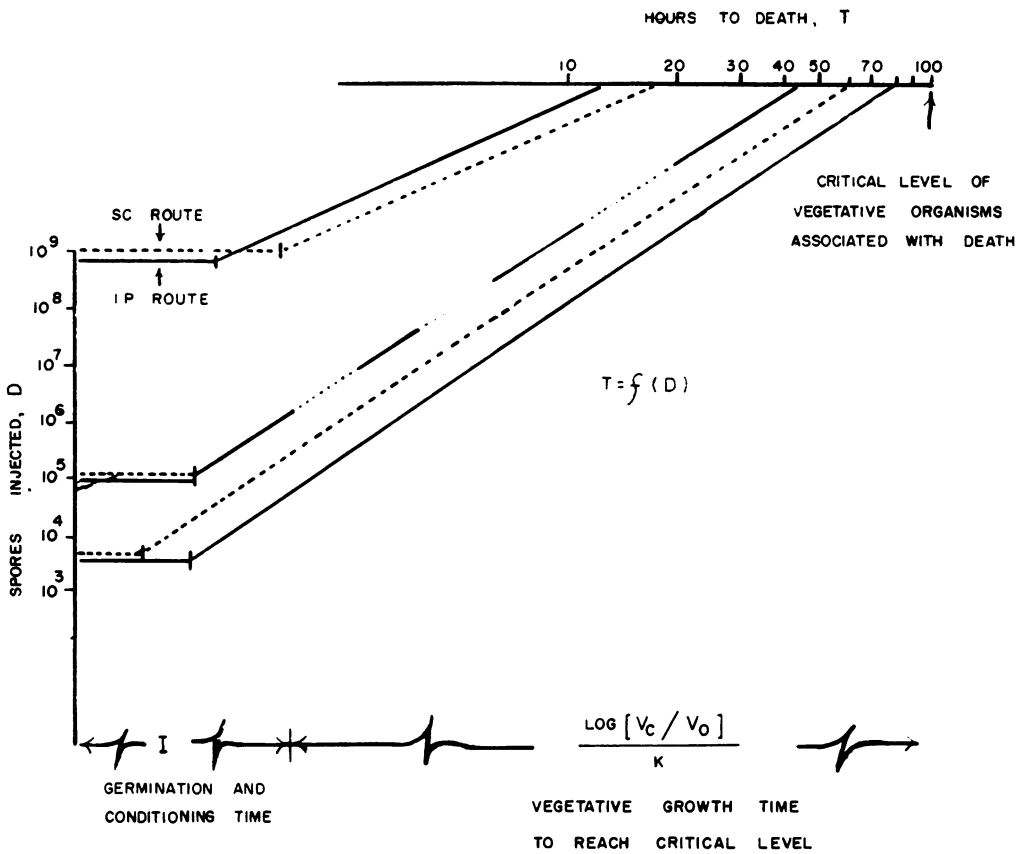

Figure 3. Schematic illustration of median time-to-death of mice challenged by two routes with various concentrations of *Bacillus anthracis* spores of high virulence.

Further interpretation of the function infers that by the subcutaneous route, the time (I) for germination and initiation of a fatal septicemia is shorter for a small inoculant than for a large inoculant. A graphic interpretation of this idea is shown in figure 3 using data shown in columns 2 and 3 of table 4 for a strain of high virulence. This model also would hold true for strains of low virulence. It is possible that some mechanism limiting germination and conditioning may be present in the subcutaneous tissue, but absent in the peritoneal cavity. Such factors could be the scarcity of amino acids essential for germination or of substrate necessary for vegetative outgrowth. A combination of these factors could result in complete germination and outgrowth of spores when the inoculum is low, but would permit only partial germination and outgrowth when large numbers of spores are introduced.

Experimental data are being gathered to resolve these theoretical concepts but much more experimental evidence is needed before any of the assumptions discussed can be accepted or rejected.

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SUMMARY

The virulence of spores of four strains or variants of *Bacillus anthracis* with characteristics of high virulence and four with low virulence were compared in mice by both an LD_{50} and a median-time-to-death method. Both intraperitoneal and subcutaneous routes of challenge were employed and compared. There was good correlation between the LD_{50} and the median-time-

to-death values for all strains or variants tested by both routes. With *B. anthracis* of high virulence, a greater number of spores was required to produce death by the intraperitoneal route than was required by the subcutaneous route. However, by the median-time-to-death method of measuring virulence, the intraperitoneal route killed mice sooner, indicating an increased virulence over that given by the subcutaneous route. This fact held true for high concentrations of spores with characteristics of high and low virulence. However, when spores of low virulence were diluted 100-fold, and injected by the subcutaneous route, shorter median-time-to-death values resulted.

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