Site of action of a gonococcal growth inhibitor produced by *Staphylococcus haemolyticus*

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Summary. The inhibitory substance produced by *Staphylococcus haemolyticus* strain no. 7 acts on growing as well as resting gonococcal cells, as shown by reductions in viable counts. The optical density of these cell suspensions was only slightly reduced. The inhibitor caused lysis of gonococcal spheroplasts at 24°C and 37°C, but was much less active at 4°C. Acting on intact gonococcal cells, the inhibitor caused a temperature-dependent release of radioactive cytoplasmic material. Electronmicroscopy showed that treated suspensions contained ghost cells with the cell envelope relatively intact. Our results suggest that the inhibitor may act on the cytoplasmic membrane of the gonococcal cell causing cytoplasmic leakage and, eventually, death.

Introduction

In-vitro inhibition of *Neisseria gonorrhoeae* by micro-organisms present in the urogenital flora has been reported by several workers (Kraus and Ellison, 1974; Kraus *et al.*, 1976; Kaye and Levison, 1977; Ingraham, 1978; Morin *et al.*, 1980; Bisailon *et al.*, 1981*b*). Kraus and Ellison (1974) have suggested that such inhibition may play a role in host resistance to gonorrhoea. Substances responsible for gonococcal inhibition have been isolated and purified (Dajani *et al.*, 1976; Morse *et al.*, 1976; Rogolski and Wiley, 1977; Simpson and Davis, 1979; Beaudet *et al.*, 1982; Dubreuil *et al.*, 1984; Morin *et al.*, 1984) but the site of action has been determined only for a staphylococcin (Morriss *et al.*, 1978) and for pyocins (Morse *et al.*, 1980).

In earlier work (Beaudet *et al.*, 1982) we purified a gonococcal growth inhibitor produced by a strain of *Staphylococcus haemolyticus* isolated from the urogenital flora. This inhibitor is a lipid-associated protein, in which the protein moiety is responsible for the activity. The protein is composed of high mol. wt aggregates formed by small peptides of mol. wt *c.* 2.5×10^3 (Frenette *et al.*, 1984). Biological characterisation showed that this inhibitor has a broad haemolytic spectrum and has some properties in common with *S. aureus* δ lysin (Frenette *et al.*, 1984). The purpose of this study was to elucidate the site of action of this inhibitor on the gonococcal cell.

Materials and methods

Bacterial strains

S. haemolyticus strain no. 7, isolated from the urogenital flora (Bisailon *et al.*, 1981*b*), was used for the production of the inhibitor. *N. gonorrhoeae* strain G-10 (Bisailon *et al.*, 1981*a*) was used as the target organism for the inhibitor. All strains were kept lyophilised or as frozen suspensions at -80°C .

Culture medium

The gonococcal strain was grown on GC agar base (Gibco Diagnostics, Madison, WI, USA) enriched with CVA 1% (Gibco) and lysed horse blood 1.5%, at 37°C in an atmosphere containing CO₂ 5% with 70% relative humidity.

Production and purification of the gonococcal inhibitor

The inhibitor was produced and purified as previously described (Beaudet *et al.*, 1982). Production was on semisolid Brain-Heart Infusion (BHI) Medium (Difco Laboratories) which had supported a 24-h growth of *S. haemolyticus* strain no. 7 at 37°C. The inhibitory substance was purified by methanol extraction, acetone fractionation and chromatography on Ultrogel AcA54. The quantitative determination of the anti-gonococcal activity has already been described (Beaudet *et al.*, 1982). The dilution of the preparation that caused a 50% decrease in the optical density (OD) of a gonococcal liquid culture, with respect to the control (without inhibitor), was taken to be the amount of arbitrary units (A.U.)/ml.

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Effect of the inhibitor on gonococcal cells

The effect of the inhibitor on growing gonococcal cells was determined by inoculating BHI medium containing inhibitor (20 A.U./ml) with *N. gonorrhoeae* G-10 to obtain 5×10^7 cfu/ml. Cultures were incubated at 37°C in CO₂ 5% in a "Psychrotherm" incubator (New Brunswick Scientific Co., NJ, USA). A control culture without the inhibitor was also included. Viable counts and the OD at 540 nm of the cultures were determined every hour for 6 h. The procedure to determine the effect of the inhibitor on resting cells was similar to the one previously described for growing cells except that BHI was replaced with 0.01 M phosphate buffer, pH 7.0, containing Proteose Peptone No. 3 (Difco) 1% and NaCl 0.85% (PPS). Each experiment was repeated four times and similar results were obtained.

Effect of the inhibitor on gonococcal spheroplasts

Gonococcal osmotic-sensitive cells were prepared by a modification of Kaback's method (Kaback, 1971). Cells grown in BHI medium were harvested in mid-exponential phase of growth, centrifuged, washed twice in 0.01 M Tris-HCl buffer, pH 8.0, containing PPS and resuspended in a small volume of 0.03 M Tris-HCl buffer, pH 8.0, containing Proteose Peptone No. 3 1%, NaCl 0.85% and sucrose 30% (PPSS) to obtain a high density population. A volume of 0.1 M Na₂EDTA corresponding to one tenth of the final volume of the cell suspension, and solid lysozyme (grade 1; Sigma) to a final concentration of 100 µg/ml, were added to the cell suspension. The cell suspension was incubated at room temperature for 30 min.

The resulting spheroplasts were dispensed in tubes and incubated at 37°C, 24°C or 4°C with various concentrations of inhibitor or with Triton X-100 35 µg/ml as a control to show maximal lysis. The OD of the suspensions was monitored at 540 nm at different time intervals for 2 h. Each experiment was repeated three times and similar results were obtained.

Effect of the inhibitor on the release of radioactive cytoplasmic material by gonococcal cells

Gonococcal cells from an 18-h culture in Neisseria Chemically Defined Medium (Gibco) were harvested by centrifugation (10 000g, 30 min, 4°C) and incubated in the same medium with uniformly labelled ¹⁴C glucose (NEN, 1 µCi/ml), for 1.5 h at 37°C in CO₂ 5% with agitation at 200 rpm. Cells were then centrifuged, washed twice and resuspended in 0.01 M phosphate buffer, pH 7.0 containing PPSS. The suspensions were incubated at 37°C, 24°C or 4°C with various concentrations of inhibitor or with Triton X-100 150 µg/ml as a control for maximal release of radioactive cytoplasmic material. At different time intervals samples of the suspensions were centrifuged at 15 000g for 8 min and the radioactivity of the supernates was measured with Bray's solution as scintillator.

Electronmicroscopy

Gonococcal cells from an 18-h culture on GC Agar Base (Gibco) enriched with CVA (Gibco) 1% v/v and lysed horse blood 1.5% v/v were centrifuged, washed and resuspended in PPSS. The inhibitor was added to the suspensions to a final concentration of 20 A.U./ml and the suspensions were incubated at 4°C for 2 h. The cells were centrifuged, washed in PPSS and fixed in glutaraldehyde 2.5% and osmic acid 2%. The cells were then embedded in Vestopal W, cut in ultra-thin sections and stained with uranyl acetate 5% followed by lead citrate and examined in a Philips EM 300 electronmicroscope.

Results

The effect of the inhibitor produced by *S. haemolyticus* strain no. 7 on growing gonococcal cells is illustrated in fig. 1. The inhibitor caused a decrease in viable counts during the first 3 h of incubation from 5.6×10^7 to 7.7×10^5 cfu/ml. Viable counts then stabilised until the end of the observation period. The OD of the treated suspension remained stable for the first 2 h of incubation and then decreased very slowly for the following 4 h. As expected, viable counts and OD of control cultures increased steadily over the incubation period.

The inhibitor was also active on resting gonococcal cells. A sharp decrease in viable counts (6.1×10^7 to 1.4×10^5 cfu/ml) of the treated suspension was seen during the incubation period while viable counts of the control suspension remained stable. The OD of the suspension decreased slightly in the presence of the inhibitor but not in controls.

The inhibitor caused lysis of gonococcal spheroplasts as suggested by a decrease in the OD of the cell suspensions (table). The extent of the reduction of OD was proportional to the concentration of the inhibitor in the suspension; the two higher doses used (5 and 7.6 A.U./ml) caused a drop comparable to that observed with Triton X-100. At 4°C, the inhibitor was still active on gonococcal spheroplasts but the decreases in OD were less than those observed at higher temperatures. The action of Triton X-100 did not seem to be affected by variations of temperature.

At 37°C the inhibitor caused an almost linear release with time of the radioactive cytoplasmic material from gonococcal cells (fig. 2). The two higher concentrations of the inhibitor were as active as Triton X-100 after incubation for 2 h. At 24°C and 4°C, the inhibitor still caused release of radioactive cytoplasmic material but its action was weaker than that observed at 37°C. Again, the action of the Triton X-100 did not seem to be modified by variations of temperature.

Fig. 1. Effect of the inhibitor (20 A.U./ml) on viable counts (A) and optical density (B) of growing (●) and resting (○) gonococcal cell suspensions; ■, □ = control experiment without inhibitor.

Fig. 2. Release of radioactive cytoplasmic material from gonococcal cells incubated in the presence of the inhibitor at 37°C (A) 24°C (B) and 4°C (C). The concentrations of the inhibitor (A.U./mL) were: 0 (1), 5 (2), 10 (3), 20 (4). Triton X-100 (150 µg/mL) was used as control for maximal lysis (5). Each value represents the mean of four experiments.

Electronmicrographs of the gonococcal cells incubated in the presence of the inhibitor showed that most of them were emptied of their cytoplasm (fig. 3). The cell walls seemed to remain intact and some diplococcoid "ghosts" were observed. Electronmicrographs of cells from the control group showed normal morphology.

Discussion

The site of action of many of the gonococcal-inhibiting substances produced by bacteria from the urogenital flora has not been studied. According to Morriss *et al.* (1978) and Morse *et al.* (1980) the staphylococcin and the pyocin they described,

Table. Effect of the inhibitor on gonococcal spheroplasts

Inhibitor concentration (A.U./ml)	Percentage reduction of OD at 540 nm* at		
	4°C	24°C	37°C
0	0	0	0
0.3	7.4	23.2	24.6
0.6	10.7	35.7	36.1
1.2	15.7	46.4	47.6
2.5	22.3	50.0	51.6
5.0	30.6	58.9	60.7
7.6	38.9	64.3	63.9
Triton X-100†	73.6	67.9	59.0

* Gonococcal spheroplasts were incubated in the presence of inhibitor for 2 h and percentage reductions were calculated by comparison with OD of spheroplasts incubated without inhibitor under the same conditions.

† Triton X-100 (35 µg/ml) was used as a control for maximal lysis.

respectively, act on the cytoplasmic membrane of gonococci. The inhibitor produced by *S. haemolyticus* was active on growing and resting gonococcal cells, suggesting that this substance acts on a cell structure of the gonococcus and does not require an active metabolism (Russel *et al.*, 1973). The OD of growing and resting suspensions of gonococci was only slightly reduced in the presence of the inhibitor but this does suggest that there could be some effect on the cell wall. However, it cannot be assumed that the inhibitor is directly responsible for this effect since autolysis is common in gonococcal cells suspended in a medium unable to maintain growth (Wegener *et al.*, 1977). The inhibiting conditions

might favour autolysis and further work on peptidoglycan hydrolysis is necessary to clarify this.

The inhibitor was also active on gonococcal spheroplasts. At 37°C and 24°C lysis was apparent at concentrations as low as 0.3 A.U./ml, which corresponded to 67 times less than that used on growing and resting cells. To show the release of cytoplasmic material caused by the antigonococcal substance, ¹⁴C-glucose was used to create a pool of radioactive material in the gonococcal cytoplasm. Morse and Bartenstein (1974) have shown that glucose is used by gonococci as the main carbon source; therefore, in our experiments, the radioactivity would be expected to become distributed in all cell components, particularly the cytoplasm. The inhibitor caused the release of radioactive cytoplasmic material from the gonococci. At 37°C, doses of 10 and 20 A.U./ml caused a release equivalent to that observed with Triton X-100 but at 24°C and 4°C release was much reduced. These results, and those obtained with the spheroplasts, suggest that the site of action of this substance is the cytoplasmic membrane.

The inhibitor was already shown to have some properties similar to those of the *S. aureus* δ lysin (Frenette *et al.*, 1984). Both substances have similar haemolytic spectra against erythrocytes of different animal species and both disrupt tissue culture cells and are inhibited by phosphatidylcholine. However, the observed effect of temperature on the activity of the gonococcal inhibitor is not shared by the δ lysin; Thelestam *et al.* (1973) and Kapral (1976) have shown the action of δ lysin on human fibroblast and human erythrocytes to be temperature independent.

Fig. 3. Electronmicrographs of normal gonococcal cells (A) ($\times 36\,000$) and gonococcal cells incubated in presence of the inhibitor (20 A.U./ml) for 2 h at 4°C (B) ($\times 20\,100$).

Electronmicrographs of gonococcal cells treated with the inhibitor showed these cells were emptied of their cytoplasm. There was no detectable ultrastructural damage to the cell envelope. Clawson and Dajani (1970) observed similar "ghost" formation with streptococcal cells treated with a bactericidal substance produced by *S. aureus*. This substance is different from the gonococcal inhibitor on the basis of its antibacterial spectrum and chemical properties.

Our results suggest that the inhibitor is acting on the cytoplasmic membrane of the gonococcus causing cytoplasmic leakage that eventually leads to cell death. We believe that the substance does not interfere with the cell wall or its synthesis, even though the OD of treated cell suspensions was slightly reduced in its presence. These results are in accord with the reported haemolytic activity of the inhibitor (Frenette *et al.*, 1984) and suggest that this substance has high membranophilic properties which are not specific to a membrane type.

Although the in-vivo activity of the inhibitor against *N. gonorrhoeae* in guinea pig subcutaneous chambers has already been shown (Frenette *et al.*, 1984), secretion has not been demonstrated *in vivo* and the role of this substance in resistance to gonorrhoea has not been established. The absence of an animal model reproducing the complexity of the urogenital tract, with the normal urogenital flora and physiological conditions of the host, including the vaginal secretions, immunity and the menstrual cycle, make this sort of study very difficult. This substance could be an interesting tool to study the structure and function of membranes; however, further work is necessary to determine the precise mechanism of action of the inhibitor.

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