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Effect of Mixing on Rifampin Bactericidal Activity Against Staphylococci

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Minimal bactericidal concentrations of rifampin were significantly increased, and serum bactericidal activity from volunteers receiving this drug was significantly decreased by vigorous mixing of microtiter plates before sampling when tested against *Staphylococcus aureus* and *Staphylococcus epidermidis* at 10^5 and 10^6 colony-forming units per ml. These results suggest that microtiter estimates of the bactericidal activity of rifampin against staphylococci should be performed after vigorous shaking.

Rifampin is among the most active anti-staphylococcal antibiotics known. In a study of 70 isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis*, Sabath et al. (9) reported that rifampin was the most active of 65 antibiotics. Bactericidal concentrations were not reported. In a report by Archer et al. (1), minimal bactericidal concentrations (MBCs) were reported for two strains of *S. epidermidis*, and the MBCs were fourfold higher than the minimal inhibitory concentrations (MICs). In that report (1) rifampin alone, after 24 h in a time-kill experiment, did not kill the two isolates at concentrations 10 to 40 times the MBC. However, there are three clinical reports (1, 3, 6) which suggest that rifampin increases serum bactericidal activity when added to other anti-staphylococcal drugs in the treatment of patients with serious staphylococcal infections.

As a prelude to a larger study of rifampin combined with many other anti-staphylococcal agents, in vitro susceptibility testing of 15 staphylococcal isolates revealed MBCs considerably higher than MICs and also higher than those reported in other studies (10). The purpose of this report is to demonstrate the importance of vigorous mixing of microtiter plates before sampling for MBC determinations of rifampin against *S. aureus* and *S. epidermidis*.

Five strains each of *S. epidermidis* and *S. aureus* susceptible and resistant to methicillin were tested for susceptibility to rifampin in microtiter plates (4). Dilutions of rifampin were made in Mueller-Hinton broth supplemented with Ca^{2+} and Mg^{2+} (50 and 20 mg/liter respectively), and antibiotic concentrations ranged from 0.0015 to 3 μg/ml. An 18-h culture was diluted to 10^8 organisms per ml against a barium sulfate standard as in the Kirby-Bauer method (2). This was further diluted so that the final inoculum in each well was either 10^5 or 10^6 organisms per ml. All organisms were studied at both inoculum levels. The total volume in each well was 100 μl. MIC was read as the lowest concentration which showed no turbidity after overnight (18 h) incubation at 37°C.

The MBC was determined with inoculations from the same microtiter plates before and after vigorous mixing of the microtiter plates for 10 s in a microshaker (Dynatech Laboratories, Alexandria, Va.). A multipoint inoculator designed to fit the microtiter plates was used to sample the wells and to transfer 0.001 ml to antibiotic-free Mueller-Hinton agar plates. The inoculations were also performed in duplicate, and the plates were incubated for 18 h at 37°C. The MBC was read as the lowest concentration which resulted in no growth for the 10^5 inoculum and which resulted in one colony or less for the 10^6 inoculum. These criteria represent, respectively, 99 and 99.9% killing of the original 10^5 and 10^6 inocula.

To estimate the effect of vigorous mixing on the determination of serum bactericidal activity, six normal volunteers, after giving informed consent, received 300 mg of rifampin in 60 ml of 5% dextrose in water by intravenous infusion over 60 min. Serum drawn 1 h after infusion was separated and stored at −20°C until used. Serum inhibitory and bactericidal activities of these individual specimens were determined as previously described (5) against two strains of methicillin-susceptible *S. aureus* that were susceptible to rifampin (MIC = <0.0015 and 0.003 μg/ml, respectively). Mueller-Hinton broth supplemented with Ca^{2+} and Mg^{2+} in 50% heat-inacti-

vated pooled human serum (GIBCO Laboratories, Grand Island, N.Y.) was used as the diluent, and bacteria were added at a final concentration of 5×10^5 organisms per ml.

The geometric mean, median, and range of MICs and MBCs obtained before and after mixing for the 10^6 inoculum are presented in Table 1. "Skip tubes" (7) were not seen in the inhibitory assays. At this inoculum, MBCs after mixing were 13 to 118 times higher than the MBCs before mixing for all three types of staphylococci studied. Similar results were obtained at a concentration of 10^5 organisms per ml.

Table 2 shows the geometric mean, median reciprocal serum inhibitory activity, and serum bactericidal activity before and after vigorous mixing. Serum rifampin concentrations ranged from 3.5 to 11 μg/ml, with a median concentration of 6.3 μg/ml. For both strains of methicillin-susceptible *S. aureus* tested, the serum bactericidal activity was considerably reduced when determined after mixing.

With increasing resistance to methicillin and the continued presence of serious infections due to staphylococci, there is a need to expand the therapeutic attack on these organisms. Although usually used in the treatment of tuberculosis, rifampin, as several recent reports have suggested, is clinically useful when added to regimens containing other anti-staphylococcal agents (1, 3, 6). Most of these reports show enhanced serum bactericidal activity attendant upon the addition of rifampin. However, the explicit details regarding the extent of mixing of the antibiotic-bacterium mixture before sampling for the bactericidal assay are not provided.

Although it is well established that rifampin is probably the most active anti-staphylococcal antibiotic known with respect to inhibitory activity (9), few data are available as to its bactericidal activity. That rifampin may be considerably less bactericidal than inhibitory against staphylococci is suggested by killing curves presented by Archer et al. (1), where 1 μg of rifampin per ml did not kill two strains of *S. epidermidis* at 24 h despite MBCs of 0.025 and 0.1 μg/ml, respectively. Tuazon et al. (10) report rifampin MBCs for 20 strains of *S. aureus* which closely resemble the MICs, but no mention is made of the mixing or agitation of the bacterium-antibiotic suspension before sampling.

This study emphasizes the importance of vigorous mixing of the contents of microtiter wells after overnight incubation for the determination of inhibitory activity and before incubation for an estimation of bactericidal activity. In the

TABLE 1. *Effect of vigorous mixing of bacterium-antibiotic suspensions on the MICs and MBCs of rifampin against staphylococci*[a]

Bacterium	MIC			MBC					
				Before mixing			After mixing		
	Geometric mean	Median	Range	Geometric mean	Median	Range	Geometric mean	Median	Range
S. aureus (methicillin resistant; $n = 5$)	<0.0015	<0.0015	All < 0.0015	0.0079[b]	0.003	<0.0015–1.5	0.934[b]	1.5	0.3–1.5
S. aureus (methicillin susceptible; $n = 5$)	0.002	0.0015	<0.0015–0.003	0.079	0.07	0.003–3	1.072	1.5	0.3–3.0
S. epidermidis ($n = 5$)	0.0023	0.003	<0.0015–0.003	0.009[c]	0.0015	<0.0015–0.07	0.501[c]	1.5	0.007–3.0

[a] Inoculum, 10^6 organisms per ml.
[b] $P < 0.01$.
[c] $P < 0.05$.

TABLE 2. *Effect of mixing on the serum inhibitory and serum bactericidal activities against two strains of S. aureus*[a] *(methicillin susceptible) after rifampin infusion (300 mg)*

Strain	No. of sera	Serum inhibitory activity[b]			Serum bactericidal activity[b]					
					Before mixing			After mixing		
		Geometric mean	Median	Range	Geometric mean	Median	Range	Geometric mean	Median	Range
1	6	1,824	>2,048	1,024–>2,048	912[c]	1,536	64–>2,048	4[c]	5	2–8
2	3	>2,048	>2,048	>2,048	1,625[c]	>2,048	1,024–>2,048	16[c]	16	8–16

[a] Inoculum, 5×10^5 organisms per ml.
[b] Expressed as a reciprocal.
[c] $P < 0.001$.

determination of both MBC and serum bactericidal activity of rifampin against *S. aureus* (both methicillin resistant and susceptible) and *S. epidermidis*, vigorous mixing in a microshaker resulted in considerably less bactericidal activity of this drug than when estimates were made before mixing. Preliminary studies with rifampin and one strain each of *Escherichia coli*, *Serratia marcescens*, and *Klebsiella pneumoniae* did not show this differential effect with mixing.

Although we have not specifically studied the mechanism responsible for this effect, it is possible that overnight incubation allows settling of the inhibited, but not dead, bacteria to the bottom of the wells, with the result that surface action prevents their inclusion in samples removed by inoculators or micropipettes.

An early description of microtiter systems for the determination of MBC recommends gentle agitation of the plate before sampling, but details are not given (4). The microshaker used here provides vigorous mixing without spilling of the contents of the wells. Another method has been reported (8) and involves the addition of sterile, stainless-steel-in-glass stirring rods to each well. Placement of the microtiter plate on a magnetic stirrer results in rapid agitation and thorough mixing of the contents of the well.

These studies show that rifampin is bactericidal for staphylococci, but at concentrations much higher than those necessary for inhibition. We suggest vigorous mixing of microtiter plates before sampling of antibiotic-bacterium suspensions for estimates of bactericidal activity of rifampin against staphylococci. Preliminary results of other studies in progress suggest that failure to do so may seriously mask antagonism or synergism of rifampin plus other antibiotics.

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