HOST RESPONSE TO INFECTION

Interaction between human polymorphonuclear leucocytes and bacteria released from in-vitro bacterial biofilm models

H. YASUDA, Y. AJIKI, J. AOYAMA and T. YOKOTA*

Biological Research Laboratories, Sankyo Co. Ltd, 2-58 Hiromachi 1-chome, Shinagawa-ku, Tokyo 140
and * Juntendo College School of Medical Technology, 2-2 Takasu, Urayasu-shi, Chiba 279, Japan

Summary. The interactions between phagocytic cells (polymorphonuclear leucocytes) and *Escherichia coli* cells released from a biofilm model formed *in vitro* on the surface of cotton threads in an artificial medium were compared with those of phagocytes and bacteria released from a newly developed in-vitro biofilm model. This new model of bacterial biofilm on the surface of cotton threads was developed by soaking cotton threads in rat carboxymethylcellulose pouch exudate and culturing *E. coli* in the exudate. The structure of the biofilm model and the surface structure of the bacteria in the biofilm resembled those observed *in vivo* in infected pouches, and they were quite different from those observed with the biofilm model in artificial medium. Both bacteria released from biofilm models in an artificial medium and those from biofilms in rat carboxymethylcellulose pouch exudate, *in vitro*, were almost equally resistant to killing by phagocytes. The sensitivity of these bacteria to phagocytosis was no different from that of normal bacteria grown in artificial medium. Bacteria from both models were also less sensitive to the killing activity of H_2O_2 . Electronmicroscopy showed that bacteria from both models had some products that interacted with ruthenium red on their surfaces, but the respective quantities of these products differed.

Introduction

Bacteria in biofilms are a major concern for clinicians in the treatment of infections because of their resistance to a wide range of antibiotics.^{1–3} Biofilms have been found on the surface of implanted biomaterials^{4–6} and tissues^{7–10} in chronic bacterial diseases that are characterised by resistance to chemotherapy^{11–14} and they are resistant to clearance by humoral or cellular host defence mechanisms.^{15–17} Despite several investigations of the increased resistance of biofilm bacteria to phagocytosis,^{16,17} the mechanisms of the resistance of biofilm bacteria to the activity of phagocytes are still obscure.

Experimental bacterial biofilms have been formed on the surface of disks of silicone latex catheter material¹⁸ or on cellulose acetate membranes^{19,20} by culturing bacteria in an artificial medium. Nickel *et al.*² formed “biofilms” on the surface of disks of urinary catheter material by culturing bacteria in artificial urine. However, more detailed investigations on the mechanisms of resistance of biofilm bacteria to antibiotics or host defence mechanisms require a com-

parative study in model systems that are different in nature from each other.

In this report, the sensitivity of bacteria released from a biofilm model formed in an artificial medium to the activity of phagocytes and the killing activity of H_2O_2 , was compared with that of bacteria released from a newly developed in-vitro bacterial biofilm model.

Materials and methods

Animals

Male Wister-Imamichi rats weighing 140–150 g purchased from Imamichi Institute for Animal Reproduction (Saitama, Japan) were used. Rats were acclimatised for 5–7 days before use in a room with an environment of controlled temperature and relative humidity, and a 12-h light–dark cycle.

Bacteria

Escherichia coli strain 704, a clinical isolate maintained in our laboratory, was cultured in Trypticase-Soy Broth (TSB; Eiken-kagaku Co., Ltd, Tokyo, Japan) at 37°C for 20 h before use.

Human polymorphonuclear leucocytes (PMNL)

Human PMNL were harvested and purified according to the methods of Zimmerli *et al.*²¹ Heparinised blood (60 ml) from a healthy volunteer was mixed with an equal volume of phosphate-buffered saline (PBS) containing dextran T500 (Pharmacia AB, Uppsala, Sweden) 3.5% and left at room temperature for 45 min. The white blood cell layer was collected and centrifuged at 1300 rpm for 5 min at 4°C. The precipitated cell fraction was washed once with 10 ml of Eagle's Basal Medium (EBM; Nissui-seiyaku Co., Ltd, Tokyo, Japan) by centrifugation at 4°C. Cells were resuspended in 4 ml of EBM. The suspension was added gently to 4 ml of Ficoll-Paque solution (Pharmacia AB, Uppsala, Sweden) and centrifuged at 1300 rpm for 30 min at room temperature. The PMNL fraction was collected; 90% of the cells in the PMNL fraction were neutrophils.

Human serum

Human serum was obtained from fresh blood of a healthy volunteer. The serum was heated at 56°C for 40 min before use.

Formation of pouches on the backs of rats, extraction of pouch exudate, induction of infection and in-vitro formation of bacterial biofilm on the surface of cotton threads

The methods used were described previously by Ajiki *et al.*¹⁰ An air pouch on the back of a rat was formed by injecting 10 ml of air subcutaneously with a 21-gauge needle after trimming off the hair with a hair clipper. Just after removal of the needle, the needle hole in the skin was sealed with an adhesive agent. The next day, under anaesthesia, the air in the pouch was aspirated and a carboxymethylcellulose (CMC) pouch was formed by injecting 10 ml of sterilised CMC (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan) 1.5% in saline. The pouch exudate which was used for the formation of biofilms *in vitro* was extracted with a syringe 7 h after the injection of CMC (about 7 ml of exudate could be extracted from a pouch). An infection was induced by inoculating 1×10^7 cfu of *E. coli* strain 704 into a pouch along with the CMC.

Sterilised cotton threads (no. 6, Suzuki-thread, Tokyo, Japan), 1 mm in diameter, were soaked in 5 ml of TSB or the pouch exudate, and 5×10^6 cfu of *E. coli* were added. The mixtures were incubated at 37°C for 24 h. The threads were then transferred into fresh TSB or pouch exudate and incubated again. Threads which had received two repeated transfers, i.e. 72 h after the beginning of incubation, were used for the experiments (fig. 1).

Preparation of biofilm bacteria

Fig. 2 is a flow chart of the experiments. The cotton threads with biofilms on their surfaces were rinsed

once with TSB, and then soaked in fresh TSB and incubated at 37°C for 30–60 min in a shaking apparatus. The critical incubation time was determined according to the turbidity of the TSB to control the number of planktonic bacteria (PB) released from the biofilm. The PB were used as biofilm bacteria in the following experiments. As a control in each experiment, bacteria which had been cultured in TSB at 37°C for 72 h were used.

Sensitivity of PB to phagocytosis and phagocytic killing

TSB (0.25 ml), containing *c.* 3×10^7 cfu of PB/ml, was mixed with EBM 0.55 ml, human serum 0.2 ml, 0.5 ml of EBM containing 4×10^7 PMNL/ml, and 0.5 ml of EBM containing gelatin (Wako-junyaku Industry Co., Ltd, Tokyo, Japan) 4%. For reaction mixtures without PMNL, 0.5 ml of EBM was added in place of the PMNL suspension. The reaction mixtures were incubated at 37°C in a rolling incubator (7 rpm). At 1 and 2 h after the beginning of incubation, the number of bacteria in a reaction mixture was counted on Heart Infusion Agar (Eiken-kagaku). Each experiment was repeated three times.

For measurement of the sensitivity of PB to phagocytosis, the reaction mixtures were centrifuged at 1300 rpm for 5 min at 4°C, 15 min after the beginning of incubation. Precipitated PMNL were resuspended in a small amount of human serum and smeared on to glass slides. Phagocytosis was observed by microscopic examination of Wright-stained cell smears. One hundred neutrophil PMNL on each smear were examined, and the percentage of phagocytosing neutrophils and the mean number of bacteria/phagocytosing neutrophil were calculated. Each experiment was repeated five times.

Sensitivity of PB to killing activity of H₂O₂

A small amount of H₂O₂ 31% solution (Iwai Kagaku Co., Ltd, Tokyo, Japan) was added to TSB containing *c.* 3×10^6 cfu of PB/ml, to make the H₂O₂ concentration 0.01% or 0.001%. The reaction mixtures were incubated at 37°C in a shaker. After 1 h, numbers of viable bacteria were counted on heart infusion agar. Each experiment was repeated three times.

Electronmicroscopy

Scanning electronmicroscopy. Pieces of subcutaneous tissue from the infected CMC pouches and the cotton threads were processed for scanning electronmicroscopy by fixing for 90 min at 4°C in glutaraldehyde 2% in 0.1 M PBS, pH 7.4, with sucrose 8%. This was followed by re-fixation for 90 min at 4°C in osmic acid 2% in the same buffer, dehydration in a series of aqueous ethanol solutions (50–100%),

Fig. 1. Flowchart of experiments, 1.

Fig. 2. Flowchart of experiments, 2.

and drying in a VFD-20 drying apparatus (Hitachi-Seisakusho Inc., Tokyo, Japan). Samples were then coated with platinum-palladium in an Ion-coater E-102 (Hitachi-Seisakusho) and examined with an S-4000 scanning electronmicroscope (Hitachi-Seisakusho).

Transmission electronmicroscopy. PB were collected by centrifugation and fixed for 2 h at 4°C in 0.66 M cacodylate buffer, pH 7.3, containing glutaraldehyde 2% and ruthenium red (TAAB Laboratories Equip-

ment Ltd, Berks) 0.005%. The samples were then rinsed with cacodylate buffer and re-fixed for 3 h at room temperature in 0.66 M cacodylate buffer containing OsO_4 2% and ruthenium red 0.005%. Samples were rinsed with cacodylate buffer, then dehydrated in a graded series of alcohols and embedded in Epon 812. Sections were made with a diamond knife on a Sorvall MT6000 (Du Pont Company, DE, USA). The sections were stained for 15 min with uranyl acetate 2% followed by a 10-min treatment with lead citrate. The

Fig. 3. In-vivo biofilm growth of *E. coli* in CMC pouches in rats. Scanning electronmicrographs of subcutaneous tissue of CMC pouches 24 (a, a') and 48 h (b, b') after inoculation of 1×10^7 cfu of *E. coli*. Bars in a, b and a', b' are equivalent to $2 \mu\text{m}$ and $0.5 \mu\text{m}$, respectively.

electronmicrographs were made with an EM-100SX transmission electronmicroscope (Nihon-Denshi Co., Ltd, Tokyo, Japan).

Results

In-vivo biofilm model of growth of *E. coli* in CMC pouches in rats

The development of biofilm with time after infection of CMC pouches of rats with *E. coli* is shown in figs. 3a and b which, respectively, show the electronmicrographs of subcutaneous tissue at 24 and 48 h after infection. It can be seen that many blood cells and

some other cells, complicated fibrous structures and bacteria, in one body, make the colonising biofilm of bacteria progressively larger and thicker. Figs. 3a' and b' show the surface structures of bacteria in the biofilms corresponding to figs. 3a and b. The surface structures of the bacteria gradually became complex.

In-vitro biofilm model of growth of *E. coli* on the surface of cotton threads in artificial medium or pouch exudate

Figs. 4a–c and 5a–c show the development of biofilm structures on the surface of cotton threads in TSB and exudate from CMC pouches, respectively. In both

Transfer repetitions

Fig. 4. In-vitro biofilm growth of *E. coli* on the surface of cotton threads in TSB. Scanning electronmicrographs of the surface of cotton threads after 0 (a, a'), 1 (b, b') and 2 (c, c') transfer repetitions in TSB. Bars in a, b, c and a', b', c' are equivalent to 2 μm and 0.5 μm , respectively.

figures, a, b, and c had undergone 0, 1 and 2 transfer repetitions, respectively. Figs. 4a'–c' and 5a'–c' are electronmicrographs of bacteria in the biofilm corresponding to figs. 4a–c and figs 5a–c.

The structure of the biofilm formed *in vitro* in CMC pouch exudate and the structure of the surface of bacteria in the biofilm resembled those observed *in vivo* in the infected pouches. However, they differed from those formed during growth in TSB.

Sensitivity of PB to killing by phagocytes

The sensitivity of PB released from biofilms formed *in vitro* on the surface of cotton threads in the pouch exudate (PB-Pouch) or TSB (PB-TSB) to killing by human PMNL is shown in fig. 6. The number of control bacteria decreased to *c.* 1% after incubation for 2 h with PMNL. However, PB from both sources showed almost the same degree of resistance to killing

Fig. 5. In-vitro biofilm growth of *E. coli* on the surface of cotton threads in pouch exudate. Scanning electronmicrograph of the surface of cotton threads after 0 (**a**, **a'**), 1 (**b**, **b'**) and 2 (**c**, **c'**) transfer repetitions in pouch exudate. Bars in figs. **a**, **b**, **c** and **a'**, **b'**, **c'** are equivalent to 2 μm and 0.5 μm , respectively.

by PMNL. The rates of growth of PB and control bacteria did not differ from each other.

Susceptibility of PB to phagocytosis

The results of phagocytosis experiments are shown in the table. The control bacteria and both PB-Pouch and PB-TSB were almost equally sensitive to phago-

cytosis by PMNL. The differences in the percentage of phagocytosing PMNL and the mean number of bacteria/phagocytosing PMNL among the three groups of bacteria were not statistically significant.

Sensitivity of PB to killing by H_2O_2

The sensitivity of PB to killing by H_2O_2 0.01 % and

Fig. 6. Susceptibility of biofilm bacteria (PB) to killing by human PMNL. PB, $c. 1 \times 10^7$ cfu, were mixed with 2×10^7 PMNL in EBM containing human serum and incubated at 37°C ; after 1 and 2 h, the numbers of viable bacteria were counted. ●, PB-Pouch + PMNL; ○, PB-Pouch; ▲, PB-TSB + PMNL; △, PB-TSB; ■, control + PMNL; □, control.

Table. Susceptibility of biofilm bacteria (PB) to phagocytosis by PMNL

Experiment no.	Pouch		TSB		Control	
	A	B	A	B	A	B
1	35	2.6	44	3.2	43	3.4
2	33	2.4	35	3.0	40	3.4
3	22	2.8	29	2.6	25	2.9
4	20	2.8	27	3.1	19	3.2
5	37	4.9	38	3.1	48	5.4
Mean (SE)	29 (3.5)	3.1 (0.46)	35 (3.1)	3.0 (0.11)	35 (5.5)	3.7 (0.44)

A, number of phagocytosing cells/100 PMNL; B, mean bacterial number/phagocytosing cell.

0.001% is shown in fig. 7. PB-Pouch and PB-TSB showed almost the same degree of resistance to killing.

Transmission electronmicroscopy of PB

Figs. 8a, b and c are, respectively, transmission electronmicrographs of PB-Pouch, PB-TSB, and control bacteria. The surfaces of both types of PB were covered with substances that bound ruthenium red, although there was much more of these substances on the surfaces of PB-Pouch than on PB-TSB. Such

substances were not found on the surface of the control bacteria.

Discussion

In the bacterial infection model of CMC pouches on the backs of rats, biofilms of complicated structure were formed on the surface of subcutaneous tissue. Electronmicrographs of the biofilms suggested that blood cells, other cells and some components of tissue

Fig. 7. Susceptibility of biofilm bacteria (PBS) to killing by H₂O₂. PB, c. 4×10^6 cfu/ml in TSB, were mixed with H₂O₂. The numbers of viable bacteria were counted after incubation at 37°C for 1 h. ■, PB-Pouch; ▨, PB-TSB; ▩, control.

Fig. 8. Transmission electronmicrograph of PB and control bacteria. a, PB released from biofilm formed in the pouch exudate; b, PB released from biofilm in TSB; c, control bacteria. Bars are equivalent to 1 μ m.

fluid have important roles in the formation of biofilms. Bacterial biofilms that result in chronic or refractory infections are probably constructed in tissues or on the surface of biomaterials by way of highly complicated mechanisms.²²

In this report, interactions between human PMNL and bacteria released from two bacterial biofilm models that were formed *in vitro* in different media were compared. One model was formed in an artificial medium, TSB, and the other in rat CMC pouch exudate. The structure of the biofilm formed in the pouch exudate and the surface structure of bacteria in the biofilm were similar to those observed in the infected CMC pouches, but they were quite different from those observed with bacteria from the biofilm model formed in the artificial medium, TSB.

Both PB-Pouch and PB-TSB showed significant resistance to killing by human PMNL, but they were as sensitive to phagocytosis by PMNL as the control bacteria. The significant increase in resistance of both PB to killing by H₂O₂ clearly suggested that the increase in resistance of PB to killing by PMNL was caused by an increase in resistance of PB to killing by

active oxygen species in PMNL. No significant differences in resistance to killing by PMNL and H₂O₂ were found between PB-Pouch and PB-TSB.

Transmission electronmicrographs showed that both PB were covered with some substances that bound ruthenium red, although PB-Pouch had more of these surface substances than PB-TSB. However, such substances were not found on the surface of the control bacteria.

From these experimental data, it may be reasonable to speculate as follows: (1) *E. coli* strain 704, cells change to produce substances (acidic polysaccharides) that bind ruthenium red on their surfaces when they adhere to the surface of threads in pouch exudate or TSB, although the quantity of such substances changes according to the medium used. (2) The differences in appearance between bacteria in biofilms formed on the surface of threads in pouch exudate and TSB observed by scanning electronmicroscopy may have resulted from quantitative differences of acidic polysaccharides, i.e., acidic polysaccharides, coupled with some components in the pouch exudate, may be necessary for the development of bacterial biofilms *in*

vivo. (3) Bacteria may acquire resistance to killing by active oxygen species produced by PMNL by production of acidic polysaccharides. However, it may be reasonable to state that the production of acidic polysaccharides is not a direct cause of the resistance, because the resistance seemed not to depend on the quantity of polysaccharide. (4) The presence of acidic polysaccharides on the surface of an individual bacterium released from a biofilm might not influence phagocytosis by PMNL. However, it is possible that a large quantity of polysaccharides influences phagocytosis by PMNL; Simon *et al.*²³ reported that a strain of *Bacteroides fragilis*, which is covered with capsular polysaccharide, was significantly more resistant to phagocytosis by neutrophils. Johnson *et al.*¹⁷ showed that bacteria adhering to a plastic surface and producing slime were more resistant to uptake by PMNL than bacteria that did not produce slime. Adherent bacteria, as well as bacteria buried in

slime, might resist phagocytosis by PMNL by some additional mechanisms.

Many reports have indicated some differences between bacteria grown *in vitro* and *in vivo*. Passage of *Neisseria gonorrhoeae* in plastic chamber implants resulted in increased resistance to complement-mediated serum killing and phagocytosis.^{18, 24} There were differences between the envelope-protein profiles of *E. coli* grown *in vivo* and *in vitro*.²⁵ However, our findings here, that bacteria grown on the surface of cotton threads in pouch exudate and in TSB had increased resistance to killing by phagocytes, indicate that the increase in resistance was caused by adhesion of bacteria and not by a difference in growth medium. The increase in resistance of biofilm bacteria to killing by phagocytes may result from some changes in the bacteria caused by adhesion. The mechanisms by which adherent bacteria change to produce polysaccharides on their surfaces are still to be investigated.

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