Enhanced Production of Cell-Bound and Extracellular Streptolysin S by Hemolytic Streptococci Pretreated with Proteases

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Z. Naturforsch. **40 c,** 166–169 (1985); received November 22, 1984

Streptolysin S, Cell-Bound Hemolysin, Protease, Streptococci

The amount of streptolysin S produced by resting streptococci was considerably increased after incubation of the washed bacteria with trypsin or pronase. Production of both cell-bound and free forms of the toxin was enhanced by the protease treatment. By addition of trypsin, streptolysin S yield was considerably increased in growing culture as well. Treatment with lysozyme was ineffective, and the toxin production was only slightly promoted by preincubation with hyaluronidase or chymotrypsin. In contrast, pretreatment with chymotrypsin caused increased production of an extracellular nuclease, whereas the yield of this enzyme was reduced after incubation of the cocci with pronase. Evidence was obtained indicating de novo synthesis of the exotoxin in the protease-treated bacteria.

Introduction

When incubated with a certain carrier substance such as yeast RNA [1], hemolytic streptococci produce streptolysin S (SLS) complex, an oxygen-labile cytolytic exotoxin composed of apotoxin (SLS peptide) and the carrier moiety [2]. In addition, the bacteria competent to produce SLS have a cell-bound hemolysin (CBH) which, in the absence of the added carrier, can lyse erythrocytes or Ehrlich ascites tumor cells by direct contact [2, 3]. The CBH activity is thought to be a cell-bound form of nascent SLS peptide. In order to elucidate the role of the bacterial envelope in CBH and SLS formation, washed streptococci were treated with several enzymes. Interestingly, the cocci digested with trypsin or pronase produced enhanced amounts of CBH and extracellular SLS, whereas the yield of an extracellular nuclease was increased in the cells pretreated with chymotrypsin. The exotoxin production by the protease-treated cells was markedly sensitive to chloramphenicol or tetracycline and partially inhibited by rifampicin. Possible mechanisms for the increased toxin yield in the protease-treated streptococci were discussed.

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0341-0382/85/0300-0166 $ 01.30/0

Materials and Methods

Chemicals

Trypsin and chymotrypsin were purchased from Worthington Biochemical Corp., whereas lysozyme, hyaluronidase and calf thymus DNA were obtained from Sigma Chemical Co. Pronase was purchased from Kaken Co., Tokyo, tetracycline hydrochloride from Takeda Chemical Ind. Ltd., Osaka, chloramphenicol from Sankyo Co., Tokyo, rifampicin from Daiichi Pharmaceutical Co., Tokyo and yeast RNA was from Kohjin Co., Tokyo. AF (guanylic acid rich oligonucleotide fraction with potent carrier activity for SLS) was prepared from RNase I core of yeast RNA by DEAE cellulose chromatography [4, 5].

Strains and media

Hemolytic streptococci, strains *Sa, Su* and *Sv* were used. The bacteria were grown aerobically, but without shaking, in a peptone-meat infusion broth at 37 °C. When SLS production was to be investigated, yeast RNA (1%) or AF (2 OD_{260} units/ml) was supplemented. For SLS production in a resting cell system [4], Bernheimer's basal medium (BBM) [6] containing AF was used.

Others

Measurement of bacterial growth, titration of SLS and assay of the nuclease activity were performed as described previously [4, 5, 7].

Results

The occurrence of SLS formation in streptococcal protoplasts [8] suggests that integrity of the bacterial envelope is not essential for the toxin production. However, the role of the streptococcal envelope in the formation of CBH and the extracellular nuclease is quite unknown, and it seems probable that the coccal outer membrane somehow modulates SLS excretion. As an approach toward this problem, streptococcal cells were treated with several enzymes and then their capacity to produce CBH, SLS and the extracellular nuclease [7] was compared in the resting cell system. As shown in Table I, the yield of CBH was slightly reduced after lysozyme treatment and somewhat increased by pretreatment with hyaluronidase or chymotrypsin. The effect of these enzymes was, however, rather marginal and might be nonspecific. On the other hand, CBH activity of the cocci was considerably increased after digestion with trypsin or pronase. Although varying to some extent, the CBH yield in the protease-treated cells was usually 2.5 to 3 times as high as the untreated control. The yield of the extracellular SLS was also increased by predigestion of the bacteria with trypsin or pronase, but not particularly affected by lysozyme, hyaluronidase or chymotrypsin. It is thus clear that the effect of the proteases on the hemolysin formation is independent of the carrier molecule. Moreover, the yield of the extracellular SLS changes nearly in parallel with the CBH titer upon treatment of the cocci with these enzymes, indicating an intimate relationship between the cell-bound and the extracellular hemolysins. In cells of strain Su as well, the yield of CBH and extracellular SLS was considerably increased after treatment with trypsin or pronase, but only slightly by chymotrypsin.

In contrast to SLS, the yield of the nuclease was slightly raised by pretreatment of the bacteria with trypsin, and rather reduced after pronase digestion. Streptococcal cells subjected to chymotrypsin treatment, however, produced significantly increased amount of the nuclease into the medium.

Besides the effect on washed resting cells, trypsin distinctly enhanced the yield of extracellular SLS in growing streptococci. As shown in Table II, the specific yield of SLS (HU/OD660 of the culture) was 6 to 8 times higher in the trypsin-supplemented culture of Strain Sa or Sv, than in the control. Similarly, the amount of SLS was increased about 11-fold in a culture of strain Su, by an addition of trypsin (data not shown). Extracellular SLS was, however, not produced even in the trypsin-supplemented culture, unless a specific carrier such as yeast RNA was added.

In the experiment shown in Table I, cells treated with each enzyme were extensively washed, suspended in BBM and then tested for production of SLS and nuclease. It is unlikely that residual trypsin or pronase stimulates processing of surface-bound nascent SLS (although insensitive to trypsin, SLS is easily inactivated by pronase). As shown in Table III, SLS production in the pronase-treated cocci was sig-

Table I. Production of CBH, SLS and nuclease in resting streptococci subjected to enzyme treatment. Cells of strain Sa were grown in peptone-meat infusion broth at 37 °C for 15 h, collected, washed three times with 0.15 M saline and suspended in BBM. The bacteria were incubated with 2 mg/ml of each enzyme, at 37 °C for 1 h, collected and washed four times with 0.15 M saline. The cells were suspended in BBM with or without AF and then tested for production of CBH and the extracellular nuclease. HU: hemolytic unit.

Enzyme (2 mg/ml)	CBH formed		SLS formed		Nuclease formed	
	HU/ml	[%]	HU/ml	[%]	Unit/ml	[%]
None	1.75×10^3	100	4.73×10^3	100	0.628	100
Lysozyme	1.24×10^3	70.9	4.43×10^3	93.7	0.741	118
Hyaluronidase	2.69×10^3	154	6.53×10^3	138	–	–
Trypsin	4.82×10^3	275	1.22×10^4	258	1.030	164
Chymotrypsin	2.92×10^3	167	6.96×10^3	147	1.482	236
Pronase	5.03×10^3	287	1.34×10^4	283	0.490	78.0

Table II. Effect of trypsin on SLS production in growing culture of streptococci. Hemolytic streptococci were grown at 37 °C for 5 h, in a peptone meat infusion broth containing 1% yeast RNA, in the presence or absence of trypsin (1 mg/ml). After measuring the turbidity (OD_{660}), each culture was centrifuged and SLS titer in the supernatant was detemined.

Strain	Addition	Growth (OD_{660})	SLS yield		
			HU/ml	HU/OD_{660}	[%]
Sa	none	0.203	6.23×10^{1}	3.07×10^{2}	100
	trypsin	0.210	4.12×10^{2}	1.96×10^{3}	637
Sv	none	0.165	5.50×10^{1}	3.30×10^{2}	100
	trypsin	0.167	4.71×10^{2}	2.79×10^{3}	837

Table III. Effect of antibiotics on SLS production in resting streptococci pretreated with pronase. Washed streptococci suspended in BBM were incubated with 2 mg/ml of pronase, at 37 °C for 1 h. The pronase-treated cells were washed four times with 0.15 M saline, suspended in BBM containing carrier AF and the indicated amount of each drug, and then incubated at 37 °C for 1 h.

Antibiotics	Concentration	SLS formed	
	μg/ml	HU/ml	[%]
None		7.35×10^{3}	100
Rifampicin	1	2.59×10^{3}	35.2
Chloramphenicol	100	6.12×10^{2}	8.33
Tetracycline	100	4.80×10^{1}	0.65

nificantly reduced by rifampicin and severely inhibited by chloramphenicol or tetracycline. These results demonstrate that the hemolysin is synthesized *de novo* after the protease treatment. Although SLS synthesis in growing streptococci is extremely susceptible to rifampicin, production of the toxin in the resting cells is refractory to the antibiotic [9]. In the pronase-treated resting cocci, moderate sensitivity to rifampicin is suggestive of increased cellular permeability to this drug. After the enzyme treatment, the amount of the ultraviolet-light absorbing material released from the resting cells was increased by 20–40%.

Digestion of streptococcal cells with certain proteases is known to result in partial liberation of surface lipoteichoic acid [10], an amphiphilic molecule having the carrier activity for SLS [11]. It seems not unlikely that endogenous lipoteichoic acid with high affinity for SLS tends to retain the toxin peptide on the coccal surface, and the increased yield of CBH and SLS after the protease treatment might be due to removal of the interfering lipoteichoic acid. In order to clarify this point, washed streptococci were extensively digested with pronase in 50 mM Tris · HCl (pH 8.0) containing 10 mM $CaCl_2$, centrifuged, and the supernatant was extracted with phenol, followed by dialysis against deionized water. When added into the resting cell system, the dialysate exhibited neither the carrier activity for SLS nor inhibitory effect on the AF-dependent production of the toxin. In addition, hemolytic activity of AF-SLS was not affected by the dialyzed phenol extract (data not shown).

In order to test possible degradation of AF-SLS by streptococci, a small amount of the toxin was mixed in BBM with the washed cells preincubated with or without pronase. After an incubation in the untreated coccal suspension, the SLS titer decreased by 34%, whereas the titer increased about 3.5-fold in the pronase-treated cell suspension. In the presence of 100 μg/ml of tetracycline, however, SLS inactivation in the resting cell system was not so efficiently prevented by the pretreatment with pronase. These results indicate requirement of protein synthesis for manifestation of the pronase effect. Hemolytic activity of AF-SLS gradually declined during incubation in the spent BBM obtained from the resting streptococcal suspension. This decline proceeded similarly in the spent medium in which the pronase-treated and extensively washed streptococci had been incubated. It seems thus unlikely that the increased SLS yield in the pronase- or trypsin-treated cells is a consequence of protection of the excreted toxin from streptococcal protease(s).

Discussion

The yield of the cell-bound and extracellular SLS significantly increased in hemolytic streptococci predigested with pronase or trypsin. Although SLS is synthesized *de novo* in the protease-treated cells, it seems unlikely that protein synthesis per se is promoted by the treatment. This increase might rather be due to facilitation of release of SLS peptide by partial removal of certain surface components such as M protein and/or lipoteichoic acid. After treating with pronase, resting streptococci became partially susceptible to rifampicin, and during subsequent incubation in BBM, leaked out a

slightly increased amount of the ultraviolet-light absorbing substance. These results suggest that the cellular permeability is somewhat raised by the protease treatment. In addition, the possibility that the putative affinity of SLS peptide to the streptococcal envelope is reduced by removal of lipoteichoic acid can not necessarily be excluded as yet. The other possibility is inactivation of certain protease (such as streptococcal proteinase) directly by degradation of the enzyme molecule or indirectly by eliminating its activator. It must be noted that the streptococcal proteinase zymogen constitutes over 95% of the extracellular proteins accumulated in the culture fluid of group A streptococci [12], and that the zymogen can be activated by the sulfhydryl groups of streptococcal cell-envelope cysteine residues [13]. The possibility that degradation of the extracellular streptococal proteinase by trypsin or pronase resulted in the increased SLS yield seems remote, as indicated by the absence of differential inactivation of the exogenously added toxin in the untreated or pronase-treated suspension or their spent media. It is, however, not improbable that nascent SLS peptide is occasionally degraded, in the intact cocci, by a cell-bound form of streptococcal protease during cotranslational export. Discrimination among the possibilities mentioned above (*i.e.* increased permeability, removal of lipoteichoic acid, and proteinase inactivation) is difficult at present stage and, in addition, these are mutually not exclusive.

In contrast to SLS, the yield of the extracellular nuclease was increased by digestion of the cocci with chymotrypsin but rather reduced after pronase treatment. The chymotrypsin-dependent increase in the nuclease yield may also be due to removal of some obstructive protein (inhibitor or protease) from the coccal envelope. The obstacle, however, must be different from the protein which interferes with SLS excretion. Present data also support the notion that different mechanisms of excretion are involved between SLS and the nuclease [14].

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