Experimental Osteomyelitis.

II. Therapeutic Trials and Measurement of Antibiotic Levels in Bone

Carl W. Norden, with the technical assistance of Elizabeth Kennedy

This study measured levels of lincomycin and cephalothin in normal and osteomyelitic bone of rabbits and compared the effect of each agent in the treatment of osteomyelitis. Proportionately more lincomycin than cephalothin was found in bone in relation to levels in serum. Levels of both antibiotics were frequently detected in higher concentrations in osteomyelitic than in normal bones. Treatment with either cephalothin or lincomycin significantly reduced the severity of bone disease; therapy begun one day after infection was more effective in this regard than therapy begun 14 days after infection. Lincomycin-treated rabbits had significantly less severe bone disease than did rabbits receiving cephalothin. Cephalothin administered for 28 days was more effective in sterilizing bone than treatment for only 14 days; lincomycin was equally effective whether given for 14 or 28 days. The data suggest that there is not a simple relationship between the level of an antibiotic in bone and its likelihood of sterilizing this tissue.

New antimicrobial agents have frequently been hailed as panaceas for chronic osteomyelitis, only to fail the test of time. The large number of uncontrolled variables seen in the human disease, i.e., age, bones involved, presence of sequestra, infecting organism, and previous therapy, makes the performance and evaluation of clinical trials difficult [1]. An experimental model of osteomyelitis in rabbits has been described [2], the pathologic and radiologic appearance of which is sufficiently similar to that of the human disease for it to be used to test the efficacy of different antimicrobial agents.

The present study was concerned with establishment of reproducible methods of detection of two antibiotics, lincomycin and cephalothin, in normal and osteomyelitic bone of rabbits. Therapeutic trials were then performed comparing the effect of immediate versus delayed institution of therapy, the effect of 14 or 28 days of treatment, and the efficacy of two different classes of antibiotics. Correlations were made of the measurements of each drug in bone and its ability to sterilize that tissue.

Materials and Methods

Measurement of antibiotics in serum and bone. The cylinder-plate method was used for all assays of antibiotics [3].

Lincomycin assay. A standard curve was constructed from the size of the zones of inhibition of reference standards tested with Sarcina lutea (ATTC 9341); zones produced by the sera being tested were compared with this curve. All sera and standards were diluted in normal lapine serum. The minimal quantity of lincomycin detectable was 0.4 μg/ml of serum.

Cephalothin assay. A standard curve was prepared using Sarcina lutea (ATTC 9341) and reference standards diluted in 50% normal lapine serum and 50% 0.1 M phosphate buffer, pH 6.0. The minimal quantity of cephalothin detectable was 0.2 μg/ml of serum.
Preparation of bone. The tibia was dissected free of soft tissue, split lengthwise, and scraped free of marrow. After drying overnight at 4 °C, any remaining marrow was removed. Using a recoil action mortar and pestle (Thermovac Industries, Copiaque, N.Y.) suspended in a dry ice-acetone bath, the frozen bone was crushed to a fine powder. The powder was weighed, suspended in 2 ml of 0.1 M phosphate buffer, pH 7.8, for lincomycin or 0.1 M phosphate buffer, pH 6.0, for cephalothin, agitated for 4 hr using a magnetic stirring bar at 4 °C, and the supernatant fluid was assayed. Longer periods of agitation did not increase the detected levels of antibiotics. Reference standards for each antibiotic gave equal zones of inhibition when suspended in either buffer or buffer plus bone powder from untreated rabbits. A standard curve relating known concentration of antibiotic to the size of the zone of inhibition was constructed. The minimal quantities of lincomycin and cephalothin detectable were 0.2 and 0.1 μg/ml of bone suspension, respectively; the amount of antibiotic was expressed in μg/g of bone. Assays performed in duplicate gave values within 0.2 μg/g of each other.

Production of osteomyelitis. The technique of inducing osteomyelitis in rabbits has been previously described [2]. In brief, 4-lb rabbits received an intramedullary injection of sodium morrhuate and 3 × 10^6 cfu of Staphylococcus aureus [2]. The minimal inhibitory concentrations of lincomycin and cephalothin for this organism were 0.4 and 0.45 μg/ml, respectively.

Conduct of therapeutic trials. All injections of antibiotic were given subcutaneously into the scruff of the neck. Rabbits received either lincomycin, 10 mg/kg at 8 AM and 4 PM or cephalothin, 50 mg/kg at 8 AM, 11 AM, 2 PM, and 5 PM. Therapy was instituted at one or 14 days after infection. Since radiologic changes of chronic osteomyelitis were present at day 14, treatment at this time was considered to represent therapy of chronic osteomyelitis.

Antibiotics were given for either 14 or 28 days. Lincomycin, when given on day one and continued for 14 days was so effective that a trial of 28 days with this agent was not performed. More rabbits received cephalothin at day one for 14 days than any other regimen since, after sacrificing the first 14 animals, we wished to see if the results were constant with a second group of animals treated in like manner.

All rabbits were killed 60 days after infection. Cultures of bone were obtained by techniques described previously [2]. Recovery of any number of cfu of S. aureus was considered to represent a positive bone culture.

Measurement of anti-alpha-hemolysin titer and whole bacterial agglutinating antibodies. These techniques were described previously [2].

Gross evaluation of severity of disease. After the diseased tibia was stripped of all soft tissue, it was coded and examined independently by two observers. The gross severity was graded from 0 to 4, and the two readings were averaged.

Radiologic evaluation of severity of disease. X-rays of each tibia were coded, read independently by two observers with a scale from 0 to 3, and the two readings were averaged.

Statistical evaluation. Differences between means were compared with student's t test; either the chi-square test or Fisher's exact test was used for comparison of differences in proportions.

Results

Measurement of cephalothin and lincomycin in serum, normal bone, and bone marrow of uninfected rabbits. Figure 1 shows mean levels of cephalothin in serum, bone marrow, and normal bone in healthy rabbits who received 100 mg/kg of cephalothin subcutaneously. The amounts of antibiotic detected in bone and bone marrow were 2% and 13% of that in serum. No cephalothin was detected in bone 120 min after injection.

Comparable studies are also shown in the figure for healthy rabbits who received 10 mg of lincomycin per kg of body weight subcutaneously. The amounts of lincomycin detected in bone and bone marrow were 18% and 37% of that in serum. No lincomycin was detected in bone 120 min after injection.

Measurement of cephalothin and lincomycin in osteomyelitic bone and sequestra. Two rabbits with chronic osteomyelitis (infected 60 days previously) received cephalothin, 100 mg/kg, 30 min before death. Levels in serum were 58 and 54 μg/ml, levels in the uninfected tibia were 1.3 and 1.2 μg/g, and the levels in the diseased tibia were 4.2 and 3.0 μg/g, respectively.
Osteomyelitic bone

Since suspensions of osteomyelitic bone powder were considerably more red than comparable suspensions from normal bone, the content of hemoglobin in such suspensions was assayed spectrophotometrically. The suspensions of normal bone contained 0.7 and 0.8 mg of hemoglobin per gram of bone, while the diseased bone suspensions contained 2.0 and 1.9 mg of hemoglobin per gram of bone. In these same two animals, levels of lincomycin in both normal bones were 0.9 µg/g, while in diseased bone they were 1.9 and 2.3 µg/g, respectively. Calculations\(^1\) revealed that only 0.07 µg of lincomycin/g could be accounted for by retained blood in diseased bone, suggesting that the increased hemoglobin content was not a sufficient explanation for the difference in levels of antibiotics in normal and diseased bones.

Results of antibiotic therapy. Table 2 shows several parameters which were followed and used to evaluate the results of therapy with each antibiotic. Comparisons were made between untreated rabbits and groups of rabbits receiving each antibiotic. Calculations\(^1\) revealed that only 0.07 µg of lincomycin/g could be accounted for by retained blood in diseased bone, suggesting that the increased hemoglobin content was not a sufficient explanation for the difference in levels of antibiotics in normal and diseased bones.

Table 1. Measurement of lincomycin in serum and bone of infected rabbits.

<table>
<thead>
<tr>
<th>Time after injection (min)</th>
<th>Amount of lincomycin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>30</td>
<td>5.4</td>
</tr>
<tr>
<td>30</td>
<td>6.5</td>
</tr>
<tr>
<td>30</td>
<td>6.5</td>
</tr>
<tr>
<td>30</td>
<td>5.0</td>
</tr>
<tr>
<td>30</td>
<td>6.0</td>
</tr>
<tr>
<td>60</td>
<td>3.2</td>
</tr>
<tr>
<td>60</td>
<td>4.5</td>
</tr>
<tr>
<td>120</td>
<td>4.5</td>
</tr>
<tr>
<td>120</td>
<td>2.5</td>
</tr>
<tr>
<td>120</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\(^1\) Blood of rabbits contains 150 mg of hemoglobin/ml of blood. The diseased bone contains 2 mg of hemoglobin/g of bone, corresponding to the amount of hemoglobin present in \(\frac{1}{10}\) ml of blood. If the concentration of lincomycin in blood were 5 µg/ml, then this amount of retained blood could contribute only 0.07 µg of lincomycin/g of bone (\(\frac{1}{10} \times 5 \text{ µg} = 0.07 \text{ µg}\)). These calculations do not exclude the possibility that a larger than usual amount of antibiotic-containing plasma might be present in the diseased bone, although this seems unlikely.
Table 2. Results of therapeutic trials of cephalothin and lincomycin for treatment of osteomyelitis in rabbits.

<table>
<thead>
<tr>
<th>Factor</th>
<th>No therapy</th>
<th>Therapy with cephalothin</th>
<th>Therapy with lincomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals†</td>
<td>26</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>41</td>
<td>15</td>
<td>26</td>
</tr>
<tr>
<td>Weight change from day 0 to day 17 (oz)</td>
<td>-12.6 ± 6.6†</td>
<td>-5.3 ± 5.1</td>
<td>-4.6 ± 5.3</td>
</tr>
<tr>
<td>Weight change from day 0 to day 60 (oz)</td>
<td>+5.9 ± 13.5</td>
<td>+24.4 ± 15.3</td>
<td>+33.5 ± 11.0</td>
</tr>
<tr>
<td>Leukocyte count (day 60)</td>
<td>18,431 ± 8,500</td>
<td>11,355 ± 5,454</td>
<td>10,785 ± 2,900</td>
</tr>
<tr>
<td>% with rise in anti-alpha-hemolysin titer</td>
<td>96</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>% with fourfold rise in bacterial agglutinating titer</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Severity of disease, gross evaluation§</td>
<td>3.4 ± 1.1</td>
<td>2.0 ± 1.4</td>
<td>1.3 ± 1.0</td>
</tr>
<tr>
<td>Severity of disease, radiologic evaluation</td>
<td></td>
<td></td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>% with sequestra</td>
<td>73</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>% with positive bone cultures</td>
<td>96</td>
<td>68</td>
<td>29</td>
</tr>
</tbody>
</table>

* Day therapy was started/duration of therapy (days).
† Surviving until day 60.
‡ Mean ± S.D.
§ Scale of 0–4.
|| Scale of 0–3.
Experimental Osteomyelitis II

Table 3. Summary of significant differences between therapeutic groups.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Cephalothin Day 1 vs. untreated</th>
<th>Cephalothin Day 14 vs. untreated</th>
<th>Lincomycin Day 1 vs. lincomycin Day 1</th>
<th>Lincomycin Day 14 vs. lincomycin Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>+*</td>
<td>NS†</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>Weight change from day 0 to day 17 (oz)</td>
<td>+</td>
<td>NS</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>Weight change from day 0 to day 60 (oz)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leukocyte count day 60</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Percent with rise in antialpha-hemolysin titer to $\geq 1:4$</td>
<td>+</td>
<td>NS</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>Severity of disease, gross evaluation</td>
<td>+</td>
<td>NS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Severity of disease, radiologic evaluation</td>
<td>+</td>
<td>NS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Percent with sequestra</td>
<td>+</td>
<td>NS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Percent with positive bone cultures</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* + = $P < .05$.
† NS = no significant difference.

Biotic one or 14 days after infection; the differences between groups are summarized in table 3.

Cephalothin. Rabbits receiving cephalothin one day after infection showed significant differences in all parameters, when compared with untreated rabbits (table 3). In contrast, rabbits receiving cephalothin 14 days after infection showed significant differences from untreated rabbits only in weight gained, count of leukocytes, and sterility of bone cultures (table 3).

Rabbits given cephalothin from day one for a 14-day course had significantly more positive bone cultures than rabbits receiving cephalothin at day one for 28 days ($P < .025$) (table 2). Similarly, rabbits receiving cephalothin at day 14 for 14 days had significantly more positive bone cultures than rabbits treated at the same time, but for 28 days ($P = .04$, Fisher’s exact test) (table 2). Thus, whether therapy with cephalothin was initiated one or 14 days after infection, significantly more bones were sterilized after 28 days of therapy than after 14 ($P < .001$).

Lincomycin. Rabbits receiving lincomycin one day after infection showed significant differences in all parameters when compared with untreated rabbits (table 3). Rabbits receiving lincomycin 14 days after infection showed increased weight-gain, lower leukocyte counts, decreased gross and radiologic severity of disease, fewer sequestra, and significantly more sterile bone cultures than did untreated rabbits (table 3).

Unlike cephalothin, lincomycin administered at day 14 and continued for 28 days did not significantly increase the number of sterile bone cultures when compared with 14 days of lincomycin treatment initiated at the same time after infection (table 2).

Comparison between cephalothin and lincomycin. Rabbits receiving lincomycin often developed severe diarrhea, and those receiving lincomycin at day 14 were significantly lighter when killed than those receiving cephalothin 14 days after infection (table 3).

Rabbits receiving lincomycin either one or 14 days after infection showed significantly less severe bone disease, as measured grossly and by x-ray, than did rabbits receiving cephalothin at comparable intervals after infection. Fewer sequestra were observed in rabbits receiving lincomycin 14 days after infection than in those receiving cephalothin at a comparable time (table 3).

The only comparably treated groups that showed significantly more sterile bones were those rabbits treated with lincomycin at day one for 14 days; 27% of rabbits in this group and 68% of rabbits receiving cephalothin at day one for
14 days had positive bone cultures \((P = .03, \text{ Fisher’s exact test})\). When cephalothin was administered at day one for 28 days, the frequency of positive cultures was reduced to 29\% (table 2).

**Discussion**

This study has shown that levels of lincomycin and cephalothin can be measured accurately in bones from normal rabbits, that antibiotic levels in bone are generally lower than in serum, and that proportionately more lincomycin than cephalothin is found in bone in relation to levels in serum. Levels of both antibiotics detected in osteomyelitic bones were frequently higher than those in normal bone. The therapeutic trials have demonstrated the following. (1) Treatment with either cephalothin or lincomycin one day after infection produced lower mortality rates, more rapid gain of weight, lower leukocyte counts, less severe bone disease, and fewer infected bones than in untreated rabbits. (2) Lincomycin-treated rabbits had significantly less severe bone disease than did rabbits receiving cephalothin. (3) Cephalothin administered for 28 days was more effective in sterilizing bone than treatment for only 14 days; in contrast, lincomycin was equally effective when given for 14 or 28 days.

The present study attempted to resolve certain technical problems inherent in the assay of antibiotics in tissue. The possibility that antibiotics could bind to bone and not be extracted, thereby lowering the level of antibiotic detected, seems unlikely since 100\% of known amounts of lincomycin and cephalothin were recovered after incubation overnight at 4 C with suspensions of normal bone powder. A second concern was that the antibiotics detected as “bone levels” might reflect the presence of retained blood and marrow in the bone. Several observations suggest that this is not so. Cephalothin was detected in blood in concentrations 10–14 times higher than lincomycin; however, levels of cephalothin in normal lapine bone were proportionately lower than lincomycin in relation to levels in blood. Furthermore, measurement of hemoglobin levels in suspensions of diseased and normal bones and calculation of the amount of antibiotic detected which might be attributed to retained blood showed that this was an insignificant portion of the total antibiotic detected.

Certain problems were encountered in the design of these therapeutic trials. First, lincomycin was excreted slowly and levels in serum were detectable for 7 hr after a subcutaneous injection; in contrast, cephalothin was detectable in serum for only 2 hr after injection. Secondly, at a peak level of lincomycin in serum of 5.4 \(\mu g/mL\), levels in healthy bone would approximate 1 \(\mu g/g\), or about twice the MIC for the studied organism. On the other hand, at a peak level of cephalothin in serum of 20 \(\mu g/mL\), levels in healthy bone would be 0.4–0.6 \(\mu g/g\), which essentially approximates the MIC of the organism used in this study. Thirdly, the daily dose of cephalothin was 10-fold greater than that of lincomycin; this dose of cephalothin was chosen to be certain that cephalothin would be detectable in bone. Finally, no cultures for proplasts were performed; the possibility of inducing these forms is real, although the experience in searching for proplasts in clinical material from patients with osteomyelitis has been generally unrewarding [4].

Despite these objections, certain facts emerge clearly. Untreated rabbits had uniformly severe disease with positive bone cultures. Treatment with either antibiotic decreased the severity of the osteomyelitis and resulted in sterilization of bones in about half the animals treated. If formation of sequestrum occurred, the likelihood of sterilization of the diseased bone declined significantly. Despite some cures seen in the presence of sequestra in these experiments, these cannot be taken to imply a recommendation for treatment of chronic osteomyelitis with antibiotics alone. The role of surgery as an adjunct to antibiotic therapy remains controversial and requires further evaluation.

A major question arises, as a result of these experiments, as to the significance of “bone levels” of an antibiotic. Five to 10-fold more lincomycin than cephalothin is present in bone when compared with simultaneous levels in serum. Additionally, in the present experimental schedule of dosage, administration of cephalothin should theoretically have been less effective than that of lincomycin since there were longer periods with no drug in serum in animals receiving cephalothin. Despite these two observations that should establish the theoretical superiority of lincomycin, cephalothin was essentially equally effective in sterilizing bone when administered for
28 days. Both antibiotics were present in bone marrow in concentrations higher than that of bone tissue, and concentrations of both agents were often higher in diseased bone than in uninfected bone. These observations suggest possible explanations for the efficacy of both drugs in treating experimental staphylococcal osteomyelitis. Osteomyelitis is a disease that involves marrow cavity as well as cortical bone, and it may be that the levels of antibiotic attained in the marrow are a better indicator of the possible efficacy of therapy than the ability of the agent to penetrate into the bone itself. Secondly, it was observed in these experiments that cephalothin was more effective when administered for 28 days, but that lincomycin was equally effective when given for only 14 days. It is possible that, with less penetration of cephalothin into bone and with lower levels of cephalothin in bone relative to the MIC of the organism used in this study, more prolonged treatment with cephalothin, assuring longer periods of effective antibiotic concentrations in bone, accounted for the effectiveness of 28 days of therapy with cephalothin. In contrast, lincomycin, with its ability to achieve higher “bone levels,” did not appear to require as long a period of treatment to assure sterilization of bone.

No definite conclusions can be drawn as to the relation of an antibiotic’s ability to penetrate into bone and its likelihood of sterilizing osteomyelitic bone. However, these experiments would suggest that the ability of an antibiotic to penetrate into bone is but a single factor in a complex therapeutic equation involving also the severity of the disease and the presence of sequestra, the duration of antimicrobial therapy and the relative sensitivity of the microorganism. This experimental model affords opportunities to perform studies to delineate further those factors responsible for the success or failure of treatment of osteomyelitis.

References