ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2000, p. 139–142 Vol. 44, No. 1
0066-4804/00/$04.00+0

Effect of Zinc-Reversible Growth-Inhibitory Activity in Human Empyema Fluid on Antibiotic Microbicidal Activity

PETER G. SOHNLE* AND BETH L. HAHN

Division of Infectious Diseases, Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, and the Research Service, VA Medical Center, Milwaukee, Wisconsin 53295

Received 3 March 1999/Returned for modification 23 June 1999/Accepted 22 October 1999

Abscess fluid supernatants have zinc-reversible microbial growth-inhibitory activity that is mediated by calprotectin, a zinc-binding protein. Because it inhibits microbial growth, this activity might interfere with killing by antibiotics that require their target organisms to be proliferating. In the present study, we cultured bacteria in human empyema fluid and used zinc to overcome the growth-inhibitory effect of calprotectin. We then compared the effect of zinc on killing by the beta-lactams ampicillin and cefazolin with that of the fluoroquinolone trovafloxacin, since the latter may be better able to kill nonproliferating organisms. In empyema fluid diluted 1:5 in normal saline, addition of zinc (30 μM) increased growth of two strains of *Staphyloccocus aureus* and two strains of *Escherichia coli* but did not affect the MICs or MBCs of the three antibiotics in Mueller-Hinton broth. For one strain of *S. aureus*, no effect of zinc was found on killing by either ampicillin or cefazolin. However, with the other strain of *S. aureus* and both strains of *E. coli*, significant enhancement of killing by both drugs was observed with zinc addition. On the other hand, no effect on the killing of any of the organisms was observed for trovafloxacin when zinc was added. These results suggest that the zinc-reversible growth-inhibitory activity of abscess fluid may interfere with the microbicidal activity of antibiotics requiring proliferating target organisms, although antibiotics better able to kill nonproliferating organisms may be less affected by this phenomenon.

Microorganisms require metals, such as iron and zinc, for growth. Sequestration of these metal ions by host metal-binding proteins can be an effective means of antimicrobial defense. This mechanism has been described primarily as a system involving host iron-binding proteins, such as lactoferrin and transferrin (7, 25). However, a similar mechanism that is based on zinc rather than iron has recently been described (17). The substance responsible for this antimicrobial effect is the calcium- and zinc-binding protein complex called calprotectin; alternative names for this complex include the L1 protein, MRP 8 and MRP 14, calgranulin A and B, and the cystic fibrosis antigen (6). Calprotectin appears to originate in the cytoplasm of neutrophils and is then released at sites of infection as these cells die and lyse; this protein has microbistatic activity against a variety of bacterial and fungal microorganisms (13, 18, 21). Abscess fluid supernatants have been shown to contain large amounts of calprotectin and to possess similar antimicrobial activity (19). In fact, purification studies have demonstrated that calprotectin accounts for almost all of the antimicrobial activity in abscess fluid supernatants (15).

Several studies have shown that the growth-inhibitory effect of abscess fluid supernatants or purified calprotectin is completely reversible when micromolar quantities of zinc ions are added to the medium (13, 15, 19). Calprotectin has been shown to directly bind zinc (19), and other studies suggest that the protein is competing with microorganisms for this metal (5, 16, 20). The antimicrobial activity of this protein is primarily microbistatic, although it does have microbicidal activity under certain circumstances (12, 21).

Some antibiotics, particularly those of the beta-lactam class, are most active against rapidly growing organisms. Because of its microbistatic activity in abscess fluids, calprotectin could interfere with the microbicidal activity of these antibiotics when they are used to treat infections that are complicated by abscesses. Indeed, Bamberger et al. have demonstrated that abscess fluid supernatants interfere with the ability of cefazolin to kill *Staphylococcus aureus*, but if zinc is added to reverse the growth-inhibitory effects of calprotectin, then killing proceeds normally (3). Thus, calprotectin appears to compromise the microbicidal effect of beta-lactam antibiotics by suppressing growth of the target microorganisms.

In contrast to the beta-lactams, quinolone antibiotics have activity in both the logarithmic and stationary phases of microbial growth, at least against certain microorganisms (26). There is also some evidence to suggest that this class of antibiotics may be relatively more effective in killing microorganisms in abscesses (4). The present study was undertaken to confirm the effects of calprotectin on microbial killing by beta-lactam antibiotics and to determine whether or not the fluoroquinolone antibiotic trovafloxacin is similarly affected.

MATERIALS AND METHODS

Calprotectin-containing fluids. A specimen of human pleural empyema fluid obtained before the patient had been treated with antibiotics was used for these studies. The fluid was centrifuged at 1,500 × *g* for 40 min and then at 38,000 × *g* for 30 min before further use. Protein concentration was determined by a dye-binding assay (Bio-Rad Laboratories, Richmond, Calif.) to be 143 mg/ml for the undiluted fluid.

Antimicrobial agents. These studies used ampicillin and cefazolin from Sigma (St. Louis, Mo.) and trovafloxacin from Pfizer. The compounds were solubilized and diluted according to standard procedures. Fresh solutions were made for each experiment.

Microorganisms. We used four microbial strains for most of the studies. These included ATCC isolates 29213 and 25923 of *S. aureus* and ATCC isolate 25922 and a local clinical isolate (LCI) of *Escherichia coli*.

MICs and MBCs. We carried out microtiter plate MIC and minimal bactericidal concentration (MBC) determinations with a modification of standard methods (9). Doubling dilutions of the different antibiotics, from 0.01 to 32 μg/ml in 0.1-ml volumes of Mueller-Hinton broth, were tested with inocula of either 10^5 or 10^3 organisms per ml. Since we previously found the effect of calprotectin to

* Corresponding author. Mailing address: Research Service/151, V.A. Medical Center, Milwaukee, WI 53295. Phone: (414) 384-2000, ext. 2878. Fax: (414) 383-8010. E-mail: psohnle@mcw.edu.

TABLE 1. Effect of zinc on antibiotic MBCs for *E. coli* and *S. aureus* in Mueller-Hinton broth

Antibiotic and organism	MBCs (μg/ml) of[a]:	
	Antibiotic alone	Antibiotic plus 30 μM zinc
Ampicillin		
S. aureus 29213	0.25–1 (0.5)	0.25–0.5 (0.5)
S. aureus 25923	0.5–4 (0.5)	1–8 (4)
E. coli 25922	1–4 (4)	0.5–4 (4)
E. coli LCI	2–2 (2)	2–2 (2)
Cefazolin		
S. aureus 29213	0.5–1 (1)	1–4 (2)
S. aureus 25923	1–4 (2)	0.5–2 (2)
E. coli 25922	1–2 (2)	0.25–2 (2)
E. coli LCI	0.5–2 (1)	0.25–1 (1)
Trovafloxacin		
S. aureus 29213	0.03–0.06 (0.03)	0.03–0.06 (0.06)
S. aureus 25923	0.03–0.1 (0.03)	0.03–0.06 (0.06)
E. coli 25922	0.01–0.03 (0.01)	0.01–0.03 (0.03)
E. coli LCI	0.01–0.03 (0.01)	0.01–0.03 (0.03)

[a] Data represent range and median of MBC data from three experiments per point for each isolate in broth alone or in broth containing 30 μM $ZnSO_4$. Differences between control and zinc-containing samples were evaluated with mean values in the unpaired *t* test, and none were found to be significant. Similar results were obtained with MIC determinations (data not shown).

be inoculum dependent, the 10^3 inoculum size was used in the experiments with antibiotics and abscess fluids. After incubation at 37°C for 18 h, the lowest dilution of antibiotic that visibly inhibited growth was taken as the MIC. After this incubation period, the samples without growth were streaked onto Trypticase soy agar and incubated for another 18 to 24 h. The MBC was taken as the lowest drug concentration that reduced growth by ≥99.9%.

Microtiter plate antagonism assay. The organisms were inoculated into 0.1-ml volumes of human empyema fluid diluted 1:5 in normal saline; various concentrations of antibiotics were added, and the samples were incubated for 18 h at 37°C. No other nutrients were added; the empyema fluid itself served as the growth medium. The inoculum used was 10^3 CFU per sample. At the end of the incubation period, the contents of the wells (0.1 ml) were removed, streaked on Trypticase soy agar, and cultured for 18 to 24 h, and the number of resulting colonies were counted. In parallel samples, 30 μM $ZnSO_4$ was added to the initial cultures to reverse the growth-inhibiting effects of calprotectin. Comparisons were made between the samples containing zinc and those that did not. Several concentrations of each antibiotic were tested in doubling dilutions around the expected MBCs in preliminary experiments; two or three appropriate antibiotic concentrations were then used in the final experiments with the concentration yielding a number of colonies closest to that of the original inoculum, but numbering at least 10, which was then compared to its zinc-containing pair for the number of colonies remaining.

Evaluation of data. To determine statistical significance, MICs and MBCs for zinc-containing samples were compared to control samples with the unpaired *t* test. Results of the assays with empyema fluid were expressed as the number of colonies remaining at the appropriate antibiotic concentration for samples containing or not containing zinc. Results were compared in these pairs with the single sample *t* test. Significance of the determinations was taken at a *P* value of <0.05.

RESULTS

MIC and MBC data were obtained for each of the two isolates of both *S. aureus* and *E. coli*. The MBC data are given in Table 1 and were generally within 1 to 2 dilutions of the MICs (data not shown). MBCs were determined with a standard high inoculum (10^5 organisms) and a lower inoculum (10^3 organisms), with the latter usually yielding smaller MBCs. As discussed above, the 10^3 inoculum size was used in the experiments with antibiotics and abscess fluids because the effect of the latter is inoculum dependent. Because zinc was used in later experiments to stimulate microbial growth in the presence of empyema fluid, we determined the effect of this metal on the MICs or MBCs of the drugs in standard media. As is also shown in Table 1, none of the MBCs between control and zinc-containing samples were significantly different. The same was also found for the MICs (data not shown). Therefore, zinc alone did not appear to affect the MICs or MBCs of the antibiotics in standard media for any of the four organisms tested.

The effect of zinc on antibiotic microbicidal activity in abscess fluid was tested with organisms grown directly in human empyema fluid diluted 1:5 in saline (final total protein concentration, 28.6 mg/ml). Different antibiotic concentrations were tested, with results from the concentration yielding a number of CFU closest to the original inoculum after incubation being compared for samples with and without added zinc. In the absence of antibiotic, zinc produced increased growth of the organisms in empyema fluid, although the amount of growth stimulation varied with the bacterial isolates (Table 2). With one isolate of *S. aureus*, 29213, such increases were found for samples containing each of the three antibiotics, as shown in Table 2. However, for the second isolate of *S. aureus* and the two isolates of *E. coli*, the numbers of CFU remaining in the samples with either cefazolin or ampicillin were significantly decreased when zinc had been added to the samples, as is also shown in Table 2. Presumably, the increase in growth caused by zinc addition made the organisms more susceptible to the microbicidal activity of the two beta-lactam antibiotics. On the other hand, addition of zinc did not significantly decrease the number of organisms remaining in the trovafloxacin-con-

TABLE 2. Enhancement of antibiotic microbicidal activity associated with zinc stimulation of microbial growth in empyema fluid[a]

Organism and antibiotic	No. of expts.	Ratio of CFU zinc/CFU no zinc	*P* value for significant decrease[b]
S. aureus 29213			
None	8	448.00 ± 193.00	
Ampicillin	4	272.00 ± 146.00	
Cefazolin	4	173.00 ± 144.00	
Trovafloxacin	8	1,162.00 ± 1,275.00	
S. aureus 25923			
None	4	40.00 ± 11.00	
Ampicillin	3	0.00 ± 0.00	<0.001
Cefazolin	4	0.05 ± 0.05	<0.001
Trovafloxacin	4	12.00 ± 4.92	
E. coli 25922			
None	8	67.60 ± 34.50	
Ampicillin	3	0.12 ± 0.05	<0.001
Cefazolin	8	0.33 ± 0.20	<0.001
Trovafloxacin	6	13.90 ± 12.70	
E. coli LCI			
None	4	3.80 ± 1.58	
Ampicillin	3	0.10 ± 0.04	<0.001
Cefazolin	4	0.01 ± 0.01	<0.001
Trovafloxacin	4	0.96 ± 0.49	NS

[a] Organisms were grown directly in the empyema fluid supernatant, with addition of either antibiotics or zinc (30 μM $ZnSO_4$) or both. Antibiotic concentrations were chosen to yield the number of colonies closest to that of the original inoculum, but numbering at least 10, with the following concentrations (in micrograms per milliliter) tested. *S. aureus*: ampicillin, 0.03, 0.06, and 0.1; cefazolin, 0.03, 0.06, and 0.1; trovafloxacin, 0.01 and 0.03. *E. coli*: ampicillin, 8, 16, and 32; cefazolin, 16 and 32; trovafloxacin, 0.03, 0.05, and 0.1. Data for ratios of CFU with zinc versus CFU without zinc are given as means ± standard error; *P* values were obtained with the single sample *t* test for reductions in CFU in zinc-containing samples versus those in control samples.

[b] NS, not significant.

taining samples for any of the isolates tested, as is also shown in Table 2. There did not appear to be a clear relationship between the stimulation of growth and the resulting enhancement of antibiotic microbicidal activity; the isolate yielding the highest stimulation with zinc was the *S. aureus* isolate that did not show the increased killing effect when zinc was added.

DISCUSSION

In these studies, addition of zinc increased bacterial growth in human empyema fluid, an effect that has previously been related to overcoming the zinc-binding antimicrobial activity of calprotectin in such fluid (19, 20). On the other hand, in the present study addition of zinc did not affect the MICs or MBCs of the three antibiotics in Mueller-Hinton broth. For one strain of *S. aureus*, no reductions of CFU were observed for any of the antibiotics when zinc was added; however, with the other isolate of *S. aureus* and the two isolates of *E. coli*, addition of zinc significantly decreased the number of CFU present in the cefazolin- and ampicillin-containing samples. In contrast, no significant reductions in CFU of any of the organisms were observed for samples containing trovafloxacin when zinc was added. Therefore, under the conditions of these experiments, stimulation of bacterial growth by addition of zinc appeared to enhance the killing ability of the two beta-lactam antibiotics, but not that of the fluoroquinolone, trovafloxacin.

Antibiotics are known to work poorly in abscesses. Often this type of infection will persist for long periods unless the abscesses are drained (14). Neutrophils appear to localize less well to chronic abscesses than to acute ones (1). The presence of abscesses appears to inhibit the bactericidal activity of blood neutrophils, and the abscess fluid milieu is inhibitory to neutrophil function (2). In addition, it has been found that there is a poorly responsive subpopulation of neutrophils in abscess cell populations and that these cells contain higher numbers of abscess-derived bacteria (10). There are a variety of substances and conditions in abscess fluids that may interfere with the activity of various antibiotics, including beta-lactamases, DNA, and acidic pH (4). It is also likely that the nondividing state of the infecting microorganisms in abscesses may be at least partly at fault. This effect of the abscess fluid milieu may be the result of an active process involving sequestration of zinc by the neutrophil protein calprotectin. It is possible that other factors relevant to chronic infections may prevent the organisms from proliferating, but as demonstrated by Bamberger et al. (3) and confirmed by the present studies, addition of zinc to abscess fluid supernatants will stimulate bacterial growth in them.

Beta-lactam antibiotics are definitely better at killing proliferating organisms than nonproliferating ones. For example, although group A streptococci are known to be exquisitely sensitive to penicillin, this drug is sometimes relatively ineffective in treating infections caused by them (23). Stevens et al. have demonstrated better results with drugs like clindamycin and erythromycin that do not depend on the proliferation of the organisms for their microbicidal effects (22). In a follow-up study, this group demonstrated the loss of penicillin-binding proteins 1 and 4 in stationary-phase streptococci (24); this finding may explain why beta-lactam drugs require rapidly growing organisms for their microbicidal effects to be manifested. Microbistatic drugs, such as tetracycline, may show antagonistic effects in assays of the microbicidal activity of beta-lactam antibiotics. Tetracycline has been reported to interfere with the efficacy of penicillin therapy for bacterial meningitis (11). As discussed above, the fluoroquinolone class of antibiotics appears to have better activity against stationary-phase antibiotics than does the beta-lactam class. Trovafloxacin, the agent used in the present studies, is a drug of this class that has a greater spectrum of activity against gram-positive and anaerobic bacteria than the earlier fluoroquinolone drugs (8).

There may be a variety of reasons why bacteria stop or slow the rate of their proliferation after the initial acute phase of an infection passes. These may involve depletion of needed nutrients, including metals or other substances, accumulation of toxic by-products within enclosed spaces, or damage from various host defense mechanisms. Within abscesses, zinc deprivation appears to be a major factor in suppressing bacterial growth. The present studies demonstrate that stimulation of growth in empyema fluid supernatants by addition of zinc enhances the bactericidal activity of the two beta-lactam antibiotics tested but does not have the same effect on the fluoroquinolone trovafloxacin. The latter drug is probably killing the organisms at a maximal rate even though they are zinc deprived and not proliferating actively. It is possible that this antibiotic may kill bacteria efficiently in infections complicated by abscesses, as has previously been shown for ciprofloxacin in other studies (4, 26).

In the present study, the results involving zinc and the beta-lactam drugs appeared to be concentration dependent inasmuch as high concentrations (two to four times the value yielding the initial inoculum) of each antibiotic would kill the organisms even without addition of zinc, and low concentrations (a half or quarter of the value yielding the initial inoculum) would not induce killing even when zinc was added (data not shown). In addition, the effect was also isolate dependent since the one isolate of *S. aureus* appeared to behave differently from the other bacterial isolates. In addition, these experiments did not show a definite relationship between stimulation of growth by zinc and enhancement of killing by the beta-lactam antibiotics. In fact, the one isolate of *S. aureus* that did not show increased susceptibility to ampicillin and cefazolin in empyema fluid with added zinc was the one with the greatest stimulation of proliferation by zinc alone. Initial growth or some other aspect of growth kinetics may be more important in modulating the microbicidal effects of the beta-lactams than the total number of organisms generated during the entire incubation period.

In summary, an effect was found whereby zinc stimulation of bacterial growth in human empyema fluid enhanced the ability of ampicillin and cefazolin to kill the growing organisms, whereas the same was not true for the fluoroquinolone trovafloxacin. This phenomenon appeared to be isolate and concentration dependent. Thus, zinc may enhance microbicidal activity by suppressing the microbistatic effect of the neutrophil protein calprotectin and increasing growth. Antibiotics that do not depend as much on growth for their killing activity, such as the fluoroquinolone trovafloxacin, apparently are not subject to this effect. It is possible that with certain bacterial isolates and antibiotic concentrations, drugs of the fluoroquinolone class may show better microbicidal activity in abscess fluids than do beta-lactams.

ACKNOWLEDGMENTS

This work was supported by Pfizer, Inc., and the Department of Veterans Affairs.

REFERENCES

1. **Bamberger, D. M., K. M. Bettin, and D. N. Gerding.** 1987. Neutrophil localization in acute and chronic experimental abscesses. J. Lab. Clin. Med. **109:**389–395.
2. **Bamberger, D. M., and B. L. Herndon.** 1990. Bactericidal capacity of neutrophils in rabbits with experimental acute and chronic abscesses. J. Infect. Dis. **162:**186–192.
3. **Bamberger, D. M., B. L. Herndon, and P. R. Suvarna.** 1993. The effect of

zinc on microbial growth and bacterial killing by cefazolin in a *Staphylococcus aureus* abscess milieu. J. Infect. Dis. **168:**893–896.
4. **Bryant, R. E., and J. A. Mazza.** 1989. Effect of the abscess environment on the antimicrobial activity of ciprofloxacin. Am. J. Med. **87**(Suppl. 5A)**:**23S–27S.
5. **Clohessy, P. A., and B. E. Golden.** 1995. Calprotectin-mediated zinc chelation as a biostatic mechanism in host defence. Scand. J. Immunol. **42:**551–556.
6. **Fagerhol, M. K., K. B. Andersson, C. F. Naess-Andresen, P. Brandtzaeg, and I. Dale.** 1990. Calprotectin (the L1 protein), p. 187–210. *In* V. L. Smith and J. R. Dedman (ed.), Stimulus response coupling: the role of intracellular calcium-binding proteins. CRC Press, Boca Raton, Fla.
7. **Finkelstein, R. A., C. V. Sciortino, and M. A. McIntosh.** 1983. Role of iron in microbe-host interactions. Rev. Infect. Dis. **5:**S759–S777.
8. **Girard, A. E., D. Girard, T. D. Gootz, J. A. Faiella, and C. R. Cimochowski.** 1995. In vivo efficacy of trovofloxacin (CP-99,219), a new quinolone with extended activities against gram-positive pathogens, *Streptococcus pneumonia*, and *Bacteroides fragilis*. Antimicrob. Agents Chemother. **39:**2210–2216.
9. **Hindler, J. (ed.).** 1992. Antimicrobial susceptibility testing, p. 5.0.1–5.25.1. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook. American Society for Microbiology, Washington, D.C.
10. **Kenny, P. A., L. K. Spencer, P. J. McDonald, and J. J. Finlay-Jones.** 1990. Functional activity of individual abscess neutrophils from mice. Infect. Immun. **58:**4004–4010.
11. **Lepper, M. H., and H. F. Dowling.** 1951. Treatment of pneumococcic meningitis with penicillin compared with penicillin plus aureomycin. Arch. Intern. Med. **88:**489–496.
12. **McNamara, M. P., J. H. Wiessner, C. Collins-Lech, B. L. Hahn, and P. G. Sohnle.** 1988. Neutrophil death as a defense mechanism against *Candida albicans* infections. Lancet **ii:**1163–1165.
13. **Miyasaki, K. T., A. L. Bodeau, A. R. K. Murthy, and R. I. Lehrer.** 1993. In vitro antimicrobial activity of the human neutrophil cytosolic S-100 protein complex, calprotectin, against *Capnocytophaga sputigena*. J. Dent. Res. **72:** 517–523.
14. **Polk, H. C., Jr., and D. E. Fry.** 1981. Infection and fever in the surgical patient, p. 1–19. *In* J. D. Hardy, (ed.), Complications in surgery and their management. W. B. Saunders, Philadelphia, Pa.
15. **Santhanagopalan, V., B. L. Hahn, B. Dunn, J. H. Wiessner, and P. G. Sohnle.** 1995. Antimicrobial activity of calprotectin isolated from human empyema fluid supernatants. Clin. Immunol. Immunopathol. **76:**285–290.
16. **Santhanagopalan, V., B. L. Hahn, and P. G. Sohnle.** 1995. Resistance of zinc-supplemented *Candida albicans* yeast cells to the growth-inhibitory effect of calprotectin. J. Infect. Dis. **171:**1289–1294.
17. **Sohnle, P. G.** 1997. Antimicrobial defence through competition for zinc. Rev. Med. Microbiol. **8:**217–224.
18. **Sohnle, P. G., C. Collins-Lech, and J. H. Wiessner.** 1991. Antimicrobial activity of an abundant calcium-binding protein in the cytoplasm of human neutrophils. J. Infect. Dis. **163:**187–192.
19. **Sohnle, P. G., C. Collins-Lech, and J. H. Wiessner.** 1991. The zinc-reversible antimicrobial activity of neutrophil lysates and abscess fluid supernatants. J. Infect. Dis. **164:**137–142.
20. **Sohnle, P. G., B. L. Hahn, and V. Santhanagopalan.** 1996. Inhibition of *Candida albicans* growth by calprotectin in the absence of direct contact with the organisms. J. Infect. Dis. **174:**1369–1372.
21. **Steinbakk, M., C. F. Naess-Andresen, E. Lingass, I. Dale, P. Brandtzaeg, and M. K. Fagerhol.** 1990. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. Lancet **336:**763–765.
22. **Stevens, D. L., A. E. Gibbons, R. Bergstrom, and V. Winn.** 1988. The Eagle effect revisited—efficacy of clindamycin, erythromycin, and penicillin in the treatment of streptococcal myositis. J. Infect. Dis. **158:**23–28.
23. **Stevens, D. L., M. H. Tanner, J. Winship, R. Swarts, K. M. Ries, P. M. Schlievert, and E. Kaplan.** 1989. Reappearance of scarlet fever toxin A among streptococci in the Rocky Mountain West—severe group A streptococcal infections associated with a toxic shock-like syndrome. N. Engl. J. Med. **321:**1–7.
24. **Stevens, D. L., S. Yan, and A. E. Bryant.** 1993. Penicillin-binding protein expression at different growth stages determines penicillin efficacy in vitro and in vivo—an explanation for the inoculum effect. J. Infect. Dis. **167:**1401–1405.
25. **Weinberg, E. D.** 1975. Nutritional immunity—host's attempt to withhold iron from microbial invaders. JAMA **231:**39–41.
26. **Zeiler, H. J., and K. Grohe.** 1984. The in vitro and in vivo activity of ciprofloxacin. Eur. J. Clin. Microbiol. **3:**339–343.