

Is In Vitro Antibiotic Combination More Effective than Single-Drug Therapy against Anthrax?

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Antibiotic combinations are used to enhance antibacterial efficacy and to prevent the development of resistance. We have tested a possible synergistic effect of several antibacterial combinations on *Bacillus anthracis*. The in vitro activities of antibiotic combinations against two strains of *B. anthracis*, strain Sterne and the Russian anthrax vaccine strain STi, were tested by the fractional inhibitory concentration (FIC) method, derived from the MICs of the agents in combination, and by measuring the rate of bacterial killing over time by several antibiotic combinations. The FIC results showed that synergism against both *B. anthracis* strains was observed only with the combination of rifampin and clindamycin. The telithromycin-amoxicillin combination showed synergism against strain Sterne only. All other combinations were either indifferent or antagonistic. The results of the bacterial time-kill study demonstrated indifferent effects for all combinations. These in vitro results demonstrate the difficulties in obtaining synergistic combinations of antibiotics against *B. anthracis*.

Anthrax has recently been the focus of attention as a potential biological warfare agent. The therapy and prophylaxis of anthrax require vaccination combined with prolonged antibacterial therapy. It is important to examine if antibiotic combinations might shorten the 60-day period recommended for postexposure prophylaxis by providing a more radical and rapid killing of the vegetative anthrax forms and thus reducing the infective load (5). Of equal interest is the question whether combinations might reduce the risk for the development of resistance by *Bacillus anthracis*. During the last bioterror attack in the United States, in 2001, many individuals received antibiotic combinations as therapy and as postexposure prophylaxis (6). Clindamycin was often used based on the assumption that it might decrease the production of bacterial toxins and thus diminish the severity of disease manifestations (3).

The aim of the present study was to investigate possible interactions among several antibiotics potentially effective against *B. anthracis*.

MATERIALS AND METHODS

Antibacterial agents. The antibiotics tested in this study were as follows: ciprofloxacin and moxifloxacin (Bayer, Leverkusen, Germany), tetracycline (Sigma, Rehovot, Israel), penicillin G (Rafa Laboratories, Jerusalem, Israel), amoxicillin (GSK, Petach Tiqva, Israel), vancomycin (Eli Lilly, Sesto Fiorentino, Italy), clarithromycin (Abbott, Promedico, Petach-Tiqva, Israel), telithromycin and quinupristin-dalfopristin (Q-D) (Aventis, Paris, France), clindamycin and linezolid [Pharmacia (Agis), Bnei Braq, Israel, and Pharmacia, Kalamazoo, Mich.], and rifampin (Sigma).

Penicillin G, vancomycin, rifampin, clindamycin, linezolid, and Q-D were

received as dry laboratory powders and were dissolved in phosphate-buffered saline (pH 7.2). Amoxicillin was dissolved in distilled water. Clarithromycin was dissolved in analytical-grade acetone, while telithromycin and tetracycline were dissolved initially in 2 drops of acetic acid and ethanol (100%), respectively, and all three of these antibiotics were subsequently diluted in distilled water to the required concentration; at the final concentrations used, these solvents had no demonstrable antibacterial activity. Prepared solutions were frozen in small aliquots and thawed before use. Antibiotic solutions were sterilized through 0.45- μ m-pore-size filters (Millipore S.A., Paris, France). Moxifloxacin and ciprofloxacin were obtained as injectable solutions.

Bacterial strains and growth conditions. The bacteria used in this study were two strains of *B. anthracis*: strain Sterne (a gift from the Colorado Serum Institute, Denver, Colo.) and the Russian strain STi, purchased in Moscow, Russia. Neither strain is a human pathogen, because both lack the plasmid necessary to produce the capsule of the vegetative form, which is necessary for human pathogenicity. Bacterial spores were stored in sterile 30% glycerol in phosphate-buffered saline and were spread on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) and incubated overnight at 37°C to obtain single colonies (vegetative form). A single colony was inoculated into 10 ml of BHI broth and incubated overnight at 37°C (use of four to five colonies instead of a single colony did not reveal a clonality effect).

Determination of MICs. MICs were determined by the microdilution technique according to the NCCLS criteria for *B. anthracis* (7, 8).

The antibacterial agents to be tested were thawed and diluted in distilled water. Twofold dilutions in 100 μ l of BHI broth were used in a concentration range from 0.015 to 1,024 mg/liter, and solutions were poured into the wells of 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). A 10- μ l volume of bacterial culture, which contains 10⁵ CFU, was then added. Following incubation of the plates for 18 h at 37°C in ambient air, the MICs were determined. *Staphylococcus aureus* ATCC 2119 was used as a control for antibiotic activity. MICs were recorded as the lowest concentrations that completely inhibited visible growth of the bacteria (8).

The effects of antibiotic combinations were determined by two methods: the fractional inhibitory concentration (FIC) and the time-kill method (2, 4, 11).

The time-kill method was performed as follows. An overnight culture of *B. anthracis* was diluted 1:1,000 with BHI broth at a final volume of 2 ml (10⁵ CFU/ml). Antibiotics at the concentrations shown in Table 3, in a volume of 12.5 to 25 μ l, were added to the bacterial suspension. As a control, similarly diluted bacterial inocula without antibiotics were used. Bacterial suspensions were incubated at 37°C, and samples (10 μ l) were removed at 0, 0.5, 2, 4, 6, 10, 12, and

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TABLE 1. MICs of antibiotics against *B. anthracis* strains STi and Sterne

Antibacterial agent	MIC (mg/liter) for <i>B. anthracis</i> :		NCCLS breakpoint (susceptibility) for <i>B. anthracis</i> ^a
	STi	Sterne	
Ciprofloxacin	0.03	0.03	≤0.5
Moxifloxacin	0.125	0.06	
Amoxicillin	0.06	0.125	
Penicillin G	0.03	0.25	≤0.12
Vancomycin	1.25	2.5	
Tetracycline	0.125	0.125	≤10
Clarithromycin	0.125	0.125	
Telithromycin	0.125	0.125	
Clindamycin	0.25	0.125	
Quinupristin-dalfopristin	0.06	0.125	
Rifampin	0.25	0.125	
Linezolid	2.0	2.0	

^a In milligrams per liter. Data are from reference 8.

24 h for viable bacterial counts. Bacterial counts were performed by diluting samples in sterile saline and plating 20 to 50 μ l on BHI agar. To improve detection in samples with low bacterial counts, 100 μ l of an undiluted specimen was also plated. In addition, the spore content at each time point and that of the inoculum were measured by the heat shock method (65°C for 30 min.). After overnight incubation at 37°C, the number of CFU (both vegetative forms and spores) were counted. All tests were performed in triplicate, and results were averaged. Data were plotted as average log₁₀ CFU per milliliter against time.

The combinations of antibiotics tested for FIC determination are presented in Table 2. The concentration range of each antibiotic in combination ranged from 1/32 the MIC to four times the MIC (4×MIC). The FIC index was calculated as [(MIC of drug A in combination)/(MIC of drug A alone)] + [(MIC of drug B in combination)/(MIC of drug B alone)]. The following definitions were used: synergism, FIC index of ≤0.5; indifference, FIC index of >0.5 and ≤4; antagonism, FIC index of >4.

The time-kill method determined the rate of killing of bacteria by each antibiotic at the concentrations of 1×MIC and 5×MIC, as well as the effects of the combination on killing at the following concentrations: both drug A and drug B at 1×MIC, A at 1×MIC and B at 5×MIC, A at 5×MIC and B at 1×MIC, and both A and B at 5×MIC. For calculation of the killing activity, the area under the curve of bacterial concentration over time (AUC) was calculated for each agent alone and for each combination, and the results were converted to a log₁₀ scale. If the AUC of the combination was 2 or more log units smaller than the AUC of each of the antibiotic constituents, the combination was considered synergistic; if the AUC of the combination was between 1 log unit or less smaller and 2 log units or less greater than the AUC of each constituent, the combination was considered indifferent; all other differences between the AUCs were considered to indicate antagonism.

RESULTS

MIC. The MICs of the antibacterial agents tested against the two strains of *B. anthracis*, as reported previously (2), are shown in Table 1.

Table 2 shows the FIC indices of the antibiotic combinations. Ciprofloxacin-tetracycline, ciprofloxacin-penicillin, ciprofloxacin-clarithromycin, ciprofloxacin-clindamycin, ciprofloxacin-Q-D, ciprofloxacin-rifampin, ciprofloxacin-linezolid, tetracycline-clindamycin, tetracycline-rifampin, tetracycline-moxifloxacin, tetracycline-telithromycin, rifampin-Q-D, rifampin-linezolid, rifampin-clarithromycin, rifampin-telithromycin, telithromycin-moxifloxacin, linezolid-clarithromycin, and penicillin-tetracycline showed indifferent effects against both *B. anthracis* strains.

The tetracycline-linezolid, penicillin-rifampin, penicillin-clindamycin, penicillin-vancomycin, and penicillin-telithromycin combinations showed indifferent activities against strain Sterne and antagonistic effects against strain STi.

TABLE 2. FICs of combination of antibiotics against *B. anthracis* strains

Antibiotic combination	FIC index (result) ^a for strain:	
	STi	Sterne
Ciprofloxacin + tetracycline	1.03 (IN)	1.03 (IN)
Ciprofloxacin + penicillin G	2.03 (IN)	2.03 (IN)
Ciprofloxacin + clarithromycin	2.48 (IN)	2.48 (IN)
Ciprofloxacin + clindamycin	2.29 (IN)	1.03 (IN)
Ciprofloxacin + Q-D	1.5 (IN)	1.25 (IN)
Ciprofloxacin + rifampin	1.125 (IN)	2.5 (IN)
Ciprofloxacin + linezolid	2.03 (IN)	2.03 (IN)
Tetracycline + clindamycin	2.48 (IN)	1.03 (IN)
Tetracycline + rifampin	0.75 (IN)	2 (IN)
Tetracycline + moxifloxacin	2 (IN)	1.48 (IN)
Tetracycline + telithromycin	0.96 (IN)	0.96 (IN)
Rifampin + Q-D	2.5 (IN)	2 (IN)
Rifampin + linezolid	0.56 (IN)	1.06 (IN)
Rifampin + Clarithromycin	0.99 (IN)	0.96 (IN)
Rifampin + telithromycin	0.72 (IN)	0.75 (IN)
Telithromycin + moxifloxacin	0.96 (IN)	1.48 (IN)
Linezolid + clarithromycin	2.125 (IN)	2.125 (IN)
Penicillin G + tetracycline	2.03 (IN)	2.03 (IN)
Tetracycline + linezolid	4.25 (AN)	2.125 (IN)
Penicillin G + rifampin	9.33 (AN)	1.5 (IN)
Penicillin G + clindamycin	9.3 (AN)	1.5 (IN)
Penicillin G + vancomycin	34 (AN)	1.06 (IN)
Penicillin G + telithromycin	5 (AN)	0.72 (IN)
Linezolid + clindamycin	9 (AN)	4.5 (AN)
Tetracycline + Q-D	6 (AN)	4.8 (AN)
Penicillin G + Q-D	50 (AN)	4.03 (AN)
Penicillin G + linezolid	33 (AN)	4.25 (AN)
Rifampin + clindamycin	0.48 (SY)	0.48 (SY)
Telithromycin + amoxicillin	0.75 (IN)	0.48 (SY)

^a IN, indifference; SY, synergism; AN, antagonism.

Antagonistic effects against both strains were demonstrated for the linezolid-clindamycin, tetracycline-Q-D, penicillin-Q-D, and penicillin-linezolid combinations. Synergistic activities were found for rifampin-clindamycin against both strains and for telithromycin-amoxicillin against strain Sterne only.

The time-kill method revealed indifferent effects for all combinations at all concentrations used (Table 3).

DISCUSSION

Clear synergism was observed only for the combination of rifampin and clindamycin by the FIC method; indifference was observed for all combinations by the time-kill method. Thus, the inclusion of clindamycin, while possibly reducing *B. anthracis* toxin production (9), does not contribute to more-rapid killing of the organism when it is used with conventional anti-anthrax agents such as penicillin, ciprofloxacin, or doxycycline. Resolution of these contradictory findings for clindamycin, with implications for its contributions to recommended therapeutic regimens, awaits animal studies using the toxin-producing *B. anthracis* strains.

There are no well-established methods for assessing the effects of antibiotic combinations on killing. Furthermore, no systematic comparison between the assay methods is available. Therefore, our results cannot be compared to a "gold standard."

A shortcoming of this study is that we did not investigate

TABLE 3. Effects of combinations of antibiotics on rates of killing of *B. anthracis* strains STi and Sterne

Antibiotic combination (concs [\times MIC]) ^a	Combination effect ^b for strain:	
	STi	Sterne
Rifampin and tetracycline		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Rifampin and ciprofloxacin		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Rifampin and Q-D		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Rifampin and linezolid		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Rifampin and clindamycin		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Penicillin G and clindamycin		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Penicillin G and tetracycline		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Q-D and tetracycline		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Q-D and ciprofloxacin		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Linezolid and clindamycin		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN
Ciprofloxacin and clindamycin		
5, 5	IN	IN
5, 1	IN	IN
1, 5	IN	IN
1, 1	IN	IN

^a Concentrations are expressed as multiples of the MIC. The concentration of the first drug in the combination is given first, followed by a comma and the concentration of the second drug in the combination.

^b IN, indifference.

whether the *B. anthracis* strains developed resistance during exposure to the combinations studied. Nevertheless, previous observations with these and other strains suggest that derepression of β -lactamases of the two types, penicillinase and cephalosporinase, may occur after a short exposure to these antibiotic classes (10). In our previous studies, resistance to

other commonly used agents (fluoroquinolones, tetracyclines, clindamycin, etc.) occurred only at passage ≥ 9 and required prolonged exposure to the antibiotic (1). An additional shortcoming of the present study is that we have not measured the effects of higher antibiotic concentrations which may be clinically achieved in serum following conventional doses of, e.g., ciprofloxacin and rifampin. It is possible that different effects on *B. anthracis* may be observed at such high concentrations.

Postexposure prophylaxis and therapy of anthrax are prolonged (≥ 60 days of therapy) because of surviving spores within the macrophages that can germinate and turn into the toxin-producing vegetative forms. In addition, all the currently recommended agents are inactive against spores (2). Our experience suggests that combinations of the antibiotics currently recommended are not likely to shorten the treatment period, due to the lack of synergistic effect. A synergistic combination might have caused rapid and radical bacterial killing, so that the organism gave rise to a smaller number of spores. The only exception was the combination of rifampin and clindamycin, which showed synergism by the FIC method. Additional studies on different *B. anthracis* strains need to be performed in order to substantiate these preliminary observations.

REFERENCES

- Athamna, A., M. Athamna, N. Abu-Rashed, B. Medlej, D. J. Bast, and E. Rubinstein. 2004. Selection of *Bacillus anthracis* isolates resistant to antibiotics. *J. Antimicrob. Chemother.* **54**:424–428.
- Athamna, A., M. Massalha, M. Athamna, A. Nura, B. Medlej, I. Ofek, D. J. Bast, and E. Rubinstein. 2004. In vitro susceptibility of *Bacillus anthracis* to various antibacterial agents and their time-kill activity. *J. Antimicrob. Chemother.* **53**:247–251.
- Centers for Disease Control and Prevention. 2001. CDC update: investigation of bioterrorism-related anthrax and interim guidelines for clinical evaluation of persons with possible anthrax. *Morb. Mortal. Wkly. Rep.* **50**:941–948.
- Eliopoulos, G. M., and R. C. Moellering. 1991. Antimicrobial combinations, p. 432–492. In V. Lorian (ed.), *Antibiotics in laboratory medicine*. The Williams & Wilkins Co., Baltimore, Md.
- Inglesby, T. V., T. O'Toole, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. M. Friedlander, J. Gerberding, J. Hauer, J. Hughes, J. McDade, M. T. Osterholm, G. Parker, T. M. Perl, P. K. Russell, K. Tonat, et al. 2002. Anthrax as a biological weapon, 2002: updated recommendations for management. *JAMA* **287**:2236–2252.
- Jernigan, D. B., P. L. Raghunathan, B. P. Bell, R. Brechner, E. A. Bresnitz, J. C. Butler, M. Cetron, M. Cohen, T. Doyle, M. Fischer, C. Greene, K. S. Griffith, J. Guarner, J. L. Hadler, J. A. Hayslett, R. Meyer, L. R. Petersen, M. Phillips, R. Pinner, T. Popovic, C. P. Quinn, J. Reefhuis, D. Reissman, N. Rosenstein, A. Schuchat, W. J. Shieh, L. Siegal, D. L. Swerdlow, F. C. Tenover, M. Traeger, J. W. Ward, I. Weisfuse, S. Wiersma, K. Yeskey, S. Zaki, D. A. Ashford, B. A. Perkins, S. Ostroff, J. Hughes, D. Fleming, J. P. Koplan, J. L. Gerberding, and the National Anthrax Epidemiologic Investigation Team. 2002. Investigations of bioterrorism related anthrax, United States, 2001: epidemiologic findings. *Emerg. Infect. Dis.* **8**:1019–1028.
- Mohammed, M. J., C. K. Marston, T. Popovic, R. S. Weyant, and F. C. Tenover. 2002. Antimicrobial susceptibility testing of *Bacillus anthracis*: comparison of results obtained by using the National Committee for Clinical Laboratory Standards broth microdilution reference and E test agar gradient diffusion methods. *J. Clin. Microbiol.* **40**:1902–1907.
- National Committee for Clinical Laboratory Standards. 2004. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. Approved standard. NCCLS publication M100-S14. National Committee for Clinical Laboratory Standards, Wayne, Pa.
- Spreer, A., H. Kerstan, T. Bottcher, J. Gerber, A. Siemer, G. Zysk, T. J. Mitchell, H. Eiffert, and R. Nau. 2003. Reduced release of pneumolysin by *Streptococcus pneumoniae* in vitro and in vivo after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. *Antimicrob. Agents Chemother.* **47**:2649–2654.
- ter Braak, E. W., P. J. de Vries, K. P. Bouter, S. G. van der Vegt, G. C. Dorrestein, J. W. Nortier, A. van Dijk, R. P. Verkooyen, and H. A. Verbrugh. 1990. Once-daily dosing regimen for aminoglycoside plus β -lactam combination therapy of serious bacterial infections: comparative trial with netilmicin plus ceftriaxone. *Am. J. Med.* **89**:58–66.
- White, R. L., D. S. Burgess, M. Manduru, and J. A. Bosso. 1996. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrob. Agents Chemother.* **40**:1914–1918.