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Mutagenicity of secondary oxidation products of 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate (8-hydroxy-2'-deoxyguanosine 5'-triphosphate)

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ABSTRACT

8-Oxo-7,8-dihydroguanine (8-hydroxyguanaine) is oxidized more easily than normal nucleobases, which can produce spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh). These secondary oxidation products of 8-oxo-7,8-dihydroguanine are highly mutagenic when formed within DNA. To evaluate the mutagenicity of the corresponding oxidation products of 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate (8-hydroxy-2'-deoxyguanosine 5'-triphosphate) in the nucleotide pool, *Escherichia coli* cells deficient in the *mutT* gene were treated with H$_2$O$_2$, and the induced mutations were analyzed. Moreover, the 2'-deoxyriboside 5'-triphosphate derivatives of Sp and Gh were also introduced into competent *E. coli* cells. The H$_2$O$_2$ treatment of *mutT* *E. coli* cells resulted in increases of G:C → T:A and A:T → T:A mutations. However, the incorporation of exogenous Sp and Gh 2'-deoxyribonucleotides did not significantly increase the mutation frequency. These results suggested that the oxidation product(s) of 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate induces G:C → T:A and A:T → T:A mutations, and that the 2'-deoxyriboside 5'-triphosphate derivatives of Sp and Gh exhibit quite weak mutagenicity, in contrast to the bases in DNA.

*Key words:* Oxidized nucleotide, 8-Oxo-7,8-dihydroguanine, 8-Hydroxyguanaine, Secondary oxidation product, Spiroiminodihydantoin, Guanidinohydantoin
**Abbreviations:** G^0^, 8-oxo-7,8-dihydroguanine; Sp, spiroiminodihydantoin; Gh, guanidinohydantoin; dG^0^TP, 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate; wt, wild-type; dSpTP, spiroiminodihydantoin-2'-deoxyriboside-5'-triphosphate; dGhTP, guanidinohydantoin-2'-deoxyriboside-5'-triphosphate; pol, polymerase
1. Introduction

Reactive oxygen species, such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen, are produced through normal cellular metabolism, and by various environmental mutagens and dietary factors [1-4]. Oxidatively damaged nucleobases in DNA are the focus of considerable interest, due to their implications in mutagenesis, carcinogenesis, aging, and neurodegeneration [5-7]. One of the major oxidized nucleobases is 8-oxo-7,8-dihydroguanine (G\textsuperscript{O}, also known as 8-hydroxyguanine), and 100–500 G\textsuperscript{O} residues are generated per cell per day [8-11]. The G\textsuperscript{O} base is susceptible to further oxidation, due to its lower redox potential (0.5 V lower than G) [12]. Spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) are two major secondary oxidation products of G\textsuperscript{O} [13-17]. Sp and Gh in single-stranded DNA are both >98% mutagenic and cause G \rightarrow C and G \rightarrow T transversions, in contrast to the ~3% mutagenicity of G\textsuperscript{O} under the same conditions [18].

Nucleobase oxidation occurs in the cellular nucleotide pool as well as in DNA, and the oxidized 2'-deoxyribonucleoside 5'-triphosphates induce mutagenic events [19]. 8-Oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate (dG\textsuperscript{O}TP, 8-hydroxy-2'-deoxyguanosine 5'-triphosphate) induces A:T \rightarrow C:G transversion mutations in living cells [20,21]. The facts that Escherichia coli MutT (NudA) catalyzes the hydrolysis of dG\textsuperscript{O}TP in vitro [22] and that the mutation frequency in mutT-deficient strains is 10- to 130-fold higher than that in wild-type (wt) strains
[23-25] indicate the importance of endogenous dG\textsuperscript{O}TP as a mutagen in cells. This oxidized form of dGTP may be further oxidized to spiroiminodihydantoin-2'-deoxyribose 5'-triphosphate (dSpTP) and guanidinohydantoin-2'-deoxyribose 5'-triphosphate (dGhTP) under physiological conditions (Fig. 1). Thus, it is important to examine the mutagenicity of the secondary oxidation products of dG\textsuperscript{O}TP, particularly dSpTP and dGhTP, in vivo.

In this study, mutT E. coli cells, in which endogenous dG\textsuperscript{O}TP is expected to spontaneously accumulate, were treated with H\textsubscript{2}O\textsubscript{2}, and the induced mutations were analyzed. In addition, dSpTP and dGhTP (Fig. 1) were introduced into competent E. coli cells. Although the H\textsubscript{2}O\textsubscript{2} treatment of mutT E. coli cells caused an increase in G:C \rightarrow T:A and A:T \rightarrow T:A transversions, the mutation frequency was not increased when dSpTP and dGhTP were incorporated into E. coli cells. These results suggested that the oxidation product(s) of dG\textsuperscript{O}TP induces G:C \rightarrow T:A and A:T \rightarrow T:A mutations. However, dSpTP and dGhTP do not seem to contribute to these induced mutations.

2. Materials and Methods

2.1. Bacterial strains

The AB1157 E. coli strain (F: thr\textsuperscript{-1}, leuB6, thi\textsuperscript{-1}, lacY1, galK2, ara\textsuperscript{-14}, xyl\textsuperscript{-5}, mtl\textsuperscript{-1}, proA2, his\textsuperscript{-4}, argE3, rpsL31, tsx\textsuperscript{-33}, supE44, flaND) was obtained
from the National Institute of Genetics, Stocks Research Center (Mishima, Japan). The MK601 (AB1157 but \textit{leu}^+) and MK602 (AB1157 but \textit{leu}^+ \textit{mutT}) strains were kindly provided by Prof. Yusaku Nakabeppu of Kyushu University [24].

2.2. Synthesis of oxidized 2'-deoxyribonucleoside 5'-triphosphates

dSpTP (the two isomers, dSpTP1 and dSpTP2) and dGhTP were synthesized by the oxidation of dGTP with Rose Bengal plus light at pH 8.0 and pH 4.4, respectively, according to the literature [26]. d\textit{G}^{\text{O}}TP and 2-hydroxy-2'-deoxyadenosine 5'-triphosphate were synthesized as described previously [27,28].

2.3. \textit{MutT}, \textit{Orf135}, and \textit{Orf17} proteins

The \textit{MutT} gene fragment was amplified by KOD DNA polymerase (pol) (Toyobo, Osaka, Japan) from a lysate of AB1157 \textit{E. coli} cells, with the primers

5'-dTAGGTTTGGATCCATGAAAAAGCTGC and

5'-dGCCTTAGTCGACCTACAGA (Life Technologies Japan, Tokyo, Japan), in which the underlined sequences correspond to BamH I and Sal I sites, respectively. The fragment was inserted into the pGEX-6P-3 plasmid (GE Healthcare Bio-Sciences, Piscataway, New Jersey, USA), which had been digested with the two restriction enzymes, as described previously [29]. This manipulation generated the gene encoding the GST-MutT fusion protein.
The MutT, Orf135, and Orf17 proteins without the GST-tag were purified after PreScission protease treatment, as described previously [29,30].

2.4. Mutant frequencies upon $H_2O_2$ treatment

A single colony (MK601 or MK602) taken from an LB agar plate was inoculated into 7 mL of LB medium. The $E. coli$ culture was incubated at 37°C until the turbidity at 570 nm reached 0.2, and then 1 mL of the culture was transferred to a microtube. After $H_2O_2$ or $H_2O$ (10 µL) was added, the culture was incubated at 37°C for 30 min. The culture was then centrifuged at 13,000 X g for 2 min at room temperature. The pellet thus obtained was resuspended in 1 mL of prewarmed LB medium, cultured at 37°C for 2 h, and then placed on ice. A portion of the suspension was diluted with ice-cold LB medium and transferred onto an LB agar plate without rifampicin (the titer plate), which was incubated at 37°C for 12 h. Another portion of the suspension was transferred onto an LB agar plate containing rifampicin (100 µg/mL) (the selection plate), which was incubated at 37°C for 20 h. The mutant frequency was calculated according to the numbers of colonies on the titer and selection plates.

2.5. Introduction of oxidized 2'-deoxyribonucleotides

A single colony (AB1157) taken from an LB agar plate was inoculated into 7 mL of LB medium. The $E. coli$ culture was incubated at 37°C until the
turbidity at 570 nm reached 0.6. The culture was placed on ice for 10 min, and was then divided into 500-μL aliquots in microtubes, which were centrifuged at 13,000 X g for 2 min at 4°C. Competent cells were prepared by a treatment with 0.1 M calcium chloride. To 50 μL of the E. coli suspension, a 2'-deoxyribonucleotide solution or H₂O (2.5 μL) was added, and then the mixture was placed on ice for 2 h. Prewarmed SOC medium (450 μL) was then added, and cells were incubated at 37°C for 1 h. The rpoB mutant frequency was calculated as described above.

2.6. MutT/Orf135/Orf17 assay

The MutT activities were assayed in a reaction mixture (50 μl) containing 20 mM Tris-HCl, pH 7.5, 8 mM MgCl₂, 5 mM dithiothreitol, 4% glycerol, and the nucleotide substrates (20 μM). Following a preincubation at 30°C for 2 min, the mixtures were incubated at 30°C for 10 min with the MutT protein (20 nM). The Orf135 activities were assayed in a reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 9.0, 5 mM MgCl₂, 1 mM dithiothreitol, and the nucleotide substrates (20 μM). Following a preincubation at 37°C for 5 min, the mixtures were incubated at 37°C for 10 min with the Orf135 protein (15 nM). The Orf17 activities were assayed in a reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 9.0, 10 mM MgCl₂, 1 mM dithiothreitol, 1% glycerol, and the nucleotide substrates (20 μM). Following a preincubation at 37°C for 5 min, the mixtures were incubated at 37°C for 15 min with the Orf17 protein (400 nM). dGTP, 5-methyl-dCTP, and dATP
were used as “standard” substrates for the MutT, Orf135, and Orf17 proteins, respectively. Reactions were terminated by the addition of EDTA. All samples were injected into a TSK-gel DEAE-2SW column (φ 4.6 mm X 250 mm, Tosoh, Tokyo, Japan), with isocratic elution by 20% CH$_3$CN, 75 mM sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. The amounts of the 2'-deoxyribonucleoside triphosphates and their hydrolyzed products were quantitated by measuring the area of UV absorbance (230 nm).

2.7. Statistical analysis

The statistical significance of the values was examined by the Student’s $t$-test (mutant frequency and survival ratio) or the $\chi^2$ test for independence (mutation spectrum). Levels of $P<0.05$ were considered to be significant.

3. Results

3.1. Hydrogen peroxide-induced mutations in mutT cells

The MutT protein seems to play a crucial role in the removal of dG$^\circ$TP from the nucleotide pool, and this oxidized form of dGTP is expected to accumulate in mutT E. coli cells [22,24]. Indeed, the A:T→C:G mutation, the typical mutation induced by dG$^\circ$TP, is frequently observed in mutT cells [23-25]. Thus, the H$_2$O$_2$ treatment of mutT cells would produce secondary oxidation
product(s) of dG\textsuperscript{9}TP in the nucleotide pool. First, we analyzed the mutations induced by H\textsubscript{2}O\textsubscript{2} in \textit{mutT} cells, to examine whether the secondary oxidation product(s) are mutagenic. Wt (MK601) and \textit{mutT} (MK602) cells were treated with H\textsubscript{2}O\textsubscript{2}, and the survival ratios and the mutant frequencies for the chromosomal \textit{rpoB} gene were calculated (Table 1 and Fig. 2).

The survival ratios of both types of cells upon H\textsubscript{2}O\textsubscript{2} treatment were similar (Table 1). The survival ratios of the wt cells were 8.8\% and 1.9\%, and those of the \textit{mutT} cells were 11.9\% and 4.3\%, upon treatments with 1.2 and 3.5 mM H\textsubscript{2}O\textsubscript{2}, respectively. Interestingly, the \textit{mutT} cells seemed to be more resistant to H\textsubscript{2}O\textsubscript{2} (3 and 3.5 mM).

The treatment with 1.2 mM H\textsubscript{2}O\textsubscript{2} increased the \textit{rpoB} mutant frequency from 1.7 \texttimes 10\textsuperscript{8} to 7.4 \texttimes 10\textsuperscript{7} in the wt strain (Fig. 2). The mutant frequency decreased to 3.1 \texttimes 10\textsuperscript{7} with the 3.5 mM H\textsubscript{2}O\textsubscript{2} treatment. Thus, the mutants corresponding to the differences in the frequencies, 7.2 \texttimes 10\textsuperscript{7} and 2.9 \texttimes 10\textsuperscript{7}, were induced by 1.2 and 3.5 mM H\textsubscript{2}O\textsubscript{2}, respectively. Meanwhile, the background (0 mM) mutant frequency was higher in the \textit{mutT} cells (1.3 \texttimes 10\textsuperscript{6}), and the H\textsubscript{2}O\textsubscript{2} treatment further promoted the mutant frequency to 4–6 \texttimes 10\textsuperscript{6}. The treatment enhanced the mutation formation in the \textit{mutT} cells relative to the wt cells, since a greater number of mutants, corresponding to the difference in the mutant frequencies (\textsim 4 \texttimes 10\textsuperscript{6}), was induced by H\textsubscript{2}O\textsubscript{2} in the \textit{mutT} cells. These results suggested that the greater efficiency of mutation induction by H\textsubscript{2}O\textsubscript{2} in the \textit{mutT}
cells is due to increased cellular accumulation of the 2'-deoxyribonucleotide MutT substrates, dGTP and its diphosphate derivative [22,31], and their further oxidation products.

We then analyzed the mutation spectra in the *rpoB* gene in each strain (Tables 2 and 3). A:T → C:G transversions (85%) were observed in the *mutT* cells without the H$_2$O$_2$ treatment, as expected. The ratio of the A:T → C:G transversions decreased with the H$_2$O$_2$ treatment (statistically significant, $P<0.01$ for 0 and 1.2 mM, $P<0.005$ for 0 and 3.5 mM) and they constituted only 32% of the total mutations upon the treatment with 3.5 mM H$_2$O$_2$. We multiplied the *rpoB* mutant frequencies by the ratio of the A:T → C:G transversion mutations to the total colonies analyzed, and expressed them as the mutation frequencies. The *rpoB* mutant frequencies were $1.3 \times 10^6$, $6.0 \times 10^6$, and $5.8 \times 10^6$ for the 0, 1.2, and 3.5 mM H$_2$O$_2$ treatments, respectively. Thus, the A:T → C:G transversion occurred with efficiencies of $1.2 \times 10^6$, $2.7 \times 10^6$, and $2.1 \times 10^6$ for the 0, 1.2, and 3.5 mM H$_2$O$_2$ treatments, respectively. These results indicated that the H$_2$O$_2$ treatment increases the A:T → C:G transversion mutation, and that other types of mutations are also more efficiently induced.

G:C → T:A and A:T → T:A transversions represented ~40% of the total mutations in the *mutT* cells treated with 3.5 mM H$_2$O$_2$, although these mutations were undetected in the control (0 mM) experiment. The increase in G:C → T:A and A:T → T:A mutations suggested that the oxidation product(s) of dGTP
produced by the H$_2$O$_2$ treatment induce these types of mutations. G:C $\rightarrow$ A:T transition was also detected in the *mutT* cells treated with 1.2 mM H$_2$O$_2$, although this mutation seemed to be decreased by 3.5 mM H$_2$O$_2$ treatment. This result suggested that secondary oxidation product(s) responsible for the transition mutation are further oxidized/degraded.

A mutational hotspot exists at position 1538 in the *mutT* cells, and the A:T $\rightarrow$ C:G transversion mutation at this position was detected for 17 of the total of 19 single-base substitutions (0 mM, Table 3). The A:T $\rightarrow$ C:G mutation at position 1538 was decreased, depending on the H$_2$O$_2$ concentration, and only three cases among the total of 18 single-base substitutions were detected for the 3.5 mM H$_2$O$_2$ treatment. These results also supported the interpretation that the intracellular dG$_{O}$TP was further oxidized by the H$_2$O$_2$ treatment.

3.2. Mutagenicity of dSpTP and dGhTP

Various damaged bases have been identified as secondary oxidation products of G$_{O}$ [32]. Among them, the Sp and Gh bases in DNA are highly mutagenic and induce transversion mutations in *E. coli* [18]. dSpTP and dGhTP are the oxidation products of dG$_{O}$TP (Fig. 1). In contrast to dG$_{O}$TP, these base structure are quite different from that of G and they might be poor substrates for DNA pols. However, the Sp and Gh bases in DNA are not absolutely blocking lesions [18], suggesting the possibility of incorporation of dSpTP and dGhTP by DNA pols.
Thus, we examined their mutagenicity as the candidates that might be responsible for the mutations observed for the H$_2$O$_2$-treated mutT cells.

It was previously demonstrated that the direct incorporation of dGTP, added to a CaCl$_2$-treated E. coli suspension, induced chromosomal gene mutations [20,33]. Therefore, we examined the mutagenicity of dSpTP1, dSpTP2, and dGhTP in E. coli cells by the direct incorporation method. These 2'-deoxyribonucleotides were synthesized by the oxidation of dGTP, according to the published method [26]. Note that the two stereo-isomers of dSpTP, dSpTP1 and dSpTP2, were separately purified, as described above [26], although their configurations have not been determined. dGhTP was purified as the mixture of two isomers in equilibrium. dSpTP and dGhTP were introduced into wt E. coli cells, and the mutant frequencies for the chromosomal rpoB gene were calculated.

The numbers of colonies on the titer plates were very similar (data not shown), indicating the minimal, if any, cytotoxicity of dSpTP and dGhTP.

Unexpectedly, the mutagenicities of dSpTP1 and dGhTP were low, in contrast to the high mutagenic potentials of Sp and Gh in DNA (Fig. 3) [18]. No mutagenicity by dSpTP2 was detected (data not shown). In contrast, the rpoB mutant frequencies upon dGTP and 2-hydroxy-2'-deoxyadenosine 5'-triphosphate treatments (12.5 nmol) were 2.4 X 10$^7$ and 3.1 X 10$^7$, respectively. Thus, the mutagenicities of dSpTP and dGhTP were at least one order of magnitude lower
than that of dG\textsuperscript{O}TP in \textit{E. coli}. Moreover, the possibility that they contributed to the H\textsubscript{2}O\textsubscript{2}-induced mutations observed in \textit{mutT} cells is excluded.

3.3. \textit{dSpTP} and \textit{dGhTP} are not hydrolyzed by bacterial MutT-type enzymes

The low mutagenic potentials of \textit{dSpTP} and \textit{dGhTP} might be due to their efficient degradation by \textit{E. coli} MutT-type protein(s). We finally examined the possibility that \textit{dSpTP} and \textit{dGhTP} are good substrates for the \textit{E. coli} MutT-type proteins, \textit{MutT}, \textit{Orf135} (NudG), and \textit{Orf17} (NudB, NtpA). \textit{dSpTP1, dSpTP2, and dGhTP} were incubated with recombinant MutT, and the reaction mixtures were analyzed by anion-exchange HPLC [34]. The MutT protein produced a significant amount of the monophosphate derivative of dG\textsuperscript{O}TP, a known substrate of MutT (Fig. 4A). In contrast, little, if any, degradation of \textit{dSpTP1, dSpTP2, and dGhTP} was observed (Fig. 4C and E, and data not shown). Thus, they are unlikely to be degraded with high efficiencies by the MutT protein in \textit{E. coli} cells. Meanwhile, the 2'-deoxyribonucleotides of Sp and Gh might be degraded by the other MutT-type nucleotide pool sanitization enzymes, \textit{Orf135} and \textit{Orf17}, in \textit{E. coli} cells. To examine this possibility, \textit{dSpTP1, dSpTP2, and dGhTP} were incubated with the \textit{Orf135} and \textit{Orf17} proteins \textit{in vitro}. However, their hydrolysis was not observed under our experimental conditions (data not shown). Therefore, the weak mutagenicity of \textit{dSpTP1, dSpTP2, and dGhTP} could not be explained by their efficient hydrolysis by the three MutT-type proteins.
4. Discussion

The objective of this study was to examine the mutagenicity of secondary oxidation products of dG$^{O}$TP. We treated *mutT* *E. coli* cells with H$_2$O$_2$ to oxidize intracellular dG$^{O}$TP, and examined the mutation spectrum. As shown in Table 2, the treatment caused an increase in G:C $\rightarrow$ T:A and A:T $\rightarrow$ T:A transversions. These results suggested that the oxidation product(s) of dG$^{O}$TP cause G:C $\rightarrow$ T:A and A:T $\rightarrow$ T:A mutations, since the degree of the increase in mutant frequency was much higher in the *mutT* strain than the wt strain (Fig. 2). G:C $\rightarrow$ T:A mutations could be generated by the incorporation of damaged dGTP opposite C in the template DNA, and dATP insertion opposite the damaged G base in DNA during the next round of replication. In addition, the incorporation of damaged dGTP opposite G in the template DNA, and dTTP insertion opposite the damaged G base in DNA, could induce the same type of mutation. A:T $\rightarrow$ T:A mutations could be generated by the incorporation of damaged dGTP opposite T in the template DNA and dATP insertion opposite the damaged G base in DNA, and by the incorporation of damaged dGTP opposite A in the template DNA, followed by dTTP insertion opposite the damaged G base in DNA.

Alternatively, dG$^{O}$TP could be incorporated opposite C and A, and further oxidation of G$^{O}$ in DNA might induce the mutations we observed. If this were the
case, then one would expect \( \text{GO}^\circ : \text{C} \) to be converted to \( \text{X}: \text{C} \) (\( \text{X} \) represents oxidized \( \text{GO}^\circ \)), and subsequent dATP incorporation opposite \( \text{X} \) would result in \( \text{G:C} \rightarrow \text{T:A} \) mutations. Likewise, if \( \text{GO}^\circ : \text{A} \) were converted to \( \text{X}: \text{A} \), then subsequent dTTP incorporation opposite \( \text{X} \) would induce \( \text{A:T} \rightarrow \text{T:A} \) mutations. Studies using plasmid DNA modified with \( \text{GO}^\circ \) at a predetermined site might be required to examine these possibilities.

Previously, we examined the effects of the UvrA- and UvrB-deficiencies on the mutations induced in \textit{mutT} cells treated with \( \text{H}_2\text{O}_2 \) [35]. The deficiency in the \textit{uvrA} or \textit{uvrB} gene resulted in fewer mutations caused by \( \text{H}_2\text{O}_2 \)-treatment of the \textit{mutT} cells. We assumed that the formation of \( \text{dGO}^\circ \text{TP} \) by the oxidation of \( \text{dGTP} \) would increase under the oxidative stress produced by the exposure to \( \text{H}_2\text{O}_2 \) in \textit{mutT} cells. However, based on the results shown in Tables 2 and 3, the increased amount of \( \text{dGO}^\circ \text{TP} \) does not explain the entire increase in the mutant frequency induced by the \( \text{H}_2\text{O}_2 \) treatment. The reduced mutant frequencies in the \textit{uvrA/mutT} and \textit{uvrB/mutT} cells, at least in part, could be due to the involvement of UvrA and UvrB in the metabolism of the putative secondary oxidation product(s) of \( \text{dGO}^\circ \text{TP} \).

The secondary oxidation products, Sp and Gh, are highly mutagenic when generated in DNA [18]. Conversely, no mutagenicities of \( \text{dSpTP} \) and \( \text{dGhTP} \) were observed (Fig. 3). Based on the assumption that they are eliminated from the nucleotide pool by MutT-type enzymes, we incubated these damaged 2'-deoxyribonucleotides with MutT, Orf135, and Orf17 \textit{in vitro}. However, no
hydrolysis products were detected under our experimental conditions (Fig. 4 and data not shown). Thus, the absence of mutagenicity by dSpTP and dGhTP might be attributable to their inability to be incorporated by the *E. coli* DNA pol(s). Alternatively, they might be incorporated in a highly “error-free” manner by DNA pol(s). For example, no mutation is induced if dGhTP is incorporated opposite G (the first round of replication) and dGTP is inserted opposite Gh (the next round of replication). Experiments using various bacterial DNA polys, including the specialized DNA polys (DNA polys II, IV, and V), would be required to understand the discrepancy.

As shown in Table 1, the survival ratios of the H$_2$O$_2$ treated wt and *mutT* cells were similar. However, the cell death ratios of the *mutT* cells were less than those of the wt cells, in the cases of the 3 and 3.5 mM H$_2$O$_2$ treatments. One possible explanation is that the intracellular H$_2$O$_2$ concentration was lower in the *mutT* cells than in the wt cells, for an unknown reason(s) triggered by the MutT-deficiency. In contrast, the resistance to H$_2$O$_2$ is difficult to explain by the amount of cytotoxic, oxidized 2'-deoxyribonucleotide(s) and/or DNA lesion(s) in the *mutT* cells. However, it might be due to a putative stress-induced response that confers resistance to reactive oxygen species.

In this study, H$_2$O$_2$-induced mutations in *mutT* cells were analyzed, and the lack of mutagenicity by dSpTP and dGhTP was demonstrated. Our results suggested that the secondary oxidation product(s) of dGTP, except for dSpTP and
dGhTP, are mutagenic. The other secondary oxidation products should be examined to reveal the mechanism(s) of the promoted mutations observed in the H$_2$O$_2$-treated mutT cells.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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mutations induced by oxidized deoxyribonucleotides, DNA Repair (Amst.) 6 (2007) 1786-1793.
Figure Legends

Fig. 1. Structures of dSpTP and dGhTP. P represents a phosphate group.

Fig. 2. Frequency of rpoB mutants upon H₂O₂ treatment. A single colony taken from an agar plate was inoculated into LB medium and incubated at 37 °C. The E. coli culture was then treated with water or H₂O₂, and was incubated at 37 °C for 30 min. After centrifugation, the pellet was resuspended in LB medium and was incubated at 37 °C for 2 hr. The rpoB mutant frequency was calculated according to the numbers of colonies on the titer and selection plates, as described in the Materials and Methods section. Open diamonds, the wt strain; closed squares, the mutT strain. Experiments were repeated at least three times, except for wt, 1.6 mM (n=1), and data are expressed as means ± standard deviation. Asterisks (**) indicate a significant difference versus the wt strain, with P<0.01.

Fig. 3. Mutant frequency in wt E. coli cells treated with damaged 2'-deoxyribonucleotides. After adding the 2'-deoxyribonucleotides to competent E. coli suspensions, the rpoB mutant frequency was calculated according to the numbers of colonies on the titer and selection plates, as described in the Materials and Methods section. The 2'-deoxyribonucleotides that were added are shown below each bar. Experiments were repeated at least six times, and the data are
expressed as means ± standard deviation. The rpoB mutant frequencies were 2.4 × 10⁷ and 3.1 × 10⁷ when E. coli cells were treated with dG⁰TP and 2-hydroxy-2'-deoxyadenosine 5'-triphosphate treatments (12.5 nmol), respectively.

**Fig. 4** HPLC analysis of reaction mixtures of oxidized 2'-deoxyribonucleotides and MutT. Each nucleotide (20 µM) was incubated with 20 nM of the MutT protein at 30°C for 10 min. (A) dG⁰TP, postincubation, (B) dSpTP1, preincubation, (C) dSpTP1, postincubation, (D) dGhTP, preincubation, and (E) dGhTP, postincubation. The reaction mixtures were analyzed by anion-exchange HPLC under the conditions described in section 2.6. dG⁰MP in panel A represents 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-monophosphate.
Fig. 1

dSpTP

dGhTP

Fig. 1
Fig. 2

Mutant frequency (X 10^-6)

[\text{[H}_2\text{O}_2\text{]} \text{ (mM)}]

Fig. 2
Mutant frequency (X $10^{-8}$)

Fig. 3
Fig. 4
Table 1. H$_2$O$_2$-induced death in wt and mutT cells

<table>
<thead>
<tr>
<th>[H$_2$O$_2$] (mM)</th>
<th>wt</th>
<th>mutT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>0.4</td>
<td>73.9 (27.0)</td>
<td>74.5 (8.8)</td>
</tr>
<tr>
<td>0.8</td>
<td>16.4 (14.7)</td>
<td>24.9 (13.7)</td>
</tr>
<tr>
<td>1.2</td>
<td>8.8 (4.5)</td>
<td>11.9 (6.5)</td>
</tr>
<tr>
<td>1.6</td>
<td>4.4</td>
<td>6.5 (3.1)</td>
</tr>
<tr>
<td>2.0</td>
<td>5.8 (3.3)</td>
<td>6.3 (3.9)</td>
</tr>
<tr>
<td>3.0</td>
<td>2.8 (2.1)</td>
<td>6.9 (5.2)*</td>
</tr>
<tr>
<td>3.5</td>
<td>1.9 (0.7)</td>
<td>4.3 (2.2)**</td>
</tr>
</tbody>
</table>

Relative E. coli colonies on the titer plates to that of 0.0 mM are shown as percentage. Experiments were repeated at least three times except for wt, 1.6 mM, and the data are expressed as the mean. Numbers in parentheses represent standard deviations. Asterisks indicate a significant difference versus the wt strain, with $P<0.05$ (*) or $P<0.01$ (**).
Table 2. Mutations induced by H₂O₂ in wt and *mutT* strains

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th></th>
<th></th>
<th>mutT</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM</td>
<td>1.2 mM</td>
<td>3.5 mM</td>
<td>0 mM</td>
<td>1.2 mM</td>
<td>3.5 mM</td>
</tr>
<tr>
<td>Single base substitution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A:T to G:C</td>
<td>7 (32)</td>
<td>2 (10)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>G:C to A:T</td>
<td>9 (41)</td>
<td>9 (43)</td>
<td>11 (48)</td>
<td>0 (0)</td>
<td>5 (25)</td>
<td>2 (9)</td>
</tr>
<tr>
<td>Transversion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A:T to T:A</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (14)</td>
</tr>
<tr>
<td>A:T to C:G</td>
<td>2 (9)</td>
<td>0 (0)</td>
<td>1 (4)</td>
<td>17 (85)</td>
<td>9 (45)</td>
<td>7 (32)</td>
</tr>
<tr>
<td>G:C to T:A</td>
<td>1 (5)</td>
<td>6 (29)</td>
<td>4 (17)</td>
<td>0 (0)</td>
<td>4 (20)</td>
<td>5 (23)</td>
</tr>
<tr>
<td>G:C to C:G</td>
<td>0 (0)</td>
<td>4 (19)</td>
<td>3 (13)</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Insertion/deletion</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>2 (9)</td>
</tr>
<tr>
<td>Others</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>No mutation</td>
<td>3 (14)</td>
<td>0 (0)</td>
<td>2 (9)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>2 (9)</td>
</tr>
<tr>
<td>Total mutation</td>
<td>22 (100)</td>
<td>21 (100)</td>
<td>23 (100)</td>
<td>20 (100)</td>
<td>20 (100)</td>
<td>22 (100)</td>
</tr>
<tr>
<td>Total colonies analyzed</td>
<td>22</td>
<td>21</td>
<td>23</td>
<td>19</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>

A portion of the *rpoB* gene (nucleotides 1519-1725) was analyzed. All data are represented as cases found (%).
Table 3. The distribution of single-base substitutions induced by H2O2 in wt and mutT strains.

<table>
<thead>
<tr>
<th>wt</th>
<th>mutT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.2 mM</td>
</tr>
<tr>
<td></td>
<td>0 mM</td>
</tr>
<tr>
<td>1534 T to C (1)</td>
<td>1536 T to C (1)</td>
</tr>
<tr>
<td>1535 C to T (1)</td>
<td>1538 A to C (2)</td>
</tr>
<tr>
<td>1546 G to A (1)</td>
<td>1547 A to G (2)</td>
</tr>
<tr>
<td>1547 A to G (5)</td>
<td>1552 A to G (1)</td>
</tr>
<tr>
<td>1576 C to T (5)</td>
<td>1576 C to A (4)</td>
</tr>
<tr>
<td>1576 C to T (2)</td>
<td>1576 C to G (3)</td>
</tr>
<tr>
<td>1715 T to G (2)</td>
<td>1691 C to T (1)</td>
</tr>
<tr>
<td>1716 C to G (1)</td>
<td>1721 C to T (1)</td>
</tr>
</tbody>
</table>

A portion of the rpoB gene (nucleotides 1519-1725) was analyzed. The sequence of the sense strand is shown. The numbers of colonies are shown on the right side in parentheses.

*aOne colony contained two base substitutions.

*bOne colony contained two base substitutions.