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Electron microscopy studies of the bactericidal effects of quinupristin/dalfopristin on *Staphylococcus aureus*

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Sir,

Quinupristin/dalfopristin is a combination of two semi-synthetic derivatives of pristinamycin which has been shown to have activity *in vitro* against a wide range of Gram-positive cocci.[1] Preliminary studies in our laboratory of its activity against staphylococci demonstrated that, following exposure to concentrations <MICs, the bacterial cells appeared larger and stained more intensely than controls. A further study of bacterial ultrastructure revealed that incubation of *Staphylococcus aureus* in the presence of quinupristin/dalfopristin resulted in two major cell alterations: increases in cell size and the thickness of the cell wall; the greatest increase in cell size was observed after exposure to a concentration equivalent to 0.5 × MIC ($P < 0.005$).[2] Electron micrographs taken after 24 h exposure to the combination at this concentration showed breaks in the walls of several cells and ghosts of lysed cells, in addition to larger cells. Our earlier study[2] was repeated with the aim of performing viable counts and quantifying breaks in the cell wall following incubation of a strain of *S. aureus* in the presence of 0.5 × MIC of quinupristin/dalfopristin.

Quinupristin/dalfopristin was provided by Rhône-Poulenc Rorer (Vitry sur Seine, France) and *S. aureus* ATCC 25923 was obtained from Difco (Detroit, MI, USA). The *S. aureus* strain was incubated without quinupristin/dalfopristin (control) and in the presence of the antibiotic at a concentration of 0.2 mg/L (equivalent to 0.5 × MIC) as described previously.[2] Aliquots were withdrawn after incubation at 36ºC for 6, 12 and 24 h and the numbers of viable bacteria were determined. Samples of the 24 h culture were also examined by electron microscopy according to a method described by Lorian *et al.*[2] The extent of the cell-wall alterations was assessed by examining photographs of fields containing 30–40 cells at ×21,000 magnification.

Following exposure of *S. aureus* to quinupristin/dalfopristin for 6 h and 12 h, the numbers of viable bacteria fell by 99% and 99.9%, respectively, compared with the control; after 24 h, the viable count increased by 0.5 $\log_{10}$. In a previous study, incubation of some strains of *Enterococcus faecium* in the presence of the combination at a concentration equivalent to 0.3 × MIC was associated with 99.9% decreases in the numbers of viable organisms.[3] The apparent paradox that exposure to concentrations <MICs produced marked bactericidal activity can be accounted for by the technique used to determine the MICs, which is based on measurements of turbidity—i.e. in common with live bacteria, dead cells produce turbidity—whereas the method of quantifying bactericidal activity determines the numbers of viable bacteria only.

The Figure shows profound structural alterations, breaks in the cell walls, extrusion of cytoplasm, and cell-wall ghosts in 10–20% of cells.

Quinupristin/dalfopristin inhibits protein synthesis as a result of its effects on bacterial ribosomes. However, unlike other antibiotics that bind reversibly to the ribosomes, the combination binds irreversibly, thereby exerting a bactericidal effect on susceptible strains of *S. aureus*.[4] The ultrastructural alterations observed following exposure to quinupristin/dalfopristin for 24 h may be lysis following death and may, as such, represent a visual record of the bactericidal effects of the drug.

References

1. Aumercier, M., Bouhallab, S., Capmou, M.-L. & Le Goffic, F. (1992). RP59500: a proposed mechanism for its bactericidal activity. *Journal of Antimicrobial Chemotherapy* **30**, *Suppl. A*, 9–14.

2. Lorian, V., Esanu, Y. & Amaral, L. (1994). Ultrastructure alterations of *Staphylococcus aureus* exposed to RP59500. *Journal of Antimicrobial Chemotherapy* **33**, 625–8.

3. Vannuffel, P. & Cocito, C. (1996). Mechanism of action of streptogramins and macrolides. *Drugs* **51**, *Suppl. 1*, 20–30.

4. Lorian, V. & Fernandes, F. (1997). Synergic activity of vancomycin–quinupristin/dalfopristin combination against *Enterococcus faecium*. *Journal of Antimicrobial Chemotherapy* **39**, *Suppl. A*, 63–6.

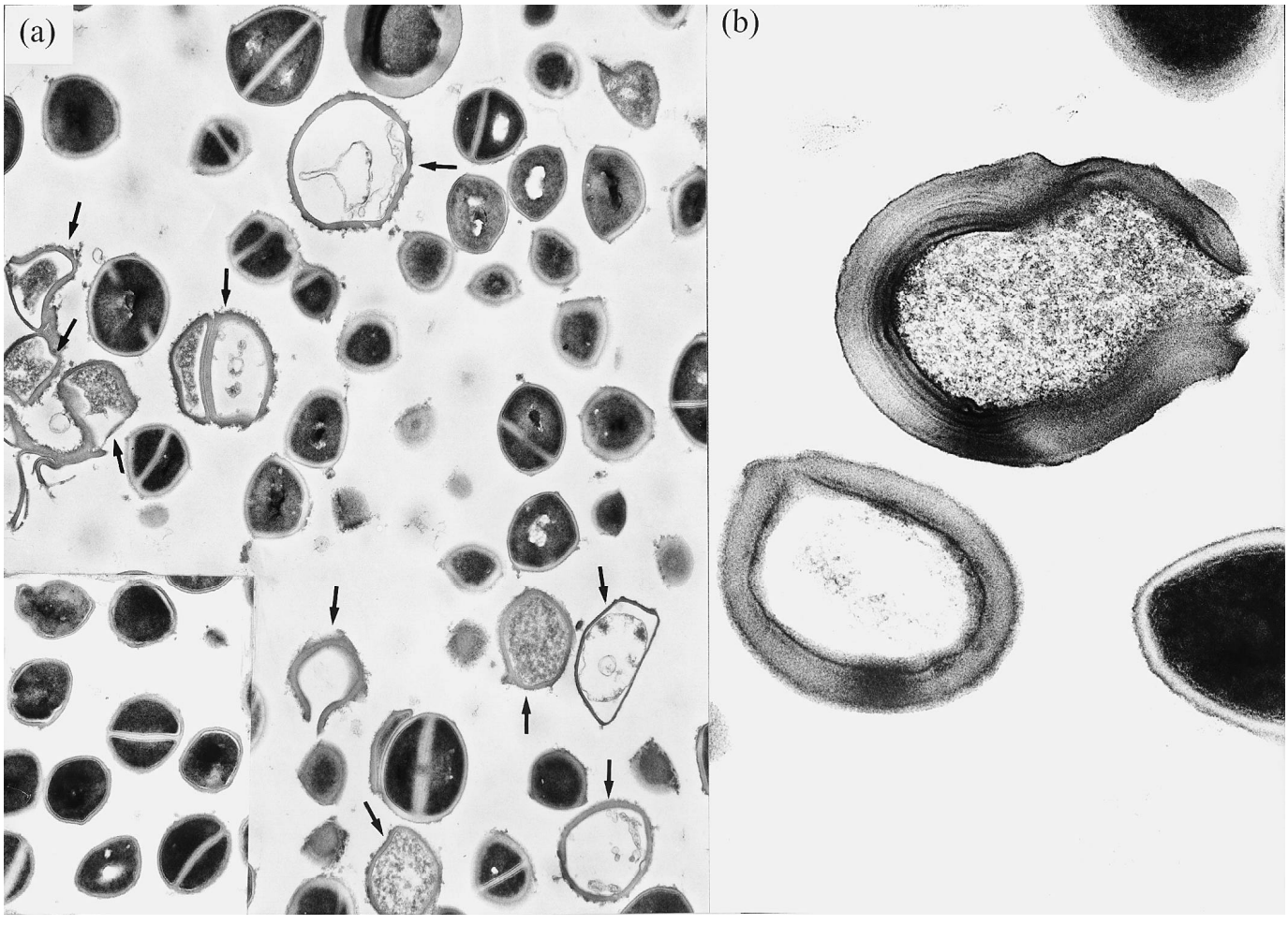

Figure. Electron micrographs of *S. aureus* following incubation for 24 h in the presence of quinupristin/dalfopristin at a concentration equivalent to 0.5 × MIC. (a) ×21,000 magnification; arrows indicate lysed cells (inset: *S. aureus* control). (b) ×82,000 magnification, showing a break in a multilayered cell wall and a cell-wall ghost.

Emergence of heterogeneous intermediate vancomycin resistance in *Staphylococcus aureus* isolates in the Düsseldorf area

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Sir,

Hiramatsu *et al.*[1–3] recently described the emergence in Japanese hospitals of heterogeneous intermediate resistance to vancomycin in clinical isolates of *Staphylococcus aureus* (hetero-VISA, also referred to as 'hetero-VRSA'). (A hetero-VISA strain is one that is susceptible to vancomycin, i.e. MIC $\leqslant$ 4 mg/L, according to breakpoints recommended by the National Committee for Clinical Laboratory Standards (NCCLS),[4] but which contains subpopulations of cells, at frequencies $\geqslant 10^{-6}$,[1] that exhibit intermediate susceptibility, i.e. MICs 8–16 mg/L.) These investigators reported the incidences of such isolates to be

20% in Juntendo University Hospital, 9.3% in seven other university hospitals and 1.3% in non-university hospitals.[1,2] All of the strains possessed the same pulsed-field gel electrophoresis (PFGE) pattern, II-A—an observation that suggests that they belonged to a single clone. Homogeneous VISA isolates have also been identified in the USA.[5] Hiramatsu *et al.*[1,3] suggested that hetero-VISA isolates are precursors of *S. aureus* strains that are resistant to vancomycin (MICs $\geqslant$ 32 mg/L). For this reason, it is important that the prevalences of VISA and hetero-VISA strains in the *S. aureus* population are closely monitored.

In order to determine the prevalences of VISA and hetero-VISA isolates locally, we studied 85 methicillin-resistant *S. aureus* (MRSA) strains randomly selected from 210 non-replicate isolates recovered between 1992 and 1998 and referred to the Institute of Medical Microbiology and Virology, University Hospital Düsseldorf, by 11 regional hospitals. The isolates were analysed by PFGE, the most discriminatory molecular technique for typing MRSA strains.[6] Carriage of the *mecA* and *coa* genes was confirmed with a multiplex PCR.

Susceptibility to vancomycin was determined by a microbroth dilution method recommended by the NCCLS,[4] with cation-adjusted Mueller–Hinton broth, and by the Etest method (AB Biodisk North America Inc., Piscataway, NJ, USA). The isolates were also screened by a method of detecting hetero-VISA previously described by Hiramatsu *et al*.[1] Each strain was inoculated on to Brain Heart Infusion (BHI) agar containing vancomycin in a concentration of 4 mg/L. The plates were incubated at 37°C for 48 h and examined after 24 h and 48 h. If there was no growth after 48 h, a strain was considered susceptible to vancomycin. If confluent growth was observed after 24 h the strain was considered to be potentially resistant to vancomycin, whereas if 1–30 discrete colonies were observed within the 48 h incubation period it was designated as potentially hetero-VISA. Hetero-VISA status was confirmed if a strain produced subclones with vancomycin MICs $\geqslant$ 8 mg/L following subculture in the presence of increasing concentrations of vancomycin and if the MIC remained stable for $<$9 days after daily subculture in a drug-free medium.

Of the 85 MRSA isolates tested, none was classified as VISA according to the above criteria. However, seven (8.2%) isolates fulfilled the criteria of Hiramatsu *et al*. for hetero-VISA;[1–3] variants exhibiting reduced susceptibility occurred at frequencies of 10^{-3}–10^{-4}. As determined by the microbroth dilution method, the MICs of vancomycin and teicoplanin for all seven strains were 1 mg/L and 4 mg/L, respectively. Thus, these strains would have been classified as susceptible to the glycopeptides on the basis of NCCLS criteria.[4]

Hanaki *et al.*[1,3] demonstrated that strains exhibiting reduced susceptibility to vancomycin (one each of VISA and hetero-VISA) produced three-fold to five-fold greater amounts of penicillin-binding proteins (PBPs) 2 and 2′ and three-fold to eight-fold greater amounts of intracellular murein monomer precursor, compared with the vancomycin-susceptible *S. aureus* control strains. These investigators also showed that amidation of glutamine residues in cell-wall muropeptides and reduced crosslinking of cell-wall peptidoglycan might contribute to reduced susceptibility to vancomycin.[7] All of these alterations produce a so-called 'trapping effect', whereby increased amounts of vancomycin are bound to the pre-existing modified cell wall, thus reducing the amount of drug that reaches its vital targets—the lipid II precursors of new cell-wall synthesis which are found in the outer leaflet of the cytoplasmic membrane beneath the pre-existing cell wall. In order to determine whether similar cell-wall modifications were present in our isolates, one strain was subjected to muropeptide analysis by high-performance liquid chromatography (HPLC), as described by Hanaki *et al.*[7] The cell wall was isolated, the teichoic acids were removed, and the remaining peptidogycan was digested by a muramidase. The resulting muropeptides were separated by reverse-phase HPLC. Peak assignments were made by comparison with previous samples in which the peaks were identified by amino acid analysis and mass spectroscopy. The cell wall of the strain contained increased amounts of glutamine-non-amidated muropeptides (although the numbers of these molecules fell following repeated subculture). The amounts of the corresponding muropeptides were even greater than those found in Mu50, the first homogeneous VISA strain described by Hiramatsu *et al*.,[1,3,7] and were greater than those of the standard amidated precursors. The strain also exhibited reduced crosslinking of cell-wall peptidoglycan. All of these data suggest that the mechanisms responsible for heterogeneous intermediate resistance to vancomycin in our *S. aureus* isolates are very similar, if not identical, to those identified in the Japanese isolates.

The seven hetero-VISA strains that we isolated were referred from three different hospitals in the Düsseldorf area. Five strains were isolated from patients on two different wards of the same hospital (three from one and two from another) and two of the five patients occupied the same room before the MRSA strains were isolated, suggesting nosocomial transmission (and, possibly, cross-infection) of these strains; a relationship in terms of acquisition/transmission could not be demonstrated for the other three isolates. PFGE revealed the seven hetero-VISA strains to be indistinguishable and to be identical to the north German epidemic strain of MRSA which is the most prevalent strain in both the Düsseldorf area and northern Germany.

The distribution of hetero-VISA strains among three geographically distinct hospitals in the Düsseldorf area suggests the potential for wider dissemination. All seven hetero-VISA isolates appear to have belonged to a single clone that was first detected in 1998, no hetero-VISA strains having been isolated in this region between 1992 and 1997; the emergence of heterogeneous intermediate

resistance to vancomycin in *S. aureus* is therefore a recent development. All patients from whose blood cultures or wound swabs hetero-VISA strains were isolated were critically ill with either carcinomatosis or severe postoperative complications. Two of the seven were treated with vancomycin before hetero-VISA strains were isolated and three received vancomycin after the strains were isolated. Thus, the isolation of a hetero-VISA strain was not clearly linked to previous vancomycin therapy and the glycopeptide appears to have been active both *in vitro* and *in vivo* against the hetero-VISA isolates. All seven patients were cured following treatment with either vancomycin or another appropriate agent. Nonetheless, isolates with reduced susceptibilities to vancomycin can still be associated with treatment failures and, as has been suggested previously, hetero-VISA strains might be precursors of *S. aureus* strains that are resistant to vancomycin.[1,3] These alone are adequate reasons to justify prospective surveillance for the purpose of identifying *S. aureus* isolates with reduced susceptibilities to vancomycin.

References

1. Hiramatsu, K., Aritaka, N., H., Kawasaki, S., Hosoda, Y., Hori, S. *et al.* (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* **350**, 1670–3.

2. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. & Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy* **40**, 135–6.

3. Hanaki, H., Kuwahara-Aria, K., Boyle-Vavra, S., Daum, R. S., Labischinski, H. & Hiramatsu, K. (1998). Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. *Journal of Antimicrobial Chemotherapy* **42**, 199–209.

4. National Committee for Clinical Laboratory Standards. (1997). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Fourth Edition: Approved Standard M7-A4.* NCCLS, Villanova, PA.

5. Centers for Disease Control. (1997). Update: *Staphylococcus aureus* with reduced susceptibility to vancomycin in the United States, 1997. *Morbiditiy and Mortality Weekly Report* **46**, 813–5.

6. Schmitz, F. J., Steiert, M., Tichy, H. V., Hofmann, B., Verhoef, J., Heinz, H. P. *et al.* (1998). Typing of methicillin-resistant *Staphylococcus aureus* isolates from Duesseldorf by six genotypic methods. *Journal of Medical Microbiology* **47**, 341–51.

7. Hanaki, H., Labischinski, H., Inaba, Y., Kondo, N., Murakami, H. & Hiramatsu, K. (1998). Increase in glutamine-non-amidated muropeptides in the peptidoglycan of vancomycin-resistant *Staphylococcus aureus* strain Mu50. *Journal of Antimicrobial Chemotherapy* **42**, 315–20.