Role of Extracellular Iron in the Action of the Quinone Antibiotic Streptonigrin: Mechanisms of Killing and Resistance of Neisseria gonorrhoeae

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The quinone antibiotic streptonigrin is believed to kill bacteria by promoting formation of oxygen radicals. This antibiotic has also been used to select resistant bacterial mutants, some of which vary in iron utilization. We examined the effects of streptonigrin on Neisseria gonorrhoeae and several types of gonococcal mutants. Streptonigrin (0.025 μg/ml) efficiently killed gonococcal strain FA1090, and this effect depended on iron. Streptonigrin-resistant mutant FA6271 had normal iron uptake but was moderately deficient in total iron. Resistance most likely resulted from failure of FA6271 to divert electrons to streptonigrin, as demonstrated by a reduction in KCN-insensitive respiration (a hallmark of the action of quinones) and superoxide formation.

Other mutants selected for inability to use human iron-binding proteins (strains FA6273 and FA6275) had no increase in streptonigrin MIC and no decrease in KCN-insensitive respiration. Mutants did not demonstrate an increase in superoxide dismutase or catalase. Streptonigrin killing of gonococci depended on a reaction(s) in which extracellular iron was important, presumably because iron was required for catalysis of hydroxyl radical. The results suggest that a membrane component may be a target for the actions of streptonigrin.

Quinone antibiotics divert electron flow from the bacterial cytochrome chain, allowing formation of O₂⁻, which ultimately leads to bacterial death (14-17, 20). Streptonigrin is of special interest because it appears to bind iron so as to promote formation of hydroxyl radical from O₂⁻ and hydrogen peroxide (7, 10, 31-34). Hydroxyl radical is a far more potent oxidizing agent than superoxide or hydrogen peroxide. Streptonigrin has been used successfully to select mutants of Escherichia coli (3) and Neisseria meningitidis (unpublished data) defective in the ability to utilize iron bound to carrier proteins, and it has been assumed that the iron deficiency in these mutants accounted for their reduced susceptibility to this drug. It has also been possible to use streptonigrin to select iron transport mutants of N. gonorrhoeae (J. Adams and P. F. Sparling, manuscript in preparation). A particularly unusual feature of N. gonorrhoeae is its failure to form superoxide dismutase (SOD) (2, 26), an enzyme believed to be important to aerotolerant organisms (9). The present study was undertaken to determine whether streptonigrin could induce gonococcal SOD, to study in more detail the effects of streptonigrin on gonococcal metabolism, and to determine the mechanism(s) for streptonigrin resistance in this organism.

MATERIALS AND METHODS

Bacterial strains and media. N. gonorrhoeae FA1090 and FA19 are serum-resistant, penicillin-susceptible clinical vaginal isolates (23, 29). These bacteria were subcultured daily on GCB agar (GC medium base; Difco Laboratories, Detroit, Mich.) containing (unless otherwise noted) 1% (vol/vol) Kellogg defined supplement I and 0.5% supplement II [Fe(NO₃)₃] (18). Nonpiliated opaque colony variants were used (30). A chemically defined medium (CDM) was prepared as described by Catlin (6) and modified by the method of West (6, West, dissertation thesis, University of North Carolina, Chapel Hill, 1986). CDM contained less than 0.2 μM iron as determined by atomic absorption spectroscopy.

Reagents. A stock solution of 10 mM ferric citrate (Fisher Scientific, Fair Lawn, N.J.) was prepared in sterile demineralized water and stored at room temperature. A stock solution of 5 mM deferoxamine mesylate (Desferal; CIBA Pharmaceutical Co., Summit, N.J.) was stored at -20°C. Streptonigrin was the gift of Matthew Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. A stock solution of streptonigrin (100 μg/ml, 0.2 mM) was prepared in sterile 0.1 M Tris hydrochloride buffer (pH 7.8) and stored in the dark at 4°C. Stock solutions of erythromycin (Abbott Pharmaceutical Co., North Chicago, Ill.), rifampin (CIBA Pharmaceutical Co.), and ciprofloxacin (Miles Pharmaceutical Co., West Haven, Conn.) were dissolved in appropriate diluents (0.1 mg/ml), filter sterilized, and stored at -20°C. Human transferrin (Sigma Chemical Company, St. Louis, Mo.) was dissolved in 40 mM Tris-150 mM NaCl-10 mM NaHCO₃ buffer, pH 8.6. Catalase, superoxide dismutase, and manniitol were purchased from Sigma Chemical Co. Dimethyl sulfoxide (Me₂SO) was purchased from Fisher Scientific Co., Pittsburgh, Pa.

Selection of resistant mutants. Log-phase cultures of FA1090 were exposed to 0.15 to 0.6 μg of streptonigrin per ml for 24 h and diluted 1:2 with fresh GCB containing the appropriate concentration of streptonigrin. Portions (0.1 ml) from each flask were plated on GCB agar plates containing streptonigrin. Colonies appearing on streptonigrin (0.3 μg/ml) plates were passed and maintained on plates containing 0.6 μg of streptonigrin per ml.

Isolation of iron transport mutants. Since the action of streptonigrin depends on the availability of iron, we were interested in obtaining mutants for use in these and other studies. To isolate iron utilization mutants, a procedure...
TABLE 1. Antioxidant enzymes of N. gonorrhoeae: effects of streptonigrin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity* (U/mg of protein)</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>SOD</td>
<td>Catalase</td>
</tr>
<tr>
<td>Parents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA1090</td>
<td>0.30 (0.16–0.81)</td>
<td>2.611.2</td>
</tr>
<tr>
<td>FA109b</td>
<td>0.31 (0.16–0.62)</td>
<td>1.169.5</td>
</tr>
<tr>
<td>FA19</td>
<td>0.31 (0.16–0.62)</td>
<td>2.830.5</td>
</tr>
<tr>
<td>Mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA6271</td>
<td>0.27 (0.06–1.08)</td>
<td>2.396.5</td>
</tr>
<tr>
<td>FA6273</td>
<td>0.93 (0.20–1.66)</td>
<td>1.213.5</td>
</tr>
<tr>
<td>FA6275</td>
<td>0.65 (0.20–1.09)</td>
<td>1.374.0</td>
</tr>
</tbody>
</table>

* Values are the mean and range for three to six experiments.

- FA1090: Both lactoferrin and transferrin were used. Briefly, gonococcal strain FA19 (which utilizes both lactoferrin and transferrin for iron) was grown overnight in CDM supplemented with 50 µM ferric nitrate (CDM-50) and mutagenized during log-phase growth by a 30-min exposure to 1% ethyl methanesulfonate. Mutant FA6273 used lactoferrin (but not transferrin) as an iron source. Mutant FA6275 could not use transferrin or lactoferrin as an iron source.

- Oxygen consumption: O2 consumption was measured with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) by using 1 ml of Hanks balanced salt solution containing 10% gonococci. The chamber was equilibrated to 100% saturation at 37°C.

- Superoxide production: Superoxide (O2-) was quantified by SOD-inhibitable reduction of ferricytochrome c (22). Organisms were grown to log phase and then passed twice through a French pressure cell (16,000 lb/in2). The resulting material was centrifuged (microfuge B Beckman Instrument Co., Palo Alto, Calif.) for 3 min. The supernatant was harvested, and the protein content determined by the method of Lowry et al. (21). The rate of O2- formation was measured after the addition of streptonigrin (30 µg/ml) and NADH (3 mM).

- SOD: SOD was measured by inhibition of nitrite formation from hydroxylamine in the presence of O2- generated by the action of xanthine oxidase on xanthine (8).

- Catalase: Catalase was quantitated spectrophotometrically (Varian DMS80) by following the consumption of 5 mM H2O2 at 240 nm in 50 mM Na2HPO4 buffer, pH 7.0 (2).

- Determination of total bacterial iron: All work and reagent preparation were done in iron-free glassware (soaked overnight in 3 N HCl) or plastic. Bacteria suspended in GCB broth were grown to log phase. Two 7-ml samples of culture were taken from each flask, centrifuged at 900 x g for 10 min at 20°C, and washed four times with 10 ml of Hanks balanced salt solution containing 0.1 M trisodium citrate, pH 7.5. Sodium citrate was required to dissolve iron precipitates at neutral pH. Bacteria were then suspended in 7 ml of demineralized water, vortexed, and maintained for 24 h at 4°C to allow lysis. The iron content of the samples was determined by the ferrozine method (5).

- Assessment of iron uptake: Human transferrin was partially saturated by adding calculated amounts of Fe3+ or 55Fe3+ (New England Nuclear Corp., Boston, Mass.). The iron was added as a solution of ferric chloride in 10-fold molar excess sodium citrate. The protein was then dialyzed for 24 h against two changes of 10 mM Tris–150 mM NaCl–10 mM sodium bicarbonate, pH 7.4. Iron uptake studies were performed by a modification of a previously described procedure (23).

- Microbial killing: Bacteria were grown to log phase, washed three times, and diluted to 106 CFU/ml. Bacteria were exposed to appropriate experimental conditions at 37°C for 0 to 120 min, during which time duplicate or triplicate samples were serially diluted and plated on GCB agar. Organisms were allowed to grow for 48 h at 37°C and 5% CO2, and survival was calculated from the number of CFU. Some experiments were conducted under anaerobic conditions in a Coy anaerobic chamber.

**Statistics.** Significance (P < 0.05) was determined by Student’s t test.

### RESULTS

- Isolation of a streptonigrin-resistant mutant: Streptonigrin (0.6 µg/ml) killed more than 106 organisms of wild-type gonococcal strain FA1090 from a starting inoculum of 105 organisms over 120 min. The growth of a resistant mutant strain FA6271, selected by exposure to increasing concentrations of streptonigrin, was not inhibited. The MICs of streptonigrin for the bacteria used in these studies are presented in Table 1. Neither this mutant nor four others (selected in an identical fashion) showed any increase in stress resistance to rifampin, erythromycin, or the quinolone ciprofloxacin, suggesting that general envelope permeability changes were unlikely to be the cause of streptonigrin resistance. FA6271 demonstrated no decrease in streptonigrin resistance after three passages on streptonigrin-free medium.

- Formation of SOD and catalase: Induction of antioxidant enzymes could reduce the sensitivity of gonococci to streptonigrin (12–16). Only small concentrations of SOD were detected in gonococcal strains FA19, FA1090, and FA6271 and mutants unable to use iron from human sources (FA6273 and FA6275, described below) (Table 1). FA1090 was grown with a concentration of streptonigrin slightly below the MIC (14). This stress did not result in an increase in SOD (Table 1). The catalase activity of all gonococcal strains examined was similar, and in some isolates was reduced by exposure to streptonigrin.

- Oxidative metabolism of streptonigrin-resistant mutants: Exposure of FA1090 to streptonigrin resulted in a slight decrease in total O2 consumption, but O2 consumption became insensitive to KCN, consistent with O2- formation (Fig. 1). Streptonigrin-resistant mutant FA6271 demonstrated resting O2 consumption rates equivalent to those of FA1090 (the parent strain); however, FA6271 expressed only 61% of the KCN-insensitive respiration of FA1090 (P < 0.01) in the presence of streptonigrin (Fig. 1). Similar results were observed with other highly streptonigrin-resistant mutants examined (data not shown).

- Formation of superoxide by gonococci: We measured O2- production by gonococci exposed to streptonigrin by ferricytochrome c reduction. With intact organisms or membrane preparations, we failed to detect O2- (data not shown). However, when membrane preparations were exposed to NADH, formation of O2- was observed (Fig. 2). The maximal rate of production of O2- by strain FA6271 was 60% of that by FA1090 (2.13 versus 5.45 µmol/mg of protein per min), as would be predicted from our studies of O2 metabolism.

- Iron metabolism: Another mechanism by which gonococci could be protected from streptonigrin is limitation of iron, required to catalyze hydroxyl radical from O2- (10, 11,
VOL. 31, FA6271. Streptonigrin (SNG) These organisms for 31-34). The iron uptake by FA6271 was equivalent to that by FA1090 regardless of the iron source used (Fig. 3). The total iron for strains FA6271 and FA1090 was 81.0 ± 6.7 and 44.3 ± 5.1 μmol of Fe per mg of protein, respectively (P > 0.05).

We also examined the metabolism of strains FA6273 (which cannot use iron bound to transferrin) and FA6275 (which cannot use iron from either lactoferrin or transferrin). These organisms had no increase in resistance to streptonigrin, developed the expected response (i.e., KCN-insensitive respiration) with exposure to streptonigrin, and demonstrated no increase in SOD or catalase (Table 1).

**Relationship of iron to the effects of streptonigrin.** We explored the effects of iron deprivation on killing of gonococci by streptonigrin. FA1090 was killed extensively by streptonigrin, whereas mutant FA6271 survived (Fig. 4). Growth of FA1090 in the presence of the iron chelator deferoxamine (Fig. 4) significantly reduced the killing of bacteria by streptonigrin, whereas addition of excess iron enhanced killing. Streptonigrin-resistant strain FA6271 was not killed even in the presence of excess iron. We initially assumed that the protection observed could be attributed to limitation of intracellular bacterial iron achieved by their growth in the presence of excess deferoxamine. However, the same magnitude of protection was noted when deferoxamine was added at the time of exposure of the organism to streptonigrin (data not shown).

To determine whether iron was required for bacterial utilization of streptonigrin, we measured streptonigrin-induced KCN-insensitive respiration of FA1090 in the presence and absence of deferoxamine; deferoxamine did not inhibit KCN-insensitive respiration by these bacteria (data not shown). To exclude a direct effect of deferoxamine, FA1090 was grown in iron-replete conditions (25 μM ferric citrate) and transferred to an iron-free medium (CDM). Iron-replete bacteria were killed better than iron-starved organisms (Fig. 5). Iron-replete organisms suspended in iron-free medium were protected somewhat from streptonigrin, but this effect could be offset by adding iron (Fig. 5).

**Effects of free-radical scavengers.** To demonstrate that the effects of streptonigrin depended on the formation of free radicals, gonococci were exposed to streptonigrin in an anaerobic environment, and virtually no killing was observed (Table 2). We also examined the protection offered by exogenous (extracellular) SOD (to scavenge $O_2^-$), catalase (to eliminate $H_2O_2$), and $Me_2SO$ (to scavenge hydroxyl radicals).

**FIG. 1.** $O_2$ consumption by gonococcal strains FA1090 and FA6271. Streptonigrin (SNG) was used at a concentration of 0.6 μg/ml.

**FIG. 2.** Formation of $O_2^-$ by *N. gonorrhoeae* by SOD-inhibitable reduction of ferricytochrome c at 37°C, OD₅₅₀. The streptonigrin (SNG) concentration was 30 μg/ml.

**FIG. 3.** Iron uptake by FA6271 and FA1090 (parent) (n = 5, mean ± standard deviation). pM, Picomoles.
FIG. 4. Microbicidal activity of streptonigrin (0.6 \( \mu \)g/ml) assessed in strain FA1090 (---) and resistant strain FA6271 (---). Organisms were grown in GCB broth without supplement II to log phase and incubated at time zero with streptonigrin (SNG), deferoxamine (150 \( \mu \)M), or 175 to 200 \( \mu \)M iron. Results are the mean and standard error of the mean for three separate determinations, each in duplicate.

Each scavenger afforded the same protection but far less than observed with deferoxamine (Table 2, Fig. 6).

**DISCUSSION**

In this study we evaluated the effects of streptonigrin on the O\(_2\) metabolism of *N. gonorrhoeae* and the mechanisms by which streptonigrin kills this organism. We found that gonococcal isolate FA1090 responded to streptonigrin with an increase in KCN-insensitive respiration, as has been reported for *E. coli* (15). Under physiological conditions, O\(_2\) consumption by gonococci and many other bacterial species results from an orderly flow of electrons through a KCN-sensitive cytochrome system (25). Diversion of electrons away from this cytochrome system appears to be critical for subsequent formation of O\(_2^-\) by bacteria exposed to quinone and quinonelike antibiotics (12-17, 20).

We isolated several mutants of *N. gonorrhoeae* to explore the effects of streptonigrin and mechanisms of resistance to this agent. Among gonococci highly resistant to streptonigrin, KCN-insensitive respiration was significantly reduced during exposure to this quinone, suggesting that these organisms were not forming superoxide. Extracellular O\(_2^-\) production by bacteria exposed to an electron donor in the presence of paraquat (15) or plumbagin (2) has been reported. Although neither intact gonococci or membrane preparations allowed detection of O\(_2^-\) in the extracellular fluid, membrane preparations incubated with NADH and streptonigrin formed this radical. Higher concentrations of O\(_2^-\) were formed by FA1090 than by FA6271. Our inability to detect O\(_2^-\) in whole organisms (in contrast to previous studies [2, 15]) may relate to differences in the methods (15) and conditions of incubation (2, 15). However, we have no firm evidence that reduced streptonigrin escapes from the intact organism to the extracellular fluid, as has been suggested as part of the action of paraquat (15). These results suggest that at least one mechanism contributing to resistance was a decrease in the diversion of electrons to streptonigrin. Kao and Hassan (17) recently reported a similar mechanism for *E. coli* resistant to paraquat.

Quinone antibiotics induce manganese SOD and catalase formation in *E. coli* (12-16). Gonococcal strain FA1090 contained negligible concentrations of SOD, as has been reported (2, 26). SOD activity was not increased in streptonigrin-resistant mutant strains grown on plates in the presence of 0.6 \( \mu \)g of this quinone per ml. Gonococcal catalase concentrations were substantially higher than reported for at least one strain of *E. coli* (15) but consistent with that found in previous work with gonococci (2, 26). The catalase concentrations of gonococcal strains FA1090, FA19, and FA6271 were equivalent. FA6273 and FA6275 had somewhat (but not significantly) less catalase. Since streptonigrin-resistant mutants were never exposed to increased concentrations of free radicals (because of their failure to reduce the quinone), stress experiments were conducted by growing susceptible organisms (e.g., FA1090) in the presence of sublethal concentrations of streptonigrin (14). This procedure did not result in an increase in SOD, and less catalase was expressed. Archibald and Duong were unable to stress *N. gonorrhoeae* to production of SOD with the quinone
EFFECT OF STREPTONIGRIN ON N. GONORRHOEAE

Growth -CDM[+Fe Growth.

\[ \text{Fe}^{3+} + \text{SNG} \]

FIG. 5. FA1090 was grown to log phase in CDM in the presence (---) and absence (---) of iron (25 \(\mu\)M ferric citrate). Bacteria were washed, suspended in CDM, and exposed to streptonigrin (0.6 \(\mu\)g/ml) with or without ferric citrate (25 \(\mu\)M). Results are the mean and standard error of the mean for three separate determinations, each in duplicate.

TABLE 2. Effect of anaerobiosis and scavenger systems on the microbicidal activity of streptonigrin

<table>
<thead>
<tr>
<th>Addition (conc)</th>
<th>Gonococcal survival (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 (\times) 10^8-9.0 (\times) 10^8</td>
</tr>
<tr>
<td>Streptonigrin</td>
<td>2.2 (\times) 10^2-7.0 (\times) 10^2</td>
</tr>
<tr>
<td>Streptonigrin (anaerobic)</td>
<td>2.0 (\times) 10^6</td>
</tr>
<tr>
<td>Streptonigrin + deferoxamine (200 (\mu)M)</td>
<td>2.6 (\times) 10^2-5.2 (\times) 10^2</td>
</tr>
<tr>
<td>Streptonigrin + SOD (1 mg/ml)</td>
<td>1.1 (\times) 10^3</td>
</tr>
<tr>
<td>Streptonigrin + catalase (100 (\mu)g/ml)</td>
<td>1.1 (\times) 10^3</td>
</tr>
<tr>
<td>Streptonigrin + SOD + catalase</td>
<td>2.0 (\times) 10^3</td>
</tr>
<tr>
<td>Streptonigrin + Me2SO (0.14 M)</td>
<td>1.2 (\times) 10^3-6.2 (\times) 10^3</td>
</tr>
</tbody>
</table>

* Results represent ranges for N. gonorrhoeae FA1090 in one to three experiments (performed in triplicate) following gonococcal incubation (120 min) in the presence of streptonigrin (0.6 \(\mu\)g/ml) and the other compounds noted. Incubations were performed under aerobic conditions except where indicated. In the absence of streptonigrin, there was no change in the number of CFU observed during the period of incubation.

\[ ^{b} P < 0.01 \text{ versus the streptonigrin-treated control.} \]

plumbagin or hyperbaric oxygen (2). It is possible that gonococci do not have the gene required for SOD production or that the SOD present in gonococci cannot be induced by the conditions explored thus far. With respect to catalase, it seems likely that gonococci constitutively produce maximal levels of this enzyme. The reduction in catalase observed among organisms grown in the presence of streptonigrin could result from \(O_2^-\)-induced damage to this enzyme.

When \(O_2^-\) and hydrogen peroxide are allowed to interact in the presence of a Haber-Weiss catalyst such as iron, hydroxyl radical is formed (11). Streptonigrin catalyzes hydroxyl radical formation in a cell-free system with NADH as an electron donor (19, 20). White and co-workers convincingly demonstrated that iron is required for the action of streptonigrin (31–34); E. coli iron transport mutants are

FIG. 6. Model for the effects of streptonigrin on bacteria. SNG, Streptonigrin; SNGH, reduced streptonigrin.
frequently resistant to the action of this antibiotic (33). Streptonigrin has also been used to select iron utilization mutants among several bacterial species (3), including pathogenic neisseriae (unpublished data). Streptonigrin (in the presence of iron) can cause nucleic acid damage (7, 10, 19), and it has been suggested that hydroxyl radical-mediated damage accounts for the bactericidal action of this antibiotic (31-34).

Our experiments demonstrated that gonococci can be protected from streptonigrin by iron deprivation. Streptonigrin-resistant mutant FA6271 demonstrated moderate reduction in total iron, which in combination with altered metabolism may explain its complete resistance to streptonigrin. However, protection of streptonigrin susceptible organisms was greatest when iron was removed from the medium. Furthermore, iron-rich bacteria added to an iron-deficient medium (even in the absence of deferoxamine) were killed poorly. These results suggest that the effects of deferoxamine do not simply relate to the ability of this compound to enter the cell and make intracellular iron unavailable. These observations are consistent with at least one set of experiments conducted by White and co-workers with E. coli (32). Since iron is believed to bind to streptonigrin (7, 31), it seems possible that iron is required in the medium for the passage of this antibiotic across the bacterial membrane. However, the ability of streptonigrin to stimulate KCN-insensitive respiration in the presence of deferoxamine disproves this hypothesis. Free-radical scavengers offered slight but insignificant protection from streptonigrin. This may reflect their inability to achieve access to critical sites of damage. Furthermore, the ability of quinone antibiotics to re-cycle (20) may allow generation of free radicals in concentrations which greatly exceed feasible application of scavengers.

Intracellular nucleic acid damage (10, 19) mediated by quinone antibiotics has been stressed. Our results, however, suggest that oxygen radicals generated by streptonigrin may have several sites of action. Figure 6 provides a scheme for the actions of streptonigrin designed to explain the existing data. An extracellular pool of iron enhances the action of streptonigrin beyond intracellular iron accumulated in organisms grown in iron-rich medium. Also, streptonigrin selects iron transport mutants (3; unpublished data). One unifying hypothesis is that membrane proteins involved in iron transport, chelation, or storage (23-25) act as a site for catalysis of hydroxyl radical. Hydroxyl radicals formed at these sites could cause a lethal increase in membrane permeability (1) or damage to iron-rich enzyme complexes (27). In the absence of extracellular iron, these sites may not be subjected to oxidation.

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LITERATURE CITED


