Effects of Norfloxacin and Rifampicin on Growth and Streptolysin S Production in Hemolytic Streptococci

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Norfloxacin, a nalidixic acid analogue, inhibited streptolysin S (SLS) production when added to young streptococcal culture. DNA synthesis was mainly affected, but increment of cell mass, RNA and protein was also significantly reduced in streptococci treated with norfloxacin. In stationary phase cells and in the washed resting bacteria, the toxin production was resistant to the drug. Pretreatment with norfloxacin did not abolish the cellular capacity to produce SLS. Although extracellular SLS was detectable at log phase of streptococcal growth, enhanced production of the toxin occurred upon cessation of coccal multiplication. In contrast to norfloxacin, lower concentration of rifampicin inhibited SLS production, even added at late log or early stationary phase. Roles of growth phase, medium and carrier in induction of SLS production were analyzed as well.

Introduction

Streptococcal growth and SLS production are not affected by nalidixic acid [1], an inhibitor of DNA gyrase. The toxin production, which requires certain carrier substance such as RNA [2] or detergent [3], is insensitive to novobiocin as well [1], and possible role of DNA gyrase in SLS messenger RNA synthesis remains to be elucidated. Norfloxacin (AM-715), an analogue of nalidixic acid recently synthesized in the laboratory of Kyorin Pharmaceutical Co. Ltd., inhibits growth of various bacteria including *Streptococcus pyogenes* [4]. Although this compound (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) rather preferentially inhibits DNA synthesis in *E. coli* [5], its effect has not been tested on macromolecular synthesis in Gram-positive bacteria.

RNA (or oligonucleotide)-dependent production of SLS is accelerated when growth of the cells entered stationary phase. Whether this enhanced production is due to increased messenger RNA synthesis or not is entirely unknown. Moreover, experimental proof for or against induction of SLS messenger synthesis by the carrier is deficient, although RNA or oligonucleotide added as the carrier for SLS is often designated as "inducer".

In order to address these questions, effects of inhibitors of nucleic acid synthesis have been tested, and influences of culture conditions such as carrier supplementation and medium shift have been examined, on macromolecular synthesis or SLS-producing capacity in hemolytic streptococci. In this report, evidences are provided indicating that norfloxacin blocks DNA synthesis and is inhibitory for SLS production in growing streptococci, and that SLS producing capacity (or SLS messenger) increasingly accumulates as the growth enters stationary phase, irrespective of presence or absence of the carrier substance.

Materials and Methods

Chemicals

Norfloxacin was generously provided by Kyorin Pharmaceutical Co. Ltd., Tokyo. Rifampicin was obtained from Daiichi Pharmaceutical Co., Tokyo, yeast RNA from Kohjin Co., Tokyo, RNase I core of yeast RNA from Sigma Chemical Co., St. Louis, and polyG was from Boehringer, Mannheim.

Strain, media and culture technique

Strain Sa, an avirulent mutant of hemolytic strep-
tococci, was grown aerobically, but without shaking,
in a peptone-meat infusion broth at 37 °C. Growth of
the bacteria was monitored by measuring turbidity at
660 nm, with a Bausch & Lomb Spectronic 20 A
spectrophotometer. Spent medium was prepared
from an early stationary culture by centrifugation
followed by filtration through a Millipore filter.
For suspending washed resting streptococci, Bern-
heimer's basal medium [6] was used. When SLS pro-
duction was to be followed, yeast RNA (1%) or the
RNase core (0.5 mg/ml) was supplemented. At inter-
vals, aliquot was withdrawn, rapidly filtered through
a Millex-HA filter (Millipore Corp., Bedford) and
hemolytic activity of the filtrate was determined as
described previously [7, 8].

Determination of nucleic acids and protein

At the specified time, aliquot of streptococcal cul-
ture was removed into ice-cold 0.15 M saline, spun
down and the cells were further washed once with
the saline to remove residual medium which might
interfere with subsequent chemical determination.
After removing acid-soluble fraction, DNA, RNA
and protein were determined as described elsewhere
[9].

Results

*Effect of norfloxacin on streptococcal macromolecu-
lar synthesis*

In order to know target metabolism of norfloxacin
in Gram-positive bacteria, exponentially growing
streptococci were incubated with varying concentra-
tion of the drug for 60 min and increments in cell
mass (as measured by turbidity), DNA, RNA and
protein were compared. As presented in Table I,
streptococcal DNA synthesis was considerably in-
hibited by 5 μg/ml of norfloxacin. Synthesis of bulk
RNA was not particularly affected, but relative
amount of cell mass and protein was somewhat de-
creased under the condition. At 10 to 20 μg/ml, in-
hibition of DNA synthesis was more than 90% and
increment of cell mass, RNA and protein was also
reduced significantly. Time course of macromolecu-
lar synthesis was next followed in streptococcal cul-
tures, in the presence and absence of 15 μg/ml of
norfloxacin. Although the effect was pronounced on

Table I. Effect of norfloxacin on macromolecular synthesis
in streptococci. Cells of streptococcal strain Sa were grown
in peptone-meat infusion broth at 37 °C and when the opti-
cal density at 660 nm reached 0.10, the culture was distrib-
uted into tubes containing varying amount of norfloxacin.
Each tube was incubated for 60 min at 37 °C and amount of
cell mass, nucleic acids and protein was determined respec-
tively.

Norfloxacin [μg/ml]	Relative increment [%]			
	Cell mass	DNA	RNA	Protein
0	100	100	100	100
5	72.5	22.1	92.9	74.8
10	64.7	7.2	85.1	57.7
15	56.7	3.0	64.8	46.9
20	56.7	2.8	64.6	46.9

DNA synthesis, the drug evidently decreased rate
and extent of synthesis of protein and, to a lesser
degree, of RNA (data not shown).

Effect of norfloxacin on SLS production

SLS yield in streptococcal culture was markedly
reduced by 8 to 10 μg/ml of norfloxacin, which still
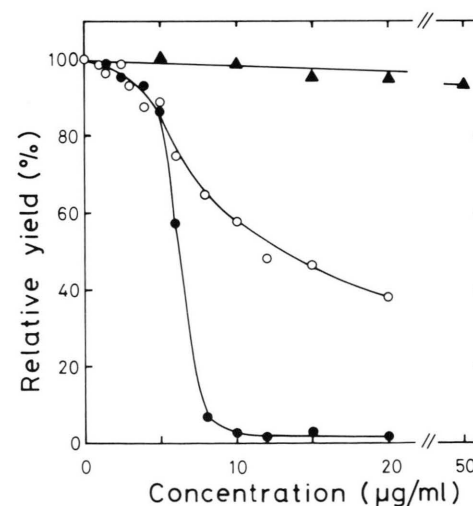allowed substantial growth of the bacteria (Fig. 1).

Fig. 1. Selective inhibition of SLS production by norfloxa-
cin in growing cells. Streptococcal cells were incubated at
37 °C in peptone-meat infusion broth or in BBM, each con-
taining indicated amount of norfloxacin and 0.5 mg/ml of
RNase core. In the broth culture, titer of SLS (●) and
extent of the growth (○) were determined after 15 h incu-
bation, whereas the toxin produced into BBM was titrated
after 60 min incubation of the resting coccal suspension
(▲).

On the other hand, production of the toxin by the resting cells suspended in BBM was practically insensitive to the drug. In streptococcal culture, degree of inhibition of SLS production varied depending on growth phase: when added at early log phase, norfloxacin reduced the toxin yield, whereas addition of the drug at late log phase was ineffective (Fig. 2A−C). Pretreatment of the early log phase culture with 20 µg/ml of norfloxacin for 30 min did not affect initiation and rate of SLS production in the subsequent culture. Prolonged pretreatment of the log phase culture caused growth inhibition, but allowed occurrence of the toxin synthesis after a considerable lag. Streptococci pretreated in BBM with 20 µg/ml of norfloxacin for 60 min grew normally and sufficiently produced SLS, after transfer into fresh culture medium containing RNase core.

Sensitivity of SLS synthesis to rifampicin

Insensitivity, to norfloxacin, of SLS production at the late stage of growth might indicate completion of SLS messenger synthesis, a possible target reaction, before entering stationary phase. To clarify this issue, effect of rifampicin, a specific inhibitor of bacterial RNA polymerase, was investigated on SLS production in streptococcal culture. As seen in Fig. 2D, addition of 5 µg/ml of rifampicin at late log phase completely blocked streptococcal growth and SLS production. Moreover, rifampicin effectively inhibited SLS production, even after initiation of the toxin synthesis at stationary phase (Fig. 2E). These results demonstrate that the observed norfloxacin resistance of SLS production at late growth stage is not due to sufficient accumulation of SLS messenger RNA.
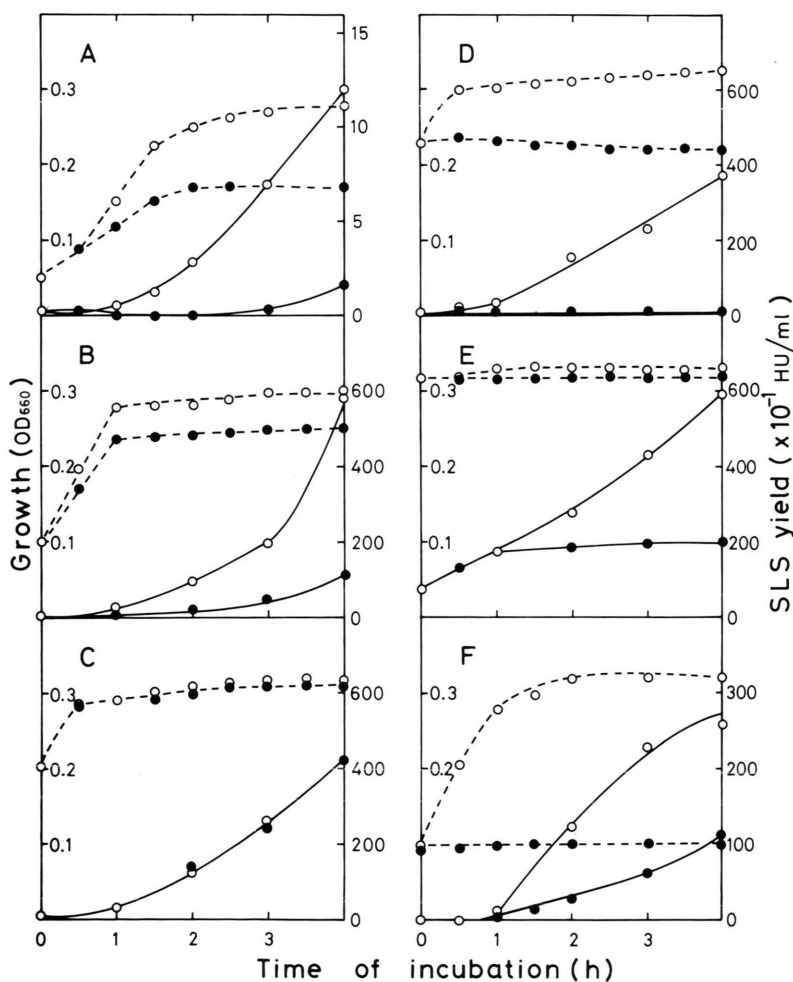

Fig. 2. Effects of norfloxacin, rifampicin and medium shift on streptococcal growth and SLS production. The bacteria were grown at 37 °C in peptone-meat infusion broth supplemented with yeast RNA (A) or the RNase core (B ~ E). Each culture was divided into two and, at the time indicated as zero, 15 µg/ml of norfloxacin (A ~ C) or 5 µg/ml of rifampicin (D, E) was added into one portion (●). The other portion was kept unadded as control (○). In the experiment shown in F, log phase culture in peptone-meat infusion broth was halved and one portion was spun down and suspended in a spent medium (●). RNase core was added to the suspension, as well as to the control (○), and each suspension was incubated at 37 °C. Cellular growth (− − − − −) and SLS yield (————) was followed as described in Materials and Methods. HU: hemolytic unit.

Relationship between growth phase and SLS production

Although detectable amount of SLS is synthesized even during log phase of growth, RNA- or oligonucleotide-dependent production of the toxin is manifested after cessation of the cellular growth, as exemplified in Fig. 2. The capacity to produce oligonucleotide-SLS in BBM was also higher in the cells harvested from stationary phase culture than in the cocci collected at mid-log phase (Table II). Moreover, when the stationary phase bacteria were transferred into fresh medium, the capacity significantly decreased again upon resumption of active growth. In addition, streptococci collected at log phase and suspended in spent medium produced, though at a reduced rate, a substantial amount of SLS, without increase in cell mass (Fig. 2F).

Table II. SLS-producing capacity of streptococci at different growth phases. Streptococcal cells (Sa) grown in meat-infusion broth were harvested at the indicated density (OD_{660}), washed and their capacity for SLS production was tested in BBM containing 0.5 mg/ml of RNase core.

Harvested at Phase of growth	OD_{660}	SLS formed HU/ml	HU/OD_{660} in BBM
Mid log	0.090	1.63×10^3	1.40×10^2
Early stationary	0.302	1.81×10^4	1.34×10^3
Stationary	0.308	2.66×10^4	1.97×10^3
Second mid-log[a]	0.450	1.47×10^4	7.59×10^2

[a] Stationary phase cells harvested at $OD_{660} = 0.308$ were transferred into fresh broth, regrown at 37 °C and collected at the mid log phase.

Development of SLS-producing capacity in the absence of the carrier

Streptococci produce only trace amount of SLS into the culture medium, unless specific carrier substance was added. The cells from the carrier-deficient culture still contain intracellular SLS [10], the precursor of the toxin, and excrete considerable amount of SLS, when washed and incubated in BBM supplemented with carrier oligonucleotides. Synthesis of bulk RNA as well as cellular growth does not occur in the washed cells and SLS production in the resting streptococcal system is insensitive to rifampicin [1]. These results indicate that SLS-producing capacity, possibly SLS messenger RNA, is endogenously formed without addition of the carrier. In order to

ascertain this view, streptococci were grown respectively in the presence and absence of carrier polyG (0.5 mg/ml), collected, washed and their capacity to produce polyG-SLS was compared in BBM. SLS titer in each filtrate was 2.2×10^3 HU/ml in the polyG-culture and less than 0.6 HU/ml in the control culture, whereas yield of the toxin in the resting streptococcal system was 6.7×10^2 HU/ml in the cells harvested from the polyG culture and 5.8×10^2 HU/ml in the control. Cellular growth was not particularly affected by supplementation of polyG. (When yeast RNA is added to culture medium, cellular growth is increased significantly and SLS-producing capacity of the resultant cells was 1.4- to 1.9-fold higher than that of the control. In this case, the RNA in the culture medium exerts its effect as a nutrient, rather than as a specific inducer.) In an additional experiment, cells grown in the presence and absence of carrier oligonucleotides were transferred into the carrier-supplemented broth and kinetics of SLS synthesis were followed. As to initiation and rate of the toxin production, no difference was found between the two second cultures.

Discussion

In *E. coli*, expression of several genes is, like DNA synthesis, sensitive to DNA gyrase inhibitor such as nalidixic acid [11]. Unavailability of gyrase inhibitor effective in Gram-positive pathogens has delayed elucidation of possible role of this enzyme in their DNA replication and gene expression. Present experiments demonstrate that streptococcal DNA synthesis and SLS production are inhibited by norfloxacin, a nalidixic acid analogue. At lower concentration, norfloxacin preferentially inhibits DNA replication (but the inhibition is incomplete), whereas synthesis of RNA as well as of protein is affected by higher concentration of the drug. Whether the decreased synthesis of RNA and protein is due to inhibition of DNA gyrase per se or to a secondary effect inherent in replication arrest is presently unknown. Inhibition of SLS synthesis is evident only when norfloxacin is added at early stage of streptococcal growth. Even at late log phase, however, addition of rifampicin or chloramphenicol suppresses the toxin production. Thus it seems less likely that the toxin synthesis is directly inhibited by this quinolone derivative. Recently, Shen and Pernet have reported

that norfloxacin inhibits gyrase action by binding to substrate DNA rather than to the enzyme molecule [12]. Norfloxacin pretreatment for 30 min at growing state or for 60 min at resting state did not affect subsequent time course of SLS production. Probably, binding of the drug to DNA is not so strong as to prevent transcription of SLS gene.

SLS-producing capacity of streptococci is enhanced after the cells cease to grow and the toxin synthesis is blocked by addition of rifampicin at early stationary phase. The accumulation of SLS-producing capacity takes place without addition of the carrier into the culture medium. Although its presence is essential for production of active SLS complex [3], the carrier seems to be devoid of inducer activity for transcription of SLS gene. Moreover, streptococcal envelope probably prevents penetration of such carrier as polyG into the cytoplasm where mRNA synthesis takes place. It seems quite reasonable that rate of SLS synthesis increases upon cessation of streptococcal growth by exhaustion of nutrients in the medium. In tissue infected with the cocci, this regulatory mechanism may assure controlled supply of the nutrients for the bacteria, through timely lysis of the host cells by the toxin. In nutritionally rich environment which permits active growth, superfluous production of the hostdamaging toxin must be disadvantageous for the parasitic cocci as well.

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