Effect of Antibiotics on Toxin Production and Viability of Clostridium perfringens

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We have recently reported (D. L. Stevens, K. A. Maier, B. M. Laine, and J. E. Mitten, J. Infect. Dis. 155:220–228, 1987) that clindamycin, rifampin, and tetracycline were more efficacious than penicillin in the treatment of fulminant gas gangrene caused by Clostridium perfringens. We hypothesize that antibiotic efficacy correlated with bactericidal or toxin-suppressing properties of these agents. To investigate the possibility that penicillin is only bacteriostatic against C. perfringens, we performed macrobroth dilution MIC and MBC determinations using C. perfringens ATCC 13124. Mean MICs were equal to MBCs for the following antibiotics (micrograms per milliliter): clindamycin, 0.07; tetracycline, 0.05; rifampin, 0.03; metronidazole, 0.69; and penicillin, 0.27. The MIC/MBCs of chloramphenicol were 1.50/3.10 (µg/ml). Because antibiotic efficacy did not correlate with bactericidal activity, we measured alpha-toxin activity and found complete suppression of alpha-toxin activity by tetracycline, metronidazole, rifampin, clindamycin, and chloramphenicol at concentrations equal to the MIC. In contrast, alpha-toxin activity persisted at concentrations of penicillin equal to and above the MIC. The dynamics of bacterial killing and kinetics of alpha-toxin production were next studied in log-phase cultures of C. perfringens with antibiotic concentrations 10 times the MIC. Clindamycin, metronidazole, and rifampin all caused rapid reductions in viability, turbidity, and alpha-toxin activity by 15 to 45 min. In contrast, penicillin demonstrated slower bacterial killing, increased turbidity (62.6% of control), and persistent alpha-toxin activity (80% of control values) for 2 h. Tetracycline and chloramphenicol were the least effective in reducing viability; however, the turbidity of cultures did not increase, and alpha-toxin activity was not detectable. Toxin suppression and rapid bacterial killing may in part explain the observed superior therapeutic efficacy of clindamycin, rifampin, and metronidazole compared with penicillin in the treatment of experimental gas gangrene.

Penicillin is the drug of choice for the prophylaxis and treatment of gas gangrene due to Clostridium perfringens (1, 5). This recommendation dates from uncontrolled clinical studies during World War II (12, 22) and the Vietnam conflict (13) as well as more recent civilian series (5, 30). Studies of experimental gas gangrene have shown that penicillin given prophylactically prevented death in nearly 100% of mice (9), sheep (21), or guinea pigs (1, 9, 10). Although Altemeier and Furste (2) demonstrated that large doses of penicillin prevented C. perfringens gas gangrene in guinea pigs, they also observed that discontinuation of penicillin 4 days after inoculation resulted in progression of infection and death owing to gas gangrene. Similarly, a 4-h delay in initiating penicillin therapy in experimental murine gas gangrene resulted in at least 40% mortality (9). These observations, the recent description by Marrie et al. (15) of decreased susceptibility of C. perfringens to penicillin, and unacceptable mortality and morbidity in humans have prompted us to investigate other forms of treatment. We have recently demonstrated survival rates of 90, 65, 40, and 15% in murine experimental gas gangrene among mice treated with clindamycin, rifampin, tetracycline, and penicillin, respectively (28a). Given prophylactically, these same antibiotics resulted in survival rates of 100, 100, 95, and 10%, respectively (28a). The current study investigated inoculum effects and bactericidal and toxin-suppressing properties of penicillin, chloramphenicol, metronidazole, clindamycin, tetracycline, and rifampin with C. perfringens in vitro. Chloramphenicol and tetracycline have been used to treat experimental gas gangrene (3), and chloramphenicol is a recommended treatment in the penicillin-allergic patient. Metronidazole and clindamycin are among the most active antibiotics in vitro and in vivo against anaerobic bacteria, and the former has been used to treat experimental gangrene (7). Rifampin has been shown to have very low MICs against C. perfringens (23). Thus, these antibiotics provide a diverse array of mechanisms of action including inhibition of cell wall synthesis (penicillin), protein synthesis (chloramphenicol, tetracycline, and clindamycin), RNA synthesis (rifampin), and electron transport (metronidazole).

MATERIALS AND METHODS
Organism. C. perfringens ATCC 13124 was purchased in lyophilized form from the American Type Culture Collection, Rockville, Md. Organisms were maintained anaerobically in chopped meat-glucose broth and recultured every 2 weeks. The purity of C. perfringens was verified before each growth experiment by inoculating blood and egg agar plates and incubating them aerobically and anaerobically with an anaerobic GasPak system (BBL Microbiology Systems, Cockeysville, Md. [Div. Becton Dickinson and Co.j]).

Inoculum preparation. The inoculum was prepared in basal Proteose Peptone medium (Difco Laboratories, Detroit, Mich.) supplemented with amino acids, salts, and vitamins to provide optimal bacterial growth and toxin production (11, 16, 17, 19). Briefly, the medium contained 2% Proteose Peptone, 0.5% yeast extract, 0.5% glucose, 0.05% NaCl, and trace amounts of MgSO4, FeSO4, L-cysteine, nicotinic acid, thiamine, riboflavin, pyridoxamine, and zinc (11, 16, 17, 19). A sample (40 ml) of an overnight
culture of C. perfringens was inoculated into 4 liters of growth medium. The culture was incubated at 37°C at pH 7 to 7.5. Nitrogen was bubbled through the culture fluid to assure proper anaerobic conditions. Dynamics of growth and toxin production were identical to those of cells grown in the BBL GasPak system. After 4 h of incubation, 600 ml of the culture fluid was removed and centrifuged at 10,000 × g for 15 min at 4°C. The bacterial pellet was washed twice with sterile saline, centrifuged for 15 min at 10,000 × g, and then resuspended in 15 ml of sterile saline. Serial dilutions were prepared in cold saline, and the absorbances of the bacterial dilutions were read at 650 nm. The number of viable bacteria or CFU in each dilution was determined by plating duplicate samples (0.01 ml) of each dilution onto blood agar plates. Plates were then incubated anaerobically at 37°C, and colonies were counted and recorded after 18 h. A standard plot of CFU against the A<sub>650</sub> of washed C. perfringens was then prepared, so that numbers of viable bacteria could be estimated from the optical density reading. In all cases the number of CFU was verified by duplicate plating of appropriate dilutions.

**Antibiotics.** Antibiotics used in this study were of assay grade and were supplied as follows: metronidazole (Searle Pharmaceuticals, Inc., Chicago, Ill.); clindamycin hydrochloride (The Upjohn Co., Kalamazoo, Mich.); rifampin (CIBA Pharmaceutical Co., Summit, N.J.); tetracycline hydrochloride (Danbury Pharmaceutical, Inc., Danbury, Conn.); and ceftoxitin (Merck Sharp & Dohme, West Point, Pa.). Penicillin G was purchased in a parenteral form under the brand name Pfizerpen (Pfizer Inc., New York, N.Y.).

**Antibiotic susceptibility tests.** All antibiotic stock solutions (625 to 0.61 μg/ml) were prepared in sterile distilled water except rifampin which was first dissolved in 0.1 ml of methanol and subsequently diluted in distilled water. Stock solutions were filter sterilized with a 0.22-μm-pore-size filter. Each assay tube contained 4.8 ml of growth medium, 0.1 ml of a 5 × 10<sup>4</sup> CFU/ml suspension, and 0.1 ml of antibiotic. Final concentrations of antibiotics ranged from 12.5 to 0.011 μg/ml. Assays were done in triplicate for each antibiotic concentration, as well as controls. Tubes were incubated at 37°C for 18 h under anaerobic conditions utilizing a BBL GasPak system. The MIC was defined as the lowest antibiotic concentration that showed no turbidity after 18 h of incubation. The MBC was defined as the lowest antibiotic concentration that killed 99.9% of the initial inoculum (4). The MBCs were determined by spreading 0.1-ml samples from each clear tube onto blood agar plates and then incubating these anaerobically at 37°C overnight. The lowest antibiotic concentration which yielded less than 10 colonies was defined as the MBC. Inoculated and uninoculated tubes containing only growth medium were used as controls. In a separate experiment the inoculum was varied between 4 × 10<sup>3</sup> and 1.2 × 10<sup>5</sup> CFU to evaluate the effect of inoculum size on antimicrobial susceptibility.

**Toxin production at various antibiotic concentrations.** After 18 h of incubation, 1-ml samples were taken from each control and antibiotic culture tube and centrifuged for 15 min at 7,000 × g. Duplicate samples (0.02 ml) of the supernatant fluid were placed in wells punched into both blood and egg yolk agar plates. Plates were incubated at 37°C aerobically for 18 h at which time the lecithinase and partial hemolysin zone diameters (millimeters) were recorded. Both of these assays are a measurement of alpha-toxin activity. This modification of the technique of Sheldon et al. (24) allowed quantitation of unknowns from a standard curve made by plotting the log concentration of lecithinase activity against the reaction zone radial diffusion diameter. A zone diameter of 5 mm represents 100 U of lecithinase (alpha toxin) activity per ml.

**Immediate effect of antibiotics on culture turbidity, bacterial viability, and alpha-toxin production.** Growth vessels consisting of 250-ml Erlenmeyer flasks fitted with rubber stoppers with ports to accommodate a sample pipette with a three-way stopper, an exhaust port, and an inlet pipette were filled with 96 ml of growth medium (see medium described above). The exhaust tubing led into a beaker containing amphotyl, and each inlet pipette was connected to a gas manifold coupled to a nitrogen tank via sterile tubing. Flasks were placed into a 37°C water bath, and nitrogen was bubbled into the medium at a rate of 25 to 50 ml/min for 30 min to assure proper anaerobic conditions. Each flask was then inoculated with 1 ml of a 10<sup>5</sup>-CFU/ml bacterial suspension resulting in a bacterial concentration of 10<sup>7</sup> CFU/ml in each flask. Immediately after bacterial inoculation, 2-ml samples were removed from each flask for base-line studies described below, and 5 ml of antibiotic was placed into each flask providing antibiotic concentrations which were 10-fold greater than their respective MICs. The remaining flask received 5 ml of distilled water and was maintained as a control. Samples were removed from each flask at 15, 30, 45, 60, 90, and 120 min after addition of the bacteria and antibiotic. Each sample was tested for A<sub>650</sub>, viability, and toxin production. Viability was determined by plating duplicate 10-μl portions of a 10<sup>−3</sup> dilution of each sample onto blood agar plates and incubating these anaerobically overnight at 37°C. Colony counts were averaged to determine CFU per milliliter present. Toxin production was assayed by placing duplicate 20-μl portions of each sample in 3-mm preformed wells in blood and lecithin agar plates. Plates were then incubated aerobically at 37°C for 18 h before zone sizes were measured.

**Statistical analysis.** Unpaired means were compared by the one-way analysis of variance and then Duncan's new multiple-range test. The level of significance was defined as P < 0.05.

**RESULTS**

MICs. The MICs of rifampin, tetracycline, and clindamycin were 0.03, 0.05, and 0.07 μg/ml, respectively. The MICs of penicillin G and metronidazole were 0.27 and 0.69 μg/ml, respectively, and within the range of values previously reported by others (6, 15, 23, 29). In contrast, chloramphenicol had an MIC of 1.50 μg/ml which is slightly higher than the MIC<sub>50</sub> (MIC for 50% of the strains tested) of 1.0 μg/ml, but less than the MIC<sub>90</sub> of 4 μg/ml reported previously (15). These values could differ from those previously reported because our medium was selected to provide optimal conditions for toxin production. Our principal goal was to correlate toxin suppression with MICs determined under conditions used for assays of toxin generation.

**MBC.** The MIC and MBC were equal for tetracycline, rifampin, clindamycin, metronidazole, and penicillin G, indicating that these antibiotics were bactericidal for C. perfringens under these assay conditions. In contrast, the MBC was only twofold higher than the MIC of chloramphenicol, indicating that it too was bactericidal. There was no evidence of tolerance (MBC/MIC > 32) to any antibiotic in this study. The actual MBCs for each drug are as follows (micrograms per milliliter); chloramphenicol, 3.1; metronidazole, 0.69; sodium penicillin G, 0.27; clindamycin hydrochloride, 0.07; tetracycline, 0.05; and rifampin, 0.03 (28a).
TABLE 1. Effect of antibiotic concentration on alpha-toxin production by C. perfringens

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>0.045</th>
<th>0.09</th>
<th>0.19</th>
<th>0.39</th>
<th>0.78</th>
<th>1.5</th>
<th>3.1</th>
<th>6.2</th>
<th>12.5</th>
</tr>
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<tbody>
<tr>
<td>Chloramphenicol</td>
<td>ND</td>
<td>ND</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>53.8</td>
<td>2.1</td>
<td>2.1</td>
<td>0</td>
<td>1.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>ND</td>
<td>62.6</td>
<td>34.9</td>
<td>9.8</td>
<td>18.5</td>
<td>18.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data represent the mean alpha-toxin activity of six samples and are depicted as the percentage of the control. Macrodilution tubes with or without antibiotic contained 10^6 CFU of C. perfringens and were incubated anaerobically in GasPak jars at 37°C for 18 h. Duplicate samples (20 μl) from supernatant fluids of three macrodilution MIC tubes were assayed for alpha-toxin activity by radial diffusion on egg yolk-lecithin agar plates.

**ND, Not determined.**

**Relationship of antimicrobial concentration and C. perfringens alpha-toxin activity.** Alpha-toxin activity was not detected at antibiotic concentrations equal to or above the MIC of clindamycin, chloramphenicol, rifampin, and tetracycline (Table 1). Small quantities of toxin activity were detected at metronidazole concentrations above the MIC. Penicillin was the least active of all agents tested since alpha toxin was detectable at the MIC and at concentrations of penicillin four to five times higher than the MIC.

**Dynamics of alpha-toxin suppression in the presence of antibiotic concentrations above the MIC.** Because the sensitivity of the alpha-toxin assay required approximately 10^6 bacteria per ml, additional experiments with 10^7 log-phase C. perfringens bacilli per ml were performed. This allowed detection of alpha-toxin activity within minutes of inoculation even if 10^2 to 10^3 bacteria were killed immediately. Finally, antibiotic concentrations which exceeded the MICs by at least a factor of 10 were selected for two reasons: (i) the inoculum size was 100-fold larger than in the standard MIC (10^5 CFU/ml) experiment; and (ii) based on the MBCs this concentration would exceed that needed for a bactericidal effect for all these antibiotics. Thus, differences in toxin activity would be due to intrinsic differences in their ability to suppress toxin expression.

Antibiotics at concentrations 10 times the MIC were incubated with 10^7 log-phase C. perfringens bacilli in fresh bacteriologic medium under anaerobic conditions. All antibiotics except penicillin completely inhibited C. perfringens alpha-toxin production within 15 min (Table 2). In contrast, penicillin-treated cultures maintained alpha-toxin activity at levels equal to 80% of those of control cultures for 45 min. Alpha-toxin activity was sustained at this level for 2 h, but became undetectable by 4 h (Table 2). There was no evidence of increased toxin release by bactericidal concentra-

**TABLE 2. Dynamics of alpha-toxin suppression by antibiotics**

<table>
<thead>
<tr>
<th>Antibiotic *</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>90</td>
<td>100</td>
<td>100</td>
<td>120</td>
<td>140</td>
<td>4.2 x 10^3</td>
<td>4.2 x 10^3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>90</td>
<td>80</td>
<td>100</td>
<td>90</td>
<td>80</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Antibiotics were added to a log-phase culture of C. perfringens ATCC 13124 to provide a concentration of drug 10-fold greater than the MIC.

* Alpha-toxin activity was measured by radial diffusion in wells punched into blood agar plates. A zone diameter of 5 mm represents 100 units/ml.

**DISCUSSION**

Penicillin has emerged as the drug of choice in treating C. perfringens gas gangrene. However, antibiotic susceptibility studies, including the present study (Table 1), indicate that chloramphenicol, tetracycline, rifampin, clindamycin, and clindamycin-resistant strains of C. perfringens are frequently encountered in patients with gas gangrene. Therefore, the use of these antibiotics in the treatment of gas gangrene should be reserved for patients who have known or suspected infections with C. perfringens.
FIG. 1. Effect of antimicrobial agents on the turbidity of log-phase cultures of C. perfringens ATCC 13124. Antimicrobial agents at concentrations 10-fold greater than the MIC were added to 10^7 log-phase C. perfringens bacilli at zero time, and the A_625 was measured at various times.

FIG. 2. Effect of antimicrobial agents on the viability of log-phase cultures of C. perfringens ATCC 13124. Antimicrobial agents at concentrations 10-fold greater than the MIC were added to 10^7 log-phase C. perfringens bacilli at zero time. Samples (0.1 ml) were removed at designated times for determination of CFU. Data points are the mean ± standard error of the mean of duplicate specimens.
and metronidazole might also be effective drugs for the treatment of C. perfringens infections (6, 15, 18, 23). Despite these in vitro similarities, we have observed striking differences in the therapeutic efficacy of these agents in the treatment of experimental gas gangrene (28a). We therefore studied the effect of various concentrations of antibiotics on alpha-toxin production and the dynamics of bacterial killing in vitro. Penicillin was the least active agent tested, and only partial inhibition of alpha toxin was apparent at antibiotic concentrations 10 times the MIC. All other antibiotics completely suppressed alpha-toxin activity by 15 min. In this study we did not directly measure toxin synthesis but rather toxin activity as a function of time. We did establish that these antibiotics did not interfere with toxin activity directly. The presence of proteolytic activity in the culture filtrate of C. perfringens is well established (28). Soda and Yamamoto (28) have demonstrated peak alpha-toxin activity during log-phase growth of C. perfringens followed by a sixfold reduction in alpha-toxin activity within 60 min of reaching the stationary growth phase. Thus, measurement of alpha-toxin activity in response to antimicrobial agents is a dynamic process, and a decline in toxin activity reflects both reduced production and continued degradation. An expected result would be reduced toxin activity induced by all agents to which the organism was susceptible. Persistent toxin activity in cultures treated with penicillin suggests either that the stationary phase was not reached or that toxin production continued despite the presence of penicillin. Similarly, turbidity was reduced to base-line levels (zero time) by 15 to 30 min for all drugs tested except penicillin and chloramphenicol. In sharp contrast, penicillin produced only a 38% reduction in turbidity by 45 min, despite a 10^5 CFU reduction in bacteria at that time.

At penicillin concentrations below the MIC (<0.27 μg/ml), filaments of C. perfringens were detectable by phase-contrast microscopy, indicating that cell wall synthesis (peptidoglycan formation) was inhibited. Such forms have been observed by others (18, 31) at penicillin concentrations below the MIC. At concentrations of penicillin equal to the MIC, filaments were not observed, and 99.9% of the bacteria were nonviable under the assay conditions used. Such bacterial forms may be viable in suitable fluid culture but nonviable when plated onto solid medium. Thus, our test conditions could falsely indicate a bactericidal effect. It would be of interest to determine whether these filaments occur in vivo and if so to evaluate their ability to produce alpha toxin. Recently, Ogata et al. (20) demonstrated that stable protoplasts of clostridial species could be produced and maintained in hyperosmolar media and that these protoplasts continued to produce macromolecules. Continued toxin production, persistent turbidity, and the existence of filamentous bacteria in the present study indicate that cell wall-deficient forms could be present. Similarly, Smith (27) has demonstrated L-forms of C. perfringens induced by penicillin and documented continued production of an incomplete hemolysin (alpha toxin) by these cells. These data with C. perfringens are in agreement with observations by Shibl (25, 26; Ph.D. dissertation, University of Glasgow, Glasgow, Scotland, 1977) and others (8, 14) using Staphylococcus aureus and Streptococcus pyogenes that clindamycin, tetracycline, and chloramphenicol are capable of suppressing alpha toxin synthesis in vitro. Specifically, suppression of staphylococcal alpha hemolysin, lipase, coagulase, and exfoliation toxins by lincomycin, clindamycin, and fucidin has been documented (8, 14, 25, 26; Shibl, Ph.D. dissertation). In the present study antibiotics which inhibit protein synthesis such as tetracycline, chloramphenicol, and clindamycin were very effective suppressors of alpha-toxin production (Tables 1 and 2). Rifampin totally suppressed alpha-toxin production, possibly by interfering with protein synthesis not at the ribosomal level, but rather at the mRNA level. Similarly, metronidazole effectively reduced alpha-toxin activity, perhaps owing to its ability to interfere with intracellular metabolism or electron transport.

In summary, this study demonstrates bactericidal activity of penicillin, tetracycline, metronidazole, rifampin, clindamycin, and chloramphenicol against C. perfringens type A (ATCC 13124). Bacterial killing was most rapid with clindamycin, rifampin, and metronidazole, whereas chloramphenicol and tetracycline were the least rapid bactericidal agents.

**TABLE 3. Effect of inoculum size on the antimicrobial susceptibility of C. perfringens**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/ml) at an inoculum size (CFU/ml) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 × 10^8</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.19</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.29</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*a Each value is the mean of duplicate determinations at each specified inoculum.

*b Inoculum size represents the mean of triplicate colony counts of appropriate dilutions of a log-phase culture of C. perfringens ATCC 13124.

**FIG. 3. Effect of penicillin on C. perfringens morphology.** C. perfringens bacilli were exposed to subinhibitory concentrations of antibiotics. Phase-contrast microscopy demonstrated long filaments of bacteria in cultures treated with penicillin (magnification, ×1,000). (a) Bacteria in the absence of penicillin.
Turbidity of *Clostridium perfringens* cultures was reduced rapidly by all antibiotics tested except penicillin. Similarly, alpha-toxin activity was completely suppressed by all antibiotics except penicillin. The combination of toxin suppression and rapid bacterial killing may be of major importance in treating infections caused by toxin-producing bacteria and may explain the superior efficacy of clindamycin, tetracycline, rifampin, and metronidazole compared with penicillin in a mouse gas gangrene model (28a).

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**LITERATURE CITED**