***In Vivo* Mutagenicities of Damaged Nucleotides Produced by Nitric Oxide and Ionizing Radiation**

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Received November 6, 2004; accepted December 4, 2004

To evaluate the *in vivo* mutagenicities of damaged DNA precursors (deoxyribonucleoside 5'-triphosphates) produced by exposure to nitric oxide (NO) and ionizing radiation, five damaged deoxyribonucleotides (deoxyxanthosine triphosphate, deoxyxanosine triphosphate, dITP, dUTP, and 8-hydroxy-dATP) were introduced into competent *Escherichia coli* cells. Their mutagenic potentials were assayed using the chromosomal *rpoB* gene as a mutagenesis target. In contrast to 8-hydroxy-dGTP and 2-hydroxy-dATP, which were examined in an earlier study, none of these damaged deoxyribonucleotides significantly increased the *rpoB* mutant frequency. These results suggest that these five damaged deoxyribonucleotides are weakly mutagenic *in vivo* if at all. Thus their contributions to mutations induced by NO and ionizing radiation may be small.

Key words damaged nucleotide; nitric oxide; ionizing radiation; mutation

Damaged DNA precursors (2'-deoxyribonucleoside 5'-triphosphates) in addition to DNA damage are important sources of mutations.¹⁾ Reactive oxygen/nitrogen species are generated endogenously and are also produced by many environmental mutagens and carcinogens. For these reasons, the modification of DNA precursors and DNA may be an important source of mutations and therefore could be one of the causative factors of carcinogenesis, aging, and neurodegeneration.

The mutagenicities of the four oxidized deoxyribonucleotides, 2-hydroxy-2'-deoxyadenosine 5'-triphosphate (2-OH-dATP), 8-hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP), 5-hydroxy-2'-deoxycytidine 5'-triphosphate, and 5-formyl-2'-deoxyuridine 5'-triphosphate were examined in previous studies and were found to be mutagenic *in vivo* upon their direct introduction into living *Escherichia coli* cells.^{2–5)} In this study, 8-hydroxy-2'-deoxyadenosine 5'-triphosphate (8-OH-dATP) which is produced by γ -irradiation,^{6,7)} and 2'-deoxyxanthosine 5'-triphosphate (dXTP), 2'-deoxyxanosine 5'-triphosphate (dOTP), dITP, and dUTP which are generated by reaction of the parent nucleotide with nitric oxide (NO),^{8,9)} were introduced into living bacterial cells. In contrast to the four oxidized deoxyribonucleotides examined previously, none of these five damaged deoxyribonucleotides caused a significant increase in *rpoB* mutant frequency. These results suggest that the five damaged deoxyribonucleotides are weakly mutagenic in *E. coli* if at all. Thus their contributions to mutations induced by NO and ionizing radiation may be weak, at least in bacterial cells.

MATERIALS AND METHODS

Materials *E. coli* strain AB1157 (F^- : *thr-1*, *leuB6*, *thi-1*, *lacY1*, *galK2*, *ara-14*, *xyl-5*, *mtl-1*, *proA2*, *his-4*, *argE3*, *rpsL31*, *tsx-33*, *supE44*, *flaND*) was used in the mutagenesis experiments. This strain was obtained from the National Institute of Genetics, Stocks Research Center (Mishima, Japan). dITP and dUTP were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). Other deoxyribonucleotides were prepared as described previously.^{10,11)}

Introduction of Nucleotides A single colony was taken

from a Luria-Bertani (LB) agar plate and 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 then divided into 0.5-ml aliquots, which were placed in microtubes. Each sample was centrifuged at 13000 *g* for 2 min at 4 °C. Competent cells were prepared by treatment with calcium chloride 0.1 M. To 50 μ l of the *E. coli* suspension, 2.5 μ l of either a 5-mM (total 12.5 nmol) solution of deoxyribonucleotide or H₂O was added, and the mixture was then placed on ice for 180 min. Prewarmed SOC medium (450 μ l) was then added, and the cells were incubated at 37 °C for 60 min.

A portion of the suspension was diluted with ice-cold LB medium, transferred to an LB agar plate (titer plate), and incubated at 37 °C for 12 h. Another portion of the suspension was transferred to an LB agar plate containing rifampicin (100 μ g/ml) (selection plate) and incubated at 37 °C for 20 h. The mutant frequency was calculated from the numbers of colonies on the titer and selection plates.

RESULTS AND DISCUSSION

Mutagenicities of the Five Deoxyribonucleotides It was previously demonstrated that the direct incorporation of an oxidized deoxyribonucleotide added to an *E. coli* suspension induced chromosomal gene mutations.^{3–5)} In this study, *E. coli* was treated with 8-OH-dATP, a deoxyribonucleotide produced by the γ -radiation of dATP (Fig. 1).⁶⁾ In addition, dOTP and dXTP, dITP, and dUTP, generated from dGTP, dATP, and dCTP, respectively, by treatment with NO were also examined.^{8,9)}

The chromosomal *rpoB* gene was chosen as a mutagenesis target because most mutations detected by phenotypic selection (rifampicin resistance) have been found to occur in limited regions, and as a result the mutations can be easily characterized by sequencing.^{12,13)} When the bacteria were treated with 2-OH-dATP as a positive control, *rpoB* mutations were elicited. The mutant frequency was 12.1×10^{-7} upon treatment with 12.5 nmol of 2-OH-dATP (Fig. 2). On the other hand, the mutant frequency was *ca.* 1×10^{-7} in the control experiment. Thus the introduction of 2-OH-dATP clearly

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Fig. 1. Structures of the Base Moieties of Damaged Deoxyribonucleotides Examined in This Study

Fig. 2. Mutant Frequency in *E. coli* Cells Treated with Damaged Deoxyribonucleotides

Competent cells were prepared, and after adding the deoxyribonucleotides to *E. coli* suspensions, the mixtures were treated as described in the Materials and Methods. The deoxyribonucleotides that were added are shown below each bar. Experiments were repeated at least three times, and the data are expressed as the mean $\pm$ standard deviation.

increased the frequency of mutation in the *rpoB* gene. On the other hand, 8-OH-dATP, dXTP, dOTP, dITP, and dUTP all failed to cause an increased *rpoB* mutant frequency (Fig. 2). Thus the *in vivo* mutagenicities of the five damaged deoxyribonucleotides were very weak or nonexistent, at least in bacterial cells.

Implications The mutagenic potentials of damaged deoxyribonucleotides *in vivo* were examined. For example, 2-OH-dATP and 8-OH-dGTP, which are formed by Fenton-type reactions, have been shown to induce G:C→T:A and A:T→C:G transversions, respectively.^{2,3} In this study, none of the deoxyribonucleotides investigated (8-OH-dATP, dXTP, dOTP, dITP, and dUTP) elicited *rpoB* gene mutations (Fig. 2). Under the same conditions, 2-OH-dATP enhanced the mutation of the *rpoB* gene more than 10-fold compared with the control experiment. Thus the mutagenicities of the five deoxyribonucleotides were at least one order of magnitude lower than that of 2-OH-dATP in *E. coli*.

The presence of hydrolase enzymes that are specific for damaged deoxyribonucleotides has been reported.^{1,14} The MutT and Orf135 proteins catalyze the hydrolysis of 8-OH-dGTP and 2-OH-dATP, respectively, and a deficiency in these enzymes results in mutator phenotypes.^{15–18} We have recently reported that the *E. coli* Orf17 protein catalyzes the hydrolysis of 8-OH-dATP.¹⁹ *E. coli* cells contain dUTPase (dUTP pyrophosphatase), which hydrolyzes dUTP.^{20,21} An inosine triphosphatase that hydrolyzes ITP, dITP, and xanthosine 5'-triphosphate is present in human cells, and a homologous gene is present in *E. coli* genomic DNA.²² Although

activity has not been demonstrated, this putative enzyme could also act on dXTP. Thus Orf17, dUTPase, and inosine triphosphatase may suppress the potential mutagenicities of 8-OH-dATP, dUTP, and dITP and dXTP, respectively. Therefore such sanitizing enzymes, which remove damaged nucleotides from the nucleotide pool, as well as the DNA repair proteins may play a pivotal role in the prevention of mutagenesis caused by reactive oxygen species and nitric oxide.

The mutagenic potentials of the damaged bases corresponding to the deoxyribonucleotides examined in this paper have been studied in bacteria and mammalian cells. 8-Hydroxyadenine is mutagenic in mammalian cells although the mutagenic potential of 8-hydroxyadenine in *E. coli* was very weak.^{23,24} Hypoxanthine (I) and xanthine frequently elicit mutations.^{25–27} Although the *in vivo* mutagenicity of oxanine has not been reported, it is potentially mutagenic since dTMP and dAMP misincorporations have been observed in *in vitro* DNA synthesis.²⁸ Uracil would be expected to be highly “miscoding.” The finding that 8-OH-dATP, dXTP, dOTP, dITP, and dUTP show only weak mutagenicities suggests that their contributions to mutations that are induced by NO and ionizing radiation are very weak, at least in *E. coli*, and that the direct modification of DNA bases is likely to be responsible.

At present, intracellular concentrations under the physiologic conditions of the nucleotides used are unknown. In this study, these damaged nucleotides were externally introduced into living *E. coli* cells. In the case of 8-OH-dGTP, the nucleotide added to a suspension of *mutT E. coli*, which lacks the 8-OH-dGTPase MutT protein, increased mutant frequency by 1.6-fold.⁵ Thus the intracellular concentrations of the damaged nucleotides after treatment may not be extremely high and may actually be physiologically relevant when cells are exposed to NO or ionizing radiation.

Acknowledgments This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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