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Disparity Between Timed-Kill and Checkerboard Methods for Determination of In Vitro Bactericidal Interactions of Vancomycin Plus Rifampin Versus Methicillin-Susceptible and -Resistant *Staphylococcus aureus*

ARNOLD S. BAYER* AND JOAN O. MORRISON

Department of Medicine, Harbor-UCLA Medical Center, Torrance, California 90509, and University of California-Los Angeles School of Medicine, Los Angeles, California 90024

Received 1 March 1984/Accepted 23 May 1984

The role of rifampin as an adjunctive agent to vancomycin in the therapy of serious systemic staphylococcal infections remains controversial. Several in vitro studies utilizing differing methodologies to define the bacteridical interactions of vancomycin plus rifampin versus *Staphylococcus aureus* have yielded markedly disparate results. The in vitro bactericidal synergistic activities of vancomycin plus rifampin were examined versus 48 clinical isolates of *S. aureus*, both methicillin susceptible and resistant. Each strain was tested simultaneously in timed-kill curve and checkerboard systems. By timed-kill curve, vancomycin plus rifampin usually had either an indifferent (67%) or synergistic (19 to 29%) effect, with a frequency dependent on sampling times; bactericidal antagonism was infrequently noted after 48 h of incubation (4%). Indifference was seen as a prevention of rifampin resistance by vancomycin. Synergy was more commonly noted at 48 than at 24 h of incubation. The bactericidal interaction results were similar for both methicillin-susceptible and -resistant strains. In contrast to the killing curve data, the checkerboard technique uniformly demonstrated bactericidal antagonism of vancomycin plus rifampin against all 48 staphylococci. We conclude that the nature of the in vitro bactericidal interactions of vancomycin plus rifampin against *S. aureus* is difficult to establish in vitro. This fact relates to the markedly disparate findings, which depended on both the synergy technique utilized and the test system conditions employed. In vivo studies are required to delineate the bactericidal interaction potentials of vancomycin plus rifampin versus *S. aureus*.

Vancomycin and rifampin are both excellent anti-staphylococcal antibiotics. These two agents have been used in combination against a variety of deep-seated staphylococcal syndromes, including endocarditis, with generally salutary clinical-bacteriological outcomes (2, 4, 5, 16, 18). However, several recent articles examining the in vitro bactericidal interactions of vancomycin plus rifampin against both methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) have yielded conflicting results. Tuazon et al. showed that 25% of MSSA strains were synergistically killed by vancomycin plus rifampin by the checkerboard technique, whereas antagonism was not seen (17). In contrast, Watanakunakorn and Guerriero demonstrated in vitro bactericidal antagonism with vancomycin plus rifampin in 86% of MRSA and MSSA isolates by timed-kill curve methodology (20). Because of these disparate findings, we have examined 48 recent clinical MSSA and MRSA isolates to define the bactericidal interactions of vancomycin-rifampin combinations as defined by two separate techniques (timed-kill curve and microtiter checkerboard techniques).

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MATERIALS AND METHODS

Isolates. Forty-eight clinical isolates of *S. aureus* were identified by standard techniques (9). The MRSA strains were defined by in vitro resistance to 5-μg methicillin and 1-μg oxacillin disks, as well as by methicillin MICs of >12.5 μg/ml (8). The 22 MSSA isolates were all recent blood isolates from the Clinical Microbiology Laboratory of the Harbor-UCLA Medical Center; the 26 MRSA isolates (kindly provided by Francisco Sapico, Downey, Calif.) were also recent clinical isolates from a variety of body sites. There were no duplicate isolates from individual patients. The isolates were maintained on Mueller-Hinton (MH) agar slants until the susceptibility studies were performed.

Antibiotics. Vancomycin was supplied by Eli Lilly & Co. (Indianapolis, Ind.), and rifampin was provided by Merrill-Dow Research (Indianapolis, Ind.). Stock solutions of each agent at a concentration of 10,000 μg/ml were prepared and kept at −70°C until thawed on the day of the in vitro studies.

Antibiotic susceptibility testing. The MICs and MBCs of vancomycin and rifampin against these 48 isolates were determined by the microtiter broth dilution technique (14). Pilot MIC and MBC determinations in our laboratory, utilizing several study staphylococcal isolates, disclosed close agreement between results in the microtiter and macrobroth dilution methods (15). Fifty-microliter volumes of each antibiotic in twofold serial dilutions were added to a separate microtiter row of wells. A 50-μl sample of each MRSA or MSSA isolate grown in MH broth (MHB) to mid-logarithmic phase (~2 h) was added to each antibiotic-containing well and mixed without splashing by a microdiluter to achieve a final concentration of ~2×10^5 CFU/ml. The final drug concentrations in the wells were 0.007 to 16 μg/ml for both drugs. These concentrations were chosen to encompass readily attainable levels in serum for each agent at standard clinical dosages (1, 13). For each isolate tested, one microtiter well contained only the organism in antibiotic-free MHB as a growth control. After inoculation and gentle mixing, all plates were incubated for 24 h at either 30°C for

* Corresponding author.

TABLE 1. In vitro susceptibilities of 48 *S. aureus* strains to vancomycin and rifampin

Drug	Strain (no. tested)	MIC (μg/ml)[a]			MBC (μg/ml)[b]		
		Range	MIC_{50}	MIC_{90}	Range	MBC_{50}	MBC_{90}
Vancomycin	MRSA (26)	0.25–1	0.39	0.41	0.25–1	0.74	0.81
	MSSA (22)	0.5–1	0.7	0.94	0.5–2	0.7	0.97
Rifampin	MRSA (26)	0.007–8	2.18	4.52	0.007–16	8	14.4
	MSSA (22)	0.007–0.31	0.016	0.027	0.007–0.062	0.017	0.029

[a] MIC_{50}, MIC required to inhibit 50% of strains; MIC_{90}, MIC required to inhibit 90% of strains.
[b] MBC_{50}, MBC required to kill 50% of strains; MBC_{90}, MBC required to kill 90% of strains.

MRSA strains or 37°C for MSSA strains (3). The MIC was then read as the lowest antibiotic concentration yielding no visible turbidity. At this time, 25-μl samples were taken from all visibly clear wells and subcultured onto antibiotic-free MH agar. After a 24-h incubation at 30 or 37°C, the MBC was read as the lowest antibiotic concentration which yielded five colonies or less on subculture (≥99.9% killing).

Synergy testing. Each isolate to be tested was grown to logarithmic phase as before. Samples of this preparation were used in parallel for synergy testing in both checkerboard and timed-kill curve systems. For the timed-kill curve, the mid-logarithmic-phase preparation was appropriately diluted in antibiotic-containing MHB test tubes (10 ml) to achieve a final inoculum of $\sim 2 \times 10^5$ CFU/ml. The final drug concentrations in the antibiotic-containing tubes were: vancomycin alone, 10 μg/ml; rifampin alone, either 0.1 or 1 μg/ml depending on the rifampin MBC; and vancomycin, 10 μg/ml, plus rifampin, 0.1 or 1 μg/ml, to represent readily attainable levels in serum for each agent. The same inoculum was added to antibiotic-free MHB as a growth control. Samples (0.1 ml) from each tube were quantitatively subcultured onto MH agar at 0, 4, 24, and 48 h of incubation at either 30°C for MRSA strains or 37°C for MSSA strains. These timed subculture plates were then incubated at 30 or 37°C for 24 h, and the $\log_{10}$ CFU per milliliter of surviving MRSA or MSSA isolates was calculated for each sampling time. Killing curves were then constructed to determine the bactericidal interaction. In vitro bactericidal synergy was considered present when at least a 2 $\log_{10}$ decline in CFU per milliliter was achieved at 24 or 48 h by the drug combination, compared with that obtained by the most active single drug constituent (12). Indifference was defined as a <2 $\log_{10}$ change in CFU per milliliter at 24 or 48 h with the drug combination versus the single agents. Antagonism was considered present when a 2 $\log_{10}$ increase in CFU per milliliter occurred at 24 or 48 h with the drug combination versus both single drug constituents.

For checkerboard synergy testing, the microtiter broth dilution technique was employed (14). For vancomycin (vertical rows), the final drug concentration range in the microtiter wells was 0.31 to 2 μg/ml. For rifampin (horizontal rows), the drug concentration range was 0.0007 to 0.5 μg/ml for highly susceptible strains and 0.062 to 16 μg/ml for moderately susceptible strains. After appropriate dilutions, 50 μl of the logarithmic-phase inoculum of each isolate was added to 50 μl of antibiotic-containing MHB in each microtiter well to achieve a final inoculum of $\sim 2 \times 10^5$ CFU/ml. One well of the microtiter plate contained only the organism plus antibiotic-free MHB as a positive growth control. All plates were incubated at 30 or 37°C for 24 h, at which time 25-μl samples were subcultured from all clear wells with a micropipette and subcultured onto antibiotic-free MH agar. After a 24-h incubation of the subculture plates at 30 or 37°C, the number of viable CFU was determined. The MBC of each agent alone and in various combinations was defined, as before, as the lowest concentration yielding five or fewer colonies on subculture. The nature of the bactericidal interaction was determined by the shape of the isobologram (14), with synergy considered present when an inward-bowing curve was observed and antagonism considered present when an outward-bowing curve was observed. To confirm the isobologram results, the fractional bactericidal index was also calculated, with ≤0.5 considered synergy and >1.0 considered antagonism (14).

RESULTS

MIC and MBC determinations. The MIC and MBC data for the 48 *S. aureus* isolates versus the individual drugs used in subsequent synergy testing are depicted in Table 1. The 22 MSSA isolates were very susceptible to the in vitro inhibitory and killing action of rifampin, with all isolates having MICs and MBCs of ≤0.062 μg/ml. In contrast, the MRSA strains exhibited a broad range of rifampin MICs and MBCs in a bimodal distribution. Of 26 MRSA strains, 6 (23%) were very susceptible to rifampin, being inhibited or killed by 0.062 μg/ml, similar to the susceptibility data for MSSA strains. The remaining 20 MRSA strains (77%) were only moderately susceptible to the inhibitory and killing action of rifampin, with MICs or MBCs between 2 and 16 μg/ml. For vancomycin, the susceptibility data for both MRSA and MSSA strains were virtually identical, with all 48 isolates tested being inhibited or killed by ≤2 μg/ml, a readily attainable level in serum. There were no instances of in vitro tolerance to either vancomycin or rifampin.

Bactericidal interaction studies. Table 2 summarizes the results of in vitro synergy testing of the 48 *S. aureus* isolates versus vancomycin-plus-rifampin combinations. By timed-kill curve, 10 of 26 MRSA strains (39%) exhibited bactericidal synergy at either 24 or 48 h; however, only 3 of these strains were synergistically killed at both sampling times (Fig. 1). Similarly, 6 of 22 MSSA strains (27%) were synergistically killed at either 24 or 48 h, with only 4 of these

TABLE 2. In vitro bactericidal interactions of vancomycin plus rifampin versus 48 *S. aureus* strains

Strain	Results	Strains responding/total strains tested in:		
		Timed-kill curve system at:		Checkerboard system
		24 h	48 h	
MRSA	Synergy	5/26	8/26	0/26
	Antagonism	6/26	2/26	26/26
	Indifference	15/26	16/26	0/26
MSSA	Synergy	4/22	6/22	0/22
	Antagonism	1/22	0/22	22/22
	Indifference	17/22	16/22	0/22

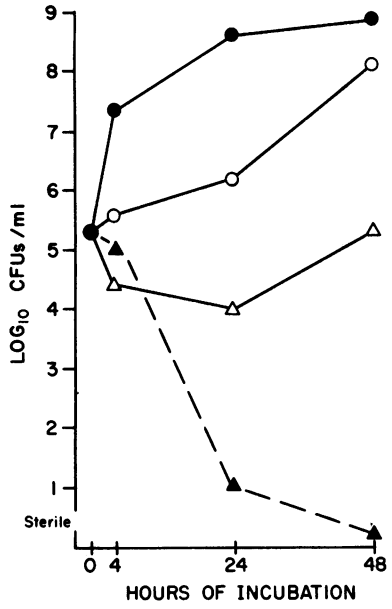

FIG. 1. In vitro bactericidal interactions of vancomycin plus rifampin by timed-kill curve versus MRSA strain 173. Symbols: ●, control; ○, rifampin (1 μg/ml); △, vancomycin (10 μg/ml); ▲, vancomycin (10 μg/ml) plus rifampin (1 μg/ml).

isolates exhibiting synergistic killing at both sampling times. The most frequent type of bactericidal interaction observed among these 48 *S. aureus* isolates by killing curve was indifference (Fig. 2), which was most commonly manifested as the prevention of development of rifampin resistance by vancomycin. Indifference occurred at a frequency of 58 to 62% for MRSA strains and 73 to 77% for MSSA strains, depending on the sampling times (Table 2). It is of note that in vitro bactericidal antagonism was relatively infrequent. For MRSA strains, rifampin antagonized the killing activity

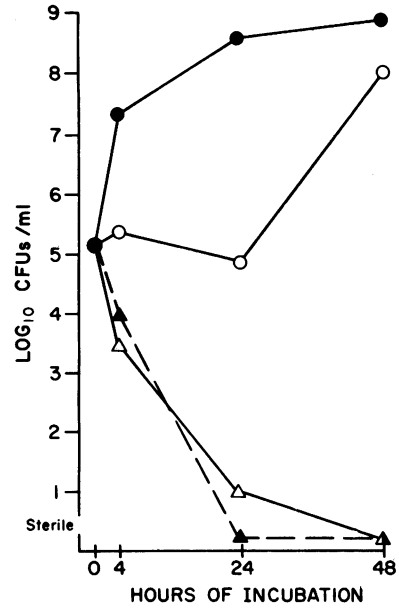

FIG. 2. In vitro bactericidal interactions of vancomycin plus rifampin by timed-kill curve versus MRSA strain 2, demonstrating indifference. Symbols: ●, control; ○, rifampin (1 μg/ml); △, vancomycin (10 μg/ml); ▲, vancomycin (10 μg/ml) plus rifampin (1 μg/ml).

of vancomycin at 24 h of incubation in 23% of interaction testing; however, with more prolonged in vitro incubation (48 h), such antagonism was seen in only two instances (8%). For MSSA isolates, bactericidal antagonism was observed in only one strain at 24 h of incubation, whereas with more prolonged incubation (48 h) of this strain with vancomycin plus rifampin, the bactericidal interaction was indifferent.

In contrast to the killing curve data, the bactericidal interaction seen with checkerboard testing of all *S. aureus* isolates was antagonistic. Isobolograms were outward bowing, with increases of vancomycin MBCs in the presence of rifampin (Fig. 3). Also, the fractional bactericidal indices were >1.0, confirming antagonism.

DISCUSSION

Vancomycin is the drug of choice in patients with infections due to MRSA, as well as the preferred therapy for systemic staphylococcal infections in patients with anaphylactoid reactions to β-lactam antibiotics (7, 19). In patients with life-threatening *S. aureus* infections requiring vancomycin therapy (such as MRSA endocarditis), the issue of adding rifampin to the antibiotic regimen often arises (19). Rifampin is an attractive agent in this regard for several reasons: (i) its in vitro inhibitory and killing activity against both MSSA and MRSA strains is excellent (20); (ii) its pharmacokinetics indicate rapid, high-level penetration into most body fluid cavities (1); and (iii) penetration of the drug into leukocytes and its bactericidal activity against intraleukocytic staphylococci is unsurpassed (11).

However, the in vitro and in vivo data concerning the efficacy of vancomycin-plus-rifampin combinations versus *S. aureus* have been conflicting. In the in vitro synergy study of Tuazon et al. (17), the addition of rifampin to vancomycin yielded partial synergy or an additive effect versus 30% of 20 MSSA strains by the checkerboard technique. In the remainder of the strains not showing an enhanced bactericidal effect, an indifferent interaction was observed; there were no examples of in vitro antagonism noted. In contrast, Watanakunakorn and Guerriero (20) found that rifampin antagonized the killing activity of vancomycin versus 100% of 20 MRSA strains and 77% of 30 MSSA strains by timed-kill curve. This latter study, however, utilized unconventional definitions of synergism and antagonism as ≥1 $\log_{10}$ decrease or increase in surviving colonies effected by the antibiotic combination versus the single-drug constituents. These definitions are in opposition to the definitions of synergy and antagonism in most other studies of at least a 2 $\log_{10}$ change in surviving colonies (12). Moreover, this study

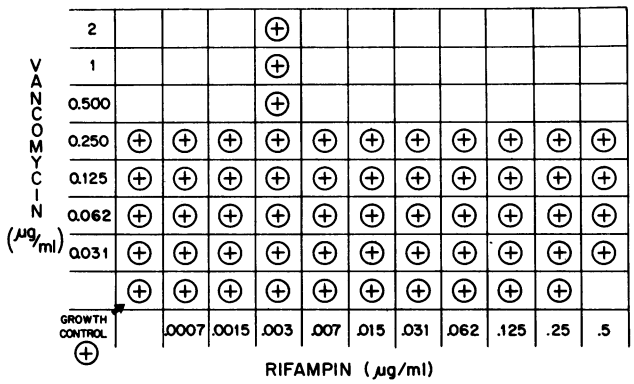

FIG. 3. In vitro bactericidal interactions of vancomycin plus rifampin by checkerboard technique versus MRSA strain 173. ⊕, More than five colonies on subculture.

utilized stationary-phase staphylococcal inocula, which may yield impaired killing by cell wall-active agents in comparison to logarithmic-phase cells (6).

Because of these conflicting in vitro data concerning the bactericidal interactions of vancomycin plus rifampin against *S. aureus*, we have systematically and simultaneously examined this interaction by two separate techniques (timed-kill curve and checkerboard). The present study delineated several interesting findings: (i) by timed-kill curve technique, rifampin generally had an indifferent effect on the killing action of vancomycin versus both MRSA and MSSA; (ii) this indifference was usually manifested as a prevention of development of rifampin resistance by vancomycin; (iii) bactericidal synergy was seen at a frequency of 27 to 39% of *S. aureus* strains tested by timed-kill curve, but these frequency figures were dependent on the sampling times chosen (24 versus 48 h); (iv) by timed-kill curve, an antagonistic interaction was infrequently observed, especially after 48 h of incubation of vancomycin plus rifampin; (v) in contrast to the killing curve data, the checkerboard technique uniformly demonstrated bactericidal antagonism of vancomycin plus rifampin versus these 48 strains.

Our finding of a poor correlation between the timed-kill and checkerboard systems for synergy testing versus *S. aureus* mirrors those reported by Norden et al. of cephalothin-plus-gentamicin interactions versus *Klebsiella pneumoniae* (14). In that study, as in ours, the timed-kill curve methodology demonstrated synergy significantly more often than did the checkerboard technique. Also, as in our study, the investigators noted that several strains found synergistically killed by timed-kill curve demonstrated antagonism by checkerboard technique. The lack of correlation between different methods of measuring synergy may be due to technical factors in the assays, as recently described by Norden et al. (14). These factors include inoculum effect, growth phase differences, and sampling times (6, 14). That these technical factors do play a significant role in the outcome of synergy testing is emphasized by the differing results of synergy testing in our study, as seen at 24 versus 48 h of incubation before subculture sampling.

A literature review of the in vivo efficacy of a combination of vancomycin plus rifampin in serious *S. aureus* infections also yields conflicting results. Levine and colleagues reported that of eight patients with MRSA endocarditis treated with vancomycin alone, six were cured, whereas two were lost to follow-up after apparent cure; however, of their seven patients receiving vancomycin plus rifampin, only three were cured, whereas three died (10). In contradistinction, Van Der Auwera et al. recently treated 13 patients with serious MRSA infections with either vancomycin alone or vancomycin plus rifampin (18). In their study, as opposed to the results of Levine et al., combination therapy with vancomycin plus rifampin was highly effective, curing all six patients. However, vancomycin failed to cure six of the seven patients receiving this agent alone ($P < 0.001$).

We conclude that the nature of the in vitro bactericidal interaction of vancomycin plus rifampin versus *S. aureus* is highly variable, and results are often disparate, depending on the methodology utilized. The in vivo significance of these in vitro observations awaits systematic in vivo studies of experimental MRSA and MSSA infections in animal models, as well as in controlled human clinical trials.

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