Antibiotic Treatment of Anthrax Infection in Mice

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Quantitative measurements of mean time to death, percentage of survivors, and viable cell populations in the whole body were employed to determine the effects of penicillin, dihydrostreptomycin, chlorotetracycline, oxytetracycline, chloramphenicol, and antiserum on the course of anthrax infection in mice. By all parameters tested, penicillin and dihydrostreptomycin were most effective in the treatment of the disease. Therapy initiated in the later stages of the disease was more effective than that initiated in the earlier stages. Quantitative studies indicated that it was more difficult to eliminate organisms from the kidney than from any other organ or tissue. These measurements for the evaluation of antibiotic therapy are suggested for the study of other bacterial diseases.

The successful treatment of bacterial infections depends upon the elimination of the organisms from the various organs of the host and, in most cases, the neutralization of metabolic products of the organism. Investigations of anthrax populations in organs thus far have been limited to the last few hours before death (7). Other attempts to measure in vivo growth of Bacillus anthracis in the whole body have met with limited success (1, 2, 8, 9, 11, 13), mainly because the techniques did not include quantitation of bacterial populations in the whole body throughout the course of the disease. We have reported (8, 10, 12) on the quantitation of anthrax septicemia in various laboratory animals, and observed differences in growth rates and terminal organism populations.

This paper evaluates the effectiveness of antibiotic and antitoxin therapy by use of measurements of mean time to death (MTD) in anthrax infection and quantitation of bacterial populations in the whole body. The effect of antibiotics administered at various stages of the disease revealed vast differences in their effectiveness not otherwise demonstrable. These differences in effect of antibiotics, we believe, enable us to understand better the dynamics of the disease and its therapy.

MATERIALS AND METHODS

Animals. Inbred mice, 18 to 20 g, of the BALB strain were used.

Challenge organism. Washed spores of the highly virulent Vollum (V1b) strain of B. anthracis were used for MTD studies. In the in vivo growth studies, spores were germinated for 30 min in a medium composed of 0.00375% L-alanine, 0.00375% L-tyrosine, 0.001875% adenosine, 0.07% Beef Extract (Difco) and 0.15% peptone (Difco), at a pH of 6.8 to 7.2. The stage of germination was determined as follows: (i) Under dark field microscopy, loss of spore refractivity was observed. (ii) Under light microscopy, resistance to spore stains and susceptibility to Gram stain was observed. There was no evidence of outgrowth of a vegetative cell.

Differential plate counts before and after the addition of sufficient phenol to obtain a final concentration of 1% (4) revealed 99.9% germination. Preliminary laboratory studies of the effect of tissue homogenates on germinated spores indicated the necessity of adding Beef Extract and peptone to effect 100% recovery of viable organisms. A dose of 10⁸ organisms was inoculated by the intraperitoneal route.

Treatment. The single-dose therapy and initial treatment, when maintenance doses were to be given, consisted of 1,000 units of penicillin, 0.4 mg of dihydrostreptomycin, 0.4 mg of chlorotetracycline, 0.4 mg of oxytetracycline, 0.4 mg of chloramphenicol, and 0.3 ml of a 1:10 dilution of anthrax antiserum prepared in horses against the Sterne strain of B. anthracis (Instituto Seroterapico Tucano, Sienna, Italy). Each milliliter of antiserum neutralized approximately 7,000 rat units of in vitro toxin (6). Maintenance therapy was administered by giving 200 units of penicillin, 0.2 mg of dihydrostreptomycin, 0.2 mg of chlorotetracycline, 0.2 mg of oxytetracycline, and 0.2 mg of chloramphenicol 12 hr after exposure to spores, and was repeated every 12 hr through 5 days. All therapy was administered intramuscularly. Animals were observed for 10 days after the last administration of antibiotics. Animals alive at that time were termed survivors.

In vitro assay of antibiotics. Three dilutions of B. anthracis spores (10⁶, 10⁵, and 10⁴) were spread on tryptose agar plates. Several levels of antibiotics,
Sensi-Discs (BBL), were placed on the inoculated agar plates. Plates were then incubated for 18 hr at 37°C. The zone of inhibition was measured as an indicator of degree of sensitivity to a given unit of antibiotic.

Preparation of tissues for in vivo growth quantitation. At each sampling period (hourly intervals unless otherwise indicated), five animals were sacrificed with CO₂ and then skinned. A longitudinal incision was made on the ventral surface of each animal, exposing the mediastinal, pleural, and abdominal cavities. Blood samples then were taken by direct cardiac puncture. The gastrointestinal tract was carefully tied off, removed, and discarded. The spleens, livers, lungs, and kidneys of the animals for each time period were excised, washed with gelatin phosphate diluent [0.5% Gelatin (Difco), 0.4% Na₂HPO₄, pH 6.8 to 7.1], weighed, and homogenized with a Tri-R stirrer (Tri-R Instruments, Jamaica, N.Y.). The carcasses less viscera were weighed, sectioned, and blended in the Omni-mixer (Ivan Sorvall, Inc., Norwalk, Conn.) by use of a gelatin phosphate diluent wash. The organ slurries were made up quantitatively to 15 ml, and carcass slurries, to 100 ml with sterile gelatin phosphate diluent.

In vivo assay technique. Several dilutions of each of the slurries were made with gelatin phosphate diluent. Samples containing 0.1 and 0.3 ml of the diluted inoculum were spread on tryptose agar plates containing 0.005% potassium tellurite, which in separate unreported tests controlled enteric contamination without affecting the growth of B. anthracis.

Analysis of data. Viable-cell count per gram of tissue was plotted graphically as a function of time after initiation of infection. Times and logarithms of viable-cell counts per gram of tissue were employed as variables to develop linear regression with a standard computer program. The linear equations obtained were then transformed in the exponential equation of best fit:

\[ N = K e^{b t} \]  (1)

where \( N \) is the number of bacteria per gram of tissue, \( K \) is a constant, and \( e^{b t} \) 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. In the treated animals, linear regressions were developed and subsequent transformations made. Changes in slope from negative to positive occurred and were plotted accordingly.

Clearance of B. anthracis in the peritoneal cavity. To estimate the number of organisms remaining in the peritoneal cavity during the early stage of infection, animals were sacrificed every 20 min through the first 2 hr, then at 4 and 6 hr. After death, 5 ml of sterile distilled water was injected into the peritoneum and 1 ml of the peritoneal wash were withdrawn and assayed by the plate count method previously described.

Results

In vitro bacterial sensitivity to antibiotics. Sensitivity tests evaluating dihydrostreptomycin, chloramphenicol, chlortetracycline, and penicillin revealed that chlortetracycline appeared the most effective agent in vitro against all levels of B. anthracis organisms (Table 1). Penicillin appeared least effective in the range tested. Dihydrostreptomycin, although effective against all bacterial levels tested, did not approximate the effectiveness shown with chlortetracycline.

Effect of antibiotics against B. anthracis grown in vivo. Examination of Fig. 1 reveals that there were differences in effectiveness not only among the various antibiotics, but also in effect due to time of antibiotic administration. In single-dose therapy, administration of 1,000 units of penicillin at 4 hr postchallenge resulted in a threefold extension of MTD with 38% survival. Treatment with chlortetracycline, dihydrostreptomycin, and oxytetracycline resulted in approximately 13% survival. The extension of the MTD was 2.5-fold for the chlortetracycline group, 1.5-fold for the dihydrostreptomycin-treated animals, and 1.0-fold for the oxytetracycline group. These differences in MTD were significant at the 0.01% level. Chloramphenicol had no effect on course of anthrax in the mouse when treatment was initiated 4 hr postchallenge.

When single dose therapy was delayed until 8 hr postchallenge, the percentage of survival was increased in all groups of animals except those treated with penicillin, which remained unchanged. Animals treated with oxytetracycline showed 20% survival with an increased MTD of 72 hr for those animals that died from anthrax. Chlortetracycline gave 38% survival, but those dying had a shorter MTD (62 hr) than was experienced when treatment was initiated 4 hr earlier. The dihydrostreptomycin-treated animals had 25% survivors with a MTD of 68 hr for those dying. MTD was only 3 hr greater than with the control group when treatment with chloramphenicol was delayed until 8 hr postchallenge. However, there was 12% survival.

The most effective antibiotics based on the above findings were then studied with stress placed on continuous therapy and time of initial treatment after onset of infection. When animals were initially treated with 1,000 units of penicillin, and subsequently every 12 hr with 200 units of penicillin, or with 0.4 mg of dihydrostreptomycin followed by 0.2 mg of dihydrostreptomycin every 12 hr, 100% of the animals survived regardless of whether therapy was initiated at 4, 8, 12, or 16 hr postchallenge (Fig. 2). Maintenance chlortetracycline treatment resulted in 75% survival when initiated at 4 or 8 hr postchallenge, and 50% when delayed to 12 hr postchallenge. The MTD of animals dying during maintenance therapy was approximately 120 hr. When treatment with any of these anti-
Table 1. In vitro sensitivity of Bacillus anthracis to antibiotics

<table>
<thead>
<tr>
<th>Antibiotic concn</th>
<th>Zone of inhibition (mm) when plate was seeded with indicated no. of organisms</th>
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<tr>
<td></td>
<td>2 X 10^4</td>
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<tr>
<td>Dihydrostreptomycin</td>
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<td></td>
<td>10 µg</td>
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<td>50 µg</td>
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<td></td>
<td>100 µg</td>
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<tr>
<td>Chloramphenicol</td>
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<td>30 µg</td>
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<td>Chlorotetracycline</td>
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<td>30 µg</td>
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<td>Penicillin</td>
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Biotics was delayed to 16 hr postchallenge, none of the animals died during the observation period.

Growth in vivo as affected by antibiotics and antiserum. An intraperitoneal injection of mice with 10^6 germinated spores produced an exponential increase in number of organisms per gram of body tissue that resulted in death at approximately 22 hr (Fig. 3). Organisms were recovered from all organs and tissues 20 min postchallenge. At that time, the spleen yielded the highest numbers of organisms per gram of tissue. Examination of regression curves in the whole body after a single dose of antibiotics or antiserum administered at 8 hr revealed that: (i) during the first 16 hr after treatment with 1,000 units of crystalline penicillin, there was a 3 log reduction of organisms in the host with death occurring some 78 hr postchallenge; (ii) during the first 4 hr after treatment with 0.4 mg of chlorotetracycline, there was a reduction of 2.5 logs in the number of organisms per gram of tissue, and this was followed by a 1-log increase in bacterial concentration over the next 12 hr, with death occurring at approximately 87 hr postchallenge; (iii) during the first 8 hr after treatment with 0.4 mg of oxytetracycline, there was a 2 log reduction of organisms in the host, and this was followed by a 1-log increase in organisms during the next 8 hr, with death at 58 hr postchallenge; and (iv) the administration of chloramphenicol and antiserum had little or no effect on the organism in the entire host.

Further examination of the growth curves of the organisms in the organs after the administra-

Fig. 1. Mean time to death and percentage of survivors after the administration of a single dose of antibiotics. Graphs were calculated by use of a minimum of 20 to 30 animals per treatment. Time refers to number of hours treatment was initiated postchallenge.

Fig. 2. Mean time to death and percentage of survivors when antibiotics were administered on a 12-hr maintenance protocol. Graphs were calculated by use of a minimum of 20 to 30 animals per treatment. Time refers to number of hours treatment was initiated postchallenge.
tion of crystalline penicillin showed that penicillin eliminated the organisms in the spleen, liver, blood, and lungs, but the carcass and kidney still had a substantial level of organisms at the end of the sampling periods. After administration of chlorotetracycline, the growth curve for the in vivo bacilli revealed that all tissues contained residual organisms at 16 hr after treatment. The growth curves for bacilli in animals treated with oxytetracycline showed that, during the 16 hr after treatment, all tissues with the exception of the carcass contained a substantial number of organisms. Penicillin was the most effective antibiotic to reduce organism numbers; it was followed by chlorotetracycline in effectiveness.

**Peritoneal cavity clearance.** Periodic examination of the peritoneal cavity for the presence of challenge organisms revealed that more than 50% of the organisms had been removed or destroyed in situ within 40 min postchallenge (Fig. 4). This reduction was a continuing process, so that at 6 hr only 0.7% of the challenge dose was found in the peritoneum.

**DISCUSSION**

Our findings showing in vitro sensitivity of *B. anthracis* to chloramphenicol and its resistance in vivo were not unusual, but, in view of the specific recommendation of chloramphenicol for treatment of external "cutaneous" anthrax (5), our results are somewhat disturbing. Only when in vitro studies of bacterial sensitivity to antibiotics are followed by in vivo tests can therapy be determined with confidence; however, chloramphenicol may have an effect in man that was not detected by the mouse test.

The in vitro sensitivity tests demonstrate that MTD was of value in determining drug of choice and therapy protocol as they correlate with MTD, percentage of survivors, and levels of organisms. When a single injection was given, chlorotetracycline extended MTD more when administered...
at 4 hr than at 8 hr postchallenge, in contrast to penicillin, dihydrostreptomycin, and oxytetracycline. When antibiotics were administered under a 12-hr maintenance protocol, organisms appeared more susceptible to chlortetracycline than when therapy was initiated 16 hr after challenge. Therapy initiated 12 hr was least effective. Dineen (3) reported a period in the course of a staphylococcal infection when the bacteria were unusually susceptible to an antimicrobial agent. He reasoned that this phenomenon was due to two distinct factors: the organism itself (its stages from portal or entry to target organ) along with secondary host responses, and the action of the drug itself. We did not find any difference in response when continuous therapy was initiated at 4, 8, 12, and 16 hr postchallenge with penicillin or dihydrostreptomycin. Both drugs resulted in complete survival during the 10-day observation period after cessation of drug therapy. The maintenance of an effective antibiotic level in the blood for a long enough time to allow the host to eliminate the pathogen, undoubtedly was the reason repeated treatment was more effective than single-dose therapy. When the bacilli were not completely eliminated, a second bloom of organisms occurred, followed by death.

In the course of our whole body studies, we were able to detect organisms in all tissues 20 min after challenge. Bonventre et al. (2), by an autoradiographic technique, observed the presence of organisms in the organs of the reticuloendothelial system 30 min after challenge. In contrast, Krazhnikov and Izrael (11), using a comparable dose, were not able to detect the presence of Bacillus anthracis in the various organs of the nonirradiated host until 36 to 48 hr after the onset of infection. We believe that the large discrepancy in the time required for dissemination of bacilli into the various organs of the host may result from differences in any or all of several factors: (i) virulence of organism, (ii) species or strain of host, (iii) route of challenge, (iv) dose, and (v) sensitivity of the assay.

The kidney is well known in medical literature for harboring chronic infections that are extremely difficult to treat, and, with anthrax, the kidney still harbors organisms after a single injection of either the effective or noneffective antibiotics. In the remaining organs, some of the less effective antibiotics eliminated the organisms more readily. We suggest that this organ should always be assayed in cases of death when antibiotics have been used and when anthrax is suspected, or when making a selection of antibiotics to be used in the treatment of anthrax and other diseases.

A 1.5 log higher level of organisms was obtained in the blood terminally after treatment with antiserum as compared to the untreated control hosts without a correspondingly higher level of organisms in the organs. Since antiserum would neutralize toxin in the blood and prevent attachment to sensitive sites, this observation suggests that the toxin found in the blood at death was produced by the organisms in the blood, and that the organisms in the organs and fixed sites have a relatively delayed effect on the host.

**Acknowledgments**

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**Literature Cited**