Bactericidal activity of vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA)

—Comparison with minocycline and ofloxacin within therapeutic levels, and factors determining their efficacy—

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Bactericidal activity of vancomycin against clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA) was assessed by time-kill curve studies. Vancomycin showed a superior bactericidal effect in comparison with minocycline or ofloxacin against all tested atrains within its therapeutic levels. Vancomycin, at concentrations above 3.13 μg/ml, was bactericidal after 24 h incubation, but the effect was weak within 6 h incubation even at higher concentrations. These results suggest that the bactericidal activity of vancomycin was critically dependent on the length of incubation, but was not dependent on its concentrations. The bactericidal activity of vancomycin was also found to be markedly influenced by the inoculum size of MRSA. From these findings, we conclude that the concentration of vancomycin in serum should be maintained in 2—4 times excess of MIC round the clock during systemic infusion and that the size of bacterial growth *in vivo* should be carefully assessed in every clinical case where its use against MRSA infection is considered.

Key words: MRSA, vancomycin, time-kill curves, time-dependency, inoculum size

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen causing severe morbidity and mortality worldwaide[1]. The first strains of MRSA were detected in 1961, shortly after methicillin had been introduced into clinical use[2]. These strains occurred sporadically, were resistant only to β-lactam antibiotics, and caused no major problem[3] until 1970 s when frequent nosocomial outbreaks of multiresistant MRSA in the United States caused a serious concern[4~6].

In Japan, a striking increase of isolates of MRSA from clinical specimens bacame an acute concern in 1980 s. Most of these strains were sensitive to minocycline or fluoroquinolons in early 1980 s[7]. However, treatment with these drugs for severe infection often ended in failure. A striking increase of the clinical strains resistant to minocycline or fluoroquinolons was also detected in late 1980 s[8,9]

In contrast, all strains isolated to date proved to be sensitive to vancomycin[8,9]. In the United States and Europe, systemic infusion of vancomycin has been used for the treatment of MRSA infections for over a decade[10~12], but a general agreement as to the optimal regimen for vancomycin therapy is yet to be attained[12]. Resistance to vancomycin has not been reported in *S. aureus* worldwide[1,10,13~15], while serious adverse effects with the use of vancomycin have been noted[12,16,18]. The following studies were performed to find theoretical grounds for a better use of vancomycin for the treatment of severe infections due to MRSA.

MATERIALS AND METHODS

Bacterial strains: Ten Clinical strains of *S. aureus*

isolated from blood cultures in our University Hospital during the recent 2 years were used. All these strains were resistant to methicillin (MIC≥ 100 μg/ml), sensitive to minocycline (MIC≤0.20 μg/ml), ofloxacin (MIC≤0.78 μg/ml), and vancomycin (MIC: 0.78−1.56 μg/ml).

Medium: Cation (Ca^{2+}, Mg^{2+})-supplemented Mueller-Hinton broth (MHB, Difco) was used in all experiments.

MICs of these strains were determined by the microdilution broth method[19].

Killing-curve tests: Studies were performed essentially by the previously described techniques[20]. All tests were done with a final volume of 10 ml of medium. Cultures of the strains and antibiotics were incubated in L-shaped glass tubes at 35°C with shaking. Glowth control tubes (medium and organism only) were also incubated. The measurement of CFU was performed at least in triplicate. Samples from each tube were collected at 0, 6, 24, and 48 h of the incubation. Colonies were counted by removing 0.1 ml of the culture with a micropipette to make serial 10-fold dilutions using sterile cold 0.85% NaCl. Aliquots of 0.1 ml of the appropriate dilution were plated onto Mueller-Hinton agar plates using a spread plate technique. Plates were incubated at 35°C for 48 h, and CFU of each plate was counted. CFU below 10^3 CFU/ml was judged as negligible. Bactericidal effect was defined as reduction of CFU by greater than 99.9% of the inoculum size.

Concentrations of each antibiotic were as follows: minocycline, 10, 2.5, (0.625) μg/ml, ofloxacin, 10, 2.5, 0.625 μg/ml. and vancomycin, (400), 100, 25, 6.25, 3.13, 1.56, 0.78, 0.39 μg/ml. Based on the previous data the peak serum concentration of each drug was assumed to be as follows: minocycline, 10 μg/ml (100 mg drip infusion)[21], ofloxacin, 2.5 μg/ml (100 mg per os)[22], and vancomycin, 25 μg/ml (500 mg drip infusion)[12,23]

The inoculum size of bacteria was increased from 10^6 CFU/ml to 10^8 CFU/ml in five strains for evaluation of the effect of inoculum size on antibacterial activity. All studies were performed by both logarithmic-phased and stationary-phased cultures, respectively.

RESULT

1. Antibiotic susceptibility of MRSA

Among strains of MRSA isolated in our University Hospital from 1987 to 1989 (N=781), the ratio of the resistant (MIC ≥6.25 μg/ml) strains to minocycline was approximately 60%, and that to ofloxacin approximaterly 50%. We have not encountered a single strain resistant to vancomycin. More than 80% of the strains were resistant to other multiple antibiotics[9]. On the basis of these results, we compared the bactericidal activity of vancomycin with those of minocycline and ofloxacin by using clinical strains known to be sensitive to all these three drugs.

2. Comparison of bactericidal effect of vancomycin, minocycline and ofloxacin within therapeutic levels

Fig. 1 shows the results of time-kill curves of two different strains. In the case of *S. aureus* No. 880, minocycline, at concentrations of both 10 and 2.5 μg/ml, gradually decreased the CFU during 48 h incubation, without reducing it below 10^4 CFU/ml.

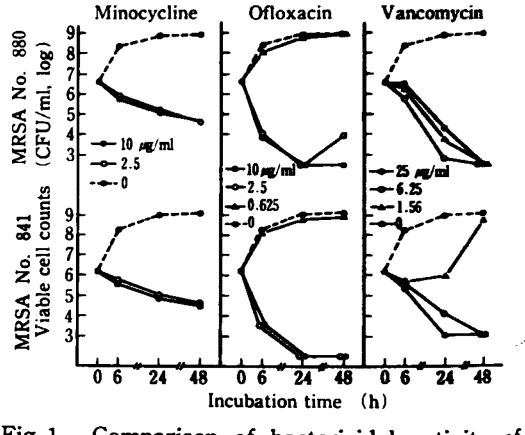


Fig. 1. Comparison of bactericidal activity of minocycline, ofloxacin, and vancomycin against MRSA. Time-kill curves for 2 different strains are shown. Concentrations of each antibiotic are as follows: of minocycline 10 μg/ml (—●—), and 2.5 μg/ml (—○—), of ofloxacin, 10 μg/ml (—●—), 2.5 μg/ml (—○—), and 0.625 μg/ml (—▲—), and of vancomycin, 25 μg/ml (—●—), 6.25 μg/ml (—○—), and 1.56 μg/ml (—▲—). Broken lines (···●···) show bacterial growth control.

Ofloxacin, at concentrations of 10 and 2.5 μg/ml, rapidly reduced the CFU, but subsequent re-increase of the CFU was apparent at a concentration of 2.5 μg/ml. Vancomycin at all tested concentrations showed an excellent bactericidal effect at 24 h incubation, but the effect was weak at 6 h incubation. The results obtained with *S. aureus* No. 841 were a little different. Ofloxacin, at a concentration of 2.5 μg/ml, reduced the CFU as rapidly as at a concentration of 10 μg/ml without a re-increase of the CFU. Furthermore, vancomycin at a concentration of 1.56 μg/ml, only weakly decreased the CFU at 6 h incubation, and a re-increase of the CFU was observed at 24 h incubation.

In Fig. 2 are shown the summary of the results of the study comparing the bactericidal activity of vancomycin with that of minocycline or ofloxacin using ten strains. While vancomycin at concentrations above 3.13 μg/ml, was bactericidal against all tested strains after 24 h incubation, the effect was weak at 6 h incubation. In contrast, minocycline decreased the CFU gradually through 48 h incubation, but bactericidal effect was not attained in any strain. No difference of antibacterial effect was detected between the concentration of 10 μg/ml and 2.5 μg/ml of minocycline. We also tested the activity at a concentration of 0.625 μg/ml, and the results were identical (data were not shown). Ofloxacin, at a concentration of 10 μg/ml, rapidly reduced CFU of all strains. However, at a concentration of 2.5 μg/ml, a re-increase of CFU was detected at 24 h incubation in 4 of the 10 strains. At a concentrations of 0.625 μg/ml, the reduction of CFU was limited to only 4 of the 10 strains, and subsequent re-increase of CFU was apparent in these strains.

3. Effect of concentration of vancomycin on bactericidal activity against MRSA

Results of study using one strain are shown in Fig. 3. Identical bactericidal activities were observed at concentrations above 1.56 μg/ml, whereas, no effect on bacterial growth was observed at a concen-

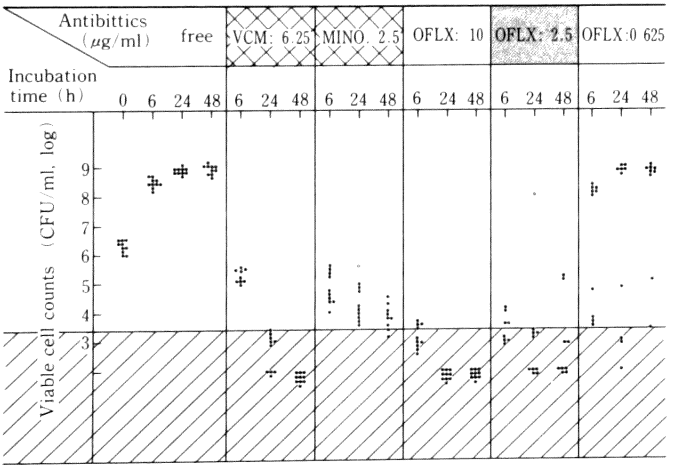


Fig. 2. Comparison of bactericidal activity of minocycline, ofloxacin, and vancomycin within therapeutic levels against MRSA. Summary of the results of the study with 10 different strains is shown. Each dot indicates CFU measured on each time of one strain, incubated with each antibiotic at the indicated concentrations. Dots of the line of antibiotics free indicate bacterial growth control for each strain. ▨: area of reduction of inoculum size more than 99.9%. ■: expected peak serum level (C_{max}), ▩: expected 1/4 C_{max} (avarege serum level) of each drug.

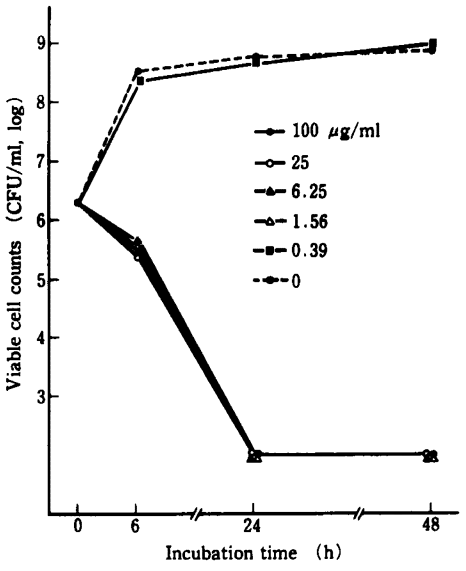
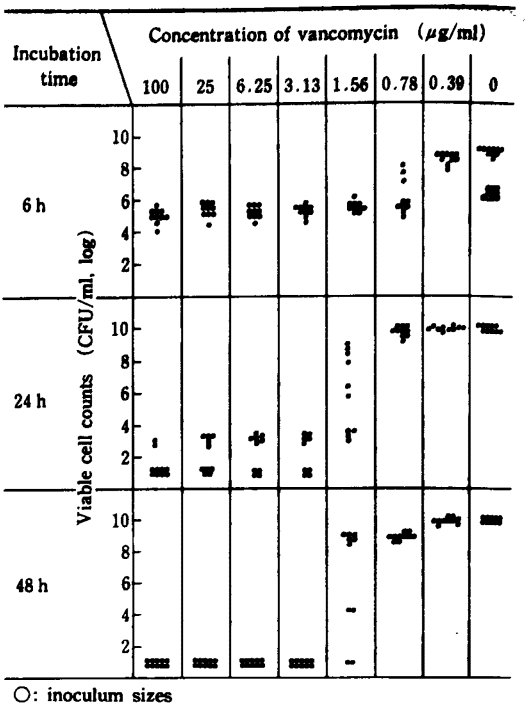


Fig. 3. Effect of concentration of vancomycin on bactericidal activity against MRSA. An exemplary strain is shown. Concentrations of vancomycin are as follows, 100 μg/ml (—●—), 25 μg/ml (—○—), 6.25 μg/ml (—▲—), 1.56 μg/ml (—△—), and 0.39 μg/ml (—■—). Broken line (···●···) shows a bacterial growth control.



○: inoculum sizes

Fig. 4. Effect of concentration of vancomycin on bactericidal activity against MRSA. Summary of the result of the study with 10 different strains is shown. Each closed circle indicates CFU measured on each time of each strain, incubated with vancomycin at the indicated concentrations. Open circles indicate inoculum size of each strain.

tration of 0.39 μg/ml.

Fig. 4 shows the summary of the results of study with the 10 strains. There was no difference of bactericidal activities at concentrations above 3.13 μg/ml in all strains. We also tested activity against 5 of the 10 strains at a concentration of 400 μg/ml, and the activities were identical (data were not shown). At a concentration of 1.56 μg/ml, the same effects were observed in 4 of the 10 strains. In the remaining 6 strains, a weak suppression of CFU was seen at 6 h incubation, but a re-increase of CFU was apparent at 24 h incubation. At concentrations below 0.78 μg/ml, there was no suppression on the growth of any tested strain.

4. Effect of inoculum size on bactericidal activity of vancomycin against MRSA

Fig. 5 shows a representative result obtained using one strain. No significant difference was detected between the inoculum size of 10^7 and 10^6 CFU/ml. However, when inoculum size was increased to 10^8 CFU/ml, a marked reduction of bactericidal activity was observed. The results of 5 different strains were identical (data were not shown).

We also tested the effect of inoculum size on the bactericidal activity of minocycline and ofloxacin against the same 5 strains. In Fig. 6 are shown the results of study comparing the antibacterial activities of minocyclin, ofloxacin and vancomycin using a strain different from the one presented in Fig. 5. With an inoculum size of 10^8 CFU/ml, no drug demonstrated bactericidal activity.

All these results were obtained with logarithmic-phased cultures of the tested strains. All experiments were repeated using the stationary-phased cultures of the same strains, and the results were

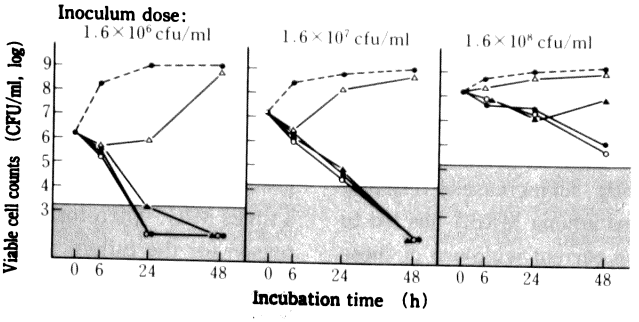


Fig. 5. Effect of inoculum size on bactericidal activity of vancomycin against MRSA. Time kill-curves of an exemplary strain is shown at different inoculum size 10^6, 10^7, and 10^8 CFU/ml. Concentrations of vacomycin are as follows: 100 μg/ml (—●—), 25 μg/ml (—○—), 6.25 μg/ml (—▲—), and 1.56 μg/ml (—△—). Broken lines (···●···) shows control growth curves. (■) is an area of reduction of inoculum size more than 99.9%.

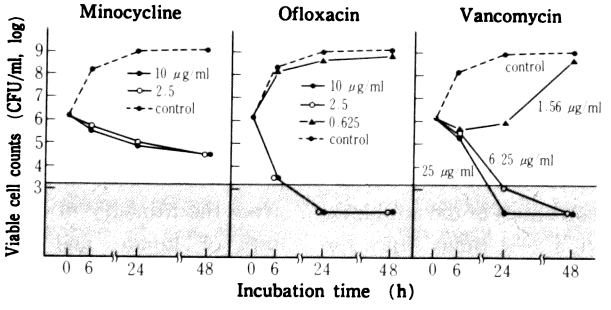


Fig. 6. Time-kill curves for minocycline, ofloxacin and vancomycin at the inoculum size of 10^8 CFU/ml. Concentrations of minocycline are 10 μg/ml (—●—), and 2.5 μg/ml (—○—), of ofloxacin are 10 μg/ml (—●—), 2.5 μg/ml (—○—), and 0.625 μg/ml (—▲—), of vancomycin are 25 μg/ml (—●—), 6.25 μg/ml (—○—), 1.56 μg/ml (—▲—), Broken lines (···●···) shows bacterial growth control. (■) is an area of reduction of inoculum size more than 99.9 %.

essentially identical (data were not shown).

DISCUSSION

Vancomycin was superior in its bactericidal activity against all tested strains of MRSA after 24 h incubation within its therapeutic levels in comparison with those of minocycline or ofloxacin. Minocycline, at all tested concentrations, reduced CFU of all strains, but, as expected, the effects were not bactericidal. Ofloxacin, at a concentration of 10 μg/ml, was bactericidal and its effect was superior to that of vancomycin, because complete reduction was attained within 6 h incubation. However, serum levels of ofloxacin as high as 10 μg/ml are not feasible with the exception of urogenital tract

after oral administration[22]. At a concentration of 2.5 μg/ml, corresponding to the maximum serum level, or at a concentration of 0.625 μg/ml, corresponding to the average serum level, the effect of ofloxacin was insufficient to be curative for severely infected patients. Recently, an increase of resistant strains to fluoroquinolons among MRSA has led to a concern in view of the current wide use of these drugs[24~26]. For these reasons, it seems reasonable to recommend systemic infusion of vancomycin as the treatment of choice for severe MRSA infection in compromised hosts.

An important feature of the bactericidal activity of vancomycin against tested strains was that the effect was dependent on the length of incubation time, but was not concentration-dependent. Concentrations above 3.13 μg/ml, corresponding to only 2—4 times MICs, showed the same effects against all strains. Earlier reports suggested that ototoxicity of vancomycin was detected among patients with high serum levels[16,27], and another report suggested that nephrotoxicity was related to high serum concentration of the drug[17]. Because the increase in bactericidal activity is not expected with higher concentrations of vancomycin, we recommend that serum concentrations of the antibiotic should be maintained within 2—4 times MICs for sustained periods during systemic infusion for its optimal therapeutic effects. Cantoni reported, in a rat model of endocarditis of *S. aureus*, that the administration of vancomycin every 6 h was more effective than every 12 h[28]. We can assume that a better therapeutic effect in this case was attributable to the maintenance of higher drug concentrations, but not because of an increase of total dosage.

Our results also suggested two shortcomings of bactericidal activity of vancomycin against MRSA. First, the effect was weak within short time incubation, and second, the effect was markedly influenced by inoculum size. These shortcomings may explain why vancomycin for severely infected cases or infections in compromised hosts often ended in failure despite *in vitro* sensitivity of the pathogens to the antibiotic. Vancomycin has been reported to fail in the treatment of Staphylococcal endocarditis in some cases[29~31]. The discrepancy between MICs and MBCs of the pathogens to vancomycin has been suggested to explain the failure[29,30]. However, our results indicate that even at concentrations much higher than MBCs, time-kill curves are identical to those obtained with lower concentrations as long as they are above 2—4 times MIC. Therefore, we suspect that the major reason for the failure of treatment by vancomycin may reside in the excess amount of bacteria in infected sites.

These limitations of antibacterial activity of vancomycin should be kept in mind when we use it clinically. Some reports suggested that addition of rifampin to vancomycin was more effective in the treatment of MRSA infections[26,27,32] Such combination therapy merits further study, if the shortcomings of vancomycin can be offset by the combination.

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Vancomycinのメチシリン耐性黄色ブドウ球菌(MRSA)に対する殺菌力の評価

—Minocycline, ofloxacinとの比較, および殺菌力に影響をおよぼす因子の検討—

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Vancomycinの MRSA 感染症に対する有用性を評価するため, 敗血症由来株を用いて殺菌曲線を minocycline, ofloxacin と比較した。常用投与量で得られる血中濃度の範囲で比較すると, vancomycinの殺菌力は他薬剤より優れていたが, その殺菌力は濃度に依存せず, 菌との接触時間に依存することが示された。すなわち, 高濃度でも短時間の殺菌力は弱く, また, 接種菌量を増加させると殺菌力は不良であった。これらのことから, vancomycinは少量頻回投与など 2～4 MIC 程度以上の濃度を長時間維持しうる投与法の方が有効であること, 巨大な感染巣を有するなどの重症例では治療効果に限界のあることが示唆された。

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