Assessment of the genotoxic potential of nitric oxide-induced guanine lesions by in vitro reactions with Escherichia coli DNA polymerase I

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It has been suggested that carcinogenesis associated with chronic inflammation involves DNA damage by nitric oxide (NO) and other reactive species secreted from macrophages and neutrophils. The guanine moiety of DNA reacts with NO, yielding two major deamination products: xanthine (Xan) and oxanine (Oxa). Oxa reacts further with polyamines and DNA binding proteins to form cross-link adducts. In the present study, we characterized the structure of the cross-link adducts of Oxa with spermine (Oxa-Sp). Spectrometric analysis of Oxa-Sp adducts showed that they are ring-opened adducts of Oxa covalently bonded to the terminal amino (major product) and internal imino (minor product) groups of spermine. To assess genotoxic potential, Xan, Oxa, Oxa-Sp and an abasic (AP) site were specifically incorporated into oligonucleotide fragments (Pol I Kf). The relative efficiency of translesion DNA synthesis catalyzed by DNA polymerase I Klenow (Pol I Kf). The relative efficiency of translesion synthesis was G (1) > Oxa (0.19) > Xan (0.12) > AP (0.088) > Oxa-Sp (0.035). Primer extension assays with a single nucleotide and Pol I Kf revealed that non-mutagenic dCMP was inserted most efficiently opposite Xan and Oxa, with the extent of primer elongation being 65% for Xan and 68% for Oxa. However, mutagenic nucleotides were also inserted. The extent of primer elongation for Oxa was 16% with dTMP and 14% with dGMP, whereas that for Oxa was 49% with dTMP. For Oxa-Sp, mutagenic dAMP (13%) was preferentially inserted. Accordingly, when generated in vivo, Xan and Oxa would constitute moderate blocks to DNA synthesis and primarily elicit G:C to T:A transitions when bypassed, whereas Oxa-Sp would strongly block DNA synthesis and elicit G:C to T:A transversions.

Introduction

It has been demonstrated by epidemiological and other studies that chronic inflammation induced by biological, chemical and physical factors are associated with increased risk of human cancer (1–3). Although the molecular mechanisms of carcinogenesis associated with inflammation have not been fully clarified, one mechanism involves DNA damage by reactive oxygen and nitrogen species that are secreted by activated macrophages and neutrophils for a prolonged period (1,2,4–6). Similar to nitrous acid, nitric oxide (NO) gives rise to reactive nitrous anhydride that can induce nitrosative deamination of DNA bases (7–10). Nitrosative deamination of cytosine and adenine results in uracil and hypoxanthine, respectively. These products are mutagenic since the hydrogen bond donor–acceptor property of the deaminated bases is reversed (11,12). Unlike cytosine and adenine, nitrosative deamination of guanine gives rise to two major products, xanthine (Xan) and oxanine (Oxa) at the ratio of Xan:Oxa = 3:1 (Figure 1) (13–16) [for a conflicting result, see Dong et al. (17)]. Xan is a simple deamination product of guanine and has been suggested to be mutagenic (12). In contrast, in Oxa, the ring N1 atom is substituted by an oxygen atom via deamination of the 2-NH2 group followed by ring opening and rearrangement (16,18,19).

Among the deaminated bases, uracil, hypoxanthine and Xan are repaired by the base excision repair pathway in prokaryotic and eukaryotic cells (20–24). Prokaryotic cells have an alternative repair pathway involving endonuclease V for these lesions (25–28). In contrast, although recent studies have shown that Escherichia coli and human DNA glycosylases excise Oxa from the DNA backbone and prokaryotic endonuclease V incises Oxa-containing DNA (29,30), the excision and incision activities of these enzymes are substantially lower than those for their physiological substrates, implying the considerably long remanence of Oxa in DNA owing to slow repair.

In our previous studies, we have shown that Oxa bearing a reactive O-acylurea structure forms bulky cross-link adducts with amino acids, polyamines and DNA binding proteins, such as histone and DNA glycosylases (31,32), which are unlikely to be repaired by the base excision repair pathway. Consistent with this notion, the sensitivity and mutation frequency of various repair-deficient E.coli strains toward NO and nitrous acid suggest that the principal pathway for coping with the cytotoxic or mutagenic effect of these agents is nucleotide excision or recombination repair rather than base excision repair (33–37). This implies that the cross-link adducts of Oxa play a role in the onset of the cytotoxicity or mutagenicity of NO and nitrous acid, which prompted us to further investigate the genotoxic effect of Oxa cross-link adducts, as well as Xan and Oxa.

In the present study, we characterized the structure of the Oxa-spermine cross-link adduct (Oxa-Sp) and assessed the

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Fig. 1. Formation of xanthine and oxanine by nitrosative deamination of guanine.

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biological relevance of Oxa-SP by analyzing the reaction products formed between Oxa-containing DNA and cell-free extracts (CFEs). Furthermore, Xan, Oxa and Oxa-SP were embedded in the same site of DNA templates, and the lethal and mutagenic potential of these lesions were compared systematically by analyzing the effect on in vitro DNA replication.

Materials and methods

Analysis of cross-link adducts formed between 2'-deoxyoxanosine and spermine

2'-Deoxyoxanosine (dOxo) was prepared from 2'-deoxyguanosine as reported previously (38). One mM dOxo was incubated with 100 mM sperm in 300 mM Tris-HCl (pH 7.4) at 37°C for 2 h. The cross-link adducts (designated as dOxo-Sp1 and dOxo-Sp2) were isolated by preparative reversed phase high-performance liquid chromatography (HPLC) and subjected to structural assignments as described below. The time courses of concentration changes in dOxo and cross-link adducts were also followed by HPLC for up to 2 h in a similar manner. For quantification of products, the following ε260 values were used [all in 50 mM ammonium acetate buffer (pH 4.7)]. The ε260 value of 2'-deoxyguanosine was 1.19 × 10^4 M⁻¹ cm⁻¹. The ε260 nm values were estimated as 5.4 × 10^4 M⁻¹ cm⁻¹ (dOxo), 3.4 × 10^4 M⁻¹ cm⁻¹ (dOxo-Sp1), and 4.9 × 10^4 M⁻¹ cm⁻¹ (dOxo-Sp2) from the integration of the sugar H-1' or base H-2 proton signal and the HPLC peak area detected at 260 nm relative to those of 2'-deoxyguanosine. The concentrations of dOxo and cross-link adducts in a reaction mixture were determined from the integrated peak areas of the HPLC chromatogram at 260 nm and the ε260 values.

**HPLC conditions**

The HPLC system consisted of Shimadzu LC-10Advp pumps and an SCL-10Avp system controller. On-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV-VIS photodiode-array detector. An Inertis ODS-3 octadeylsilane column (4.6 × 250 mm, particle size 5 μm, GL Science) was used for reversed phase HPLC. The eluent was 50 mM ammonium acetate buffer (pH 4.7) containing methanol. The methanol concentration was increased from 0 to 40% for 20 min in a linear gradient mode. The column temperature was 40°C and the flow rate was 1.0 ml/min.

**Spectrometric measurements**

NMR spectra were measured on a JEOL INE-ECAS500 spectrometer in DMSO-d6 at 30°C. The chemical shifts (p.p.m.) were referenced to tetramethylsilane (TMS) as an internal standard. The signal assignments were performed by COSY, ESI-MS/MS measurements were performed on an API 2000 triple-quadrupole mass spectrometer (Applied Biosystems). The purified sample was dissolved in 50% methanol containing 0.1% formic acid. The sample was directly infused into the MS system by a syringe pump without a column at a flow rate of 5 μl/min. The sample was selected in the positive mode with an electron spray capillary potential of 5.0 kV. Spectrometric data of dOxo-Sp1: 1H NMR (500 MHz, DMSO-d6, at 30°C): δ (p.p.m./TMS) 7.84 (s, 1H, H-2), 6.38 (s, 2H, NH2), 5.83 (dd, 1H, H-1'), 4.28 (m, 1H, H-3), 3.78 (dd, 1H, H-4'), 3.52 (ABX, 2H, H-5'), 3.40 (m, 2H, CH2), 3.34 (m, 2H, CH2), 2.72 (m, 4H, CH2), 1.51 (m, 2H, CH2), 1.45 (m, 4H, CH2); UV: shoulder 250 nm (pH 7.0); ESI-MS (positive) m/z: 471 [M + H]+, ESI-MS/MS (positive, daughter ions of m/z 471) 454, 338, 264, 129. Spectrometric data of dOxo-Sp2: 1H NMR (500 MHz, DMSO-d6, at 30°C): δ (p.p.m./TMS) 8.00 (dd, 1H, J = 5.9 and 5.9 Hz, CONHCH3), 7.83 (s, 1H, H-2), 6.41 (s, 2H, NH2), 5.81 (dd, 1H, H-1'), 4.28 (m, 1H, H-3), 3.77 (dd, 1H, H-4'), 3.52 (ABX, 2H, H-5'), 3.25 (m, 2H, CONHCH3), 2.74 (m, 2H, CH2), 2.64-2.54 (m, 8H, CH2), 2.28 (m, 2H, H-2', 2'), 1.66-1.61 (m, 4H, CH2), 1.46 (m, 4H, CH2); UV: λmax 237 nm (pH 7.4); ESI-MS (positive) m/z: 471 [M + H]+, ESI-MS/MS (positive, daughter ions of m/z 471) 454, 338, 264, 129.

**Oligonucleotides**

The oligonucleotides used in this study are listed in Table I. The oligonucleotides with normal constituents (11PRM, 51TEMP, 41G, 13PRM, 14PRM, 23 M and 25COM) were synthesized by the standard phosphoramidite method. 41AP containing a model abasic (AP) site (tetrahydrofuran) was synthesized using the corresponding phosphoramidite monomer (Gen Research). 41OXA containing Oxa was prepared by using the DNA polymerase reaction in a manner similar to that reported previously (31). Briefly, 11PRM was 5'-end labeled with 32P and annealed to 51TEMP. The template/primer (51TEMP/11PRM) was incubated with 2'-deoxyoxanosine 5'-triphosphate (39) and E.coli DNA polymerase I Klenow fragment deficient in 3'-5' exonuclease [Pol I Kf (exo-)] (New England Biolabs) at 25°C for 5 min. Subsequently, normal dNTPs (dCTP, dTTP, and dATP) were added to the reaction mixture, and incubation was further continued at 25°C for 40 min. DNA was purified by phenol extraction and ethanol precipitation. The fully extended product containing Oxa (41OXA) was separated by 16% denaturing PAGE, extracted from the gel and purified by a Sep-Pak cartridge (Waters). The template containing Xan (41XAN) was similarly prepared using 2'-deoxyxanthosine 5'-triphosphate (39) except that the products containing single and two consecutive Xan residues produced in the first reaction were separated by 16% denaturing PAGE. The former was used as a primer for the second polymerase reaction. The preparation of 25OXA was reported previously (31). For the preparation of 41OXA-SP and 25OXA-SP containing Oxa-Sp, 41OXA and 25OXA were incubated with 2'-deoxyoxanosine in 10 mM Tris-HCl (pH 7.5) and products were purified by PAGE as described for 41OXA-SP and 25OXA-SP migrated more slowly than parental 41OXA and 25OXA, respectively, in the PAGE separation.

**Cross-link reactions with HeLa CFEs**

CFEs were prepared on ice or at 4°C. The harvested HeLa cells were suspended in three volumes of 50 mM Tris-HCl (pH 7.5), 3 mM EDTA, 5 mM Mg(CH3COO)2, 3 mM 2-mercaptoethanol, 300 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml pepstatin. The cells were disrupted with 20 strokes of a tight-fitting Dounce homogenizer and centrifuged at 65,000 g for 30 min. The supernatant was recovered and used as CFEs. The protein concentration was determined with the BCA protein assay kit (Pierce). The duplex of 25OXA/25COM (2 mM, 25OXA was 5'-end 32P-labeled) was incubated with CFEs (1-20 μg) in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (total 20 μl) at 37°C for 1 h. The sample was mixed with gel loading buffer (0.05% xylene cyanol, 0.05% bromophenol blue, 20 mM EDTA and 98% formamide), heated and separated by 16% denaturing PAGE.

**Analysis of translesion DNA synthesis**

The reactions to assay translesion synthesis (TLS) were performed under running-start conditions using templates (41OXA, 41XAN, 41OXA-SP, 41AP and 41G) and a 5'-end 32P-labeled primer (11PRM) whose terminus was 10 nt shorter than the lesion site. The template and primer were annealed by briefly heating at 80°C and cooling to room temperature. The template/primer (10 nM) was incubated with E. coli DNA polymerase I Klenow fragment (Pol I Kf) or Pol I Kf (exo-) (both 0.0001 U, New England Biolabs) and 4 dNTPs (50 μM each) in buffer A (66 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 50 μg/ml BSA and 6.6 mM MgCl2; total 5 μl) at 37°C for 210
3–60 min. The reaction was terminated by adding gel loading buffer. Products were separated by 16% denaturing PAGE, and the radioactivity in the gel was analyzed on a phosphorimaging analyzer Fuji BAS 2000. Alternatively, the gel was autoradiographed at −80°C.

**Analysis of nucleotides inserted opposite lesions**

The nucleotides inserted opposite the lesions were analyzed by primer extension assays under standing-start conditions. The template (41OXA, 41XAN, 41OXA-SP, 41AP and 41G) was primed by 5′-end 32P-labeled 14PRM (10 nM as template/primer) and incubated with Pol I Kf (0.0001–0.01 U) and a single dNTP (100 μM) in buffer A (5 mM) at 37°C for 5 min. Products were analyzed as described above.

**Results**

**Characterization of dOxo-spermine cross-link adducts**

We have shown previously that Oxa in DNA produces cross-link adducts with amines and polyamines (31), but the detailed nature of the adducts has not been clarified. Here, the structure of the adducts was elucidated by analyzing the product formed between dOxo and spermine. When dOxo was incubated with spermine for 2 h, two product peaks designated as dOxo-Sp1 (retention time = 3.8 min) and dOxo-Sp2 (5.2 min) appeared in the HPLC chromatogram (Figure 2A). The products were isolated by preparative HPLC and subjected to structural assignment. Positive ion ESI-MS of both products showed a signal with m/z = 471 for the molecular ion. The 1H NMR spectrum of both products in DMSO-d6 showed signals indicative of an intact 2′-deoxyribosine moiety, an aromatic proton, a primary amino group and ten methylene groups. In addition, a double-doublet imino proton signal (8.00 p.p.m.) was observed only for dOxo-Sp2. A correlation between the imino proton signal and a methylene proton signal (3.25 p.p.m.) was observed in the COSY spectrum. The imino proton signal disappeared when D2O was added to the sample. Combining these data, we have concluded that dOxo-Sp1 and dOxo-Sp2 are ring-opened adducts of dOxo covalently bonded to the internal imino and the terminal amino groups of spermine, respectively (Figure 2C). We also measured the 1H NMR spectra of dOxo-Sp1 and dOxo-Sp2 in the range −1–17 p.p.m. to observe exchangeable protons. The double-doublet imino proton signal of dOxo-Sp2 and the amino proton signal of the primary amide group of dOxo-Sp1 and dOxo-Sp2 were present, but no other exchangeable proton signals were evident. It is likely that the

![Fig. 2. Analysis of cross-link adducts (dOxo-Sp1 and dOxo-Sp2) formed between dOxo and spermine. (A) HPLC profile of the reaction mixture. dOxo was incubated with spermine at 37°C for 2 h and the reaction mixture was subjected to reversed phase HPLC analysis. The peak with an asterisk indicates a void peak. (B) Concentration changes in dOxo (circles), dOxo-Sp1 (triangles) and dOxo-Sp2 (squares) with reaction time. The reaction between dOxo and spermine was performed as described above, and an aliquot of the sample was taken at 0, 30, 60, 90 and 120 min and analyzed by HPLC. (C) Production and structures of dOxo-Sp1 and dOxo-Sp2. The numbers on dOxo-Sp1 and dOxo-Sp2 indicate atom positions for 1H NMR assignment.](image-url)
signals were obscured by broadening owing to an exchange with H2O in the sample.

Figure 2B shows the time course of the reaction of dOxo with spermine. The concentration ratio for dOxo-Sp1:dOxo-Sp2 was ~1:5 throughout the reaction. The concentrations of dOxo-Sp1, dOxo-Sp2 and dOxo after 2 h of incubation were 133, 691 and 133 μM, respectively. The yields of dOxo-Sp1 and dOxo-Sp2 relative to consumed dOxo were 15.2 and 79.3%, respectively. The stability of the adducts was also examined. No decomposition was observed by HPLC when isolated dOxo-Sp1 and dOxo-Sp2 were incubated in 300 mM Tris-HCl (pH 7.4) at 37°C for 2 days (data not shown).

Cross-link adducts formed with HeLa CFEs

In a previous study, we showed that Oxa in DNA forms cross-link adducts with purified proteins and with those in CFEs (31). However, similar reactions with low molecular weight species, such as amino acids and polyamines, were demonstrated only for pure reactants and not for CFEs. Thus, cross-link formation with low molecular weight species was examined using cell extracts. A duplex oligonucleotide containing Oxa (25OXA/25COM) was incubated with HeLa CFEs at 37°C for 1 h, and products were analyzed by 16% denaturing PAGE. When products were separated by PAGE for 40 min, two types of cross-linked species were detected (Figure 3, left gel). The high molecular weight species retained in the well were probably cross-link adducts with proteins as inferred from previous results (31). The low-molecular weight species appeared as a smear band over 25OXA, which was separated into several distinct products when PAGE separation was extended to 4 h (Figure 3, right gel). The mobilities of the separated products were comparable with or somewhat slower than that of the Oxa-Sp adduct (25OXA-SP) run as a marker. The amount of low molecular weight cross-link products increased with increasing amounts of CFEs. Although no further attempt was...
made to identify the individual cross-link products, this result shows that Oxa in DNA forms cross-links with low molecular weight constituents in cells, possibly including polyamines and other amines.

**Translesion synthesis**

To elucidate the influence of guanine lesions induced by nitrosative stress on DNA synthesis, Xan, Oxa, Oxa-Sp and an AP site were incorporated in the same site of oligonucleotide templates [G (41G), Oxa (41OXA), Xan (41XAN) and an AP site (41AP)]. The templates were primed by 13PRM and replicated by Pol I Kf or Pol I Kf (exo⁻) under running-start conditions for up to 60 min. Primer extension catalyzed by Pol I Kf (Figure 4) was arrested by Oxa-Sp (lanes 12 and 13), Oxa (lanes 7 and 8), Xan (lanes 24 and 25) and an AP site (lanes 17 and 18) in the incubation for up to 10 min. The primary termination site of DNA synthesis commonly occurred one nucleotide before the lesion in the templates. In contrast, primer elongation on the intact template containing G was rapid, and products that were almost fully extended were observed after 10 min of incubation (lane 3). With the template containing Oxa-Sp, products resulting from TLS were seen only faintly after 60 min of incubation (lane 15). In contrast, TLS products for Oxa and Xan accumulated more rapidly (after 30–60 min incubation; lanes 9, 10, 26 and 27). TLS for an AP site (lanes 19 and 20) was less efficient than that for Oxa and Xan, but more efficient than that for Oxa-Sp. Thus, TLS for Oxa-Sp was the least efficient among the tested lesions. Similar patterns of termination and TLS were observed with Pol I Kf (exo⁻) (data not shown). To compare the TLS efficiency, the amount of bypassed products was quantified and is plotted against incubation time in Figure 5. The slope of the plots for Pol I Kf (Figure 5A), the relative efficiency of TLS was roughly estimated as G (1) > Oxa (0.19) > Xan (0.12) > AP (0.088) > Oxa-Sp (0.035). Pol I Kf (exo⁻) exhibited slightly better TLS efficiencies than Pol I Kf (Figure 5B). The distinct TLS efficiencies for Oxa, Xan, an AP site and Oxa-Sp suggest that the lethal potential of these lesions differs significantly.

**Nucleotides inserted opposite lesions**

To analyze the nucleotide inserted opposite the lesions during TLS, the templates were primed by 14PRM and replicated by Pol I Kf (0.0001, 0.001 and 0.01 U) under standing-start conditions with a single dNTP for 5 min. When the template/primers were incubated with 0.0001 U of Pol I Kf, insertion of nucleotides was not observed opposite Oxa-Sp, whereas dCMP, a cognate nucleotide, was preferentially inserted opposite G, Xan and Oxa (Figure 6, top gels). dAMP was inserted opposite an AP site (Figure 6, top gel). The primer extension was almost complete for G under these conditions, but was partial for Xan, Oxa and an AP site, indicating that nucleotide insertion opposite Xan, Oxa and an AP site is less efficient than that opposite G. Incubations with larger amounts of Pol I Kf (0.001 and 0.01 U) revealed the insertion of non-cognate nucleotides, which was clearly seen with 0.01 U of Pol I Kf (Figure 6, bottom gels). The major non-cognate nucleotides inserted were dAMP for Oxa-Sp, dTMP and dGMP for Xan, dTMP for Oxa, and dAMP and dGMP for an AP site. Note that both dAMP and dGMP are non-cognate nucleotides if an AP site is derived from the depurination of Xan with an acid labile N-glycosidic bond (24,38,40) or from the direct depurination of guanine by NO (13,17). The insertion of dAMP and dGMP opposite an AP site (dAMP > dGMP) by Pol I Kf is consistent with our previous study (41). The amount of Pol I Kf (0.001 and 0.01 U) used to reveal the insertion of non-cognate nucleotides for the lesions was excessive for the template containing normal guanine so that various nucleotides (dGMP, dCMP and dTMP) were multiply incorporated (Figure 6, two bottom gels for G).

To compare the efficiency of nucleotide insertion opposite individual lesions, the amount of inserted nucleotide (i.e. the percentage of extended primer) was quantified for the assays with 0.001 U of Pol I Kf (Figure 6, middle gels). The amount of inserted nucleotides and the insertion efficiency relative to that of dCMP are summarized in Table II. The relative insertion efficiency for Oxa-Sp was dAMP (4.3) > dGMP (1) = dCMP (1) > dTMP (0.67), the order of which is similar to that of a non-instructive AP site [dAMP (13) > dGMP (4.2) > dCMP (1) > dTMP (0.4)]. Concerning the nucleotide insertion opposite Oxa, Hitchcock et al. (30) have recently reported that Pol I Kf (exo⁻) inserts all four dNMPs with the same efficiency (the degree of primer extension was 35% for all dNMPs), showing no preferences for nucleotides, whereas human Pol β inserts nucleotides in the order of dCMP (1) > dTMP (0.8) = dAMP (0.8) > dGMP (0.16) (29). Thus, the present result with Pol I Kf [dCMP (1) > dTMP (0.72) > dAMP (0.12) > dGMP...
Chronic inflammation has long been recognized as a risk factor for many human cancers. In addition to mechanisms that are independent of genotoxicity, accumulated evidence suggests carcinogenesis associated with chronic inflammation involves DNA damage mediated by NO and other reactive species that are generated by macrophages and neutrophils (1,2,4--6). It has been shown that NO, an NO carrier (N-nitrosoindole) and nitrous acid mediate nitrosative deamination of DNA bases, and that Xan and Oxa are produced from guanine (13--16). Furthermore, Oxa but not Xan reacts with polyamines and DNA binding proteins, yielding cross-link adducts (31). Although the genotoxic potential of Xan and Oxa has been assessed by several laboratories including ours (24,29,30,39,42--44), that of Oxa cross-link adducts has not been elucidated previously. Furthermore, the genotoxic potential of Xan, Oxa and Oxa cross-link adducts has not been compared systematically using common DNA templates. In the present study, we have characterized the structure of Oxa-Sp as a representative cross-link adduct of Oxa with low molecular weight species and compared its genotoxic potential with that of Xan and Oxa under common conditions. The reaction of dOxo with spermine gave rise to two types of adducts (dOxo-Sp1 and dOxo-Sp2, Figure 2C). We tentatively assume that the same adducts are formed with Oxa in DNA, although oligonucleotides prepared by a similar method (25OXA-SP and 41OXA-SP) could not be separated into two species containing dOxo-Sp1 and dOxo-Sp2 under the present PAGE conditions. The formation of cross-link adducts with low molecular weight species in CFEs, including spermine, has also been confirmed in the present study (Figure 3).

The lethal potential of Oxa-Sp as well as of Xan, Oxa and an AP site [as a depurination product of Xan or a direct depurination of G by NO, Table II] is substantially different from that reported for Pol Kf (exo-), and more like that for Pol b. Moreover, the present result for Xan [dCMP (1) > dTMP (0.24) ≈ dGMP (0.21) > dAMP (0.07), Table II] agrees qualitatively with that reported by Wuenschell et al. (24) for Pol I Kf (exo-) [dCMP (1) > dTMP (0.25) ≫ dGMP and dAMP (insertion not observed)], but is quantitatively different with respect to the relative insertion efficiency of dGMP.

**Table II.** Nucleotide insertion opposite guanine lesions induced by nitrosative stress

<table>
<thead>
<tr>
<th>Damage</th>
<th>Inserted dNMP</th>
<th>Insertion (%)</th>
<th>Major mutations expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xan</td>
<td>dAMP</td>
<td>5 (0.08)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dGMP</td>
<td>14 (0.22)</td>
<td>G:C→C:G transversion</td>
</tr>
<tr>
<td></td>
<td>dCMP</td>
<td>65 (1.0)</td>
<td>No mutation</td>
</tr>
<tr>
<td></td>
<td>dTMP</td>
<td>16 (0.25)</td>
<td>G:C→A:T transition</td>
</tr>
<tr>
<td>Oxa</td>
<td>dAMP</td>
<td>8 (0.12)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dGMP</td>
<td>2 (0.03)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dCMP</td>
<td>68 (1.0)</td>
<td>No mutation</td>
</tr>
<tr>
<td></td>
<td>dTMP</td>
<td>49 (0.72)</td>
<td>G:C→A:T transition</td>
</tr>
<tr>
<td>Oxa-Sp</td>
<td>dAMP</td>
<td>13 (4.3)</td>
<td>G:C→T:A transversion</td>
</tr>
<tr>
<td></td>
<td>dGMP</td>
<td>3 (1.0)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dCMP</td>
<td>3 (1.0)</td>
<td>No mutation</td>
</tr>
<tr>
<td></td>
<td>dTMP</td>
<td>2 (0.67)</td>
<td>—</td>
</tr>
<tr>
<td>AP site</td>
<td>dAMP</td>
<td>67 (13)</td>
<td>G:C→T:A transversion</td>
</tr>
<tr>
<td></td>
<td>dGMP</td>
<td>21 (4.2)</td>
<td>G:C→C:G transversion</td>
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<td></td>
<td>dCMP</td>
<td>5 (1.0)</td>
<td>No mutation</td>
</tr>
<tr>
<td></td>
<td>dTMP</td>
<td>2 (0.40)</td>
<td>—</td>
</tr>
</tbody>
</table>

aThe percentage of extended primer obtained in the primer assays with 0.001 U of Pol I Kf (Figure 6). The values in parentheses are the insertion efficiencies relative to that of non-mutagenic dCMP.

bFor those with dNMP insertion efficiencies of >10%.

cAP sites are assumed to be derived from depurination of Xan or direct depurination of G by NO.

**Fig. 6.** PAGE analysis of nucleotides inserted opposite G, Xan, Oxa, Oxa-Sp and an AP site. Templates containing G (41G), Xan (41XAN), Oxa (41OXA), Oxa-Sp (41OXA-SP) and an AP site (41AP) were primed by 5’-end labeled 14PRM. The primer (10 nM as template/primer) was extended with a single dNTP (100 μM) and the indicated amount of Pol I Kf at 37°C for 5 min. Products were separated by 16% denaturing PAGE. The lesion present in the template and dNTP added into the reaction mixture are indicated at the top and bottom of the gels, respectively. Parts of the sequences of the templates (X = G, Xan, Oxa, Oxa-Sp or AP) and a primer (14PRM) are also shown at the top. The original primer (14PRM) and the extended one are indicated on the right by solid and broken arrows, respectively.

**Discussion**

Chronic inflammation has long been recognized as a risk factor for many human cancers. In addition to mechanisms that are independent of genotoxicity, accumulated evidence suggests carcinogenesis associated with chronic inflammation involves DNA damage mediated by NO and other reactive species that are generated by macrophages and neutrophils (1,2,4--6). It has been shown that NO, an NO carrier (N-nitrosoindole) and nitrous acid mediate nitrosative deamination of DNA bases, and that Xan and Oxa are produced from guanine (13--16). Furthermore, Oxa but not Xan reacts with polyamines and DNA binding proteins, yielding cross-link adducts (31). Although the genotoxic potential of Xan and Oxa has been assessed by several laboratories including ours (24,29,30,39,42--44), that of Oxa cross-link adducts has not been elucidated previously. Furthermore, the genotoxic potential of Xan, Oxa and Oxa cross-link adducts has not been compared systematically using common DNA templates. In the present study, we have characterized the structure of Oxa-Sp as a representative cross-link adduct of Oxa with low molecular weight species and compared its genotoxic potential with that of Xan and Oxa under common conditions. The reaction of dOxo with spermine gave rise to two types of adducts (dOxo-Sp1 and dOxo-Sp2, Figure 2C). We tentatively assume that the same adducts are formed with Oxa in DNA, although oligonucleotides prepared by a similar method (25OXA-SP and 41OXA-SP) could not be separated into two species containing dOxo-Sp1 and dOxo-Sp2 under the present PAGE conditions. The formation of cross-link adducts with low molecular weight species in CFEs, including spermine, has also been confirmed in the present study (Figure 3).

The lethal potential of Oxa-Sp as well as of Xan, Oxa and an AP site [as a depurination product of Xan or a direct depurination of G by NO, Table II] is substantially different from that reported for Pol Kf (exo-), and more like that for Pol β. Moreover, the present result for Xan [dCMP (1) > dTMP (0.24) ≈ dGMP (0.21) > dAMP (0.07), Table II] agrees qualitatively with that reported by Wuenschell et al. (24) for Pol I Kf (exo-) [dCMP (1) > dTMP (0.25) ≫ dGMP and dAMP (insertion not observed)], but is quantitatively different with respect to the relative insertion efficiency of dGMP.
depurination product of G by NO (13,17)] was estimated systematically by analyzing the effect on in vitro DNA replication. Oxa, Xan and an AP site blocked DNA synthesis catalyzed by Pol I Kf and Pol I Kf (exo−) with varying efficiencies (Figure 5). The TLS efficiency of Pol I Kf was G (1) > Oxa (0.19) > Xan (0.12) > AP (0.088) > Oxa-Sp (0.055). To our knowledge, this is the first comprehensive report on TLS efficiencies for nitrosative damages of guanine. If replicative or TLS DNA polymerases respond in a similar manner, Oxa-Sp would constitute a strongly lethal lesion in vivo, whereas Oxa and Xan would be moderately lethal.

We have also assessed the mutagenic potential of Oxa-Sp, Xan and Oxa by analyzing the nucleotide inserted opposite the lesions by Pol I Kf (Table II). For Oxa-Sp, mutagenic dAMP was preferentially inserted over non-mutagenic dCMP. Thus, Oxa-Sp would mainly give rise to G:C to T:A transversions, but with a limited frequency, since TLS for this lesion is inefficient and the efficiency of mutagenic insertion is lower than those for Oxa and Xan (Table II). Conversely, non-mutagenic dCMP was inserted most efficiently for Oxa and Xan, but mutagenic nucleotides were also incorporated. According to the preference for mutagenic nucleotides (Table II), Oxa would primarily yield G:C to A:T transitions, whereas Xan would yield G:C to A:T transitions and G:C to C:G transversions with comparable frequencies. The comparison of the insertion efficiencies of mutagenic nucleotides for Oxa (49% for dTMP) and Xan (16% for dTMP and 14% for dGMP) suggests further that the intrinsic mutagenic potential of Oxa is higher than that of Xan. Routledge et al. (45) treated plasmid DNA with NO in vitro and analyzed the mutation spectra after the transfection of the plasmid DNA into E.coli or human cells. The mutations were almost exclusively G:C to A:T and A:T to G:C transitions. The former mutations are consistent with the present result that Oxa and Xan direct the incorporation of dTMP during DNA replication. The involvement of uracil derived from nitrosative deamination of cytosine in G:C to A:T transitions was virtually ruled out by Schmutte et al. (46), who showed that the transfection of the NO-treated plasmid into an E.coli strain deficient in uracil-DNA glycosylase did not result in a significant increase in mutations. The additional A:T to G:C transitions obtained in the transfection assay are possibly attributable to hypoxanthine generated by nitrosative deamination of adenine that directs the incorporation of dCMP exclusively (11,12). Although AP sites could result from the depurination of Xan in DNA under acidic conditions or at high temperatures, it has been shown recently that the depurination rate of Xan is very low under physiological conditions of pH and temperature (24,40). Thus, the role of AP sites in NO-induced mutagenesis may be relatively inefficient and the efficiency of mutagenic insertion is lower than those for Oxa and Xan (Table II). Conversely, non-mutagenic dCMP was inserted most efficiently for Oxa and Xan, but mutagenic nucleotides were also incorporated. According to the preference for mutagenic nucleotides (Table II), Oxa would primarily yield G:C to A:T transitions, whereas Xan would yield G:C to A:T transitions and G:C to C:G transversions with comparable frequencies. The comparison of the insertion efficiencies of mutagenic nucleotides for Oxa (49% for dTMP) and Xan (16% for dTMP and 14% for dGMP) suggests further that the intrinsic mutagenic potential of Oxa is higher than that of Xan. Routledge et al. (45) treated plasmid DNA with NO in vitro and analyzed the mutation spectra after the transfection of the plasmid DNA into E.coli or human cells. The mutations were almost exclusively G:C to A:T and A:T to G:C transitions. The former mutations are consistent with the present result that Oxa and Xan direct the incorporation of dTMP during DNA replication. The involvement of uracil derived from nitrosative deamination of cytosine in G:C to A:T transitions was virtually ruled out by Schmutte et al. (46), who showed that the transfection of the NO-treated plasmid into an E.coli strain deficient in uracil-DNA glycosylase did not result in a significant increase in mutations. The additional A:T to G:C transitions obtained in the transfection assay are possibly attributable to hypoxanthine generated by nitrosative deamination of adenine that directs the incorporation of dCMP exclusively (11,12). Although AP sites could result from the depurination of Xan in DNA under acidic conditions or at high temperatures, it has been shown recently that the depurination rate of Xan is very low under physiological conditions of pH and temperature (24,40). Thus, the role of AP sites in NO-induced mutagenesis may be relatively inefficient. However, the mutagenic effect of Oxa-Sp has not been assessed in the reported studies and hence the mutation spectrum of Oxa-Sp in similar transfection assays remains to be determined.

In the present study, we have shown that Oxa-Sp constitutes a strong block to DNA synthesis and would elicit G:C to T:A transversions when bypassed. Although we could not assess the influence of the two forms of Oxa-Sp adducts (dOxo-Sp1 and dOxo-Sp2) on DNA replication separately, both Oxa-Sp adducts are unlikely to form functional hydrogen bonds with incoming dNTPs because the Watson–Crick face is severely altered. It is also possible that the bulky structures of Oxa-Sp adducts cause steric clashes with incoming dNTPs and hence further inhibits the binding of dNTP to the polymerase active site. Thus, we tentatively assume that similar to AP sites (47,48) and the ring-fragmentation products of thymine (49–51), Oxa-Sp adducts are non-instructive and that the observed preference of nucleotide insertion reflects the intrinsic property of Pol I Kf.

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