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Cutaneous Biology

Confocal laser scanning microscopic observation of glycocalyx production by *Staphylococcus aureus* in mouse skin: does *S. aureus* generally produce a biofilm on damaged skin?

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Summary

Background Bacteria that adhere to damaged tissues encase themselves in a hydrated matrix of polysaccharides, forming a slimy layer known as a biofilm. This is the first report of detection of glycocalyx production by *Staphylococcus aureus* using confocal laser scanning microscopy (CLSM) on damaged skin tissues.

Objectives To analyse glycocalyx production by *S. aureus* cells on damaged skin tissues and the influence of polymorphonuclear leucocytes (PMNs) and various antimicrobial agents on its production using CLSM in cyclophosphamide (Cy)-treated (neutropenic) or non-Cy-treated (normal) mice.

Methods *S. aureus* cells were inoculated on damaged skin tissues in neutropenic or normal mice with or without topical application of antimicrobial agents. *S. aureus* cells were stained with safranine, and positive staining with fluorescein isothiocyanate-conjugated concanavalin A was considered to indicate the presence of glycocalyx.

Results All *S. aureus* cells tested on damaged skin tissues formed microcolonies encircled by glycocalyx. The colony counts of *S. aureus* cells on croton oil dermatitis in normal mice treated with 2% fusidic acid ointment were about 100 times lower than those in neutropenic mice (control).

Conclusions As *S. aureus* cells can generally produce a biofilm on damaged skin tissues, antimicrobial agents may not eradicate *S. aureus* cells without the help of PMNs. *S. aureus* glycocalyx may play a crucial role in colonization and adherence to damaged skin tissues.

Key words: antimicrobial agent, biofilm, confocal laser scanning microscopy, glycocalyx, polymorphonuclear leucocyte, *Staphylococcus aureus*

Bacterial glycocalyx is a polysaccharide-containing material produced by bacteria.[1] Bacteria that adhere to implanted medical devices or damaged tissues can become the cause of persistent infections. These bacteria encase themselves in a hydrated matrix of polysaccharide and protein, forming a slimy layer known as a biofilm.[2] Various terms such as glycocalyx, slime or exopolysaccharide have been used to designate the biofilm produced by bacteria.[3] While the relationship between glycocalyx, slime and exopolysaccharides is unclear, the term glycocalyx is general and may also refer to bacterial slime and exopolysaccharides. Biofilm architecture has been difficult to determine because images obtained by fluorescence microscopy are degraded by out-of-focus flare from focal planes above or below objects of interest. This problem has been overcome by the use of confocal laser scanning microscopy (CLSM), which allows horizontal and vertical optical thin sectioning of hydrated biofilms.[4] The real structure of the bacterial glycocalyx can only be determined by CLSM and after the glycocalyx has collapsed during dehydration to produce electron-dense accretions.[5] CLSM analysis is non-destructive, and

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CLSM makes it possible to analyse live biofilm structures. In our previous studies, *Staphylococcus aureus* cells (furuncle 1 strain) were inoculated on cut wounds in the skin[6] and the surface of the skin inflamed by application of croton oil[7] in cyclophosphamide (Cy)-treated (neutropenic) mice. Because electron microscopy (EM) revealed fibril-like structures (glycocalyx) and membrane-like structures around the *S. aureus*, we suggested that *S. aureus* cells may form a biofilm on damaged skin tissues in a neutropenic condition.[6,7] Concanavalin A (ConA) is used as a marker for detecting slime on staphylococcal cells; its mechanism of action involves the reaction of ConA and mannose.[8] However, there are no reports of detection of glycocalyx associated with *S. aureus* using CLSM on damaged skin tissues. In the present study, we analysed glycocalyx production by *S. aureus* cells in damaged skin tissues and the influence of polymorphonuclear leucocytes (PMNs) and various antimicrobial agents on its production using CLSM in neutropenic or non-Cy-treated (normal) mice.

Materials and methods

Bacterial strains

Five strains of *S. aureus* (furuncle 1, 2, 3, 4 and 5 strains) isolated from furuncle lesions (all of coagulase type IV), five (impetigo 1, 2, 3, 4 and 5 strains) isolated from impetigo lesions [two strains of coagulase type I (exfoliative toxin B producers) and three strains of coagulase V (exfoliative toxin A producers)], and five [atopic dermatitis (AD) 1, 2, 3, 4 and 5 strains] isolated from AD lesions (two strains of coagulase type III and three strains of coagulase type VII) were used and their levels of glycocalyx production *in vivo* and *in vitro* were studied. The minimum inhibitory concentration (MIC) of fusidic acid (FA; Sankyo Co., Tokyo, Japan) against impetigo 1 strain was 0·25 μg mL^{-1}. Bactericidal activity of povidone-iodine (PVP-I; Meiji Seika Co., Tokyo, Japan) against impetigo 1 strain was observed at a concentration of $\geqslant$ 0·1% in sterile saline solution. Of the 15 strains of *S. aureus*, seven were methicillin sensitive (oxacillin MIC $\leqslant$ 2 μg mL^{-1}) and eight were methicillin resistant (oxacillin MIC $\geqslant$ 4 μg mL^{-1}).

Bacterial suspension for inoculation

The strains of *S. aureus* were grown in 8 mL of tryptic soy broth (TSB; Nissui Pharmaceutical Co., Tokyo, Japan) at 37 °C overnight without shaking. Following incubation, the bacterial cells were harvested by centrifugation at 6000 *g* for 10 min at 4 °C, then resuspended in sterile saline solution, and centrifuged as described above. The process was repeated three times. The washed bacteria were resuspended in polypropylene microcentrifuge tubes (1 mL; Iuchi Bio-Systems, Tokyo, Japan) and were used in the following experiments.

Observation of glycocalyx production by confocal laser scanning microscopy in neutropenic mice

Five-week-old female mice of the ddy strain, weighing approximately 20 g, were purchased from Keari Co. (Osaka, Japan). They were made leucocytopenic by one intraperitoneal injection of Cy (Shionogi Pharmaceutical Co., Osaka, Japan) at a dose of 3 mg per mouse at 2 days before bacterial inoculation. The mean peripheral white blood cell count ($n = 3$) was 1275 mm^{-3} (normal 6505). The backs of the neutropenic mice were shaved and a cut wound 1 cm in length was produced by surgical blades (Feather no. 15) in one group (cut wound group) and a round area 1·5 cm in diameter was painted once with a cotton swab soaked in croton oil (Wako Pure Chemicals Co., Osaka, Japan) in another group (croton oil dermatitis group). One hundred microlitres of the bacterial suspension (about 10^8 colony-forming units, c.f.u.) of furuncle 1, 2, 3, 4 and 5 strains was slowly applied through a micropipette on the cut wound area in the cut wound group. Eighteen hours after croton oil application in the croton oil group, 0·1 mL of the bacterial suspension (about 10^8 c.f.u.) of impetigo 1, 2, 3, 4 and 5 strains and AD 1, 2, 3, 4 and 5 strains was slowly applied through a micropipette on the area treated with croton oil while the applied suspension was being spread with the tip of the pipette. Biopsy specimens ($n = 3$) were obtained from the skin lesions at 4, 8, 12, 24, 48 and 72 h after inoculation with furuncle 1 strain in the cut wound group, at 4, 8 and 12 h from those inoculated with impetigo 1 strain in the croton oil dermatitis group, and at 12 h from those inoculated with the other 13 strains in the cut wound group and croton oil dermatitis group. Specimens were embedded in OCT Compound (Sakura Finetechnical Co., Tokyo, Japan) for frozen tissue specimens, and cut to a thickness of 10 μm using cryostat HM 505 E (Micron, Tokyo, Japan). The specimens were washed three times with 0·01 mol L^{-1} phosphate-buffered saline (PBS; pH 7·4) for 5 min at room temperature and immersed in 0·1% safranine (pH 7·4; Wako) for 5 min. After washing

with PBS, the specimens were immersed in fluorescein isothiocyanate-conjugated ConA (FITC-ConA; Funakoshi Co., Tokyo, Japan; 50 μg mL^{-1}) for 5 min at room temperature. After washing with PBS, the specimens were embedded using the Prolong™ antifade kit (Molecular Probes, Leiden, the Netherlands) and were observed using an LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany).

Topical application of antimicrobial agents in neutropenic mice

In the croton oil dermatitis group inoculated with impetigo 1 strain, 2% FA ointment and 10% PVP-I solution were topically applied at 4 and 8 h after bacterial inoculation, respectively. In one group ($n = 3$), biopsy specimens were obtained from the skin lesions at 12 h after the inoculation and were observed by CLSM. In the other group ($n = 5$), the infected skin areas 1·5 cm in diameter were excised *en bloc* just above the muscle layer at 12 h after bacterial inoculation. The excised skin specimens were chopped using a sterile pair of scissors and homogenized with 3 mL of sterile saline solution in sterile mortars. Then, 1·5 mL of 5% sodium thiosulphate (Wako) was added to the sample solution for bacterial count in the PVP-I-treated group.[7] One millilitre of each homogenate was taken in a small sterile tube and a 10-fold dilution method was employed to count the c.f.u. Two separate 0·1-mL aliquots of each diluted specimen were cultured on Mueller–Hinton agar plates (Difco, Detroit, MI, U.S.A.) at 37 °C and the c.f.u. were counted at 24 h after the inoculation. The counts obtained were converted into counts per unit lesion area. Under these conditions the background of the normal resident flora was negligible.

Observation of glycocalyx production by confocal laser scanning microscopy in normal mice

In the croton oil dermatitis group of normal mice, biopsy specimens ($n = 3$) were obtained from the skin lesions at 4, 8 and 12 h after bacterial inoculation with impetigo 1 strain and at 12 h from those inoculated with impetigo 2, 3, 4 and 5 strains, and the specimens were observed by CLSM.

Topical application of antimicrobial agents in normal mice

In the croton oil dermatitis group of normal mice, 2% FA ointment and 10% PVP-I solution were topically applied at 4 and 8 h, respectively, after bacterial inoculation with the impetigo 1 strain. Biopsy specimens ($n = 3$) were obtained from the skin lesions and were observed by CLSM, and the numbers of bacteria ($n = 5$) in the lesions (1·5 cm in diameter) were counted at 12 h after bacterial inoculation.

Observation of glycocalyx production by confocal laser scanning microscopy

Attachment on coverslips. Cell suspensions of the 15 *S. aureus* strains (about 10^8 c.f.u.) were inoculated separately into 2 mL of Pl-TSB [rabbit plasma (Denka Seiken, Tokyo, Japan): TSB 1 : 1] on 1·0-cm^2 coverslips (Sumitomo Bakelite, Tokyo, Japan). After incubation for 12 h at 37 °C, the coverslips were immersed in safranine and FITC-ConA, and were observed by CLSM.

Tube adherence. The 15 strains were inoculated separately into polypropylene microcentrifuge tubes containing 1 mL of TSB. After 48 h at 37 °C, each tube was decanted and 1 mL of 0·4% aqueous solution of trypan blue (Sigma, St Louis, MO, U.S.A.) was added. Each tube was then gently rotated to ensure uniform staining and the contents were gently decanted. The tubes were then placed upside down to drain. The colour of the inner surfaces of the tubes was observed. A positive result was defined as the presence of an adherent layer of stained material on the inner surfaces of the tubes (Christensen method).[8–11]

Congo red agar method. The 15 strains were inoculated into laboratory dishes (Nissui) containing 20 mL of brain heart infusion (Nissui) agar with 0·08% Congo red (Sigma) and 5% glucose (Nakarai Tesque, Kyoto, Japan), and discrete colonies were formed. After 24 h at 37 °C, the colour of the colonies was observed. Black colonies were interpreted as glycocalyx producers.[10,11]

Statistical analysis

Data were analysed using a *t*-test for unpaired comparisons.

Results

Microcolonies stained positive with safranine alone or with safranine and FITC-ConA, and were encircled by materials stained strongly positive with FITC-ConA alone at 4, 8 and 12 h in those inoculated with furuncle 1 strain in the cut wound group, at 4, 8 and 12 h (Fig. 1) in those inoculated with impetigo 1 strain

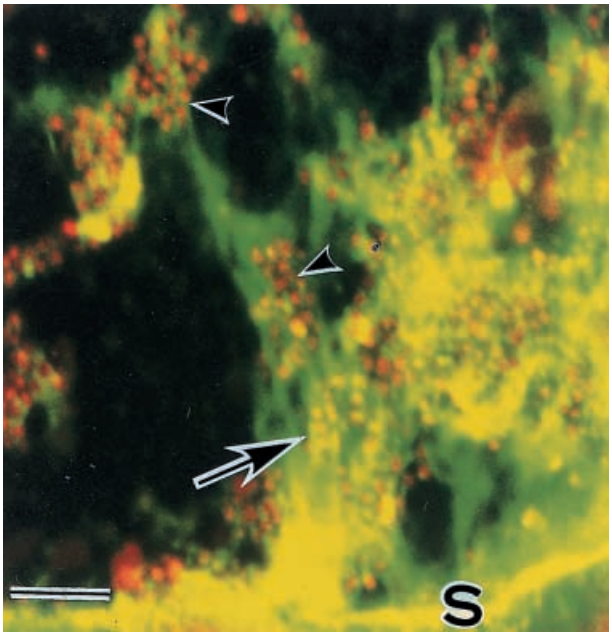

Figure 1. The double-staining patterns of safranine and fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA) from tissues inoculated with impetigo 1 strain in the croton oil dermatitis group of neutropenic mice after incubation for 12 h at 37 °C. Microcolonies were stained with safranine alone (arrowhead; red) or safranine and FITC-ConA (arrow; yellow), and were encircled by materials stained strongly positive with FITC-ConA alone (green). S, surface of the epidermis. Bar = 10 μm.

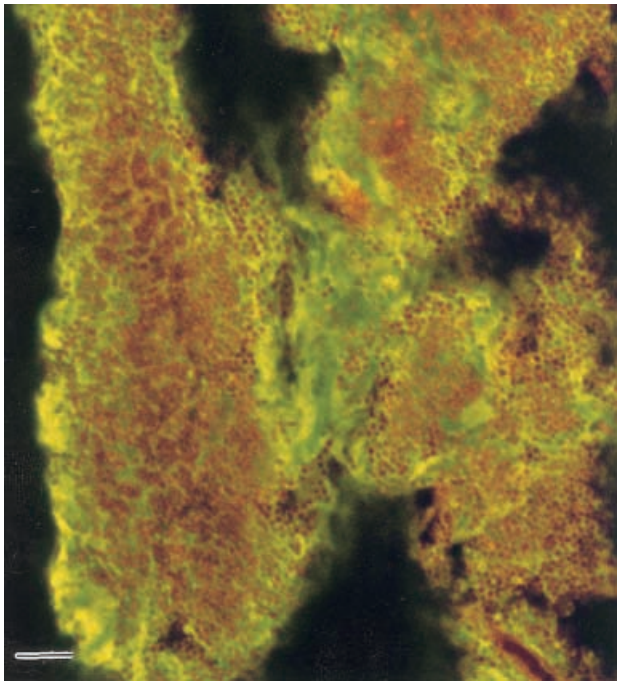

Figure 2. The double-staining patterns of safranine and fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA) from tissues inoculated with furuncle 1 strain in the cut wound group of neutropenic mice after incubation for 24 h at 37 °C. Microcolonies were stained with safranine alone (red) and were encircled by materials stained strongly positive with FITC-ConA alone (green). Bar = 10 μm.

in the croton oil group, and at 12 h in those inoculated with the other 13 strains in the cut wound and croton oil dermatitis groups of neutropenic and normal mice. Microcolonies stained positive with safranine alone, and were encircled by materials stained strongly positive with FITC-ConA alone at 24 h (Fig. 2), 48 h and 72 h in furuncle 1 strain in the cut wound group. Table 1 shows glycocalyx production observed by CLSM and colony counts in the croton oil dermatitis group inoculated with impetigo 1 strain at 12 h after bacterial inoculation in neutropenic and normal mice. The colony counts of *S. aureus* cells in the croton oil dermatitis group of normal mice treated with 2% FA ointment were about 100 times lower than those in neutropenic mice (untreated control). Table 2 shows glycocalyx production as observed by CLSM at 12 h after bacterial inoculation in neutropenic mice and by attachment on coverslips in Pl-TSB, tube adherence, and the Congo red agar method in 15 *S. aureus* strains. Microcolonies were encircled by materials stained strongly positive with FITC-ConA in 15 of 15 strains in neutropenic mice and bacterial cells stained positive with FITC-ConA were recognized in a different positive proportion in 15 of 15 strains in attachment on coverslips in Pl-TSB, while glycocalyx production was not always recognized by two *in vitro* methods (tube adherence and Congo red agar method).

Discussion

Susceptibility tests with *in vitro* biofilm models have shown survival of bacterial biofilms after treatment with antibiotics at concentrations hundreds to a thousand times the MIC for bacteria in a suspension culture. *In vivo*, antibiotics might suppress symptoms of infection by killing free-floating bacteria shed from the attached population, but fail to eradicate those bacterial cells embedded in the biofilm. When antimicrobial therapy stops, the biofilm can act as a nidus for recurrence of the infection. Biofilm infections can linger for months, years or even a lifetime and usually persist until the colonized surface is surgically removed from the body.[2] Cramton *et al.* reported that an anaerobic environment stimulated polysaccharide intercellular adhesin/poly-*N*-succinyl-β-1,6-glucosamine production in both *S. aureus* and

Table 1. Glycocalyx production observed by confocal laser scanning microscopy (CLSM) and colony counts in the croton oil dermatitis group inoculated with impetigo 1 strain at 12 h after bacterial inoculation in neutropenic and normal mice

	Neutropenic mice		Normal mice	
Group	CLSM finding[a]	Colony counts	CLSM finding[a]	Colony counts
Untreated control	Positive	7·2 ± 0·29	Positive	6·13 ± 0·25
2% fusidic acid ointment	Positive	6·04 ± 0·1*	Positive	4·02 ± 0·35**
10% povidone-iodine solution	Positive	6·87 ± 0·18	Positive	6·07 ± 0·18

[a] Organisms were inoculated on croton oil dermatitis in mice. Positive: microcolonies were encircled by materials stained strongly positive with fluorescein isothiocyanate-conjugated concanavalin A. Colony counts ($\log_{10}$ colony-forming units): mean ± SD per unit area. Fusidic acid 2% ointment and povidone-iodine 10% solution were topically applied at 4 and 8 h after bacterial inoculation. *$P < 0·01$ (compared with untreated control in neutropenic mice); **$P < 0·01$ (compared with untreated control in neutropenic and normal mice).

Table 2. Glycocalyx production observed by confocal laser scanning microscopy (CLSM) at 12 h after bacterial inoculation in neutropenic mice and attachment on coverslips in Pl-TSB, tube adherence, and the Congo red agar method in 15 *Staphylococcus aureus* strains

Finding by CLSM							
Neutropenic mice[a]		Attachment on coverslips[b]		Tube adherence		Congo red agar method	
Positive	Negative	1	2	Positive	Negative	Positive	Negative
15/15	0/15	9/15	6/15	6/15	9/15	6/15	9/15

[a] Organisms were inoculated on a cut wound or on croton oil dermatitis in neutropenic mice. Positive: microcolonies were encircled by materials stained strongly positive with fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA). Negative: microcolonies were not encircled by materials stained positive with FITC-ConA. [b] Organisms were directly inoculated into Pl-TSB (rabbit plasma/tryptic soy broth 1 : 1) with coverslips from the agar plates. Valuations of staining patterns of FITC-ConA: 1, weakly or strongly positive in about 50% of cells and negative in about 50% of cells; 2, strongly positive in about 50% of cells and weakly positive in about 50% of cells.

S. epidermidis.[12] This production leads to cell–cell adhesion and is required for biofilm formation.[12]

Because lectins are plant proteins with a high affinity for specific sugar residues, they are used as probes to detect these residues. For example, ConA is known specifically to bind to α-mannose (2-epimer of D-glucose).[13] We considered that the *S. aureus* cells stained with safranine and that the materials staining positive for FITC-ConA were sugars, probably glycocalyx, which were produced by *S. aureus* cells.[14] We also considered that a reaction of ConA and mannose was a marker for detecting *S. aureus* glycocalyx in the present study.

Baselga *et al*. reported that the majority of *S. aureus* cells (85%) in slime-producing strains (using the tube adherence and the Congo red agar method) and a minority of cells (5%) in non-slime-producing strains showed a condensed exopolysaccharide matrix (slime) surrounding the bacterial cell wall, as revealed by EM and immunofluorescence.[15] In the present study, 100% (15 of 15) of *S. aureus* strains produced glycocalyx according to CLSM findings in neutropenic mice, while the proportion decreased to 40% (six of 15) in two *in vitro* methods (tube adherence and the Congo red agar method; Table 2). These differences in the detection of glycocalyx production agree with those found by Watson,[16] who observed that in bovine mastitis, > 80% of *S. aureus* cells produced exopolysaccharide after *in vivo* growth and that the proportion decreased to < 10% when bacteria were grown in regular laboratory media. We considered that the findings of microcolonies encircled by materials (glycocalyx) stained strongly positive with FITC-ConA were those of biofilm, because EM revealed membrane-like structures around the *S. aureus* cells[6,7] and antimicrobial agents such as 2% FA ointment and 10% PVP-I solution failed to eradicate *S. aureus* cells especially in neutropenic mice. Although *S. aureus* cells could produce biofilm on damaged skin tissues at 4 h after bacterial inoculation, the production of glycocalyx by individual cells was reduced at 24 h. In previous studies,[6,7] fibril-like structures (glycocalyx) around *S. aureus* cells on damaged skin tissues were also reduced at 24 h after bacterial inoculation. Thus, we suggest that *S. aureus* cells that are attached to damaged skin tissues can rapidly produce glycocalyx but stop production after completion of the biofilm.

As mice are well defended by PMNs against *S. aureus* invasion,[17] the animals in our previous studies[6,7] were made neutropenic by intraperitoneal administration of Cy[18] to reduce neutrophilic infiltration. Recently, we reported that a combination of roxithromycin and imipenem was a potentially effective treatment for *S. aureus* biofilm-associated skin infections as roxithromycin can induce the invasion of PMNs into the biofilm.[19] In the present study, numerous PMNs invaded the epidermal and dermal skin tissues and contained cocci in normal mice at 12 h after bacterial inoculation, but did not invade those in neutropenic mice. Necrosis of the epidermis was observed in normal mice but not in neutropenic mice at 12 h after bacterial inoculation (data not shown). The colony counts of *S. aureus* cells in the croton oil dermatitis group treated with 2% FA ointment were much lower in normal mice than in neutropenic mice (Table 1). FA ointment can kill many more *S. aureus* cells due to damage of the biofilm by PMNs. This suggests that PMNs play an important role in the treatment of *S. aureus* biofilm-associated skin infections and their inflammation. From our observations in the present study, we suggest that the effect of antimicrobial agents against the *S. aureus* biofilm in normal mice is mainly due to the invasion of PMNs into the biofilm.

The presence of a fibrin-rich biofilm is a well-known factor responsible for prolonging *S. aureus* infections. The biofilm of *S. aureus* is reinforced with fibrin fibres, making it more resistant to physical effects than are other bacterial biofilms.[20] We previously reported that the attachment of *S. aureus* cells to coverslips, the conversion of fibrinogen to fibrin, and the production of glycocalyx by *S. aureus* cells are minimum requirements for the production of a biofilm on coverslips *in vitro*.[21] We considered that the existence of fibrin was essential for glycocalyx production and biofilm formation by *S. aureus* cells to aid in the attachment of *S. aureus* cells *in vitro*, because *S. aureus* cells attached on coverslips and fibrin alone can produce glycocalyx.[14] In the present study, no fibrin was evident in the *S. aureus* biofilm produced on damaged skin tissues and none was detected in the area around the biofilm (data not shown). The fibril-like structures were suggested to be fibrin on the basis of the staining pattern of fibrin formed by the culture supernatant of a *S. aureus* strain acquired using the sterile syringe filter *in vitro* (data not shown). Thus, we propose that fibrin may not always be necessary for biofilm formation on damaged skin tissues and that the place of attachment and biofilm formation may involve floating bacteria.

In conclusion, the results of the present study indicate that *S. aureus* cells can generally produce a biofilm on damaged skin tissues such as dermatitis and cut wounds relatively quickly, which can make the infection hard to eradicate using an antimicrobial agent alone, without the help of PMNs. Further, *S. aureus* glycocalyx may play a crucial role in the colonization and adherence of infections on damaged skin tissues. The clinical approach using CLSM is relevant to the estimation of patients with low neutrophil counts and infection by *S. aureus* in damaged skin. The use of CLSM may be a new area to some dermatologists and may help in the diagnosis of bacterial skin infections.

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