Damage to Chromosomal and Plasmid DNA by Toxic Oxygen Species

By MAJA ROZENBERG-ARSKA,^{1,2*} B. SWEDER VAN ASBECK,¹
THEODORUS F. J. MARTENS¹ AND JAN VERHOEF^{1,2}

¹Department of Microbiology, Catharijnesingel 59, 3511 GG Utrecht, The Netherlands

²Department of Clinical Bacteriology, University Hospital, Catharijnesingel 101,
3511 GV Utrecht, The Netherlands

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Bacterial DNA was incubated with xanthine plus xanthine oxidase plus excess iron as an oxygen-species-generating system, and DNA injury was measured by agarose gel electrophoresis and by the ability of the DNA to transform competent bacteria. After 5 to 10 min incubation, the covalently closed circular form of plasmid DNA was converted into the open circular form, and after 30 min, to some extent into the linear form. Biological activity, measured as the number of transformed bacteria, decreased rapidly after 10 min incubation. Incubation of chromosomal DNA with the enzymic oxygen-species-generating system resulted in the degradation of DNA to small fragments within about 1 h. Excess iron was essential for the damaging effect of xanthine plus xanthine oxidase. Damage to DNA could be prevented by oxygen scavengers such as superoxide dismutase, catalase, mannitol and thiourea. Our results suggest that hydroxyl radical is the injurious oxidant for bacterial DNA, and that it can mediate physicochemical as well as biological alterations in DNA.

INTRODUCTION

Toxic oxygen species play an important role in the microbicidal activity of phagocytic cells, such as polymorphonuclear leucocytes and monocytes (Klebanoff, 1975; McPhail *et al.*, 1981). Not much is known about the precise mechanism by which toxic oxygen species kill and contribute to the degradation of micro-organisms after phagocytosis (Elsbach, 1980). Recently we presented evidence that bacterial DNA is degraded by nucleases present in serum (Rozenberg-Arska *et al.*, 1984a) and in mononuclear leucocytes (Rozenberg-Arska *et al.*, 1984b). We have now extended these studies to answer the question as to whether toxic oxygen metabolites are capable of reacting with bacterial nucleic acid and whether they contribute to the degradation of DNA. Damage to DNA was visualized in gels and the loss of biological activity of DNA was determined by the capacity of DNA to transform recipient bacteria. Our results show a rapid change in the structure of bacterial DNA during incubation with the oxygen-species-generating system xanthine plus xanthine oxidase (Beauchamp & Fridovich, 1970). These changes, which lead to loss of biological activity of the DNA, occurred only in the presence of excess iron, suggesting involvement of $\cdot\text{OH}$.

METHODS

Reagents. Xanthine was from Sigma. Xanthine oxidase (Sigma; buttermilk; specific activity approximately 0.7 units mg protein⁻¹) was prepared at a concentration of 0.1 units ml⁻¹ in TE buffer (10 mM-Tris/HCl with 0.1 mM-EDTA; pH 8.0). Catalase [Sigma; bovine liver; EC 1.11.1.6.; 33900 units (mg protein)⁻¹] was used at a concentration of 500 µg ml⁻¹ in TE buffer. Superoxide dismutase (SOD) [Sigma; bovine blood type I; EC 1.15.1.1.; 2.8 units (mg protein)⁻¹] was used at a concentration of 100 µg ml⁻¹. Mannitol (OPG, Utrecht, The

Abbreviation: SOD, superoxide dismutase.

Netherlands), and thiourea (BDH), were dissolved in TE buffer and used at a concentration of 100 mM. Fe(III) citrate (Merck) was dissolved immediately before use in distilled water at a concentration of 0.2 M.

Isolation of chromosomal and plasmid DNA. Chromosomal and plasmid DNA were isolated from *Escherichia coli* K12 strain PC2166 harbouring plasmid RSF1030 (molecular weight 5.5×10^6) coding for ampicillin resistance. Plasmid DNA was isolated by the rapid alkaline extraction procedure described by Birnboim & Doly (1979), and subsequently dissolved in 100 μ l distilled water. Chromosomal DNA was isolated according to the method of Cosloy & Oishi (1973), and subsequently dissolved in an appropriate volume of 1/10 strength standard saline citrate (15 mM-NaCl/1.5 mM-trisodium citrate).

Incubation of chromosomal and plasmid DNA in xanthine plus xanthine oxidase. The standard reaction mixture consisted of 2 mM-xanthine, 0.1 units xanthine oxidase ml^{-1} , with or without 0.2 mM-Fe(III) citrate in TE buffer in a total volume of 0.5 ml at pH 7.5.

Breakdown of DNA assay. Chromosomal DNA at a concentration of $644 \mu\text{g ml}^{-1}$, or plasmid DNA in concentration of $450 \mu\text{g ml}^{-1}$, was incubated in xanthine plus xanthine oxidase with or without iron for different time intervals. The extent of degradation of the DNA molecules was measured by electrophoresis in agarose gels. The effect of the treatments on the biological activity of DNA was demonstrated by the capacity of DNA to transform competent recipient bacteria.

Agarose gel electrophoresis. This was used to visualize plasmid and chromosomal DNA after they had been incubated with toxic oxygen species for various time intervals. Electrophoresis was done in horizontal slab gels (gel dimensions 14.5 cm $\times$ 27 cm $\times$ 3 mm, with 16 slots), containing 0.6% (w/v) agarose (type IV, Sigma) dissolved in electrophoresis buffer (40 mM-Tris, 20 mM-sodium acetate, 2 mM-EDTA, adjusted to pH 7.8 with acetic acid). Both agarose and electrophoresis buffer contained ethidium bromide at a final concentration of $2 \mu\text{g ml}^{-1}$. Electrophoresis was done either for 4 h at 30 mA or overnight at 12 mA. Gels were photographed under UV light (360 nm) using Polaroid type film (Kodak 667) with an orange 22 filter.

Transformation by chromosomal DNA. *E. coli* AM1095 (Rec⁺ Leu⁻) was transformed by chromosomal DNA which had been incubated with the oxygen-species-generating system. The procedure was described by Bergmans *et al.*, (1981).

Transformation by plasmid DNA. *E. coli* K12C600 was transformed by plasmid DNA which had been incubated with the oxygen-species-generating-system. The procedure was a modification of that described in detail by Bergmans *et al.* (1981) and by Cosloy & Oishi (1973).

Effect of the addition of scavengers to the oxygen-species-generating system on chromosomal and plasmid DNA. The influence of SOD, catalase, mannitol and thiourea on the toxic effects of the oxygen-species-generating system was studied. Chromosomal and plasmid DNA were incubated with xanthine plus xanthine oxidase in the presence of Fe(III) citrate with and without various oxygen radical scavengers for 2 h at 37 °C at pH 7.5. After incubation, part of the treated DNA was visualized in gels and the remainder was used to transform recipient bacteria.

Statistical analysis. The SEM was taken as an estimate of variance.

RESULTS

Effect of the xanthine-xanthine oxidase system on the biological activity of plasmid and chromosomal DNA

Xanthine plus xanthine oxidase had no injurious effect on plasmid DNA. After incubation, no changes in the physicochemical structure of the DNA were observed in gels. Also, plasmid DNA retained its biological activity. Plasmid DNA incubated with xanthine plus xanthine oxidase was able to produce 301 ± 2.5 (mean $\pm$ SEM) transformed colonies compared with 296.2 ± 7.8 transformed colonies produced by control plasmid DNA (Table 1). However, in the presence of Fe(III) citrate, xanthine plus xanthine oxidase reduced the capacity of plasmid DNA to transform recipient bacteria significantly (27.7 ± 7.8 vs 296.2 ± 7.8).

To find out which toxic oxygen species generated by the system was responsible for the change in configuration and biological activity of plasmid DNA, scavengers of different oxygen species were used. When plasmid DNA was incubated with xanthine plus xanthine oxidase plus Fe(III) citrate in the presence of catalase or SOD, the number of transformants was 235.7 ± 7.2 and 159.3 ± 9.2 , respectively, compared to 27.7 ± 7.8 when no catalase or SOD was added. This indicates that catalase or SOD prevents at least some toxic effect of xanthine and xanthine oxidase. Addition of either mannitol or thiourea also protected against the toxic effect of the oxygen-species-generating system on plasmid DNA (Table 1). Similar results were found when chromosomal DNA was incubated with xanthine plus xanthine oxidase (Table 1). The extent of the change in the DNA depended on the duration of the incubation period. After 15 min

Fig. 1. Effect of xanthine plus xanthine oxidase plus Fe(III) citrate on plasmid DNA. A reaction mixture containing 2 mM-xanthine, 0.1 units xanthine oxidase ml⁻¹ and 0.2 mM-Fe(III) citrate in a total volume of 0.5 ml TE buffer, pH 7.5, was incubated with 9 µg plasmid DNA at 37 °C. Tracks 1 to 4, plasmid DNA after 30, 60, 90 and 120 min incubation; track 5, plasmid DNA incubated in the absence of the xanthine-xanthine oxidase-Fe(III) citrate system. Xanthine, xanthine oxidase or the combination of xanthine and xanthine oxidase without Fe(III) citrate had no effect on plasmid DNA. Abbreviations: CCC, covalently closed circular DNA; OC, open circular DNA; Lin, linear DNA; Chr, chromosomal DNA.

Table 1. *Effect of xanthine, xanthine oxidase and Fe(III) citrate with and without scavengers on the capacity of plasmid and chromosomal DNA to transform recipient bacteria*

Plasmid DNA (18 µg) or chromosomal DNA (9 µg) was incubated for 2 h at 37 °C with the complete reaction mixture (2 mM-xanthine, 0.1 units xanthine oxidase ml⁻¹, 0.2 mM-Fe(III) citrate, pH 7.5; total vol. 1 ml) with additions and omissions as stated; the DNA was then used to transform recipient bacteria.

Incubation conditions	No. of transformants*	
	Plasmid DNA	Chromosomal DNA
Complete reaction mixture	27.7 ± 7.8 (3)	50.0 ± 10.8 (4)
- Xanthine, xanthine oxidase and Fe(III) citrate	296.2 ± 7.8 (9)	784.4 ± 38.8 (9)
- Xanthine	308.3 ± 9.2 (3)	ND
- Xanthine oxidase	304.0 ± 8.7 (3)	ND
- Fe(III) citrate	301.0 ± 2.5 (3)	ND
+ Catalase (500 µg ml ⁻¹)	235.7 ± 7.2 (3)	763.0 ± 37.1 (3)
+ Heated catalase (15 min, 100 °C)	33.3 ± 3.3 (3)	33.3 ± 3.3 (3)
+ SOD (100 µg ml ⁻¹)	159.3 ± 9.2 (3)	773.3 ± 31.8 (3)
+ Heated SOD (15 min, 100 °C)	31.6 ± 1.6 (3)	35.0 ± 2.9 (3)
+ Mannitol (100 mM)	161.3 ± 5.5 (3)	742.5 ± 23.3 (4)
+ Thiourea (100 mM)	228.0 ± 15.6 (3)	760.0 ± 23.6 (4)

ND, Not determined.

* Mean ± SEM of (n) experiments.

incubation of plasmid DNA with Fe(III) citrate plus xanthine plus xanthine oxidase, a fall in the number of transformants from 435.3 ± 15.4 to 174.0 ± 6.4 colony forming units was observed. After 60 min incubation the number of transformants decreased to 52.5 ± 4.7 colony forming units (data not shown). A time-dependent breakdown of plasmid DNA could also be shown in agarose gels (Fig. 1). Degradation of DNA started with the change of the covalently closed circular (CCC) form into the open circular (OC) form; upon further incubation all DNA

Fig. 2. Effect of xanthine plus xanthine oxidase plus Fe(III) citrate on plasmid DNA in the presence of oxygen radical scavengers. Plasmid DNA was incubated with the reaction mixture described in the legend to Fig. 1, in the presence or absence of various oxygen radical scavengers. Track 1, no added scavenger; track 2, plus catalase ($100 \mu\text{g ml}^{-1}$); track 3, plus SOD ($100 \mu\text{g ml}^{-1}$); track 4, plus mannitol (100 mM); track 5, plus thiourea (100 mM); track 6, plasmid DNA incubated in the absence of the xanthine-xanthine oxidase-Fe(III) citrate system.

Fig. 3. Effect of xanthine plus xanthine oxidase plus Fe(III) citrate on chromosomal DNA. The reaction mixture described in the legend to Fig. 1 was incubated with $4.5 \mu\text{g}$ chromosomal DNA at 37°C . Tracks 1 to 6, chromosomal DNA after 180, 120, 60, 30, 15 and 5 min incubation; track 7, chromosomal DNA incubated in the absence of xanthine oxidase; track 8, chromosomal DNA incubated in the absence of the xanthine-xanthine oxidase-Fe(III) citrate system.

changed to the OC form and, to some extent, to the linear form. It could also be observed that scavengers of toxic oxygen species diminished the effect of xanthine plus xanthine oxidase plus Fe(III) citrate on DNA degradation (Fig. 2). The addition of scavengers inhibited breakdown of plasmid DNA to different extents. Inhibition of DNA degradation was most pronounced when SOD was added to the xanthine-xanthine oxidase system, but thiourea, catalase and mannitol also showed significant effects.

Degradation of chromosomal DNA was observed after about 15–30 min incubation. After 1 h, only a smear of DNA was visible in the gel (Fig. 3). Addition of scavengers such as catalase, SOD, mannitol and thiourea inhibited this damaging effect (data not shown).

DISCUSSION

Reactive oxygen species have been implicated as a possible cause of bacterial cell death during phagocytosis (Babior *et al.*, 1973; Klebanoff, 1975); they may also play an important role in the pathogenesis of tissue damage induced by radiation, inflammation (McCord, 1974) and ischaemia (McCord, 1985). They are known to be produced by the action of certain enzymes such as xanthine oxidase (Beauchamp & Fridovich, 1970), and by polymorphonuclear and mononuclear phagocytes during the metabolic burst (Babior *et al.*, 1973; Badwey & Karnovsky, 1980; Klebanoff, 1975). The true contribution of reactive oxygen species to natural processes and in the development of pathological conditions is still largely unknown.

DNA is generally considered to be the most critical cellular target with regard to the lethal, carcinogenic and mutagenic effects of drugs, radiation and environmental chemicals; in several studies, the involvement of reactive oxygen species in DNA damage caused by irradiation (X-ray, ionizing irradiation) or drug treatment has been inferred (Armel *et al.*, 1977; Lesko *et al.*, 1980; Myers, 1973; Rowley & Halliwell, 1983). It has been shown that the hydroxyl radical leads to the formation of alkali-labile lesions in the DNA strands and to single strand breaks (Armel *et al.*, 1977; Myers, 1973). Lesko *et al.* (1980) found that the degradation of DNA depended on the presence of Fe^{2+} ions. DNA binds Fe^{2+} and this complex is effective in catalysing H_2O_2 breakdown to yield $\cdot\text{OH}$. Chelation of iron prevented damage to the DNA (Sausville *et al.*, 1978). In most studies, DNA damage was measured by assaying for nicks in the DNA molecule. We studied the effect of toxic oxygen species, generated by xanthine oxidase, on the physicochemical and biological activity of bacterial (chromosomal and plasmid) DNA. The xanthine–xanthine oxidase system reduces oxygen to O_2^- and H_2O_2 , and these two products can react to form $\cdot\text{OH}$ (Beauchamp & Fridovich, 1970). Without the catalytic effect of trace metals this is a sluggish process, but the reaction proceeds briskly in the presence of Fe(III) complexes, due to reduction of the latter by O_2^- and the further reaction of Fe^{2+} with H_2O_2 (Weinstein & Bielski, 1979). Damage to DNA by the xanthine–xanthine oxidase–iron system could be prevented with SOD and catalase, suggesting that both O_2^- and H_2O_2 are essential for its injurious effect. Also, since iron was required, and mannitol and thiourea are scavengers with specificity for $\cdot\text{OH}$ (Dorfman & Adams, 1973), we concluded that this oxidant was the most injurious toxic oxygen species for bacterial DNA. We have shown that toxic oxygen species (especially $\cdot\text{OH}$) can induce extensive breakdown of bacterial (chromosomal and plasmid) DNA. When chromosomal DNA was incubated with xanthine plus xanthine oxidase plus excess iron degradation of DNA into numerous nucleotides was observed. Nucleases were not needed for this breakdown.

In addition, xanthine plus xanthine oxidase plus iron interfered rapidly with the biological activity of plasmid and chromosomal DNA: both kinds of DNA were unable to transform recipient cells after incubation with the above system. In humans, virtually every cell contains systems in which oxygen species are generated (e.g. the mitochondrial system or enzymes such as xanthine oxidase). Polymorphonuclear and mononuclear leucocytes especially possess a potent toxic oxygen-species-generating system. Therefore, it is possible that in these cells, as well as in other cell types, damage to DNA can occur (Birnbom, 1982). Indeed, it has recently been shown that leucocyte-derived toxic oxygen species can induce mutations in bacterial DNA (Weitzman & Stossel, 1982).

Our studies suggest that the beneficial effects of toxic oxygen species not only include the killing of bacteria, but also the protection of the host by preventing bacteria from acquiring or maintaining drug resistance by destroying of transferable information on plasmid DNA.

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