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Methicillin Potentiates the Effect of Gentamicin on Methicillin-Resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) strains show an unusual type of resistance at 37°C; only a small subpopulation of cells is resistant to the β-lactam antibiotics. Incubation in the presence of methicillin (MET), however, results in the emergence of a homogenous population highly resistant to MET. The purpose of the present study was to determine whether MET, despite its lack of killing effect on MRSA, would promote the bactericidal effect of gentamicin (GM), as demonstrated for MET-susceptible strains of *S. aureus*. Eleven epidemiologically distinct strains of MRSA were incubated with MET at various concentrations, in the presence or absence of sub-bactericidal concentrations (⅕ or ⅒ of the minimal bactericidal concentration) of GM, and tested for the synergistic action of both antibiotics by three different methods. Population analysis of the 11 strains in the presence of high concentrations of MET showed that the addition of GM at ⅕ or ⅒ of its minimal bactericidal concentration resulted in marked killing of the 11 strains. Time-kill curves obtained with 32 μg of MET per ml and GM at ⅕ or ⅒ of its minimal bactericidal concentration confirmed this synergistic killing at 24 h. These results were further documented by the checkerboard method on two strains. We conclude that the synergism between MET and GM, previously demonstrated for MET-susceptible *S. aureus*, holds true for MRSA as well and that it can be demonstrated at sub-bactericidal concentrations of GM.

Several studies published recently have conclusively shown that the bactericidal action of penicillins on *Staphylococcus aureus* can be increased by the concomitant administration of various aminoglycosides. Experiments demonstrating this potentiating effect were performed either by the checkerboard method or by the time-kill curve method, with gentamicin (GM) concentrations superior to individual minimal bactericidal concentrations (MBCs) (3, 22-24, 28, 29).

The explanation for the synergism between aminoglycosides and penicillins on staphylococci is as yet unknown. However, some information regarding a probably synergistic mechanism has been obtained with other organisms. Work by Moellering et al. on enterococci has suggested that cell wall modifications induced by the penicillins might allow better intracellular penetration and ribosomal binding of the aminoglycosides (7, 14, 31). The above-mentioned synergism between bactericidal concentrations of penicillins and aminoglycosides on *S. aureus* is reminiscent of such an effect and would be in agreement with this model.

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There are several experimental approaches by which such a model could be further tested for *S. aureus*. For instance, if penicillins modify the cell wall of these organisms without exerting a marked bactericidal effect, the hypothesis could be tested by adding GM to the system at small concentrations that by themselves would be inactive. If permeability changes are induced by the penicillins in the absence of a bactericidal effect, one should observe a clear-cut bactericidal effect mediated by GM at these low concentrations.

Methicillin (MET)-resistant, GM-susceptible organisms offer a unique opportunity to test this hypothesis, since an 18-h incubation with high doses of MET does not reduce the number of these organisms, but nevertheless alters their cell walls, as evidenced by their decreased susceptibility to lysostaphin (19-21). We therefore submitted 11 strains of methicillin-resistant *S. aureus* (MRSA) to well-defined, sub-bactericidal concentrations of GM and investigated the potentiating effect of high concentrations of MET. Our results show that, in spite of its lack of killing action, MET favors the bactericidal effect of GM at these low sub-bactericidal concentrations. These results are consistent with

the hypothesis of an increased cell wall permeability induced by MET. Furthermore, our results show that the synergism between MET and GM also applies to MRSA at concentrations well below the therapeutic levels, offering possibly a new treatment rationale for infections due to these organisms.

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MATERIALS AND METHODS

***S. aureus* strains.** The 11 *S. aureus* strains used were obtained from various microbiology laboratories in different countries. Their MET resistance was demonstrated by the disk diffusion method at 30 and 37°C, by the disk diffusion method with addition of 5% NaCl, and by the agar dilution method (13, 27). All belonged to phage group III and most to serotype 18 (18). The 11 strains were plasma coagulase and catalase positive and produced yellow pigment on blood agar plates. They showed various antibiotic susceptibility patterns (4), but were all susceptible to GM according to the disk diffusion and agar diffusion methods.

All 11 strains of MRSA were repeatedly tested as to their MET resistance in Mueller-Hinton broth (Difco), using an initial inoculum of 10^6 organisms per ml. Minimal inhibitory concentration (MIC) values ranged from 32 to more than 256 μg/ml, whereas MBC values were all above 256 μg/ml. All 11 strains showed normal growth at 256 μg/ml when the agar dilution method was used.

Antibiotics. MET, GM, kanamycin, tobramycin, and amikacin were commercially obtained. Sisomicin standard powder was a gift from Schering Corp. All antibiotics were dissolved in Mueller-Hinton broth, and solutions were used fresh.

GM MICs and MBCs. Tube dilution susceptibility tests were performed on each MRSA with Mueller-Hinton broth. To insure minimal variations in Ca and Mg concentrations, all experiments were performed in Mueller-Hinton broth prepared from the same batch of powder (no. 606130). GM concentrations ranged from 0.25 to 8 μg/ml in a volume of 2 ml. Inocula of 10^6 colony-forming units (CFU) per ml were prepared from an overnight incubation in 5 ml of Mueller-Hinton broth at 37°C. Control tubes without antibiotic were included in each series of dilutions.

After incubation for 18 to 20 h, the MIC was recorded, and 0.1-ml samples were subcultured from each clear tube on Mueller-Hinton plates. After 24 h of incubation, the MBC was determined, defined as the lowest concentration of GM that resulted in a killing of at least 99.9% of the initial inoculum. Carry-over effect of GM was ruled out as shown below.

Population analysis in the presence of MET and MET plus GM. MET resistance of *S. aureus* was determined by population analysis as proposed by Chabbert et al. (6). Each strain was added, at an initial concentration of 10^6 organisms per ml, to Mueller-Hinton tubes containing increasing concentrations of MET, ranging from 0.25 to 256 μg/ml. Inocula, antibiotic dilution, incubation time, and control tubes were used as described above. After 18 h of incubation, residual CFU were counted after appropriate dilutions and 24 h of subculture on Mueller-Hinton plates. The sensitivity of the method allowed the counting of the residual CFU up to a 99.999% killing of the initial inoculum.

In simultaneous experiments, population analysis of our 11 strains of MRSA was carried out in the presence of the same increasing concentrations of MET, but with the addition of GM (or another aminoglycoside) at 1/10 of its MBC (for the strains whose MBC was higher than 1 μg/ml) or 1/5 of its MBC (for the strains whose MBC was 1 μg/ml). Control experiments showed that these low concentrations of GM administered alone did not decrease the number of CFU after an 18-h incubation.

Time-kill curves. Time-kill curves were performed on the 11 MRSA strains. For each strain, four time-kill curves were simultaneously performed: one control without antibiotic, one with 32 μg of MET per ml (a concentration more than twice the generally accepted cutoff point of 12.5 μg/ml for MRSA [8, 11, 21]), one with GM at 1/10 or 1/5 of its MBC, and one associating MET and GM at the above concentrations. An inoculum of 10^6 organisms per ml was added to each 4-ml incubation culture; the tubes were incubated at 37°C, and 0.1-ml portions were removed from each incubation mixture at 2, 4, 6, 12, and 24 h, diluted in sterile distilled water, and plated on Mueller-Hinton plates for CFU counts.

Checkerboard method. Two strains were tested by the two-dimensional dilution checkerboard method, using Mueller-Hinton broth and an inoculum of 10^6 organisms per ml. Concentrations of MET ranging from 0 to 128 μg/ml, of GM ranging from 0 to 0.5 μg/ml, and all possible combinations thereof were used. The MIC was recorded after incubation for 18 h, and the MBC was read after subculturing 0.1 ml of each clear tube for 24 h on Mueller-Hinton plates.

Exclusion of carry-over effect. Control experiments were carried out on seven strains of MRSA to exclude a carry-over effect of the antibiotics onto the Mueller-Hinton plates, which could have led to an artificial decrease in the number of residual CFU. A solution (0.1 ml) of 128 μg of MET per ml and GM at the highest concentrations used in our experiments was spread at 1/1, 1/10, and 1/100 dilutions on Mueller-Hinton plates. Thereafter, 10^2 MRSA were added to each agar plate with absorbed antibiotic solution and to a control plate. All plates showed identical numbers of residual CFU after a 24-h incubation, thus ruling out any significant carry-over effect mediated by the antibiotic solutions.

Exclusion of osmotic lysis by the diluting solutions. Diluting the samples with distilled water before the plating on Mueller-Hinton plates could have resulted in lysis of some microorganisms, since they had been previously incubated with MET. Such an effect was ruled out by testing three strains in four different diluting solutions, i.e., distilled water, Mueller-Hinton broth, 0.9% NaCl, and iso-osmotic 0.6 M sucrose. No difference in the number of residual CFU was observed.

RESULTS

Determination of GM MICs and MBCs against MRSA. The MICs and the MBCs obtained for the 11 strains of MRSA are shown in Table 1. Five strains showed an MBC of 1.0 μg/ml, and all the 11 strains were susceptible to 4.0 μg/ml or less. Unlike other investigators, we did not observe any strain outgrowing these concentrations after 24 h under our experimental conditions (16, 22, 30). To rule out a possible development of progressive GM resistance, several strains had their MBC redetermined periodically; no change in the MIC or in the MBC could be demonstrated on repeated incubation in the presence of GM.

Population analysis in the presence of antibiotics. Population analysis was used as the method of choice to test 11 strains of MRSA in the presence of increasing concentrations of MET, since it has been shown that for a given strain high concentrations of MET can kill part of this heterogenous population of microorganisms. MET had no bactericidal effect at all at 18 h on 10 of 11 strains at a concentration of 32 μg/ml and had a modest effect on strain 69 (Fig. 1). It should be remembered that most strains of *S. aureus* are susceptible to <1 μg of MET per ml and that the cutoff point of MET resistance has been set by most authors at 12.5 μg/ml (8, 11, 21). As demonstrated in Fig. 1 and as shown by other authors (4), even very high concentrations of MET (256 μg/ml) were unable to eradicate all the microorganisms after an 18-h incubation, the number of residual CFU being, with one exception, higher than 10^4/ml. In summary, only very high concentrations of MET were able to affect a small subpopulation of MRSA.

To investigate whether this absence of any significant antibacterial effect induced by MET was nevertheless accompanied by an increased

TABLE 1. *MICs and MBCs of GM for 11 MRSA strains*

MRSA strain	MIC (μg/ml)	MBC (μg/ml)[a]
13	0.5	1.0
15	0.5	1.0
50	1.0	1.0
56	0.5	1.0
63	0.5	1.0
12	0.5	2.0
45	0.5	2.0
69	0.25	2.0
3	1.0	4.0
72	1.0	4.0
Nov	1.0	4.0

[a] Defined as a >99.9% decrease in CFU after 18 h of incubation.

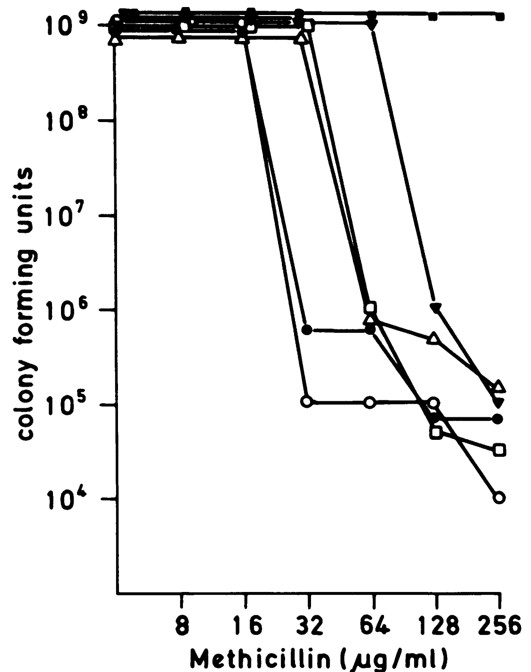

FIG. 1. *Population analysis of the 11 strains of MRSA after 18 h of incubation in Mueller-Hinton broth in the presence of increasing concentrations of MET. Symbols: (■) strains Nov and 3; (△) strains 13 and 45; (▲) strains 12 and 50; (●) strains 15, 63, and 72; (□) strain 56; (○) strain 69.*

susceptibility of MRSA to GM, population analyses in the presence of MET were run in parallel with or without GM. The GM concentration chosen was ⅕ to ⅒ of the MBC as determined in Table 1. Figure 2 shows a typical result of such a population analysis performed in the presence of MET, with or without sub-bactericidal concentrations of GM. The addition of 0.2 μg of GM per ml resulted in a marked shift of the profile towards the left and towards the base line. The first antibacterial effect was no longer detected at 128 μg of MET per ml but at 4 μg/ml, and the lowest number of residual CFU per milliliter dropped from 5×10^4 to 10^1 under the new experimental conditions.

The combined effects of sub-bactericidal concentrations of GM on the bacterial population incubated with high doses of MET were therefore twofold and could be expressed by two factors, f_1 and f_2; f_1 measures the displacement of the profile to the left, and f_2 measures the displacement of the profile towards the base line. These two factors were therefore defined as follows. f_1 = MET dose showing first demonstrable decrease in CFU at 18 h without GM/MET dose showing first demonstrable decrease in

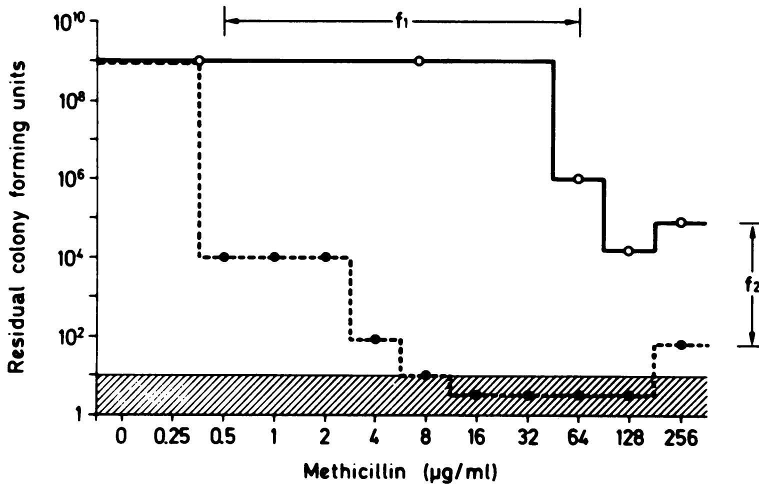

FIG. 2. *Population analysis of MRSA strain 50 in Mueller-Hinton broth after incubation with increasing concentrations of MET without* (○) *and with* (●) *GM at ⅕ of its MBC (0.2 µg/ml). The hatched area shows the limit of the sensitivity of the method. For definition of factors* f_1 and f_2 see the text.

CFU at 18 h with GM; f_2 = number of residual CFU after 18 h with MET/number of residual CFU after 18 h with MET and GM. Values above 1 for f_1 and f_2 therefore denote an additional antibacterial effect mediated by GM.

Table 2 summarizes the results obtained with 11 strains of MRSA incubated either with MET or with MET and sub-bactericidal concentrations of GM, expressed by the two factors, f_1 and f_2. For all strains, there was a marked enhancement of the bactericidal effect of GM at sub-bactericidal concentrations; factor f_1 ranged from 8 to 256 and more, and f_2 ranged from 10^2 to 10^8.

Time-kill curves. To confirm these results, to demonstrate that the observed effects were fulfilling the criteria of synergism, and to get some insight into the kinetics of this combined antibiotic effect, time-kill curves were performed on the 11 strains of MRSA. Figure 3 shows the mean values and the standard error of the mean obtained with the 11 strains in the presence of MET (32 µg/ml), GM (⅕ to ⅒ of the MBC, i.e., 0.2 to 0.4 µg/ml), and both. As shown previously, high concentrations of MET exerted a partial, transient killing action which could be observed during the first 6 h of incubation (6). GM at ⅒ or ⅕ of its MBC showed a similar decrease in the residual CFU. At 12 h of incubation, growth had resumed, achieving eventually the values observed in the controls. Addition of MET (32 µg/ml) to GM at 0.2 to 0.4 µg/ml resulted in a marked killing effect at 12 and 24 h, respectively, for all 11 strains tested. Synergism, defined as a more than 2-log difference in residual CFU, could be demonstrated with MET and GM at 24 h of incubation. Care was taken to rule out possible growth of dwarf or deficient colonies by keeping and checking the plates after prolonged incubation.

TABLE 2. *GM plus MET effect on 11 MRSA strains, expressed by factors* f_1 *and* f_2[a]

No. of MRSA strains	f_1	f_2[b]
2	>256	10^8, 10^6
1	256	10^3
1	64	2×10^2
4	32	10^2 (1), 10^3 (2), 10^4 (1)
2	16	2×10^4, 5×10^2
1	8	10^2

[a] For formulas and further details, see the text.
[b] Parentheses indicate number of strains.

Checkerboard method. Isobolograms were obtained by testing two strains according to the checkerboard method. Both curves obtained showed synergism according to the criteria of Barry and Sabath (3).

Additional experiments with other aminoglycosides. To demonstrate that the bactericidal effect of GM at these low concentrations could be reproduced with other aminoglycosides, the same experiments were repeated with GM, kanamycin, amikacin, tobramycin, and sisomycin, after determination of their respective MICs and MBCs. Strain 69 was chosen for these studies; the results of the population analysis in the presence of MET without or with other aminoglycosides at ⅒ of their MBC are summarized in Table 3. Although the effect was maximal

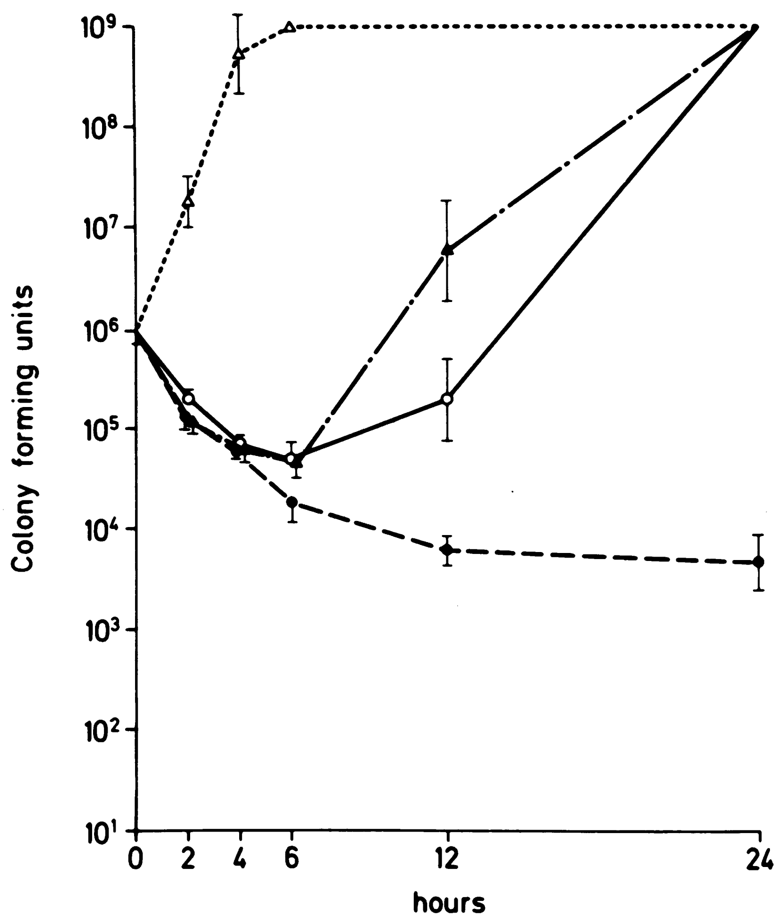

FIG. 3. *Time-kill curves obtained with MRSA in the presence of MET (32 µg/ml), GM (⅛ or 1/10 of MBC), or both. Increased killing in the presence of MET and GM could be demonstrated at 12 and 24 h, and synergism (>2-log decrease in CFU) could be demonstrated at 24 h. Each curve represents the mean ± standard error of the mean of 11 strains tested. Symbols:* (△) *control;* (○) *MET;* (▲) *GM;* (●) *MET + GM.*

with GM, it could be reproduced with all four aminoglycosides tested; the number of residual CFU was reduced by 2×10^4 by concentrations as low as 0.2 µg of sisomycin or 0.8 µg of amikacin per ml.

Demonstration of synergism at 30°C. Although MET resistance is heterogenous at 37°C, this phenomenon affects all cells of a given strain when incubation is performed at 30°C (1, 9, 10, 26). In fact, this property forms the basis of the demonstration of MET resistance in the clinical laboratory (13). It was therefore of interest to repeat the above experiments at an incubation temperature at which MET would be unable to exert any bactericidal effect at all. Strain 69 was again chosen for these experiments, and time-kill curves, performed at 37 and 30°C in the absence of antibiotics or in the presence of MET (32 µg/ml), GM (0.2 µg/ml), or both, confirmed the synergism observed at 37°C.

TABLE 3. *Bactericidal effect on MRSA strain 69 of various aminoglycosides at sub-bactericidal concentrations in the presence of MET*

Antibiotics (concn,[a] µg/ml)	f_1	f_2
MET + GM (0.2)	32	2×10^4
MET + KM (0.8)	8	2×10^4
MET + AK (0.8)	8	2×10^4
MET + TM (0.2)	4	1×10^3
MET + SM (0.2)	8	2×10^4

[a] All aminoglycoside concentrations are 1/10 of MBC, MBC being defined as a >99.9% decrease in CFU after 18 h of incubation. KM, Kanamycin; AK, amikacin; TM, tobramycin; SM, sisomycin.

DISCUSSION

MRSA strains show an unusual type of resistance towards β-lactam antibiotics at 37°C: only a subpopulation of microorganisms is resistant to MET, and incubation of 10^6 organisms per ml

in the presence of this antibiotic results in an initial decrease of the cell population (due to the killing action of the MET on the susceptible subpopulation), followed by a progressive increase of the bacterial counts (due to the emergence of MET-resistant variants) (2, 6, 8, 25). Depending on the experimental conditions, this initial decrease in the cell population can be minimized, and in our time-kill curves it never affected more than 1 log of our cell population after 6 h of incubation. The end result is the emergence of a homogenous population, highly resistant to MET, but with biochemical cell wall modifications as described by Sabath et al. (21).

The purpose of the present study was twofold. First, we wanted to investigate whether MET, in spite of its lack of killing action, might favor the intracellular accessibility of GM, an observation that would be in favor of a possible induction of increased cell wall permeability. Second, and more pragmatically, we wanted to find out whether the synergism between MET and various aminoglycosides, which has been previously described on MET-susceptible *S. aureus,* also held true for MRSA (22-24, 28, 29).

Our results show that MET at 32 μg/ml, a concentration at which it remained without effect on 10 of the 11 strains of MRSA tested, potentiates the bactericidal effect of GM at concentrations as low as 0.2 to 0.4 μg/ml, i.e., at ⅕ or ⅒ of its MBC determined in the same medium. These observations illustrating synergism between the two agents were obtained with three different methods proposed by Barry and Sabath (3): (i) population analysis in the presence of increasing concentrations of MET, with or without sub-bactericidal concentrations of GM, showed marked killing of the 11 strains when incubated with both antimicrobial agents; (ii) time-kill curves performed on 11 strains confirmed the synergism after 24 h of incubation; and (iii) serial dilutions performed with the checkerboard method on two strains of MRSA, for which a measurable MET MBC could be used as an end point, demonstrated similar evidence for synergism. The observed effects were not specific for GM, since we were able to reproduce them with other aminoglycosidic antibiotics such as kanamycin, amikacin, sisomycin, and tobramycin. These results are in accordance with recent data obtained with other aminoglycosides used at higher concentrations on MET-susceptible *S. aureus,* as demonstrated by Watanakunakorn and Glotzbecker (29).

A major technical aspect pertaining to our experiments should be discussed. Since high doses of MET were used in our incubation experiments, a carry-over effect could have occurred when MET-containing broth was subcultured onto Mueller-Hinton plates, thereby decreasing artificially the number of residual CFU. In some preliminary experiments, indeed, we noticed a carry-over effect on Mueller-Hinton agar plates in the presence of MET- and GM-susceptible *S. aureus,* when the subcultured Mueller-Hinton broth contained more than 64 μg of MET or 8 μg of GM, respectively, per ml (unpublished data). We therefore eliminated this problem by 10- and 100-fold dilutions and appropriate plating experiments. Experiments performed with distilled water, 0.9% NaCl, Mueller-Hinton broth, and 0.6 M sucrose as the diluting solutions showed identical results.

Synergism between MET and various aminoglycosides on *S. aureus* has so far been demonstrated with MET-susceptible strains and/or concentrations of antibiotics that were, either one or both, bactericidal in their own right (22, 28). Thus, in a recent study, synergism between nafcillin or oxacillin and sisomicin or netilmicin was demonstrated at aminoglycoside concentrations equal to or higher than their MBCs. Similar concentrations have been used in animal models as well (22). Our experiments expand this previous information by showing that synergism is possible for MRSA as well and that it can be demonstrated with very low, sub-bactericidal GM concentrations. They therefore provide experimental in vitro data in favor of a combined therapeutic approach to infections caused by MRSA.

Several authors have obtained similar results with enterococci, where synergism has been demonstrated by combining penicillin with aminoglycosides at concentrations inferior to the previously determined MIC (15). Since solid experimental data obtained with enterococci have permitted the theory that synergism is due to cell wall permeability changes (14, 31), a similar mechanism could be postulated for *S. aureus.* MRSA strains offer an indirect way to test this hypothesis, since their survival is little affected during incubation with high concentrations of MET, in spite of cell wall modifications (19-21). Such a hypothesis is indeed supported by our observations showing that incubation of MRSA during 18 h with MET produces no net bactericidal effect, but results in an increased susceptibility of the microorganisms to sub-bactericidal concentrations of GM. It could be argued that, since our time-kill curves in the presence of MET still show a small but finite decrease in the cell population at 2, 4, and 6 h, the high susceptibility to GM observed might involve only a small, new, actively dividing cell subpopulation. However, synergism between MET and GM was observed at 24 h, when the initial decrease in cell population had been largely compensated

for; moreover, time-kill curves performed at 30°C, a temperature at which MET at concentrations up to 50 μg/ml has no effect at all on the cell population (1, 10, 17), confirmed that active growth of a subpopulation was not a prerequisite for the expression of synergism.

Time-kill curves performed with sub-bactericidal concentrations of GM showed an initial decrease and subsequent rise of the *S. aureus* population. This surprising phenomenon has already been described by Sande and Courtney (22), but on MET-susceptible strains and at higher GM concentrations, so that it could be attributed to the development of GM-resistant strains (12). Such an explanation was ruled out in our experiments, since we submitted the strains of MRSA incubated for 20 h in GM to a new susceptibility test; no change in MBC or appearance of variant forms was noticed, as described by authors using higher GM concentrations (22, 30). Rapid uptake of GM by the bacteria with intracellular accumulation and delayed bactericidal effect after subculturing seems to be a more likely explanation. Such a hypothesis is supported by some recent evidence of rapid intracellular uptake of GM by *Pseudomonas aeruginosa* and *Escherichia coli* (5).

Our results expand previous information on the synergism between beta-lactam antibiotics and aminoglycosides by showing that it applies also to MRSA and that it can be demonstrated at very low, sub-bactericidal concentrations of the aminoglycosides. Population analysis and time-kill curves offer in vitro support for the use of this combination in infections due to MRSA, which respond poorly to beta-lactam antibiotics (6). Finally, these experiments offer indirect evidence for possible increased uptake of GM by MRSA whose cell walls have been modified by MET, as demonstrated by Sabath et al. (20, 21).

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