Lack of Miscoding Properties of 7-(2-Oxoethyl)guanine, the Major Vinyl Chloride-DNA Adduct

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ABSTRACT

Chloroethylene oxide, an ultimate carcinogenetic metabolite of vinyl chloride, was reacted with poly(deoxyguanylate-deoxycytidylate); the nucleic acid base adducts, 7-(2-oxoethyl)guanine and 3,N4-ethenocytosine, were analyzed by reverse-phase high-performance liquid chromatography. Chloroethylene oxide-modified poly(deoxyguanylate-deoxycytidylate) was assayed as template in a replication fidelity assay with Escherichia coli DNA polymerase I, and the newly synthesized product was subjected to nearest-neighbor analysis. Misincorporation of deoxyadenosine monophosphate and thymidine monophosphate were found to increase with the level of template modification. About 80% of the mispairing events were located opposite minor cytosine lesions. 7-(2-Oxoethyl)guanine, the major adduct identified (>98% of the adducts), did not miscode for either thymine or adenine, failing to support an earlier hypothesis that the cyclic hemiacetal form, O6,7-(1'-hydroxyethano)guanine, could, by analogy with O6-methyl- and O6-ethylguanine, simulate adenine. Our results indicate that direct miscoding of 7-(2-oxoethyl)guanine may contribute only slightly to the induction of mutations by chloroethylene oxide or vinyl chloride.

INTRODUCTION

The mutagenic and carcinogenic activity (4, 9, 15) of vinyl chloride has been shown to depend upon its metabolic activation into CEO (Ref. 3; see Chart 1). The carcinogenicity of CEO was demonstrated in mice (27), but no evidence has been obtained to date that chloroacetaldehyde, or adenine, failing to support an earlier hypothesis that the cyclic hemiacetal form, O6,7-(1'-hydroxyethano)guanine, could, by analogy with O6-methyl- and O6-ethylguanine, simulate adenine. Our results indicate that direct miscoding of 7-(2-oxoethyl)guanine may contribute only slightly to the induction of mutations by chloroethylene oxide or vinyl chloride.

DNA adducts (3, 16, 21, 27). Previous reports (2, 7, 23, 25) indicated a slightly decreased fidelity of replication of synthetic polynucleotides with Escherichia coli DNA polymerase I, when the templates contained 1,N4-ethenocytidine or 7-cytosine. As an extension of these studies, we have now investigated the coding properties of oxet-G, which has been proposed to miscode for thymine, following the formation of a cyclic hemiacetal at the O6-position (Ref. 21; see Chart 1).

MATERIALS AND METHODS

Treatment of Templates

Poly(dG-dC) (d20w = 8.0, 20.3 AMO units/mg, from P-L Biochemicals GmbH, St. Goar, Federal Republic of Germany) was prepared by dissolving 4.35 mg in 14.5 ml 50 mM sodium cacodylate buffer (pH 7.0):0.2 mM sodium chloride. CEO [purity >99.5%, prepared and stored as described previously (3)] in 1- to 5-μl aliquots was added under constant stirring at room temperature (21°C). A pH of 7.0 was maintained carefully by addition of 2 N NaOH using an autoburet and an automatic titrator. Under these conditions, CEO reacted very rapidly (half-life, about 3 min). The end of the reaction, after each addition, was indicated by a stable absorbance was monitored at 254 nm. Mobile phases were prepared with Milli-Q water (Millipore water purification system); methanol (LiChrosolv, E. Merck, Darmstadt, FRG) was used as eluant, and 80% of the mispairing events were located opposite minor cytosine lesions. 7-(2-Oxoethyl)guanine, the major adduct identified (>98% of the adducts), did not miscode for either thymine or adenine, failing to support an earlier hypothesis that the cyclic hemiacetal form, O6,7-(1'-hydroxyethano)guanine, could, by analogy with O6-methyl- and O6-ethylguanine, simulate adenine. Our results indicate that direct miscoding of 7-(2-oxoethyl)guanine may contribute only slightly to the induction of mutations by chloroethylene oxide or vinyl chloride.

By analogy to the promutagenic lesion O6-alkylguanine (22), it was suggested that the genetic and possibly carcinogenic effects of vinyl chloride might result from the persistence of miscoding
enzyenic hydrolysis. The former was performed in 200 μl 0.1 M hydrochloric acid (45 min, 75°C), and samples were analyzed by HPLC without further treatment. For enzymic hydrolysis, samples were dissolved in 200 μl 50 mM Tris-HCl (pH 6.75) containing 4 mg (8000 units/ml) DNase (EC 3.1.4.1; Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany) and incubated for 3 h at 37°C. Subsequently, 10 μg (0.015 units with bis-4-nitrophenyl phosphate as substrate) snake venom phosphodiesterase (EC 3.1.21.1; Boehringer Mannheim) and 10 μg (0.06 unit) acid phosphatase (EC 3.1.3.2; Boehringer Mannheim) were added, and incubation was continued for a further 15 h. Precipitated proteins were removed by centrifugation, and the supernatant was analyzed by HPLC.

Oxet-G was analyzed following either enzymatic or acid hydrolysis (14). Enzymatic hydrolysis was used to release 3',N'-ethenodeoxycytidine because it is unstable under acidic conditions. N2',3'-Ethenoguanine was analyzed by acid hydrolysis.

The nucleosides and bases generated by hydrolysis of CEO-modified poly(dG-dC) were separated by gradient elution on a 4.6-mm x 25-cm RP-18 Spheri-5 reverse-phase column (Brownlee Labs, Inc., Santa Clara, CA). Eluant A was 0.025 M ammonium formate (pH 5.5), and Eluant B was methanolic water (80:20, v/v). The gradient program was: Step 1, 0% Eluant B (isocratic, 10 min); Step 2, 0 to 2% Eluant B (linear, 5 min); Step 3, 2 to 9% Eluant B (linear, 6 min); Step 4, 9 to 13% Eluant B (linear, 6 min); Step 5, 13 to 30% Eluant B (linear, 10 min); Step 6, 30 to 0% Eluant B (linear, 5 min). The flow rate was set at 1 ml/min, and the column temperature was set at 30°C. Nucleosides and nucleobases used as optical markers for the test chromatograms were purchased from Sigma Chemical Co. (St. Louis, MO). 3',N'-Ethenodeoxycytidine and oxet-G were synthesized as described (14). N2',3'-Ethenoguanine was a generous gift of Dr. G. Doerjer (Institute of Toxicology, University of Mainz, Federal Republic of Germany) and was synthesized as described (16).

Analysis of Nucleotides. Nucleotides were resolved by anion-exchange chromatography on a MicroPak AX-10 column (4 mm x 30 cm; Varian, Saint Priest, France). The gradient consisted of Eluant A, water, and Eluant B, 11 mM orthophosphoric acid, pH 2.3 (analytical reagent grade). The program was: Step 1, 20 to 100% Eluant B (linear, 8 min); Step 2, 100% Eluant B (15 min); Step 3, 100 to 20% Eluant B (linear, 5 min). Runs were made at ambient temperature and at a flow rate of 4 ml/min.

Replication Fidelity Assays

Each reaction mixture (170 μl) contained 12.5 nmo/ untreated or CEO-treated poly(dG-dC) (nucleotide phosphorus); 400 pmol each of [8-³²P]-dGTP (185 dpm/pmol), dCTP, dATP, or dTTP, 5'-[α-³²P]dUTP (12,300 dpm/pmol), or 5'-[α-³²P]dATP (17,800 dpm/pmol) (free nucleoside 5'-triphosphates were obtained from P-L Biochemicals; radiolabeled nucleotides were from Amersham France, Versailles, France), and 1 unit E. coli MRE 600 DNA polymerase I (EC 2.7.7.7, Grade I; Boehringer Mannheim GmbH); 7 mM magnesium chloride; and 50 mM Tris-HCl buffer (pH 7.8). After 1 h incubation at 30°C, the solutions were spotted onto Whatman GF/C glass microfilter filters (Whatman S.A., Ferrières, France), and DNA was precipitated in cold 5% (w/v) trichloroacetic acid. The filters were rinsed 3 times in cold 5% trichloroacetic acid containing 2% (w/v) sodium pyrophosphate, twice in 5% trichloroacetic acid alone, and once in ethanol; the radioactivity present on the filters was determined by scintillation counting using Aquasol 2 (NEN Chemicals GmbH, Dreieich, Federal Republic of Germany).

Nearest-Neighbor Analysis

Template Mixture IX was used for nearest-neighbor analysis and for nicking by an AP endonuclease.

Replication of Control Template and Template IX, by E. coli DNA Polymerase I. The incubation medium (1.30 to 1.35 ml) consisted of 82.5 nmo/ control template or 95 nmo/ Template IX; 2 nmo/ each of dGTP, dCTP, dATP, or dTTP; [32P]dATP, or [32P]dTTP; 20.4 units E. coli DNA polymerase I; 4.5 mM magnesium chloride; and 50 mM Tris-HCl buffer (pH 7.8).

Purification of the Replicated Templates. After 1 h incubation at 30°C, the polynucleotides were purified from excess nucleotides on Elutip-d columns (Schleicher & Schuell GmbH, Dassel, Federal Republic of Germany). Samples were charged on the columns and washed repeatedly with a low-salt solution [0.2 M sodium chloride:1 mM EDTA:20 mM Tris-HCl buffer (pH 7.3 to 7.5)]. Templates were then eluted in a high-salt solution [1 M sodium chloride:1 mM EDTA:20 mM Tris-HCl buffer (pH 7.3 to 7.5)], desalted on Sephadex G-50F (same conditions as under "Treatment of Templates"), and lyophilized.

Enzymatic Hydrolysis (10). Each of the 4 templates (control template and Template IX labeled with 5'-[α-³²P]dAMP or with 5'-[α-³²P]dTMP (13 to 25 μg) was dissolved in 975 μl 5 mM Tris-Cl buffer (pH 8.6) with 2 mM calcium chloride. Micrococcal endonuclease (EC 3.1.31.1, from Staphylococcus aureus; Grade VI; Sigma) was added at 125 μg (0.1 unit) calf spleen phosphodiesterase (EC 3.1.16.1; Boehringer Mannheim) were added, and the mixture was incubated at 37°C. At the end of 1 h, the same amount of enzyme was added, and incubation was continued for another 2 h.

Determination of the Specific Activities of 3'-dCMP and 3'-dGMP. Enzymatic hydrolysates were brought to pH 2.3 by the addition of diluted orthophosphoric acid and centrifuged for 5 min at 10,000 x g to remove any particulate. The solutions were dried under a stream of nitrogen; the residues were then redissolved in water for HPLC analysis, and the radioactivity was determined in the fractions.

Nicking of AP Sites

Micrococcus luteus AP endonuclease B (a generous gift from Drs. J. Pierre and J. Laval, Institut Gustave Roussy, Villejuif, France) was used to nick phosphodiester bonds at AP sites (20). The reaction mixture (110 μl) contained 50 mM Tris-HCl buffer (pH 8.0), 2.5 mM magnesium chloride, 6.25 nmo/ poly(dG-dC) (nucleotide phosphorus), and 1 or 2 units AP endonuclease B. After 2 h at 37°C, the mixtures were heated at 50°C.

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RESULTS

HPLC Analysis of Nucleobase Adducts in CEO-treated Poly(dG-dC). Treatment of poly(dG-dC) (double-stranded form) with increasing concentrations of CEO resulted in the dose-dependent formation of oxet-G as the main alkylation product in poly(dG-dC) (Table 1, Column 2). Only trace amounts of 3,N\(^{-}\)-ethenodeoxycytidine were formed after treatment with the highest concentrations of CEO (Templates VI, VII, VIII; Table 1, Column 3). \(N^2\)-3-Ethenoguanine could not be detected.

Misincorporation of dTMP and dAMP in CEO-treated Poly(dG-dC) Templates. When poly(dG-dC) templates containing increasing amounts of oxet-G and cC were incubated in the presence of \(E. coli\) DNA polymerase I and the 4 deoxynucleoside 5\(-\)triphosphates, the rate of replication decreased, and the frequencies of dAMP and dTMP incorporation increased (Table 1, Columns 4, 5, and 6).

Nearest-Neighbor Analysis of Replicated Templates. In order to determine the sites of misincorporation of dAMP and dTMP, control poly(dG-dC) and Template IX were replicated with \(E. coli\) DNA polymerase I in the presence of \([\text{SP}]\text{dATP},\) or \([\text{SP}]\text{dTTP} \) and the 3 other nucleotides (Table 1). Following incubation, the templates were purified, hydrolyzed to nucleoside 3\(-\)mono-

- phosphates, and analyzed by HPLC and scintillation counting (Table 2). A major part of adenine was incorporated opposite cytosine, whereas thymine was distributed equally opposite cytosine and guanine; the 2 distributions varied by only 4 to 5% from one template to the other (Table 2, Columns 4 and 7). The frequencies of cytosine and guanine lesions to the misincorporation events were evaluated by their mispair frequencies (Table 2, Columns 5 and 8) calculated as error rate (Table 1, Columns 5 and 6) multiplied by the fraction of CpY or GpY sequences (Table 2, Columns 4 and 7; Y stands for A or T). The frequencies of mispairings induced by CEO treatment were calculated by subtracting the background frequencies (Table 2, Column 5) from the frequencies observed with Template IX (Table 2, Column 8): C:A, 13.2 \(\times\) 10\(^{-3}\); G:A, 3.3 \(\times\) 10\(^{-3}\); C:T, 0.9 \(\times\) 10\(^{-3}\); G:T, 1 \(\times\) 10\(^{-3}\). Thus, 80% of the CEO-induced dAMP incorporation occurred opposite cytosine residues; but the proportions of new C:T and G:T mismatches were roughly equal. The contributions of cytosine and guanine lesions to the overall dAMP and dTMP misincorporations induced by CEO treatment were 77 and 23%, respectively; therefore, cytosine lesions were about 3 times more efficient than were guanine lesions in decreasing the fidelity of DNA synthesis.

Misincorporation of dAMP and dTMP following Nicking of AP Sites. In order to assess the role of AP sites in the replication errors observed with CEO-treated poly(dG-dC), Template IX was nicked with \(M. luteus\) AP endonuclease B and replicated by \(E. coli\) DNA polymerase I in the presence of the 4 deoxynucleoside 5\(-\)triphosphates, including \([\text{PH}]\text{dGTP}\) and either \([\text{SP}]\text{dATP},\) or \([\text{SP}]\text{dTTP}\). After Template IX had been nicked by an AP endonuclease, the efficiency of DNA synthesis was enhanced. Taking the rate of replication of control poly(dG-dC) as 100% (see Table 1), the efficiency of replication of Template IX was

### Table 1

**CEO-modified poly(dG-dC) templates: HPLC analysis and misincorporation assays**

Poly(dG-dC) templates (Column 1) were obtained by reaction of poly(dG-dC) with various concentrations of CEO. After purification, the nucleotides were hydrolyzed either enzymatically at neutral pH (in presence of DNase I, snake venom phosphodiesterase, and acid phosphatase) or chemically at acid pH (in 0.1 N hydrochloric acid for 45 min at 75°C). The supernatants containing nucleotides and/or nucleobases were analyzed by HPLC. DNA adducts were identified by their retention volume and quantitated by their absorbance at 254 nm. All experiments were repeated at least twice; the data presented are representative. The contribution of noncomplementary nucleotides was measured by counting the radioactivity of \([\text{PH}]\text{GMP}\) (Column 4), \([\text{SP}]\text{dTMP}\) (Column 5), and \([\text{SP}]\text{AMP}\) (Column 6) in acid-insoluble material; blank values, obtained in the absence of polymerase or template, have been subtracted.

<table>
<thead>
<tr>
<th>Template</th>
<th>Oxet-G:guanine</th>
<th>cC:cytosine</th>
<th>dGMP incorporation (pmol)</th>
<th>dTMP:dGMP</th>
<th>dAMP:dGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(dG-dC)</td>
<td>0.014 ± 0.002</td>
<td>ND(^{a})</td>
<td>128.9 ± 12.6(^{\text{a}})</td>
<td>8.8</td>
<td>12</td>
</tr>
<tr>
<td>I</td>
<td>0.047 ± 0.002</td>
<td>ND</td>
<td>48.2 ± 5.3</td>
<td>4.0</td>
<td>13</td>
</tr>
<tr>
<td>III</td>
<td>0.123 ± 0.006</td>
<td>ND</td>
<td>22.8 ± 2.6</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>IV</td>
<td>0.185 ± 0.005</td>
<td>ND</td>
<td>33.2 ± 1.7</td>
<td>8.7</td>
<td>57</td>
</tr>
<tr>
<td>V</td>
<td>0.290 ± 0.012</td>
<td>ND</td>
<td>12.5 ± 1.6</td>
<td>7.3</td>
<td>57</td>
</tr>
<tr>
<td>VI</td>
<td>0.337 ± 0.009</td>
<td>0.0025</td>
<td>11.8 ± 1.6</td>
<td>16</td>
<td>98</td>
</tr>
<tr>
<td>VII</td>
<td>0.381 ± 0.007</td>
<td>0.0005</td>
<td>3.23 ± 0.35</td>
<td>24</td>
<td>150</td>
</tr>
<tr>
<td>VIII</td>
<td>0.361 ± 0.012</td>
<td>0.0005</td>
<td>3.02 ± 0.60</td>
<td>32</td>
<td>200</td>
</tr>
<tr>
<td>IX</td>
<td>0.360(^{b})</td>
<td>0.0005</td>
<td>3.53 (\times) 10(^{-3})</td>
<td>28</td>
<td>177</td>
</tr>
</tbody>
</table>

\(^{a}\) Roman numerals refer to poly(dG-dC) treated with various amounts of CEO.

\(^{b}\) Molar ratio, oxet-G determined by HPLC, following acid hydrolysis.

\(^{c}\) Molar ratio, determined as nucleobases by HPLC, following enzymatic hydrolysis.

\(^{d}\) Expressed as the noncomplementary-complementary nucleotide molar ratio incorporated on the templates.

\(^{e}\) Mean ± SD; \(n = 3\).

\(^{f}\) ND, not detected.

\(^{g}\) Mixture of equimolar amounts of Templates VI, VII, and VIII. These templates were pooled in order to facilitate nearest-neighbor analyses (see Table 2). Composition, replication rate, and error rates were calculated as the means of the corresponding values observed with Templates VI, VII, and VIII.
EFFECTS OF CEO-INDUCED LESIONS ON DNA REPLICATION

Table 2
Nearest-neighbor analyses of poly(dG-dC) templates and mispair frequencies

<table>
<thead>
<tr>
<th>Nearest-neighbor sequence</th>
<th>Poly(dG-dC)</th>
<th>Template IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAMP:dGMP</td>
<td>0.90</td>
<td>1.0</td>
</tr>
<tr>
<td>dTMP:dGMP</td>
<td>0.80</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Control poly(dG-dC) and CEO-treated poly(dG-dC) (Template IX) were replicated in the presence of E. coli DNA polymerase I and the 4 nucleoside 5'-triphosphates, including [α-32P]dATP and [α-32P]dGTP. Templates were then hydrolyzed to nucleoside 3'-monophosphates, which were separated by HPLC and counted by liquid scintillation. Fractions (Columns 4 and 7) of the nearest-neighbor sequences were calculated from the specific activities of 3'-dCMP and 3'-dGMP (Columns 3 and 6). Each XpY sequence corresponds to a X:Y mispair formed between template and daughter strand (X stands for G or C, Y for A or T). Data from the misincorporation assays (Table 1, Columns 5 and 6) were combined with data from nearest-neighbor analyses, and the mispair frequencies (Columns 5 and 8) were calculated as

X:Y mispair frequency = [X:pY, nearest-neighbor sequence fraction] x [dYMP/dGMP, error rate].

The error rates were: for poly(dG-dC), dAMP:dGMP = 1.2 × 10^-3, dTMP:dGMP, 0.89 × 10^-3, for Template IX, dAMP:dGMP, 17.2 × 10^-3, dTMP:dGMP, 2.8 × 10^-3.

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We wish to thank Dr. A. Crosey (Institut Curie, Orsay, France) for the preparation of CEO, Drs. J. Pierre and J. Laval (Institut Gustave Roussy, Villejuif, France) for the kind gift of AP endonuclease B, and J.-C. Bérelzé for skilful technical assistance. We are indebted to M. Wriscez for typing the manuscript and to E. Heseltine for editorial assistance.

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was 3%; it was 12% following incubation with 1 unit of AP endonuclease and 20% when 2 units of enzyme were used.

The error rates of dTMP:dGMP and dAMP:dGMP were decreased by factors of 1.7 and 2.4, respectively, after incubation of Template IX with 2 units of endonuclease. There were different effects on dAMP incorporation and replication rate; the replication rate was doubled when 2 units of enzyme were used instead of 1, but there was no further (or only a marginal) reduction in the dAmp:dGMP ratio.

DISCUSSION

In HPLC analyses, following the reaction of CEO with poly(dG-dC) in a double-stranded form (Table 1), oxet-G amounted to >98% of the total adducts identified. This finding is consistent with those obtained by analysis of liver DNA from vinyl chloride-treated rodents (13, 14, 19). A specific ethenoguanine may represent only a small proportion of the total adducts identified. This finding is consistent with its nucleophilic selectivity [Swain-Scott's constant, $s = 0.83$ (8)]; by comparison with a nonmiscoding adduct (25), was not investigated. This differential selectivity of CEO, Drs. J. Pierre and J. Uval (Institut Gustave Roussy, Villette, France) for the possible role of minor vinyl chloride:DNA adducts (1,13,17,18,24).

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