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TECHNICAL MANUSCRIPT 372

IN VIVO GROWTH AND DISTRIBUTION
OF ANTHRAX BACILLI IN RESISTANT,
SUSCEPTIBLE, AND IMMUNIZED HOSTS

William I. Jones, Jr.

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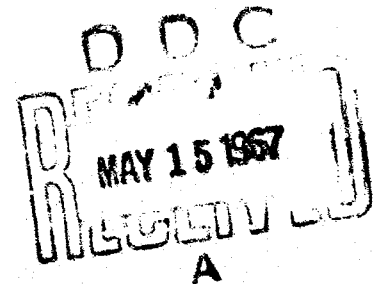
Ralph E. Lincoln

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APRIL 1967



DEPARTMENT OF THE ARMY

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Process Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORY

Project 1C522301A059

April 1967

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

In vivo growth curves of Bacillus anthracis in the body and various tissues in susceptible and resistant hosts have been developed. The effect of two levels of immunity imposed on these hosts was demonstrated. Differences in growth rates of organisms in the bodies of infected rats and guinea pigs showed the effect of innate resistance. As the degree of immunity was increased, the rate of organism buildup decreased and was accompanied by a decreased terminal population. The blood best reflected the population growth in the host. Data generated from the naturally resistant rat that had been immunized with both protective antigen and live vaccine demonstrated the role of "toxins" in terminal anthrax. Gross morphological studies pointed out the errors regarding terminal population of organisms when a sample was taken postmortem rather than at death.

I. INTRODUCTION

In vivo quantitation of bacterial growth in anthrax has largely been limited to the septicemic stage. In guinea pigs Keppie et al.¹ observed that during the pre-septicemic stage, bacilli were largely (78%) in the spleen, and that as septicemia progressed the distribution shifted so that most (80%) of the bacilli were in the blood. Septicemic growth was logarithmic. This observation has been verified under a wider range of conditions, i.e., immunized hosts, different species of animals challenged with avirulent as well as virulent strains, and with virulence-enhanced (egg yolk) spores, by Klein et al.,² Lincoln et al.,³ and Mahlandt et al.⁴ This American group also noted that animals naturally resistant to the establishment of anthrax or actively immunized have a lower number of organisms in the blood at death than do susceptible species or nonimmunized controls. They noted a positive relationship between free toxin and the number of bacilli in the blood at death. Their data are interpreted to suggest a characteristic rate of septicemic growth and level of organisms in the blood at death for each species.

Their work raises questions about population growth as related to immunity and resistance, because it would seem that more organisms would be needed in vivo to kill the resistant or immunized host than would be required in the susceptible or nonimmunized host. It is certain that removal of bacilli from the blood stream or the sequestering of bacilli in an organ is not synonymous with their destruction. It is classically believed that most pathogenic disease organisms show an affinity for certain sites in the host because of (i) nutritional requirements, (ii) relative lack of vascularity that protects the pathogen against antibodies and blood phagocytes, or (iii) a concentration in sessile phagocytes that provide conditions for parasitic growth or sequestering of blood-carried organisms. This report describes the total in vivo growth characteristics of Bacillus anthracis in tissues of both the immunized and nonimmunized rat (resistant) and guinea pig (susceptible). Gross cell morphology in the blood and spleen also was observed at death and at 3 hours postmortem.

II. MATERIALS AND METHODS

A. ANIMALS

The Hartley strain guinea pig (250 to 350 g) from the Fort Detrick Animal Farm and the Norvegicus black rat (200 to 250 g) from Long-Evans stock obtained from the National Institutes of Health Animal Farm were used. These two species differ greatly in their resistance to the establishment of anthrax and in their response once the disease becomes established.⁵

B. IMMUNIZATION

To obtain animals with varied degrees of immunity, one group each of guinea pigs and rats were immunized with the Belton-Strange protective antigen (PA) prepared by the method described by Haines et al.⁶ and diluted 1:10. The PA was administered by intraperitoneal injection of 0.1 ml on days 1, 3, 5, 8, and 11 (first level of immunity). In addition, a second group of guinea pigs and rats, after receiving the initial (above) PA protocol, was given a booster of 1×10^8 spores of the low-virulence 30R strain of B. anthracis (second level of immunity). Both of these immunization procedures are described more fully by Klein et al.⁷

C. CHALLENGE

One week after completion of their immunizations, animals were challenged with 1×10^7 B. anthracis spores of the highly virulent Vollum strain (Vlb), which were enhanced by treatment with egg yolk as described by Kaga.⁸ All challenges were by the subcutaneous route.

D. EXPERIMENTAL PROCEDURES

For both the guinea pig and the rat, three groups of animals were challenged:

- Group A - Nonimmunized or controls
- Group B - Immunized (PA5)
- Group C - Immunized (PA5 + LV)

Animals from each group were randomly assigned two per cage. Numbers were assigned to cages, and animals were sacrificed at 0, 45, 90, and 120 minutes, then every 6 hours for a period of time corresponding to a previously determined mean time to death. Two replicates were run. Serially sacrificed animals were immediately skinned and weighed; heart blood samples were taken; and tissues were separated and weighed.

The spleen, kidneys, liver, and lungs were homogenized in gelatin phosphate diluent with a Tri-R Stirring Apparatus.* The intestinal tract was discarded to avoid enteric contamination. The remaining carcass of the animal then was weighed and ground in a Hamilton Beach** heavy-duty meat grinder and homogenized in the Sorvall Omni-mixer*** To avoid growth of contaminants, tissue samples were diluted immediately in gelatin phosphate diluent and plated on tryptose agar containing 0.005% potassium tellurite. Colonies were counted 24 hours after incubation at 34 °C.

E. QUANTITATION OF IMMUNITY

The immunity index (I)⁹ was used to measure the immunity developed in both hosts by the two immunizing protocols. The virulence enhancement of the inoculum by egg yolk treatment was calculated by the same formula and represented as RI.

F. ASSAY OF RESIDUAL BLOOD IN EXCISED ORGANS AND TISSUES

The number of bacilli in the residual blood in the various organs was estimated by centrifuging the homogenized tissues and determining the amount of hemoglobin in the supernatant fluid. The hemoglobin values, obtained by using the cyanomethemoglobin method, were compared with those found in a given unit of the animal blood. The ratio thus obtained was corrected proportionally to the residual blood in the organ.

G. MORPHOLOGICAL STUDIES

Spleen and heart blood samples were taken at death and at 3 hours postmortem. Emulsions of the spleen were prepared with the hand-operated standard Ten Broeck tissue grinder. Samples of blood and/or homogenized tissue were placed on a standard microscope slide and spread by streaking with the end of another glass slide. After air-drying, the samples were stained with Wright's stain and examined and photographed under oil immersion.

H. ANALYSIS OF DATA

Total viable cell count per gram of tissue was plotted graphically as a function of time after initiation of infection. Times and logarithms of cell counts were used as variables to develop a linear regression using

* Tri-R Instruments, Jamaica, New York.

** Hamilton Beach Co., Div. of Scovill Manufacturing Co., Racine, Wisconsin.

*** Ivan Sorvall Inc., Norwalk, Conn.

a standard computer program. The linear equations obtained were then transformed in the exponential equation of best fit,

$$N = Ke^{bt}$$

where N is the number of bacteria per gram of tissue, K is a constant, and e^{bt} is an exponential function in which b is the hourly rate of increase of organisms in the body and t is the sampling time in hours.

III. RESULTS

A. NONIMMUNIZED HOST

The virulence enhancement effect of egg yolk treatment on the anthrax spore has been well established.^{8,9,10} In an effort to determine the effect on the host of egg yolk enhancement of the growth and distribution of organisms, in vivo growth studies were initiated with the highly susceptible guinea pig and the naturally resistant rat.

1. Guinea Pig

When the guinea pig was infected with 10^7 untreated spores of B. anthracis, death occurred at 30 hours with a terminal concentration of $10^{8.8}$ organisms per gram of tissue (Fig. 1). The rate of growth of organisms in the blood was 50% greater than that in remaining tissues [blood ($b = 0.32$) vs. tissue ($b = 0.21$)], and the terminal concentration of $10^{8.6}$ organisms per milliliter was obtained.

When spore inoculum was treated with egg yolk, the initial destruction of organisms 1 hour postchallenge was not evident. The growth rate in the whole body was decreased so that terminal concentration was 10^8 organisms per gram of tissue. This decrease in rate was the greatest in the carcass; the growth rate remained essentially of equal magnitude in the remaining tissues. It should be noted that growth rate in the blood was the greatest regardless of inoculum treatment.

2. Rat

The animals died at 43 hours with a terminal concentration of $10^{7.1}$ organisms per gram of tissue (Fig. 2). The growth rates of organisms in the carcass, spleen, and kidney were approximately of the same magnitude [carcass ($b = 0.06$), spleen ($b = 0.05$) and kidney ($b = 0.06$)] but, the growth rates in the remaining tissues (liver, lung, and blood) were two to three times as great ($b = 0.11$ to 0.16).

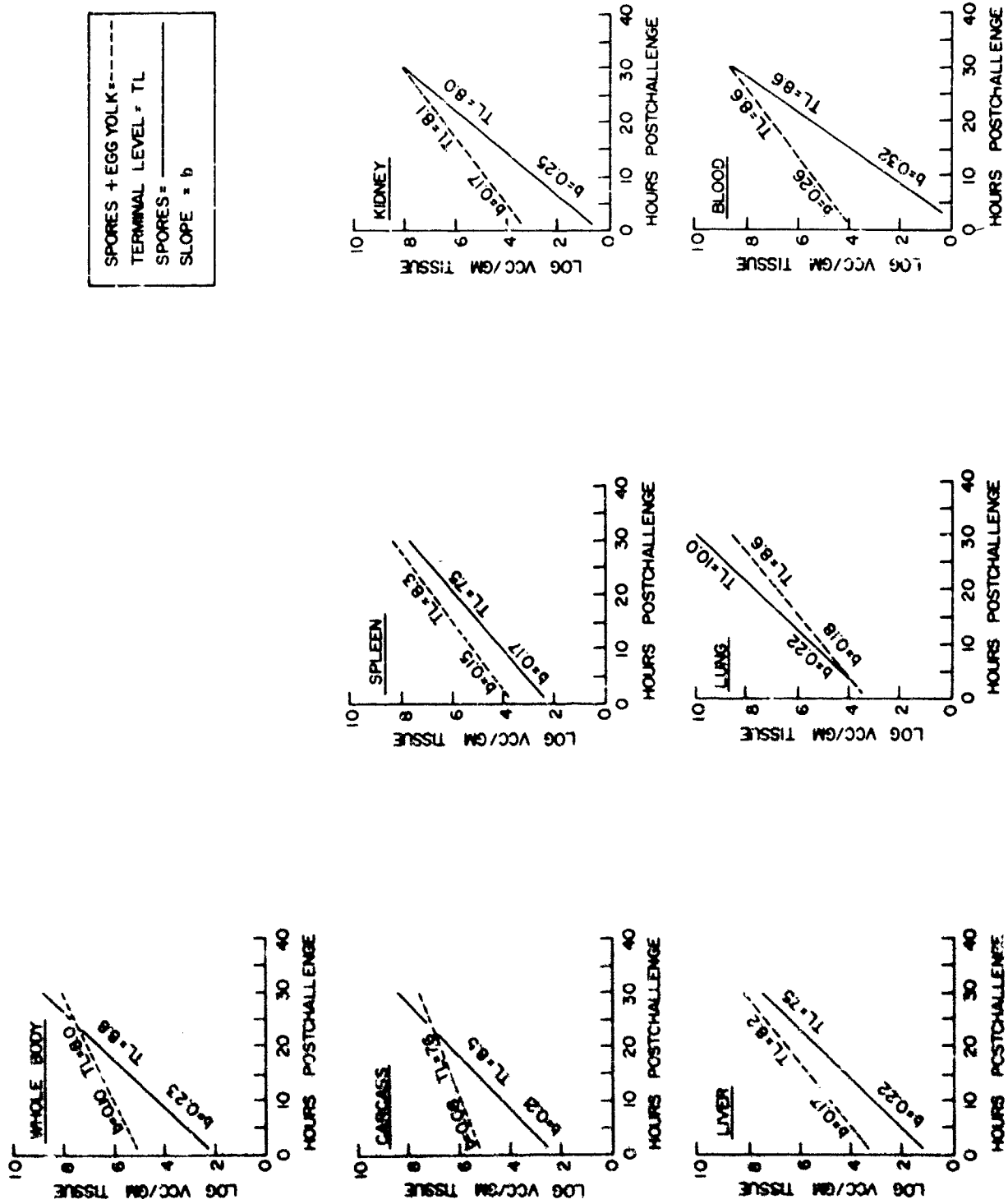


Figure 1. In Vivo Growth of *B. anthracis* in the Nonimmunized Guinea Pig.

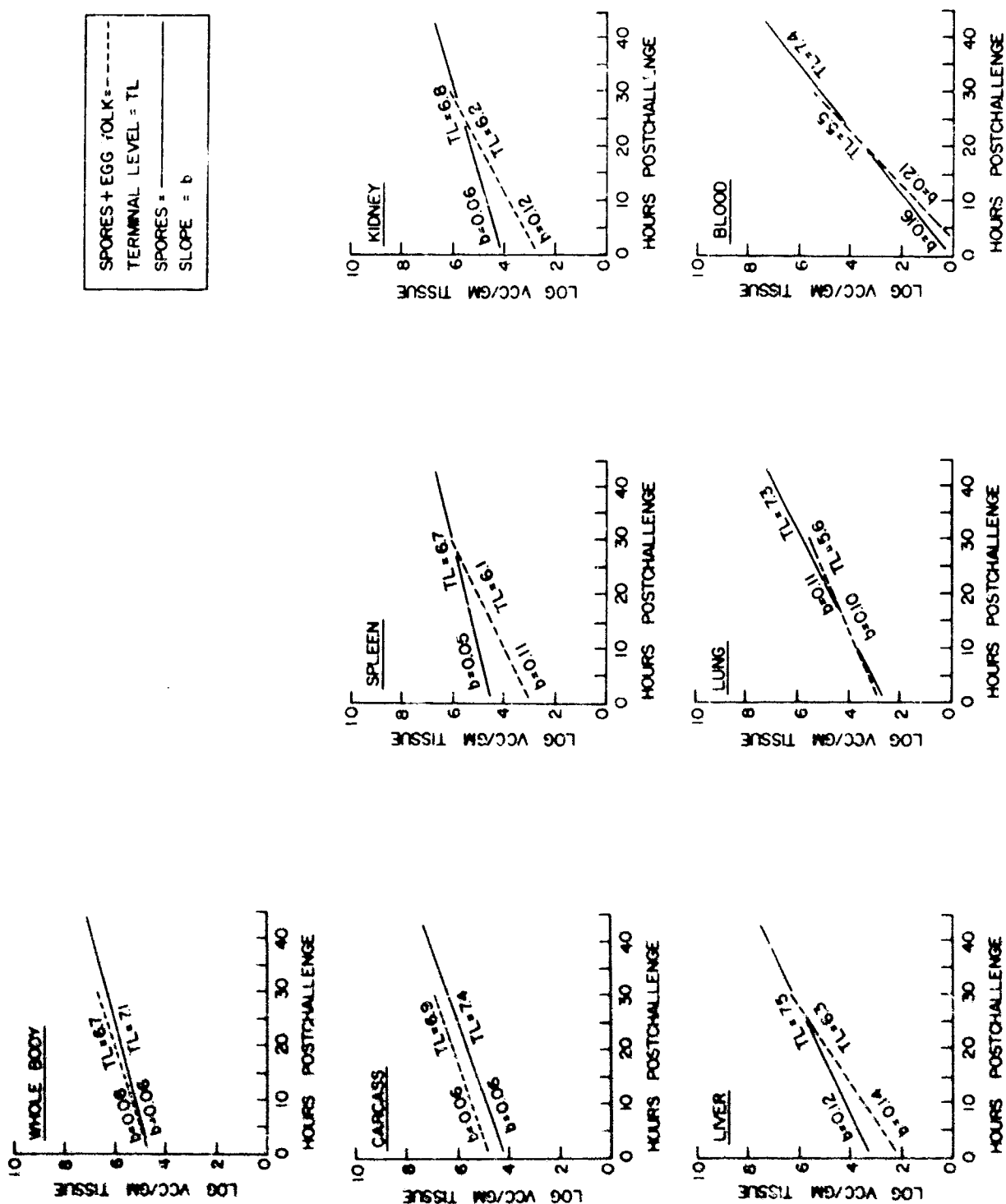


Figure 2. In Vivo Growth of *B. anthracis* in the Nonimmunized NIH Rat.

Rats challenged with spores plus egg yolk demonstrated an increased rate of growth in the whole body. With untreated spores $b = 0.06$ vs. $b = 0.07$ for egg yolk-treated spores. The increased growth rate was mainly attributable to the growth rates of organisms in the spleen ($b = 0.11$), kidney ($b = 0.12$), and blood ($b = 0.21$). The accelerated growth rate together with the lack of initial destruction of organisms resulted in a shorter time to death (30 hours). The terminal concentration of organisms in the host was $10^{6.7}$ per gram of tissue. The decrease in time to death after challenge with treated inoculum indicated enhanced virulence, most probably attributable to early capsule formation.⁵

We were not able to show a parallel effect in the guinea pig because of its greater susceptibility to establishment of the disease and because of the large dose of organisms used. Our data show that egg yolk treatment of inoculum produced contrasting effects in the growth rate of organisms in the susceptible guinea pig and the resistant rat. Egg yolk minimizes the initial destruction of organisms and decreases the rate of organism growth in the whole body of the susceptible host. In the resistant rat, in which there was little evidence of initial destruction of organisms, there was a definite increase in the rate of growth of organisms throughout the body. This produced the RI of 1.39.

B. IMMUNIZED HOST

To explore the effect of immunization on population changes, two immunization protocols were used that resulted in two levels of immunity and resistance to the disease.⁴ Egg yolk inoculum was used to reduce the high variability of response to challenge associated with the induced resistance resulting from immunization.

Population growth in both animals was affected by immunization. The guinea pig had the greater response, as demonstrated by $I = 3.9$ compared with $I = 1.25$ for the rat. Again, because of the conditions under which the experiment was conducted, quantitative measurements of the second level of immunization (PA5 + LV) in the guinea pig were not possible. However, the added resistance afforded by this protocol was demonstrated by reduction in numbers of organisms in the blood and tissue homogenates. This is in agreement with our earlier reports.^{2,3} The data also showed no extended lag period, indicating that anthrax was easily established in the immunized guinea pig.

1. Guinea Pig

Figure 3 shows that in the PA5-treated guinea pigs, the rate of growth in the whole body was decreased 60% [controls ($b = 0.10$) vs. PA5 ($b = 0.04$)] resulting in an increase in the time to death to 43 hours, accompanied by a terminal concentration of $10^{6.7}$ organisms per gram of tissue. The rate of growth in all organs was decreased as a result of immunization. The organ least affected was the spleen. Immunization reduced the rate of growth in the carcass of the guinea pig 75% compared with that of controls [control ($b = 0.08$) vs. PA5 ($b = 0.02$)]. The remaining organs showed reduction in growth rates compared with those of controls, varying from 20 to 50% [controls (avg $b = 0.19$) vs. PA5 ($b = 0.11$)]. The terminal concentration of organisms in the various organs was decreased 1 to 3 logs compared with controls.

In the PA5 + LV immunized guinea pigs both the rate of growth and concentration of organisms were further reduced in the whole body. In general, growth of organisms in the body of the guinea pigs was inversely proportional to the degree of immunization obtained.

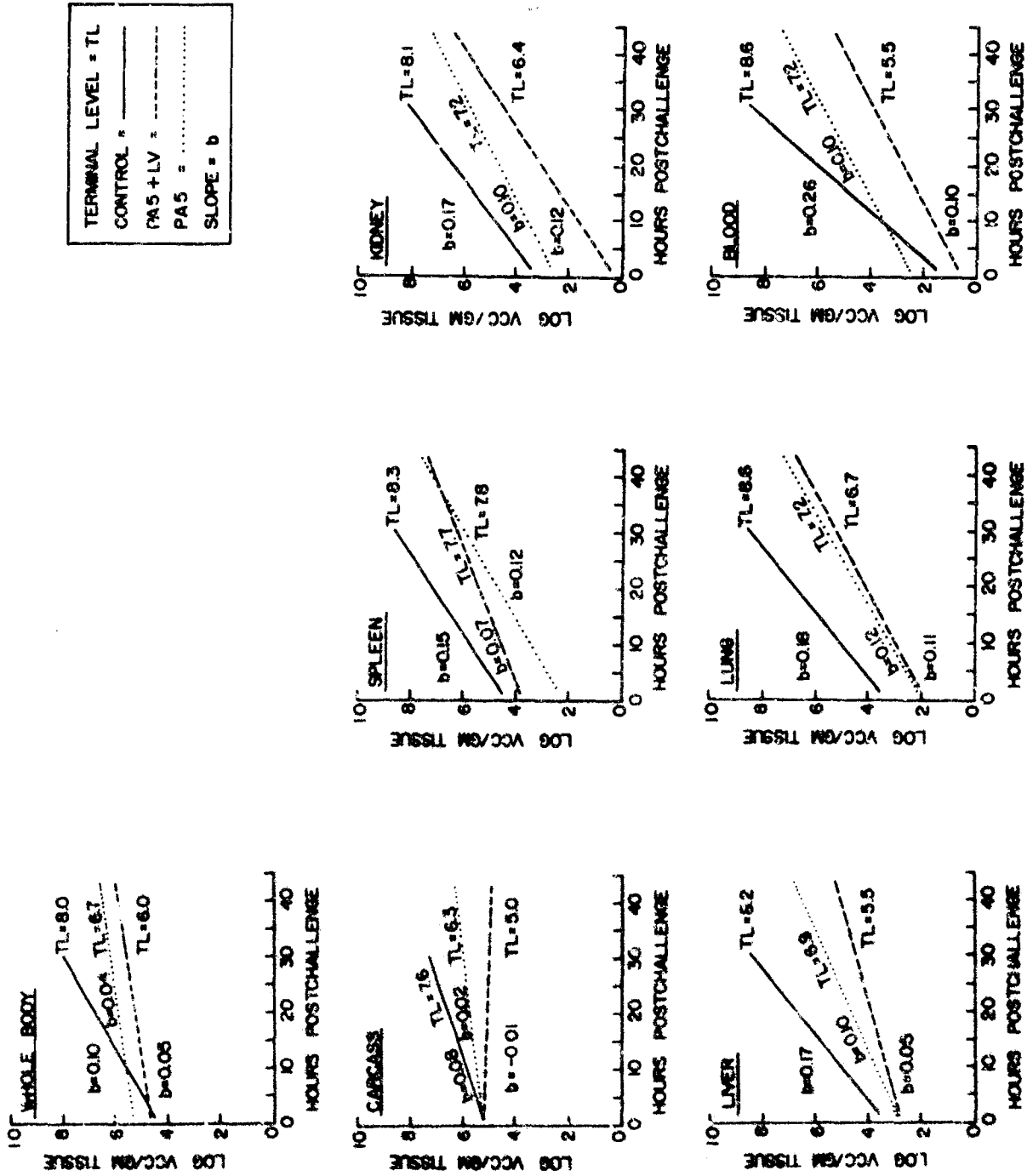
2. Rat

Immunization of the rat, which is naturally resistant to establishment of the disease, results in only a slight increase in resistance as shown by an $I = 1.25$ for the maximum immunity developed (PA5 + LV).

Figure 4 shows that the growth rate of organisms in the whole body of the PA5-immunized rat was 25% slower than that in the control [control ($b = 0.08$) vs. PA5 ($b = 0.04$)]. Further immunization to the second level (PA5 + LV) produced little if any organism buildup in the whole body and its organs. The only growth noted was in the carcass and spleen, as evidenced by slopes of $b = 0.01$.

Terminal levels of organisms were similarly reduced by immunization. The terminal concentration of organisms in the body of the PA5-immunized animals was $10^{6.5}$; it was $10^{4.1}$ in the PA5 + LV group. These reductions in terminal concentration were reflected primarily in the blood.

Immunization of the guinea pig and rat produced similar results when the disease was once established. There was a decrease in rate of growth or organism buildup in all organs, accompanied by reduced terminal concentration in the whole body and its organs inversely proportional to the degree of immunity imposed. However, it should be noted that by virtue of its innate resistance, the rat, after imposition of the second level of immunity (PA5 + LV), did not exhibit any organism buildup. It subsequently died with a terminal level of organisms in the body less than the number of challenge organisms.

Figure 3. In Vivo Growth of *B. anthracis* in the Immunized Guinea Pig.

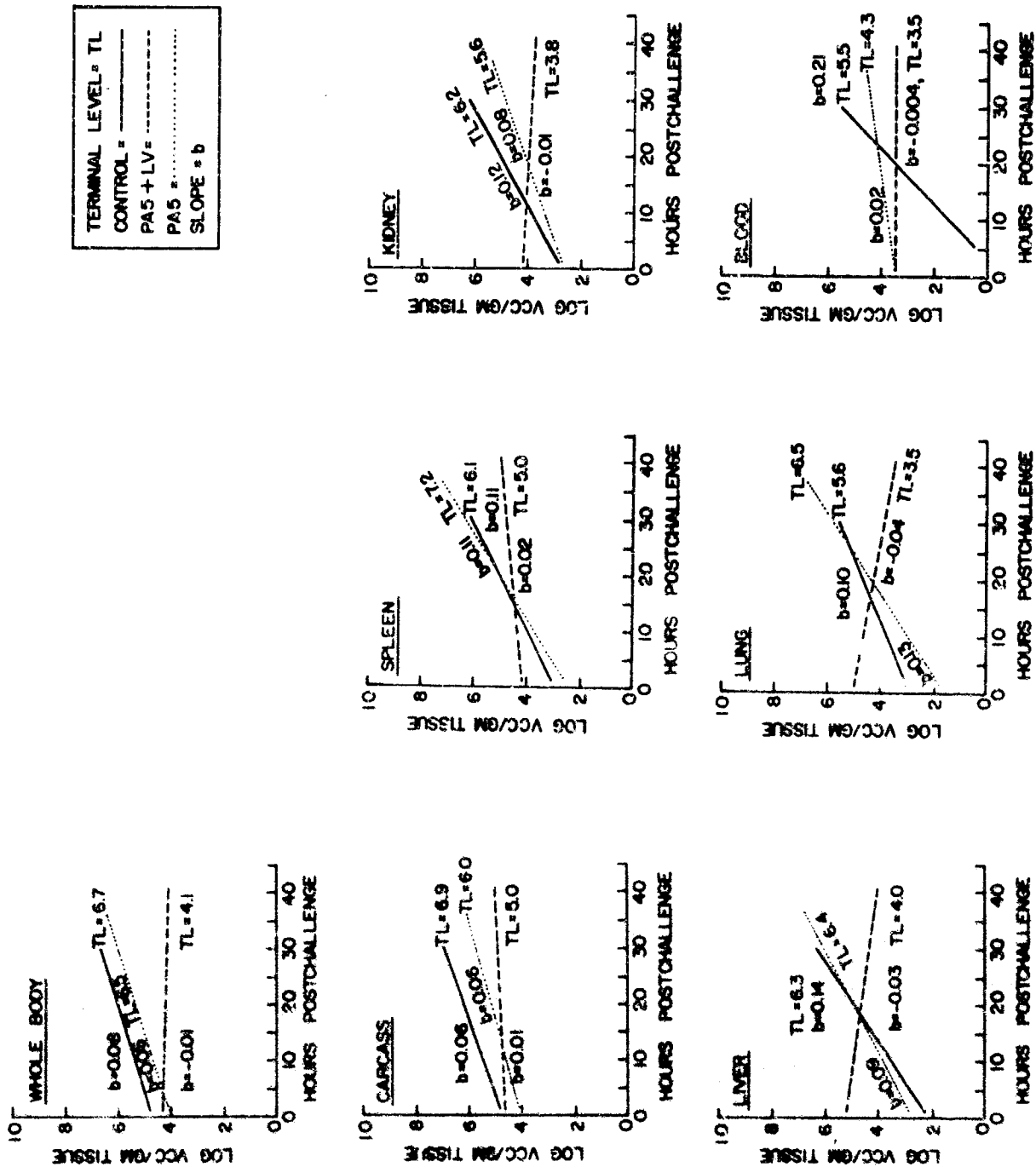


Figure 4. In Vivo Growth of *B. anthracis* in the Immunized NIH Rat.

C. MORPHOLOGICAL STUDIES

The gross morphology of in vivo bacterial cells from the blood at death of the host was not detectably different in either the guinea pig or rat, and morphology was not affected by egg yolk treatment of the inoculum in either host or by immunisation (guinea pig only was tested) (Fig. 5). Bacilli were observed as single cells or chains of two cells. It was rare to observe a 3- to 4-cell chain in the rat, but such chains occurred with a frequency of about 10% in the guinea pig.

In the spleen, as in the blood, bacilli occurred typically as one or two cells. In the guinea pig, the size was the same as observed in the blood, but in the rat (Fig. 6) the cells were much smaller, and in addition only one-third stained normally in the spore challenge with egg yolk. This observation was interpreted as the initial stage of lysing.

At 3 hours postmortem, chain length increased so that some chains might extend through two or three microscopic fields and increased the size of individual bacterial cells as seen with the light microscope. Tissue homogenates of both the immunized and nonimmunized guinea pigs showed similar responses. Essentially the same pattern was observed in the rat except that increased lysing of the cells was observed.

These studies extend our experience with nonimmunized susceptible animals to resistant and immunized ones. In all cases, phagocytosis of anthrax bacilli by blood phagocytes in vivo is rarely observed. In extensive earlier work on septicemic anthrax, we rarely observed a phagocytized bacillus. This observation can now be extended to the blood of both immunized and resistant hosts.

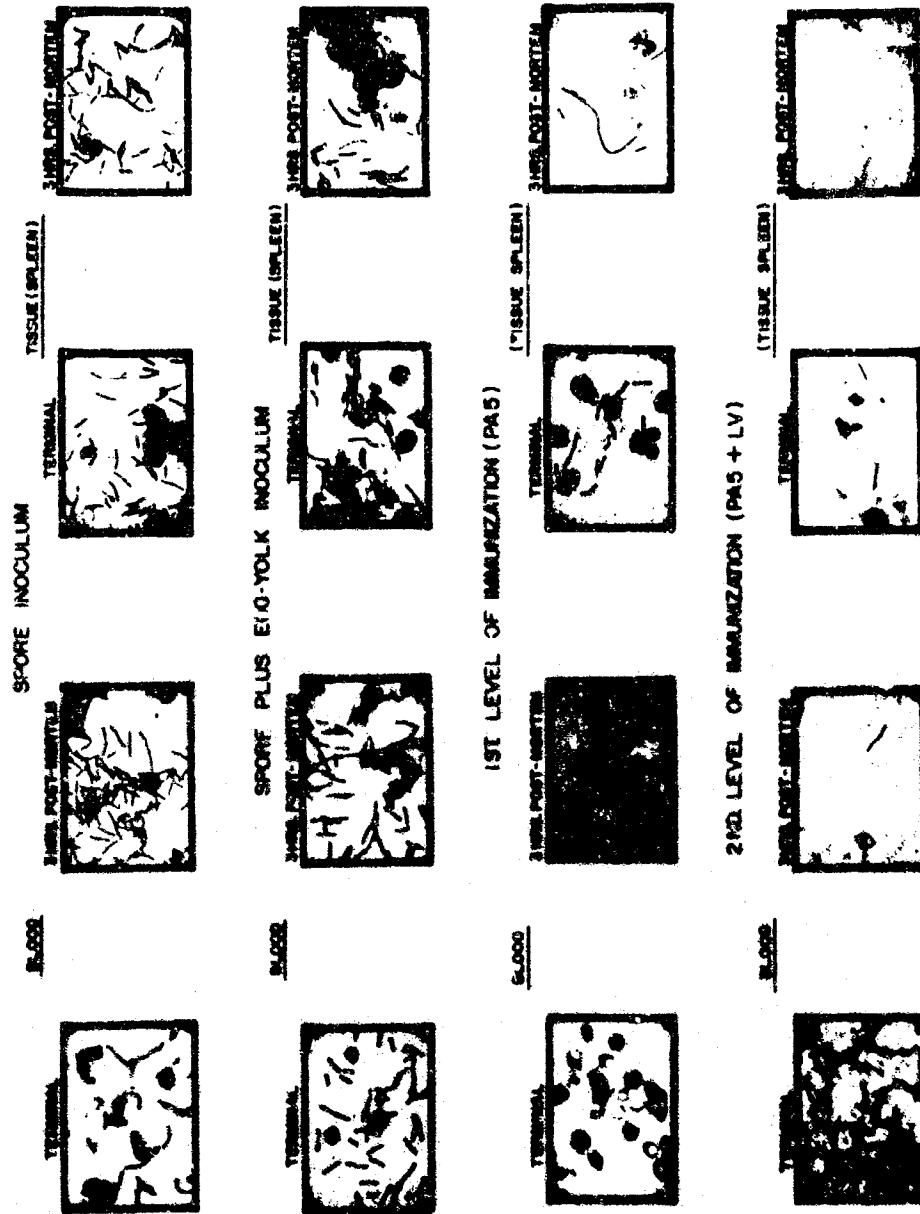
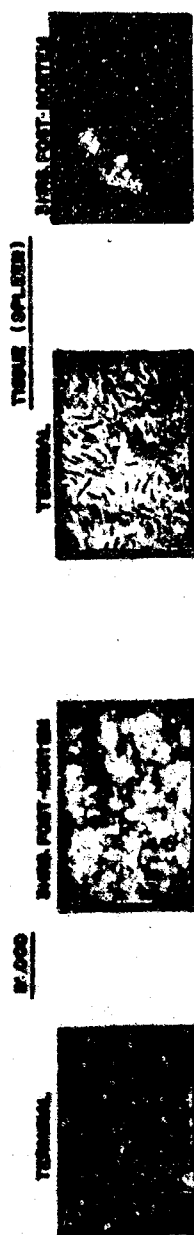


Figure 5. Morphology of Anthrax Bacilli in the Guinea Pig.

SPORE INOCULUM



SPORE PLUS EGG YOLK INOCULUM

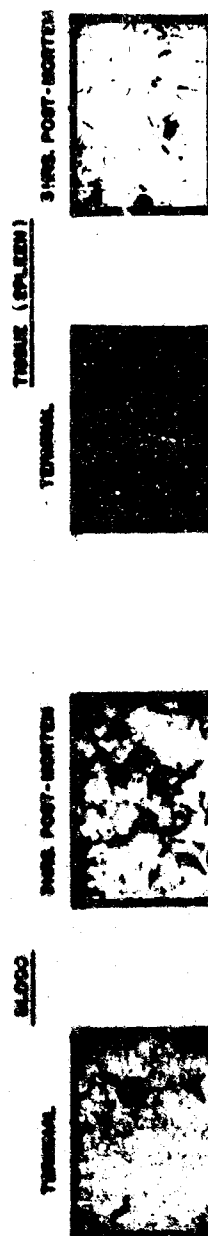


Figure 6. Morphology of Anthrax Bacilli in the MIN Rat.

IV. DISCUSSION

Challenge of the susceptible guinea pig and the resistant rat produced contrasting effects attributable to the degree of innate resistance. Contrary to popular belief, our observations showed that the more resistant the host, i.e., the immunized rat, the less the amount of initial destruction of disease organisms. It appears that degree of resistance should not be based on the amount of stimulation needed to activate the host's phagocytic defenses.

However, it should be re-emphasized that resistance to anthrax is of a dual nature with separate resistance to the establishment of infection and to the toxin.⁵ This was adequately demonstrated by data observed with the innately resistant rat subjected to the PA5 + LV immunization protocol. The rate of organism buildup was negative, i.e., the number of organisms destroyed at any given time was greater than the number being produced. As the organisms multiplied or were destroyed, toxin was released. Thus, even though the organism population in the body of the immunized host was decreasing, the amount of effective toxin was increasing. Finally, the animal succumbed from a toxemia accompanied by a reduced bacteremia.

Mahlandt et al.⁴ showed a definite relationship between the host resistance and the different components of the toxin (PA, LF and EF) after challenge with B. anthracis toxin and spores. Such a response to the antigenic activity in the production of antibodies and other substances was also evident by the first- and second-level immunizing protocols used here. Terminal populations of organisms were definitely lowered in the body of immunized hosts. This was reflected in the blood and tissues. The immune process of both test animals was highly effective in changing the infectious processes, as evidenced by the changes in rates of organism buildup.

The rat immunized with PA5 + LV had a negative slope for organism buildup. Both levels of immunization in the guinea pig provided slower rates of buildup and lower terminal levels. Thus, we conclude that immunization causes the host to respond to challenge in a way that produces a bactericidal effect. The amount of bactericidal activity appears to be dependent upon the degree of innate and induced immunity of the host.

Since the naturally resistant rat subjected to the PA5 + LV protocol possessed a terminal population less than the number of organisms utilized to produce infection, it was apparent that there was some minimum amount of toxin needed to cause death of the host and that this toxin must act on the body for some critical period of time. That a minimum amount of toxin is required to kill each species was recently established. Therefore, if a host is immunized, there is a decrease in buildup of organisms in the body that lowers the terminal concentration of organisms, and time to death is extended. It follows, then, that hosts that live the longest will have a lower amount of toxin and organisms in the body at death.

Thus, it is apparent that immunization per se was effective against the establishment of the disease and the rate of growth of organisms in the body.

The difference in gross morphology of the anthrax organisms growing in the live compared with the dead or postmortem animal was striking. Ward et al.¹¹ observed elongation of bacilli in the immune animal that was not observed in these studies. However, elongations were repeatedly noted in postmortem animals in which growth conditions were markedly changed. It was apparent that not only the physical state but the nutrient conditions existing in the host were changed. The effect on morphology of the organisms clearly paralleled that of in vitro growth and not in vivo growth as seen in the live animal. This observation points out the necessity when sampling septicemic growth, especially in treated or immune animals, of taking samples before and at death, not after death when new growth conditions exist. Such postmortem observations would be erroneous and give misleading results for numbers of anthrax organisms present when the animal succumbed to the infection.

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