steady-state kinetic analyses were also performed to measure the relative bypass frequencies past the 8-Cl-dG.

Materials and methods

General

Ultrapure dNTPs were from GE Healthcare Corp. Exonuclease-free Klenow fragment and *Eco*RI restriction endonuclease were purchased from New England Biolabs, Inc. (Ipswech, MA). 2′-Deoxyguanosine-5′-triphosphate (dGTP) and Blue Dextran were obtained from Sigma–Aldrich Corp. (St. Louis, MO). DNA templates, primers, AlexaFlour-546 dye (Alexa546)-labelled primers and standard markers were purchased from Japan Bio Service Corp. (Saitama, Japan). Alexa546 was conjugated at the 5′-terminus of primers and standard markers. These oligodeoxynucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE) before use. Human pol α (1200 units/mg of protein) was purchased from CHIMERx (Milwaukee, WI). Human pol κ was prepared as C-terminal truncations with 10 His-tags, as previously described (38). Human pol η was kindly provided by Drs. Chikahide Masutani and Fumio Hanaoka.

Synthesis of 8-Cl-dGTP

8-chloro-2′-deoxyguanosine-5′-triphosphate (8-Cl-dGTP) was synthesised as previously described (39). Briefly, 10 mM dGTP was reacted with 1 mM sodium hypochlorite (NaOCl) in 250 mM sodium phosphate buffer (pH 8.0) containing 20 μM nicotine at 37°C for 1 h (Figure 1a). The reaction was terminated by adding 10 mM methionine. The reaction mixture containing 8-Cl-dGTP was fractioned by high-performance liquid chromatography (HPLC) on a reversed phase SunFire C18 column (0.46 × 15 cm; Waters Corp.), eluted over 40 min at a flow rate 1.0 ml/min with a linear gradient of 50 mM triethylamine acetate (pH 7.0) containing 0–15% acetonitrile. Separation and purification were performed by using HPLC LC-20AB pump, SPD-M20A photodiode array detector and CBM-20A system control unit (Shimadzu Corp., Kyoto, Japan).

Enzymatic preparation of 8-Cl-dG-modified oligodeoxynucleotide

The modified 38-mer template (5′CATGCTGATGAATTCCTTCXCTTCTTT CCTCTCCCTTT, X = 8-Cl-dG) containing a single 8-Cl-dG was prepared by enzymatic incorporation of 8-Cl-dGTP using a 19-mer oligodeoxynucleotide (5′CATGCTGATGAATTCCTTC) and exonuclease-free Klenow fragment followed by ligation to an 18-mer (5′CTTCTTTCCTCTCCCTTT) with T4 DNA ligase (Figure 2), as described in our previous work (40).

Briefly, to prepare a 20-mer (5′CATGCTGATGAATTCCTTCX. X = 8-Cl-dG) containing 8-Cl-dG at the 3′-terminus, 8-Cl-dGTP (20 μM) was put into the reaction mixture that contains an unlabelled or Alexa546-labelled 19-mer primer (250 ng,5′CATGCTGATGAATTCCTTC), a 38-mer template (500 ng, 5′AAAGGGAGAGGAAAGAAZCGAAGGAATTCATCAGCATG, Z = AP-site), and exonuclease-free Klenow fragment (10 units) in 20 μl of 1× NEBuffer 2 [10 mM Tris–HCl (pH 7.9), containing 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT)]. After the incubation at 25°C for 1 h, the reaction was stopped by heating the sample to 75°C for 10 min. The AP-site neighbouring dCMP:8-Cl-2′-deoxycytidine monophosphate (dGMP) base site inhibited >2-mer extension by 8-Cl-dGTP incorporation at the 3′-turminus of primer (Figure 3a). We subjected the reaction sample to 20% denaturing PAGE (30 × 40 × 0.05 cm) containing 7 M urea and recovered the 8-Cl-dG-incorporated 20-mer from gel. The purified 8-Cl-dG-modified 20-mer was ligated with 5′-phosphorylated 18-mer (2 μg, 5′CTTCTTTCCTCTCCCTTT) at 4°C overnight using T4 DNA ligase (1400 units) (Takara Bio, Inc., Shiga, Japan), 0.1 mM Adenosine triphosphate (ATP) and a 24-mer template (3 μg, 5′AGGAAAGAAGCGAAGGAATTCATC) in 50 μl of 66 mM Tris–HCl (pH 7.6) containing 6.6 mM $MgCl_2$ and 10 mM DTT. The resultant 8-Cl-dG 38-mer was also purified by using 20% denaturing PAGE (Figure 2).

Liquid chromatography tandem mass spectrometry analysis of oligodeoxynucleotides

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses were performed using a Quattro Ultima triple stage quadrupole MS (Waters-Micromass, Manchester, UK). Full-scan mode with the range of *m/z* 400-800 was conducted to monitor multicharged ion of the oligonucleotide. The oligonucleotides were dissolved with 50% methanol (approximately 6 μg/ml) and infused into mass spectrometer by using syringe pump at the flow rate of 600 μl/min. MS analyses were carried out in negative ion mode with nitrogen as the nebulizing gas. The ion source temperature was 100°C, the desolvation gas temperature was 200°C and the cone voltage was operated at a constant 35 V. Nitrogen gas was also used as the desolvation gas (700 l/h) and cone gas (35 l/h).

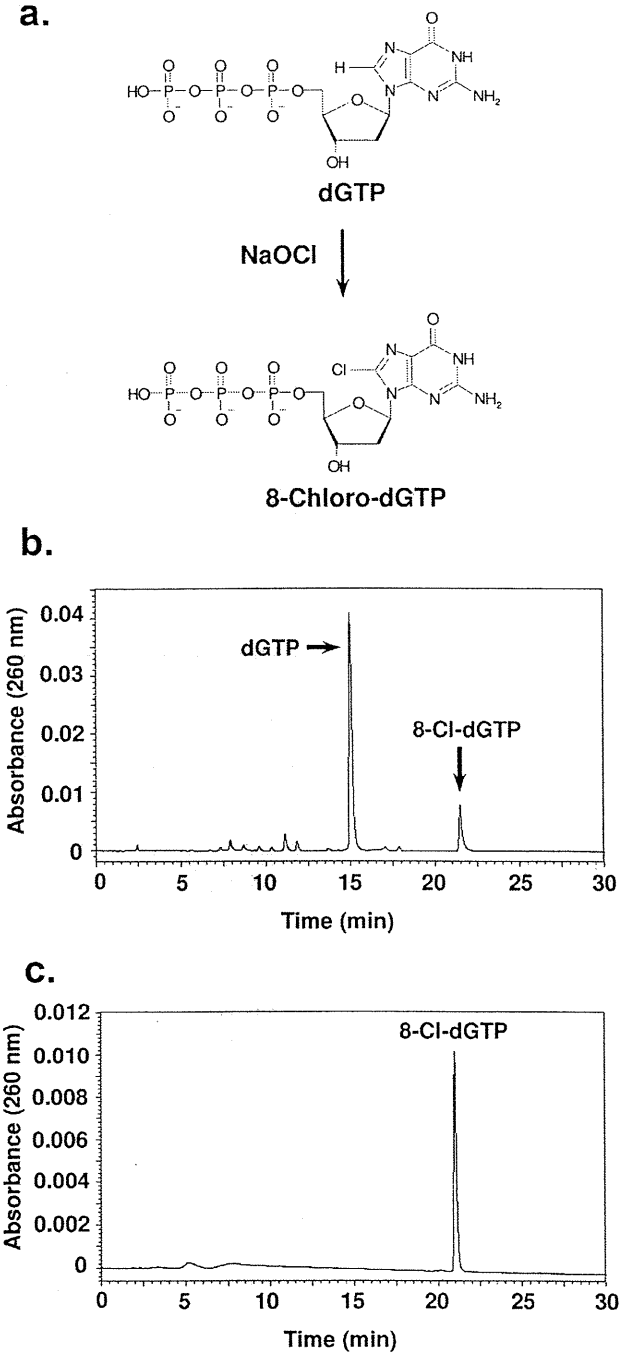

Fig. 1. Formation of 8-Cl-dGTP. (a) Synthesis of 8-Cl-dGTP. dGTP was reacted with NaOCl in the presence of nicotine in sodium phosphate buffer (pH 8.0) as described in Materials and methods. (b) HPLC separation of 8-Cl-dGTP. (c) HPLC analysis of purified 8-Cl-dGTP. 8-Cl-dGTP was isolated from dGTP on a reversed phase SunFire C18 column (0.46 × 25 cm; Waters) using a linear gradient of 50 mM triethylamine acetate (pH 7.0) containing 0–15% acetonitrile over 40 min at a flow rate 1.0 ml/min.

Enzymatic digestion of oligodeoxynucleotides

A 20-mer oligodeoxynucleotide-containing 8-Cl-dG at the 3′-terminus was incubated at 37°C for 2 h in 100 μl of 30 mM sodium acetate (pH 7.0) containing 1 mM zinc sulphate, P1 nuclease (8 units) (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and *E. coli* alkaline phosphatase (5 units) (Sigma–Aldrich Corp.). Then 20 μl of 0.5 M Tris–HCl (pH 8.5) was added

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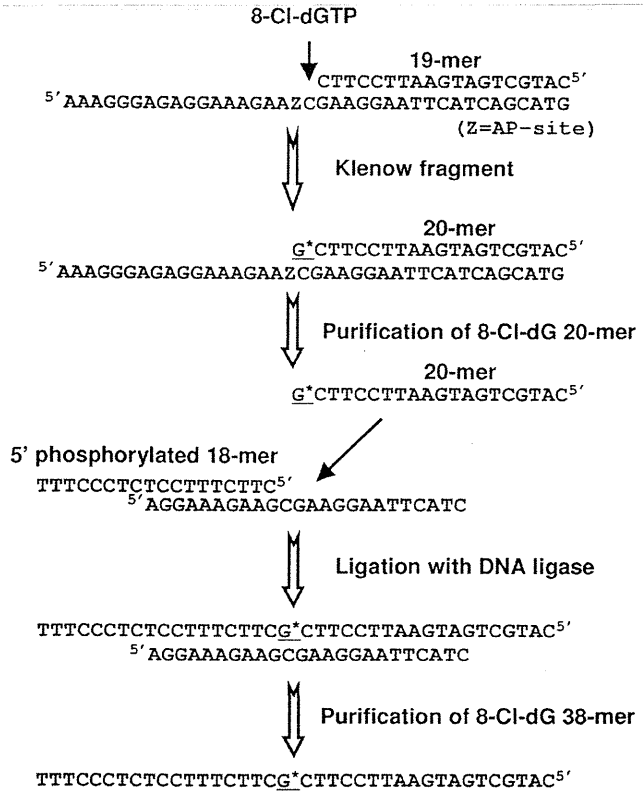

Fig. 2. Diagram of enzymatic preparation of 8-Cl-dG-modified 38-mer oligodeoxynucleotide.

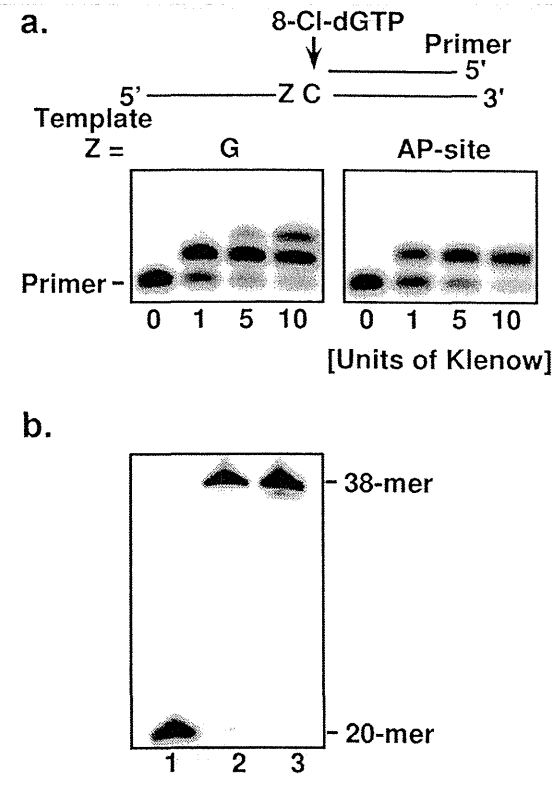

Fig. 3. Preparation of 8-Cl-dG-modified oligodeoxynucleotide. (a) Incorporation of 8-Cl-dGTP by exonuclease-free Klenow fragment (0, 1, 5 or 10 units) on without (left) and with (right) AP-site on the templates. Accumulation of 1-mer-extended 20-mer containing 8-Cl-dGMP at the 3′-terminus was observed on AP-site-introduced templates. The AP-site inhibited >2-mer extension by 8-Cl-dGTP incorporation at the 3′-turminus of the primer, resulting in the efficient recovery of 8-Cl-dG-modified 20-mer. (b) Ligation of 8-Cl-dG-modified 20-mer with 18-mer. Alexa546-labelled 20-mer-containing 8-Cl-dG was ligated with 5′-phosphorylated 18-mer at 4°C overnight using T4 DNA ligase (1400 units). The reaction mixture was analysed by 20% denaturing PAGE. Lane 1, 8-Cl-dG-modified 20-mer; lane 2, ligation product of 8-Cl-dG-modified substrate; lane 3, 38-mer standard.

to the reaction mixture and further incubated at 37°C for 2 h. The mixture was evaporated to dryness, and the resultant deoxynucleosides were extracted three times each with 100 µl of ethanol. Ethanol extracts were evaporated to dryness, dissolved in distilled water and analysed by HPLC, using COSMOSIL 5C18-MS-II column (0.46 × 25 cm; Nacalai Tesque, Inc., Kyoto). Elution was carried out using a linear gradient of 50 mM triethylamine acetate (pH 7.0) containing 0–16% acetonitrile over 40 min at a flow rate of 1.0 ml/min.

Primer extension reactions

Primer extension reactions catalysed by pol α, κ and η were conducted at 25°C for 30 min in a buffer (10 µl) containing all four dNTPs (100 µM each), using 8-Cl-dG-modified and unmodified 38-mer templates (750 fmol) primed with an Alexa546-labelled 10-mer (500 fmol, 5′AGAGGAAAGA) (Figure 5). The reaction buffer for pol α contains 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 60 mM KCl, 10 mM DTT, 250 µg/ml bovine serum albumin (BSA), and 2.5% glycerol. The reaction buffer for pol η and κ contains 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM DTT, 250 µg/ml BSA, 60 mM KCl and 2.5% glycerol. Reaction was stopped by addition of 2 µl of formamide dye containing Blue Dextran (100 mg/ml) and ethylenediaminetetraacetic acid (50 mM) and incubation at 95°C for 3 min. The whole amount of the reaction sample was subjected to 20% denaturing PAGE (30 × 40 × 0.05 cm). The separated products were visualised by using Molecular Imager FX Pro and Quantity One software (BioRad Laboratories).

Quantitation of miscoding specificities and frequencies

Using the 8-Cl-dG-modified and unmodified 38-mer oligodeoxynucleotide (750 fmol) primed with an Alexa546-labelled 10-mer (500 fmol, 5′AGAGGAAAGA), we conducted primer extension reactions catalysed by pol α (2000 fmol), κ (50 fmol) or η (50 fmol) at 25°C for 30 min in a buffer (10 µl) containing all four dNTPs (100 µM each) and subjected them to 20% denaturing PAGE (30 × 40 × 0.05 cm) (Figure 5). We used the amount of these pols so that fully extended products (approximately 28–34 mer) were 70–90% of the starting primer in primer extension reactions. The fully extended products were extracted from the gel. The recovered oligodeoxynucleotides were annealed with the unmodified 38-mer, cleaved with *Eco*RI and subjected to two-phased PAGE (20 × 65 × 0.05 cm) containing 7 M urea in the upper phase and no urea in the middle and bottom phases (each phase containing 18, 20 and 24% polyacrylamide, respectively). The phase width is approximately 10, 37 and 18 cm from the upper phase. To quantify base substitutions and deletions, we compared the mobility of the reaction products with those of Alexa546-labelled 18-mer standards containing dC, dA, dG or dT opposite the lesion and one- (Δ^1) or two-base (Δ^2) deletions (Figure 5) (29–31). The separated products were visualised by Molecular Imager FX Pro, and the bands were quantified by using Quantity One software (BioRad Laboratories). The percentage of 2′-deoxynucleoside monophosphate (dNMP) incorporation was normalised to the amount of the starting primer.

Steady-state kinetic studies of nucleotide insertion and extension

Kinetic parameters associated with nucleotide insertion opposite the 8-Cl-dG lesion and chain extension from 3′ primer terminus were determined at 25°C, using varying amounts of single dNTPs. For insertion kinetics, reaction mixtures containing dNTP (0–1000 µM) and pol κ (7–20 fmol) were incubated at 25°C for 0.5–3 min in 10 µl of Tris–HCl buffer (pH 8.0), using a 38-mer template (750 fmol) primed with an Alexa546-labelled 12-mer (500 fmol; 5′AGAGGAAAGAAG). To measure chain extension, reaction mixtures containing a 38-mer template (750 fmol) primed with an Alexa546-labelled 13-mer (500 fmol; 5′AGAGGAAAGAAGN, where N is C, A, G or T), with varying amounts of dGTP (0–500 µM) and pol κ (10 fmol) were incubated at 25°C for 0.5–2 min. The reaction samples were subjected to 20% denaturing

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PAGE (30 × 40 × 0.05 cm). The Michaelis contents (K_m) and maximum rates of reaction (V_{max}) were determined by Enzyme Kinetics Module 1.1 of SigmaPlot 2001 software (SPSS, Inc.). Frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) were determined relative to the dC:dG base pair according to the following equation: (41,42)

$$F = (V_{max}/K_m)_{[\text{wrong pair}]}/(V_{max}/K_m)_{[\text{correct pair = dC:dG}]}.$$

Results

Enzymatic preparation of site-specifically modified oligodeoxynucleotide containing a single 8-Cl-dG

dGTP was incubated with NaOCl in phosphate buffer and then subjected to HPLC. 8-Cl-dGTP was efficiently formed and completely isolated from dGTP (Figure 1b). The retention time of 8-Cl-dGTP (21.3 min) was longer than that of dGTP (14.8 min). We purified twice the fraction of 8-Cl-dGTP (Figure 1c) and characterised it by LC–MS scanning the molecular ion peak of 8-Cl-dGTP (*m/z* 539.5). The 8-Cl-dG-modified 20-mer oligodeoxynucleotide was prepared by enzymatic incorporation of 8-Cl-dGTP by exonuclease-free Klenow fragment (Figure 3a), as described in Materials and methods. The 8-Cl-dG-modified and unmodified 20-mers were digested with P1 nuclease and alkaline phosphatase, and the resultant product was subjected to HPLC (Figure 4). The obtained molar ratio was consistent with the theoretical value of the nucleoside composition (dC:dA:dG:dT:8-Cl-dG = 5:4:3:7:1) of the 20-mer. Furthermore, the 8-Cl-dG-modified 20-mer (molecular weight 6117.4) was characterised by using LC–MS/MS analysis (Supplementary Figure S1 is available at *Mutagenesis* Online). The characterised 8-Cl-dG-modified 20-mer was ligated with 5′-phosphorylated 18-mer as described in Materials and methods. Most of the 8-Cl-dG-modified 20-mer was ligated with the 18-mer (Figure 3b, lane 2), resulting in the formation of 38-mer containing 8-Cl-dG adduct. The 8-Cl-dG-modified 38-mer was purified from the 20% denaturing PAGE.

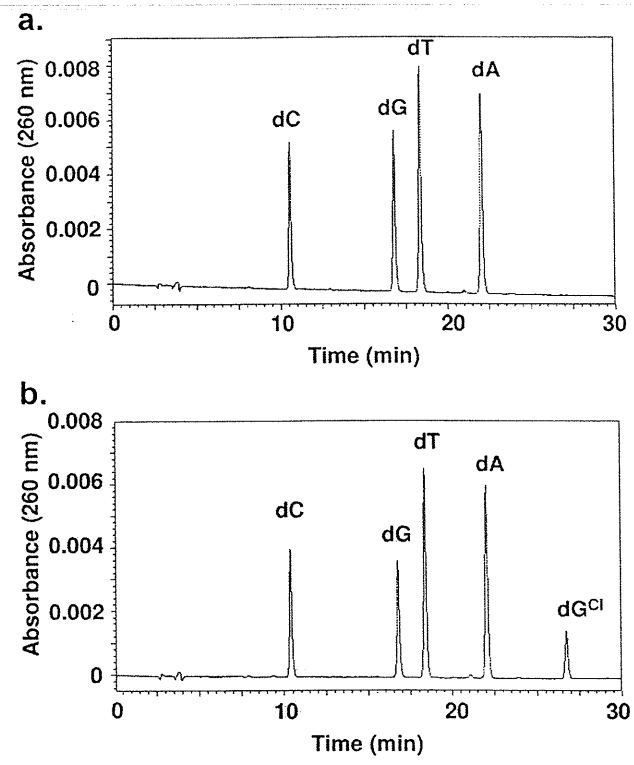

Fig. 4. HPLC elution profiles of enzymatic digests of unmodified and 8-Cl-dG-modified 20-mer. Three micrograms of unmodified (a) or 8-Cl-dG-modified (b) 20-mer (5′CATGCTGATGAATTCCTTCX, X = dG or 8-Cl-dG) was digested to nucleosides as described in Materials and methods. Ethanol extracts of the digested samples were evaporated to dryness and placed on a COSMOSIL 5C18-MS-II column (0.46 × 25 cm; Nacalai Tesque Inc., Kyoto). Elution was carried out using a linear gradient of 50 mM triethylamine acetate (pH 7.0) containing 0–16% acetonitrile over 40 min at a flow rate of 1.0 ml/min.

Primer extension reactions catalysed by human DNA pols on 8-Cl-dG-modified DNA template

Primer extension reactions were carried out using 8-Cl-dG-modified 38-mer templates in the presence of all four dNTPs and varying amounts of pol α, κ and η (Figure 5). As shown in Figure 6, the primer extension on the unmodified dG template rapidly occurred to form fully extended products by pol α, κ and η. When the 8-Cl-dG-modified template was used, the primer extension reactions catalysed by pol α and κ were slightly retarded prior to and opposite the lesion (Figure 6a and 6b). Pol η easily bypassed the 8-Cl-dG as efficiently as unmodified dG (Figure 6c). Blunt-end addition to the fully extended product (33–34 mers) was observed in all primer extension reactions, as reported earlier for *E.coli* and mammalian DNA pols (43,44).

Miscoding specificities of 8-Cl-dG adduct

Translesion synthesis catalysed by pol α, κ and η was conducted in the presence of all four dNTPs. The fully extended products (approximately 28–34-mers) past 8-Cl-dG lesion site were recovered from gel, digested by *Eco*RI and subjected to two-phased PAGE for quantitative analysis of base substitutions and deletions, as described in Materials and methods. A standard mixture of six Alexa546-labelled oligomers containing dC, dA, dG or dT opposite the lesion or one- and two-base deletions can be resolved by this method (Figure 5). When unmodified dG template was used, the incorporation of dCMP, the correct base, was observed opposite dG at 72.8%, 76.8% and 67.3% of the starting primers for pol α, κ and η, respectively (Figure 7). Using 8-Cl-dG-modified template, pol κ incorporated dCMP (63.6%) as the primary product opposite the 8-Cl-dG. Moreover, pol κ promoted one-base deletion (6.4%), associated with dGMP (5.5%), dAMP (3.7%) and dTMP (3.5%) misincorporations opposite the lesion (Figure 7b). Pol α and η exclusively incorporated dCMP opposite 8-Cl-dG lesion (Figure 7a and 7C). Small amounts of unknown products were also detected in the extension by pol η on both dG-unmodified and modified templates (arrowheads in Figure 7c). The migration of these unknown products differed from those of standard markers, implying that pol η might misinsert nucleotide on undamaged bases other than the position of the adduct site, as reported previously (45).

Steady-state kinetic studies on 8-Cl-dG-modified DNA template

Using steady-state kinetic methods, the frequency of dNTP incorporation (F_{ins}) opposite 8-Cl-dG and chain extension (F_{ext}) from the primer terminus by pol κ were measured. The F_{ins} value for dGTP (1.24×10^{-2}) was 9.7 times lower than that for dCTP (0.12), the correct base, but 2.5 and 3.7 times higher than that for dATP (5.02×10^{-3}) and dTTP (3.39×10^{-3}),

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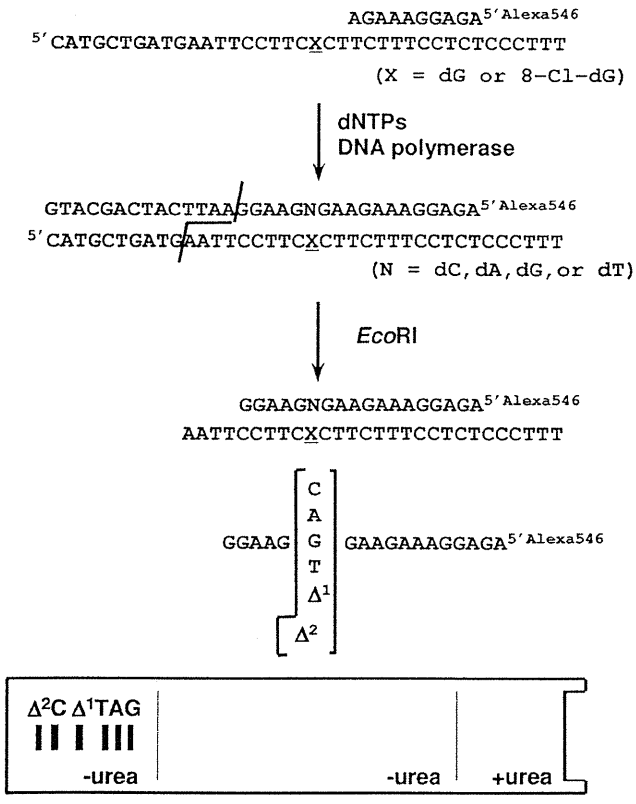

Fig. 5. Diagram of the method used to determine miscoding specificity. Unmodified and 8-Cl-dG-modified 38-mer templates were annealed to Alexa546-labelled 10-mer primer. Primer extension reactions catalysed by pol α, κ, and η were conducted in the presence of all four dNTPs. Fully extended products formed during DNA synthesis were recovered from the denaturing 20% polyacrylamide gel, annealed with the complementary 38-mer, cleaved with *Eco*RI, and subjected to two-phased PAGE. To determine miscoding specificities, mobilities of the reaction products were compared with those of 18-mer standards containing dC, dA, dG or dT opposite the lesion, and one-base- (Δ^1) or two-base (Δ^2) deletions.

respectively (Table 1). F_{ext} for dG:8-Cl-dG (2.47×10^{-3}) was 19.8 and 1.3 times lower than that for dC:8-Cl-dG (4.89×10^{-2}) and dT:8-Cl-dG (3.13×10^{-3}), but 1.4 times higher than that for dA:8-Cl-dG (1.76×10^{-3}). Therefore, the relative bypass frequency ($F_{ins} \times F_{ext}$) past the dG:8-Cl-dG pair was 192 times lower than that for dC:8-Cl-dG, but 3.5 and 2.9 times higher than that for dA:8-Cl-dG and dT:8-Cl-dG.

Discussion

Enzymatic preparation method of site-specifically modified oligodeoxynucleotide containing a single DNA adduct is a valuable tool for studying mechanisms of DNA repair and mutagenesis involving adducts (40). The conventional method for preparing such adduct-modified oligomers requires the phosphoramidite derivative of the adduct. However, it generally takes a long time to establish its synthetic method, and the number of commercially available phosphoramidites is limited. We employed the enzymatic preparation using 8-Cl-dGTP as a starting material to make modified oligomer because 8-Cl-dG phosphoramidite was also unavailable commercially. Actually, we now can purchase many types of synthetic dNTPs containing a damaged base and an analogue from chemical companies.

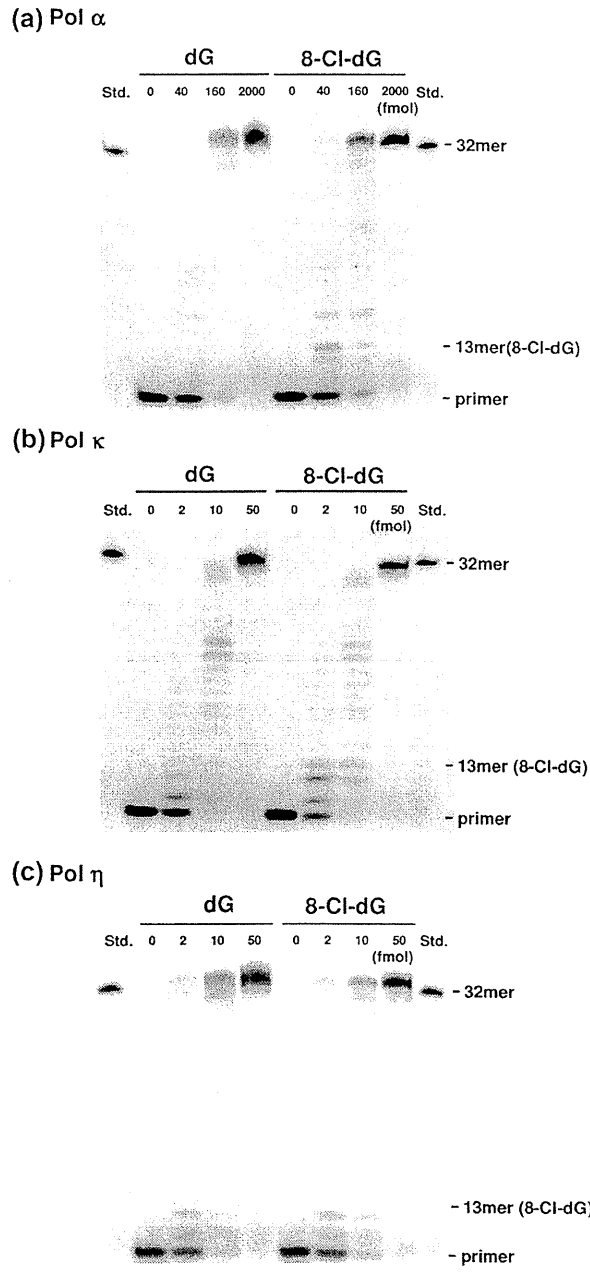

Fig. 6. Primer extension reactions catalysed by DNA polymerases on 8-Cl-dG-modified DNA templates. Unmodified and 8-Cl-dG-modified 38-mer templates were annealed to Alexa546-labelled 10-mer primer. Primer extension reactions catalysed by variable amounts (0–2000 fmol) of pol α, κ, and η were conducted at 25°C for 30 min in the presence of four dNTPs. Whole amount of the reaction mixture was subjected to 20% denaturing PAGE. 13-mer(8-Cl-dG) marks the location opposite the DNA adducts.

Thus, the enzymatic preparation method may be a useful addition to the phosphoramidite method when preparing DNA adduct-modified oligomers.

The miscoding specificities of 8-Cl-dG was determined by using an *in vitro* experimental system that can quantify base substitutions and deletions formed during the replication by pol α, κ and η in the presence of all four dNTPs (Figure 5). When pol α and η was used, both pols preferentially incorporated dCMP opposite 8-Cl-dG (Figure 7a and 7C). In contrast, pol

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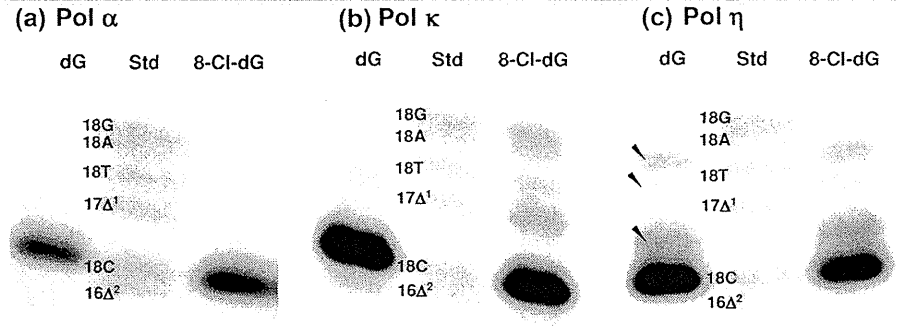

Fig. 7. Miscoding specificities of 8-Cl-dG lesion in reactions catalysed by pol α, κ, and η. Using unmodified and 8-Cl-dG-modified 38-mer templates primed with an Alexa546-labelled 10-mer, we conducted primer extension reactions at 25°C for 30 min in a buffer containing four dNTPs (100 μM each) and either pol α (2000 fmol for unmodified and 8-Cl-dG-modified templates), pol κ (50 fmol for unmodified and 8-Cl-dG-modified templates), or pol η (50 fmol for unmodified and 8-Cl-dG-modified templates), as described in Materials and methods. The extended reaction products (>28 bases long) produced on the unmodified and modified templates were extracted following 20% denaturing PAGE. The recovered oligodeoxynucleotides were annealed to the complementary unmodified 38-mer and cleaved with *Eco*RI restriction enzyme. The entire product from the unmodified and 8-Cl-dG-modified templates was subjected to two-phased PAGE (20 × 65 × 0.05 cm). Mobilities of reaction products were compared with those of 18-mer standards (Figure 5) containing dC, dA, dG or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions.

κ promoted one-base deletion with lesser amounts of dGMP, dAMP and dTMP misincorporation (Figure 7b). Thus, 8-Cl-dG can form mismatches with all wrong bases in DNA duplexes that can cause mutations. The steady-state kinetic analyses also supported these results (Table 1). This indicates that the broad miscoding spectrum of 8-Cl-dG may be generated in inflamed tissues.

Recently, 8-Cl-dG adduct has been detected in DNA from LPS-treated rat liver by LC-MS/MS analysis (27). The study showed that the relative abundance of 8-Cl-dG in hepatic DNA and urine of rat was at least 2 orders of magnitude lower than that of 8-oxo-dG. In the present study, $F_{ins} \times F_{ext}$ ratio for the dG:8-Cl-dG/dC:8-Cl-dG of pol κ was 0.0052, which is ~6200-fold lower than that for the dA:8-oxo-dG/dC:8-oxo-dG (Table 2). Overall, 8-Cl-dG is apparently less-mutagenic lesion than 8-oxo-dG.

In spite of the chemical similarity, the miscoding specificities of 8-Cl-dG were slightly different from those of 8-bromo-2′-deoxyguanosine (8-Br-dG), another major halogenated adduct (46). Pol α could bypass 8-Cl-dG in error-free manner (Figure 7a). By contrast, pol α promoted one-base deletion during the bypass of 8-Br-dG (46). Moreover, $F_{ins} \times F_{ext}$ ratio for the dG:8-Cl-dG/dC:8-Cl-dG of pol κ was 23-fold lower than that for the dG:8-Br-dG/dC:8-Br-dG (Table 2). The difference of the miscoding potential between 8-Cl-dG and 8-Br-dG may be explained by the destability effect of each halogenated base in DNA duplex. Actually, 8-Br-dG:dC base pair is less stable than 8-Cl-dG:dC in DNA duplexes as observed in the melting temperature assay (28,47). The covalent atomic radii of bromine and chlorine atoms are 114 and 99 picometre, respectively (48). The greater atomic radius of C8 atom may have more steric hindrance between the deoxyribose and C8 atom of modified guanine to destabilise dG:dC base pair, enhancing the slippage of DNA polymerases, such as one-base deletion by pol α and κ, as well as other bulky DNA adducts (49–52).

At sites of inflammation, 8-Cl-dG must be repaired by cellular DNA repair enzymes because urinary excretion of 8-Cl-dG in LPS-treated rats has been observed (27). Actually, 8-Cl-dGTP is degraded by human mutT homologue *in vitro* (39). Thus, C8-chlorinated guanine molecule in DNA may be recognised and removed by DNA repair mechanism such as base excision repair, as well as 8-oxo-dG. To investigate this possibility, we examined the incision of 8-Cl-dG paired with dC in DNA duplex by DNA glycosylases and endonucleases including human 8-oxoguanine DNA glycosylase 1 (hOGG1), endonuclease VIII-like 1 (hNEIL1), alkyladenine DNA glycosylase (hAAG), *E. coli* formamidopyrimidine DNA

Table I. Kinetic parameters for nucleotide insertion and chain extension reactions catalysed by human DNA pol κ

N:X	Insertion dNTP			Extension dGTP			$F_{ins} \times F_{ext}$
	↓GAAGAAAGGAGA			↓NGAAGAAAGGAGA			
	5′CCTTCXCTTCTTTCCTCTCCCTTT			5′CCTTCXCTTCTTTCCTCTCCCTTT			
	K_m (μM)[a]	V_{max} (%/min)[a]	F_{ins}	K_m (μM)[a]	V_{max} (%/min)[a]	F_{ext}	
C:G	9.04±2.95	8.37±1.09	1	1.63±0.16	7.82±0.40	1	1
C:X	47.8±25.3	5.10±1.16	0.12	19.1±3.84	4.48±0.39	4.89×10^{-2}	5.87×10^{-3}
A:X	409±96.2	1.90±0.20	5.02×10^{-3}	180.0±32.3	1.52±0.12	1.76×10^{-3}	8.84×10^{-6}
G:X	53.2±20.3	0.61±0.10	1.24×10^{-2}	229.3±38.3	2.72±0.21	2.47×10^{-3}	3.06×10^{-5}
T:X	1939±655	6.09±1.48	3.39×10^{-3}	115.7±15.0	1.74±0.08	3.13×10^{-3}	1.06×10^{-5}

Kinetics of nucleotide insertion and chain extension reactions were determined as described in Materials and methods. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the following equation: $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair\ =\ dC:dG]}$. X = 8-Cl-dG lesion.
[a]Data were expressed as mean ± SD obtained from three independent experiments.

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Table II. $F_{ins} \times F_{ext}$ past DNA adducts by human DNA pol κ

	X	8-Cl-dG (Table I)	8-oxo-dG (32)	8-Br-dG (46)
Pol κ	C:X	5.87×10^{-3}	6.21×10^{-4}	3.00×10^{-3}
	A:X	8.84×10^{-6}	**2.00×10^{-2}**	8.55×10^{-6}
	G:X	**3.06×10^{-5}**	7.65×10^{-8}	**3.56×10^{-4}**
	T:X	1.06×10^{-5}	5.98×10^{-5}	1.41×10^{-5}

Values in boldface indicate a primarily misincorporated base opposite the DNA adduct.

glycosylase (FPG) and endonuclease V (EndoV). Surprisingly, 8-Cl-dG is hardly cleaved by any enzymes (Supplementary Figure S2 is available at *Mutagenesis* Online). This suggests that 8-Cl-dG may be removed by the DNA repair machinery other than those DNA repair enzymes in cells. Further investigation is expected to elucidate the mechanism of repair for the inflammation-associated DNA adduct.

In conclusion, two-phased PAGE analysis and steady-state kinetic studies were performed to determine the miscoding specificities of the chlorinated DNA adduct 8-Cl-dG. 8-Cl-dG is a mutagenic lesion; the miscoding frequency and specificity varies depending on the DNA polymerase used. Thus, HOCl-induced 8-Cl-dG adduct may generate mutations at sites of inflammation.

Supplementary data

Supplementary Figures S1 and S2 are available at *Mutagenesis* Online.

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A combination of *in vitro* comet assay and micronucleus test using human lymphoblastoid TK6 cells

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The comet assay has been widely used as a genotoxicity test for detecting primary DNA damage in individual cells. The micronucleus (MN) test is also a well-established assay for detecting clastogenicity and aneugenicity. A combination of the comet assay (COM) and MN test is capable of detecting a variety of genotoxic potentials as an *in vitro* screening system. Although the *in vitro* MN test has a robust protocol and Organisation for Economic Co-operation and Development (OECD) test guideline, the *in vitro* COM does not. To establish a robust protocol for the COM and to compare its sensitivity with that of the MN, we conducted COM and MN concurrently for five genotoxic agents (ethyl methanesulfonate, methyl methanesulfonate, hydrogen peroxide, gamma-rays and mitomycin C) and one non-genotoxic agent (triton X-100), using human lymphoblastoid TK6 cells. Relative cell count (RCC), relative population doubling (RPD), relative increase in cell count (RICC) and relative cell viability determined by trypan blue dye-exclusion assay (TBDE) were employed as cytotoxic measurements. However, the relative cell viability determined by TBDE just after the treatment was not an appropriate parameter of cytotoxicity for the genotoxic agents because it remained constant even at the highest doses, which showed severe cytotoxicity by RCC, RPD and RICC. The results of the COM showed qualitative agreement (positive or negative) with those of the MN except for mitomycin C, which is an interstrand cross-linker. The COM always required higher doses than the MN to detect the genotoxic potential of the genotoxic agents under the test conditions applied here. The doses that induced a comet tail always yielded <50% RICC, and do not accord to the OECD test guideline for MN because of their high cytotoxicity. These results are helpful for interpreting the results of the COM and MN in *in vitro* genotoxic hazard assessments. Further investigation is required to standardise the COM.

Introduction

The single cell gel electrophoresis assay, also known as the comet assay, was developed by Ostling and Johanson (1) and its methodology was later optimised by Singh *et al.* (2) and Olive and Banath (3). The comet assay can detect DNA strand breaks and alkali-labile sites, which are considered primary DNA damage. Because the comet assay is a rapid and sensitive test for detecting genotoxic potential that has been used in genetic toxicology, radiation biology, and medical and environmental studies (3,4), it is a promising genotoxicity assay *in vitro* and *in vivo*. However, there is no official international guideline for its conduct. The Organisation for Economic Co-operation and Development (OECD) test guideline for the *in vivo* comet assay (COM) is now under development and will be published soon. The draft guideline is available on the website (5). On the other hand, there is no action to develop the OECD guideline for the *in vitro* COM although its guideline and the standard procedure had been proposed at International Workshop on Genotoxicity Test Procedure (IWGTP) in 1999 (4). This situation may prevent the *in vitro* COM from becoming widely used for regulatory purposes.

We previously developed a simple *in vitro* COM method using hydrophilic glass slides (MAS-coat type) instead of an agarose bottom layer (6). A simple method is necessary to avoid experimental errors and to produce reproducible and reliable results and will contribute towards establishing a robust protocol applicable to nearly any cell type. The *in vitro* micronucleus (MN) test is a well-established test for detecting clastogenicity and aneugenicity (7–9). A combination of the COM and MN as an *in vitro* screening system might be capable of detecting a variety of genotoxic chemicals. An OECD test guideline for MN (TG487) was recently developed and now is under revision (10).

To establish a robust protocol for the COM and to investigate the possibility of combining the COM and MN as a genotoxicity detection system with multi-endpoints, we concurrently conducted the COM and MN for five genotoxic and one non-genotoxic agents using human lymphoblastoid TK6 cells. Of the five genotoxic agents, ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) are alkylating agents, hydrogen peroxide (H_2O_2) induces oxidative damage, gamma-rays break DNA strands and cause oxidative damage, and mitomycin C (MMC) cross-links DNA interstrands. Triton X-100 (TRX), a detergent, was selected as a non-genotoxic chemical. Discriminating between cytotoxicity and genotoxic effects in the COM is of great importance because extensive endonucleolytic DNA degradation can occur very rapidly upon cell death, resulting in DNA fragments migrating out of the nuclei during electrophoresis. To evaluate cytotoxicity, relative cell viability (RV) determined by trypan blue dye-exclusion assay (TBDE), which detects dead or dying cells with a highly disrupted membrane, is frequently employed for the COM (11). Cytostatic measurements are recommended for the MN: relative cell count (RCC), relative population doubling (RPD) and relative increase in cell count (RICC) (12). We evaluated cytotoxicity multilaterally using all these parameters in this study.

Materials and methods

Cell line and culture conditions

We used human lymphoblastoid cell line TK6 (13). The cells were maintained in RPMI-1640 medium (GIBCO, Invitrogen Corp., CA, USA) supplemented with 10 vol% heat-inactivated horse serum (Cell Culture Bioscience, Nichirei Biosciences, Inc., Tokyo, Japan), 200 μg/ml sodium pyruvate, 100 U/ml

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penicillin and 100 μg/ml streptomycin, in a culture flask at 37°C, 5% CO_2 and 100% humidity.

Test chemicals and agent

EMS (CAS 62-50-0) and TRX (CAS 9002-93-1) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). MMS (CAS 66-27-3) and H_2O_2 (CAS 7722-84-1) were purchased from Sigma-Aldrich Inc. (CA, USA) and Wako Pure Chemical Industries, Ltd (Osaka, Japan), respectively. Caesium 137 from a Gammacell® 40 Exactor (Best Theratronics Ltd, Ontario, Canada) was the source for gamma-ray radiation. MMC (CAS 50-07-7) was purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan). EMS was dissolved in dimethyl sulfoxide (DMSO; guaranteed grade, Wako), H_2O_2 was dissolved in phosphate-buffered solution (Takara Bio Inc., Shiga, Japan) and other chemicals were dissolved in saline (for medical use, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). All test chemicals were prepared immediately prior to treatment.

Treatment conditions

We started cell culture by thawing a frozen cell stock in 50 ml medium with TS-75 culture flask and expansively cultured the cells for experimental use. Cell density was determined by an automatic cell counter (Coulter Counter Z2, Beckman Coulter, Inc., CA, USA), and the cells were appropriately diluted to prevent overgrowth ($<1.5 \times 10^6$ cells/ml). Logarithmic growth was normally maintained with population doubling times of 13 h for TK6 cells. Cell suspensions were prepared at 2×10^5 cells/ml and treated with serial dilution with one of the chemicals described above for 4 h at 37°C with gentle shaking or irradiated with gamma-rays at a dosage rate of 1 Gy/min. Vehicle or serial dilution of the chemicals (0.1 ml) was added to 9.9 m; of cell suspension in a 15-ml centrifuge tube (single cell culture). Cell suspensions (10 ml) in a 25-cm^2 culture flask were irradiated for 0 (control) to 8 min on ice (single cell culture).

MAS-coat type glass slides

Hydrophilic MAS-coat type glass slides (Cat TF0215M) were provided by Matsunami Glass Ind., Ltd (Osaka, Japan). The slides had two wells whose surfaces were designed to be hydrophilic to promote agarose adherence. The wells were externally coated with a water-repellent barrier allowing easy application of the samples.

Alkaline comet assay

Immediately after the end of treatment with the chemical or agent, 1 ml of the cell suspension was transferred to a new tube. The tube was centrifuged and the supernatant removed. The cells in the tube were washed once with 1 ml of cold HBSS (Hank's Balanced Salt Solutions, GIBCO) containing 20 mM EDTA and 10 vol% DMSO and resuspended in 0.5 ml of cold HBSS. In accordance with our previously reported method (6), specimens were prepared using an MAS-coat type glass slide instead of the conventional agarose bottom layer. The cells were thoroughly mixed with 0.5 w/v% low-melting agarose (NuSieve™ GTG™ Agarose, Lonza Group Ltd, BSL, Switzerland) and an aliquot of the mixture was pipetted onto a glass slide. After the gel had completely solidified, the slide specimens were immersed in chilled lysing solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 10 vol% DMSO and 1 vol% Triton X-100 in ultrapure water (pH 10)] overnight in a refrigerator (4°C). The slide specimens were rinsed and left in the electrophoresis solution [0.3 M NaOH and 1 mM EDTA in ultrapure water (pH >13)] for 20 min at <10°C to allow the duplex DNA to unwind. Electrophoresis was performed in the same alkaline buffer at 0.3A for 15 min at <10°C. The slide specimens were then rinsed with 0.4 M Tris-base (pH 7.5) to neutralise and immersed in anhydrous ethanol for 5 min to dehydrate. The slide specimens were stained with SYBR-Gold (Molecular Probes, Invitrogen Corp., CA, USA) in Tris-EDTA buffer (pH 7.5–8.0), and 100 cells per dose were examined at ×200 magnification under an Olympus BX50 Fluorescence Microscope (Olympus Corp., Tokyo, Japan) connected to the comet assay scoring system and equipped with a U-MNIBA3 filter (excitation: 470–495 nm, absorbing: 510–550 nm; Olympus Corp., Tokyo, Japan). The Comet Assay IV (Versions 4.11 and 4.2, Perceptive Instruments Ltd, Suffolk, UK) image analyzer was used as the analysis (scoring) software. DNA damage was quantified automatically as % tail DNA (percentage of tail intensity in total comet intensity). Statistically significant differences in % tail DNA between the chemical-treated groups and the vehicle-treated group were evaluated by Dunnett's multiple comparison test ($P < 0.05$).

Micronucleus test

In accordance with the previously reported method (14), the MN was conducted 48 h after the treatment, which is the optimal sampling time for TK6 cells. A small portion of the cell culture ($\sim 1 \times 10^6$ cells) from the flask was transferred to a centrifuge tube and then centrifuged. The pellet was suspended in hypotonic KCl solution for 10 min at room temperature, rinsed twice with ice-cold fixative (glacial acetic acid:methanol of 1:3) and then resuspended in methanol containing 1 vol% acetic acid. A drop of the suspension was placed on a clean glass slide, which was dried in air, and the cells were stained with 40 μg/ml acridine orange solution. The slides were covered with coverslips, and the cells (only those showing well-outlined interphase and mononucleated cells) were immediately examined at ×400 magnification under an Olympus BX50 Fluorescence Microscope equipped with a U-MWB2 filter (excitation: 460–490 nm, absorbing: 520 nm; Olympus Corporation, Tokyo, Japan). One thousand cells per dose were examined and the frequency (%) of micronucleated cells was determined. Statistically significant differences in the frequency of micronucleated cells between the chemical-treated groups and the vehicle-treated group were evaluated by Kastenbaum and Bowman's method ($P < 0.05$).

Cytotoxicity assay

Cytotoxicity was evaluated by cell viability from the TBDE and cytostatic activity on the basis of cell growth. The TBDE was conducted just after the treatment (Day 0) and 1 and 2 days later (Days 1 and 2). The cells in an aliquot of cell suspension were counted just after the treatment and on Days 1 and 2. RCC, RPD and RICC for Day 1 (RPD1 and RICC1) and Day 2 (RCC2 and RICC2) were calculated (15,16).

$$\text{RCC} = \frac{\text{Final count treated cultures}}{\text{Final count control cultures}} \times 100 \quad (1)$$

$$\text{RPD} = \frac{\text{Number of population doubling in treated cultures}}{\text{Number of population doubling in control cultures}} \times 100 \quad (2)$$

Where

$$\text{Population doubling} = \frac{[\log (\text{post-treatment cell number} / \text{initial cell number})]}{\log 2} \quad (3)$$

$$\text{RICC} = \frac{\text{Increase in number of cells in treated cultures (final} - \text{starting)}}{\text{Increase in number of cells in control cultures (final} - \text{starting)}} \quad (4)$$

Results

Ethyl methanesulfonate

The effect of EMS was examined at six dose levels: 62.5, 125, 250, 500, 1000 and 2000 μg/ml (Figure 1a). The COM showed a significant dose-related increase in % tail DNA at up to 1000 μg/ml. At 2000 μg/ml, the % tail DNA showed a downturn because of severe cytotoxicity. The MN showed a significant increase in micronucleated cells at 62.5 μg/ml and greater. At 1000 and 2000 μg/ml, the MN could not be assessed because of severe cytotoxicity. In the cytotoxicity assay, the cell viability determined by TBDE on Day 0 remained at almost 100% for all doses, but it decreased in a dose-related manner on Days 1 and 2. All cytostatic parameters were decreased in a dose-related manner and reached nearly their lowest level.

Methyl methanesulfonate

The effect of MMS was examined at five dose levels: 2.5, 5, 10, 20 and 30 μg/ml (Figure 1b). The COM and MN showed dose-related increases in % tail DNA and the frequency of micronucleated cells, respectively. However, the MN gave significant positive responses from the lowest dose. In the cytotoxicity assay, the cell viability determined by TBDE on Day 0 remained at almost 100% for all doses. All cytostatic

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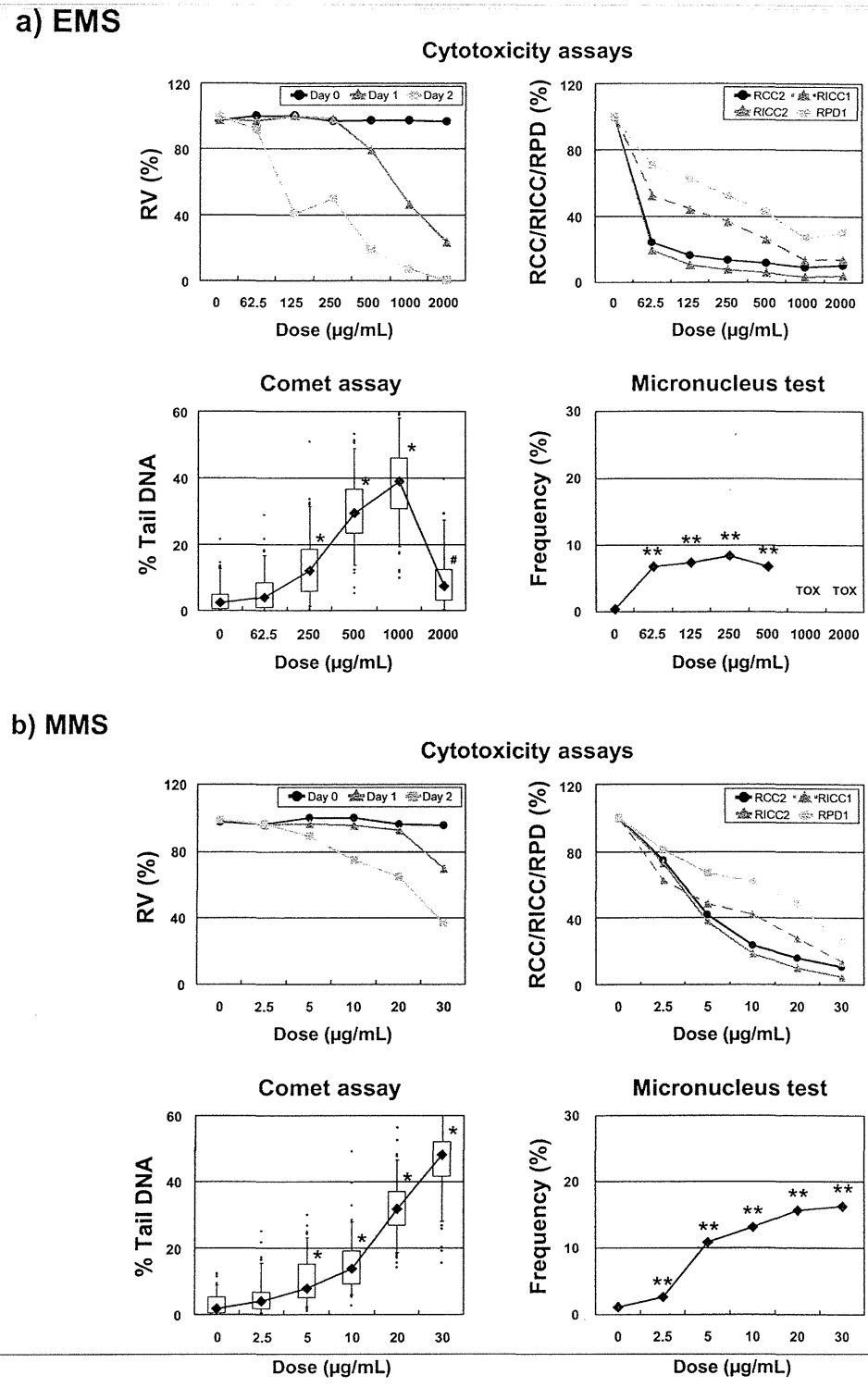

parameters were decreased moderately in a dose-related manner.

Hydrogen peroxide

The effect of H_2O_2 was examined at five dose levels: 20, 40, 80, 160 and 320 μM (Figure 1c). Although cell viability determined by TBDE on Day 0 remained at 100% at up to the highest dose, all cytostatic parameters decreased drastically and reached the cytotoxic level (55±5% of these parameters) at 40 μM and greater. At 40 μM, H_2O_2 induced a positive response in the MN, but not in the COM, which exhibited a positive response at a severely cytotoxic dose (>80 μM).

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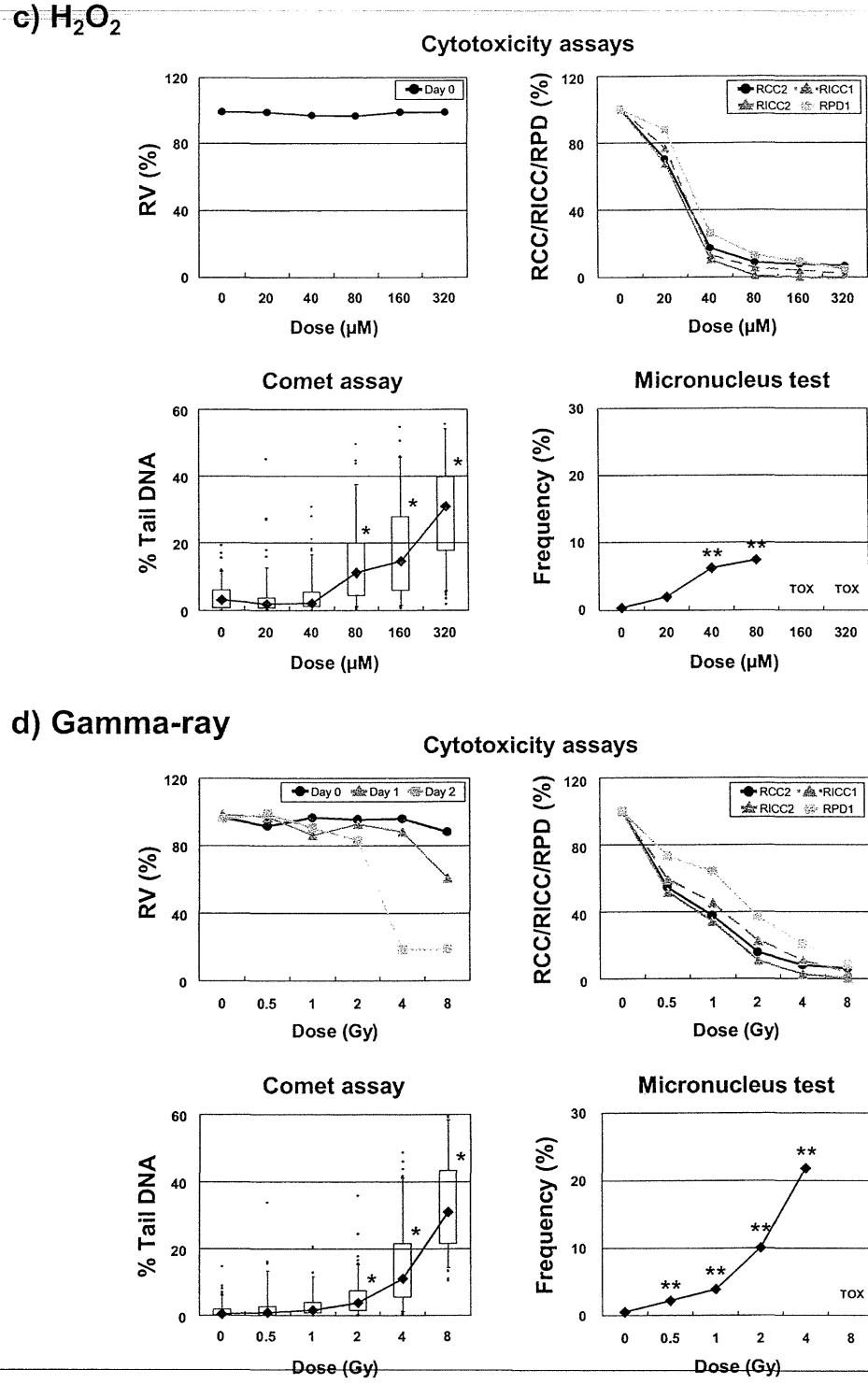

Gamma-ray

The effect of gamma-ray radiation was investigated at five dose levels: 0.5, 1, 2, 4 and 8 Gy (Figure 1d). Both the COM and MN showed positive dose-related responses. The MN showed statistically significant responses at 0.5 Gy and greater; however, the COM required 2 Gy to produce a positive result and was a cytotoxic level according to cytostatic parameters (55 ± 5% of these). The cell viability determined by TBDE on Day 0 remained at almost 100% for all doses, but it decreased on Days 1 and 2 in a dose-related manner. All cytostatic parameters decreased in a dose-related manner.

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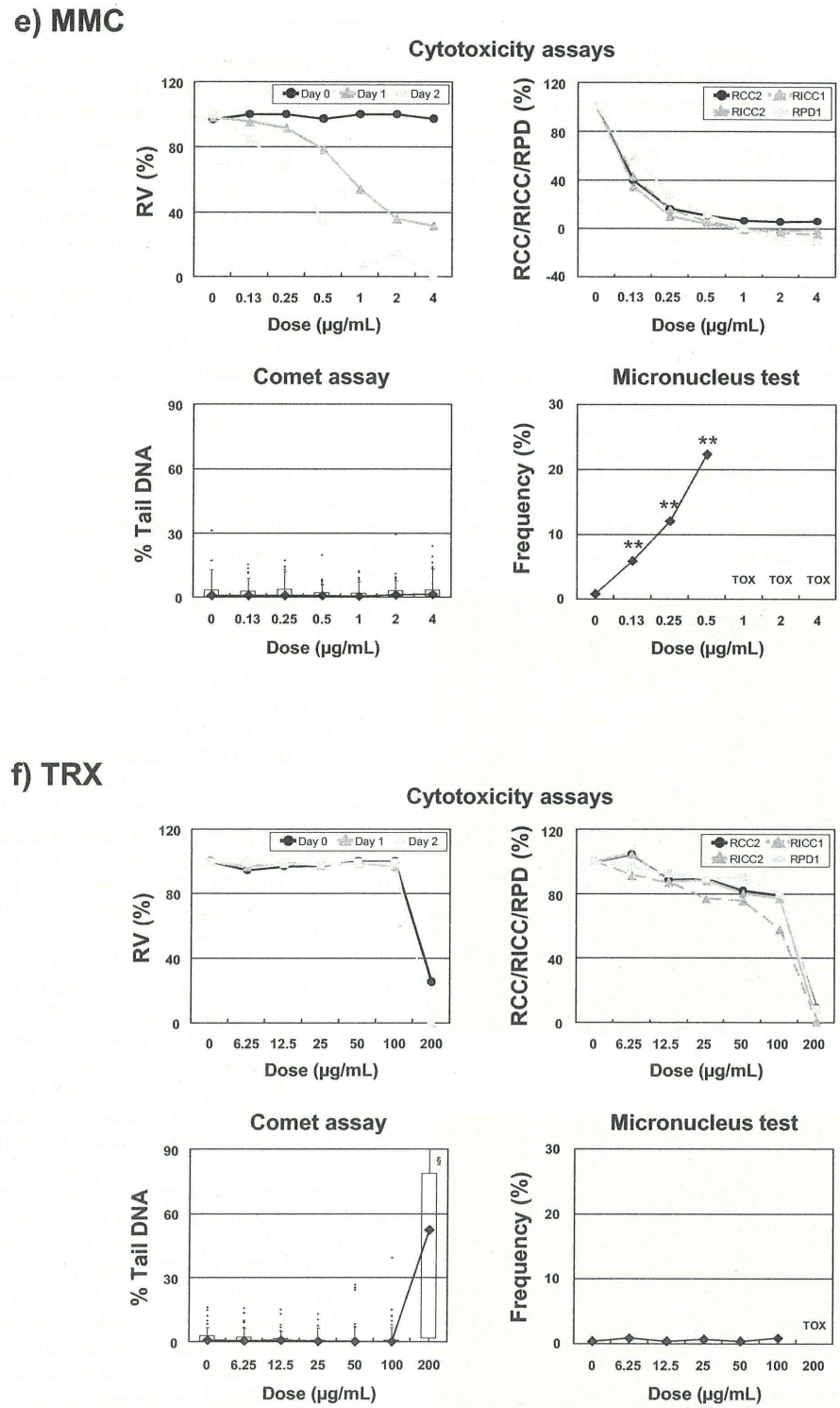

Fig. 1. Cytotoxic responses, comet % tail DNA and MN induction in TK6 cells after treatment with the test chemical or agent. TK6 cells were treated for 4 h with EMS (**a**), MMS (**b**), H_2O_2 (**c**), gamma-ray (**d**), MMC (**e**) or TRX (**f**). Cytotoxicity assays: RV was calculated from TBDE just after the treatment (Day 0) as well as 1 and 2 days later (Days 1 and 2). RCC, RPD and RICC for Day 1 (RPD1 and PICC1) and Day 2 (RCC2 and RICC2) were calculated. Comet assay: 100 cells per dose were examined and DNA damage was quantified as % tail DNA. In the box-and-whisker plot, the box represents interquartile range (IQR), the whisker represents cells ±1.5 IQR, and the dotted points outside the box-and-whisker plot represent outlier. Each data point in the box plot represents the median of 100 cells for each dose. *Significant increase from vehicle control found with Dunnett's multiple comparison test ($P < 0.05$). #Downturn because of severe cytotoxicity. MN test: frequency of micronucleated cells. One thousand cells (those exhibiting well-outlined interphase and mononucleated cells) were examined 2 days after treatment (Day 2). TOX: the cells could not be examined because of severe cytotoxicity. **Significant increase from vehicle control found with Kastenbaum and Bowman's method ($P < 0.05$). §Only 33 comet images could be analysed because of severe cytotoxicity and its images showed hedgehog (Figure 2b).

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Mitomycin C

The effect of MMC was examined at six dose levels: 0.13, 0.25, 0.5, 1, 2 and 4 μg/ml (Figure 1e). MMC showed dose-related cytotoxicity in cytostatic measurements and a dose-related increase in the frequency of micronucleated cells. No increase was noted in the comet tail at any dose. Although the MN could not be assessed at 1 μg/ml or greater because of severe cytotoxicity, the cell viability determined by TBDE on Day 0 remained at almost 100% for all doses.

Triton X-100

The effect of TRX was examined at six dose levels: 6.25, 12.5, 25, 50, 100 and 200 μg/ml (Figure 1f). No significant increase in the comet tail was observed at up to 100 μg/ml. At 200 μg/ml, only 33 comet images could be analyzed and a significant increase in % tail DNA was noted. This was considered an irrelevant positive response because severe cytotoxicity was indicated by the cell viability determined by TBDE on Day 0. The % tail DNA is due to a hedgehog but not a real comet tail (Figure 2). TRX showed no increase in the frequency of micronucleated cells at any doses.

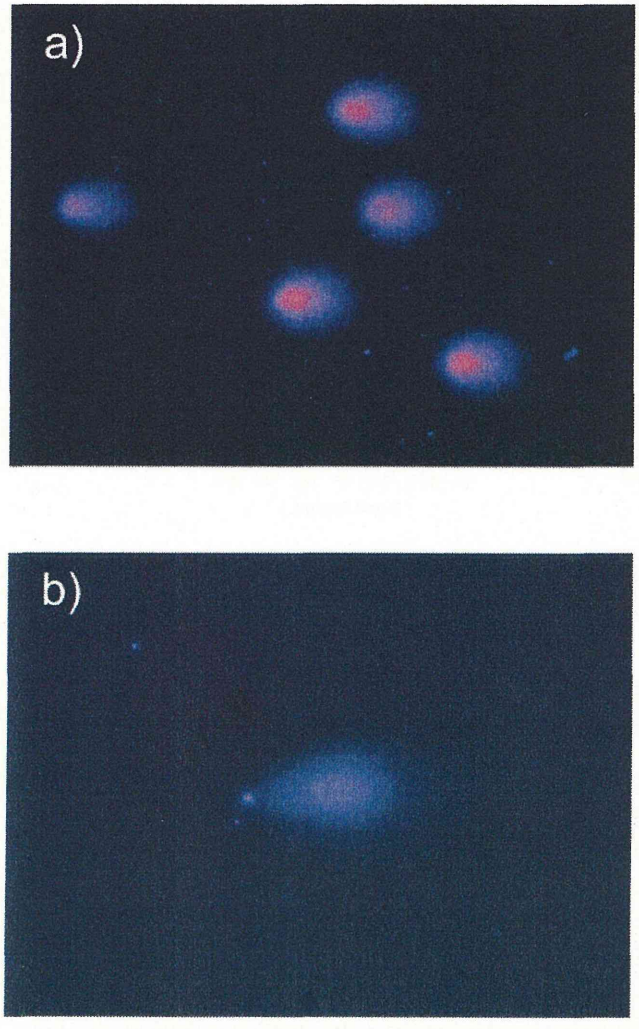

Fig. 2. Representative comet images of TK6 cells treated with EMS (**a:** 1000 μg/ml) and TRX (**b:** 200 μg/ml). b is a hedgehog.

Discussion

Human lymphoblastoid cell line TK6 was originally developed for the thymidine kinase gene mutation assay (13). This cell line is now becoming popular for the MN because it is both a human cell line and *p53* proficient (17). The draft revised OECD test guideline for MN suggests that the *p53* status, genetic (karyotype) stability, DNA repair capacity and origin (rodent versus human) of the cells should be considered in choosing cell lines for the MN (10,18). Because TK6 cells can be used for other *in vitro* genotoxicity tests, including chromosome aberration (CA) tests (19), COM (20), microarray analysis (21) and GreenScreen HC assay (22), it has great advantages for *in vitro* genotoxicity studies. The combination of these genotoxic endpoints by TK6 cells is capable of detecting a variety of genotoxic chemicals as an *in vitro* screening system as well as being an important approach to elucidate genotoxic mechanisms.

The COM is also a widely used *in vitro* genotoxicity test (4,23). Because of the many advantages of the COM, such as sensitivity, speed, cost, the small number of cells required and its applicability to all eukaryotic cells (3), it could be an alternative to other standard *in vitro* genotoxicity tests. To investigate the possibility of a combination of the COM and MN altering each other, we compared their sensitivities and responsiveness under the same treatment protocols. EMS, MMS, H_2O_2 and gamma-ray challenges exhibited clear positive responses in both the COM and MN, indicating that both assays had definitely detected these genotoxic agents. However, the COM required doses >2- to 4-fold to show significant positive responses compared with the MN (Table I). The low sensitivity of the COM has also been reported by others. Giannotti *et al.* (24) treated Chinese hamster lung cells with several genotoxic chemicals for 3 h and then conducted the COM and CA. They found that positive results in the COM always appeared at higher doses than those in the CA. Using TK6 cells, Kawaguchi *et al.* (25) also demonstrated that the lowest genotoxic dose of alkylating agents in the COM was always higher than that in the MN. These results indicate that in comparison with others, the COM is not sensitive for *in vitro* genotoxicity tests under the test conditions applied here. MMC, an interstrand cross-linker, was negative in the COM but positive in the MN. It has been reported that MMC yielded negative results by the standard alkaline comet assay both *in vitro* and *in vivo* (26). MMC is considered to induce DNA–DNA cross-links, thus preventing fragmentation during alkaline electrophoresis. The combination of the MN and COM could be useful for identifying genotoxic chemicals with DNA–DNA cross-linking properties.

In the COM, the RV determined by TBDE is usually employed for assessing cytotoxicity (11). To avoid non-specific DNA fragmentation by apoptosis under severely cytotoxic conditions, treatments with <75% RV are not generally evaluated (4,26). The positive response at the highest dose of TRX showing 30% RV should be ignored. We observed that most of the cells at the highest dose in TRX showed hedgehogs but not real comet tails (Figure 2). The RV determined by TBDE and the criterion of the highest concentration (<75% RV) would be appropriate for assessing cytotoxicity in the TRX study. However, the RV determined by TBDE just after the treatment remained at almost 100% for the other chemicals but decreased 1 or 2 days later. This is probably because loss of membrane integrity and function is a late event in apoptosis. TBDE,

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Table I. Lowest observed genotoxic effect level (LOGEL)

Mutagens	COM LOGEL	RCC2 (%)	RICC2 (%)	RICC1 (%)	RPD1 (%)	MN LOGEL	RCC2 (%)	RICC2 (%)	RICC1 (%)	RPD1 (%)
EMS (μg/ml)	250	13.2	7.5	36.4	52.3	62.5	24.3	19.3	52.2	70.5
MMS (μg/ml)	5	42.1	38.0	48.3	67.3	2.5	74.7	72.9	62.2	80.8
H_2O_2 (μM)	80	8.8	1.0	5.4	12.9	40	17.4	10.0	13.1	26.0
Gamma-ray (Gy)	2	15.8	10.8	22.5	37.0	0.5	54.4	51.7	59.1	73.1
MMC (μg/ml)	–	–	–	–	–	0.13	39.0	34.0	43.0	59.0
TRX (μg/ml)	–	–	–	–	–	–	–	–	–	–

therefore, may not be an appropriate cytotoxic measurement for the COM (27), in particular for TK6 cells (28). The doses giving positive responses in the COM represent severe cytotoxic conditions as determined by cytostatic analyses.

For *in vitro* cytogenetic tests, the OECD test guideline for the MN (TG487) recommend RICC or RPD to assess cytotoxicity and 55±5% of the cytostatic parameters as a top dose (10,16,18). We calculated four cytostatic parameters: RCC2, RICC2, RICC1 and RPD1. RICC2 may be a suitable parameter for the MN with long-term treatment or expression time (29). RICC1 and RPD1 are recommended for short-term cytogenetic tests (12,15). The statistically significant positive responses in the COM were always observed at <50% of RCC2, RICC2 and RICC1 for genotoxic agents other than MMC (Table I). This indicates that the comet tail is usually induced under severe cytotoxic conditions, which should be excluded by other cytogenetic tests. If we take the same criteria (55±5% of the cytostatic parameters) for choosing the top dose for the COM, even strong mutagens will be missed. Appropriate cytotoxic measurements and a criterion for the selection of the maximum dose for the COM are needed. We should also reconsider the biological meaning of the COM results for genotoxic hazard assessment.

In conclusion, the % tail DNA in the COM is clearly increased in a dose-related manner by the treatment of strong mutagenic agents except for interstrand cross-linker. However, the positive results were always observed under severely cytotoxic conditions as assessed by cytostatic parameters. It may be difficult to combine the MN and COM because of the differences in dose ranges for positive reactions. Further studies, including cytotoxic measurements, maximum dose setting and investigation of various types of genotoxic agent are needed to standardise the COM using TK6 cells.

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Environmental and Molecular Mutagenesis 54:229–239 (2013)

Commentary

Genotoxicity of Nanomaterials: Refining Strategies and Tests for Hazard Identification

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A workshop addressing strategies for the genotoxicity assessment of nanomaterials (NMs) was held on October 23, 2010 in Fort Worth Texas, USA. The workshop was organized by the Environmental Mutagen Society and the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute. The workshop was attended by more than 80 participants from academia, regulatory agencies, and industry from North America, Europe and Japan. A plenary session featured summaries of the current status and issues related to the testing of NMs for genotoxic properties, as well as an update on international activities and regulatory approaches. This was followed by breakout sessions and a plenary session devoted to independent discussions of in vitro assays, in vivo assays, and the need for new assays or new approaches to develop a testing strategy for NMs. Each of the standard assays was critiqued as a resource for evaluation of NMs, and it became apparent that none was appropriate without special considerations or modifications. The need for nanospecific positive controls was questioned, as was the utility of bacterial assays. The latter was thought to increase the importance of including mammalian cell gene mutation assays into the test battery. For in-vivo testing, to inform the selection of appropriate tests or protocols, it was suggested to run repeated dose studies first to learn about disposition, potential accumulation, and possible tissue damage. It was acknowledged that mechanisms may be at play that a standard genotoxicity battery may not be able to capture. Environ. Mol. Mutagen. 54:229–239, 2013. © 2013 Wiley Periodicals, Inc.

Key words: nanomaterials; workshop; genotoxicity

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Summary of a workshop of the Environmental Mutagen Society (EMS).
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INTRODUCTION

The term "nanomaterials" (NMs) is used as an umbrella term for a diverse group of nanosized particulate materials. NMs are designed to have unique properties different from the bulk material. NMs are found in an increasing number of products, including clothing, drug-delivery systems, wound dressings, cosmetics, and paint, as well as many other products. The novel properties of NMs have resulted in questions about their potential effects on human health and the environment. The ability of the standard genotoxicity assays to predict potential hazards of NMs has not been established. This workshop did aim to evaluate the usefulness of the standard regulatory genotoxicity tests, identify gaps for potential research, and if feasible, develop recommendations for the genotoxicity testing of NMs.

There are many published studies on the genotoxicity of NMs indicating they can be genotoxic in vitro as well as in vivo [Gonzalez et al., 2008; Landsiedel et al., 2009; Singh et al., 2009]. Yet for the purposes of designing an appropriate testing strategy, it is clear there are key data gaps and confounding factors that need to be addressed, e.g., as discussed in Doak et al. [2009]. For instance, as is typical in a new focus area, many publications are directed at reporting positive results, even when the results include a minimal enhancement over the controls. There are insufficient data on NMs that are negative in genotoxicity tests. Also, positive results for NMs tested in nonstandard assays at high, non-physiologically relevant concentrations/doses may not contribute meaningfully to the risk assessment of these materials. There is a great deal of attention given to the unique aspects of NMs, which often fuels a sense of concern that these materials may be associated with adverse toxic effects not observed with non-NMs of the same composition. Although there are a number of initiatives underway that are designed to help address issues related to NM safety assessment, the growing interest in developing new products that utilize NMs creates an immediate need for a testing approach that can be used today by industry, regulatory agencies, and academic scientists.

To promote progress in this direction, a workshop was held on October 23, 2010 in Fort Worth Texas, USA, in connection with the annual meeting of the Environmental Mutagen Society (EMS). The workshop was organized by the Relevance and Follow-up of Positive Results in In Vitro Genetic Toxicity Testing Project Committee (IVGT) at the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) with the intention of developing recommendations for a genotoxicity testing scheme for NMs, as well as the identification of potential research needs. The workshop was attended by over 80 participants representing stakeholders from academia, regulatory agencies and industry from a wide range of geographies (list of attendees: Appendix A). The workshop was designed to give all participants the opportunity to actively contribute to the discussion during the breakout sessions. These sessions followed a plenary session featuring summaries of the current status of testing of NMs for genotoxic properties in vitro and in vivo, as well as an update on international activities.

Workshop Focus

The workshop, limited by time constraints, made the deliberate decision to focus on the following topics:

- Evaluation of the usefulness of standard in vitro and in vivo assays to capture genotoxic effects caused by NMs.
- Discussion on whether standard protocols are suitable to detect the anticipated modes of action.
- Discussion of the need for integration of "new technologies" (e.g. "omics") into the genotoxicity assessment of NMs.
- Definition of gaps left by the standard assays.
- Identification of additional techniques that might fill gaps.
- Identification of research needs in this area.

What Was Not Discussed

- It was recognized that there are many definitions of what is to be considered as "nanosized" and we did not seek to define the term at this workshop. The term "NMs" was considered as an umbrella term for a diverse group of nanosized particulate materials. We want to emphasize, though, that the discussion was focusing on particulate materials that do not solubilize under physiological conditions or leach ions, as the genotoxic hazard of those may be adequately captured by standard testing approaches.
- The importance of appropriate characterization of NMs was acknowledged, and there was agreement that the characterization should generally cover morphology and metric related characteristics (form, chemical composition, shape, specific surface area, primary particle size, and size distribution), as well as physico-chemical properties such as purity, state (amorphous vs. crystalline), charge, and solubility. This has been discussed before by many authors [e.g., Powell et al., 2006]. Thus we decided not to address this issue directly, although it was raised in several breakout groups.

Workshop Structure

The breakout sessions were organized into three groups charged with discussion of core questions that were defined by the steering team ahead of the meeting.

- Group 1 evaluated the usefulness of standard in vitro assays (both bacterial and mammalian) and whether standard protocols are suitable to detect the anticipated modes of action.

- Group 2 assessed the utility of standard in vivo approaches and which assays could be recommended.
- Group 3 discussed the need for integration of new technologies into the genotoxicity assessment of NMs, as well as the question which tests or additional research would be useful.

After summarizing and discussing the outcome of the breakout group activities, the final plenary brought everyone back together to seek consensus towards the goal of defining a strategy for genotoxicity testing of NMs. The proceedings of this workshop provide some initial recommendations and considerations on a testing approach for NMs that reflect the available information and experience of the global expert participants. It was beyond the scope of a 1-day workshop to develop definitive recommendations, but it is hoped that these proceedings help lay the foundation for future research and more formal discussions on testing guidelines.

WORKSHOP SUMMARY CONCLUSIONS

- All of the standard genotoxicity assays require modification in methods or special considerations for the assessment of NMs.
- Bacterial assays may be limited in utility due to the apparent lack of uptake of NMs but additional studies were deemed necessary before final conclusions can be drawn.
- If bacterial assays are not performed, then the inclusion of mammalian cell gene mutation assays into the test battery would become more important.
- None of the protocols of in vitro standard assays was considered appropriate without special considerations or modifications like: possible interference of cytochalasin B treatment with the uptake of NMs, potential lack of uptake of agglomerated NMs by cells, and possible interference of NMs with endpoint measurement, e.g., for assays measuring fluorescence.
- Issues with exposure and removal of the NM test articles may result in complications in both monolayer and suspension cell cultures.
- The in vitro micronucleus assay was seen to be advantageous as it is capable of detecting chromosomal damage in the form of clastogenicity (e.g., triggered by ROS), aneugenic effects such as physical disturbance of spindles/mitotic apparatus).
- Because NMs may exhibit unusual ADME characteristics, such as organ-specific accumulation, it was recommended that other testing, such as repeat dose toxicity (RDT) studies be performed prior to in vivo genotoxicity testing to aid in design of an informative test.
- Thus, it was suggested that the testing paradigm should be changed so that other in vivo mammalian studies would be conducted prior to conducting an in vivo genotoxicity test. Alternatively, genotoxicity endpoints could be integrated into RDT testing.
- NM-specific positive controls were not considered to be necessary for genotoxicity assays, because positive controls relate to assay performance.
- Among the research needs identified were methods to measure DNA interaction and distribution of NMs, and methods to differentiate effects of substances in different physical states.
- Unconventional in vivo systems, such as Drosophila or Zebra fish, might be useful in the study of NM effects on both somatic and germ cells.
- It was acknowledged by the workshop participants that mechanisms may be at play for NMs that a standard genotoxicity battery may not be able to capture; therefore it was recommended that additional tools be sought which should help unravel potential modes and mechanisms of action.

SUMMARIES OF THE PRESENTATIONS FROM THE PLENARY SESSION

Approaches and Caveats to Investigating the Genotoxicity of NMs: Shareen H. Doak, Swansea University, UK

This presentation introduced a number of the confounding factors that are likely to account for some of the inconsistencies in the current literature, which contribute to the difficulty in reaching firm conclusions on the safety of NMs. The importance of complete physico-chemical characterization of NMs under investigation was highlighted, in addition to considering their secondary structure and form under experimental conditions, as this is what biological test systems are actually exposed to. In addition, sample preparation techniques used can influence this secondary structure and the degree of NM agglomeration. Thus, to facilitate comparison between studies and full interpretation of the biological responses observed, full detail with regards to physico-chemical features and sample preparation technique is vital. The presentation also highlighted a number of sources for variability in genotoxicity test systems including the selection of cell types (for in vitro studies); NM interaction with assay components including serum, colorimetric and fluorometric dyes, the use of cytochalasin B in the cytokinesis blocked micronucleus assay [Doak et al., 2009, 2012]; and the applicability of the Ames test for NM genotoxicity testing [Doak et al., 2012].

Accounting for such confounding factors within the experimental system when initiating nano(geno)toxicology investigations should improve the quality of in vitro assays, providing more reliable and reproducible data sets.

Testing for Genotoxic Effects of NMs In Vitro and In Vivo: Robert Landsiedel, BASF, Germany

This presentation focused on the use of standard genotoxicity assays to identify NM hazards. Generally, NMs are not expected to cause a new quality of genotoxic effects other than the well-established gene mutations, clastogenic and aneugenic effects by direct or indirect

mechanisms. Therefore, the battery of standard genotoxicity assays should be generally applicable. The questions are, what modifications of these assays are needed to meet special requirements of NMs, which assay (or combination of assays) is predictive for NM mutagenic potential in humans, and is there a need for additional data (genetox or other assays) to allow a valid hazard identification for humans? This was explicated by results of selected NMs in various test systems in vitro and in vivo. These included several Ames bacterial tests, HPRT gene mutation tests, micronucleus tests (MNT), and comet assays in vitro, as well as MNT and comet assays in vivo, published elsewhere [Landsiedel et al., 2010a,b; Schulz et al., 2011].

In vitro methods for genotoxicity testing require the dispersion of the NM in the culture media. The use of efficient, reproducible and well-documented dispersion protocols is crucial. Various protocols to prepare dispersions from 10 different NMs were tested; methods and pitfalls in preparing and characterizing the dispersions were presented. In summary, the agglomeration state and the surface coating of particles in dispersion may vary with the surrounding fluid (water, dimethylsulfoxide [DMSO], fetal calf serum, lung surfactant). The particle size distribution in the dispersions was analyzed by light scattering and analytical ultracentrifugation. The methods and their limitations were evaluated. In general, the highest amounts of ultrafine particles, i.e., the least agglomerated particles, were observed when using fetal calf serum [Schulze et al., 2008]. Several instances of interference of NMs with in vitro test systems have been described, e.g., in the in vitro chromosome aberration test, precipitation of (agglomerated) particles on the slides interfered with scoring for chromosome breakage.

The selection of appropriate in vitro genotoxicity tests for a NM requires information on its material properties and potential fate in the body. The particle size distribution and surface coating of the NMs in vitro may be very different from the ones found in the body. We have assessed the genotoxic potential of two differently sized amorphous silica NMs with potential inhalation exposure, by use of the in vitro single cell gel electrophoresis (Comet Assay) in a permanent cell line (V79 cells), and in Precision Cut Lung Slices (PCLS) from Wistar rats [Schulz et al., 2011].

In addition to in vitro methods, in vivo methods include uptake, distribution, excretion, and modification processes and allow the observation of concurrent general toxicity. Both of the amorphous silica NMs were instilled into the lungs of Wistar rats and genotoxic effects in the lung (Comet Assay) and bone marrow (Micronucleus Test) were examined along with general toxicity (e.g., inflammation of the lung). The comparison of the in vitro and in vivo methods revealed that while some genotoxic effect were observed in vitro those could not be reproduced in vivo [Schulz et al., 2011].

Developing a Coordinated International Research Effort in Nano-Genotoxicology: Tim Singer, Health Canada

This presentation highlighted several international initiatives currently underway, which are expected to produce data that will be informative in the context of: (a) better understanding the genotoxicity of a selection of NMs, and (b) better understanding the relevance and reliability of genotoxicity tests for NMs. The Organization for Economic Cooperation and Development (OECD)'s Working Party on Manufactured Nanomaterials has initiated nine projects, of which three were noted to be particularly relevant for the two objectives above, including the Safety Testing of a Representative Set of Manufactured Nanomaterials (Project 3), Manufactured Nanomaterials and Test Guidelines (Project 4), and the Role of Alternative Methods in Nano Toxicology (Project 7). Project 3 intends to test thirteen representative NMs across 59 endpoints for which there are OECD Test Guidelines; nine of these NMs were to be tested for genotoxicity using one or more of the tests in the standard test battery. Project 4 intends to review existing OECD test guidelines for their applicability to NMs and to identify the need for new or revised guidelines. This project has produced *Preliminary Guidance Notes on Sample Preparation and Dosimetry* as guidance to researchers [OECD, 2010]. Project 7 intends to support and advice on further developments of alternative test methods and integrated testing strategies. Furthermore, in Europe, a multilateral project called *Nanogenotox* was launched in 2010 with the aim of obtaining sound and reliable methods for detecting the potential genotoxicity of manufactured NMs within 3 years. This project is currently undertaking round-robin genotoxicity tests of titanium dioxide, silicon dioxide, and carbon nanotubes.

Scientific Issues Related to Genotoxicity Safety Assessment of NMs: Rosalie K. Elespuru, USFDA

This talk presented (1) some general concerns for safety assessment of NMs, (2) a description of the varying regulatory contexts for NM assessment at FDA, and (3) discussion points on the adequacy of current genotoxicity assays for NM assessment. Besides NM characterization, other concerns for safety assessment include product variability as related to manufacturing, stability, shelf life, and sample preparation. The regulatory basis for product assessment at FDA varies both between and within centers, reflecting the varying legal contexts. For example, for some products the assessment is risk/benefit based, whereas for other product categories the assessment is related to risk only. Concerning genotoxicity testing, we are aware that NM modes of action may be unknown and thus not always predictable. Thus, we seek tests that will detect a broad set of genotoxic mechanisms. We need to address whether the standard tests are appropriate and sufficient. Can we detect reactive oxygen species efficiently? If not, what other tests might be considered? Generally we need to determine how

to deal with the broad scope of NM effects, as well as variations based on differences in particle size, composition, and properties.

The Principal Approaches to Genotoxic Hazard Assessment of NMs in Japan: Masamitsu Honma, NIHS, Japan

This presentation introduced "Research on Hazard Characterization and Toxico-Kinetic Analysis of the Manufactured NMs for the Establishment of Health Risk Assessment Methodology," a project supported by the Ministry of Health, Labor, and Welfare in Japan. In accordance with the OECD sponsorship program on the testing of manufactured NMs, this project examined mainly fullerene and carbon nanotubes. The presentation highlighted principal genotoxicity test strategies. The in vitro tests used were (1) a chromosome aberration test to detect structural aberrations as well as polyploidy, and (2) a cell transformation or micronucleus assay to detect non-DNA interacting genotoxic mechanisms, including aneugenicity. OECD test guidelines and good laboratory practices (GLPs) are needed to ensure reproducible and reliable data. In the in vivo tests, target organs (e.g., lung and mesothelium), as demonstrated by histopathological observation and toxicokinetics analysis are the ones generally examined. A gene mutation assay using transgenic rodents is useful for the target organs. As a follow-up, oxidative DNA damage is measured. Finally, weight of evidence (WOE) and mode of action (MOA) approaches are considered for both in vitro and in vivo results. Negative results are not dismissed and should be published.

BREAKOUT GROUP DISCUSSIONS

Characterization of NMs

All groups emphasized the importance of characterization of NMs. Characterization generally covers morphology and metric related characteristics (form, chemical composition, shape, specific surface area, primary particle size, and size distribution) but also physico-chemical properties such as purity, state (amorphous vs. crystalline), charge, and solubility.

Breakout Group 1: Standard In Vitro Assays: Assessment/Strategy Development

Question: If you had to conduct genotoxicity testing on a nanoparticle today using only in vitro tests, or a battery of in vitro and in vivo tests, what in vitro tests would you choose and why?

Outcome of discussion

The Salmonella typhimurium Bacterial Reverse Mutation Assay (Ames Assay)

On the basis of existing data as well as practical aspects of the Ames assay, it is not clear whether bacterial assays add significantly to a testing approach. Since bacteria do not have active systems facilitating particle uptake, there is the question of whether nanoparticles are taken up into the bacteria at all. Thus, bacterial assays might not be suitable for genotoxicity assessment of nanoparticles, particularly considering agglomeration, and thus could lead to false negative results due to exclusion of nanoparticles from the bacterium. The vast majority of studies utilizing the standard Ames assay have demonstrated negative results [Gonzalez et al., 2008; Landsiedel et al., 2009; Singh et al., 2009]. One study examining iron–platinum nanoparticles capped with tetramethylamonium hydroxide did report a weak positive using the TA100 strain [Maenosono et al., 2007]. This nanoparticle has not been tested in any other cytogenetic system and thus the value of the single positive result cannot be determined. It must be noted that a number of recent reports utilizing the Ames assay along with other in vitro and in vivo genotoxicity assays indicated that all tests were negative for genotoxicity when examining fullerenes [Shinohara et al., 2010] and both TiO_2 and ZnO [Landsiedel et al., 2010a,b]. However, Al_2O_3 provides an example of a NM that is negative in the Ames test [Balasubramanyam et al., 2010] while being positive in the in vitro micronucleus assay [Di Virgilio et al., 2010], and in vivo micronucleus and comet assays [Balasubramanyam et al., 2009]. Of course, with the limited data currently available, additional studies are necessary to arrive at a definitive conclusion on the utility of the bacterial assays in a test battery. It was recommended that a coordinated study comparing a set of NMs prepared in the same manner and tested concurrently in the other in vitro genotoxicity assays be conducted to address this issue.

In vitro Micronucleus Assay

The existing data as well as mechanistic considerations indicating that the in vitro micronucleus assay responds to a variety of relevant mechanisms involved in NM genotoxicity indicate that this is a useful test for the evaluation and investigation of NMs. For example, the in vitro micronucleus assay is able to detect chromosomal damage in the form of clastogenicity induced by ROS, one of the main mechanisms of NM genotoxicity. In addition, the test system is capable of detecting potential aneugenic effects such as physical disturbance of spindles/mitotic apparatus [Sargent et al., 2009]. These different mechanisms can be discriminated by performing kinetochore or centromere staining on the resultant cell preparations. Importantly, the in vitro micronucleus assay has been shown to be useful for addressing linear versus nonlinear dose–response relationships [Elhajouji et al., 1995; Doak et al., 2007] which appear to be relevant to understanding the biological relevance of NMs. The group felt that if a