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ARTICLE

The role of polysaccharide intercellular adhesin (PIA) in *Staphylococcus epidermidis* adhesion to host tissues and subsequent antibiotic tolerance

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Abstract The aim of this study was to determine the role of polysaccharide intercellular adhesin (PIA) in *Staphylococcus epidermidis* adhesion to host tissues and subsequent antibiotic tolerance. The adherence of *S. epidermidis* 1457 and the mutant defective in PIA production (1457-M10) to urinary epithelium and endothelium was estimated by colony counting. Minimum bactericidal concentration and mean reduction of cellular activity (XTT) following antibiotic exposure was determined for planktonic and adhered bacteria. *S. epidermidis* 1457 adhered to a greater extent to both cells than the mutant strain. The adhered strains had a significantly higher antimicrobial tolerance than their planktonic counterparts. The mutant strain was, in general, the most susceptible to the antibiotics assayed. In conclusion, PIA may influence *S. epidermidis* adherence to host tissues and their antimicrobial susceptibility. Initial adhesion may be the main step for the acquisition of resistance in *S. epidermidis*.

Introduction

In the last few decades, coagulase-negative staphylococcus (CoNS), in particular *Staphylococcus epidermidis*, have been associated with an increasing number of nosocomial infections involving indwelling devices [1, 2]. Additionally, these bacteria also demonstrate the ability to adhere to host tissues, leading to serious infections [3]. Despite the increased clinical relevance of CoNS, their virulence factors are not completely known [1, 4], especially those involved with host tissue infections. It should also be highlighted that adherence to host tissue might be influenced by distinct factors of those mediating materials adhesion and biofilm formation [5].

The ability to adhere and subsequently form biofilm on indwelling devices is among the potential virulence factors associated with *S. epidermidis* [4, 6, 7]. Indeed, biofilm formation frequently compromises the efficacy of implanted medical devices [8–10] and is also known to occur in native tissues, such as cartilage and cardiac tissue for *S. aureus* [11]. Biofilm formation can be divided into two main phases: initial adherence to the implant or tissue surface and biofilm accumulation involving cell proliferation and intracellular adhesion [12–14]. The molecule polysaccharide intercellular adhesin (PIA, synthesised by the *ica* operon [15, 16]) has a very important role in the establishment of the biofilm structure being involved in cell-to-cell adhesion [17–21]. Gram-positive bacteria infections have become increasingly problematic due to the increased acquisition of resistance to antimicrobial agents [22, 23], hindering their treatment [24, 25]. This aspect contributes to the increased importance of CoNS as pathogens, and is particularly relevant when biofilm formation occurs, since it was observed that bacteria embedded in this structure can tolerate antibiotic levels significantly higher than planktonic cells, exhibiting a dramatically increased resistance (up to 1,000-fold) [26–28]. Several mechanisms have been suggested to explain this phenomenon, all based on the biofilm structure. However, the study of antimicrobial resistance has been neglected for bacteria in the first phase of biofilm formation (initial adhesion). In the adhered state, bacteria demonstrate deep physiologic and morphologic alterations comparatively to their planktonic phenotype, which may modify, and

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increase, antibiotic tolerance [29]. One of the purposes of this work was, therefore, to determine the susceptibility of adhered *S. epidermidis* cells to host tissues to five different antibiotics with different action mechanisms (the cell wall synthesis inhibitors vancomycin, cefazolin and dicloxacillin, the protein synthesis inhibitor tetracycline and the RNA synthesis inhibitor rifampicin).

It was also a goal of this study to determine the role of PIA in *S. epidermidis* adhesion to epithelium and endothelium and subsequent antibiotic tolerance.

Materials and methods

Bacterial strains and growth conditions

In this work, two strains of *S. epidermidis* were used: *S. epidermidis* 1457, a biofilm-forming strain [30], and its mutant *S. epidermidis* 1457-M10 [31]. This mutant strain was produced by the insertion of transposon Tn*917* in the *ica*A gene of *S. epidermidis* 1457, which led to the inactivation of the *ica*ADBC gene and, consequently, to the complete abolition of PIA production and biofilm formation [32, 33]. Both strains were kindly provided by Professor Gerald Pier from Harvard University.

Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) plates were prepared according to the manufacturer's instructions (Sigma-Aldrich). Strains were grown for 18 h (for adhesion and minimum bactericidal concentration [MBC] assays) or for 24 h (for XTT colorimetry assay) at 37°C with agitation (110 rpm), in 30 ml of TSB inoculated with bacterial cultures prepared in the previous day on TSA plates. Cells were harvested by centrifugation (for 5 min at 9,000 rpm and 4°C), washed twice with phosphate-buffered saline (PBS, prepared with 10 mM KH_2PO_4 (Sigma-Aldrich), 10 mM K_2HPO_4 (Sigma-Aldrich) and 150 mM NaCl (Sigma-Aldrich, pH 7.0), resuspended in PBS and cellular concentration adjusted depending on the assay.

Animal cells and growth conditions

For the adhesion assays, primary human umbilical vein endothelial cells (HUVEC, C-003-5C, Cascade Biologics) and the established urinary epithelial cell line TCC-SUP (DSMZ) were used. HUVEC cells were grown in Medium 200 (Cascade Biologics) supplemented with 10% low serum growth supplement (Cascade Biologics). TCC-SUP cells were grown in D-MEM medium supplemented with 15% fetal bovine serum (FBS, Gibco-Invitrogen) and 1% antibiotic (Penicillin-Streptomycin, Gibco-Invitrogen). For the adherence studies, endothelial and epithelial cells were grown to confluence in 96-well microplates (100 µl), at 37°C and 5% CO_2, with the medium described above for the appropriate cell type, without antibiotic. Approximately 5×10^3 HUVEC cells/well or 4×10^4 TCC-SUP cells/well were obtained at confluence.

Adhesion assay

Monolayers of HUVEC and TCC-SUP cells were washed with PBS and then inoculated with 100 µl of the bacterial suspensions, yielding 1×10^8 bacteria/well. A control was performed with 100 µl of PBS. After 2 h of contact at 37°C and 5% CO_2, monolayers were washed three times with PBS to remove non-adhered bacteria. Endothelial and epithelial cells were detached by the addition of trypsin/EDTA (Cascade Biologics for HUVEC cells and Gibco-Invitrogen for TCC-SUP cells). The number of adherent bacteria was determined by colony forming units (cfu) enumeration, plating 10-fold serial dilutions of well suspensions onto TSA plates, which were inoculated overnight at 37°C. This experiment was repeated three times, in duplicate.

Antibiotics

In this study, five antibiotics were used, namely, cefazolin, vancomycin, dicloxacillin, tetracycline and rifampicin (Sigma-Aldrich). For the antimicrobial testing, solutions of 20, 10, 5, 2.5, 1.25 and 0.75 mg/L (planktonic cells) and 1,000, 500, 250, 100, 50 and 5 mg/L (adhered cells) were prepared, from stock solutions with concentrations of 2,000 mg/L (in sterile water), in Mueller Hinton Broth (Sigma-Aldrich), immediately before each experiment.

MBC for planktonic cells

For each strain, 5 µl of bacterial suspension yielding 1×10^7 cfu/well for 1457 and 4×10^6 cfu/well for strain 1457-M10 (concentrations are identical to those obtained for adhered bacteria) were added to each well of a 96-well plate containing 100 µl of different dilutions of each antibiotic and incubated at 37°C with agitation (110 rpm) for 24 h. Then, 10-µl samples of each well were spread in TSA plates, which were incubated at 37°C for another 24 h. MBC was determined as the minimal antibiotic concentration that did not allow colony formation. Experiments were repeated three times, in triplicate.

MBC for adhered cells

Antibiotic dilutions (100 µl) were added to 96-well microplates containing bacteria adhered to HUVEC and TCC-SUP cells and were incubated at 37°C and 5% CO_2 for 24 h. Then, aliquots of 10 µl from each well were spread in TSA plates and incubated at 37°C for 24 h for MBC

determination. MBC was determined as described previously. Experiments were repeated three times, in triplicate.

XTT colorimetry for planktonic cells

Bacteria were exposed to each antibiotic (in the respective MBC) by the addition of 100 μl of bacterial suspension (yielding 1×10^7 cfu for strain 1457 and 4×10^6 cfu for strain 1457-M10) to each well of a 96-well plate containing 100 μl of antibiotics (2 times concentration). The plate was incubated at 37°C and 110 rpm for 3 h, after which 50 μl of a solution containing 100 mg/L of XTT (Sigma-Aldrich) and 10 mg/L of PMS (Sigma-Aldrich) were added to each well. The plate was then incubated at 37°C for 3 h in the dark, with agitation (110 rpm). Cells were allowed to settle for 15 min before spectrophotometric readings and 150 μl of the supernatant from each well were transferred to a new well plate and the absorbance was measured at 490 nm. XTT solutions resulting from cell suspensions not exposed to antibiotics were used as controls. All experiments were repeated two times, in triplicate. Results of the reduction of cellular activity were expressed as the difference in absorbance readings at 490 nm between controls without antibiotic and antibiotic-treated strains, per μg of antibiotic used, in order to standardise the antimicrobial agents concentration.

XTT colorimetry for adhered bacterial cells

Antibiotics (200 μl) at the MBC were added to a 96-well microplate containing bacteria adhered to HUVEC or TCC-SUP cells and incubated at 37°C and 110 rpm for 3 h. Then, 50 μl of a solution containing 100 mg/L of XTT and 10 mg/L PMS were added to each well. Microplates were incubated for additional 3 h at 37°C in the dark. Cells were allowed to settle before spectrophotometric readings and 150 μl of the supernatant from each well were transferred to a new well plate, with absorbance measured at 490 nm. Bacteria adhered to HUVEC or TCC-SUP cells and not exposed to antibiotics were used as controls. All experiments were repeated two times, in triplicate. Results of the mean reduction of cellular activity were expressed as the difference in absorbance readings at 490 nm between controls without antibiotic and antibiotic-treated strains, per μg of antibiotic used.

Statistical analysis

Statistical analysis was performed using SPSS software (Statistical Package for the Social Sciences). Adhesion results were analysed using one-way analysis of variance (ANOVA) and XTT colorimetry assays were analysed using one-way ANOVA with Bonferroni test. All tests were performed with a confidence level of 95%.

Results

Adhesion to endothelium and urinary epithelium

For the mutant strain, *S. epidermidis* 1457-M10, the number of bacteria adhered to both tissues (Fig. 1) was significantly lower ($P=0$) than for strain 1457. Both strains demonstrated significantly higher ($P=0$) extent of adhesion to endothelial cells when compared with urinary epithelial cells. Figure 1 allows the comparison of the strains' adherence to endothelium and epithelium.

Antibiotic susceptibility assessed by MBC

Table 1 presents the MBC of five antibiotics for *S. epidermidis* strains on the planktonic or adhered states. Adhered bacteria, either to endothelium or epithelium, had

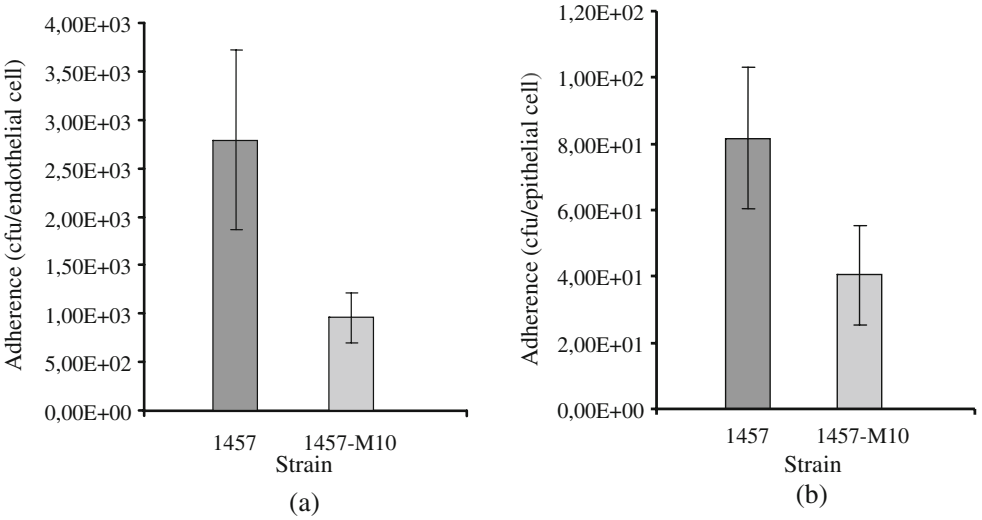

Fig. 1 Mean number of *Staphylococcus epidermidis* 1457 and 1457-M10 colony forming units (cfu) adhered to: **a** endothelial cells and **b** urinary epithelial cells

Table 1 Minimum bactericidal concentration (MBC) of different antibiotics for *Staphylococcus epidermidis* 1457 and *S. epidermidis* 1457-M10 on the planktonic and adhered states

Antibiotic	MBC (mg/L)					
	Planktonic		Adhered to endothelial cells		Adhered to urinary epithelial cells	
	1457	1457-M10	1457	1457-M10	1457	1457-M10
Cefazolin	<0.75	<0.75	500–1,000	500–1,000	500–1,000	500–1,000
Vancomycin	10–20	10–20	500–1,000	500–1,000	500–1,000	500–1,000
Dicloxacillin	1.75–2.5	1.75–2.5	500–1,000	500–1,000	500–1,000	500–1,000
Tetracycline	10–20	10–20	100–250	100–250	100–250	100–250
Rifampicin	0.75–1.25	0.75–1.25	5–50	5–50	5–50	5–50

higher MBC values than planktonic bacteria, indicating a lower susceptibility. The RNA inhibitor, rifampicin, was the most efficient antibiotic against adhered bacteria.

Antibiotic susceptibility assessed by XTT colorimetry

The mean reduction in metabolic activity for both *S. epidermidis* strains, after exposure of planktonic or adhered cells to the MBC of the antibiotics, was also assayed (Table 2). Cellular activity reduction for adhered bacteria was significantly lower than for planktonic bacteria, confirming the MBC results that indicate a higher tolerance for adhered cells.

Table 3 shows the significance values obtained by the statistical comparison of susceptibility between *S. epidermidis* strains for the different antibiotics and bacterial states. Significant differences were observed for vancomycin, dicloxacillin and tetracycline for bacteria in the planktonic and adhered states. For cefazolin, significant differences were detected for adhered bacteria, while for rifampicin, strains susceptibility was significantly different only when they were adhered to urinary epithelium.

Discussion

S. epidermidis has been established as one of the most important pathogens associated with nosocomial infections, particularly those involving indwelling devices [1, 4]. Furthermore, these microorganisms have also demonstrated the ability to adhere to human tissues, causing high-mortality infections [3]. Bacterial adhesion is thought to be one of the critical steps for host tissue infections, but the current knowledge about this phenomenon, and the factors influencing it, is still very limited.

In this study, it was evaluated the adherence of two *S. epidermidis* strains that differ only on their ability to produce PIA, an important cell component on biofilms

Table 2 Mean reduction of cellular activity, assessed by XTT, after 3 h of exposure to the MBC of the antibiotics, of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 on planktonic and adhered states, expressed as the difference in absorbance values with and without antibiotic treatment per μg of antibiotic

Antibiotic	$ABS_{490\ nm}$ (± SD)					
	Planktonic		Adhered to endothelium		Adhered to urinary epithelium	
	1457	1457-M10	1457	1457-M10	1457	1457-M10
CFZ	4.35×10^{-1} $\pm 1.58\times10^{-2}$	4.16×10^{-1} $\pm 2.22\times10^{-2}$	1.70×10^{-4} $\pm 7.07\times10^{-7}$	1.84×10^{-4} $\pm 5.00\times10^{-6}$	4.39×10^{-5} $\pm 6.35\times10^{-6}$	5.92×10^{-5} $\pm 5.17\times10^{-6}$
VAN	1.50×10^{-2} $\pm 1.24\times10^{-3}$	1.64×10^{-2} $\pm 7.07\times10^{-4}$	1.51×10^{-4} $\pm 1.44\times10^{-5}$	2.66×10^{-4} $\pm 1.84\times10^{-5}$	1.09×10^{-4} $\pm 1.50\times10^{-5}$	6.18×10^{-5} $\pm 7.74\times10^{-6}$
DCX	8.75×10^{-2} $\pm 6.41\times10^{-3}$	1.13×10^{-1} $\pm 4.48\times10^{-3}$	1.61×10^{-4} $\pm 5.13\times10^{-6}$	1.98×10^{-4} $\pm 2.65\times10^{-6}$	5.14×10^{-5} $\pm 4.89\times10^{-6}$	3.27×10^{-5} $\pm 5.82\times10^{-6}$
TET	7.14×10^{-3} $\pm 7.69\times10^{-4}$	8.29×10^{-3} $\pm 8.49\times10^{-4}$	6.24×10^{-4} $\pm 2.20\times10^{-5}$	8.73×10^{-4} $\pm 4.06\times10^{-5}$	1.44×10^{-4} $\pm 3.08\times10^{-5}$	4.78×10^{-4} $\pm 2.60\times10^{-5}$
RIF	2.18×10^{-1} $\pm 1.32\times10^{-2}$	2.14×10^{-1} $\pm 1.30\times10^{-2}$	2.38×10^{-3} $\pm 1.36\times10^{-4}$	2.36×10^{-3} $\pm 1.22\times10^{-4}$	4.10×10^{-3} $\pm 2.80\times10^{-4}$	5.59×10^{-3} $\pm 2.98\times10^{-4}$

CFZ: cefazolin; VAN: vancomycin; DCX: dicloxacillin; TET: tetracycline; RIF: rifampicin

Table 3 Significance (*P*) values obtained for the comparison of metabolic activity reduction between *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 for the different antibiotics and bacterial states

Antibiotic	Planktonic	Adhered to endothelium	Adhered to urinary epithelium
Cefazolin	0.121	**0.030**	**0.001**
Vancomycin	**0.041**	**0.001**	**0.000**
Dicloxacillin	**0.000**	**0.000**	**0.000**
Tetracycline	**0.034**	**0.001**	**0.000**
Rifampicin	0.580	0.859	**0.000**

Significant *P*-values ($P<0.05$) are represented in **bold**

formation, whose influence in the initial adhesion is not entirely understood. *S. epidermidis* 1457, the PIA producer, demonstrated a higher adherence to host cells than the mutant strain (unable to produce PIA), with significant differences of about 3-fold to endothelium and 2-fold to urinary epithelium (Fig. 1). Previously, Rupp et al. [34] reported a lower adherence of this mutant strain to subcutaneous catheters implanted in mice, which, in conjugation with the results presented, suggest that the 1457-M10 strain adheres in a lower extension than the 1457 strain to both biomaterials and host cells, either in vivo or in vitro.

Adherence to substrates is known to be influenced by several aspects, including bacterial and host cells, and even environmental factors. Among them are the specific interactions established between microorganism ligands and complementary receptors in host cells. Extracellular polymers, such as, for example, PIA, may act as ligand and, consequently, influence the rate and extension of microbial adhesion [35]. Hence, the lower adherence of the mutant strain to endothelial and urinary epithelial cells may be explained by its inability to produce PIA, preventing possible interactions between this exopolymer and host cell surface components.

PIA's role on initial adhesion has been an object of discussion for years: several authors [18–21] support the view that this polymer is only responsible for biofilm cell accumulation; others [10, 36] have a divergent opinion, suggesting that PIA influence on adhesion is not well established, showing that the assays' conditions (such as dynamic or static conditions; long- or short-term adherence) are responsible for the extent of adhesion. Furthermore, most studies are related to biomaterials adhesion, with a very small knowledge about adherence to host tissues. The results obtained in the present study corroborate the PIA relation with the early stages of adherence to host tissues.

Comparing the adhesion to both tissues (Fig. 1), the *S. epidermidis* strains demonstrated a significantly higher adherence to endothelial cells. This may be a consequence of the host cell's own characteristics, such as surface hydrophobicity or surface proteins expression (fibronectin, fibrinogen, vitronectin), that may influence interactions with microbial surfaces. However, the percentage of bacterial cells that adhered to host cells in relation to the initial inoculum was very small for both *S. epidermidis* strains, as demonstrated by the percentage of initial inoculum adhered (3.5% and 14.7% for the 1457 strain, and 1.9% and 5.4% for the 1457-M10 strain). This is in accordance with previous studies, which indicate a lower *S. epidermidis* propensity to adhere to urinary tissue [37, 38] and to endothelial cells [39].

In the last few years, antimicrobial resistance has been established as a serious problem for the treatment of infections, especially those involving biofilms. For this reason, the most recent antimicrobial resistance studies concern bacteria embedded in a biofilm structure. However, some authors [9, 27, 40] contradict this approach, highlighting the importance of the evaluation of bacterial resistance in the first phase of biofilm formation, which consists of initial adhesion. Furthermore, antimicrobial susceptibility is usually assessed by determining the minimal inhibitory concentration (MIC). However, Williams et al. demonstrated that adhered bacteria may be able to evade the bactericidal effect of antibiotics [29], and, therefore, although more resistant, they exhibit no MIC alterations, which may lead to the misinterpretation of susceptibility. Additionally, there had been an increased number of immunocompromised patients with CoNS infections, and treatment with antibiotics at their MIC seemed to be ineffective, since, at this concentration, the efficiency of the antibiotic relies on the collaboration of the immune system to completely eliminate the infection. Therefore, these aspects emphasise the need to evaluate the bactericidal effect of the antibiotics, and not their MIC, which may be assessed by MBC determinations [41].

MBC results for *S. epidermidis* strains (Table 1) demonstrate that bacteria which adhered to both host tissues have a much lower susceptibility to antibiotics than planktonic bacteria. This is particularly true for cell wall synthesis inhibitors (cefazolin, vancomycin and dicloxacillin), for which differences of about two times more were detected. Furthermore, it was also detected a higher

resistance of adhered bacteria to both tetracycline (protein inhibitor) and rifampicin (RNA inhibitor), although the susceptibility to antibiotics appeared to be less affected by the sessile phenotype. The results obtained are in agreement with a previous study of Cerca et al. concerning *S. epidermidis* biofilms and involving the same antibiotics [27]. It was also observed that rifampicin displayed the best efficacy against bacteria adhered to host cells, and that it was the only one with a possible clinical application for the treatment of CoNS infections, since its *peek serum* concentration (10 mg/L [27]) is the only one within the MBC range determined.

The XTT results (Table 2) allowed the same conclusions as MBC, indicating a significantly higher resistance for adhered bacteria, with rifampicin showing the lowest reduction of activity.

Therefore, both MBC and XTT results provide evidence that the adhered phenotype offers protection against several antibiotics, suggesting that biofilm structure is not the main factor leading to increased tolerance. Indeed, the initial adhesion may be the most important step for the acquisition of this low susceptible state. Such observations contradict the mechanisms previously proposed to explain the increased resistance, which are mainly based on biofilm structure. These include a lower or incomplete penetration of the antibiotic due to a diffusion barrier, phenotypic variability within the biofilm and slow cell growth within the biofilm [9, 27, 28]. Slow growth within the biofilm could be a possible explanation for the higher resistance displayed by adhered bacteria, which was observed by Williams et al. for *S. aureus* in the adhered state (without biofilm formation) [29]. However, since this could only be valid for antibiotics whose action depends on the cell growth rate, such as cell wall inhibitors, the fact that rifampicin activity is also dependent on the growth rate and no significant differences regarding this antibiotic susceptibility were detected between adhered and planktonic bacteria implies that it is not a valid explanation for the increased tolerance. Hence, other mechanisms must be involved in the acquisition of antibiotic tolerance by adhered bacteria, which may rely on the physiological state of individual bacteria and not on the physical effect of the biofilm matrix [29]. Furthermore, the susceptibility of the adhered phenotype appears to be affected by the antibiotic mechanism of action, since cell wall inhibitors present a reduced activity against adhered bacteria, and protein and RNA inhibitors remain relatively efficient.

The XTT results allow a correct comparison between strains susceptibility, since it allows the analysis of the antibiotic effect on bacteria viability for a specific antibiotic concentration. Although with some exceptions, significant differences (Table 3) were observed between strains, with the mutant 1457-M10 presenting a higher susceptibility (Tables 2 and 3). Since the only distinction between the strains consists of their ability to produce PIA, this exopolymer might be the explanation for this difference. It could confer some sort of protection against antibiotics, and, consequently, the inability of the mutant strain to produce PIA might cause it to be more susceptible.

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