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THE ROLE OF HYDROXYL RADICAL IN CHROMOSOMAL AND PLASMID DAMAGE IN *NEISSERIA GONORRHOEAE IN VIVO*

DANIEL J. HASSETT[1], KAREN BEAN[1], GOUR BISWAS[1], and MYRON S. COHEN*[1,2]

Departments of Microbiology and Immunology[1] and Medicine[2], University of North Carolina at Chapel Hill, Chapel Hill, NC 27514

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Viable *Neisseria gonorrhoeae* exposed to streptonigrin generate intracellular hydroxyl radical detected by spin-trapping with 5,5-dimethyl-1-pyrroline-N-oxide; gonococci exposed to paraquat generate primarily superoxide (*J. Biol. Chem.*, **262**: 13404-143048, 1987). The use of streptonigrin and paraquat provide a model with which to examine the action and site(s) of hydroxyl radical-mediated damage. *N. gonnorrhoeae* exposed to streptonigrin, but not paraquat, developed extensive chromosomal, plasmid, and RNA damage. Addition of excess Fe^{+3} to the reaction mixture enhanced intracellular hydroxyl radical formation by paraquat, detectable as DNA damage. Desferal and dimethyl sulfoxide allowed approximately 25% of protection of plasmid DNA damage as judged by linear scanning densitometry. These results demonstrate DNA and RNA damage in viable organisms exposed to intracellular redox stress and confirm the critical role of hydroxyl radical in this process.

KEY WORDS: Hydroxyl radical, *in vivo* DNA damage.

INTRODUCTION

Redox-active antibiotics are able to divert cellular electron flow, leading to the formation of oxygen-centred radicals.[1-5] Using concentrations of streptonigrin which allowed *N. gonnorrhoeae* to generate comparable levels of reactive oxygen intermediates,[4] we have shown that streptonigrin demonstrates remarkable ability to kill gonococci relative to paraquat (PQ^{+2}). The antimicrobial activity of streptonigrin could be attributed to the formation of hydroxyl radical ($HO^{\cdot}$).[1,4] Streptonigrin-mediated killing of bacteria has been attributed to both extracellular and intracellular events,[1,4] with emphasis on the likelihood of DNA damage.[6,7] Oxygen radicals can result in DNA strand scission[8-13] and inhibition of the transforming ability of bacterial DNA.[14] However, nearly all studies have been conducted *in vitro* with isolated DNA. In this work, we have examined the effects of oxygen radicals generated *in vivo* by viable *N. gonorrhoeae* responding to streptonigrin or PQ^{+2}. *N. gonorrhoeae* was chosen because its vigorous aerobic metabolism[15] allows formation of superoxide in

Address All Correspondence To: Myron S. Cohen, M.D., Division of Infectious Disease, CB # 7030, 547 Burnett-Womack 229H, University of North Carolina, Chapel Hill, NC 27514.

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Abbreviations used: PQ^{+2} (paraquat) 1,1'-dimethyl-4,4'-bipyridinium dichloride; HBSS, Hank's balanced salt solution; GCB, gonococcal culture broth.

response to redox-active antibiotics; the organism possesses little or no superoxide dismutase,[1,16,17] the presence of which might confuse interpretation.

MATERIALS AND METHODS

Streptonigrin was a gift of Dr. Matthew Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute and was prepared fresh prior to each experiment. All reagents used were of the finest grade available. *Neisseria gonorrhoeae* strain FA 1090[18] contains only a 4.2 Kb "cryptic" plasmid.[19] Growth and maintenance of this organism were as previously described.[4]

Late log phase gonococci were pelleted by centrifugation and resuspended in a 5.0 ml reaction mixture containing various combinations of *N. gonorrhoeae* (approximately 5×10^9 cells), Kellogg's defined supplement one[20] (2%), 5 mM sodium bicarbonate, ferric citrate (Fe^{+3}, 10^{-4} M), diethylaminetriaminepentaacetic acid (DETAPAC, 10^{-4} M), desferrioxamine mesylate (desferal, 200 μM), dimethyl sulfoxide (DMSO, 0.14 M), PQ^{+2} (90 mM), streptonigrin (64 μM), gonococcal culture broth (GCB)[14], and Hanks' balanced salts solution (HBSS), pH 7.4. Aerobic exposure to streptonigrin and PQ^{+2} was performed at 37°C for 60 minutes. The reaction was terminated by washing the cells twice with 30 mM Tris-10 mM EDTA-50 mM NaCl, pH 7.4. Gonococcal plasmid and chromosomal DNA, and cellular RNA, (in Figure 1) were isolated[21] from each preparation and equivalent amounts were electrophoresed on 0.8% (w/w) agarose gels as previously described.[22] Plasmid damage was observed as a significant conversion from covalently closed circles to the open circular form. This was measured by linear scanning densitometry (LKB 222 UltroScan XL, Bromma, Sweden equipped with background adjustment program), a technique able to descriminate small differences in plasmid type and increase precision of interpretation. Chromosomal damage was observed as significant conversion to lower molecular mass fragments, exhibited as a smear[12] in the gels. RNA damage was observed as the complete destruction of gonococcal 23 and 16 S ribosomal RNA subunits.

RESULTS AND DISCUSSION

Exposure of cells to streptonigrin led to plasmid and chromosomal damage as well as complete destruction of 23 and 16 S ribosomal RNA subunits (Figure 1a, lanes 2 vs. 3). By comparison, PQ^{+2} had minimal effect on chromosomal or plasmid DNA or RNA (Figure 1, lane 6). Linear scanning densitometry of agarose gel photographic negatives confirmed the magnitude of streptonigrin plasmid DNA damage relative to PQ^{+2} (Figure 1b). Addition of *exogenous* Fe^{+3} did not increase streptonigrin-mediated plasmid damage but enhanced the destruction of chromosomal DNA (Figure 1, lane 4) and PQ^{+2}-mediated plasmid damage to a level which was still less than half that observed with streptonigrin (Figure 1, lane 7). The enhancement of chromosomal damage in streptonigrin-treated cells with excess Fe^{+3} may account for the decrease in viability demonstrated in our earlier work.[1] We have previously shown that excess Fe^{+3} allows PQ^{+2} to generate some intracellular $HO^{\cdot}$.[4] The addition of DETAPAC only slightly increased streptonigrin-mediated plasmid damage but markedly enhanced chromosomal damage (Figure 1, lane 5). PQ^{+2}-mediated plasmid damage in the presence of DETAPAC was diminished three-fold (Figure 1b, lane 7

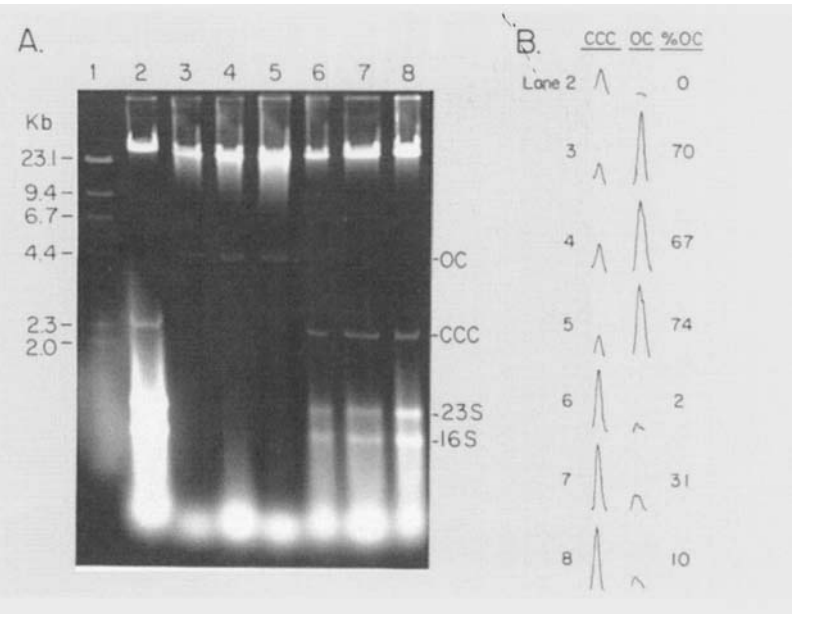

FIGURE 1A Effect of streptonigrin and PQ^{+2} on the integrity of chromosomal and plasmid DNA and RNA isolated from intact *N. gonorrhoeae*. Bacteria were grown in GCB at 37°C as in METHODS until late-log phase, pelleted, and resuspended in the following medium in the order in which they appear: lane 1; 250 ng biotinylated lamda Hind III DNA standards, lane 2; approximately 5 ug of gonococcal DNA (chromosomal and plasmid) and RNA, lane 3; + streptonigrin (64 μM), lane 4; streptonigrin + Fe^{+3} (0.1 mM), lane 5; streptonigrin + Fe^{+3} + DETAPAC (0.1 mM), lane 6; as in lane 2 except PQ^{+2} (90 mM) replaced streptonigrin, lane 7; PQ^{+2} + Fe^{+3} + DETAPAC. The data presented are typical of at least 5 additional experiments.

FIGURE 1B Densitometric scanning tracings of the open and covalently closed circular plasmid portions of agarose gel shown in Fig. 1A. The ratio of open(OC)/closed(CCC) is an indicator of the extent of damage evoked by streptonigrin or paraquat to the 4.2 Kb plasmid of *N. gonorrhoeae*. Percent OC was calculated (lanes 3–8) after subtracting the % OC in control experiments (no streptonigrin or PQ^{+2}, lane 2).

vs. 8). In this experiment it seems likely that DETAPAC slows[23] redox-cycling of Fe^{+3}, which is apparently of greater importance to PQ^{+2} than to streptonigrin mediated DNA damage. Desferal, an Fe^{+3} chelator which blocks $HO^{\cdot}$ formation, diminished streptonigrin-Fe^{+3} -mediated chromosomal and plasmid damage (Figure 2b, lane 3 vs. 4) more than DETAPAC. In previous works,[1,4] desferal diminshed the toxicity of streptonigrin, at least in part by depleting iron from the reaction medium,[1] thus limiting the binding of streptonigrin to iron[24] involved in catalysis of $HO^{\cdot}$. It is also possible that desferal decreases the intracellular iron pool by actually entering the cell, a phenomenon suggested to protect endothelial cells from neutrophil attack.[25] Dimethylsulfoxide (DMSO) traverses cell membranes and scavenges $HO^{\cdot}$ at all sites. Inclusion of DMSO in the system provided approximatly 50% protection of plasmid DNA similar to that observed with desferal (Figure 2, lane 5).

These results are consistent with earlier experiments suggesting that part of the killing observed in gonococci exposed to streptonigrin could be attributed to DNA

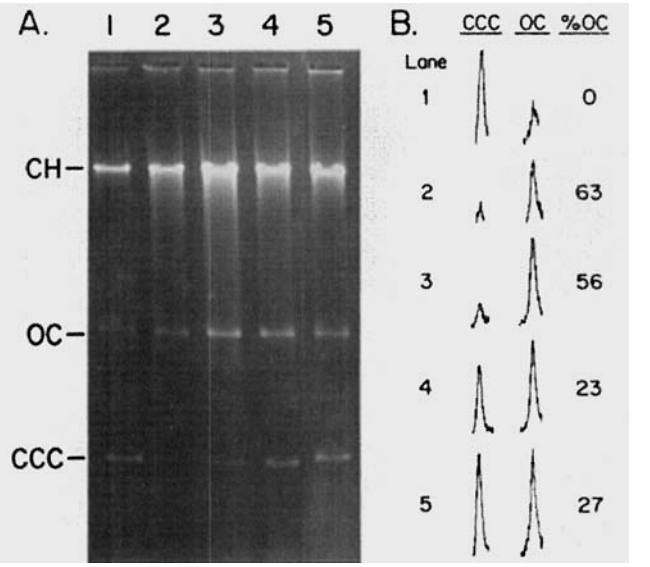

FIGURE 2A Effect of $HO^{\cdot}$, and Fe^{+3} scavengers on damage to chromosomal and plasmid DNA of *N. gonorrheae*. Bacteria were grown under the identical conditions outlined in Figure 1 except than 100 ug/ml RN-ase was added before final precipitation of DNA. Reaction mixtures consisted of the following: lane 1, GC + GCB + supplement I + 5 mM sodium bicarbonate as in Fig. 1a minus RNA; lane 2, lane 1 = streptonigrin (64 uM); lane 3, lane 2 + Fe^{3+} (0.1 mM); lane 4, lane 3 = desferal (200 uM), lane 5, lane + DMSO (0.14 M). After 1 hour of incubation under aerobic conditions, the cells were washed, and the DNA extracted as previously described.[21] The data are typical of 3 additional experiments.

FIGURE 2B Densitometric scanning tracings of the open (OC) and covalently closed circular (CCC) plasmid portions of agarose gel shown in Figure 2A. Ratios of OC/CCC are calculated as in Figure 1B.

damage.[4] The conditions which protected DNA allowed considerable protection against cell death.[1,4] The ability of streptonigrin to bind to DNA, and more specifically, to cytosine,[26] may also represent an important part of its action, and raises the possibility of site specific DNA damage. In addition, streptonigrin may act as a carrier for iron into the cell,[1] thereby further enhancing the liklihood of interaction with cellular DNA.[27] Identical plasmid degradation products were repetitively observed in streptonigrin-treated bacteria (Figure 1, lanes 3–5), supporting this hypothesis. Our results confirm DNA damage in viable organisms exposed to quinone and viologen antibiotics, as suggested by exposure of isolated DNA to oxygen-centered radicals.[8] Working with viable *E. coli*, Denq and Fridovich[28] observed plasmid damage caused by PQ^{2+} and by the more active napthoquinone plumbagin, measured as chromosomal smearing, single strand DNA breakage, and endonuclease III-sensitive sites, through the generation of O_2- and H_2O_2. This latter study[27] and others using isolated DNA have inferred a role for $HO^{\cdot}$ because of protection afforded by superoxide dismutase and catalase. The use of streptonigrin paired with spin trapping[11] allows more direct demonstration of the nucleic acid damage attributable to $HO^{\cdot}$ formation.

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