Combination of minocycline and rifampicin against methicillin- and gentamicin-resistant *Staphylococcus aureus*

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SUMMARY Methicillin- and gentamicin-resistant *Staphylococcus aureus* may remain sensitive to minocycline and to rifampicin. A study of growth curves has shown that at inhibitory concentrations (0.4 µg/ml), minocycline prevents the development of mutants resistant to rifampicin.

Staphylococcus aureus resistant to methicillin and gentamicin is responsible for an increasing number of hospital infections, some of which are severe.¹⁻⁷ A number of treatments have been suggested although vancomycin is often the only major antibiotic which is active against these strains. However, it is necessary to assess the effect of the "second choice" antibiotics. The risk of rapid development of resistance to rifampicin is well known,^{8,9} and in spite of the excellent penetration particularly in polymorphonuclear cells of this antibiotic,^{10,11} its use alone is contraindicated. Minocycline is active against *Staph aureus*,¹²⁻¹⁴ including multi-resistant strains, particularly those resistant to tetracycline and methicillin.¹⁵⁻¹⁷ The minocycline minimal inhibitory concentrations (MICs) are compatible with a therapeutic effect.

A combination of minocycline and rifampicin was investigated in vitro (a) to demonstrate whether a synergistic or antagonistic effect was present, and (b) to determine the protective effect of minocycline against rifampicin resistance.

Material and methods

MICROBIAL STRAINS

Between January and September 1979, most strains of *Staph aureus* isolated at the Brugmann University Hospital showed a high resistance pattern: they were resistant not only to β -lactam antibiotics (penicillin, methicillin, cloxacillin, cephalosporins) but also to gentamicin (the gentamicin MICs were > 12.5 µg/ml) but all these strains were susceptible to minocycline according to the Kirby Bauer method.¹⁸ Three

strains of different phage type were selected for this investigation.

Microbial strains

Minocycline HCL (Cyanamid Benelux, batch no 7116B-172). Rifampicin (Lepetit, batch no P/4) (solution in dimethyl formamide). The MICs of minocycline (tube dilution method in Mueller Hinton medium, inoculum 10^6 micro-organisms/ml) were 0.2 µg/ml for all the strains. Rifampicin showed minimal inhibitory activity in liquid medium up to a concentration of 0.01 µg/ml. However, this value was uncertain because of the possible occurrence of the "skip phenomenon." This one-step resistance was not predictable and might occur at any concentration between 0.01 µg/ml and 6.4 µg/ml of rifampicin.

GROWTH CURVES

Densitometric measurement of bacterial growth was carried out using an Abbott MS-2 apparatus¹⁹ and a simple multichambered cuvette (5996-01 research cartridges).

CULTURE MEDIA

Iso-sensitest (Oxoid, Basingstoke, Hampshire, England).

STANDARDISATION OF CULTURES

Before the cultures were introduced into the multichambered cuvette, each strain was pre-incubated in culture with agitation for six hours. The microbial density was determined with a spectrophotometer (Spekol, Jena) and adjusted by dilution to 0.5 on the McFarland scale of bacterial density. The culture was then diluted 1/100 to obtain 2×10^6 viable units/ml.

Samples of the bacterial suspension (1.4 ml) were introduced into each chamber of the multichambered cuvette, and these were then transferred to the measuring unit of the MS-2 apparatus.

The antibiotic combination (0.1 ml in iso-sensitest medium) was added to the chambers at time zero during the exponential phase of growth so as to obtain concentrations of 6.4 µg/ml; 3.2 µg/ml; 1.6 µg/ml; and so on. Each concentration of minocycline in the combination was studied against all the concentrations of rifampicin. The growth curve was recorded from the time that the antibiotic was added to the chamber (time zero on the abscissa of figure).

The growth curves were produced on a video screen (Tektronix 4006-1) from densitometric values obtained at five-minute intervals for 20 hours on magnetic tape. The biomass was expressed in logarithms N_2 of the increase in optical density.

DETECTION OF ONE STEP RESISTANCE TO RIFAMPICIN AS WELL AS BACTERICIDAL ACTIVITY

After 20 hours' incubation, 0.1 ml from each chamber was inoculated over the entire surface of a 9 cm diameter Petri dish containing 10 ml of Iso-sensitest and 1.5% agar. A disc containing 10 µg rifampicin was placed in the centre of the Petri dish. The number of colonies developing after 24 hours on the Petri dish was assessed semi-quantitatively. Absence of growth (or < 10 colonies) was given the score ±; 10 to 100 colonies +; 100 to 1000 colonies ++; and > 1000 colonies (or a confluent culture) +++. Strains whose colonies were seen up to the rifampicin disc were regarded as resistant. The MIC of these was > 100 µg/ml. No case of "one step" resistance to minocycline was seen.

Results

In spite of the fact that the three strains of *Staph aureus* were of different lysotypes, the results were very similar. The figure shows the profile of the growth curves obtained. Minocycline produced total inhibition of the growth of the microbial strains at a concentration of 0.4 µg/ml. At lower concentrations growth was slow and became even slower as the concentration of minocycline approached the MIC. No antibacterial effect has been detected at 0.01 µg/ml minocycline concentration and the curve was similar to the control. In the case of rifampicin, a regrowth of cultures was observed at concentrations between 0.04 and 6.4 µg rifampicin/ml and was seen after 14 ± 2 hours incubation. This time was independent of the rifampicin concentrations. The regrowth did not always occur ("skip phenomenon" due to "one step" resistance). The rate of bacterial

Growth curve of *Staphylococcus aureus* in the presence of different concentrations of minocycline, rifampicin, and a combination of the two antibiotics (shown as figures lying on the curves). The antibiotics were introduced at time zero (abscissa indicated with an arrow). A transitory increase in the optical density may occur at concentrations of minocycline above the MIC.

multiplication was comparable to the control culture. No bacterial regrowth was seen in the presence of both antibiotics, at concentrations above the MIC of minocycline (0.4 µg/ml). Strains resistant to rifampicin appeared at concentrations below this

When the two drugs were used at concentrations below their respective MICs, regrowth occurred. So we cannot speak of synergism because there is no inhibitory activity of minocycline at concentrations below the MIC even in the presence of rifampicin, also added at concentrations below the MIC. The presence of rifampicin did not reduce the sensitivity of the strains to minocycline.

BACTERIAL VIABILITY AND ONE STEP RESISTANCE TO RIFAMPICIN

The table shows the results obtained by subculturing the different chambers of the multichambered cartridges on Petri dishes containing blood agar. When the control culture was subcultured at time zero on blood agar plate, we obtained > 1000 colonies (heavy growth +++) and these strains were always susceptible to rifampicin. Any decrease in the number of colonies was described with reference to that initial point (initial inoculum).

When the concentration of minocycline was below its MIC (< 0.4 µg/ml), we observed:

(a) a possible regrowth (not always) even when the concentration of rifampicin was above its MIC (absence of synergism) and it was impossible to predict the regrowth ("skip phenomenon"); in every case of regrowth, the strain was resistant to rifampicin (+++R)

(b) a constant growth at concentration of rifampicin below its MIC; in these cases, however, all the strains were susceptible to rifampicin (+++S). When the concentration of minocycline was higher than the MIC (> 0.4 µg/ml) we observed:

(a) at concentrations of rifampicin higher than its MIC, the number of colonies were smaller than the initial inoculum; the decrease may be important (±)

when the two antibiotics are present at high concentrations—for example, minocycline ≥ 1.6 µg/ml and rifampicin ≥ 0.4 µg/ml—and all the strains isolated by subculture were susceptible to rifampicin.

(b) at concentration of rifampicin below its MIC, the density of colonies was smaller (+++) than the control (+++) and the decrease in the number of micro-organisms seemed to be due to minocycline alone because any subculture realised from chambers without rifampicin gave the same results (+++); all the strains isolated by subculture from chambers containing minocycline at concentrations above the MIC (0.4 µg/ml) remained susceptible to rifampicin.

When the concentrations of both antibiotics were below their MICs (0.4 µg/ml for minocycline and 0.01 µg/ml for rifampicin) all the subcultures were confluent and the bacteria remained susceptible to both antibiotics (+++S).

Discussion

This study is of limited value since it was carried out using stains isolated at the same hospital which, although of different lysotypes, showed the same profile of sensitivity to the antibiotics, and which probably had the same resistance plasmid. Nevertheless the antimicrobial activity of minocycline on multiresistant strains of *Staph aureus* (particularly resistance to methicillin) has been shown in other countries. Resistance to minocycline (MIC ≥ 3 µg/ml) associated with certain lysotypes has, however, been described.^{17 20-22} We have shown by the study of growth curves that only minocycline at MIC prevented the regrowth of bacteria resistant to rifampicin. The fall in the number of viable units after 20 hours' contact with a combination of the two antibiotics

Viability and rifampicin sensitivity of *Staphylococcus aureus*

Rifampicin (µg/ml)	Minocycline (µg/ml)									
	0.0	0.02	0.05	0.1	0.2	0.4*	0.8	1.6	3.2	6.4
6.4	(+++R)	(+++R)	(+++R)	(+++R)	(++R)	+S	+S	+S	±S	±S
3.2	(+++R)	(+++R)	(+++R)	(+++R)	(++R)	+S	+S	+S	±S	±S
1.6	(+++R)	(+++R)	(+++R)	(+++R)	(++R)	+S	+S	±S	±	±S
0.8	(+++R)	(+++R)	(+++R)	(+++R)	(+++R)	+S	+S	±S	±S	±S
0.4	(+++R)	(+++R)	(+++R)	(+++R)	(+++R)	+S	+S	±S	±S	±S
0.2	(+++R)	(+++R)	(+++R)	(+++R)	(+++R)	+S	+S	+S	+S	+S
0.1	(+++R)	(+++R)	(+++R)	(+++R)	(+++R)	+S	+S	+S	+S	+S
0.05	(+++R)	(+++R)	(+++R)	(+++R)	(+++R)	+S	+S	+S	+S	+S
0.02	(+++R)	(+++R)	(+++R)	(+++R)	(+++R)	+S	+S	+S	+S	+S
0.01*	(+++R)	(+++R)	(+++R)	(+++R)	(+++R)	+S	+S	+S	+S	+S
0.005	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S
0.002	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S
0.0	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S	+++S

*Minimum inhibitory concentrations (MICs).

In parentheses: non-reproducible data ("skip phenomenon").

Colony count: ± = 0-10; + = 10-100; ++ = 100-1000; +++ = > 1000 or confluent growth.

R = rifampicin resistance.

S = rifampicin sensitivity.

was greater than that seen with minocycline alone. The bactericidal activity of rifampicin alone against the strains used in this study was uncertain after 20 hours' incubation, because of the frequent emergence of "one step" resistant bacteria. This in vitro study has shown that a combination of minocycline and rifampicin is of potential value in cases of *Staph aureus* infection. Further studies will be necessary to define the true therapeutic effect of the combination and the risk of the emergence of resistance in vivo to one or both antibiotics. In animals infected by *Staph aureus* the development of resistant strains to rifampicin is not so high as predicted in other studies realised in vitro.²³ This anomaly may be due to the inoculum of the stage in the growth cycle—that is, the exponential phase or not.²⁴

Bacterial density in infected subjects is a determining factor (the usual incidence of resistant mutants is 10^{-8} organisms/ml). Failures have been described in experimental endocarditis in dogs.²⁵ The combination of rifampicin with other antibacterial agents has been studied against a number of bacterial species,²⁶ particularly with tetracycline,²⁷ nafcillin,²⁸ vancomycin,²⁸ oleandomycin,²⁹ erythromycin,³⁰ trimethoprim,^{31–35} nalidixic acid,³⁶ and polymyxin B.³⁷ Rifampicin is of special interest in staphylococcal infection because the good intracellular penetration of the compound results in the death of phagocytosed bacteria.^{10–11} The high degree of lipophilicity of rifampicin and of minocycline^{37–38} is important in obtaining active concentrations of both compounds at the same site.

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