EXPERIMENTAL ANTHRAX IN THE RAT

I. The Rapid Increase of Natural Resistance Observed in Young Hosts

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The degree of natural resistance of an individual host to infectious disease reflects both the genetically determined development of cellular and humoral defense mechanisms and the responses of these mechanisms to a variety of experiences. Thus, it is generally true that mature hosts have greater resistance to many infectious organisms than do immature members of the same species. A measure of the rate of increase in resistance following birth, and of the age at which a high level of resistance is attained for any given host-parasite system, should provide useful information in the continuing search for host mechanisms that contribute to adult resistance. Few, if any, investigations have supplied data adequate for calculating such a rate over any extensive period after birth.

There are published reports that correlate the increasing age of the rat with its resistance to a wide variety of pathogens.[1-7] In 1890 Charrin and Roger[8] reported that the resistance of the white rat to infection with *Bacillus anthracis* varied with the age and weight of the host. This organism is unusually convenient for long-term studies because of its easily stored, relatively stable, highly virulent spores. Furthermore, the remote likelihood that specific immunity to anthrax would be acquired by chance encounter with the anthrax bacillus is advantageous.

Preliminary experiments, employing relatively wide variations in both age of host and dose of spores, indicated that *B. anthracis* and the albino rat might constitute a useful host-parasite system for the study of natural resistance. This paper reports an investigation of dose-age relationships in maturing rats. Survival values and hours between injection and death were determined for a number of litters of the genetically homo-

geneous Fischer rat tested at 1 to 40 days of age with different numbers of spores. An average rate of increase in resistance was calculated from probit regression analysis estimates of the ages at which each dose level corresponded to 50 per cent survival. The data have been examined for certain host factors in addition to age that might have influenced survival.

MATERIAL AND METHODS

Breeding and Care of Hosts

Four pedigreed litters (filial generations 62 and 63) of the Fischer 344 strain of albino rat were obtained in 1957 from the Division of Research Services, National Institutes of Health, through Dr. George E. Jay, Jr. The system of brother by sister mating was continued for our colony. Filial generations 63, 64, 65, and 66 contributed the test rats for these experiments. Beginning with matings in generation 65, all animals of the same filial generation were derived from 1 brother-sister pair 3 generations earlier. Parentage, date of birth, sex, and weight at the time of injection for every rat were recorded.

Although there was no attempt to raise "pathogen-free" rats, certain precautions were observed: (1) Ultraviolet-lighted air locks separated the breeding room from laboratories and from rooms that held experimentally infected animals. (2) Ultraviolet lights burned continuously in the breeding room. (3) Other rats were excluded from the building, and other species from the rat rooms. (4) Human contact was restricted. Individuals working with the rats showered after handling other species, and dressed in freshly laundered clothes before entering the rat rooms. Purina Laboratory Pellets for Rats and tap water were available *ad libitum* to animals of all ages. Sucklings which had been inoculated were nursed by their mothers until death, or until they survived at least 2 weeks. Litters in the group tested with 10^7 spores were weaned on the day before injection, at 19 to 29 days of age. All others were weaned at 28 days.

Groups of Hosts

Four large groups of litters, for testing with 10^4, 10^7, 10^8, or 10^9 spores, were selected to provide ranges in age which responded with low through high survival per cents for each of these 4 doses. Two smaller groups were used to check responses of rats whose ages ranged between 10 and 20 days to doses of 10^5 and 10^6 spores. The age of rats at the time of inoculation was usually known to within 12 hours, always to within 24. There were nearly equal numbers of males and females in all except the oldest group plotted in Text-figure 1.

Pathogen, Dose, Injection Route

All spore inoculums were harvested from a single culture of a highly virulent strain of *B. anthracis*. This culture was prepared from the same parent stock as that used by Roth, DeArmon and Lively.[9] This stock is known as the 1B sib-progeny of the Vollum strain. The concentrated spore suspension, which contained 1 per cent phenol, was diluted 1/10 in distilled water, heat-shocked at 60° C. for 30 minutes, and stored in the refrigerator. (Viable counts varied between 7×10^9 and 9×10^9 per ml. during the experimental period.) The suspension was subjected to 3 types of tests to characterize its virulence: (a) the LD_{50} by intradermal route for guinea pigs, (b) the proportion of smooth (capsule-forming) colonies,[10] (c) the median survival time for mice inoculated intraperitoneally.[9] All responses were characteristic of highly virulent populations. The stock suspension was diluted in distilled water to the concentration required for the test. Each inoculum was counted for viable spores within 24 hours of injection, and did not differ from the intended dose by more than 20 per

cent. All doses, given in a volume of o.1 ml., were injected either subcutaneously or intradermally. On the average, 3 independent tests were run for each combination of dose and age.

Time to Death

Inspection at 12 to 18 hours after injection revealed that all animals were alive and eating well. Frequent subsequent surveys insured a record of time to death that was accurate to plus or minus 6 hours.

Diagnosis

The first 366 rats that died were necropsied. The cut surfaces of liver, spleen, or subcutaneous edema at the inoculation site were touched to glass slides. The slides
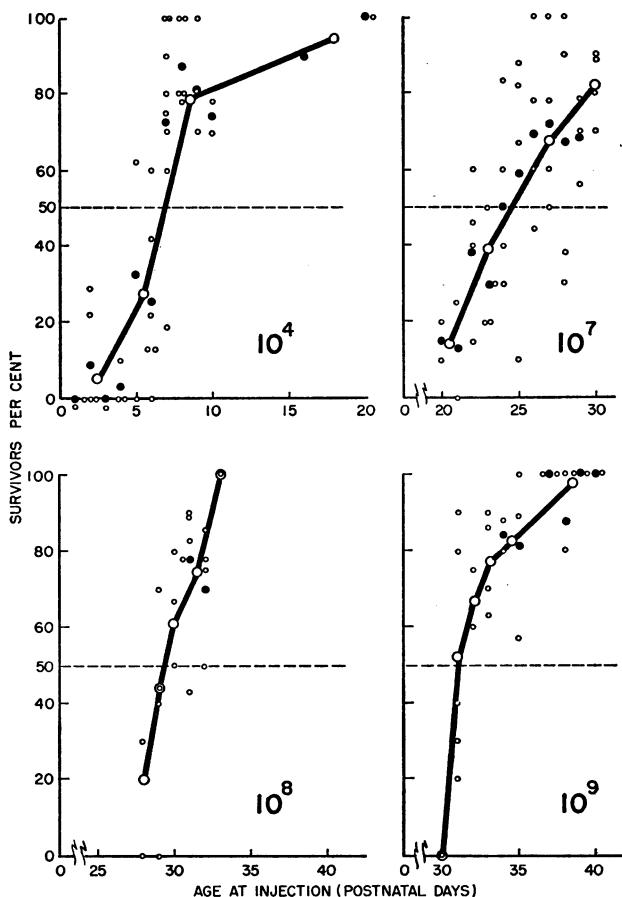
TEXT-FIGURE 1. The relationships between age at injection and proportion of Fischer rats surviving the indicated numbers (10^4, etc.) of spores of *B. anthracis*. The curves are drawn through points (large circles) obtained by combining data from litters of similar ages, and plotting per cent survivors at the mid-point of the ages of those litters combined. There were nearly equal numbers of males and females tested for each of these points except one. In the group plotted at 38.5 days and tested with 10^9 spores, there were 5 males and 35 females. If ages of combined litters differed by more than 24 hours, the per cent of survivors within each 24-hour group is shown by closed circles. The extent of variability among litters is indicated by small circles—one for each litter.

were then placed in 10 per cent formalin (phosphate-buffered at pH 7) overnight, washed in running water, air-dried and stained with the Jenner-Giemsa stain. Microscopic examination consistently revealed short chains of large, square-ended, thick-walled, encapsulated bacilli, morphologically characteristic of *B. anthracis* in tissue. Tissues from more than 200 of the same rats were streaked on plates of Difco blood agar base enriched with 5 to 7 per cent whole, fresh rabbit blood. Colonies characteristic of the injected organism were always recovered, usually in pure culture. Because of these consistent results, and the rarity of spontaneous deaths in the rat colony, necropsy and culture were discontinued. Death between 18 hours and 1 week following injection of spores was subsequently considered sufficient evidence of specific infection.

RESULTS
Age and Per Cent Survival

The relationships between injection age and per cent survival for groups of rats of similar ages within each of the 4 large groups of litters tested with 10^4, 10^7, 10^8, or 10^9 spores are shown in Text-figure 1. These results indicated that with increasing age the rats rapidly acquired the ability to survive larger numbers of spores. From the graphs, 50 per cent

TABLE I
AVERAGE WEIGHTS AND NUMBERS OF FISCHER RATS FOR THE GROUPS PLOTTED IN TEXT-FIGURE I

Log of spore dose	Age * (days)	Weight † (gm.)	No. of litters tested	No. of individuals	
				Tested	Surviving
4	2.5	6.7	10	97	5
	5.5	9.7	8	72	20
	8.5	12.6	18	170	133
	18.0	25.8	2	18	17
5	12.5	19.3	5	48	29
6	16.5	23.9	8	75	55
7	20.5	31.2	4	35	5
	23.0	35.4	12	120	47
	27.0	50.5	19	174	117
	30.0	62.4	4	39	32
8	28.0	57.8	2	15	3
	29.0	58.9	4	34	15
	30.0	62.9	3	18	11
	31.5	70.9	9	71	53
	33.0	74.8	1	10	10
9	30.0	62.0	1	10	0
	31.0	70.7	5	50	26
	32.0	74.9	2	18	12
	33.0	80.8	4	35	27
	34.5	83.0	5	40	33
	38.5	92.4	7	40	39

* Mid-point of injection ages represented. For days included, when more than one, see closed circles, Text-figure 1.

† Average weight per rat at injection.

survival would be expected when 10^4 spores are administered to 7-day-old rats, 10^7 spores to 24.5-day-old rats, 10^8 spores to 29.25-day-old rats, and 10^9 spores to 31-day-old rats. Additional information regarding these groups is recorded in Table I.

Rate of Resistance Increase

The methods of probit analysis[11] were used to compute the age at injection corresponding to 50 per cent survival for the dose used to challenge each of 6 groups of rats. The computed ages are plotted in Text-figure 2 against the log of the number of spores administered. These points were weighted for the unequal numbers of litters tested with each dose, and a straight line was fitted * to them for the interval from day 5.8 (10^4) to day 31.3 (10^9). The antilog (1.5) of the slope of this line estimates the average rate of increase in resistance during this interval. Thus, for each day of increasing age from 5.8 to 31.3 days, the 50 per cent survival level corresponded to 1.5 times the number of spores administered the previous day.

Time to Death

Animals of all ages died promptly (96 per cent in 72 hours, 99.8 per cent by the end of one week). With one exception, the deaths that occurred under 24 hours were in the groups of younger animals.

### Variations Among Litters in Replicate Experiments

When litters the same age were tested with the same number of spores, there was considerable variation in the proportions of each litter that died of anthrax (Text-figure 1). Known variables among these genetically uniform litters were average weight per rat, numbers of each sex, age of mother at parturition, different preparations of inoculums, and degree of infestation with pinworms and of infection with an unidentified agent(s) of chronic respiratory disease (CRD). The severity of the CRD and pinworm infestation was not recorded for individuals or litters, but both conditions were observed to be widespread in the colony. Records did permit a compilation of data dealing with the other known variables listed above.

Weight. The close correlation between weight and age in the period of rapid growth makes it difficult to distinguish between their separate influences on increase in resistance. Table I shows the relationship between weight and age in the Fischer rat. Instances in which weight does

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* Method of least squares. Equation: $Y = 2.77 + 0.1823X$, where $Y =$ log of dose corresponding to 50 per cent survival, and $X =$ age at injection in days.

not correlate with per cent survival follow:

(1) Within each dose group, the average weight per rat for each litter was calculated and plotted against per cent survival. No correlation could be detected between heavy litters and high per cent survival unless the heavy litters were also older. The responses and distribution
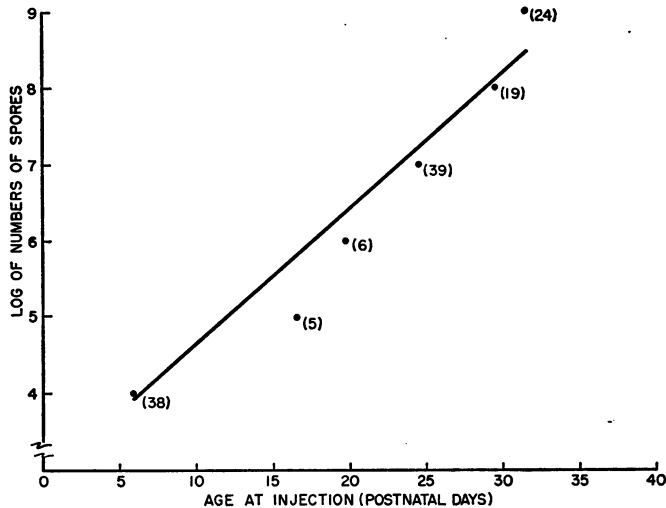
TEXT-FIGURE 2. Relationship, at 50 per cent survival for each dose group, between numbers of *B. anthracis* spores and injection age of Fischer rats. Each plotted point (obtained by regression analysis of probit per cent survival on age of all litters that were tested with each dose) was weighted for the number of litters, and the straight line fitted by the method of least squares. Numbers of litters are in parentheses. See text for further details, including use of the equation for this line to estimate the average rapidity of increase in resistance over this period.

of weights of litters included in the 10^7 dose group made possible the examination of the effect of age without regard to weight on the per cent survival of the 20 litters weighing between 25 and 45 gm. Again, the importance of age in determining response to challenge is apparent (Text-figure 3).

(2) A comparison of the weights of survivors with the average weight per rat for the litter revealed that among 94 litters there were 6 litters in which all survivors were average or below average weight, 85 litters in which the survivors were both above and below average weight, and 3 litters in which all survivors were average or above average weight. Furthermore, there was no correlation between time to death and heavy or light individuals within a litter.

(3) Table II presents several instances of closely equivalent average weights for groups of male and female litter mates with differences in

proportions of survivors varying from plus 21 to minus 37 per cent. These differences may or may not be due to sex, but they certainly are not due to weight.

Sex. The data for comparison of per cent survival among males and females (Table III) are from tests of each litter that fulfilled the following requirements: (a) The age-dose combination was one for which the
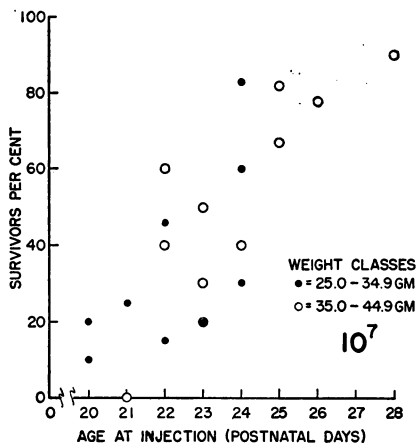
TEXT-FIGURE 3. Relationships of age at injection to per cent survivors among litters of closely similar weights (average weight per rat at time of injection).

average survival had been observed to be 10 to 90 per cent; (b) no less than 10 and no more than 90 per cent survived; and (c) at least two of each sex were included. Within each dose group, the data were combined from litters of similar age. It is concluded that (a) the expected correlation occurred within each sex between age and increase in per cent survival; (b) different sex ratios among litters the same age could not account for the observed variation among litters in survival per cent; and (c) statistically significant (chi-square) difference in response can be correlated with sex only among rats more than 4 weeks old at injection, at which time a larger proportion of females survived. This means that sex must be controlled in testing rats 4 weeks old or older, although when survival is high for both sexes, a difference need not show up (Table III, 10^9 dose).

Age of Mother. All litters were identified by age of mother at parturition. Table IV summarizes the number of litters, by mother's age, for which the per cent survival fell either below or above the average curves in Text-figure 1. No differences could be related to ages at parturition of 3 through 8 months. The number of litters with 9- to 13-month-old mothers was too small to render their superior survival values significant.

TABLE II

INJECTION WEIGHTS COMPARED WITH PER CENT SURVIVORS AMONG
GROUPS OF FISCHER RATS INJECTED WITH SPORES OF *B. anthracis*

Group				Average wt. (gm.)	Survivors (%)
Log of spore dose	Age * (days)	No. tested			
4	5.5	M	25	10.1	48
		F	30	9.4	27
		Difference		+0.7	+21
7	21.5	M	57	34.8	37
		F	55	33.3	24
		Difference		+1.5	+13
8	29.5	M	16	60.8	38
		F	23	58.8	61
		Difference		+2.0	−23
8	31.5	M	32	75.6	63
		F	29	68.5	86
		Difference		+7.1	−23
9	31.5	M	31	82.4	36
		F	37	72.1	73
		Difference		+10.3	−37

* Mid-point of injection ages for groups in Table III which showed a difference between per cent survivals of males and females.

TABLE III

COMPARISON OF SURVIVAL OF MALE AND FEMALE FISCHER RATS
INJECTED WITH SPORES OF *B. anthracis* AT DIFFERENT AGES

Log of spore dose	Age * at injection (days)	Litters †	No. tested M	F	Total	Survivors (%) M	F	Total	Chi square	P ‡
4	5.5	6	25	30	55	48	27	36	2.85	0.053
	7.0	6	28	28	55	59	64	62	0.15	
	9.0	5	23	25	48	78	72	75	0.22	
7	21.5	11	57	55	112	37	24	30	2.33	0.125
	25.0	10	46	47	93	54	58	56	0.86	
	28.5	11	52	53	105	64	64	64	0.01	
8	29.5	4	16	23	39	38	61	51	2.05	0.150
	31.5	7	32	29	61	63	86	74	4.41	0.035
8 §	30.5	11	48	52	100	54	75	65	4.79	0.029
9	31.5	7	31	37	68	36	73	56	9.30	0.002
	34.0	8	33	36	69	76	81	78	0.21	

* Mid-point of different ages represented.

† See text for bases for choosing the included litters.

‡ The probability that the observed proportions are no more different than could have occurred by chance, from those expected if males and females survived in the same proportions as the total population.

§ Data in this line are the result of combining the two preceding lines.

TABLE IV

NUMBERS OF LITTERS WITH BELOW- OR ABOVE-AVERAGE * PER CENT SURVIVAL (FOR THE PARTICULAR AGE-DOSE COMBINATION), CLASSIFIED BY AGE OF MOTHER AT PARTURITION

Age of mother at parturition (mo.)	Number of litters with per cent survivors	
	Below average	Above average
3	23	26
4	8	2
5	5	9
Total	36	37
6	5½ †	5½
7	8	7
8	3	3
Total	16½	15½
9	1½	4½
10	1½	3½
11	0	1
12	0	2
13	0	1
Total	3	12

* Here defined as falling below or above the lines drawn through the open circles on Text-figure 1.

† One-half used when a litter's response coincided with the average.

The observed trend, however, was above the average survival for litters from older mothers.

Inoculums. The ±20 per cent variation in viable spore counts among replicate dilutions of inoculums did not correlate with variations in survival responses. Experiments with above-average counts gave high survival as often as did experiments with below-average counts, and vice versa.

DISCUSSION

A rapid increase in the resistance of Fischer rats to spores of *B. anthracis* has been demonstrated during the first 40 postnatal days. This increase in resistance, as measured by per cent survival of 1- to 40-day-old rats after injection with differing numbers of spores, appears to be primarily an age-related phenomenon. Between ages 5.8 and 31.3 days the average rate of increase in resistance was estimated to be 1.5-fold per day. For the period between 10 and 20 days, the responses of the 13 litters tested on 10^5 or 10^6 spores confirmed a continuing increase in resistance between the ages of 5.8 and 24.5 days. The data are not sufficient to ascertain whether the rate during this age period is the same as the over-all average. Likewise, there were insufficient data to determine a rate for the observed increase in resistance from birth to 6 days, or after 31 days. It is of interest to note that Whitman,[12] studying yellow

fever in Swiss mice, demonstrated an age-correlated rate of resistance increase.

The high resistance exhibited by the adult laboratory rat for a miscellany of pathogens has usually been reported as being attained by age 21 to 30 days.[1-7] Unfortunately, high resistance means only that the animal is resistant to the highest practicable dose of the micro-organism in question. Full resistance to 10^9 spores of *B. anthracis* Vollum 1B injected intradermally appeared in females of the Fischer strain by the 40th postnatal day.[13] (Only 5 males were available for this oldest test group of 40 rats whose average age was 38.5 days.) The oldest group in which there were equal numbers of each sex averaged 34.5 days old and had a survival value of 83 per cent. A larger dose, another injection route, and equal numbers of each sex in the test group might reveal that Fischer rats are not fully resistant at age 40 days.

In spite of the close correlation between age and weight, there is good reason to believe that increase in resistance of the rat is not primarily related to weight (Text-figure 3 and Table II). Recently, studies with the germ-free rat demonstrated that increased weight does not enable 5-week-old rats to survive injection with anthrax spores.[14] Moreover, among species highly susceptible to anthrax, body weight as such bears no relationship whatsoever to lethal dose. The LD_{50} of intradermally or subcutaneously injected spores is approximately the same for adult albino mice, grasshopper mice, hamsters, guinea pigs, and rabbits.[15]

While it has been demonstrated that resistance of the rat to *B. anthracis* challenge does increase rapidly with age, the basic mechanisms involved in this phenomenon have not been investigated. One can only speculate on the possible importance of such factors as spontaneously occurring CRD, contact with other aerobic, spore-forming bacilli known to have antigens in common with the anthrax organism, and the little-understood processes of physiologic and morphologic maturation. Certain important stages in the maturation of the reticuloendothelial system of the rat have been reported,[16-19] and are compatible in time with the responses reported in this investigation.

Furthermore, the variations in response of individuals within a given litter, and among litters the same age, could not be related to sex, weight, or slight variations in spore doses when examined by multiple probit regression analysis,[11] and are without explanation. The possible effects of the age of the mother, and variations in protection afforded to suckling littermates by mothers' milk are suggested by reports of work using different host-parasite systems.[20-22]

Despite the paucity of information regarding the mechanisms involved, the responses over the age range reported are sufficiently pre-

dictable to indicate that the Fischer rat and *B. anthracis* may be useful as a standard testing system in which to evaluate factors that alter the resistance of the host or the virulence of the pathogen. Caution should be exercised regarding extrapolation from the data reported to untested age-dose combinations and routes of infection.

SUMMARY

Responses have been determined for the genetically uniform Fischer 344 strain of albino rat, at different ages, to graded doses of the highly virulent Vollum 1B strain of *Bacillus anthracis*.

A rapid increase in resistance was related to age within each of 4 groups tested with appropriate doses. When the age at injection was increased by 4 to 10 days, the proportion of survivors at each dose level rose from less than 20 to more than 80 per cent.

Calculation of an average increase in resistance revealed that for every 1-day increase in injection age from the sixth through the 31st day, an increment of 1.5-fold in spore dose was required to maintain 50 per cent mortality.

The data were examined for possible influence on survival of the factors of age, weight, and sex of host, and of age of mother at parturition. No differences in per cent survival between litters could be related to mothers' age at parturition of 3 through 8 months. No significant differences between the responses of males and females were noted among animals tested during the first 4 weeks of life, but among animals tested during the 5th week, females exhibited a significantly higher per cent survival. Evidence was presented that indicated that increasing weight does not account for the increasing resistance between 1 and 40 days of age.

It was suggested that the information obtained provides a reliable basis for selecting useful age-of-host and dose-level combinations to screen various factors for their influence upon the virulence of the bacillus or upon the resistance of the rat.

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