A triazine dye, cibacron blue 3G-A induces Staphylococcus aureus to form giant clusters

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1. SUMMARY

Cibacron blue 3G-A (CB), one of the triazinyl dyes commonly used as a ligand for affinity chromatography, induced staphylococci to form giant clusters without affecting cell viability. It was demonstrated that the cluster formation was not a mechanical aggregation of cells by CB, but a consequence of its biological effect on staphylococcal growth. Clusters induced by CB did not form a regular arrangement, and were different from clusters induced by SDS or polyanethole sulfonate.

2. INTRODUCTION

It has been widely reported that peptidoglycan hydrolases or so called autolytic enzymes might be involved in septum formation, cell separation, cell elongation and wall turnover [1,2]. Recently we found that an endo-β-N-acetylglucosaminidase in the culture fluid of Staphylococcus aureus has affinity to a dye ligand affinity gel [3]. Cibacron blue 3G-A, which is the affinity ligand of Blue Toyopearl, is one of the triazinyl dyes widely used for enzyme purification. The specific interaction of CB with dehydrogenases and kinases has been reported, and the enzyme activity of some of them was inhibited by CB [4,5]. Our observation that a peptidoglycan hydrolase has affinity to CB led us to investigate the effect of CB on S. aureus cell growth. In this communication, we described that CB blocked staphylococcal cell separation without a bactericidal effect.

3. MATERIALS AND METHODS

3.1. Bacterial strains

Staphylococcus aureus FDA 209P (ATCC 6538) was used to determine the effect of Cibacron Blue 3G-A (CB; Fluka AG, Chemische Fabrik, Buchs, Switzerland) (Fig. 1) on cell separation. To survey the effect of CB on other staphylococci, several other strains were also used: S. capitis ATCC 27840, S. cohnii ATCC 29974, S. epidermidis ATCC 12228, S. haemolyticus ATCC 29970, S. intermedius ATCC 29663, S. saprophyticus ATCC 15305, S. simulans ATCC27848, S. warnei ATCC 27836 and S. xylosus ATCC 29971.

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3.2. Determination of MIC

Minimal inhibitory concentration (MIC) of CB against several microorganisms was determined by a two fold serial agar dilution method. Cells (10^6 cfu) were inoculated onto Trypticase soy agar (Becton Dickinson and Co., Cockeysville, MD) plates containing serially diluted CB. The plates were incubated at 37°C for 24 h and the development of visible growth on agar was investigated.

3.3. Effect of CB on cell growth

Exponentially growing *S. aureus* FDA 209P (10^6 cfu) in Trypticase Soy Broth (TSB: Becton Dickinson and Co.) were cultured in TSB containing CB with standing at 37°C to study the pattern of cell growth. For surveying the effect of CB on growth pattern of other strains, small portions (10^6 cfu) of exponentially growing culture were added to serial dilutions of CB in TSB (150 µl) in two steps on a microtiter plate (Becton Dickinson and Co.). The concentration of CB was within the range 3.7 μM–1 mM. The plates were incubated at 37°C for 24 h and the pattern of cell growth was determined through observation of the bottom of wells. For morphological observation, cells were

![Figure 2: Appearance of *S. aureus* FDA 209P cultured in Trypticase soy broth containing 60 μM of cibacron blue 3G-A. (a) Clusters grown as visible particles sizes (×10). (b, c) Phase contrast microscopic observation of a cluster (b, ×200; c, ×2500). (d) Thin section of a cluster resulting from inhibition of cell separation (×32000).](image-url)
monitored with phase contrast microscopy with photographs taken to document observations.

3.4. Electron microscopy

Bacterial cells were prepared for thin sectioning by fixation with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 1 h at room temperature. After washing, they were postfixed with 1% OsO₄ in 0.1 M cacodylate buffer for 1 h at room temperature. After dehydration using a series of increasing ethanol concentrations, the material was embedded in Epon and polymerized for 17 h at 60°C. This sections were cut with a diamond knife on a ULTROTOME III (LKB8800, LKB Instruments, Inc. Rockville, MD), additionally stained with uranyl acetate and lead citrate, and examined under a Hitachi H 500H electron microscope (Hitachi Co. Ltd, Tokyo, Japan).

4. RESULTS AND DISCUSSION

MICs of CB against all microorganisms employed in this study were over 1600 µg/ml (2 mM). In the presence of 30 µM CB, S. aureus FDA 209P cells grew in giant clusters visible to the naked eye (Fig. 2a). Phase contrast microscopic observations demonstrated that cells cultured with CB grew in giant clusters without separation (Fig. 2b–d), while control cells appeared as well-separated cocci with a few clusters (data not shown). The effect of CB on cell growth was also studied using a microtiter plate method. The cells which formed a giant cluster showed diffuse pattern in the well, whereas control cells grown in TSB without CB settled to form a white compact button at the bottom of the well (Fig. 3). This method was useful for the determination of the

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration (µM)</th>
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<tbody>
<tr>
<td>S. capitis ATCC 27840</td>
<td>3.7</td>
</tr>
<tr>
<td>S. haemolyticus ATCC 29970</td>
<td>7.5</td>
</tr>
<tr>
<td>S. xylosus ATCC 29971</td>
<td>7.5</td>
</tr>
<tr>
<td>S. simulans ATCC 27848</td>
<td>15</td>
</tr>
<tr>
<td>S. aureus FDA 209P</td>
<td>30</td>
</tr>
<tr>
<td>S. epidermidis ATCC 12228</td>
<td>30</td>
</tr>
<tr>
<td>S. warnei ATCC 27836</td>
<td>63</td>
</tr>
<tr>
<td>S. intermedius ATCC 29663</td>
<td>63</td>
</tr>
<tr>
<td>S. cohnii ATCC 29974</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>S. saprophyticus ATCC 15305</td>
<td>&gt;1000</td>
</tr>
</tbody>
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2 Determined by a microtiter plate method described in MATERIALS AND METHODS.

minimum concentration needed for cells to induce visible clusters. The CB effect on growth pattern was also surveyed for another 10 strains of clinically isolated S. aureus. All strains formed visible clusters in the CB concentration range 30 µM–2 mM.

Studies were conducted to determine how widespread this effect of CB on bacterial growth was among *Staphylococcus* spp using the microtiter plate method. The strains which formed giant clusters when cultured with were as follows: S. capitis, S. haemolyticus, S. xylosus, S. simulans, S. epidermidis, S. warnei and S. intermedius. The minimum concentration of CB needed for the formation of visible clusters varied among strains as shown in Table 1. On the other hand, S. saprophyticus and S. cohnii did not form clusters in 1 mM CB.

Experiments using S. aureus FDA 209P were conducted to determine whether CB would directly cause cell aggregation. The incubation of washed cells in PBS containing CB (1 mM) at 37°C resulted in no aggregation. Clusters induced by CB showed characteristics analogous to those of a cluster forming mutant reported by Chatterjee et al. [6]. Our attempt to disaggregate CB-induced clusters by addition of the following detergents: 2% sodium dodecyl sulphate (SDS), 2% Triton X-100 and proteases, trypsin (1 mg/ml), pronase (1 mg/ml), was unsuccessful. On the
other hand, clusters could be disaggregated by mild sonication, and viable cells recovered with an increase in CFU. These data suggested that cluster formation by CB was not merely mechanical aggregation of cells, but consequence of biological action on _S. aureus_ growth. CB action seems to be specific, since individual cells of a cluster were well divided and the size and shape of them were not affected by CB treatment (Fig. 2c, d). An indication that cells remained joined by their cell walls was observed by electron microscopy (Fig. 2d). This implies that CB specifically block cell separation at the last stage of the division cycle.

Several factors influencing cell separation have been identified [7,8,9]. When _S. aureus_ was cultured in the presence of a sublethal concentration of SDS, cells were induced to form packets [7]. The packet formation was suggested to be due to an inactivation of cell separation enzyme(s) by SDS. The packets formed in this way were not as large as clusters induced by CB. Since SDS is a potent denaturant of proteins, it might be critical for cells to grow in higher concentration of SDS enough to inhibit cell separation enzyme activity completely. On the other hand, the inhibitory action of CB seems to be rather specific. Cell separation of _S. aureus_ was completely blocked by CB at a concentration ten times lower than that of SDS, at which cell viability was not affected by CB. _S. aureus_ cells treated with CB in the range tested did not form regularly arranged clusters. These results were different from those obtained from treatment with SDS by Koyama et al. [7] and with polyanethole sulphonate by Wecke et al. [8].

We have recently demonstrated that staphylococcal 51kD endo-β-N-acetylglucosaminidase, purified from Blue Toyopearl, caused separation of cell clusters of _S. aureus_ without bacteriolysis and its activity was completely blocked by CB (manuscript submitted). The physiological function of this enzyme in vivo has not yet determined, however these data imply some interaction between CB and enzyme(s) responsible for cell separation. The exact mechanism of inhibition of cell separation by CB is not clear. Further studies using CB should eventually disclose the molecular mechanism of cell separation.

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**REFERENCES**


