Phagocytosis and intracellular killing of heterogeneous vancomycin-intermediate *Staphylococcus aureus* strains

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Risk factors for invasive infections by heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) may involve resistance to opsonophagocytosis and bacterial killing. hVISA strains typically have a thickened cell wall with altered peptidoglycan cross-linking. To determine whether hVISA may be endowed with an increased resistance to phagocytosis, this study assessed the characteristics of uptake and killing by granulocytes of three hVISA strains. All isolates were analysed by multilocus sequence typing and staphylococcal chromosome cassette *mec* typing. One of the strains belonged to the Hungarian methicillin-resistant *S. aureus* (MRSA) clone ST239-MRSA-III and the other two to the New York/Japan MRSA clone ST5-MRSA-II. In the presence of 10% normal serum, the extent of phagocytosis and killing by blood granulocytes was equivalent for hVISA, MRSA and methicillin-sensitive *S. aureus* (MSSA) strains. Using granulocytes and serum from one patient who survived hVISA infection, the rate of phagocytosis and killing was also found to be comparable to that by control cells in the presence of 10% serum. However, phagocytosis and killing of hVISA and MRSA (ATCC 25923) strains by normal granulocytes was markedly decreased in the presence of low concentrations (1 and 2.5%) of serum from the patient who survived hVISA infection compared with that found with normal human serum. These data suggest that hVISA and MRSA isolates may be more resistant to opsonophagocytosis and bacterial killing than MSSA isolates, at least in some cases.

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INTRODUCTION

Over the past decade, infections by methicillin-resistant *Staphylococcus aureus* (MRSA) strains have become increasingly prevalent, and vancomycin has remained the drug of choice for treatment of patients with MRSA infections (Garnier *et al.*, 2006; Jones, 2006; Gould, 2008). However, due to selective pressure, *S. aureus* isolates with decreased susceptibility to vancomycin have emerged (Song *et al.*,

2004). The Clinical and Laboratory Standards Institute (CLSI) designates staphylococci requiring concentrations of vancomycin $\leq 2 \text{ mg l}^{-1}$ for growth inhibition as susceptible, those requiring $4\text{--}8 \text{ mg l}^{-1}$ as intermediately sensitive and those requiring $\geq 16 \text{ mg l}^{-1}$ as resistant (CLSI, 2009). Heterogeneous vancomycin-intermediate *S. aureus* (hVISA) strains appear to be sensitive to vancomycin with a susceptibility range of $1\text{--}2 \text{ mg l}^{-1}$ but contain subpopulations of organisms with intermediate sensitivity to vancomycin (Garnier *et al.*, 2006; Sakoulas *et al.*, 2006). The first staphylococcal infection caused by hVISA was detected in Japan, which was followed by reports of a number of cases from other countries (Goldblum *et al.*, 1978; Hiramatsu *et al.*, 1997a, b; Garnier *et al.*, 2006; Gould, 2008). Research

Abbreviations: AUC, area under the curve; CLSI, Clinical and Laboratory Standards Institute; hVISA, heterogeneous vancomycin-intermediate *S. aureus*; MLST, multilocus sequence typing; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; PAP, population analysis profile; SCC, staphylococcal chromosomal cassette.

on phagocytosis and killing by professional phagocytes may provide new insight into the pathogenicity of hVISA strains. We report here on three patients with severe, invasive hVISA infection. We propose that hVISA strains, as well as MRSA strains, may be more resistant than methicillin-sensitive *S. aureus* (MSSA) strains to opsonophagocytosis and killing by granulocytes.

METHODS

Patients

Patient 1. This 56-year-old man was diagnosed with a parapharyngeal abscess requiring incision and tracheotomy. Two days after admission, haemodialysis was started because he developed sepsis and acute renal failure and he remained dialysis dependent. Three months later, his jugular vein catheter was removed because of bacterial colonization and amikacin administration was started. Routine nasal and throat cultures and repeated blood cultures yielded MRSA that was susceptible to vancomycin; therefore, amikacin was combined with vancomycin and later with rifampicin. A chest X-ray revealed bilateral basal pneumonia and computed tomography revealed multiple pulmonary and spleen abscesses. Progression of the infection, ventricular arrhythmia and circulatory failure led to a fatal outcome. Post-mortem blood culture yielded MRSA with reduced vancomycin susceptibility.

Patient 2. This patient had Little's disease complicated by tetraplegia, somatomental retardation and epilepsy. She was admitted to hospital aged 24 years with pneumonia and acute respiratory failure after an epileptic attack. Empiric antibiotic therapy with clindamycin and ceftriaxone was started and a tracheotomy was performed. *Pseudomonas aeruginosa* and *Candida* species were detected in bronchial secretions and the antibiotic regimen was changed to ceftazidime, amikacin and fluconazole. In addition, parenteral nutrition had to be instituted. Later, nasal culture, blood culture and bronchial secretions yielded MRSA and treatment with vancomycin was started. The strain isolated from the bronchial culture showed reduced susceptibility to vancomycin and was confirmed as hVISA. She developed sepsis and bone marrow failure and died, despite 3 months of intensive treatment.

Patient 3. This 27-year-old woman was admitted to hospital for burns over ~40 % of the total body surface area. Repeated necrotomy and skin grafting were performed. During hospitalization, she developed pneumonia. Samples were taken for culture from the throat, nose, wounds and genitalia. hVISA was cultured from skin lesions and she was treated with tigecycline. Two months after admission, she was discharged from hospital with healed skin lesions.

Antibiotic susceptibility testing. Susceptibility to different antibiotics was tested by the disc diffusion method with Oxoid discs according to CLSI guidelines (CLSI, 2009; Szabó *et al.*, 2009). Methicillin resistance was determined by oxacillin Etest (AB Biodisk). MICs of vancomycin, teicoplanin, erythromycin, clindamycin, ciprofloxacin, gentamicin, amikacin, rifampicin, tigecycline, linezolid, daptomycin and quinupristin/dalfopristin were defined by Etest according to CLSI guidelines (Brown *et al.*, 2005; Tóth *et al.*, 2008; CLSI, 2009; Szabó *et al.*, 2009). The suspected hVISA strains were examined using the macro Etest method (Tóth *et al.*, 2008).

Population analysis profile (PAP). Fifty microlitres of the starting bacterial cell suspension (prepared by diluting a culture grown overnight to an OD₅₇₈ of 0.3) was diluted to 1:10 and 1:10⁴ and spread on brain–heart infusion agar plates (Oxoid) containing 0, 0.5,

1.0, 2.0, 2.5, 4 and 8 mg vancomycin l⁻¹. Colonies were counted after 48 h of incubation at 37 °C using a semi-logarithmic graph of counted c.f.u. versus vancomycin concentration. The area under the curve (PAP-AUC) was calculated for each strain (Walsh *et al.*, 2001). To distinguish VISA, hVISA and vancomycin-susceptible MRSA (ATCC 29213), a ratio of the AUC of the test MRSA divided by the corresponding AUC for *S. aureus* strain Mu3 used as a positive control was calculated. The criteria used for detection of hVISA and VISA strains were AUC ratios of ≥0.9 and ≥1.3, respectively. The ratio of test MRSA AUC divided by the corresponding Mu3 AUC was calculated as described previously (Walsh *et al.*, 2001; Tóth *et al.*, 2008).

Molecular typing methods. The hVISA/VISA strains were typed by multilocus sequence typing (MLST) and staphylococcal chromosome cassette (SCC) *mec* typing (Enright *et al.*, 2000; Oliveira & de Lencastre, 2002). Allele sequences and sequence types were verified at the <http://www.mlst.net> website.

Granulocytes. Granulocytes were separated from heparinized (10 U ml⁻¹) venous blood as described previously (Maródi *et al.*, 1984, 2001). Cells were washed and resuspended to a concentration of 10⁷ ml⁻¹ in Krebs–Ringer phosphate buffer with 5 % glucose (KRPBG). The granulocyte suspension contained >98 % neutrophils (band form and segmented) as demonstrated by May–Grünwald–Giemsa staining in cytocentrifuge preparations. Cells were finally resuspended in KRPBG to a concentration of 10⁷ cells ml⁻¹.

Preparation of sera. Whole blood was obtained from eight healthy adults. Blood was allowed to clot at room temperature for 1 h, followed by centrifugation at 4 °C (Maródi *et al.*, 1993). Serum was removed and stored in aliquots at –20 °C until use (Maródi *et al.*, 1998).

Bacteria. MSSA (ATCC 43300), MRSA (ATCC 25923) and three hVISA isolates from patients 1, 2 and 3 were cultured overnight at 37 °C in 100 ml nutrient broth (Oxoid). Bacteria were harvested by centrifugation at 1500 g for 10 min, washed twice with KRPBG and resuspended in KRPBG to a concentration of 10⁷ bacteria ml⁻¹ (Maródi *et al.*, 1995).

Phagocytosis. Phagocytosis of bacteria by granulocytes was measured as the percentage decrease in the number of viable extracellular bacteria in the phagocytic cell suspension (Maródi *et al.*, 1983, 2000). Briefly, 200 µl granulocyte suspension (10⁷ cells ml⁻¹) was incubated with 200 µl bacterial suspension (10⁷ bacteria ml⁻¹) in the presence of various concentrations of serum at 37 °C under slow rotation (4 r.p.m.). After 60 or 120 min incubation, 50 µl aliquots were removed and added to 450 µl cold KRPBG and the cells were centrifuged for 6 min at 600 r.p.m. in a bench microfuge. The number of viable extracellular bacteria was determined by serial tenfold dilutions in KRPBG and plating on nutrient agar plates. Colonies were counted after incubation of the plates at 37 °C for 18 h.

Bacterial killing. Bacterial killing was measured as the percentage decrease in the number of viable extracellular and intracellular bacteria in the phagocytic cell suspension (Maródi *et al.*, 1984, 1995). Briefly, 2 × 10⁶ granulocytes (concentration 5 × 10⁶ ml⁻¹) and bacteria (concentration 5 × 10⁶ ml⁻¹) were incubated in the presence of various concentrations of serum at 37 °C under slow rotation (4 r.p.m.). After 60 or 120 min of incubation, 50 µl aliquots were removed and added to 450 µl cold distilled water to disrupt the granulocytes. The total number of viable bacteria was determined by serial dilutions in KRPBG and plating on nutrient agar plates. A control experiment without serum showed a 24 and 52 % increase in the

Fig. 1. Modified PAPs for the hVISA strains hVISA1, hVISA2 and hVISA3 from patients 1 (■), 2 (▲) and 3 (---), respectively. Strain Mu3 (◆) was used as a positive control, and MRSA ATCC 29213 (●) was used as a negative control.

number of *S. aureus* in the presence of granulocytes over 60 and 120 min incubation, respectively.

RESULTS

Antibiotic sensitivity profile of the hVISA strains

We identified three new hVISA isolates from three different patients, which increases the number of *S. aureus* isolates with the same vancomycin resistance spectrum in Hungary to five (Tóth *et al.*, 2008, this report, and one unpublished case). In patient 1, the MRSA strain was isolated from blood and was confirmed by a macro Etest and population analysis as hVISA. On the basis of the macro Etest, the inhibiting concentrations of vancomycin and teicoplanin were 8 and 12 mg l⁻¹, respectively. The PAP-AUC ratio was 1.01 as shown in Fig. 1. The MIC values for other antibiotics are shown in Table 1. According to the MLST and SCCmec typing results, this strain belonged to the Hungarian clone (ST239-MRSA-III).

The MRSA strain isolated from patient 2 was shown to be hVISA by macro Etest and population analysis. Inhibiting concentrations of vancomycin and teicoplanin were both

8 mg l⁻¹. The PAP-AUC ratio was 0.97 (Fig. 1). The MIC values for other antibiotics are listed in Table 1. The isolate was also tested by MLST and SCCmec typing, and was found to belong to the New York/Japan MRSA clone (ST5-MRSA-II).

The MRSA strain isolated from patient 3 also had intermediate vancomycin sensitivity according to the macro Etest. By macro Etest, the inhibiting concentrations of vancomycin and teicoplanin were both 8 mg l⁻¹. The PAP-AUC ratio was 0.93 (Fig. 1), confirming that it was a hVISA strain. Testing of the isolate by MLST and SCCmec typing confirmed that it also belonged to the New York/Japan MRSA clone (ST5-MRSA-II).

Phagocytosis and intracellular killing of hVISA, MRSA and MSSA strains by granulocytes

We studied the phagocytosis of opsonized hVISA, MRSA and MSSA strains by freshly isolated peripheral blood granulocytes from healthy donors. In the presence of 10% normal human serum as a source of opsonins, granulocytes displayed a comparable degree of ingestion of these strains after 60 min incubation (Table 2). Over a period of 120 min, >90% of bacteria were ingested, independent of their antibiotic sensitivity.

We also compared the killing of hVISA, MRSA and MSSA strains by blood granulocytes in the presence of 10% serum over a period of 120 min. Similar to the uptake of staphylococcal strains with different susceptibility for antibiotics, killing of these bacteria by normal granulocytes was also comparable at both 60 and 120 min time points (Table 2). The opsonic activity of serum from patient 3, who survived the hVISA infection, was tested on two occasions over a 2-month period. The ingestion and killing by normal granulocytes of hVISA at 60 and 120 min in the presence of 10% normal human serum or 10% serum from patient 3 was comparable (Fig. 2). Phagocytosis and killing of hVISA by the patient's granulocytes in the presence of 10% normal serum was also comparable to that mediated by the patient's serum of normal granulocytes.

Next, we compared phagocytosis and killing of hVISA, MRSA and MSSA by normal granulocytes in the presence

Table 1. MICs for the hVISA strains isolated from the patients in this study

Oxa, Oxacillin; Ery, erythromycin; Cli, clindamycin; Cip, ciprofloxacin; Gen, gentamicin; Ami, amikacin; Rif, rifampicin; Tig, tigecycline; Dap, daptomycin; Qui/Dal, quinupristin/dalfopristin; Lin, linezolid.

hVISA	MIC (mg l ⁻¹)										
	Oxa	Ery	Cli	Cip	Gen	Ami	Rif	Tig	Dap	Qui/Dal	Lin
hVISA1	>256	>256	>256	>32	24	16	0.006	0.50	1	1	1.5
hVISA2	>256	>256	>256	>32	64	64	>32	0.50	1	1	1
hVISA3	>256	>256	>256	>32	32	16	0.006	0.25	1	2	0.75

Table 2. Comparison of phagocytosis and killing of *S. aureus* strains with varying antibiotic sensitivities by peripheral blood granulocytes

Phagocytosis and bacterial killing of MSSA (ATCC 43300), MRSA (ATCC 25923) and hVISA strains by peripheral blood polymorphonuclear neutrophil granulocytes from healthy donors. Phagocytic cells were incubated for 120 min with bacteria in the presence of 10% normal human serum. Data represent means $\pm$ SEM of at least three experiments.

<i>S. aureus</i> strain	Phagocytosis (%)		Staphylococcal killing (%)	
	60 min	120 min	60 min	120 min
ATCC 43300	92 $\pm$ 3	97 $\pm$ 2	82 $\pm$ 2	85 $\pm$ 4
ATCC 25923	85 $\pm$ 4	95 $\pm$ 3	76 $\pm$ 5	87 $\pm$ 6
hVISA1	82 $\pm$ 3	93 $\pm$ 3	71 $\pm$ 6	80 $\pm$ 7
hVISA2	80 $\pm$ 5	94 $\pm$ 4	79 $\pm$ 3	83 $\pm$ 5
hVISA3	81 $\pm$ 6	92 $\pm$ 4	77 $\pm$ 6	86 $\pm$ 4

of low concentrations of serum (1 and 2.5 %) as a source of opsonins from patient 3 and healthy individuals, respectively (Fig. 3). We found that the extent of both ingestion and killing of hVISA and MRSA was markedly decreased compared with that found with MSSA (Fig. 3). These data indicated that the capacity of patient 3's serum to promote killing of hVISA and MRSA by blood granulocytes was impaired.

DISCUSSION

We reported recently that the prevalence of MRSA in patients with invasive staphylococcal infections was as high as 25 % in Hungary, and hVISA strains have emerged that may have been the result of the extensive use of glycopeptides, especially vancomycin, for the treatment of patients with severe MRSA infections (Tóth *et al.*, 2008; Szabó, 2009). hVISA infections are usually associated with poor disease outcome with a mortality rate of ~75 % (Szabó, 2009). Risk factors for the emergence of hVISA/VISA strains may include haemodialysis, burn wounds and prolonged use of indwelling foreign devices (Goldblum *et al.*, 1978; Boelaert, 1994; Szabó, 2009). In the patients

described in this report, chronic haemodialysis, prolonged use of indwelling catheters and severe burns, respectively, may have contributed to the occurrence of invasive hVISA infection. In each case, MRSA nose colonization might have also contributed to the emergence of hVISA.

hVISA strains have been reported from a number of countries, and most of the isolates belonged to the New York/Japan MRSA clone (CDC, 1997; Hiramatsu *et al.*, 1997a, b; Ploy *et al.*, 1998). To date, five cases of hVISA infection have been detected in Hungary. Two of the hVISA strains described in this report belonged to the New York/Japan MRSA clone ST5-MRSA-II and one to the Hungarian clone ST239-MRSA-III. We believe that if the vancomycin MIC is >1 mg l⁻¹, alternative therapies should be considered to avoid the possibility of treatment failure and the selection of hVISA/VISA strains.

Staphylococci display cell-bound protein A, which may interfere with opsonophagocytosis by binding the Fc part of IgG and inhibiting uptake by phagocytic cells through their Fc receptors. It has been described previously that several hVISA strains were phagocytosed by polymorphonuclear neutrophil leukocytes similarly to MRSA and

Fig. 2. Comparison of phagocytosis and killing by normal peripheral blood granulocytes of the opsonized hVISA strain isolated from patient 3. Phagocytosis (left) and bacterial killing (right) were studied by incubation of bacteria (10^7 ml⁻¹) with normal granulocytes (10^7 ml⁻¹) and 10 % serum from patient 3 (open columns), normal granulocytes and normal serum (filled columns) or granulocytes from the patient and normal human serum (shaded columns) at 60 and 120 min of incubation under slow rotation (4 r.p.m.). The data are representative of two separate experiments (SEM <6 % for each).

Fig. 3. Phagocytosis (a) and killing (b) of the hVISA strain isolated from patient 3, MRSA (ATCC 25923) and MSSA (ATCC 43300) in the presence of 1 and 2.5% serum. At both concentrations, the patient's serum (shaded columns) promoted markedly decreased ingestion and killing of hVISA and MRSA compared with that found with MSSA, in contrast to the results using normal serum from a healthy individual (filled columns). The data represent means of at least three experiments performed in duplicate (SEM <11% for each).

MSSA strains (Gemmell, 2004). Our findings with ATCC strains of MRSA and MSSA and the hVISA strains isolated from our patients further confirm that glycopeptide resistance and cell-wall thickening do not result in altered opsonophagocytosis and killing in the presence of a high concentration (10%) of serum. However, by using more sensitive serum concentrations (1 and 2.5%), we found markedly decreased phagocytic uptake and bacterial killing of hVISA and MRSA in the presence of serum from one patient with invasive hVISA infection compared with that with normal serum. The impaired capacity of the patient's serum to promote opsonophagocytosis of hVISA and MRSA strains may have contributed to the severity of the disease caused by hVISA in this case.

To our knowledge, this is the first report on intracellular killing of *S. aureus* with various antibiotic resistance profiles. In particular, we investigated the hypothesis that both hVISA and MRSA strains may be more resistant than MSSA strains to phagocytic killing by neutrophil granulocytes. This hypothesis was based on the observation that hVISA strains have a thickened cell wall with altered peptidoglycan cross-linking and a higher resistance to killing by professional phagocytes. The data presented in

this report suggest that hVISA as well as MRSA strains are more resistant than MSSA strains to killing by granulocytes in the presence of low concentrations of serum. Based on these data, we propose that the structural changes in the hVISA and MRSA cell wall, which may be related to resistance to antibiotics, may also endow these staphylococci with an increased resistance to opsonophagocytosis and killing by professional phagocytes.

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