A Novel, Sensitive Assay for \(O^6\)-Methyl- and \(O^6\)-Ethylguanine in DNA, Based on Repair by the Enzyme \(O^6\)-Alkylguanine-DNA-Alkyltransferase in Competition with an Oligonucleotide Containing \(O^6\)-Methylguanine

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ABSTRACT

A novel assay for \(O^\alpha\)-alkylguanine-type adducts in DNA is reported. It is based on the use of the suicide repair enzyme \(O^\alpha\)-alkylguanine-DNA-alkyltransferase (AGT) to repair such adducts in DNA in competition with an oligonucleotide containing a single residue of \(O^\alpha\)-methylguanine, end labeled to high specific activity. The stoichiometric mode of action of AGT results in decreased amounts of oligonucleotide being repaired in the presence of increasing levels of adducts in the competing DNA. The extent of oligonucleotide repair is determined by immunoprecipitation of the unrepaired form with rabbit antiserum directed against \(O^\alpha\)-alkyldesoxyguanosine and radioisotopic. The amount of \(O^\alpha\)-methylguanine in competing DNA is calculated by reference to a standard curve constructed using DNA of known alkylation. In view of its relatively wide spectrum of alkyl group specificity, use of AGT from rat liver permits the determination of both \(O^\alpha\)-methyl- and \(O^\alpha\)-ethyIguanine (detection limits, 0.8 fmol and 3 fmol, respectively). On the other hand, the restricted specificity of Escherichia coli AGT to repair of \(O^\alpha\)-methylguanine makes the assay based on it specific for this type of lesion (detection limit, 0.5 fmol). The maximum amount of DNA which can be included in the assay is 15 \(\mu\)g and 10 \(\mu\)g for the rat liver and \(E.\ collar AGT-based assays, respectively, leading to a limit of sensitivity of \(8 \times 10^{-8}\) mol \(O^\alpha\)-methylguanine/mol guanine (50 fmol/mg DNA) (both enzymes) and 3 \(\times\) \(10^{-8}\) mol \(O^\alpha\)-ethyIguanine/mol guanine (200 fmol/mg DNA) (rat liver AGT-based assay) and making this one of the most sensitive assays for these important precarcinogenic adducts. The new assay has been validated by assaying DNA from rat liver methylated \textit{in vivo} with dimethylaminozium to a known extent and has been found to give results in close agreement with those of radioimmunoassay. Six h after i.p. administration of dimethylaminozine (0.01-1 mg/kg) to rats, \(O^\alpha\)-methylguanine was detectable by the competitive-repair assay in liver or lymphocyte DNA at levels of 0.14-14.4 \(\mu\)mol/mol guanine.

INTRODUCTION

The current awareness of the significance of carcinogen-DNA adducts in the mechanism of chemical mutagenesis and carcinogenesis has led to much interest in the possibility of their detection in human tissues. It is believed that such detection may serve as a qualitative index of exposure to mutagens and carcinogens, while it is hoped that the quantitative determination of carcinogen-DNA adducts present in human tissues may ultimately be useful in risk assessment (1–3).

The detection of the extremely low levels of carcinogen-DNA adducts expected to be derived from human exposure to environmental genotoxic agents has necessitated the development of assays of corresponding sensitivity and specificity. Assays primarily based on immunochemical techniques or on \(32\)P-post-labeling of nucleoside adducts have been developed for an increasing number of specific adducts and many of them are capable of extremely high sensitivities, thus permitting the detection of the corresponding adducts at levels of environmen-
examined for use in an assay for $O^\alpha$-methyl- and $O^\alpha$-ethylguanines in DNA.

**MATERIALS AND METHODS**

Oligonucleotides and Antibodies. The oligonucleotide d(CGCC($O^\alpha$-meG)AGCTCCC)G as well as the corresponding nonalkylated oligonucleotide were generously provided by Drs. B. F. Li and P. F. Swann, Department of Biochemistry, The University College and Middlesex Hospital Medical School, London. They were synthesized by the phosphotriester method and purified by HPLC (20). They were 5' labeled with $[^3]P$ATP (approximately 5000 Ci/mmol) using T4-poly nucleotide kinase under conditions of the exchange reaction (21). 5' Labeling with $[^3]P$adenosine 5'- (thy) triphosphate (800-1200 Ci/mmol; New England Nuclear, Dreieich, FRG) was carried out by a modification of the same method involving two additions of T4-poly nucleotide kinase (10 units each, one at the beginning of the reaction and one 30 min later. Typical specific activities achieved were 2000-3500 cpm/fmol with $[^3]P$ and 100-250 cpm/fmol with $[^3]S$. Following labeling, the oligonucleotides were purified by gel filtration through Sephadex G15 using for elution 50 mM ammonium bicarbonate/10 mM DTT. The specific activity of the labeled oligonucleotides was determined by titration with AGT preparations of known concentration.

The production and properties of rabbit antisera against $O^\alpha$-methyl-deoxyguanosine have already been described (13).

Sources of AGT. Purified AGT from E. coli (ada protein) (3000 pmol/mg protein) was obtained from Applied Genetics, Freeport, NY. Partially purified rat liver AGT was prepared as described by Scicchitano and Pegg (22), and purified by chromatography on DNA-cellulose to a specific activity of 14.5 pmol/mg protein (148-fold purification relative to the crude extract). The specific activity of rat liver AGT was determined by titration of $^{3}H$-methylated DNA of known $O^\alpha$-methylguanine content which had been depleted of $N^\alpha$-methylated purines by heating to 80°C for 16 h in neutral buffer.

Alkylated DNA. Calf thymus DNA (Sigma) was methylated with $N$-[methyl-$^3$H]-$N$-nitrosourea (1.1 Ci/mmole) (New England Nuclear) (22) and purified by repeated precipitation with ethyl alcohol. It was analyzed for methylation by hydrolysis in 0.1 N HCl at 70°C and HPLC and found to contain 76.8 pmol $O^\alpha$-methylguanosine/mol guanine. Samples of rat liver DNA, methylated in vivo with dimethyl nitrosamine and accurately analyzed by radioimmunoassay (16), were kindly provided by Dr. A. M. Camus, The International Agency for Research on Cancer, Lyon, France. They contained 0.28, 0.39, 0.50, and 0.90 pmol $O^\alpha$-methylguanosine/mol guanine. Salmon sperm DNA, ethylated with $N$-[ethyl-$^3$H]-$N$-nitrosourea and containing 13.5 pmol $O^\alpha$-ethylguanosine/mol guanine, was kindly provided by Prof. A. A. van Zeeland, Department of Radiation Genetics and Chemical Mutagenesis, The State University of Leiden, The Netherlands.

Competitive Repair Assay. The c.r.a. for $O^\alpha$-methylguanine was set up using AGT derived from rat liver or E. coli. In view of the different reaction kinetics as well as stabilities of these enzymes, different conditions were used in each case. With AGT from rat liver, the assay was carried out as follows: To a series of vials containing alkylated DNA (0-10 fmol $O^\alpha$-alkylguanine, up to 15 µg total DNA) in transferase buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.8, 1 mM EDTA, 10 mM DTT, 50 µM spermidine, 5% glycerol, 50 µg/ml bovine serum albumin), 7 fmol AGT in transferase buffer were added and the mixture (volume 36 µl) was incubated for 10 min at 37°C. Following this incubation, 10 fmol of labeled $O^\alpha$-methylguanine-containing oligonucleotide in 4 µl transferase buffer were added and incubation continued for a further 2 h. The mixture was then placed on ice, 10 µl of 5 × PBS (1 × PBS: 1 mM NaHPO$_4$, pH 7.2, 0.15 NaCl) and 25 µl of an appropriate dilution of rabbit anti-$O^\alpha$-methyldeoxyguanosine antisemur added, and the mixture further incubated on ice for 30 min. Protein was precipitated with an equal volume of saturated ammonium sulphate solution and washed with 50% saturated ammonium sulphate solution, the precipitate was dissolved in 0.1 N sodium hydroxide, mixed with scintillation cocktail, and counted. The assay with E. coli AGT was carried out in a similar way except that (a) incubation with the methylated DNA (first stage) was prolonged to 2 h and that with the oligonucleotide (second stage) to overnight and (b) the total amount of DNA per assay was limited to a maximum of 10 µg. The optimal conditions of each assay were determined by systematic variation of all the relevant parameters as described under "Results."

In order to confirm that the inhibition of repair of the labeled oligonucleotide observed during the second stage of the assay was due to repair of $O^\alpha$-alkylguanine-type of damage in the DNA used during the first-stage incubation, a series of mixtures containing alkylated DNA as above, 100 fmol rat liver AGT and no labeled oligonucleotide, were first incubated overnight at 37°C (to remove all $O^\alpha$-alkylguanine present in the DNA), then heated to 60°C for 10 min to inactivate all residual AGT. Finally AGT and labeled oligonucleotide were added as described in the previous paragraph to assay any remaining $O^\alpha$-alkylguanine.

Animal Experiments. Pairs of Sprague-Dawley rats (140-180 g), fed normal rat chow and water ad libitum, were injected i.p. with dimethyl nitrosamine dissolved in physiological saline at doses of 0.01-1 mg/kg. Blood was collected by cardiac puncture under ether anesthesia 6 h later. For the isolation of circulating lymphocytes, freshly collected blood was centrifuged on Lymphoprep (Nycomed AS, Oslo), the lymphocyte layer collected and washed with PBS. This preparation was used for the isolation of DNA by a standard method involving treatment with protease K and ribonucleases A and T1 and a series of extractions with phenol and chloroform (21). DNA was isolated from rat liver by a modification of a published procedure involving phenol extractions, with the additional inclusion of treatment with protease K and ribonuclease A (23).

**RESULTS**

Immunoprecipitation with antibodies against $O^\alpha$-methyl-deoxyguanosine was found to offer a rapid and convenient method for the separation of repaired and unrepaired oligonucleotide. Substrate precipitation was always greater than 80% and was not significantly affected by the presence of nonmethylated oligonucleotide. The background due to nonspecific precipitation of the latter was less than 10%.

Examination of the kinetics of repair of the oligonucleotide by the E. coli and rat liver AGT preparations indicated that, at enzyme and substrate concentrations of 10 fmol per 40 µl, the reaction was complete in well under 2 h. Full details of these kinetic studies are reported elsewhere (24). Repair of $O^\alpha$-methylguanine in macromolecular DNA (corresponding to the first stage of the assay) is known never to proceed to 100% completion (22), and for this reason no attempt was made to analyze in detail the kinetics of this reaction under the conditions likely to hold during the assay. However, preliminary examination confirmed that repair of fmol quantities of $O^\alpha$-methylguanine in DNA by similar amounts of rat liver AGT would require a few hours to proceed to any significant degree and, at the lower fmol end of the concentration scale, would result in only limited repair.

In view of the prolonged periods of incubation which were anticipated to be required during the assay, the stability of the AGT preparations used was examined by incubation of 7 fmol enzyme in 40 µl reaction buffer for different times at 37°C followed by addition of 10 fmol $^{35}$S-labeled oligonucleotide substrate and incubation for a further 2 h. The results obtained (Fig. 1) indicated that the rat liver AGT preparation lost most of its activity within the first hour of incubation, while E. coli AGT was significantly more stable. No significant stabilization could be achieved by inclusion in the incubation medium of various concentrations of the protease inhibitors phenyl-methylsulphonyl fluoride, disopropylfluorophosphate, and soybean trypsin inhibitor, or of increased amounts of DNA,
and the incubation continued for a further 2 h.

ence of the amount of alkylation initially present. Instead, it
protein (bovine serum albumin), DTT, and EDTA.

for the periods indicated. Subsequently, 10 fmol of labeled oligonucleotide added
was necessary to construct a standard curve relating the extent of oligonucleotide repair to the amount of O^-alkylguanine

was to use the E. coli AGT in an assay for ethylation in
view of the greatly reduced ability of this enzyme to repair ethylated adducts (20, 25).

The limited stability of the AGT preparations and the less
than 100% repair of O^-methylguanine in macromolecular
DNA achievable, meant that the proposed assay could not be
based on quantitative repair of O^-alkylguanine in the unknown
DNA (and corresponding consumption of the enzyme), fol-
lowed by back-titration of the latter and calculation by differ-
ence of the amount of alkylation initially present. Instead, it
was necessary to construct a standard curve relating the extent
of oligonucleotide repair to the amount of O^-alkylguanine
present in DNA during the first-stage incubation and calculate
the level of alkylation of unknown DNA samples by reference
to it. With these considerations in mind, a standard assay
protocol was set up involving two stages: During the first stage,
increasing quantities of DNA, containing known amounts of
O^-alkylguanine, were incubated with a fixed amount of AGT.
After this incubation, a fixed amount of labeled oligonucleotide
was added and the second-stage incubation carried out, followed
finally by immunoprecipitation and counting of the unrepaired
oligonucleotide. The effects on the extent of oligonucleotide
repair of a number of parameters were systematically examined
in order to achieve optimization of the assay. Apart from the
lengths of the two incubation periods, the absolute as well as
the relative concentrations of AGT and the oligonucleotide
substrate used in the assay are critical for achieving maximum
sensitivity. The optimal concentration of AGT would have to
constitute a balance between a value sufficiently high so as to
yield significant repair of the DNA under examination under
the conditions of the assay but, at the same time, sufficiently
low so as to be significantly consumed in this process. Similarly,
the concentration of the radiolabeled oligonucleotide would
have to be sufficiently low for its consumption by the AGT
remaining after the first-stage incubation to be significant, while
its lower limit would be determined by (a) its specific radioac-
tivity and (b) its rate of repair by AGT.

The parameters examined included the lengths of the two
incubations (0–30 min and 2–16 h, respectively), the amount of
AGT (2–12 fmol per assay) and the amount (0.2–12 fmol per assay) as well as the specific radioactivity (^32P- or ^35S-
labeling) of the oligonucleotide substrate. Furthermore, as the
rate of repair of methylated DNA is known to depend on its
level of methylation (i.e., on the frequency with which the
methyl groups are distributed along the DNA molecule) (22), the effects of the extent of methylation as well as the size of the
DNA (i.e., the number of methyl groups per DNA molecule)
were examined, by using DNA methylated to different degrees,
both in the form of macromolecular DNA (average size, 40
kilobase pairs) and of DNA exhaustively digested with the
restriction enzyme MboI (average size, 246 base pairs).

The optimal conditions finally selected are described in “Ma-
terials and Methods.” As can be seen in Fig. 2A, following
preincubation of AGT with DNA containing increasing quan-
tities of O^-methyl- or O^-ethy1guanine, the amount of labeled
oligonucleotide remaining unrepai red increases steadily. A
practically linear relationship with the amount of competing O-
methylguanine is observed while, in the case of O^-ethy1guan-
ine, the corresponding relationship is clearly sigmoidal, prob-
ably due to the slower rate of repair of this adduct. Under the
optimal conditions, as little as 0.8 fmol O^-methylguanine (rat
liver AGT-based assay) or 0.5 fmol O^-methylguanine (E. coli
AGT-based assay) in DNA caused a significant reduction in
oligonucleotide repair. The corresponding limit of detection of
O^-ethy1guanine using rat liver AGT was 3 fmol. [No attempt
was made to use the E. coli AGT in an assay for ethylation in
view of the greatly reduced ability of this enzyme to repair
ethylated adducts (20, 25).] No significant improvement in the
sensitivity of the assay was seen when ^32P-labeled substrate (261
cpm/fmol) was used in place of the ^35S-labeled one (63 cpm/
fmol), indicating that the sensitivity of the assay was limited by
the rate of the first-stage reaction (data not shown). Finally,
the extent of oligonucleotide repair in competition with a given
amount of O^-methylguanine was not significantly affected by
either the size of the DNA or its degree of methylation (within
the range 0.9–76.8 (pmol O^-methylguanine/mol guanine) (Fig.
2B). In view of these findings, undigested DNA and ^35S-labeled
oligonucleotide were routinely employed.

It is well known that the repair of DNA by AGT is inhibited
by nonalkylated DNA. This inhibition was reflected in the effect
of increasing total DNA concentration on the sensitivity of the
assay and resulted in decreased sensitivity which in practice
became limiting at 15 (g per assay for the rat liver enzyme and
10 (g per assay for the E. coli enzyme (data not shown).

Validation of the c.r.a was carried out by analysis of four
samples of rat liver DNA obtained from animals which had
been dosed with dimethylnitrosamine and containing known
amounts of O^-methylguanine. These samples were analyzed
using both the rat liver and the E. coli AGT-based assays. Two
different quantities of each sample were analyzed while keeping
the total amount of DNA constant at 10 (g per assay. The
results obtained (Table 1) show excellent agreement with those

![Fig. 1. Examination of the stability of rat-liver (O) and E. coli (●) AGT. Seven
fmol AGT were incubated in the absence of substrate in transferase buffer at 37°C
for the periods indicated. Subsequently, 10 fmol of labeled oligonucleotide added
and the incubation continued for a further 2 h.](image)

![Fig. 2. Standard curves for the inhibition of oligonucleotide repair by increasing
amounts of DNA containing O^-methyl- or O^-ethy1guanine. The conditions
of incubation are described in “Materials and Methods.” A. undigested DNA
containing 76.8 (pmol O^-methylguanine/mol guanine (open symbols) or 13.5
(pmol O^-ethy1guanine/mol guanine (filled symbols). Circles, E. coli AGT; squares,
rat liver AGT. The differences in the vertical intercepts are due to the use of
radiolabeled oligonucleotides of different specific activities. B. DNA containing
76.8 (pmol O^-methylguanine/mol guanine, undigested (filled symbols) or exhaus-
tively digested with MboI (open symbols). The regression line is drawn through the data for undigested DNA containing 76.8 (pmol of O^-methyl-
guanine/mol guanine.](image)
The ability of rat liver AGT to recognize and repair O6-alkylguanine adducts in DNA makes the E. coli AGT-based c.r.a. in practice specific for O6-methylguanine. Therefore, the combined use of the two c.r.a.’s reported here for the analysis of DNA exposed to unknown environmental alkylating agents would permit the specific detection and quantitation of both O6-methylguanine and O4-ethylguanine. It should be noted, in this context, that there is evidence that both kinds of adducts may be formed in humans by exposure to as yet unknown environmental chemicals (16–19).

Analysis of a series of rat liver DNA samples, methylated in vivo with dimethylnitrosamine, indicated that the c.r.a. gives results in excellent agreement with those obtained by radioimmunoassay ($r = 0.99$) (Table 1). The negative results obtained following separate pretreatment of the same samples with excess AGT verified that the assay specifically measured O6-alkylguanine adducts.

Following i.p. administration of dimethylnitrosamine to rats, O6-methylguanine could be detected in lymphocyte DNA at levels only slightly lower than those found in the DNA of liver, the main site of DMN metabolism. The extent of methylation found is comparable with that reported recently by Degan et al. (27) and supports the suggestion of these workers that methylation of lymphocyte DNA may serve as a useful index of human exposure to dimethylnitrosamine. By extrapolating from Fig. 3 it can be calculated that O6-methylguanine could be detected by the c.r.a. in rat lymphocytes 6 h after exposure to a dose of dimethylnitrosamine as low as 13 $\mu$g/kg.

The c.r.a. reported here appears to be one of the most sensitive assays currently available of O6-methylguanine and should prove useful for studies of molecular epidemiology of this precarcinogenic adduct. Its limits of sensitivity, which are in practice determined by the amount of DNA which can be usefully included in it, the stability of AGT and the rate of DNA repair, are summarized in Table 2. These limits compare favorably with those of immunochemical techniques which have been employed in the past for the detection of O6-alkylguanine adducts (12–15). Most immunoassays for O4-ethyldeoxyguanosine have a limit of detection of a few femtomoles or more, while the lowest extent of DNA-methylation that can be detected is well over $10^{-7}$ mol O6-methyldeoxyguanosine/mol guanine. The performance of immunoassays for O6-ethyldeoxyguanosine is better owing to the higher avidity of the corresponding antibodies. Higher sensitivities can be achieved by combination of HPLC of enzymatically hydrolyzed DNA and immunoassay. In a study of DNA methylation in specimens of esophageal DNA obtained from individuals from a region in China with high incidence of esophageal cancer, Umbenhauer et al. (16) reported levels of O6-methylguanine in the region of 20–200 fmol/mg DNA, detected by combined HPLC/radioimmunoassay analysis of approximately 3 mg DNA in each

### Table 1: Analysis of methylated DNA samples by c.r.a. and radioimmunoassay

<table>
<thead>
<tr>
<th>DNA sample no.</th>
<th>O6-Methylguanine content ($8 \times 10^{-15}$ mol/molG)</th>
<th>Radioimmunoassay</th>
<th>c.r.a. rat liver AGT</th>
<th>c.r.a. E. coli AGT</th>
<th>O6-Ethylguanine, Rat liver AGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>32 (+14.3%)</td>
<td>26 (~8.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>40 (+2.6%)</td>
<td>40 (+2.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>54 (+8.8%)</td>
<td>46 (~7.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>46 (+8.8%)</td>
<td>54 (+8.8%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Deviation from radioimmunoassay values.

### Table 2: Sensitivity of the competitive-repair assay

<table>
<thead>
<tr>
<th>O6-Methylguanine</th>
<th>Rat liver AGT</th>
<th>E. coli AGT</th>
<th>O6-Ethylguanine, Rat liver AGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum DNA per assay ($\mu$g)</td>
<td>15</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Limit of detection (fmol)</td>
<td>0.8</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>Sensitivity fmol/mg DNA</td>
<td>50</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>mol/mg guanine</td>
<td>$8 \times 10^{-4}$</td>
<td>$8 \times 10^{-4}$</td>
<td>$3 \times 10^{-7}$</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

We are grateful to Drs. B. F. Li and P. F. Swann, Department of Biochemistry, The University College and Middlesex Hospital, London, for a generous gift of the oligonucleotides, and to Dr. A-M. Camus for gifts of DNA of known O'-alkylguanine content.

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