DUAL NATURE OF RESISTANCE MECHANISMS AS REVEALED BY STUDIES OF ANTHRAX SEPTICEMIA[1]

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ABSTRACT

KLEIN, FREDERICK (Fort Detrick, Frederick, Md.), BERTRAM W. HAINES, BILL G. MAHLANDT, IRA A. DEARMON, JR., AND RALPH E. LINCOLN. Dual nature of resistance mechanisms as revealed by studies of anthrax septicemia. J. Bacteriol. **85**:1032–1038. 1963.—From studies of septicemic anthrax, resistance was described in relation to toxin and to growth of bacilli. This description was based on the observations that the terminal concentrations of bacilli in the blood were influenced primarily by the susceptibility of the host to toxin, whereas the death-response time of the host was dependent on both resistance to bacilli and toxin susceptibility. Resistance to the establishment and growth of infecting organisms and susceptibility to the toxin produced by growth of the bacilli are separate aspects of pathogenesis. A complete description of pathogenesis must accordingly treat of both these phenomena as individual entities.

Although it is generally agreed that a septicemia, i.e., a progressing bacteremia, is observed with anthrax, quantitative data are scarce. Keppie, Smith, and Harris-Smith (1955) and Klein et al. (1960) presented data for guinea pigs, and Trnka et al. (1958) gave data for sheep. Observations on four chimpanzees and four rhesus monkeys, respectively, were given by Albrink and Goodlow (1959) and Middleton and Standen (1961). In contrast, Stockman (1911) and Bloom et al. (1947) suggested that a bacteremia remains slight in immunized animals. Thus, complete detailed data on the occurrence of bacteremia, septicemia, or both in anthrax are currently available only for the disease in guinea pigs. There has been a logical reluctance to assume that the observations on guinea pigs apply to other species, and particularly species that seem to react differently to infection by *Bacillus anthracis* than the guinea pig does.

Bennett and Beeson (1954) showed that the septicemic course of a disease is altered by several factors, including the parasite's virulence and the host's ability to remove bacteria. They pointed out the need for accurate comparison of results in normal animals with those in animals subjected to various types of treatment or manipulation.

In line with this thought, Klein et al. (1960) showed that guinea pigs immunized with alum-precipitated antigen differed from control guinea pigs in two ways. One was an increased resistance which allowed immunized animals to withstand 1,600 times more challenge organisms than controls. The other was that the terminal concentration of bacilli in the blood of immunized animals succumbing to challenge was only about one-sixth that of controls and was independent of the size of the challenge dose. It was also shown that resistance, as measured by time to death after challenge, was reduced by egg yolk treatment of the challenge spores. Yet the septicemic course of anthrax, including the terminal concentration of bacilli, was unaltered by this treatment of the spores.

Observations are recorded on the rate of septicemic development and the terminal level of organisms in the blood of the naturally resistant rat and the naturally susceptible mouse. Egg yolk enhancement of virulence was studied in each host, while age and strain were varied in the rat. These studies show that resistance to anthrax must be described both in relation to the ability to establish growth in a host and to the susceptibility of the host to toxin produced during growth of the bacilli.

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

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MATERIALS AND METHODS

Infecting organism. The Vollum strain (Vlb) of *B. anthracis* was used and grown in N-Z-Amine-Type A (Sheffield Chemical Co., Norwich, N.Y.) medium at 34 C for 24 hr.

Animals. Black rats obtained from the National Institutes of Health (NIH) Animal Farm, where they were developed from Long-Evans stock, and Fischer 344 strain of albino rat (Taylor, Kennedy, and Blundell, 1961) were used in this investigation. Swiss mice were obtained from the Fort Detrick Animal Farm.

Virulence enhancement. This was achieved by treating anthrax spores with egg yolk (Kaga, 1956).

Blood assay of septicemia. Concentration of organisms per ml of whole blood was obtained by microscope counts of the stained organisms from a measured amount of blood smeared on a 1-cm^2 area of a slide (Keppie et al., 1955). Viable counts were also made from serial dilutions of blood in gel-phosphate buffer on Tryptose agar fortified by glucose (0.9%) and agar (0.5%). Colonies were counted after 24 hr of incubation at 34 C.

In vitro toxin. This was prepared as described by Thorne, Molnar, and Strange (1960).

Antiserum. Hyperimmune antiserum was prepared in a horse by injecting washed spores of the Sterne strain. This was the same antiserum described by Thorne et al. (1960) and was designated as DH-1-4A.

In vivo toxin. This was obtained from the lymph and terminal blood of monkeys challenged with anthrax spores of the Vollum strain. Processing of the lymph and blood was the same as described by Smith, Keppie, and Stanley (1955).

Challenge. All animals were challenged by intraperitoneal injection of organisms treated as described.

Description of studies. The results reported in this article came from three separate areas of work. One was designed to study the effect of virulence enhancement on septicemia in two host species. In a second experiment, differences in septicemia between different strains and ages of rats were studied. The third area provided information on the interaction between egg yolk treatment and host age on the dose-response relationship of the rat.

In the first study, trials were repeated until techniques were established and observations on an adequate number of animals were built up for each treatment condition. In each trial, groups of animals were randomly assigned to treatments. The number of animals assigned to each treatment condition at the finish of the study was as follows: 30 rats received a high dose of spores only; 30 rats, a low dose of spores only; 8 rats, a high dose of spores mixed with egg yolk; 8 rats, a low dose of spores mixed with egg yolk; 12 mice, spores only; and 12 mice, spores mixed with egg yolk.

Starting at 10 hr prior to expected death time of the rat and 6 hr prior to expected death time of the mouse, blood was withdrawn from the tail of each rat every 3 hr, and from each mouse every 2 hr, until death occurred. The time-to-death was recorded, and the sampling time prior to death calculated.

The study to determine how the variables of the septicemia were influenced by age and strain was replicated. Ten rats of each strain (NIH Black and Fischer 344) and each age (23, 44, 65, and 86 days) were challenged. Since the data from rats 65 and 86 days old were not significantly different in any respect, these two groups were pooled and are referred to throughout the remainder of this paper as 76-day-old rats. Blood samples were drawn and assayed as described above.

The third study was an extension of the work of Taylor et al. (1961) to include Fischer and NIH rats at 30, 60, and 90 days of age. Three replicates of this study were run, and data were obtained on 261 animals divided among the treatments. Each animal was challenged with 10^8 egg yolk-treated or nontreated spores; observations on death were made every 0.5 hr over a 48-hr period, after which the animals were observed every 6 hr for 8 days.

Analysis. For all these studies, the number of organisms per ml of blood was plotted according to the time prior to death for each infected animal. Preliminary plots of these data indicated that the bacilli in the blood increased exponentially with time. This relationship could be expressed mathematically as:

$$V_t = V_d\, 10^{-bt}$$

where V_t is the number of bacilli per ml of blood at any time t in hours prior to death, V_d is the number of bacilli per ml of blood at death, and b is the hourly rate of increase of organism in the host's blood.

Least squares estimates ($\hat{V}_d$ and $\hat{b}$) of the un-

known parameters in the equation were made from the data.

RESULTS

The concentration of bacilli in the blood of the NIH rats during the interval from 14 to 2 hr prior to death is shown in Fig. 1. Spores were not present as determined by testing for the presence of heat-resistant bacteria. Since there was a variable number of observations at each time period, data were combined to give geometric mean values for time intervals of 2 hr. The estimates $\hat{V}_d$ and $\hat{b}$, calculated from the data for each of the challenge methods, are shown in the same figure, beside the set of data to which they refer. Neither terminal concentration nor doubling time (slope) differed significantly among the three challenges. Thus, the average terminal concentration of organisms was estimated to be $\hat{V}_d = 1 \times 10^6$. The 95% confidence limits around this estimate extended from 0.6×10^6 to 1.6×10^6 organisms. The average doubling time of the bacilli in the blood, calculated from the estimated slope, was 120 min, with 95% confidence limits from 102 to 139 min. Direct counts were comparable with plate counts.

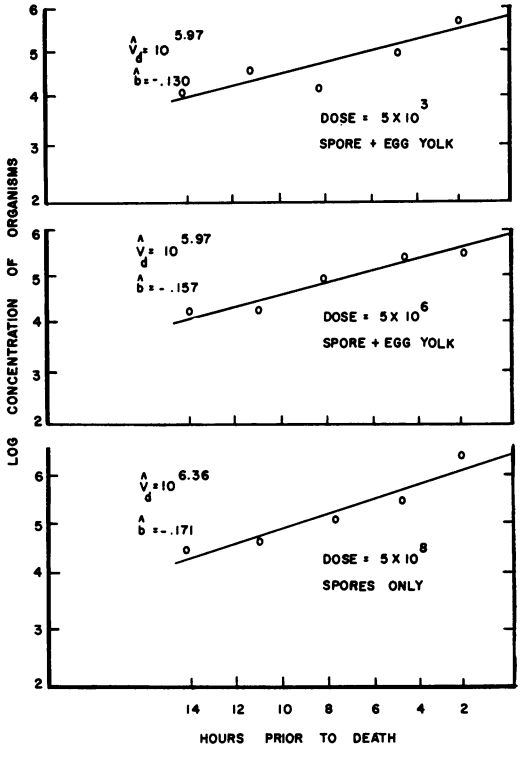

FIG. 1. *Effect of dose and treatment on regression of log concentration of organisms in rat blood plotted against time prior to death.*

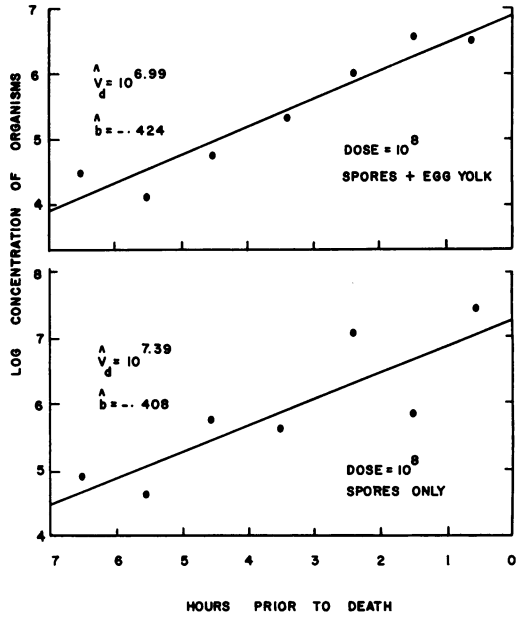

FIG. 2. *Effect of treatment on regression of log concentration of organisms in mouse blood plotted against time prior to death.*

In mice, when the challenge dose consisted of untreated spores, heat-resistant spores were found in the blood samples throughout the course of the disease. When the challenge dose consisted of egg yolk-treated spores, heat-resistant spores were rarely observed in the blood even early in the development of the disease. To distinguish heat-resistant spores from germinated spores and vegetative cells (the latter two forms are collectively referred to as bacilli in this paper), two counts were made on each blood sample. The first was a count of total organisms in untreated blood. The second was a count for heat-resistant spores made from a heat-shocked sample of blood. The difference between these two counts represents an estimate of the concentration of vegetative anthrax organisms in the blood. Thus, bacilli/ml = total organisms/ml − heat-resistant spores/ml.

The concentration of bacilli in the blood of mice during the interval from 7 hr to 30 min prior to death is presented in Fig. 2. These data have been combined as geometric means associated with a time interval of 1 hr. As with rats, there

were no significant differences among terminal concentrations or doubling times of bacilli regardless of treatment conditions. Estimates of the constants for each treatment condition are shown in Fig. 2. The average for all three treatments of the concentrations of bacilli in the blood at death was $\hat{V}_d = 10 \times 10^6$ with a 95% confidence interval from 4×10^6 to 25×10^6. The average doubling time of 45 min, with a 95% confidence limit of 37 to 59 min, was significantly reduced relative to the situation with rats. As with rats, direct counts were comparable with plate counts.

It became apparent from these data and experience with guinea pigs (Klein et al., 1960) that the bacterial generation time and terminal concentration were not altered by egg yolk treatment of the challenge anthrax organism. It appears from our observation on mice that (i) virulence enhancement after egg yolk treatment is, in part, the result of early germination of the spores. This observation supplements other observations of this phenomenon; namely, (ii) early and massive encapsulation, which Mesrobeanu and Slavescu (1942) observed when spores plus egg yolk were inoculated into animals, and which we demonstrated in vitro when egg yolk-treated spores and whole blood were incubated and examined microscopically; and (iii) a greatly increased retention of the inoculum in the peritoneum when egg yolk is present in the inoculum, a phenomenon that we also observed through microscopic studies of peritoneal fluid.

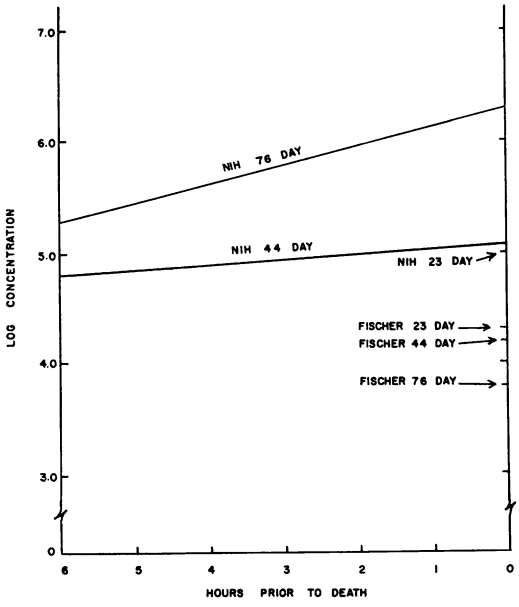

FIG. 3. *Concentration of Bacillus anthracis organisms per ml of blood, by age of rat and time prior to death.*

TABLE 1. *Estimates of slopes of septicemia and terminal concentration of organisms in the blood of rats, by type and age*

Type of rat	Age of rat (days)	V_d	SE	b	SE
National Institutes of Health	76	6.3	0.26	0.37	0.06
	44	5.1	0.14	0.17	0.01
	23	5.0	0.44	0.05	0.30
Fischer	76	3.8	0.11	0.01	0.02
	44	4.2	0.11	0.02	0.01
	23	4.3	0.14	0.05	0.03

Any one or a combination of these three factors would enhance the invasive capacity of the organisms by increasing their ability to overcome the initial resistance of the host. Thus, it appears that egg yolk exerts its effect before bacterial multiplication and spread proceed to the septicemic stage. By this time, the host defenses have been overcome, and the resulting septicemia then progresses at a fixed bacterial generation rate to the same terminal concentration of bacilli/ml of blood, regardless of preliminary spore treatment. The fact that generation time and terminal concentration of bacilli in the blood appear to be constants, with a characteristic rate or level for each host species, may indicate that the degree of phagocytosis and multiplication of bacilli in tissues prior to septicemia are two important factors in the differences in resistance to anthrax among different host species.

In the second study, we observed differences in the septicemic stage of anthrax dependent upon the age of NIH and Fischer rats. Regression lines showing concentration of organisms in the blood of NIH rats are shown in Fig. 3. Slopes of these lines plotted from plate counts are statistically significant at the 95% level. Slopes for 23-day NIH rats and for all Fischer rats were not significantly different from zero, so are not shown. Terminal concentrations of bacilli per ml of blood from all ages of both strains are shown on the vertical axis (as log concentration). All these estimates with their standard errors are presented in Table 1.

From this study, it was evident that there were marked differences in the terminal concentration of bacilli between these two strains of rats. The NIH black rat died with a higher terminal level and at a later time than did the Fischer 344 rat. From observations of terminal concentration and doubling time, we learned that at the same time that the 76-day-old Fischer rats were dying with a terminal concentration of $10^{3.8}$ organisms/ml, the blood of the 76-day-old NIH rats contained a significantly higher concentration (about $10^{5.5}$ organisms/ml).

In monkeys infected with anthrax, we (Klein et al., 1962) showed that, at least during the 10 hr before death, toxin production is continuous and proportional to the concentration of organisms. This observation, if translatable to rats, indicates that the mature NIH rat is both less resistant to infection and less susceptible to toxin than the mature Fischer rat. The former must be true, since the concentration of organisms built up faster and higher in the NIH than in the Fischer rat. The latter must be true because the concentration of organisms in the blood of the NIH rat at the time the Fischer rat died was high enough to have released an amount of toxin that would have killed the Fischer rat. Thus, the concentration of toxin that killed the Fischer rat was not enough to kill the NIH rat. These observations were supported by the results of experiments in which Fischer rats were killed with in vitro-produced toxin but NIH rats were killed only with a much higher concentration of the same material (Table 2). In addition, terminal blood from 21 monkeys which died from anthrax was tested in 107 rats. The mean time to death of the 73 rats that died was 303 min (range, 54 to 1,260 min). In only three of the monkeys was the blood found to be nontoxic. Similar results with toxin were shown by Stanley and Smith (1961) between guinea pigs and mice. Thus, we interpret a host's pattern of septicemic response as indicative both of resistance to infection and of susceptibility to anthrax toxin.

TABLE 2. *Mean time to death (in min) of rats challenged with anthrax toxin produced in vitro, by strain of rat and concentration of toxin*

Toxin	Strain of rat					
	Fischer			National Institutes of Health		
	Total no.	No. survived	MTD*	Total no.	No. survived	MTD*
			min			*min*
10 X	0	—	—	22	12	2,894
8 X	0	—	—	2	2	S†
5 X	0	—	—	2	2	S
4 X	6	0	62	2	2	S
2 X	6	0	76	0	—	—
Unconcentrated	8	0	105	4	4	S
0.5X	8	1	446	4	4	S
0.25X	8	8	S	2	2	S

* Mean time to death.

† Indicates survival.

In the third study, we demonstrated that the effects on mean response time discussed in the previous two studies of egg yolk treatment held true over the age range tested for both strains of rats. In Table 3, we show by strain and age of rat and by egg yolk treatment the harmonic mean times-to-death of the 261 animals tested. From this table, it is seen that animals of both strains and all ages challenged with egg yolk-treated spores died significantly sooner than animals challenged with nontreated spores. Since egg yolk is known to enhance the invasive capacity of the organism and block the resistance of the host, one would expect the effect of this treatment to be greater in a relatively more resistant host. This phenomenon is illustrated in Fig. 4 as a greater distance between egg yolk and nonegg yolk lines for Fischer than for NIH rats. Thus, the effect of egg yolk treatment on the relatively more resistant (to infection) Fischer rat was greater than the effect of this treatment on the relatively less resistant NIH rat.

Perhaps this association between differential responses to egg yolk treatment and resistance, as illustrated here between host strains, also holds true between host ages. If this is true, then we must consider the young NIH rats to be more resistant to infection than the older NIH rats since the effects of egg yolk treatment of the spores were greater in the young NIH rats. These deductions agreed with some of our septicemic data on ages, since among NIH rats the young die with a lower terminal concentration than the old. However, our data were too few and varied to answer this question adequately.

TABLE 3. *Harmonic mean times to death (in hours) of 261 rats, by type and age of rat and challenge treatment*

Type of rat	Challenge treatment*	Age of rat (days)					
		30		60		90	
		MTD†	SE	MTD	SE	MTD	SE
Fischer	Spores + egg yolk	5.7	0.15	8.3	0.15	9.1	0.15
	Spores	17.0	0.15	25.0	0.15	25.0	0.15
National Institutes of Health	Spores + egg yolk	8.0	0.18	13.0	0.18	17.0	0.19
	Spores	21.0	0.18	29.0	0.19	31.0	0.19

* Dose: 1×10^9 spores.
† Mean time to death.

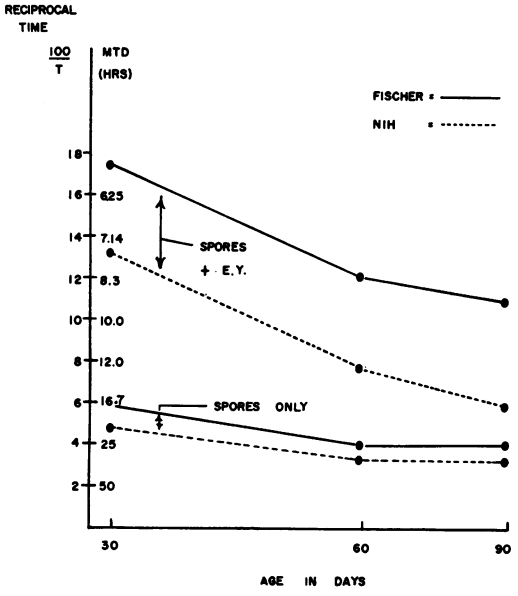

FIG. 4. *Time to death of Fischer and National Institutes of Health (NIH) black rats, by challenge treatment and age of rat. Abbreviations:* MTD, *mean time to death;* E.Y., *egg yolk.*

DISCUSSION

Three important concepts arise from these studies: a reaffirmation of Bennett and Beeson's (1954) estimation of the value of a study of septicemia; the development of the idea that resistance to anthrax consists not only of resistance to the organism but also susceptibility to toxin; and the possibility that resistance to the bacilli and toxin need not be concurrent.

Egg yolk enhancement of the anthrax spores was shown to change parameters that were relatively independent of toxin, but to leave unchanged those response parameters strongly influenced by toxin. This phenomenon was revealed in comparisons between control and treated animals and between the two rodent species.

Conclusions about resistance to anthrax based on observations of response time alone are incomplete and may be misleading. That is, one species of animal which appears from its greater time-to-death to be more resistant to anthrax than another species may actually not be less resistant to infection by the organism but actually less susceptible to toxin. This is indicated by the greater terminal concentration of bacilli in the blood of the longer-lived animals than in those animals that succumb relatively soon. The results clearly indicate the complexity of resistance and its separability into distinct categories for even a single disease such as anthrax. This concept of both a bacterial and toxin resistance to anthrax can explain the fact that the effects of egg yolk treatment of the spores on the mean time to death were greater on relatively resistant hosts (Fischer rats) than on relatively nonresistant hosts (NIH rats).

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