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Dermatopathology

Confocal laser scanning microscopic observation of glycocalyx production by *Staphylococcus aureus* in skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus

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Summary

Background Glycocalyx collapses during dehydration to produce electron-dense accretions. Confocal laser scanning microscopy (CLSM) may be used to visualize fully hydrated microbial biofilms.

Objectives Using CLSM, to analyse glycocalyx production by *Staphylococcus aureus* cells in skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus. A second objective was to compare numbers of *S. aureus* cells in tissue sections prepared by different methods for routine light microscopy.

Methods *S. aureus* cells in skin lesions of impetigo, atopic dermatitis and pemphigus were stained with safranin, and positive staining with fluorescein isothiocyanate-conjugated concanavalin A was considered to indicate the presence of glycocalyx.

Results All *S. aureus* cells tested in skin lesions of impetigo, atopic dermatitis and pemphigus were covered with glycocalyx and formed microcolonies. The numbers of *S. aureus* cells in a routine light microscopy section were significantly lower than those in a frozen section that had not been dehydrated with ethanol.

Conclusions *S. aureus* cells generally produce glycocalyx in skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus, which accounts for the difficulty of removing *S. aureus* cells from these skin lesions. The glycocalyx may collapse during dehydration and most of the *S. aureus* cells may be carried away during preparation of routine light microscope sections.

Key words: atopic dermatitis, bullous impetigo, confocal laser scanning microscopy, frozen section, glycocalyx, pemphigus foliaceus, *Staphylococcus aureus*

Bacteria sometimes adhere to surfaces *in vitro* and *in vivo* and multiply there, forming a thin bacterial mass. Such bacterial colonization, in the form of a thin film-like structure, has been referred to as a biofilm.[1] It has been reported that biofilm-forming bacteria are more resistant to antibiotics and that they are even resistant to immunological attack.[2,3] Electron microscopy (EM) techniques require that biofilm specimens be dehydrated, a process known to reduce the total volume of exocellular matrix material significantly and to lead to collapse of the matrix, compression of the cells, and distortion of the architecture. To eliminate this problem, a confocal laser scanning microscopy (CLSM) technique was developed to study the hydrated biofilm.[4] CLSM makes it possible to observe biofilms without destruction of their structure, and reveals live biofilm structures. CLSM also allows observation of horizontal and vertical optical thin sections of hydrated biofilms. Concanavalin A (ConA) is used as a marker for detecting glycocalyx on staphylococcal cells; its

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mechanism of action involves the reaction of ConA and mannose.[5] Previous studies using CLSM have shown that *Staphylococcus aureus* cells attached on coverslips produce glycocalyx *in vitro*.[6] Another study using CLSM revealed that *S. aureus* cells isolated from bullous impetigo, furuncle and atopic dermatitis formed microcolonies encircled by glycocalyx on damaged mouse skin tissues, which conventional antimicrobial agents could not eradicate without the help of polymorphonuclear leucocytes (PMNs).[7] In our previous studies, we reported that fibrin formation by *S. aureus* cells was inhibited in a low pH medium containing acetic acid, hydrochloric acid and acidic hot-spring water.[8,9] However, there are no reports of the detection by CSLM of glycocalyx production by *S. aureus* in human skin lesions such as bullous impetigo, atopic dermatitis and pemphigus foliaceus. We suggest that antimicrobial agents cannot eradicate *S. aureus* cells from skin lesions of atopic dermatitis because they produce glycocalyx, and that the numbers of *S. aureus* cells seen in routine light microscope sections are few in comparison with those in cultures from lesions. In the present study, we used CLSM to analyse glycocalyx production by *S. aureus* cells in human skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus. We also analysed the decrease in the number of *S. aureus* cells during preparation of routine light microscope sections and the effect of acetic acid and hydrochloric acid on *S. aureus* glycocalyx production *in vitro*.

Materials and methods

Patients

Nine patients with bullous impetigo were included:

1 Patient 1, a 3-year-old girl, 0·5 days after onset, with *S. aureus* coagulase type V producing exfoliative toxin (ET) A.
2 Patient 2, a 3-year-old boy, 1 day after onset, coagulase type VII and ETA.
3 Patient 3, a 7-year-old boy, 2 days after onset, coagulase type V and ETA.
4 Patient 4, a 67-year-old woman, 5 days after onset, coagulase type I and ETA.
5 Patient 5, a 2-year-old girl, 7 days after onset, coagulase type I and ETB.
6 Patient 6, a 4-year-old girl, 10 days after onset, coagulase type V and ETA.
7 Patient 7, a 4-year-old boy, 0·5 days after onset, coagulase type I and ETA.
8 Patient 8, a 2-year-old girl, 2 days after onset, coagulase type I and ETB.
9 Patient 9, a 3-year-old girl, 7 days after onset, coagulase type V and ETB.

Eleven patients with atopic dermatitis were included:

1 Patient 10, a 24-year-old man, coagulase type I.
2 Patient 11, a 22-year-old man, coagulase type I.
3 Patient 12, a 22-year-old man, coagulase type VII.
4 Patient 13, a 20-year-old man, coagulase type VII.
5 Patient 14, a 21-year-old man, coagulase type V.
6 Patient 15, a 24-year-old man, coagulase type VIII.
7 Patient 16, a 30-year-old man, coagulase type II.
8 Patient 17, a 61-year-old woman, coagulase type I.
9 Patient 18, a 18-year-old man, coagulase type I.
10 Patient 19, a 27-year-old woman, coagulase type II.
11 Patient 20, a 23-year-old man, coagulase type VIII.

Two patients with pemphigus foliaceus were included:

1 Patient 21, a 49-year-old woman, coagulase type II.
2 Patient 22, a 60-year-old woman, coagulase type III.

Written informed consent was obtained from all subjects or their guardians. The background of the patients is presented in Table 1. We chose pemphigus foliaceus in this study because pemphigus foliaceus shows identical tissue specificity and histology to staphylococcal scalded skin syndrome (SSSS) and bullous impetigo, which are caused by ET.[10]

Treatment

Patient 5 had been treated with oral amoxicillin 20 mg kg^{-1} daily in three doses (Meiji Seika Co., Tokyo, Japan) between 2 and 7 days after onset at another clinic; we then treated her with oral cefdinir 10 mg kg^{-1} daily in three doses (Fujisawa Pharmaceutical Co., Tokyo, Japan) and topical 2% fusidic acid (FA) ointment (Sankyo Co., Tokyo, Japan) applied to the affected area twice daily between 8 and 10 days after onset. However, the impetigo lesions on the face were aggravated further and widespread erythematous eruptions on the trunk followed, which progressed rapidly to blister formation at 10 days after onset, suggesting SSSS. Therefore, we changed the treatment to oral faropenem 15 mg kg^{-1} daily in three doses (Yamanouchi Pharmaceutical Co., Tokyo, Japan) and

Table 1. Background of patients

	Bullous impetigo	Atopic dermatitis	Pemphigus foliaceus
Male	3	9	0
Female	6	2	2
Age (years), mean (range)	10·5 (2–67)	26·5 (18–61)	54·5 (49–60)
Days after onset, mean (range)	3·9 (0·5–10)		
Infecting strains coagulase type			
I	4	4	0
II	0	2	1
III	0	0	1
V	4	1	0
VII	1	2	0
VIII	0	2	0

topical 2·5% acetic acid ointment [hydrophilic petrolatum containing 2·5% acetic acid (Ishizu Seiyaku, Kyoto, Japan); self-manufactured product; applied to affected area two times daily] between 11 and 17 days after onset, and the SSSS was cured. The minimum inhibitory concentrations (MICs) of amoxicillin, cefdinir, FA, faropenem and acetic acid (as percentage concentration) against *S. aureus* isolated from impetigo lesions at 7 days after onset were 4, 1, 0·06 and 0·5 μg mL^{-1}, and 0·12% (pH 5·0), respectively. The isolates tested in this study were all sensitive to FA. In our area, the MIC range, MIC_{50} and MIC_{80} of FA against *S. aureus* strains isolated from various skin infections were 0·06–128, 0·25 and 2 μg mL^{-1}, respectively.[11]

Observation of glycocalyx production by confocal laser scanning microscopy

Blister lesions from nine patients with bullous impetigo and two with pemphigus foliaceus were cultured on sheep blood agar using a sterile swab, and were stripped for specimens. Eczematous skin lesions from 11 patients with atopic dermatitis were also cultured on sheep blood agar, and were stripped using transparent double-sided tape (Scotch™) for 5 s; the opposite side was stripped using a glass slide. An eczematous skin lesion of one atopic dermatitis patient (patient 20) was biopsied. Blister lesions of pemphigus foliaceus and eczematous skin lesions of atopic dermatitis were stamped simultaneously using a Nissui Food Stamp (Nissui Pharmaceutical Co., Tokyo, Japan) with a 10 × 10 cm contact plate for 5 s and the colony forming units (c.f.u.) were counted 48 h after being cultured.[12] Eleven blister specimens from bullous impetigo and pemphigus foliaceus and one biopsy specimen of atopic dermatitis (patient 20) were embedded in OCT Compound (Sakura Finetechnical Co., Tokyo, Japan) for frozen tissue specimens, and were cut to a thickness of 10 μm using a cryostat HM 505 E (Micron, Tokyo, Japan). Scotch tape and frozen 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% safranin (pH 7·4; Wako, Osaka, Japan) for 5 min. After washing with PBS, one specimen was immersed in fluorescein isothiocyanate-conjugated ConA (FITC-ConA; Funakoshi Co., Tokyo, Japan; 50 μg mL^{-1}) for 5 min at room temperature. Another specimen was immersed in bovine serum albumin/PBS for 30 min, and then immersed in × 50 anti-*S. aureus* antibody (mouse IgM; Cosmo Bio Co., Tokyo, Japan) for 60 min. After washing with PBS, the specimen was immersed in × 100 FITC-labelled antimouse IgM antibody (rat IgG; Cosmo Bio Co.) for 60 min. After washing with PBS, these paired 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).

Comparison of routine light microscope section and frozen section

In patients 4, 5, 20 and 21, the blister and biopsy specimens were divided into three sections. The first section was fixed with formalin, dehydrated with ethanol and prepared for routine light microscopy by haematoxylin and eosin and Gram staining; the second section was frozen but not dehydrated with ethanol, and similarly prepared for light microscopy; and the third section was frozen and prepared for CLSM as described above.

Effect of fusidic acid, acetic acid and hydrochloric acid against Staphylococcus aureus *cells in fibrin clots* in vitro

S. aureus strains with MICs for FA and acetic acid of 0·06 μg mL^{-1} and 0·12% (pH 5·0), respectively, were isolated from patient 5. Cell suspensions containing $5·3 \times 10^8$ c.f.u. were used to inoculate 2·5 mL of Pl-TSB broth [rabbit plasma (Denka Seiken Co., Tokyo, Japan)/tryptic soy broth (Nissui) 1 : 1] on 1·77 cm^2 coverslips (Sumitomo Bakelite Co., Tokyo, Japan) in tissue culture dishes (35 × 10 mm; Becton Dickinson Co., Lincoln Park, NJ, U.S.A.). After incubation for 6 h

at 37 °C, fibrin clots had begun to form on the coverslips. The coverslips with fibrin clots ($n = 5$) were then placed in 2·5 mL of Pl-TSB broth either alone (control) or supplemented with 2% (20 000 μg mL^{-1}) FA, 2·5% acetic acid (0·41 mol L^{-1}, pH 4·2), or hydrochloric acid (pH 4·2; Nacalai Tesque, Kyoto, Japan). After incubation for 24 h at 37 °C, the coverslips were gently washed 10 times with sterile saline solution (1 mL). The coverslips were suspended in 4 mL of sterile saline solution and sonicated (Heatsystems–Ultrasonics Inc., Plainview, NY, U.S.A. Model W-225R) at 60% power for 50 s at 4 °C. *S. aureus* cells were washed by suspending them three times in sterile saline solution with centrifugation at 6000 ***g*** for 10 min at 4 °C to eliminate the acetic acid and hydrochloric acid. The washed bacteria were resuspended in 1 mL sterile saline solution and counted (as c.f.u.) on soybean–casein digest agar plates with lectin and polysorbate 80 (SCDLP agar) using the 10-fold dilution method. The coverslips were also stained with safranin and FITC-ConA, and were observed by CLSM. We were unable to determine the colony counts of *S. aureus* cells in the FA group accurately because the effect of 2% FA (equal to about 330 000 MIC against this strain) could not be completely excluded. Thus, we only used the FA group in the CLSM study.

Statistical analysis

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

Results

Confocal laser scanning microscopy study

The staining patterns of safranin were completely consistent with those of FITC-labelled antimouse IgM antibody (or anti-*S. aureus* antibody) in all specimens tested.

A few microcolonies were located on the horny layer (Fig. 1A) and many microcolonies were located on the upper or lower part of the blister cavity in all patients with bullous impetigo (Fig. 1B) and pemphigus foliaceus (Fig. 2). In all patients with atopic dermatitis, several microcolonies were located on the surfaces of the horny cells acquired using Scotch tape (Fig. 3), and were located on or among the horny cells acquired by biopsy in one atopic dermatitis patient (patient 20) (Fig. 4C). All *S. aureus* cells detected stained positive with safranin alone or safranin and FITC-ConA (Figs 1A,B, 2, 3 and 4C). Bullous impetigo showed the same staining patterns with safranin and FITC-ConA regardless of the number of days after onset (Table 1). Using this method, *S. aureus* cells alone stained positive with safranin alone (red) and *S. aureus* cells covered with glycocalyx stained positive with safranin and FITC-ConA (yellow).[6,7] Microcolonies encircled by material staining positive with FITC-Con A were far more frequent and dense in two bullous impetigo patients (patients 1 and 5) who were difficult to treat, and in two atopic dermatitis patients (patients 10 and 20) and one pemphigus foliaceus patient (patient 21), with a great number of *S. aureus* cells according to the stamp method.

Comparison of routine light microscopy sections and frozen sections

In patients 4, 5, 20 and 21, there were markedly fewer *S. aureus* cells in routine light microscope sections (Fig. 4A) than in the frozen sections that were not dehydrated with ethanol (Fig. 4B,C).

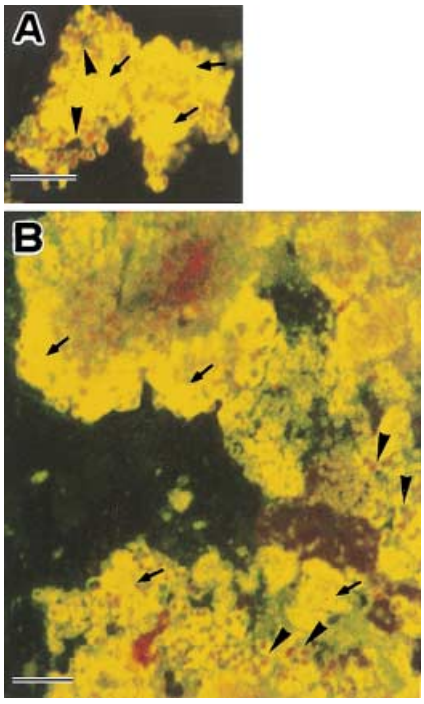

Figure 1. The double-staining patterns of safranin and fluorescein isothiocyanate–concanavalin A (FITC-ConA) from a blister lesion of bullous impetigo (patient 5). A few microcolonies were located on the horny layer (A), and many microcolonies were located at the upper or lower part of the blister cavity (B). *Staphylococcus aureus* cells stained positive with safranin alone (arrowheads; red) or safranin and FITC-ConA (arrows; yellow). Bar = 10 μm.

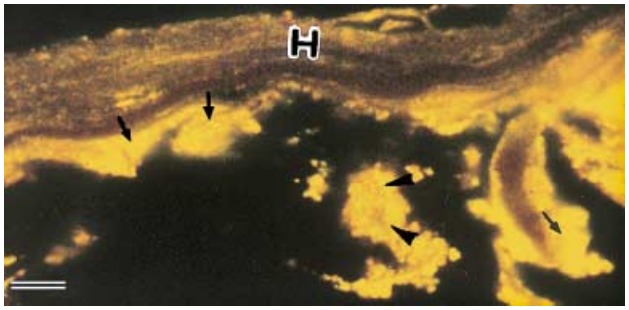

Figure 2. The double-staining patterns of safranin and fluorescein isothiocyanate–concanavalin A (FITC-ConA) from a blister lesion of pemphigus foliaceus (patient 21). Many microcolonies were located at the upper part of the bullous cavity. *Staphylococcus aureus* cells stained positive with safranin alone (arrowheads; red) or safranin and FITC-ConA (arrows; yellow). H, horny layer. Bar = 10 μm.

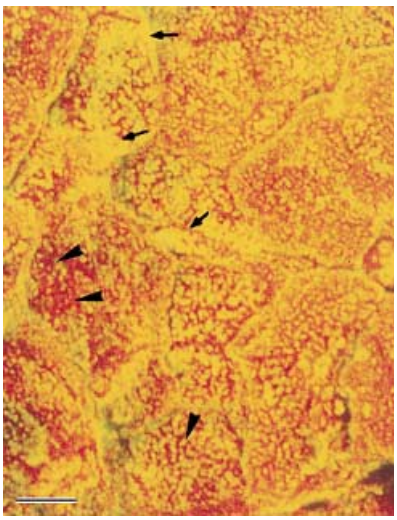

Figure 3. The double-staining patterns of safranin and fluorescein isothiocyanate–concanavalin A (FITC-ConA) from an eczematous lesion of atopic dermatitis acquired using Scotch tape (patient 10). All microcolonies were located on the surfaces of horny cells. *Staphylococcus aureus* cells stained positive with safranin alone (arrowheads; red) or safranin and FITC-ConA (arrows; yellow). Bar = 10 μm.

Effect of fusidic acid, acetic acid and hydrochloric acid against Staphylococcus aureus *cells in fibrin clots* in vitro

Several microcolonies were encircled by large quantities of material staining strongly positive with FITC-ConA in Pl-TSB alone (control) and Pl-TSB with 2% FA, whereas few microcolonies were encircled by small quantities of material staining positive with FITC-ConA in Pl-TSB with 2·5% acetic acid (pH 4·2) and hydrochloric acid (pH 4·2). Table 2 shows the colony counts of *S. aureus* cells in fibrin clots in Pl-TSB with or without 2·5% acetic acid and hydrochloric acid (pH 4·2) at 24 h after the bacterial inoculation. The colony counts of *S. aureus* cells were, respectively, about 10^4 or 10^5 times lower in Pl-TSB with 2·5% acetic acid or hydrochloric acid (pH 4·2) than in Pl-TSB alone (control; $P < 0{\cdot}01$). The colony counts of *S. aureus* cells were about 10 times lower in Pl-TSB with 2·5% acetic acid than in Pl-TSB with hydrochloric acid (pH 4·2; $P < 0{\cdot}01$).

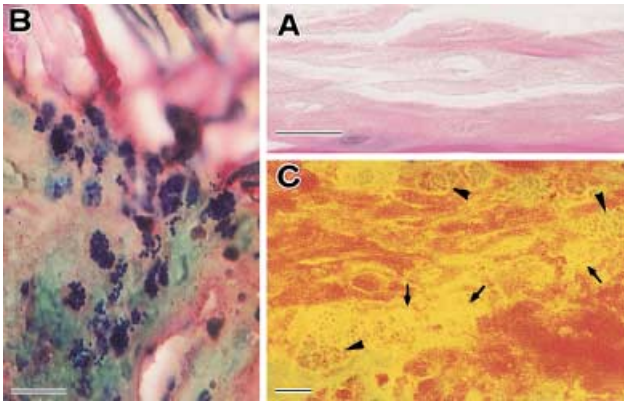

Figure 4. Horny layer of atopic dermatitis acquired via a biopsy (patient 20). (A) Microcolonies were not observed among the horny cells in routine haematoxylin and eosin staining. Bar = 10 μm. (B) In the frozen section, microcolonies among horny cells were Gram positive. Bar = 10 μm. (C) *Staphylococcus aureus* cells stained positive with safranin alone (arrowheads; red) or safranin and fluorescein isothiocyanate–concanavalin A (arrows; yellow) among the horny cells in the frozen section. Bar = 10 μm.

Table 2. Colony counts of *Staphylococcus aureus* cells in fibrin clots in plasma/tryptic soy broth (Pl-TSB; 1 : 1) with or without 2·5% acetic acid (pH 4·2) and hydrochloric acid (pH 4·2) at 24 h after bacterial inoculation. Organisms (isolated from patient 5) attached on coverslips with fibrin clots after incubation for 6 h were directly inoculated into Pl-TSB

	Colony counts per unit area (mean ± SD $\log_{10}$ c.f.u.; $n = 5$)
Before incubation	6·93 ± 0·15
After incubation for 24 h	
Pl-TSB alone (control)	8·18 ± 0·2
Pl-TSB with 2·5% acetic acid (pH 4·2)	3·03 ± 0·23*
Pl-TSB with hydrochloric acid (pH 4·2)	4·46 ± 0·35*

c.f.u., colony-forming units. *$P < 0{\cdot}01$ compared with Pl-TSB alone (control).

Discussion

Adherent bacteria are protected from host defence mechanisms and the activity of antimicrobial agents.[13]

The antibiotics in current use were first identified on the basis of their activity against growing cultures of individual cells. 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.[3]

Because lectins are plant proteins with high affinity for specific sugar residues, they are used as probes to detect these residues. For example, ConA is known to bind specifically to α-mannose (2-epimer of D-glucose).[14] Because the staining patterns of safranin were completely consistent with those of FITC-labelled antimouse IgM antibody (or anti-*S. aureus* antibody), we considered that materials staining positive with safranin in this study were *S. aureus* cells. We also considered that the material staining positive for FITC-ConA was sugar, probably glycocalyx, produced by *S. aureus* cells.[6,7]

Because the structure of bacterial glycocalyx is highly hydrated (> 99% water), it was difficult to visualize using a light microscope, and the advent of EM produced only further confusion concerning glycocalyx, as EM involves dehydration of the specimen, and the glycocalyx was only seen as the tangled wreckage of its original and intact hydrated structure.[15] One of the first bacterial structures lost during repeated culture *in vitro* is the glycocalyx, whose protective functions are not necessary in a single species culture. CLSM uses a laser beam to image fully hydrated living bacterial populations and has confirmed that almost all bacterial cells are surrounded by an extracellular layer of exopolysaccharide material (glycocalyx) that forms a highly structured, highly hydrated matrix when the cells adhere to a surface and proceed to produce a biofilm.[15] In the present study, we confirmed that all *S. aureus* cells detected were covered with glycocalyx and formed microcolonies on skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus. Further, we found that the standard preparation of a section for light microscopy caused collapse of the glycocalyx during dehydration and showed fewer *S. aureus* cells. We also determined that the findings (Fig. 1A,B) of microcolonies encircled by material (glycocalyx) staining positive with FITC-ConA were those of biofilm, because antimicrobial agents such as cefdinir and 2% FA failed to eradicate *S. aureus* cells in patient 5 with bullous impetigo, regardless of the drug sensitivity of the cells.

Eczematous skin lesions of atopic dermatitis are usually densely colonized with *S. aureus*.[16,17] We previously reported that *S. aureus* cells were found arranged linearly along the horny cells of atopic dermatitis, and that amorphous or fine material staining positive for ruthenium red surrounded almost all *S. aureus* cells, suggesting an abundant production of glycocalyx by *S. aureus* cells in EM.[17] Although *S. aureus* colonization on atopic dermatitis lesions can be eliminated to some degree after treatment with antibiotic therapies,[18,19] it is very difficult to remove *S. aureus* from atopic dermatitis skin lesions completely by any therapeutic method. These findings suggest that *S. aureus* cells produce a biofilm on eczematous skin lesions of atopic dermatitis. From the present study, we consider that antimicrobial agents cannot eradicate *S. aureus* cells on the skin lesions of atopic dermatitis because *S. aureus* cells attach on horny cells and multiply among them, producing glycocalyx.

Acetic acid solution (5%) has been reported to eradicate *Pseudomonas aeruginosa* from superficial wounds.[20] Nihei *et al*. reported that hydrophilic petrolatum containing 3% acetic acid effectively eradicated methicillin-resistant *S. aureus* on skin lesions of pemphigoid.[21] Hansson and Faergemann reported that 0·25% acetic acid solution shows surprisingly good effects against *S. aureus* cells when applied to venous leg ulcers.[22] The present study showed that glycocalyx production by *S. aureus* cells was inhibited in lower pH media (pH 4·2). The effect of acetic acid against sessile *S. aureus* cells was thought to be due not only to its low pH but also to the chemical action of acetic acid itself, as shown by a comparison with the colony counts of *S. aureus* cells in Pl-TSB alone (control) and Pl-TSB containing hydrochloric acid under the same pH conditions (Table 2). Acetic acid 2·5% ointment remarkably reduced the number of *S. aureus* cells colonizing the skin lesions of pemphigus foliaceus and bullous pemphigoid, as shown by the stamp method (data not shown), so we think that acetic acid ointment may have some therapeutic advantage against these diseases because of its antistaphylococcal action.

We recently reported that a combination of roxithromycin and imipenem was a potentially effective treatment for *S. aureus* biofilm-associated skin infections, as roxithromycin could induce the invasion of PMNs into biofilm.[23] We also reported that 2% FA ointment could kill many more *S. aureus* cells due to the damage of biofilm by PMNs.[7] In the bullous impetigo of patients 4 and 5, numerous PMNs invaded the blister cavity and phagocytosed some *S. aureus* cells surrounded by glycocalyx which stained positive with FITC-ConA in the frozen section (data not shown). These findings suggest that PMNs play an important role in

the treatment of *S. aureus* biofilm-associated skin infections. We think that antibiotics may not work effectively in a dermatological situation such as colonization in the absence of invasion of PMNs.

In conclusion, the present study suggests that *S. aureus* cells generally produce glycocalyx on skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus, and that this accounts for the difficulty of removing *S. aureus* cells from these skin lesions. These findings also explain why conventional antimicrobial agents alone may not eradicate *S. aureus* cells from these skin lesions. Acetic acid may be a useful topical application for use in clinical dermatology for the prevention and treatment of the staphylococcal biofilm. Further, we should be aware that glycocalyx may collapse during dehydration and that most *S. aureus* cells may be carried away during routine preparation of light microscopic sections from skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus. The use of CLSM may be helpful for analysing the real structure of biofilms in skin bacterial infections.

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