Flow cytometric analysis of chlorhexidine action

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Abstract

The mechanism by which chlorhexidine kills bacteria is still ill defined. We have investigated the action of chlorhexidine on Escherichia coli JM101/psb311 using a combination of flow cytometry and traditional methods. Chlorhexidine-induced uptake by E. coli cells of bis-(1,3-dibutylbarbituric acid) trimethine oxonol and propidium iodide, which monitor membrane potential and membrane integrity respectively, was shown to be concentration dependent for the range 0.003–0.3 mmol L⁻¹. In addition, cells in log phase growth were more susceptible to 0.03 mmol L⁻¹ chlorhexidine than those in stationary phase. There was, however, no direct correlation between dye uptake and decline in colony forming units.

Keywords: Flow cytometry; Chlorhexidine; Membrane potential; Membrane integrity; Escherichia coli

1. Introduction

Chlorhexidine has been shown to cause changes to the cell membrane integrity with the resulting loss of cell constituents to the suspending medium [1–4]. At high concentrations chlorhexidine-induced leakage of cytoplasmic constituents is reduced and coagulation of the cytoplasm occurs [3,5]. Correlation between the amount of cell constituents released and loss in bacterial viability, however, has not been established [2]. In addition, it has been suggested that chlorhexidine is a specific inhibitor of membrane bound ATPase [6] but ATPase inhibition is only seen with high concentrations of chlorhexidine and is thus not believed to be the primary mechanism of action [7,8].

We have observed the effects of chlorhexidine on individual bacterial cells from stationary and log phase cultures of Escherichia coli, using flow cytometry. Particular physiological functions of the cell of interest can be probed with specific fluorescent dyes. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)) was chosen to monitor changes in membrane potential. This dye enters a cell only if the membrane potential has collapsed [9], and has previously been used with flow cytometry to detect bacterial antibiotic susceptibility [10,11]. Membrane integrity was assessed using propidium iodide. This small cationic molecule binds to nucleic acids, provided it has access to them through a damaged cell membrane. The vital dye-like property of this dye has been exploited by Gant et al. [12], to detect anti-
biotic induced changes in bacterial membrane permeability.

In this study flow cytometric techniques have been compared with traditional colony counting methods in assessing the biocidal activity of chlorhexidine.

2. Materials and methods

2.1. Bacterial strain and medium

The organism used was a genetically engineered bioluminescent strain of *E. coli* JM101 containing the lux plasmid psb311 (courtesy of Amersham International), previously used by Sheppard et al. [13]. It was cultured in Luria Betani broth (LB broth) containing 10 g of tryptone, 5 g yeast extract, 5 g NaCl, 4 ml of 1 mol l\(^{-1}\) NaOH to 1000 ml of distilled water and included tetracycline 10 µg ml\(^{-1}\) to maintain psb311. Cultures of *E. coli* were grown overnight (18 h) without agitation at 37°C to stationary phase. For log phase cultures a 1:100 dilution of the overnight culture was made in LB broth and incubated for 3 h at 37°C on an orbital shaker at 200 rpm.

2.2. Experimental procedure

Prepared cultures were spun at 4000 rpm for 10 min in an IEC (Dunstable, UK) Centra-4R centrifuge, the supernatant fluid discarded and the pellet resuspended in PBS. A stock solution (0.9 mmol l\(^{-1}\)) of chlorhexidine acetate (ICI, Macclesfield, UK) in distilled water was diluted in PBS to give solutions of 0.3 mmol l\(^{-1}\), 0.03 mmol l\(^{-1}\), 0.015 mmol l\(^{-1}\) and 0.003 mmol l\(^{-1}\). Organisms, suspended in PBS, were added to 5 ml volumes of the chlorhexidine solutions to give an initial colony count of 10^8 cfu ml\(^{-1}\) (determined by bioluminescent measurements (Lab M luminometer, Oldham, UK) using an established bioluminescence versus cfu ml\(^{-1}\) calibration curve [13]). Samples (0.5 ml) were removed from the test suspensions (held at room temperature) at regular intervals over 60 min. Two aliquots (0.2 ml each) were removed from each sample, and stained with DiBAC\(_4\)(3) or propidium iodide for flow cytometric analysis as outlined below. The remainder of the sample was used for plate counts.

Each experimental protocol was repeated at least twice; median values from pooled data are shown.

2.3. Viable counts

A 10 µl aliquot from each sample was added to 90 µl of neutralised peptone water (NPW) consisting of 1.5% w/v peptone water base (Oxoid CM9), 0.3% w/v lecithin (BDH, Poole, Dorset), 3.0% w/v tween 80 in water to neutralise the action of chlorhexidine. Serial dilutions were made in NPW and aliquots of each dilution were added to LB agar plates (LB broth containing 15 g l\(^{-1}\) bacteriological agar) using the method described by Miles et al. [14]. The agar plates were incubated for 48 h at 37°C prior to counting.

2.4. Bacterial staining

DiBAC\(_4\)(3) (excitation 493 nm, emission 516 nm) and propidium iodide (excitation 536 nm, emission 617 nm) were supplied by Molecular Probes Inc., USA and Sigma Chemical Co., UK respectively. DiBAC\(_4\)(3) was dissolved in acetone to give a stock solution of 1 mg ml\(^{-1}\). This was diluted 1:10 in 70% ethanol to give a working solution of 100 µg ml\(^{-1}\). Propidium iodide was dissolved in deionised water to a concentration of 100 µg ml\(^{-1}\). DiBAC\(_4\)(3) or propidium iodide were added to 0.2 ml of cell suspensions to give a final concentration of 10 µg ml\(^{-1}\). Cell suspensions were incubated with the dye for 3 min at room temperature prior to flow cytometric analysis.

Dye performance was determined by analysing the uptake of DiBAC\(_4\)(3) or propidium iodide after treating cultures of *E. coli* JM101/psb311 in log phase and stationary phase with 70% ethanol prior to washing and resuspending in fresh PBS.

In the following experiments the proportion of cells in a bacterial culture which exhibited cell-associated fluorescence with DiBAC\(_4\)(3) or propidium iodide equivalent in intensity to that of alcohol fixed organisms is expressed as a percentage as described previously [10].

2.5. Flow cytometric analysis

This was carried out using a Blyte HS (Bio-rad, Hemel Hempstead, UK) dual parameter flow cytom-
eter equipped with a xenon-mercury arc lamp. The instrument was used as previously described by Mason et al. [10]. In brief, fluorescence measurements were made using the FITC filter block with the following characteristics: excitation 470–490 nm, emission 520–560 nm (beam splitter at 510 nm), sheath flow pressure and sample flow were set to 0.7 bar and 2 µl min⁻¹, respectively. At least 5000 events were collected per sample.

3. Results

3.1. Viable counts

No detectable decrease in viability was observed in control cultures (Fig. 1). Chlorhexidine-treated stationary phase cultures, however, exhibited a concentration dependent decline in viable counts. Viable counts from log phase cultures exposed to 0.03 mmol l⁻¹ chlorhexidine decreased more rapidly than those from similarly treated stationary phase cultures.

3.2. Light scattering properties

The forward and side angle light scatter of stationary phase cells increased within 2–5 min after exposure to chlorhexidine (0.3 mmol l⁻¹) (Fig. 2). This change in light scattering properties was not observed with the other concentrations of chlorhexidine.

3.3. Changes in bacterial fluorescence subsequent to exposure to chlorhexidine

The proportion of bacteria from control cultures fluorescing with DiBAC₄(3) or propidium iodide did not exceed 10%. Chlorhexidine-induced uptake of the dyes was concentration dependent (Fig. 3). A comparison of log and stationary phase cultures exposed to 0.03 mmol l⁻¹ chlorhexidine revealed that loss of membrane potential and integrity was more rapid in log phase cultures. Interestingly, histograms of data accumulated from stationary and log phase cultures after treatment with chlorhexidine (0.03 mmol l⁻¹) revealed two distinct peaks of propidium iodide fluorescence, which resolved to a single peak later in culture (Fig. 4). This phenomenon was consistently observed after 15–30 min in the case of log phase cultures and at 60–90 min in stationary phase cultures. These unusual patterns of propidium iodide uptake were not observed from stationary phase cultures treated with chlorhexidine at any of the other concentrations used.
tential and integrity can provide an indication of chlorhexidine-induced membrane damage.

Despite the percentage of fluorescent bacteria increasing as the number of cfus declined, the expected 99.9% cell-associated fluorescence (corresponding to a 3 log order decrease in cfus) was not obtained with either dye (compare Figs. 1 and 3). Thus, consistent with previous findings [2] these results indicate that there is no direct relationship between the membrane perturbative events caused by chlorhexidine and recovery on agar.

Flow cytometric measurements are carried out whilst the cells are suspended in the chlorhexidine environment. In comparison, there is a delay of 24-48 h prior to observation of colonies. Moreover neutralisation of the chlorhexidine and dilution of the sample, to ensure that the colonies are countable, is essential. Using a similar neutralisation procedure Ismael et al. [15] demonstrated that some chlorhexidine remains bound to cells. Therefore, biocidal action may continue during incubation of the plate or bound chlorhexidine may cause a biostatic effect.

The precise events of chlorhexidine-induced membrane disruption is still unclear. Hugo and Longworth [5] reported that an initial chlorhexidine-induced leakage of cytoplasmic constituents is followed by a second phase of leakage at low concentrations, or at high concentrations coagulation of the cytoplasm occurs. Our results indicate that the membrane potential of cells collapses prior to the loss of membrane integrity. Furthermore, the two peaks of propidium iodide—associated fluorescence

Fig. 3. Median percentage of fluorescent E. coli JM101/psb311 cells stained with DiBAC₄(3) (A), or PI (B). Results from stationary phase cultures exposed to CHDE at 0.003 mmol l⁻¹ (○), 0.015 mmol l⁻¹ (*), 0.03 mmol l⁻¹ (□), 0.3 mol l⁻¹ (♦) and log phase cultures exposed to 0.03 mmol l⁻¹ (■) for 60 min at 24°C.

4. Discussion

These results demonstrate that flow cytometric monitoring of bacterial cytoplasmic membrane po-

Fig. 4. Dual parameter histograms of forward angle scatter versus PI fluorescence from cultures of E. coli JM101/psb311. Histograms to the right of the bold line represent those cells with PI-associated fluorescence. Results from control culture in log phase (i), and log phase culture exposed to CHDE at 0.03 mmol l⁻¹ for 30 min at 24°C (ii).
observed with 0.03 mmol l⁻¹ chlorhexidine may represent biphasic damage to membrane integrity. It was also notable that log phase cultures which showed a high uptake of DiBAC₄(3) within 2 min of exposure to this concentration subsequently showed a decline in their cell-associated fluorescence when exposed to chlorhexidine for a further 5 to 10 min. Kuyyakanond and Quesnel [8] demonstrated the restoration of a membrane potential by cells following exposure to biocidal concentrations of chlorhexidine, possibly through the generation of protons by the injured cells.

The increased light scattering properties of organisms exposed to the higher chlorhexidine concentration (0.3 mmol l⁻¹) suggest there are major changes to the internal cellular structure, possibly coagulation. This would be consistent with the observation that rapid biocidal activity occurs when the cytoplasm is coagulated [5]. Cell surface swellings, occurring at high chlorhexidine concentrations, which are thought to result from the accumulation of biocide molecules [3] could also affect the light scattering properties of treated bacteria.

Finally, these results indicate that the biocidal activity of chlorhexidine is dependent on the phase of growth from which cells are harvested, with log phase cells showing a greater sensitivity towards chlorhexidine than stationary phase cells. Similar findings have been reported for other membrane-active biocides [16]. Log phase cells, in contrast to stationary phase cells, are actively dividing when harvested and thus these results may indicate that sensitisation to chlorhexidine occurs during the cell division cycle [17]. Furthermore, the greater resistance of stationary phase cells could result from synthesis of starvation proteins at the onset of this phase [18].

In conclusion, our results have demonstrated that flow cytometry can be used to detect chlorhexidine action against individual cells. Whether flow cytometry in combination with fluorescent probes relates more closely to biocide efficacy than cfus remains to be determined. Flow cytometry, however, could continue to have a role in elucidating the mechanism of action of antimicrobial agents.

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