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COMPARISON OF REPAIR OF METHYLATED PYRIMIDINES IN POLY(dT) BY EXTRACTS FROM RAT LIVER AND ESCHERICHIA COLI

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SUMMARY. Partially purified preparations of O^6-alkylguanine-DNA alkyltransferase from rat liver and E. coli were tested for their ability to repair O^4-methylthymine in a methylated poly(dT).poly(dA) substrate. The bacterial preparation readily carried out this reaction, but no loss of O^4-methylthymine was obtained with the rat liver protein. These results indicate a significant difference in specificity between the mammalian and bacterial proteins which could have important consequences for carcinogenesis and mutagenesis by alkylating agents in mammalian cells.

The initiation of tumors by alkylating agents appears to involve structural changes in target cell DNA. A number of alkylation sites in DNA are observed under physiological conditions including alkylation at the O^6-position of guanine and the O^4-position of thymine (1-5). These adducts are potentially promutagenic and are known to miscode when copied by DNA polymerases (4,6-8). Mammalian and bacterial cells contain a protein termed O^6-alkylguanine-DNA alkyltransferase which repairs the O^6-alkylguanine lesion by transferring the alkyl group to one of its own cysteine residues restoring the guanine in the DNA in a single step (9-12). The alkyltransferase is inactivated in this process and re-activation is very slow if it occurs at all. Therefore, rapid repair of O^6-alkylguanine in DNA can occur after exposure to doses of alkylating agent which produce fewer adducts than the number of molecules of the alkyltransferase, but higher doses exhaust the alkyltransferase capacity and lead to the accumulation of O^6-alkylguanine. In some but not all cases, a reasonable correlation between the capacity of cells to repair O^6-alkylguanine by this

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method and resistance to carcinogenesis and mutagenesis has been documented (3,13).

Although most of the work in this field has concentrated on the possibility that O^6-alkylguanine is the critical lesion, it has been pointed out that other adducts such as O^4-alkylthymine could be equally important (4,14). This is particularly relevant with respect to ethylating agents since these produce a higher proportion of such adducts than their methylating counterparts (3,4,14). However, there has been little work on the enzymology of repair of O^4-alkylthymine Recently, three laboratories have reported preliminary data that E. coli contains a methyltransferase protein which can act on O^4-methylthymine (15-17). In the present work, we tested the abilities of partially purified O^6-alkylguanine-DNA alkyltransferases from E. coli and rat liver to repair methylated thymine residues in DNA. The substrate used was prepared by reacting poly(dT) with N-[^{3}H]methyl-N-nitrosourea and mixing this labeled poly(dT) with an equal amount of poly(dA). The substrate termed [^{3}H]methylated poly(dT)·poly(dA) contained labeled O^2-methylthymine, O^4-methylthymine and 3-methylthymine as well as methylphosphate triesters. The rat liver preparation did not repair any of these methylated bases, but the E. coli preparation efficiently repaired the O^4-methylthymine.

MATERIALS AND METHODS

Polynucleotides and enzymes were obtained from Sigma Chemical Co., St. Louis, MO. Deoxyribonuclease I was purchased from Worthington Biochemical Corp., Freehold, NJ. N-[^{3}H]Methyl-N-nitrosourea (2.9 Ci/mmol) was purchased from Amersham/Searle, Arlington Heights, IL. N-[^{3}H]Ethyl-N-nitrosourea (0.123 Ci/mmol) was obtained from NEN, Boston, MA. E. coli strain BS21 was kindly provided by Dr. S. Mitra, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN. DNA containing radioactive alkylated bases was prepared by the reaction of calf thymus DNA (6 mg/ml) with [^{3}H]methylnitrosourea or [^{3}H]ethylnitrosourea in 0.05 M Tris buffer, pH 8.0, as previously described (11). Methylated poly(dT).poly(dA) was prepared by reacting 25 units of poly(dT) with 0.1 mCi of [^{3}H]methylnitrosourea in 0.05 M Tris, pH 8.0 at 37°C for 30 min. The alkylated poly(dT) was dialyzed extensively against 0.05 M Tris, pH 8.0 to remove remaining unbound radioactivity. The alkylated poly(dT) was incubated with 25 units poly(dA) in this same buffer at room temperature for two h. O^6-Alkylguanine-DNA alkyltransferase was isolated from rat liver (11) or E. coli (12). One unit was defined as the amount of protein needed to remove 1 pmol of O^6-methylguanine from DNA.

Assay for alkylated base removal. Assays of the E. coli protein were carried out by incubation at 37°C for 1 h of a reaction mixture containing the methylated poly(dT).poly(dA) substrate (about 0.5 pmol of O^4-methylthymine),

15 mM NaCl, 50 mM Hepes, pH 7.8, 1 mM EDTA, 10 mM dithiothreitol , 0.5 mM 2-mercaptoethanol, 5% glycerol and the protein. Assays of the rat liver extracts were carried out for 90 min at 37°C using the same amount of the substrate, 50 mM Tris, pH 7.8, 0.1 mM EDTA, 5 mM dithiothreitol, 0.02% Brij 35. Control incubations without added protein were carried out for the same time in the same buffers. At the end of the incubation, the reaction mix was cooled to 0°C and adjusted to pH 6.5 and 0.01M magnesium acetate. Deoxyribonuclease I (660 units/ml) was added and the mixture incubated for 1 h at 37°C. The pH was then adjusted to 8.0 by the addition of 0.1 volumes of 1M Tris, pH 8.6 and E. coli alkaline phosphatase (8 units/ml) and snake venom phosphodiesterase (0.2 units/ml) were added. After incubation at 37°C for 16-18 h, proteins were precipitated by boiling the samples for 5 min, centrifuging at 16,000 x g for 1 h and recentrifuging the supernatant. This was then analyzed by HPLC after addition of authentic markers of methylated thymidine derivatives (6,8). HPLC was carried out using a Rainin Lichrosorb RP18 column (4.6 x 300 mm) fitted with a precolumn (4.6 x 40 mm) of the same material. The samples were eluted with 20% aqueous methanol at 35°C for 30 min and the columns then washed with 100% methanol for 40 min. The flow rate was 1 ml/min and the deoxynucleotide markers were detected using a Waters UV detector at a wavelength of 254 nm. Elution times were 9.5 min for O^2-methylthymidine, 14.7 min for 3-methylthymidine and 21.7 min for O^4-methylthymidine. For the assay of O^6-methylguanine removal, calf thymus methylated DNA containing 0.54 pmoles of O^6-methylguanine was incubated as described above with E. coli, rat liver extracts, or the appropriate buffer. After the incubation period, samples were acid hydrolyzed with 0.1N HCl at 70°C and analyzed on a Whatman Partisil 10SCX column (13). The assay of ethylated base removal was carried out by incubation of ethylated DNA (about 15 pmol of O^6-ethylguanine; 4.8 pmol of O^4-ethylthymine) with 20 units of rat liver protein for up to 8 h in the assay buffer for rat liver protein described above. The ethylated DNA was then analyzed for 7-ethylguanine, O^6-ethylguanine, and ethylated pyrimidines after enzymic digestion to nucleosides at pH 6.8 (14).

RESULTS AND DISCUSSION

Analysis of the methylated substrate prepared by reacting poly(dT) with N-[^{3}H]-methyl-N-nitrosourea was carried out by enzymatic degradation to deoxynucleosides and separation by reverse phase HPLC. This indicated that about 10% of the incorporated radioactivity was present in the three methylated thymidine derivatives. This was distributed as about 2.2% as O^2-methylthymidine, 4.5% as 3-methylthymidine and 3.3% as O^4-methylthymidine. The remaining radioactivity was not characterized, but presumably represents methylation on phosphates which would yield predominantly dTp(Me)dT after hydrolysis. The majority of this labeled material remained bound to the column during the gradient separation and was eluted with 100% methanol. When the substrate was incubated with O^6-alkylguanine-DNA alkyltransferase from E. coli and then analyzed (Figure 1c and d) there was a complete loss of O^4-methylthymidine, but 3-methylthymidine was not affected. When relatively large amounts of the bacterial protein were used, there also appeared to be some loss of O^2-methyl-

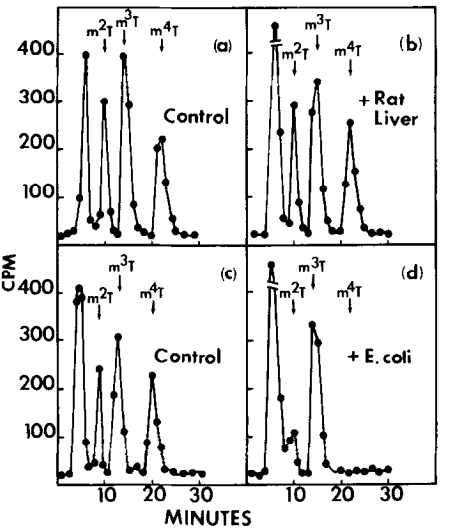

Figure 1. HPLC analysis of methylated thymine derivatives from methylated poly(dT).poly(dA) after incubation with rat liver or E. coli O^6-alkylguanine-DNA alkyltransferase. Results in panels (a) and (c) show analysis of the substrate incubated without protein, in panel (b) for incubation for 90 min with 1.7 units of rat liver alkyltransferase and in panel (d) for incubation for 60 min with 11.2 units of E. coli alkyltransferase. The elution positions of authentic O^2-methylthymidine (m^2T), 3-methylthymidine (m^3T) and O^4-methylthymidine (m^4T) are indicated by the arrows.

thymidine as indicated in Figure 1d, but in other experiments with lower amounts of the protein, this was not observed (Table 1). In all experiments, the O^4-methylthymidine was virtually all removed by the bacterial protein and there was no loss of 3-methylthymidine (Table 1). In contrast, when the O^6-alkylguanine-DNA alkyltransferase from rat liver was used, there was no loss of any of the methylated thymidines including O^4-methylthymidine (Figure 1a and 1b). As shown in Table 1, the rat liver protein was active in removing O^6-methylguanine from DNA in a parallel experiment in which exactly the same protein preparation and assay conditions were used except for the substrate which in this case was methylated calf thymus DNA.

These results confirm recent reports (15-17) that O^4-methylthymidine is repaired by extracts from E. coli. Although the bacterial preparation used by us was partially purified, there were a number of protein bands when assayed by polyacrylamide gel electrophoresis in addition to that at a M.W. of about 18,000 which corresponds to the O^6-alkylguanine-DNA alkyltransferase. Therefore, it is possible that the removal of O^4-methylthymidine was due to a contaminating

TABLE 1

REMOVAL OF METHYL GROUPS FROM O^6-METHYLGUANINE AND O^4-METHYLTHYMINE

Extract added	Methylated bases remaining in substrate (pmol)				
	O^2-methyl-thymine	3-methyl-thymine	O^4-methyl-thymine	O^6-methyl-guanine	7-methyl-guanine
Experiment A					
None	0.38	0.76	0.56	0.58	4.6
3.8 units alkyltransferase from E. coli	0.38	0.77	0.05	0.05	4.4
3.8 units alkyltransferase from rat liver	0.41	0.83	0.57	0.06	4.7
Experiment B					
None	0.30	0.61	0.44	N.D.	N.D.
1.7 units alkyltransferase from rat liver	0.29	0.57	0.42	N.D.	N.D.
11.2 units alkyltransferase from E. coli	0.30	0.66	0.01	N.D.	N.D.
22.4 units alkyltransferase from E. coli	0.19	0.43	0.00	N.D.	N.D.

In experiment A, the extract shown was incubated with the appropriate DNA substrate (either methylated poly(dT).poly(dA) for analysis of methylated thymines or methylated calf thymus DNA for methylated guanines) and the resulting loss in the methylated bases shown was determined. In experiment B, only the methylated poly(dT).poly(dA) substrate was used, but the number of units of the alkyltransferase added was based on the capacity to remove O^6-methylguanine from DNA under published assay conditions (11,12). One unit of O^6-alkylguanine-DNA alkyltransferase activity corresponds to the ability to remove 1 pmol of methyl groups from O^6-methylguanine in DNA.

N.D., not determined.

protein, but our results are consistent with the report (16) that the same protein can repair either O^6-methylguanine or O^4-methylthymine. However, it is certainly possible that E. coli contains multiple proteins which carry out repair of O^4-methylthymine.

Our results indicate clearly that the rat liver O^6-alkylguanine-DNA alkyltransferase does not repair O^4-methylthymidine in the methylated poly(dT).poly(dA) substrate used. It is remotely possible that the liver alkyltransferase would work on O^4-methylthymidine in a methylated DNA substrate rather than a homopolymer, but this possibility is difficult to test because such substrates contain much more O^6-methylguanine than O^4-methylthymine [7.5% of the total methylation on the O^6-position of guanine versus 0.1% on the O^4-position of thymine after reaction of calf thymus DNA with N-methyl-N-nitrosourea (3)]. However, DNA reacted with N-ethyl-N-nitrosourea contains a higher proportion

of the O^4-ethylthymine adduct than DNA alkylated by N-methyl-N-nitrosourea [2.5% of the total reaction at O^4-ethylthymine and 7.8% of the total at O^6-ethylguanine (3)]. Therefore, ethylated calf thymus DNA was tested as a substrate for the rat liver O^6-alkylguanine-DNA alkyltransferase. The repair protein was allowed to act for up to 8 h without any detectable loss in the O-ethylpyrimidines although virtually all of the O^6-ethylguanine was lost within 1 h (results not shown). Furthermore, it is known that the rat liver O^6-alkylguanine-DNA alkyltransferase is active in repairing O^6-methylguanine in the random polymer, poly(dG,dC,m^6dG) (11) and the methylated poly(dT).poly(dA) substrate is shown in the present paper to be a good substrate for O^4-methylthymine repair by the bacterial extract. Therefore, the most probable conclusion is that the rat liver O^6-alkylguanine-DNA alkyltransferase does not act on O^4-alkylthymine under conditions in which O^6-alkylguanine is repaired rapidly. The rate of repair of O^4-methylthymine must be at least 100 times lower than the repair of O^6-methylguanine which is complete in 10 minutes under the conditions used.

The finding that the rat liver O^6-alkylguanine-DNA alkyltransferase does not react with O^4-alkylthymine is consistent with recent reports that O^4-ethylthymine is not lost from a variety of rat tissues including liver over a 48 h period following treatment with N-ethyl-N-nitrosourea (18,19) and that during acute or chronic exposure to diethylnitrosamine, O^4-ethylthymine accumulates in rat liver DNA whereas O^6-ethylguanine does not (20,21). The fact that the rat O^6-alkylguanine-DNA alkyltransferase is apparently specific for O^6-alkylguanine could explain how in cells containing this protein the O^4-alkylthymine may rapidly become the predominant mis-coding lesion (4,6,8) thus contributing to the biological effects of alkylating agents, including tumor initiation. The amount of O^6-alkylguanine-DNA alkyltransferase present in cells is both species and cell-type specific (3). It is quite possible (and certainly not ruled out by our experiments which used a partially purified rat liver extract) that repair of O^4-alkylthymine can be brought about by other mammalian proteins which may also be cell and species specific. There is published evidence that

O^4-ethylthymine is removed from mammalian cell DNA, although the mechanism of removal is unknown (19,22). The relative activities of this system and the O^6-alkylguanine-DNA alkyltransferase would then determine the relative persistence of these adducts. Further studies of this possibility are clearly needed.

Finally, it appears from this work that despite the close similarities between the bacterial and rat liver O^6-alkylguanine-DNA alkyltransferase (9-12,23), the bacterial protein is not invariably a good model for extrapolations to situations involving mammalian cells.

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REFERENCES

1. Margison, G. P. and O'Connor, P. J. (1979) In: Chemical Carcinogens and DNA, edited by P. L. Grover, pp. 111-159, CRC Press, Florida.
2. Pegg, A. E. (1977) Adv. Cancer Res. 25, 195-267.
3. Pegg, A. E. (1983) In: Reviews in Biochemical Toxicology, edited by E. Hodgson, J. R. Bend and R. M. Philpot, Vol. 5, pp. 83-133, Elsevier, Biomedical, New York.
4. Singer, B. and Kusmierek, J. T. (1982) Ann. Rev. Biochem. 52, 655-693.
5. Lawley, P. D. (1976) In: Chemical Carcinogens, ACS Monograph 173, edited by C. E. Searle, pp. 83-244, American Chemical Society, Washington, D.C.
6. Singer, B., Sagi, J. and Kusmierek, J. T. (1983) Proc. Natl. Acad. Sci. 80, 4884-4888.
7. Abbott, P. J. and Saffhill, R. (1977) Nucleic Acids Res. 3, 761-769.
8. Singer, B., Abbott, L. G. and Spengler, S. J. (1984) Carcinogenesis, in press
9. Olsson, M. and Lindahl, T. (1980) J. Biol. Chem. 255, 10569-10571.
10. Foote, R. S., Mitra, S. and Pal, B. C. (1980) Biochem. Biophys. Res. Commun. 97, 654-659.
11. Pegg, A. E., Wiest, L., Foote, R. S., Mitra, S. and Perry, W. (1983) J. Biol. Chem. 258, 2327-2333.
12. Demple, B., Jacobsson, A., Olsson, M., Robins, P. and Lindahl, T. (1982) J. Biol. Chem. 257, 13776-13780.
13. Montesano, R. (1981) J. Supramol. Struct. 17, 259-273.
14. Singer, B., Bodell, W. J., Cleaver, J. E., Thomas G. H., Rajewsky, M. G. and Thon, W. (1978) Nature (London) 276, 85-88.
15. McCarthy, J. G., Edington, B. V. and Schendel, P. F. (1983) Proc. Natl. Acad. Sci. 80, 7380-7384.
16. McCarthy, T. V., Karran, P. and Lindahl, T. (1984) EMBO J. 3, 545-550.
17. Ahmmed, Z. and Laval, J. (1984) Biochem. Biophys. Res. Commun. 120, 1-8.
18. Müller, R. and Rajewsky, M. F. (1983) Z. Naturforsch 38c, 1023-1029.
19. Singer, B., Spangler, S. and Bodell, W. J. (1981) Carcinogenesis 2, 1069-1073.
20. Swendberg, J. A., Dryoff, M. C., Bedell, M. A., Popp, J. A., Huh, N., Kirstein, U. and Rajewsky, M. F. (1984) Proc. Natl. Acad. Sci. 81, 1692-1695.
21. Scherer, E., Timmer, A. P. and Emmelot, P. (1980) Cancer Letters 10, 1-6.
22. Bodell, W. J., Singer, B., Thomas, G. H. and Cleaver, J. E. (1979) Nucleic Acids Res. 6, 2819-2829.
23. Cairns, J. (1980) Proc. R. Soc. Lond. B 208, 121-133.