Reduced Susceptibility to Host-Defense Cationic Peptides and Daptomycin Coemerge in Methicillin-Resistant *Staphylococcus aureus* From Daptomycin-Naive Bacteremic Patients

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(See the editorial commentary by Kelley et al on pages 1153–6.)

**Background.** We hypothesized that, for methicillin-resistant *Staphylococcus aureus* (MRSA), in vitro daptomycin susceptibility could be influenced by exposures to endogenous host defense peptides (HDPs) prior to clinical exposure to daptomycin.

**Methods.** Two endovascular HDPs were used: thrombin-induced platelet microbicidal protein (tPMP) and human neutrophil defensin-1 (hNP-1) from neutrophils. Forty-seven unique MRSA isolates obtained from bacteremic patients in multicenter prospective clinical trials were studied. Clinical characteristics, microbiologic parameters, prior vancomycin therapy, and susceptibilities to tPMP, hNP-1, and daptomycin were compared using univariate and multivariate analyses.

**Results.** All strains were daptomycin susceptible. Daptomycin minimum inhibitory concentrations (MICs) were inversely related to in vitro tPMP (but not hNP-1) killing. Strains with a daptomycin MIC of 1 mg/L exhibited significantly less killing by tPMP, compared with strains with an MIC of ≤0.5 mg/L. Prior vancomycin therapy did not influence this relationship. Regression tree modeling confirmed that reduced tPMP-induced killing in vitro was the strongest predictor of higher daptomycin MICs within the daptomycin-susceptible range.

**Conclusions.** Among daptomycin-susceptible MRSA isolates from patients who had never received daptomycin, higher daptomycin MICs tracked with increased resistance to killing by platelet-derived but not neutrophil-derived HDPs. These findings support the notion that endogenous exposure of MRSA to specific HDPs may play a role in selecting strains with an intrinsically higher daptomycin MIC phenotype.

*Staphylococcus aureus* presents significant clinical challenges because of its rising prevalence of antimicrobial resistance and its wide repertoire of virulence factors that have coevolved with the human immune response [1]. The progressive development of antimicrobial resistance has traditionally been thought to be driven by the selection pressure of exposure of microorganisms to exogenously administered antibiotics [2]. However, little attention has been paid to the potential “priming” role(s) of exposures of such organisms to endogenous cationic host defense peptide (HDP) molecules in this regard. Recent data from our laboratories have shown that methicillin-resistant *Staphylococcus aureus* (MRSA) strains that, after failed daptomycin therapy, developed in vitro resistance to calcium-daptomycin, a cationic antimicrobial, frequently exhibit “cross-resistance” to killing by prototypical HDPs from platelets and neutrophils [3]. Of importance, preliminary data suggest that MRSA isolates...
passaged through animal models with localized infections may also demonstrate such cross-resistance in the absence of prior daptomycin therapy [4].

In the current investigation, we examined the relationship of in vitro susceptibilities to vancomycin and daptomycin in the presence of 2 prototypical HDPs, one derived from platelets and the other derived from neutrophils, that are thought to be important in the innate immune response to S. aureus endovascular infections [5, 6]. We focused on: MRSA strains from patients without endocarditis; in vitro susceptibility profiles of daptomycin and HDP in strains from patients obtained before daptomycin exposures; and potential impacts of recent prior vancomycin treatments on daptomycin-HDP susceptibility outcomes, given the putative link between prior vancomycin exposure in vivo and in vitro and HDP resistance in vitro [7]. By using a previously well-characterized group of MRSA isolates from bacteremic patients without endocarditis who had never received daptomycin [8–10], we provide the first evidence that endogenous exposures to cationic HDPs may promote a “minimum inhibitory concentration (MIC) creep” to calcium-daptomycin among bacteremic MRSA isolates prior to daptomycin exposures.

**METHODS**

**Clinical Strains**

Forty-seven well-characterized MRSA isolates were obtained from bacteremic patients in 4 multicenter clinical trials performed during 1998–2002 at multiple US hospitals, as previously described [8–10]. For each study, appropriate ethical regulations were followed and approval from the ethics committee or institutional review board at each participating institution was received. None of these clinical trials involved the preclinical evaluation of daptomycin, and, given that all the cases were obtained prior to the US Food and Drug Administration approval of daptomycin in 2003, none of the patients had ever received this agent. The strains studied represented unique bloodstream isolates from different patients. To maintain relative clinical homogeneity, patients with clinical and echocardiographic parameters, were excluded. The clinical and demographic characteristics of the patients from whom the MRSA isolated were obtained have been detailed previously [8–10].

Prior studies from our laboratory and from other laboratories have suggested a relationship between the genotype and function of the accessory gene regulator (agr) locus and outcomes of antibiotic therapy, particularly vancomycin [10–12]. Thus, for the current bacteremia isolates, multiplex polymerase chain reaction (PCR) was used to determine agr genotypes, as described previously [10, 13]. In addition, a semiquantitative δ-hemolysin–production assay was performed to assess agr function on a scale of 0 to 4, relative to production by agr group II prototype strain RN6607. A score of 2 represented a δ-hemolysin phenotype comparable to that of RN6607, while a score of 0 denoted no δ-hemolysin activity, which is comparable to that of agr knockout RN9120. A score of 1 denoted minimal δ-hemolysin production, a score of 3 denoted production higher than that of RN6607, and a score of 4 denoted production markedly higher than that of RN6607 [14]. Scoring of δ-hemolysin assays was performed by one of the authors (G. S.), who was blinded to strain identifications. Staphylococcal cassette chromosome mec (SCCmec) genotyping was also performed using multiplex PCR. Vancomycin and daptomycin MICs were determined in duplicate on different days by broth microdilution, using Clinical and Laboratory Standards Institute (CLSI) methods [15]. Calcium chloride supplementation (50 mg/L) was routinely used for determining daptomycin MICs. Isolates were also screened for the presence of heterogeneous vancomycin-intermediate S. aureus (hVISA), using glycopeptide-resistance detection Etest (AB BioMerieux).

**HDP In Vitro Susceptibilities**

Two prototypical HDPs felt to be involved in innate host defense against S. aureus infections were selected for analysis. These HDPs differ in source (neutrophils vs platelets), size, structure, charge, and putative mechanisms of action [16]. The α-defensin human neutrophil defensin-1 (hNP-1) was purchased from Peptide International (Louisville, KY). The thrombin-induced platelet microbicidal protein (tPMP) preparation was isolated from freshly collected, thrombin-stimulated rabbit platelets, and its bioactive equivalency was determined as described previously [5, 6]. For HDP killing assays, each MRSA isolate was grown to stationary phase (16–20 hours) in Luria-Bertani broth, pelleted, and then washed in assay buffer (phosphate-buffered saline for tPMP assays and protein binding buffer for hNP-1 assays). The final HDP concentrations chosen for study were 1 and 2 mg/L of tPMP and 10 and 20 mg/L of hNP-1. These concentrations were selected because they caused <50% killing over the 2-hour assay period when tested against several of the MRSA study isolates in extensive pilot investigations. The initial bacterial inoculum was 10^3 colony-forming units/mL, as described previously [3]. The percentage of surviving bacteria (±SD) after 2 hours of incubation at 37°C with HDPs of interest was calculated by quantitative culture plating on blood agar plates. Results represent 3 separate experiments performed in duplicate on separate days.

**Statistical Analyses**

Clinical and microbiological characteristics associated with tPMP and hNP-1 susceptibility profiles were compared using...
univariate and multivariate analyses. Continuous and ordinal variables were compared using Kruskal-Wallis analysis of variance and Mann-Whitney U tests. Categorical variables were compared using \( \chi^2 \) tests or Fisher exact tests, when appropriate. Correlation analysis was used to examine the relationships between tPMP and hNP-1 susceptibilities. Multivariate analyses to detect potential correlations among HDP and daptomycin in vitro susceptibility patterns were performed using classification and regression tree modeling and logistic regression. Differences were considered to be statistically significant at \( P \) values of < .05. All analyses were performed using Systat 11 software (Systat Software).

RESULTS

Patient Demographic Characteristics
Forty-seven initial MRSA isolates obtained before therapy from 47 unique patients with MRSA bacteremia were studied. Patient ages ranged from 24 to 87 years, with a median age of 70 years; 57% were male, and 43% were in an intensive care unit at the onset of their infection. The source of bacteremia was vascular-catheter related in 12 patients. In the remaining patients, the bacteremia source was a soft-tissue abscess (for 17 patients), the lower respiratory tract (for 6), an osteomyelitic site (for 3), a device (for 1), and an intra-abdominal site (for 1); sources for 7 patients were unknown. Twenty-five patients (53%) had received intravenous vancomycin therapy \( \leq 30 \) days before the documented MRSA bacteremic episode.

In Vitro Susceptibilities to Vancomycin and Daptomycin
All isolates were susceptible to vancomycin and daptomycin, with vancomycin MICs of 0.5 mg/L (for 10 isolates), 1.0 mg/L (for 33), and 2.0 mg/L (for 4) and daptomycin MICs of 0.25 mg/L (for 4), 0.5 mg/L (for 31), and 1.0 mg/L (for 12). The MICs obtained in the duplicate, separate-day experiments were identical for each isolate. In total, only 6% of the isolates (3 of 47) were categorized as hVISA by the glycopeptide-resistance detection Etest.

In Vitro Susceptibilities to HDPs
Table 1 shows the tPMP and hNP-1 susceptibility profiles in the context of the presence or absence of an intravascular catheter at the time of bacteremia detection and of recent prior use of vancomycin. No significant relationship was noted between the source of bacteremia or previous vancomycin use with tPMP or hNP-1 susceptibility profiles.

A statistically significant relationship was noted between tPMP and hNP-1 susceptibility profiles for the overall MRSA strain set [3]. Thus, bacterial survival in the in vitro assays for the 2 HDPs tracked together when 1 mg/L tPMP and 10 mg/L hNP-1 were compared (\( P < .001 \)) (Figure 1). Similar results were seen when bacterial survival in 2 mg/L tPMP was compared with survival in 20 mg/L hNP-1 (data not shown). The relationship of tPMP and hNP-1 susceptibility profiles with 2 genotypic markers (\( \text{agr} \) type and SCC\( \text{mec} \) type) are displayed in Table 2. The majority of strains (89%) were SCC\( \text{mec} \) type II, with no statistically significant relationship between HDP

Table 1. Host Defense Peptide Susceptibility, by Bacteremia Source and Previous Vancomycin Therapy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>tPMP Susceptibility</th>
<th>hNP-1 Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/L</td>
<td>2 mg/L</td>
</tr>
<tr>
<td>Bacteremia source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catheter related ((n = 12))</td>
<td>35 ± 30 (26)</td>
<td>.951</td>
</tr>
<tr>
<td>Non–catheter related ((n = 35))</td>
<td>33 ± 25 (25)</td>
<td>24 ± 26 (17)</td>
</tr>
<tr>
<td>Prior vancomycin treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use within 30 d ((n = 25))</td>
<td>31 ± 25 (19)</td>
<td>.481</td>
</tr>
<tr>
<td>No use within 30 d ((n = 22))</td>
<td>37 ± 27 (34)</td>
<td>34 ± 32 (23)</td>
</tr>
</tbody>
</table>

Data are mean ± SD (median) percentage survival after 2-h exposure in vitro. Abbreviations: hNP-1, human neutrophil defensin-1; tPMP, thrombin-induced platelet microbicidal protein.
susceptibility profiles and this genotypic marker. In contrast, although representing a minority of isolates, agr type III strains were significantly more resistant to killing by tPMP at 1 and 2 mg/L and by hNP-1 at 10 mg/L, compared with agr type I or II isolates, when comparing mean survival data. Virtually identical data were observed when comparing median survival data sets (for 1 mg/L tPMP: 70% for group III, 39% for group I, and 16% for group II [P = .002]; for 2 mg/L tPMP: 67% for group III, 22% for group I, and 8% for group II [P = .001]; and for 10 mg/L hNP-1: 53% for group III, 36% for group I, and 34% for group II [P = .044]).

**Table 2. Host Defense Peptide Susceptibility Among Distinct Methicillin-Resistant Staphylococcus aureus Genotypes and Vancomycin-Daptomycin Minimum Inhibitory Concentrations**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Strains, No. (%) (n = 47)</th>
<th>tPMP Susceptibility&lt;sup&gt;a&lt;/sup&gt;</th>
<th>hNP-1 Susceptibility&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/L</td>
<td>2 mg/L</td>
<td>10 mg/L</td>
</tr>
<tr>
<td>agr group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10 (21)</td>
<td>39 ± 24 (39)</td>
<td>.002</td>
</tr>
<tr>
<td>II</td>
<td>29 (62)</td>
<td>23 ± 19 (16)</td>
<td>14 ± 16 (8)</td>
</tr>
<tr>
<td>III</td>
<td>8 (17)</td>
<td>64 ± 26 (70)</td>
<td>62 ± 36 (67)</td>
</tr>
<tr>
<td>SCCmec type&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>39 (89)</td>
<td>33 ± 26 (21)</td>
<td>NS</td>
</tr>
<tr>
<td>IV</td>
<td>5 (11)</td>
<td>43 ± 28 (41)</td>
<td>30 ± 28 (20)</td>
</tr>
<tr>
<td>Vancomycin MIC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mg/L</td>
<td>10 (21)</td>
<td>38 ± 30 (38)</td>
<td>NS</td>
</tr>
<tr>
<td>1.0 mg/L</td>
<td>23 (70)</td>
<td>32 ± 26 (19)</td>
<td>22 ± 25 (14)</td>
</tr>
<tr>
<td>2.0 mg/L</td>
<td>4 (9)</td>
<td>35 ± 21 (31)</td>
<td>27 ± 28 (22)</td>
</tr>
<tr>
<td>Daptomycin MIC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 0.5 mg/L</td>
<td>35 (74)</td>
<td>28 ± 24 (16)</td>
<td>.009</td>
</tr>
<tr>
<td>1.0 mg/L</td>
<td>12 (26)</td>
<td>50 ± 25 (46)</td>
<td>37 ± 26 (32)</td>
</tr>
</tbody>
</table>

Data are mean ± SD (median) susceptibility, unless otherwise indicated.

Abbreviations: hNP-1, human neutrophil defensin-1; NS, not significant; SCCmec, staphylococcal cassette chromosome mec; tPMP, thrombin-induced platelet microbicidal protein.

<sup>a</sup>Three MRSA strains were SCCmec nontypeable.

**Relationship Between HDP Susceptibilities and agr Function**

As noted above, MRSA with agr group III genotype exhibited decreased HDP susceptibility as compared to group I or II isolates. The interrelationships among agr function, daptomycin MICs, and tPMP susceptibilities were assessed by classification and regression tree modeling. Among the 12 strains in which agr function was preserved or enhanced as assessed by δ-hemolysin + assay, higher daptomycin MICs tracked with reduced killing by tPMPs, irrespective of the δ-hemolysin profile (ie, agr functionality) (Figure 2B and 2C). In contrast, there were no statistically significant relationships identified between hNP-1 susceptibility profiles and daptomycin MICs detected in these studies (eg, median survival in hNP-1 was 34% and 47% for strains with daptomycin MICs of ≤ 0.5 mg/L and 1.0 mg/L, respectively; P = .168).

**DISCUSSION**

The virulence of *S. aureus* in a wide spectrum of clinical settings is attributable to its elegant host-invasion machinery.
plus its ability to evade the host’s innate defenses [1, 17]. One major component of the host’s innate defense system is the repertoire of cationic HDPs contained within or elaborated by a number of cell types, including neutrophils and platelets [16, 18, 19]. These latter HDPs are particularly relevant to bloodstream infections. Our laboratories have previously demonstrated in a number of experimental models and clinical studies that *S. aureus* strains that exhibit reduced killing by tPMPs, irrespective of δ-hemolysin profile (ie, *agr* functionality). The median 2-hour survival in 1 mg/L tPMP was 16% for MRSA with a daptomycin MIC of ≤ 0.5 mg/L (n = 35) and 46% for MRSA with an MIC of 1 mg/L (n = 12) (P = .009) (A). For MRSA with reduced or absent *agr* function [n = 35], the median 2-hour tPMP survival was 16% for an MIC of ≤ 0.5 mg/L (n = 25) and 44% for an MIC of 1 mg/L (n = 9) (P = .057) (B). For MRSA with *agr* function present or enhanced (n = 12), the median tPMP survival was 17% for an MIC of ≤ 0.5 mg/L (n = 9) and 48% for an MIC of 1.0 mg/L (n = 3) (P = .012) (C).
Similar to many endogenous HDPs, daptomycin contains a significant peptide moiety that can be positively charged by calcium decoration during in vivo use. Therefore, one potential driver of such HDP-daptomycin cross-resistance phenotypes may be the capacity of innate HDPs to impact organisms before daptomycin therapy, facilitating increased daptomycin MICs on subsequent daptomycin exposure. To study this, we required a cadre of daptomycin-susceptible strains that were relatively homogeneous in terms of their recent clinical characterizations, microbiologic phenotype (ie, MRSA only), clinical syndrome (ie, bacteremia without endocarditis), and isolation from patients who had never received prior daptomycin therapy (ie, daptomycin-naive individuals).

A number of interesting findings emerged from this study. First, as expected, a substantial proportion of isolates came from patients with indwelling intravascular catheters. Of interest, there were no differences in HDP susceptibility profiles between patients with and patients without intravascular catheters. These data are consistent with prior studies from our laboratories, in which the greatest differences in HDP killing profiles were detected between S. aureus isolates from patients with endocarditis versus those from patients with either catheter-associated or soft tissue abscess-associated bacteremias [20, 28]. This relationship is felt to reflect the capacity of staphylococcal strains with reduced susceptibility to HDP killing to seed, persist, and proliferate at sites of endovascular damage, especially abnormal cardiac valves [28].

Second, a number of in vitro and clinical studies have identified prior vancomycin exposures as an important potential risk factor for development of increased daptomycin MICs [29]. The mechanism by which this occurs is not well-defined but may include increased cell wall thickness and/or reduced autolysis phenotypes, as commonly exhibited by strains with reduced susceptibility to vancomycin [30, 31]. In the current study, we detected no relationships between prior vancomycin treatment and increased resistance to in vitro killing by the prototypic HDPs tested. This inability to detect a vancomycin impact may reflect the following differences between the past and current investigations: the intervals between vancomycin therapy and MRSA bacteremia, the duration of prior vancomycin use, and/or the clinical syndrome studied (eg, endocarditis vs nonendocarditis).

Third, the major finding from this study was that, even in the absence of prior daptomycin therapy, modestly increased daptomycin MICs in the study strain set were significantly correlated with reduced capacity to kill the organism in vitro by selected HDPs. Of note, although the tPMP and hNP-1 susceptibilities for individual organisms tracked together, only the tPMP susceptibilities correlated with increases in daptomycin MICs (ie, comparing isolates with daptomycin MICs of 0.25–0.5 mg/L vs 1 mg/L). We hypothesize that the principle HDPs to which these bacteremic organisms were exposed were platelet derived, resulting in an adaptive enhancement of resistance to tPMP killing. If so, these findings would also suggest that platelet-mediated defenses are critically important in protection against staphylococcal bacteremia [17, 18]. Alternatively, one could speculate that a certain proportion of this bacteremic patient population was initially colonized and infected by a cohort of isolates intrinsically more resistant to tPMP-mediated killing. However, the studies presented herein were not designed to determine such potential cause-and-effect relationships. Nonetheless, it is remarkable that HDP-daptomycin cross-resistance appeared to be relatively HDP selective. In contrast, in our prior HDP-daptomycin cross-resistance study [3], tPMP and hPN-1 resistance statistically tracked together with increases in daptomycin MICs. This latter difference may well be related to the presumed manner in which the increased daptomycin MICs were induced (ie, by exposure to HDPs in daptomycin-naive patients in the current study vs prior exposures to daptomycin in the previous study [3]). Another potential explanation for the differences in outcomes between platelet-derived tPMPs and neutrophil-derived hNP-1 may be the well-known differences in their primary, secondary, and tertiary structures and/or their distinct mechanisms of action [16].

Fourth, clinical correlations of agr genotype and function with reduced susceptibilities to both cationic calcium–daptomycin and HDPs have been inconsistent. In our recent analysis of daptomycin-HDP cross-resistances, 7/10 MRSA strains showing the cross-resistant phenotype were agr type II [3]. In another recent investigation from our laboratories of 36 MRSA strains [32], the vast majority were either agr types I or II; there was no apparent linkage of agr type II with HDP in vitro susceptibility profiles in this latter study. In contrast, we recently found that tPMP resistance in vitro tracked with agr type III and reduced agr functionality [8]. In the present study, we also found a significant relationship between agr type III and reduced killing by tPMP. In this regard, the recent finding that S. aureus isolates of the clonal complex 30 genetic background are highly associated with endocarditis is of particular interest [33]. This clonal complex generally falls within the agr group III genotype [34]. Given the link between tPMP resistance and endocarditis [21, 28], these data suggest a genotypic predisposition of these strains to establish endovascular infection. However, there was no clear interrelationship between HDP profiles and agr functional status in the present investigation. Thus, the relationship among agr types, agr function, and HDP susceptibilities may well be strictly specific to strain or clonal complex type; moreover, specific agr types and/or functionality may merely be “biomarkers” for daptomycin-HDP cross-resistance and not causal in this phenotype [12].

Last, although all MRSA strains in this study were within the “daptomycin-susceptible” range, the significant correlation of reduced in vitro killing by prototypical HDPs with a
modest daptomycin "MIC creep" (0.25–0.5 mg/L vs 1 mg/L) is quite reminiscent of the "vancomycin creep" scenario. In this latter circumstance, a 2-fold increase in vancomycin MICs within the "susceptible" range (≤1 vs 2 mg/L) appears to identify a S. aureus strain cohort (both methicillin-susceptible and MRSA) with increased likelihood of suboptimal clinical outcomes [35, 36]. Whether MRSA strains with modestly increased daptomycin MICs and reduced capacities to be killed by HDPs (as in the current study) are associated with less favorable treatment outcomes remains to be determined.

The results of our investigations have interpretive limitations. For example, the in vitro HDP susceptibility testing was performed in austere media, in the absence of host factors (eg, serum proteins). Moreover, both neutrophils and platelets contain a large cohort of HDPs that were not individually tested in this study. Additional nonbloodstream HDPs (eg, HDPs of cutaneous origin) were not explored, but future investigations are planned. In addition, it is likely that bloodstream bacteria are simultaneously exposed to multiple HDPs. Finally, the concentrations of HDPs used in our in vitro assays are undoubtedly lower than those encountered physiologically by organisms in vivo and were chosen for their ability to discriminate different populations of MRSA in our cohort.

Present investigations are in progress to further address the phenomenon of daptomycin-HDP "cross-resistance" and to adjudicate several of the above limitations. However, on the basis of our current data, the development of cationic antimicrobial peptides as novel therapeutics, designed on endogenous antimicrobial templates, should be screened against well-characterized cohorts of "antibiotic-naive" bloodstream isolates for the potential endogenous priming phenomena.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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