Paradoxical Enhancement of the Toxicity of 1,2-Dibromoethane by
O^6-Alkylguanine-DNA Alkyltransferase*

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The presence of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) paradoxically increases the mutagenicity and cytotoxicity of 1,2-dibromoethane (DBE) in Escherichia coli. This enhancement of genotoxicity did not occur when the inactive C145A mutant of human AGT (hAGT) was used. Also, hAGT did not enhance the genotoxicity of S-(2-haloethyl)glutathiones that mimic the reactive product of the reaction of DBE with glutathione, which is catalyzed by glutathione S-transferase. These experiments support a mechanism by which hAGT activates DBE. Studies in vitro showed a direct reaction between purified recombinant hAGT and DBE resulting in a loss of AGT repair activity and a formation of an hAGT-DBE conjugate at Cys145. A 2-hydroxyethyl adduct was found by mass spectrometry to be present in the Gly^136-Arg^147 peptide from tryptic digests of AGT reacted with DBE. Inclusion of AGT with DBE and oligodeoxyribonucleotides led to the formation of covalent AGT-oligonucleotide complexes. These results indicate that DBE reacts at the active site of AGT to generate an S-(2-bromoethyl) intermediate, which forms a highly reactive half-mustard at Cys145. In the presence of DNA, the DNA-binding function of AGT facilitates formation of DNA adducts. In the absence of DNA, the intermediate undergoes hydrolytic decomposition to form AGT-Cys^145-SCH\_2CH\_2OH.

The repair of O^6-alkylguanine adducts in DNA by O^6-alkylguanine-DNA alkyltransferase (AGT) provides an important means of defense against the mutagenic, carcinogenic, and cytotoxic effects of many simple alkylating agents (1–6). It was therefore quite unexpected when it was found that the overexpression of OgT (one of the two Escherichia coli AGTs) actually increased the toxicity of dibromoethane (DBE) and dibromomethane (DBM) toward E. coli (7). The enhancement of the mutagenicity and toxicity of DBE in E. coli has been confirmed and extended to AGTs from other species, including human AGT (hAGT) and other mammalian AGTs (8–10), the E. coli Ada (9) and the Salmonella typhimurium OgT, although the latter was only weakly active (11). The expression of the E. coli Ada was also reported to increase the killing of mammalian cells by DBE (12).

There has been much interest in the toxicity of 1,2-dihaloethanes because of the potential widespread exposure to these agents. The use of DBE as a fumigant and a gasoline additive was drastically reduced because it was found to be carcinogenic in rats and mutagenic in many species. Mutagenicity has been observed in cells from microorganisms, yeast and other fungi, plants, insects, humans, and other mammals (13–15). The majority of the metabolism of DBE occurs via a cytochrome P450 (P450)-mediated oxidation forming 2-bromoacetaldehyde (16, 17), which can react with DNA to generate genotoxic products, although the kinetics of the reaction are unfavorable (18–20). A well established pathway in which DBE generates DNA adducts is via metabolism by glutathione-S-transferases (GSTs) (21–27). The initial product of this reaction is a glutathionyl half-mustard that undergoes a non-enzymatic dehalogenation to generate an episulfonium ion, which reacts rapidly with water or a cellular nucleophile, including DNA (28). The major product of this reaction is S-[2-(N\_7-guanyl)ethyl]glutathione, but N\_2- and O\_6-guanyl adducts are also formed, and all three adducts are potentially mutagenic (29).

The interaction, if any, of AGT with the pathways and adducts described above is not currently understood. AGT is not an enzyme but acts in a stoichiometric manner to bind to DNA and transfers the alkyl group from the O\_6 position of guanine to a cysteine residue on the protein (1–3). AGT is not specific for O\_6-methylguanine in DNA, because the protein can repair larger adducts attached to this position, including ethyl, 2-chloroethyl, butyl, benzyl, and pyridyloxobutyl adducts (3, 30, 31).

Several possibilities for the enhancement of the toxic effects of DBE by AGT can be envisaged. It is possible that AGT recognizes a DNA adduct generated from DBE by the action of P450s or GSTs but fails to repair it correctly and actually increases its mutagenic and toxic effects. There are some precedents for this with other types of DNA damage. First, AGT can become cross-linked to DNA that has been treated with 1,3-bis(chloroethyl)-1-nitrosourea in vitro (32, 33). This occurs because of the reaction of AGT with the cyclic adduct 1-O\_6-ethanoguanine. Attack by the AGT protein on the carbon of the ethane linkage attached to the O\_6 position leads to the AGT protein becoming covalently bound to the DNA via a 1-guan-1-yl-2-(cysteine-S-yl)ethane linkage.

Second, it is known that even non-covalent binding of AGT to
DNA lesions can enhance their genotoxicity. Thus, hAGT binds to O\(^4\)-methylthymine in DNA but repairs this lesion very slowly and actually increases mutations caused by O\(^4\)-methylthymine by preventing their repair by other pathways (34). A similar phenomenon was found to occur when the C145A mutant of hAGT was expressed in E. coli. This mutant cannot repair O\(^4\)-methylguanine, because the Cys\(^{145}\) acceptor site is lost but its binding to methylated DNA in cells exposed to N-methyl-N'-nitro-N-nitrosoguanidine increases both mutations and decreases cell survival due to shielding of the O\(^4\)-methylguanine from other repair pathways (35). However, this second pathway is rendered unlikely, because the AGT-mediated increased toxicity of DBE was abolished when cells were treated with O\(^4\)-benzylation that inactivates AGT by forming S-benzyllycysteine at the active site (9, 10). Thus, it appears that the alkyl transfer function of the AGT protein is needed.

Another possible mechanism for AGT to mediate toxicity of DBE would be for the protein to act in the same way as GST and convert DBE to a low molecular weight reactive intermediate that is then released and can diffuse away and react with cellular components. However, this possibility is argued against by the findings that hAGT mutants having mutations in the DNA binding domain were not able to activate DBE (10).

The studies to date, which have all been carried out in vivo, suggested that a functional AGT being able to bind to DNA is essential to observe the increased toxicity of DBE. In the experiments described in this report, we have studied the reaction of hAGT and relevant mutants with DBE and 2-bromoethanol (BE) in vitro. We have obtained evidence that supports a model in which DBE reacts at the active site of AGT to generate a reactive intermediate at Cys\(^{145}\), and this then forms a covalent AGT adduct in DNA. This is a novel mechanism for the formation of genotoxic damage in which the DNA-binding function of the AGT protein facilitates formation of DNA adducts from a reactive intermediate.

**EXPERIMENTAL PROCEDURES**

**Materials**—DBE and BE were purchased from Sigma Chemical Co. (St. Louis, MO). Isoamyl-\(\beta\)-thiogalactopyranoside, kana-
mycin, and reagents used in bacterial culture were obtained from Sigma. \({}^{[14}C\)DBE (111 mCi/mmol), adenosine \(5'\)-\(\beta\)-thiotriphosphate, triethyl ammonium salt (\(\gamma\)-\(S\)-ATP, \(S\)), and \(\gamma\)-\(S\)-methylated protein molecular weight markers were purchased from Amersham Biosciences (Piscataway, NJ). T4 polynucleotide kinase (PNK) was from New England BioLabs (Beverly, MA). All oligodeoxyribonucleotides were synthesized by Invitrogen (Gaithersburg, MD). The 18-mer 5'-\(\mathrm{TGCGTCAAGGGTACCGAG}\)-3' containing an S-2(\(\alpha\)-ethylnylguanine)gluthatine at the G* site were synthesized and purified as described previously (36). S-2(\(\alpha\)-fluoroethyl)gluthatine and S-2(\(\alpha\)-chloroethyl)gluthathione were synthesized and purified as described previously (37). \(\mathrm{H}^3\)Methylated calf thymus DNA (ctDNA) was prepared by reaction of N-(\(\mathrm{H}\)-methyl-N-nitrosourea (18.8 mCi/mmol from Amersham Biosciences) with ctDNA as described previously (38, 39).

**Bacterial Strains and Plasmids**—The TRG8 cells were derived from GWR109 cells by selection for cells with an altered cell membrane to withstand for the measurement of cell survival and mutation frequency.

**Purification of Recombinant AGT**—Recombinant C-terminal histidine-tagged wild type hAGT, AGT C145S mutant and N-terminal his-
tidine-tagged synthetic enzyme were cloned in a pQE30 vector and expressed in XL-1 blue cells and purified as described previously (41).

**Measurement of Cell Survival and Mutation**—TRG8 cells were grown in 20 ml of Luria-Bertani (LB) medium supplemented with 50 \(\mu\)g/ml ampicil-
in and kanamycin and 0.2 \(\mu\)mol isopropyl \(\beta\)-D-thiogalactopyranoside until the optical density of the bacterial culture reached 0.5 at 600 nm. Cells were pelleted and resuspended in 1\(\times\) M9 minimal salts. Aliquots (0.5 ml) of the cell suspension were treated with 0–1\% of DBE, BE, or \(\mathrm{Me}_2\)SO vehicle with shaking at 37 °C for 0–90 min. The cells were pelleted, washed with 1 ml, and resuspended in 0.5 ml of 1\(\times\) M9 salt solution after the treatment. The cell suspensions (100 \(\mu\)l, in 1:10\(^6\) dilution) were plated on M9 minimal plates supplemented with 0.4% glucose (\(\mathrm{w/v}\)), 40 \(\mu\)g/ml histidine, 50 \(\mu\)g/ml ampicillin, and 50 \(\mu\)g/ml kanamycin for survival measurement. Undiluted cell suspensions (100 \(\mu\)l) were plated on M9 minimal plates not supplemented with histidine for the measurement of hAGT activity. A colony from the plates was counted after incubating at 37 °C for 36–40 h. The survival rate of DBE- or BE-treated cells was expressed as a percentage of survival compared with vehicle-treated cells. The mutation frequency was calculated by the number of his revertants over 10\(^6\) survivors grown on M9 minimal plates provided with histidine.

**Transformations**—The TRG8 pIN and pIN vectors were transformed into TRG8 cells by standard electroporation procedures as indicated previously (36). γ\(^3\)H]Methylated calf thymus DNA (ctDNA) was prepared by a 30-min reaction measuring the removal of \(\beta\)-ethylation and the formation of genotoxic damage in which the DNA-binding function of the AGT protein is needed.

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analyzed with a PhosphorImager (29). The migration of the unmodified 18-mer was about 12 cm, and that of the modified oligonucleotide was about 13.5 cm.

Mass Spectrometry Analysis—Wild type and C145S hAGT protein (80 μg) were incubated with 20 mM DBE or BE in AGT buffer at 37°C for 1 h. Trypsin (50 μg) was added to unreacted AGT protein or the reaction mixtures and the trypsin digestion was carried out in 100 mM (NH₄)₂CO₃ (pH 8.0) overnight with shaking at 37°C.

MALDI-TOF spectra were recorded over a range of m/z 250–6800 on a PerSeptive Voyager Elite instrument in the positive reflector mode (PerSeptive Biosystems, Framingham, MA). The accelerating voltage, grid voltage, and guide wire voltages were 20,000 V, 74.5%, and 0.05%, respectively. A 10 mg/ml solution of Bradykinin and insulin were used as external references.

Electrospray ionization-MS studies were performed on a Finnigan TSQ-7000 triple quadrupole mass spectrometer (San Jose, CA) equipped with a standard API-1 electrospray ionization source with a deactivated fused silica capillary, heated to 200°C. The tube lens voltage was set to 80 V. N₂ was used as the sheath gas (70 p.s.i.) and as the auxiliary gas (10 p.s.i.). Spectra were acquired in the positive ion mode with a needle voltage of 4 kV. For MS/MS experiments, daughter ions were generated using ~35-eV collision-induced dissociation.

The autosampler and high performance liquid chromatography system consisted of an Alliance 2990 Separations Module from Waters (Millford, MA). A Zorbax Rx octylsilane (C8) column (2.1 × 150 mm) (MacMod, Chadds Ford, PA) was used for the LC/MS experiments. 20-μl aliquots of samples were injected, and a 0.2 ml/min flow rate was maintained. Solvent A was 0.1% formic acid in water, and solvent B was acetonitrile. The gradient was the following: 95% A and 5% B at time zero, 95% A and 5% B at 5 min, 95% A and 5% B at 50 min 10% A and 90% B at 60 min, 95% A and 5% B at 60 min, and 95% A and 5% B at 70 min.

RESULTS

Role of hAGT in Cytotoxicity and Mutagenicity of DBE and BE—Previous studies have shown that the expression of hAGT in E. coli TRG8 cells lacking endogenous alkyltransferase greatly increased the genotoxicity of exposure to 1 mM DBE (10). A more detailed investigation of the time course and concentration dependence of this effect confirmed and extended these findings (Fig. 1). Genotoxicity and mutagenicity of DBE

FIG. 1. Effects of DBE, BE, and hAGT on the survival and mutation of E. coli TRG8 cells. Studies were made on cells carrying pIN vector (No AGT, filled circle), the wild type hAGT (+hAGT, filled square), mutant C145A (+C145A, filled triangle). The TRG8 cells were treated with 0–0.1 mM DBE or 0–0.5 mM BE for 90 min or the time shown. Cells were diluted and plated for determination of survival and mutation as described under “Experimental Procedures.” A and B, the effect of time of exposure to 0.1 mM DBE, C and D, effects of DBE concentration; E and F, effects of BE concentration. Percentage survival (A, C, and E) was determined by the number of colonies obtained from bacteria exposed to DBE or BE divided by that of bacteria exposed to the Me₂SO vehicle alone. Mutation frequency (B, D, and F) was calculated as the number of his revertants on M9 minimal plates lacking histidine over the 10⁸ survivors on M9 plates supplemented with histidine.

FIG. 2. Effects of GST-derived metabolites from DBE on survival and mutation of E. coli TRG8 cells expressing and lacking hAGT. TRG8 cells were exposed to S-(2-fluoroethyl)glutathione or S-(2-chloroethyl)glutathione as described under “Experimental Procedures.” A and B, results for cells exposed to 10 mM S-(2-fluoroethyl)glutathione. C and D, results for cells exposed to 5 mM S-(2-chloroethyl)glutathione. Percentage survival is shown in A and C, and mutation frequency is given in B and D. Studies were made on cells carrying empty pIN vector (No AGT, empty circle) and expressing the wild type hAGT (+hAGT, filled circle).
increased significantly as the treatment was extended from 15 to 90 min (Fig. 1, A and B) and as the DBE dose was increased to 0.1 mM (Fig. 1, C and D). After 90-min exposure to 0.1 mM DBE, killing of >98% of the cells was observed with ~1800 mutants in 10^8 survivors. The DNA repair-inactive hAGT C145A mutant and transfection with the empty pIN vector were completely ineffective in bringing about these increases with <5% cell killing and <10 mutants in 10^8 survivors (Fig. 1, A–D). The C145A mutant is known to be able to bind to O6-methylguanine and protect it from repair by other pathways (35), and this result therefore provides further support to the hypothesis that the enhanced toxicity of DBE is not due to shielding of DBE-induced lesions by AGT binding.

In contrast to the results with DBE, 2-bromoethanol (BE), a P450-derived metabolite of DBE (16), was not cytotoxic at levels up to 0.5 mM irrespective of AGT content and produced only a minuscule increase in mutations in hAGT-expressing cells with less than 20 mutants in 10^8 survivors at 0.1 mM BE (Fig. 1, E and F). The presence of hAGT did not enhance (and in fact slightly protected against) the genotoxicity of compounds analogous to those produced by the activation of DBE by GST (Fig. 2). The results of treatment with S-(2-fluoroethyl)glutathione, a surrogate of the DBE reaction intermediate with glutathione catalyzed by GST (26), are shown in Fig. 2 (A and B). Exposure to 10 mM S-(2-fluoroethyl)glutathione reduced cell survival to less than 6%. The presence of hAGT produced a modest level of protection from killing, but the differences in hAGT-positive and -negative
DBE toxicity mediated via hAGT expression seen in Fig. 1 is not mediated by interactions with DNA lesions caused by GST-derived metabolites. The slight protection against mutagenesis seen in Fig. 2 may be due to the ability of hAGT to repair the bulky $O^6$-alkylguanine lesion produced by the glutathione half-mustard. As shown in Fig. 3, $S\{2-(O^6$-ethylguananyl)$\}$glutathione is a substrate for hAGT. Incubation of an 18-mer oligonucleotide containing this adduct with excess hAGT resulted in the partial removal of the lesion.

**Reaction of AGT with DBE or BE**—The possibility that hAGT reacts directly with DBE to generate a reactive intermediate that is responsible for the increased genotoxicity was examined in vitro using purified recombinant hAGT and its C145S mutant. The proteins were purified to homogeneity using a C-terminal histidine tag (41). Studies were carried out to determine whether or not hAGT reacts directly with DBE and BE, the effect of this reaction on hAGT alkyltransferase activity, and the nature of the product formed in the reaction. The results showed a direct reaction between hAGT and DBE. The DNA repair activity of hAGT was inactivated by DBE in a time- and dose-dependent manner (Fig. 4). This reaction was retarded by addition of ctDNA to the reaction (Fig. 4A). The $ED_{50}$ values for DBE were 1.1 and 3.2 mM, respectively, in the absence and presence of ctDNA, suggesting that hAGT in a DNA-bound form may become less reactive with DBE. Exposure to BE also produced a dose-dependent inhibition of alkyltransferase activity of hAGT with an $ED_{50}$ of 2.2 mM (Fig. 4A). The hAGT inactivation by 5 mM DBE or BE appeared to be a first order time-dependent reaction under these conditions where DCE is in a vast excess (Fig. 4B).

To investigate whether the hAGT inhibition by DBE involved a DBE-AGT conjugation and the role of alkyl acceptor Cys$^{145}$ residue in the reaction, hAGT or the C145S mutant was incubated with 0–20 mM of [14C]$S\{2-(O^6$-ethylguananyl)$\}$glutathione, and the activity and the nature of the product formed in the reaction. The results showed a direct reaction between hAGT and DBE. The DNA repair activity of hAGT was inactivated by DBE in a time- and dose-dependent manner (Fig. 4). This reaction was retarded by addition of ctDNA to the reaction (Fig. 4A). The $ED_{50}$ values for DBE were 1.1 and 3.2 mM, respectively, in the absence and presence of ctDNA, suggesting that hAGT in a DNA-bound form may become less reactive with DBE. Exposure to BE also produced a dose-dependent inhibition of alkyltransferase activity of hAGT with an $ED_{50}$ of 2.2 mM (Fig. 4A). The hAGT inactivation by 5 mM DBE or BE appeared to be a first order time-dependent reaction under these conditions where DCE is in a vast excess (Fig. 4B).
It is known that the binding of DNA to the hAGT protein restricts access to the Cys145 acceptor site, which is located in a pocket buried within the protein structure (42).

More detailed kinetic studies of the reaction of hAGT with \[^{14}C\]DBE showed that there was a stoichiometric binding of DBE to hAGT when no more than 0.5 nmol of hAGT was included (Fig. 5). The rate of the adduct formation studied using increasing concentrations of DBE (0–25 mM) and 20 µg of hAGT protein over a 30-min reaction course suggested a second order reaction with a k of \(-24 \text{ m}^{-1} \text{ min}^{-1}\).

The structure of the adduct formed at Cys145 was investigated by mass spectrometry. MALDI-TOF, LC/MS, and LC/MS/MS analyses were performed to analyze tryptic digests of unreacted hAGT, hAGT, and the C145S mutant protein isolated after reaction with 20 mM DBE or BE for 1 h (Fig. 6). Four of the 16 possible trypsin fragments, including the fragment Gly136-Arg147 containing the active site Cys145, were observed (Fig. 6A) in the MALDI spectrum of the unreacted wild type hAGT. Five peptide fragments were identifiable in the MALDI spectra of trypsin-digested hAGT reacted with DBE or BE (Fig. 6A). Small peaks were observed at an m/z of 1360.5, corresponding to the mass for AGT tryptic fragment Gly136-Arg147 and a 2-hydroxyethyl group, suggesting the addition of a 2-hydroxyethyl on this AGT fragment. No signals corresponding to adduct formation at any other residues were observed in either spectrum. In an LC/MS experiment, peaks with an m/z ratio corresponding to the \(M+1\) or \((M+2)/2\) signals of 13 of the expected fragments of unmodified wild type hAGT were seen. An LC peak had a signal at an m/z ratio of 658.5, which corresponds to the \((M+2)/2\) signal of the fragment containing residues Gly136-Arg147.

To determine if the signals with an m/z of 1360.5 observed in the MALDI spectra were due to an adduct on Cys145, LC/MS and LC/MS/MS experiments were performed on these samples. Both the DBE and the BE spectra had a small peak at approximate 31 min with a signal at an m/z of 680.5, which was absent for the unreacted hAGT sample (Fig. 6B). Because the LC peak in the BE sample had a more significant signal at an m/z of 680.5 than the DBE sample, the former was selected for LC/MS/MS experiments. Daughter ions were observed that corresponded to several b- and y-type ions of the Gly136-Arg147 residue fragment containing a 2-hydroxyethyl adduct at Cys145.

In the MALDI spectrum of the unreacted C145S mutant, five fragments were identified, including the fragment containing Cys145 (results not shown). Also, LC/MS experiments of the C145S mutants indicated a strong signal at \(-30.5\) min with an m/z corresponding to the \((M+2)/2\) ion of the unmodified fragment containing Cys145. LC/MS/MS experiments confirmed the assignment of this signal to the unmodified fragment. No new signals were observed by MALDI or LC/MS for the samples containing C145S with DBE or BE, suggesting that adduct formation does not occur in this mutant. These results are consistent with the formation of a 2-hydroxyethyl adduct at Cys145 of hAGT directly after reaction with BE or indirectly via the reaction with water of an unstable 2-bromoethyld in the MALDI spectra of tryptic-digested hAGT reacted with DBE or BE (Fig. 6A).

**Fig. 8.** Effect of DBE and BE on formation of cross-links between hAGT and oligodeoxyribonucleotides of different composition. After incubation of hAGT, the oligonucleotide indicated and 20 mM DBE (A) or BE (B) for at 37°C for 1 h the mixtures were separated, and autoradiographs were produced as for Fig. 7. The oligonucleotides used were 5'-end-\[^{35}S\]-labeled 5'-d(TTCTGGCTTTTGGCCC)-3' (I), 5'-dAACAGCCAGGGGCC)-3' (II), 5'-d(TTCTGGCTTTTGGCCC)-3' (III), 5'-dAAAGCCAGGGGCC)-3' (IV), 5'-d(TTCTGGCTTTTGGCCC)-3' (V), and 5'-dAAAGCCAGGGGCC)-3' (VI). The amounts of oligonucleotide present in the free and conjugated forms were quantified by ImageQuaNT application software. Some of the oligonucleotides (particularly II, III, and VI) contained a small impurity band running slightly slower than the main band. The extent to which this was conjugated with AGT did not differ from the main band, and it was not included in the calculation.

**Fig. 9.** Current working hypothesis for enhancement of DBE toxicity by AGT. Reaction of hAGT with either DBE or BE is shown.
nucleotide (4.5 kDa). Several weaker bands corresponding to larger complexes were also seen (Fig. 7). Complexes 2A and 2B may correspond to two hAGT molecules bound to the 35S-labeled oligonucleotide (expected molecular mass, 49.7 kDa). As shown in the upper panel of Fig. 7, this band could be detected in a gel stained with Coomassie Blue and is slightly more retarded than spermine synthase (42 kDa) (Fig. 7A). (The resolution of this gel and the fact that there is an excess of unconjugated hAGT in these experiments did not allow the resolution of complex 1 from the unconjugated hAGT or of complexes 2A and 2B from each other.) No changes in mobility of the 35S-labeled oligonucleotide occurred when the C145S mutant was substituted for the wild type hAGT indicating that cross-linking of hAGT with DNA requires an intact active site Cys145 residue (Fig. 7B).

To examine the reactivity of all four deoxyribonucleotides in DNA with AGT and DBE, wild type hAGT was incubated with oligonucleotide 16-mers containing only thymine, adenosine, or cytosine bases. Formations of complex 1 and complex 2 were observed from all three oligonucleotides (Fig. 8A). The reaction was strongest with the 5'-d(T16)-3'. Because of problems with secondary structure, it was not possible to use a 16-mer substrate with all guanine residues but the comparison of 5'-d(A16)-3' with 5'-d(GGAGGAGGAGGAG)-3' and of d(5'-A-ACAGCCAGAGGGCC)-3' with 5'-d(TTTCTCCTTTTTTTCC)-3' showed that guanine is also a target for reaction and is more reactive than adenine or cytosine.

Under comparable conditions BE did not promote significant covalent attachment of hAGT to oligonucleotide 16-mers (Fig. 8B). Under the conditions used in the experiment shown in Fig. 8, the reaction of hAGT in the presence of DBE resulted in the conversion of 30–48% of oligonucleotide to the conjugate form. In contrast, BE led to <4% conjugation (Fig. 8B).

### Lack of Direct Reaction of DBE with Oligodeoxyribonucleotides

The possibility that the cross-linking of hAGT to the oligonucleotides in the presence of DBE was due to the initial direct reaction of the DBE with the oligonucleotide to generate an adduct such as 1,057-ethanoguanine whose “repair” by hAGT leads to the covalent link with the protein was also examined. However, there was no detectable reaction of 5'-d(TGGGGGTTTGG)-3' with [14C]DBE in the absence of hAGT. An aliquot (5 μg) of this oligonucleotide was incubated without hAGT at 37 °C for 1 h in the same buffer as used for the AGT labeling experiments in the presence of 20 μM [14C]DBE. The free [14C]DBE was then removed by using a MicroSpin G-25 column run at 3750 rpm for 2 min. Less than 0.1% of the radioactivity was present in the fraction that contains the oligonucleotide, and this value was not different when no oligonucleotide was added or when the separation was carried out without any incubation.

### DISCUSSION

DBE was used widely until being reported as a mutagen in many species and a carcinogen in rat in the 1970s (13, 44). Although the industrial use of DBE has been curtailed, it is of importance to understand the mechanism of genotoxicity of DBE and related dihaloalkanes and the role of cellular defense mechanisms, including DNA repair pathways in protecting against such agents.

Early studies reported that mutagenicity of DBE in E. coli was reduced by the UV light-induced excision repair system but unaffected by the loss of a major apurinic/apyrimidinic site repair function and enzymes that repair alkylation lesions (15). However, more recently, it was reported that the presence of alkyltransferase DNA repair proteins increased the toxicity of DBE in E. coli and mammalian cells (7, 8, 10, 12). One mechanism that was suggested to explain this increase was abortive repair of DNA lesions produced from an activated intermediate of DBE that led to an increase rather than a decrease in their toxicity.

One pathway by which DNA damage can be caused by DBE is well established. Mutagenicity and cytotoxicity can be mediated via conjugation with glutathione. Such bioactivation catalyzed by GST can lead to mutagenic DNA adducts (24, 29). S-(2-Bromoethyl)glutathione, the GST-catalyzed DBE metabolite with glutathione, transforms into an electrophilic episulfonium ion that attacks nucleoside bases and forms mostly (95%) S-[2-(N2-guanyl)ethyl]glutathione (29). This DNA adduct blocks DNA polymerase activity and leads to G:C to A:T transition mutations. It was suggested that abortive repair of DNA adducts formed from a GST-derived metabolite by Ogt may be responsible for the increased genotoxicity in cells expressing this bacterial AGT (7).

Our studies show that it is unlikely that incorrect repair of such lesions explains the hAGT-mediated increase in genotoxicity. We used S-(2-fluoroethyl)glutathione (t1/2 = 37 min) in 0.2 M phosphate buffer and S-(2-chloroethyl)glutathione (t1/2 = 5.3 min) as surrogates for S-(2-bromoethyl)glutathione (t1/2 = 0.4 min) (26). As shown in Fig. 2, the cytotoxicity and mutagenicity of these S-(2-haloethyl)glutathiones was not enhanced but actually slightly reduced by expression of hAGT. This result is consistent with our finding (Fig. 3) that the S-[2-(O2-guanyl)-ethyl]glutathione adduct, which is known to be formed albeit in small amounts from the reaction with DNA, is a substrate and is repaired accurately by wild type hAGT protein. S-(2-Fluoroethyl)glutathione and S-(2-chloroethyl)glutathione were only weakly mutagenic in E. coli strain TRG8, which lacks all alkyltransferase activity and even less so when hAGT is expressed, but they are strongly mutagenic in Salmonella typhimurium TA1535 (26, 37). This result suggests that the two chemicals cause a mutation distinct from that of hAGT-mediated DBE mutagenicity.

A second pathway for metabolism of DBE is via P450-mediated oxidation forming 2-bromoacetaldehyde, which is rapidly reduced to BE (16). As shown in Fig. 1, BE is neither cytotoxic nor mutagenic in TRG8 cells irrespective of their AGT status, suggesting that the observed DBE toxicity in E. coli expressing high levels of Ogt, Ada, or hAGT (7, 8, 10, 12) and Chinese hamster lung fibroblasts expressing Ogt (12) results from unmetabolized DBE rather than from BE. The difference of DBE and BE in inducing toxicity in AGT-expressing cells indicates that having two good leaving groups is critical for bringing about this toxicity.

Our comparison of the effects of wild type AGT and the inactive C145A mutant (Fig. 1) indicate that the Cys145 residue is critical for the mechanism. Previous studies (9, 10), showing that the ability of AGT to enhance the genotoxicity of DBE is prevented by treatment with the AGT inhibitor, O6-benzylguanine, which reacts with Cys145 (45), also support this conclusion. The C145A mutant has an intact DNA binding domain and is known to bind to DNA in vitro as well as does wild type AGT (42). Also, the expression of this mutant in E. coli increases the mutagenicity of methylating agents by binding to O6-methylguanine adducts formed in the DNA and preventing their repair (35). The inability of the C145A mutant to enhance the genotoxicity of DBE therefore rules out the mechanism by which non-covalent binding of hAGT protein to a DNA lesion prevents damage repair by other DNA repair mechanisms. It also focuses attention on the interaction of DBE with Cys145 as a critical part of the mechanism.

As shown in Figs. 4–6, DBE and BE do react readily with the Cys145 residue. This is shown unequivocally by the loss of AGT activity, by the covalent attachment of radioactivity from [14C]DBE, and by the conversion of Cys145 to its hydroxyethyl...
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derivative seen in the analysis by MS. The facile reaction of this Cys\textsuperscript{145} residue but not others is confirmed by the lack of labeling and of adducts detectable by MS when the C145S mutant was used. The enhanced reactivity of Cys\textsuperscript{145} is probably due to its conjugation as part of a hydrogen-bond network involving a water molecule, His\textsuperscript{146} and Glu\textsuperscript{172} (42). This activates Cys\textsuperscript{145} to allow the rapid nucleophilic attack on O\textsuperscript{6}-alkyl adducts in DNA for its physiological reaction and also renders hAGT very sensitive to inactivation by NO (41). However, the rate of hAGT reaction with DBE calculated from conjugation study showed that it is 40 times lower (24 M\textsuperscript{-1} min\textsuperscript{-1}) than that of GST-catalyzed glutathione conjugation (1000 M\textsuperscript{-1} min\textsuperscript{-1}) (26).

The detection of a 2-hydroxyethyl adduct on AGT-Cys\textsuperscript{145} is consistent with an initial formation of a 2-bromoethyl adduct on Cys\textsuperscript{145}. The S-(2-bromoethyl)-Cys\textsuperscript{145} hAGT would be expected to form an episulfonium ion, a potent electrophile that in the absence of other reactants would react with water to form S-(2-hydroxyethyl)-Cys\textsuperscript{145} hAGT (Fig. 9). BE, which was also able to inactivate hAGT (Fig. 4), could form this derivative directly, and it was found in the MS analysis (Fig. 6).

As shown in Figs. 7 and 8, if DNA is present when the S-(2-bromoethyl)-Cys\textsuperscript{145} hAGT is formed, there is a formation of a cross-linked DNA-hAGT. This would be expected by the reaction of the episulfonium ion with DNA, which is placed in proximity to it by the DNA-binding properties of the hAGT protein. The absence of cross-linking by BE (Fig. 8) is expected due to the absence of a second leaving group. These data are all consistent with the scheme shown in Fig. 9 in which the hAGT-enhanced genotoxicity of DBE occurs via the initial formation of S-(2-bromoethyl)-Cys\textsuperscript{145} hAGT, its conversion to an episulfonium ion intermediate, and its reaction to form DNA adducts. Further work is needed to establish the nature of these adducts and their biological effects.

Structural and biochemistry studies on hAGT have provided a plausible mechanism for its ability to repair O\textsuperscript{6}-methylguanine adducts in DNA. The DNA is bound via a winged helix-turn-helix motif with little sequence specificity and the target O\textsuperscript{6}-methylguanine nucleotide is flipped out of the helix into a binding pocket that contains Cys\textsuperscript{245} (44, 46). As described above, the interaction of this Cys\textsuperscript{245} with other residues at the active site activates it for an efficient attack on the alkyl group of O\textsuperscript{6}-methylguanine to form S-alklyleysteine and restore the original DNA structure. AGTs in general are known to also be expected to form an episulfonium ion, a potent electrophile that in the reaction of the episulfonium ion with DNA, which is placed in proximity to it by the DNA-binding properties of the hAGT protein. The absence of cross-linking by BE (Fig. 8) is expected due to the absence of a second leaving group. These data are all consistent with the scheme shown in Fig. 9 in which the hAGT-enhanced genotoxicity of DBE occurs via the initial formation of S-(2-bromoethyl)-Cys\textsuperscript{145} hAGT, its conversion to an episulfonium ion intermediate, and its reaction to form DNA adducts. Further work is needed to establish the nature of these adducts and their biological effects.

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