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Fosfomycin Reduces CD15s-Related Antigen Expression of *Streptococcus pyogenes*

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We have previously shown the immunological mimicry of human sialyl-Lewis^x (CD15s) by a surface antigen of *Streptococcus pyogenes*. This mimicking surface antigen may act as a ligand to the selectin family and may induce antibody production against CD15s on host cells, suggesting a possible role in the pathogenesis of *S. pyogenes*. In this study, the effects of antibiotics on the CD15s-related antigen expression of *S. pyogenes* were examined at a concentration below the MIC (sub-MIC). The amounts of CD15s on the surfaces of *S. pyogenes* cells and on the surfaces of *S. pyogenes* biofilms were determined by a whole-cell enzyme-linked immunosorbent assay and by laser scanning fluorescence microscopy, respectively, by using an anti-CD15s monoclonal antibody. At the sub-MICs, fosfomycin (1*R*,2*S*-1,2-epoxypropyl phosphonic acid), its enantiomer (1*S*,2*R*-1,2-epoxypropyl phosphonic acid), and benzylpenicillin significantly inhibited the CD15s expression of all strains studied. The effects of fosfomycin and its enantiomer on biofilms were also observed by scanning electron microscopy. Incubation of *S. pyogenes* with the sub-MIC of fosfomycin or its enantiomer, which has no antibacterial activity, reduced the amount of CD15s on the biofilm surface and made it smooth. These results suggest that fosfomycin or its enantiomer might be useful for preventing *S. pyogenes* adherence to human CD15s receptors and the resulting immunological pathogenicity.

Sialyl-Lewis^x (CD15s; Neu5Ac alpha 2-3 Gal beta 1-4 [Fuc alpha 1-3] GlcNAc beta 1-R; a receptor for the selectin family) and related antigens are expressed on human neutrophils, monocytes (6, 22, 26), various adenocarcinomas (13–15, 23), *Schistosoma mansoni* (29), *Helicobacter pylori* (1, 3), and *Streptococcus gallolyticus* (12). We previously demonstrated that an anti-CD15s monoclonal antibody (MAb) (26) reacted with a cell surface antigen of *Streptococcus pyogenes* (11). The role of the CD15s-related antigen in the pathogenesis of *S. pyogenes* has not been studied in detail. The expression on *S. pyogenes* of an antigen that mimics the host structure may camouflage *S. pyogenes* after infection (16, 19), thereby aiding survival and successful colonization. *S. pyogenes* possesses multiple adhesins: lipoteichoic acid, fibronectin-binding protein, M protein, vitronectin-binding protein, and C carbohydrate (9). In addition, CD15s on the streptococcal surface may act as an adhesin to the selectin family expressed on host cells such as endothelial cells and neutrophils. Infection with a bacterium expressing CD15s-related antigen possibly induces antibody specific for CD15s, which has a potential role in autoimmunity, as suggested for *H. pylori* and *S. mansoni* (1, 29).

Brook et al. (2) reported that sub-MICs of penicillin and clindamycin reduce the level of expression of the *S. pyogenes* capsule. However, the effects of antibiotics on CD15s-related antigen expression have not been studied. In this study, therefore, the effects of sub-MICs of antibiotics on CD15s-related antigen expression by *S. pyogenes* and on *S. pyogenes* biofilms were determined by an enzyme-linked immunosorbent assay (ELISA) and laser scanning fluorescence microscopy. The morphological changes in *S. pyogenes* biofilms as a result of treatment with antibiotics at sub-MICs were studied by scanning electron microscopy.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. pyogenes* ATCC 19615 was isolated from a patient with a sore throat. *S. pyogenes* TDP-1 (M type 1), TDP-3 (M type 3), and TDP-4 (M type 12) are isolates from children with acute glomerulonephritis (11). *S. pyogenes* TDP-11 (M type 6) is an isolate from an upper pharynx tumor. *S. pyogenes* A374 (M type 12) is an isolate from a patient with poststreptococcal glomerulonephritis (24). Serotypes M1 and M3 are reported to be particularly associated with invasive disease and fatal infections (4, 17), and M6 and M12 are potentially nephritogenic types (7). All strains were cultured in brain heart infusion broth (BHI; Difco, Detroit, Mich.) supplemented with 0.5% glucose for 18 h at 37°C.

Antibiotics and MIC determination. The antibiotics used in this study were fosfomycin (1*R*,2*S*-1,2-epoxypropyl phosphonic acid), the enantiomer of fosfomycin (1*S*,2*R*-1,2-epoxypropyl phosphonic acid), benzylpenicillin, cefditoren, streptomycin (all five antimicrobial agents were from Meiji Seika Kaisha Ltd., Tokyo, Japan), minocycline (Lederle Japan, Tokyo, Japan), ofloxacin (Daiichi Seiyaku, Tokyo, Japan), and erythromycin (Wako Jun-yaku, Osaka, Japan). Antibiotic susceptibility was determined by a broth microdilution method. Briefly, 10 μl of an *S. pyogenes* whole-cell suspension was added to 100 μl of a serial twofold dilution of antibiotics in Anaerobe Broth MIC (Difco) in wells of a microtiter plate to achieve a final concentration of 10^6 organisms per ml. The plate was incubated overnight at 37°C in an atmosphere of 5% CO_2. The MIC was the lowest concentration of antibiotic which yielded no bacterial growth.

Whole-cell ELISA. CD15s expression on bacterial surfaces was measured as described previously (11). CD15s-polyacrylamide polymer (CD15s-PA; monosaccharide composition; Neu5Ac, 9.3 mol%; Fuc, 10.1 mol%; Gal, 9.6 mol%; GlcNAc, 9.5 mol%; Seikagaku Kogyo, Tokyo, Japan) was used as a positive control. Briefly, 50 μl of a whole-cell suspension (2.0×10^8 CFU/ml) or CD15s-PA suspension was divided into aliquots, and each aliquot was placed into individual wells of an ELISA plate (MS-8696F; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and allowed to dry overnight at 37°C. The wells were pretreated with 1% bovine serum albumin in 0.01 M phosphate-buffered saline (PBS; pH 7.2) for 1 h at room temperature. After washing three times with PBS, anti-CD15s MAb SNH-3 (diluted to 1:200; Wako Pure Chemical, Osaka, Japan) (24) was added to each well and the plate was allowed to stand at room temperature for 1 h. The plate was again washed as described above, horseradish peroxidase-labeled goat anti-mouse immunoglobulin M (IgM; diluted to 1:2,000; μ chain; Cappel Research Products, Durham, N.C.) was added to each well, and the plate was stored at room temperature for 1 h. After washing, 50 μl of a mixture of

TABLE 1. Antibiotic susceptibility of *S. pyogenes*

Antibiotic	MIC (μg/ml)				
	ATCC 19615	TDP-1	TDP-3	TDP-4	A374
Fosfomycin	32	32	32	32	32
Enantiomer of fosfomycin	>128	>128	>128	>128	>128
Benzylpenicillin	0.016	0.016	0.016	0.031	0.016
Cefditoren	0.008	0.008	0.008	0.016	0.008
Erythromycin	0.016	0.016	0.016	0.016	0.031
Minocycline	0.031	0.031	0.031	0.016	0.016
Ofloxacin	0.125	0.125	0.125	0.125	0.125
Streptomycin	0.063	0.063	0.016	0.031	0.031

H_2O_2 and 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulfonate) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added. The absorbance at 414 nm of each well was measured with an immunoreader (model 2550; Bio-Rad Laboratories, Richmond, Calif.). Control wells contained the second antibody and the substrate mixture alone.

To investigate the effects of antibiotics on CD15s expression, bacterial cells were incubated in the presence of sub-MICs of antibiotic at 37°C for 1 h. Bacterial cells were harvested by centrifugation, washed, resuspended, and used for whole-cell ELISA.

Biofilm formation and antibiotic treatment. We examined the effects of fosfomycin and its enantiomer on an *S. pyogenes* biofilm with an in vitro system. *S. pyogenes* was grown overnight in BHI (Difco) at 37°C. Bacterial cells were harvested by centrifugation and washed with PBS. The cells were resuspended at a concentration of 2.0×10^9 CFU/ml, a 100-μl aliquot was inoculated into each well of a 24-well multiplate containing 900 μl of fresh BHI and a plastic coverslip (cell desk; diameter, 13.5 mm; Sumitomo Bakelite Co., Ltd.), and the plates were incubated for 4 days at 37°C. After *S. pyogenes* formed a biofilm on the cell desk, antibiotics or BHI, as a control, was added to each well and the plates were incubated for an additional 1 h at 37°C. Biofilms were observed by laser scanning fluorescence microscopy and scanning electron microscopy.

Laser scanning fluorescence microscopy. The *S. pyogenes* biofilm was observed by laser scanning fluorescence microscopy (ACAS 570; Meridian Instruments, Okemos, Mich.) with MAb SNH-3. Samples were reacted with MAb SNH-3 (200 μg/100 μl, diluted to 1:500; Wako Pure Chemical), followed by a reaction with fluorescein isothiocyanate-labeled goat anti-mouse IgM (diluted to 1:2,000; μ chain; Cappel Research Products) at room temperature for 1 h. The fluorescence was measured at 488 nm by laser scanning fluorescence microscopy. Data were processed by image analysis and line analysis by using the complement data program of the ACAS software system (Meridian Instruments). A complementary image which replicated the real immunohistomorphology was obtained.

Scanning electron microscopy. *S. pyogenes* biofilms formed on a plastic coverslip were fixed in 0.1 M cacodylate buffer (pH 7.2) with 2.5% glutaraldehyde for 1 h at room temperature. The samples were dehydrated with a series of ethanol solutions which ranged in 10% increments from 50% (vol/vol) ethanol in distilled water to absolute ethanol. All samples were dried to the critical point with a critical point drier, coated with gold, and examined by scanning electron microscopy (Hitachi S-800; Hitachi, Tokyo, Japan).

RESULTS

The MICs of the antibiotics for the *S. pyogenes* strains used in this study are presented in Table 1. The MIC of fosfomycin was 32 μg/ml for all strains, and the enantiomer of fosfomycin had no growth-inhibitory activity at 128 μg/ml. No strains were found to be resistant to any of the other antibiotics tested. Fosfomycin, its enantiomer, and benzylpenicillin significantly reduced the amount of CD15s antigen of *S. pyogenes* at concentrations lower than their MICs (Table 2). The level of reduction of CD15s as a result of treatment with fosfomycin and its enantiomer was greater than that as a result of treatment with benzylpenicillin.

The effect of fosfomycin and its enantiomer on an *S. pyogenes* biofilm was studied in an in vitro system. *S. pyogenes* ATCC 19615 formed a biofilm well under the conditions that we used (Fig. 1). CD15s expression on the biofilms detected by laser scanning fluorescence microscopy was shown by image analysis and line analysis (Fig. 2, left and center). Anti-CD15s MAb-reactive sites were demonstrated as white, red, and yellow areas contrasted with a blue background. The scale to the right of the image gives the intensity of fluorescence (color values of 0 to 4,095). Line analysis indicates the staining intensities of the surface of the *S. pyogenes* biofilm in a cut line. The intensity of the control biofilm was an integrated value of 308,810 with a query length of 135.80 μm, the intensity of the biofilm treated with fosfomycin at the sub-MIC was an integrated value of 186,076 with a query length of 141.60 μm, and the intensity of the biofilm treated with the fosfomycin enantiomer was an integrated value of 187,164 with a query length of 112.81 μm. Strong fluorescence was seen on the surface of the control biofilm. Treatment with fosfomycin or its enantiomer at their sub-MICs significantly reduced the amount of CD15s on the biofilm surface, as shown either by image analysis or by line analysis. The fosfomycin enantiomer demonstrated slightly less of an effect compared with the effect of fosfomycin.

When observed by scanning electron microscopy, the surfaces of the bacterial cells in the control biofilm seemed rough and had tiny particle-like substances. The bacterial cells in an antibiotic-treated biofilm appeared to be smoother than those of the control biofilm, but they were still covered with a glycocalyx (Fig. 2, right). Within the antibiotic treatment period used in this study sub-MICs of fosfomycin and its enantiomer significantly reduced the level of expression of CD15s, although they produced no pronounced change in the biofilm structure.

TABLE 2. Effects of antibiotics on CD15s of *S. pyogenes*

Antibiotic	Amt of CD15s (absorbance at 414 nm)				
	ATCC 19615	TDP-1	TDP-3	TDP-4	A374
Control	0.870 ± 0.014	0.864 ± 0.006	0.829 ± 0.016	0.852 ± 0.011	0.842 ± 0.013
Fosfomycin	0.623 ± 0.027[a]	0.614 ± 0.011[a]	0.610 ± 0.009[a]	0.631 ± 0.017[a]	0.606 ± 0.006[a]
Enantiomer of fosfomycin	0.625 ± 0.020[a]	0.614 ± 0.011[a]	0.603 ± 0.010[a]	0.606 ± 0.009[a]	0.614 ± 0.009[a]
Benzylpenicillin	0.714 ± 0.009[a]	0.690 ± 0.041[a]	0.685 ± 0.030[a]	0.676 ± 0.022[a]	0.713 ± 0.019[a]
Cefditoren	0.871 ± 0.019	0.865 ± 0.007	0.804 ± 0.012	0.821 ± 0.010	0.817 ± 0.011
Erythromycin	0.822 ± 0.030	0.790 ± 0.027	0.768 ± 0.038	0.801 ± 0.033	0.801 ± 0.048
Minocycline	0.781 ± 0.044	0.818 ± 0.014	0.795 ± 0.029	0.798 ± 0.016	0.813 ± 0.010
Ofloxacin	0.803 ± 0.011	0.808 ± 0.020	0.799 ± 0.016	0.794 ± 0.038	0.802 ± 0.024
Streptomycin	0.803 ± 0.012	0.823 ± 0.027	0.802 ± 0.018	0.797 ± 0.014	0.806 ± 0.018

[a] $P < 0.01$.

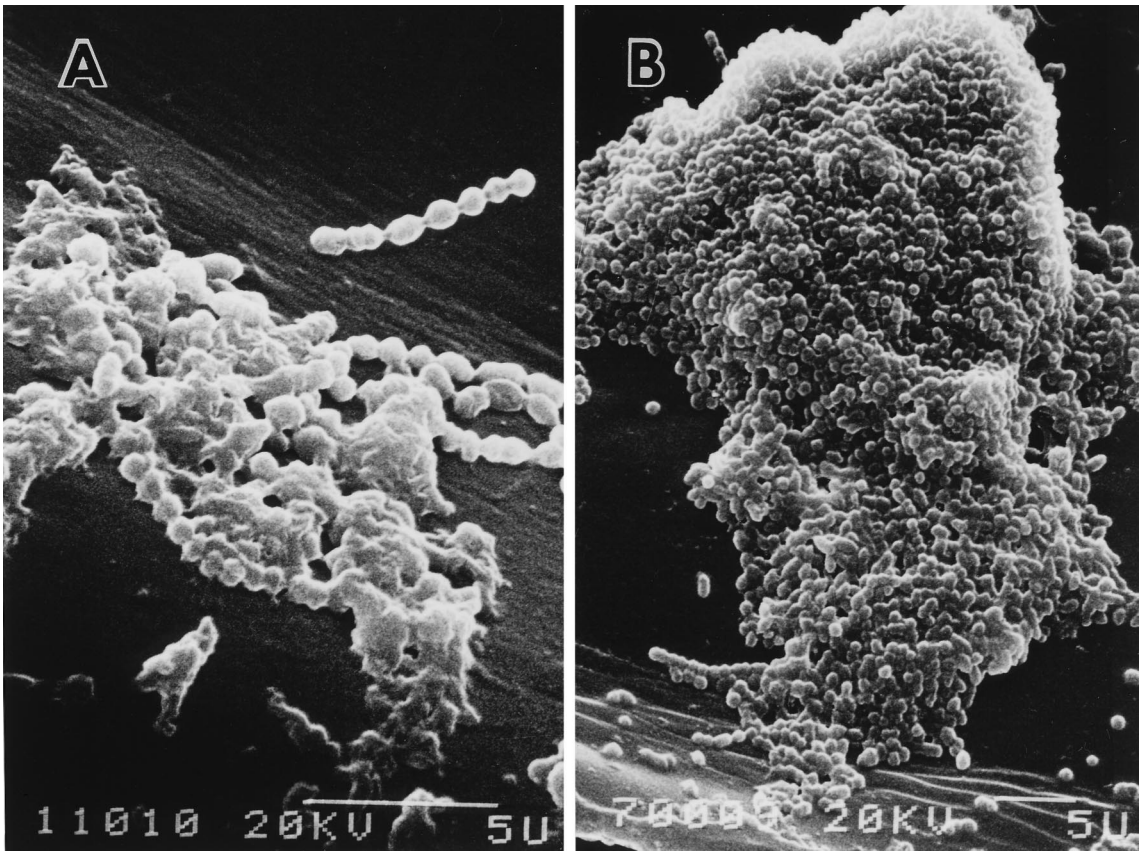

FIG. 1. *S. pyogenes* ATCC 19615 biofilm in vitro. (A) One-day culture in antibiotic-free medium. (B) Four-day culture in antibiotic-free medium.

DISCUSSION

Bacterial adherence is an essential step in the initiation of bacterial infection. Many kinds of molecules on the bacterial cell surface mediate bacterial adherence to the host, and these are called adhesins. It is well known that CD15s is a ligand for the human selectin family. The selectin family is mainly expressed on an inflamed endothelium, activated platelets, and lymphocytes. It is therefore possible that CD15s on the streptococcal surface acts as an adhesin to human cells and plays a role in initial adherence and bacterial translocation.

In the present study we demonstrated that fosfomycin, the enantiomer of fosfomycin, and benzylpenicillin reduced the amount of CD15s expressed on the surfaces of *S. pyogenes* cells. Benzylpenicillin and fosfomycin are cell wall inhibitors that act during different steps of cell wall synthesis. These agents may affect the surface structure by affecting the cell wall structure. Penicillin was reported to inhibit the formation of *S. pyogenes* capsules, although the effect was not as strong as that of clindamycin (2). Cefditoren, a cephem, however, did not reduce the amount of CD15s, although that agent is also a cell wall inhibitor.

It is of interest that treatment with the enantiomer of fosfomycin, which has no detectable effect on bacterial growth, resulted in a significant change in the amount of CD15s. The fact that the enantiomer of fosfomycin has an effect almost equivalent to that of fosfomycin suggests that their respective activities, other than growth inhibition, play a role in the suppression of CD15s. Fosfomycin is reported to possess a wide variety of biological activities other than bacterial growth inhibition or bactericidal action. These include an effect on human T-lymphocyte function (20), an effect on cytokine production by human monocytes (21), and an effect that reduces the toxicity caused by cisplatin (30). Some of these activities are also shown by the enantiomer of fosfomycin. Fosfomycin is similar in structure to phosphoenol pyruvate, which is involved in several biosynthetic pathways (17). The fact that the enantiomer is not a functional inhibitor of one class of enzymes (such as those involved in cell wall biosynthesis) does not necessarily mean that it cannot inhibit other putative classes (such as those involved in capsular biosynthesis).

Although antibiotic treatment of planktonic cells was assessed by ELISA, bacteria infecting the host usually exist as sessile or biofilm cells. It is therefore of importance to investigate the effects of sessile or biofilm cells on CD15s. Experiments with biofilms focused on the effects of fosfomycin and its enantiomer. When used to treat *S. pyogenes* biofilms in vitro, fosfomycin and its enantiomer at their sub-MICs reduced the level of antigen expression on the biofilm surface, although these agents did not cause significant morphological changes in the biofilm. High IgM and IgG titers against *S. pyogenes* surface antigens were observed in the sera of *S. pyogenes*-infected patients (8, 10). CD15s-bearing glycolipids were quite abundant on neutrophils, with 2×10^7 copies/cell (27) and a calculated density of CD15s-bearing glycolipids on the neutrophil surface of 44,000 molecules/μm (5, 22, 28). Anti-CD15 antibodies are produced in patients with infections caused by *S. mansoni*, which expresses CD15, and these, together with complement, cause lysis of human neutrophils (29). A potential role of autoimmunity from molecular mimicry of the human Lewis blood group antigen has also been suggested for *H.*

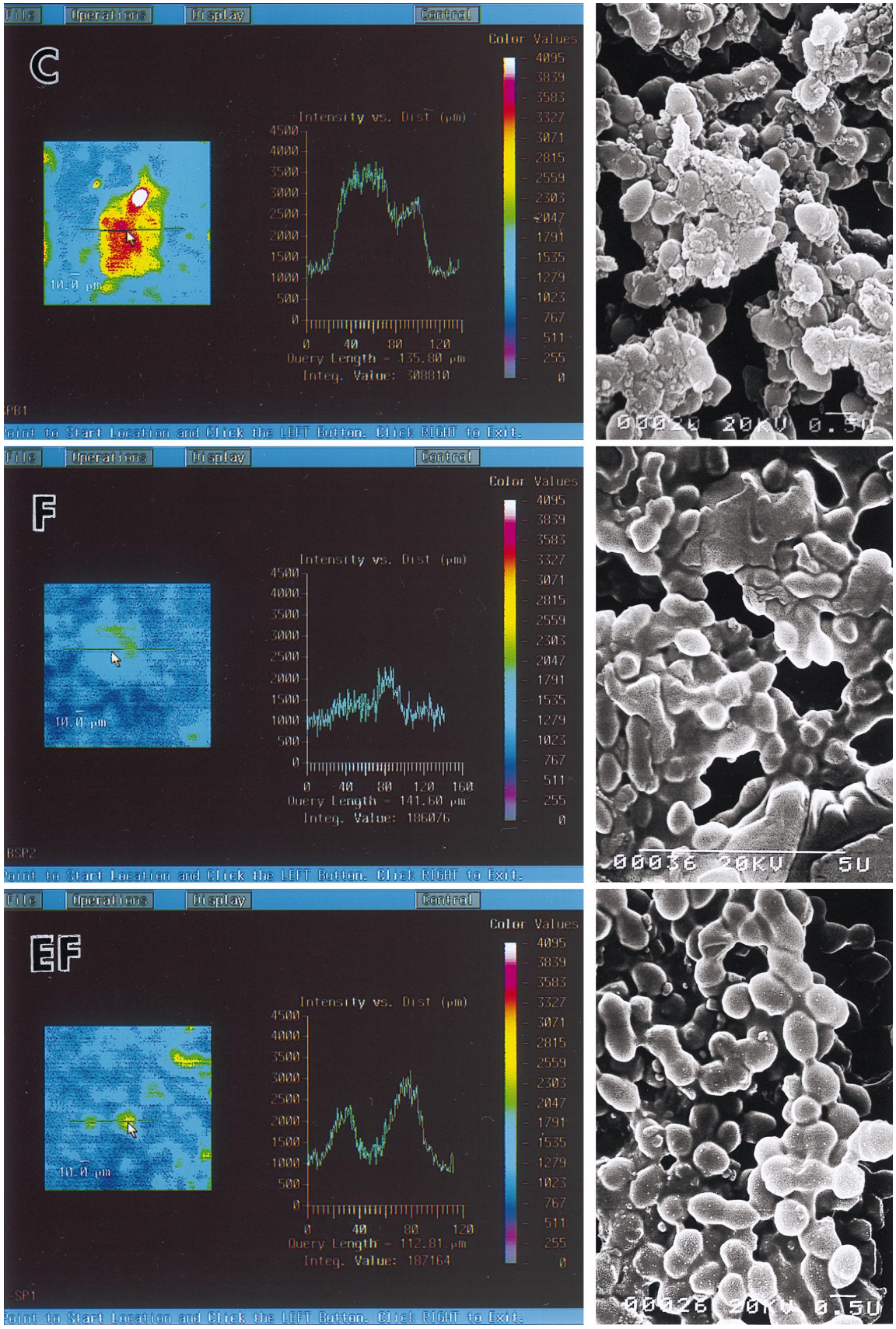

FIG. 2. Effects of antibiotics on CD15s expression in *S. pyogenes* biofilms in vitro. (Left) Image analysis by laser scanning fluorescence microscopy; (center) line analysis; (right) scanning electron microscopy. C, control; F, fosfomycin; EF, enantiomer of fosfomycin. The scale to the right of the image gives the intensity of fluorescence (color values of 0 to 4,095). An *S. pyogenes* biofilm was treated with fosfomycin or its enantiomer at the sub-MIC (5 μg/ml). Biofilms were observed by laser scanning fluorescence microscopy to demonstrate the amount of CD15s and by scanning electron microscopy. A sample for laser scanning fluorescence microscopy reacted with an anti-CD15s MAb and fluorescein isothiocyanate-labeled goat anti-mouse IgM. Bars, 10.0 μm.

pylori (1). It is probable that human neutrophils and other cells that express CD15s might be recognized by anti-CD15s antibodies produced against *S. pyogenes*. The effect of fosfomycin and its enantiomer on the streptococcal antigen may alter the immunological pathogenesis of *S. pyogenes.*

Although further experiments are needed to elucidate the pathogenesis of the streptococcal CD15s antigen, because of their suppressive effects, fosfomycin and its enantiomer could be used to treat streptococcal infections. Specifically, the enantiomer of fosfomycin is a unique agent that reduces the amount of streptococcal CD15s without affecting human commensal bacteria.

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