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Assessment of *Streptococcus pyogenes* microcolony formation in infected skin by confocal laser scanning microscopy

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Summary ***Background:*** *Streptococcus pyogenes* and *Staphylococcus aureus* are often simultaneously detected from many cases of non-bullous impetigo with atopic dermatitis. ***Objectives:*** Using confocal laser scanning microscopy (CLSM), to investigate formation of *S. pyogenes* microcolonies in skin lesions. ***Methods:*** The *S. pyogenes* cells in the stationary growth phase alone were strongly stained with fluorescein isothiocyanate-concanavalin A (FITC-ConA), and this staining was reduced by pretreatment with amylase. Although the components of sugars in glycocalyx produced by *S. pyogenes* cells are unknown, we suggested that the materials stained by FITC-ConA were consistent with the presence of ConA-reactive sugars in glycocalyx produced by *S. pyogenes* cells. ***Results:*** *S. pyogenes* cells associated with streptococcal impetigo skin and croton-oil inflamed mouse skin formed microcolonies encircled by materials (glycocalyx) that stained strongly with FITC-ConA, and these findings were consistent with those in biofilms. In croton-oil inflamed mouse skin, polymorphonuclear leukocytes (PMNs) infiltrated to just below the epidermis in the cefdinir-treated group but only to the middle dermis in the cefdinir-non-treated group. In this case *S. pyogenes* and *S. aureus* cells formed separate microcolonies and existed independently in the outer walls of pustule lesions of streptococcal impetigo. ***Conclusion:*** In skin infections, *S. pyogenes* and *S. aureus* formed aggregates of microcolonies (similar to that in biofilms) encircled by glycocalyx, which can make the infection hard to eradicate using an antimicrobial agent alone. The effect of conventional antimicrobial agents against biofilm is mainly due to the increase of the invasion of PMNs into the biofilm.

1. Introduction

Bacterial glycocalyx is a polysaccharide-containing material produced by bacteria [1]. Bacteria sometimes adhere to certain surfaces in vitro and

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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 biofilm [2]. It has been reported that biofilm-forming bacteria are more resistant to antibiotics and even resistant to immunologic attack [2,3]. Electron microscope (EM) techniques required that biofilm specimens be dehydrated, a process known to significantly reduce the total volume of exocellular matrix material (glycocalyx) and lead to collapse the matrix, compression of the cells, and distortion of the architecture. This has been obviated by the use of the confocal laser scanning microscopy (CLSM), which allows horizontal and vertical optical thin sectioning of hydrated biofilms [4]. CLSM analysis is non-destructive, and CLSM makes it possible to analyze live biofilm structures. Concanavalin A (ConA) is used as a marker for detecting glycocalyx on staphylococcal cells and its mechanism of action involves the reaction of ConA and mannose [5–8]. Previous studies using CLSM have shown that *Staphylococcus aureus* cells in the stationary growth phase alone attached on coverslips produced glycocalyx in vitro [6]. Another study using CLSM revealed that *S. aureus* cells isolated from bullous impetigo, furuncle, and atopic dermatitis lesions formed microcolonies encircled by glycocalyx on damaged mouse skin tissues, which conventional antimicrobial agents could not eradicate without the help of polymorphonuclear leukocytes (PMNs) [7]. Recent study using CLSM revealed that *S. aureus* cells generally produced glycocalyx in skin lesions of bullous impetigo, atopic dermatitis, and pemphigus foliaceus, and the glycocalyx might collapse during dehydration and most of the *S. aureus* cells might be removed in a routine light microscope section [8]. In the present study, we used CLSM to investigate the status of microcolonization by attached *Streptococcus pyogenes* and *S. aureus* cells in streptococcal impetigo lesions, *S. pyogenes* cells in infected skin lesions, and the influence of an antimicrobial agent in croton-oil inflamed mouse skin. Production of materials that stained with fluorescein isothiocyanate-conjugated (FITC)-ConA by *S. pyogenes* cells in vitro were also assessed.

2. Materials and methods

2.1. Patients

Specimens were obtained from one patient (a 16-year-old man) with streptococcal impetigo (2 days after onset) and atopic dermatitis. The patient's face and upper extremity were covered with thick-walled pustules on an erythematous base with crusts, and he had regional adenopathy with fever ($> 38.5\,^{\circ}C$). *S. pyogenes* (T-2 type) and *S. aureus* (oxacillin susceptibility 0.5 μg/ml; coagulase type II producer and toxic shock syndrome toxin-1 non-producer) strains were codetected in pustules on the upper extremity.

2.2. Bacterial strains

Two strains of *S. pyogenes* (T-1 and T-2 type) isolated from streptococcal impetigo with atopic dermatitis were used and their levels of production of the materials that stained positive for FITC-ConA in vivo and in vitro were studied.

2.3. Bacterial suspension for inoculation

The *S. pyogenes* strains were grown in 8 ml of Brain Heart Infusion (BHI; Nissui, Tokyo) broth at 37 °C overnight without shaking. Following inoculation, the bacterial cells were harvested by centrifugation at $6000 \times g$ for 10 min at 4 °C, then resuspended in 0.01 M phosphate buffered saline (PBS; pH 7.4), and centrifuged as described above. The process was repeated three times. The washed bacteria were resuspended in polypropylene microcentrifuge tube (1 ml; Iuchi BioSystems, Tokyo) and were used in the following experiments. The minimum inhibitory concentration (MIC) of cefdinir (CFDN; Fujisawa Pharmaceutical Co., Osaka) against these strains were 0.12 μg/ml.

2.4. CLSM and light microscopy of streptococcal impetigo specimens

Pustule lesions of upper extremity of streptococcal impetigo were stripped for use as specimens. Specimens were embedded in O.C.T. Compound (Sakura Finetechnical Co., Tokyo) for frozen, and were cut to a thickness of 10 μm using a chryostat HM 505 E (Micron, Tokyo). Frozen specimens were divided into four sections. The first was prepared for CLSM and stained with safranine (Wako Pure Chemicals Co., Osaka) and FITC-labeled anti-*S. pyogenes* antibody (goat IgG: Cosmo Bio Co., Tokyo), the second also for CLSM was stained with safranine and anti-*S. aureus* antibody (mouse IgM: Cosmo Bio Co., Tokyo), and then with FITC-labeled anti-mouse IgM antibody (rat IgG: Cosmo Bio), the third also for CLSM was stained with safranine and FITC-ConA (Funakoshi Co., Tokyo), and the fourth (not dehydrated with alcohol) for light microscopy was stained with

hematoxylin–eosin (H&E). Frozen sections for CLSM were washed three times with 0.01 M phosphated buffered saline (PBS; pH 7.4) for 5 min at room temperature. The first and second sections were immersed in 0.1% safranine (pH 7.4) for 5 min. After washing with PBS, these sections were immersed in 1% bovine serum albumin/PBS for 30 min to prevent non-specific bindings, and then the first was immersed in × 50 FITC-labeled anti-*S. pyogenes* antibody and the second immersed in × 50 anti-*S. aureus* antibody for 60 min. After washing with PBS, the second section was again immersed in × 100 FITC-labeled anti-mouse IgM antibody for 60 min. The third section was immersed in 0.1% safranine for 5 min. After washing with PBS, the section was immersed in FITC-ConA (50 μg/ml) for 5 min at room temperature. After washing with PBS, these three arranged sections were embedded using the Prolong TM antifade kit (Molecular Probes, Leiden, The Netherlands) and were observed using an LSM 510 CLSM (Carl Zeiss, Germany).

2.5. Informed consent

Written informed consent was obtained from the study subject.

2.6. CLSM and light microscopy in mouse skin

Five-week-old female mice of the ddy strain, weighing approximately 20 g, were purchased from Keari Co. (Osaka). The backs of the mice were shaved and a round area 1.5 cm in diameter was painted once with a cotton swab soaked in croton oil (Wako). At 18 h after croton oil application, 0.1 ml of the bacterial suspension (about 10^7 c.f.u.) were 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. The mice ($n = 6$) were divided to two group at random. In half the group ($n = 3$), CFDN (20 mg/kg) was administered orally at 4, 10, and 18 h after bacterial inoculation. Biopsy specimens ($n = 3$) were obtained from the skin lesions at 24 h after inoculation. Specimens were embedded in O.T.C. compound for frozen, and three sections were cut from each biopsy specimen. The first for CLSM was stained with safranine and FITC-labeled anti-*S. pyogenes* antibody, the second also for CLSM was stained with safranine and FITC-ConA, and the third (not dehydrated with ethanol) for light microscopy was stained with H&E.

2.7. Observation of cells in the exponential growth phase and the stationary growth phase, and the floating cells in vitro by CLSM

Cell suspensions of the two *S. pyogenes* strains were inoculated into 2 ml of BHI broth. After incubation for 3 and 24 h at 37 °C, cell suspensions washed by PBS were resuspended in 2 ml of BHI broth and 1.0 cm^2 coverslips (Sumitomo Bakelite, Tokyo) were immersed in this suspension. After incubation for 3 h at 37 °C, the coverslips were immersed in safranine and FITC-ConA, and were observed as described above. Bacterial cells pretreated 3 and 24 h in BHI broth were considered to be in the exponential growth phase and the stationary growth phase, respectively.

Cell suspensions of the two *S. pyogenes* strains were also inoculated into 2 ml of BHI broth without immersin of coverslips. After incubation for 3 h at 37 °C, bacterial cells were stained with safranine and FITC-ConA, recovered by centrifugation, washed and then observed as described above. Bacterial cells after 3 h incubation in BHI broth without coverslips were considered to be the floating cells. The floating cells were used as negative control.

2.8. Effect of amylase on cells attached to coverslips

Cell suspensions of the two *S. pyogenes* strains were inoculated into 2 ml of BHI broth with 1.0 cm^2 coverslips. After incubation for 3 h at 37 °C, the coverslips were washed by PBS and immersed separately in 2 ml of PBS with or without 0.1% amylase (Sanko, Tokyo) for 1 h at 37 °C. The coverslips were immersed in safranine and FITC-ConA, and were observed as described above.

3. Results

3.1. Appearance of *S. pyogenes* in pustules of streptococcal impetigo

In pustule lesions of streptococcal impetigo, light microscopy using H&E staining showed necrosis with the infiltrations of numerous PMNs (Fig. 1A), and a few microcolonies were located on the lesion's outer walls that PMNs did not infiltrate (Fig. 1A–C). In this method (Appendix A) [6–8], *S. aureus* cells without glycocalyx were found to stain only with safranine (red), *S. aureus* covered with glycocalyx to stain with safranine and FITC-ConA (yellow; Fig. 1F), and *S. aureus* to stain with

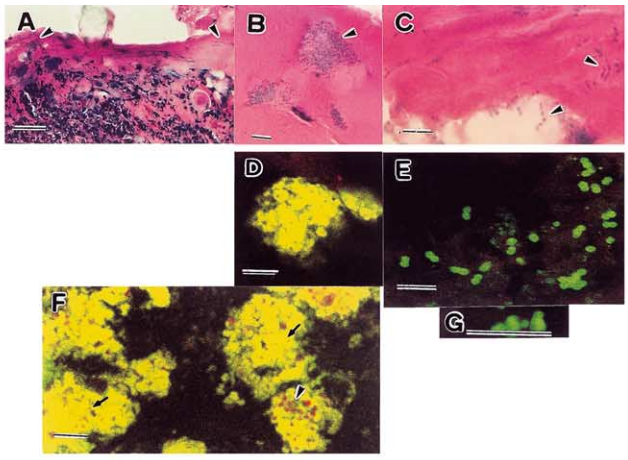

Fig. 1

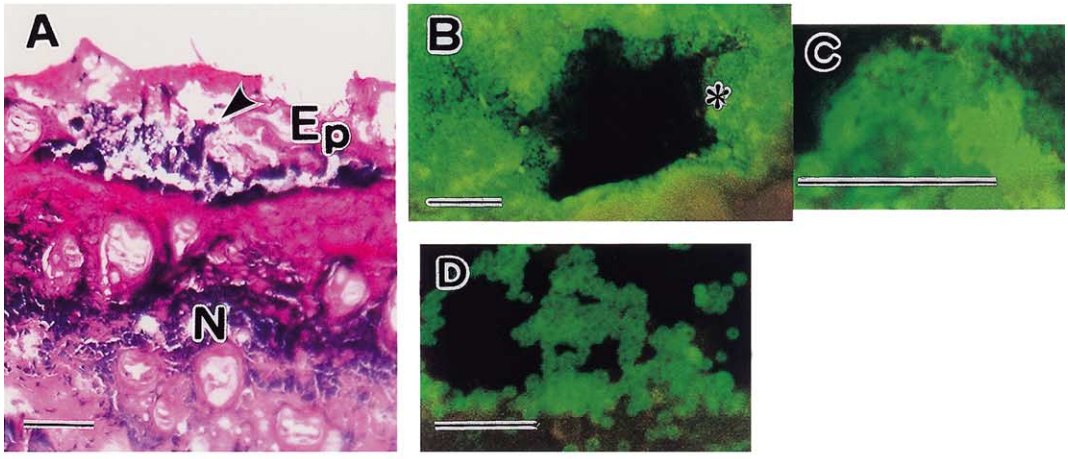

Fig. 2

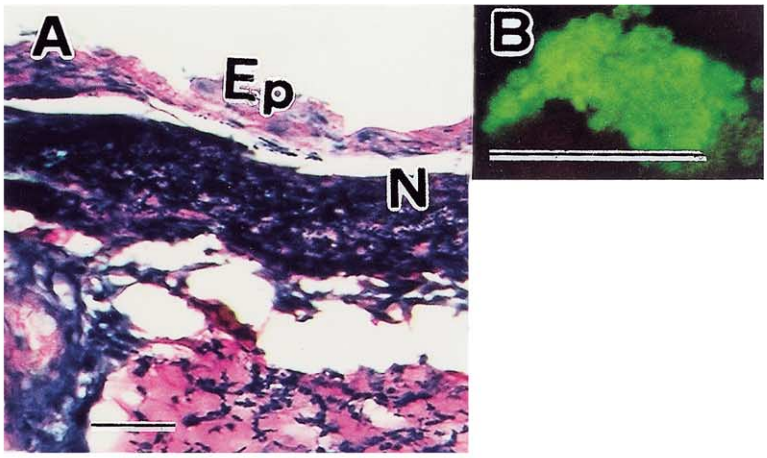

Fig. 3

safranine and FITC-labeled anti-mouse IgM antibody (or anti-*S. aureus* antibody; yellow; Fig. 1D). Because *S. pyogenes* cells were not stained by safranine in the present study, but were stained by FITC-ConA alone (Fig. 2B, C) and FITC-labeled anti-*S. pyogenes* antibody alone (Fig. 1E, Fig. 2D), *S. pyogenes* cells showed green fluorescence. Microcolony clusters (Fig. 1B) demonstrated by H&E staining were consistent with those stained with FITC-labeled anti-mouse IgM antibody (or anti-*S. aureus* antibody; yellow; Fig. 1D), indicating the clustered microcolonies were composed of *S.*

aureus cells. The microcolony clusters were stained with safranine alone (red) or safranine and FITC-ConA (yellow; Fig. 1F). Microcolony chains (Fig. 1C) were seen when stained with FITC-labeled anti-*S. pyogenes* antibody alone (green; Fig. 1E), indicating these microcolonies consisted of *S. pyogenes* cells. These microcolony chains also were stained with FITC-ConA alone (green; Fig. 1G).

3.2. Appearance of *S. pyogenes* in croton-oil inflamed mouse skin

In the CFDN-untreated mice, light microscopic examination of H&E staining found necrosis, numerous PMNs that infiltrated to the middle dermis, and numerous microcolonies located on the epidermis not-yet-infiltrated with PMNs (Fig. 2A). In the CFDN-treated mice, our examination revealed necrosis, numerous PMNs that infiltrated to just below the epidermis (Fig. 3A), and a few microcolonies located on the outer epidermis not-yet-infiltrated with PMNs (Fig. 3B). In the both groups, the *S. pyogenes* cells were stained with FITC-labeled anti-*S. pyogenes* antibody alone (green; Fig. 2D, Fig. 3B) and also with FITC-ConA alone (green; Fig. 2B, C). The similar results were obtained in the two strains and each of mice.

3.3. In vitro study

Table 1 shows the FITC-ConA staining patterns of two *S. pyogenes* strains in the exponential growth phase and the stationary growth phase, and the floating cells after incubation for 3 h. Cells in the stationary growth phase were stained strongly or weakly with FITC-ConA, whereas the exponential phase cells attached to a coverslip or the floating did not at all.

Table 2 shows the FITC-ConA staining patterns of two *S. pyogenes* strains attached to coverslips in PBS with or without amylase after incubation for 1 h. The staining pattern of FITC-ConA in PBS with amylase was reduced in comparison with control preparations untreated with amylase. The similar results were obtained in the two strains.

4. Discussion

S. pyogenes is an important organism of bacterial infections in both adults and children [9]. Impetigo is the most common bacterial skin infection in children [10]. All cases of bullous impetigo are caused by *S. aureus* and non-bullous impetigo are usually due to *S. pyogenes* [11], although recently in Japan *S. pyogenes* and *S. aureus* are often simultaneously detected from many cases of non-bullous impetigo with atopic dermatitis. Almost all lesions of streptococcal impetigo begin with vesicles, which rapidly become pustules and then rupture to form characteristic, thick, honey-colored crusts [12]. Impetigo lesions where the infecting organisms were predominantly *S. pyogenes* strains showed thick-walled pustules on an erythematous base with or without crusts; these skin lesions were an early manifestation of streptococcal impetigo. Thick-crusted lesions infected by more *S. aureus* than *S. pyogenes* cells were a late manifestation [13]. From the present study, we found that microcolonies of *S. pyogenes* and *S. aureus* cells in the outer walls of pustule lesions of streptococcal impetigo existed independently and formation of aggregates between *S. aureus* and *S. pyogenes* cells was not detected, and streptococ-

Fig. 1 Pustule lesion of streptococcal impetigo. (A) An H&E-stained section showing necrosis with the infiltration of numerous PMNs and a few microcolonies (arrowhead) located on the outer walls of the pustule. Bar = 100 μm. (B) An H&E-stained section showing a microcolony cluster (arrowhead) located on the outer walls of the pustule. Bar = 10 μm. (C) An H&E-stained section showing microcolony chains (arrowhead) located on the outer walls of the pustule. Bar = 10 μm. (D) Microcolony clusters were stained with safranine and FITC-labeled anti-mouse IgM antibody (or anti-*S. aureus* antibody; yellow). Bar = 10 μm. (E) Microcolony chains were stained with FITC-labeled anti-*S. pyogenes* antibody alone (green). Bar = 10 μm. (F) Microcolony clusters were stained with safranine alone (arrowhead; red) or safranine and FITC-ConA (arrow; yellow). Bar = 10 μm. (G) Microcolony chains were stained with FITC-ConA alone (green). Bar = 10 μm.

Fig. 2 *S. pyogenes* cells in croton-oil inflamed skin from the CFDN-non-treated mice. (A) An H&E-stained section showing necrosis, infiltration of numerous PMNs (N) in the middle dermis, and numerous microcolonies (arrowhead) located in the epidermis (Ep). Bar = 100 μm. (B) Microcolonies were stained with FITC-ConA alone (green). Bar = 10 μm. (C) Microcolonies were stained with FITC-ConA alone (green). A high magnification view of one part (*) of B. Bar = 10 μm. (D) Microcolony chains were stained with FITC-labeled anti-*S. pyogenes* antibody alone (green). Bar = 10 μm.

Fig. 3 *S. pyogenes* cells in croton-oiled inflamed skin from the CFDN-treated mice. (A) An H&E-stained section showing necrosis, infiltration of numerous PMNs (N) in just below the epidermidis (Ep). Bar = 100 μm. (B) Microcolonies on the outer epidermis were stained with FITC-labeled anti-*S. pyogenes* antibody alone (green). Bar = 10 μm.

Table 1 FITC-ConA staining patterns of two *S. pyogenes* strains in the exponential growth phase and the stationary growth phase, and the floating cells after incubation for 3 h

	Organisms		
	On coverslip		In valuations of staining patterns
Culture conditions	Exponential growth phase[a]	Stationary growth phase[b]	Floating cells[c]
BHI broth	0	3	0

Valuations of staining patterns of FITC-ConA. 0, Negative in all cells; 3, strongly positive in more than 80% of cells and weakly positive in less than 20% of cells.
[a] Organisms were inoculated into BHI broth with coverslips after pre-incubation in BHI broth for 3 h.
[b] Organisms were inoculated into BHI broth with coverslips after pre-incubation in BHI broth for 24 h.
[c] Organisms were directly inoculated into BHI broth without coverslips from the agar plates.

cal impetigo lesions were infected with *S. aureus* cells that formed microcolonies encircled by glycocalyx.

Because lectins are plant or animal proteins with high affinity for specific sugar residues, they are used as probes to detect these residues. For example, ConA is known to specifically bind to α-mannose (2 epimer of D-glucose) [14]. Amylases are a group of hydrolases that split complex carbonhydrates constituted of α-D-glucose units on adjacent glucose residues [15]. Although the components of sugars in glycocalyx produced by *S. pyogenes* cells are unknown, we suggested that the materials stained by FITC-ConA were consistent with the presence of ConA-reactive sugars in glycocalyx produced by *S. pyogenes* cells. This suggestion is supported by the binding-character of ConA to α-mannose, the finding that FITC-ConA is reduced after treatment with amylase, and the finding that FITC-ConA staining occurs in only the stationary growth phase cells attached to coverslips. ConA also binds to lipoteichoic acid (LTA) present on the surface of *S. pyogenes* cells [16]. Because cells in the stationary growth phase alone were stained with FITC-ConA (Table 1), we suggested that ConA did not react to LTA but to glycocalyx in the present study. From the present study, we suggested that ConA reacting with mannose is a marker for detecting *S. pyogenes* glycocalyx. We also considered that *S. pyogenes* cells on streptococcal impetigo skin and croton-oil inflamed mouse skin formed microcolonies encircled by (glycocalyx) substance, which stained with FITC-ConA, and these findings were consistent with findings of microcolony formation in biofilms.

Staphylococci exposed to penicillin or streptomycin are phagocytized faster than control staphylococci [17]. Recently, we reported that a combination of roxithromycin and imipenem was a potentially effective treatment for *S. aureus* biofilm-associated skin infections since roxithromycin could induce the invasion of PMNs into biofilm [18]. We also reported that 2% fusidic acid ointment could kill much more *S. aureus* cells owing to biofilm damage by PMNs [7]. In the bullous impetigo, numerous PMNs invade the blister cavity and phagocytose some *S. aureus* cells surrounded by glycocalyx. We thought that antibiotics might not work effectively in a dermatological situation in which colonization occurs without PMNs infiltration [8]. In the present study of croton-oil inflamed mouse skin, PMNs infiltrated to epidermis in the CFDN-treated group but only to the middle dermis in the CFDN-non-treated group. These findings suggest that the effect of conventional antimicrobial agents against biofilm is mainly due to the increase of the invasion of PMNs into the biofilm.

In conclusion, the present study suggests that *S. pyogenes* and *S. aureus* cells form separate microcolonies and exist independently in the outer walls of pustule lesions of streptococcal impetigo. *S. pyogenes* and *S. aureus* cells in skin infections form aggregates of microcolonies (biofilm) encircled by glycocalyx, which can make the infection hard to eradicate using an antimicrobial agent alone, without the help of PMNs. The use of CLSM may be helpful for assessing the real structure of biofilms in skin bacterial infections.

Table 2 FITC-ConA staining patterns of two *S. pyogenes* strains attached to coverslips in PBS with or without amylase after incubation for 1 h

	Organisms on coverslips
PBS alone (control)	2
PBS with 0.1% amylase	1

Coverslips with attached organisms after incubation for 3 h in BHI broth were submerged in PBS with or without amylase. Valuations of staining patterns of FITC-ConA. 1, Weakly positive in more than 50% of cells and negative in less than 50% of cells; 2, strongly positive in more than 50% of cells and weakly positive in less than 50% of cells.

Appendix A

Fluorescence patterns of *S. aureus* and *S. pyogenes* strains.

	S. aureus		*S. pyogenes*	
Safranine	red		not stained	
Safranine and FITC-labeled anti-mouse IgM antibody (or anti-*S. aureus* antibody)	yellow			
Safranine and FITC-labeled anti-*S. pyogenes* antibody			green	
Safranine and FITC-ConA	glycocalyx with yellow	without red	glycocalyx with green	without not stained

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