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Studies of the Repair of O^6-Alkylguanine and O^4-Alkylthymine in DNA by Alkyltransferases from Mammalian Cells and Bacteria

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O^6-Methylguanine in DNA is repaired by the action of a protein termed O^6-alkylguanine-DNA alkyltransferase (AT) which transfers the methyl group to a cysteine residue in its own sequence. Since the cysteine which is methylated is not regenerated rapidly, if at all, the capacity for repair of O^6-methylguanine is limited by the number of molecules of the AT available within the cell. The level and inducibility of the AT differed greatly in different mammalian cell types and species with the highest levels in human tissues and in liver and the lowest levels in brain. Only a small induction occurred in rat liver in response to exposure to alkylating agents. In *E. coli* such exposure increased the activity more than 100-fold. The AT was not specific for methyl groups but also removed ethyl, 2-hydroxyethyl, *n*-propyl, isopropyl and *n*-butyl groups from the O^6-position in DNA. The protein isolated from *E. coli* removed methyl groups much more rapidly than the larger alkyl groups but the mammalian AT isolated from rat liver showed much less difference in rate with adducts of different size. Ethyl and *n*-propyl groups were removed by the rat liver AT only three to four times more slowly than methyl groups. Another important difference between the bacterial and mammalian ATs is that the bacterial protein was also able to remove methyl groups from the O^4-position of thymine in methylated DNA or poly(dT) but the AT from rat liver or human fibroblasts did not repair O^4-methylthymidine. These results indicate that the results obtained with the *E. coli* system may not be a suitable model for extrapolation to predictions of the effects of alkylating agents in initiating tumors or mutations in mammalian cells.

Introduction

Dimethylnitrosamine and related carcinogens are converted enzymatically into reactive alkylating agents (*1,2*). Such alkylating agents act as mutagens and tumor initiators by interaction with the cellular DNA. At least 12 sites in DNA are targets for alkylation by dialkylnitrosamines and *N*-alkyl-*N*-nitroso-ureas, but there is evidence that attacks on the oxygen atoms of guanine and possibly also pyrimidines may be of particular importance in mutagenesis and carcinogenesis (*2–7*).

O^6-Methylguanine in DNA is repaired by an unusual mechanism involving a protein termed O^6-alkylguanine-DNA alkyltransferase (AT). AT reacts with DNA containing O^6-alkylguanine residues and catalyzes the transfer of the alkyl group to a cysteine acceptor site contained within its protein sequence (*8–16*). This transfer restores the DNA structure to normal within a single step but the AT protein becomes stoichiometrically inactivated because the alkylcysteine is not regenerated rapidly if at all. Therefore, the number of O^6-alkylguanine adducts which can be repaired without waiting for new AT to be synthesized is limited to the number of molecules of AT present. The present paper describes measurements of the amount of AT activity in various mammalian cells types, studies of the inducibility of AT in rodent liver and comparisons of the specificity of the *E. coli* and rat liver AT with respect to the size of the alkyl group which can be removed and the alkylated base which can be repaired.

Materials and Methods

Detailed descriptions of the preparation of mammalian cell extracts containing AT and the assay of AT activity using radioactive [^{3}H]methylated-DNA substrate are given in the literature (*10,11,13,17–19*). AT was purified from *E. coli* strain BS21 as described by Demple et al. (*12*). Alkylated DNA substrates were prepared by reaction of the appropriate *N*-alkyl-*N*-nitrosourea with calf thymus DNA (*13,20*). Quantitation

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Table 1. O^6-Alkylguanine-DNA alkyltransferase activity in human and rodent tissues.[a]

Tissue	Alkyltransferase activity, fmole/mg	
	Rat	Human
Liver	115	940
Colon	21	260
Lung	54	120
Esophagus	29	220
Brain	10	76
Meningeoma	N.M.	207
Neurinoma	N.M.	143

[a] Experimental details are given in the literature (*14,17,18*).

of unlabeled O^6-alkylguanines by fluorescence detection after separation by HPLC was as described by Pegg et al. (*20*). The competition assay for AT activity in which a known amount of AT is incubated with an unlabeled alkylated DNA substrate for various times and the remaining AT activity quantitated by addition of [^{3}H]methylated DNA was carried out as described elsewhere (*20*). The preparation of [^{3}H]methylated poly (dT), analysis of methylated poly (dT) or DNA for methylated pyrimidine bases and assay for loss of O^4-methylthymine was as described by Dolan et al. (*21*).

Results and Discussion

Species and Tissue Distribution of AT

AT has been detected in all normal and tumor tissues examined from both rats and humans but the amount of activity found varies substantially (*13–18,22–25*). Even allowing for the much greater individual variations in the human samples (*14,17,18,22–25*), there is general agreement that human tissue samples are much more active than their rodent equivalents. Typical values from assays carried out in our laboratory are shown in Table 1. It should be noted that these values are based on homogenates of tissues which may contain many different cell types and results are averages which may disguise substantial differences between individual cells. Also, mammalian cells vary substantially in size and protein content and since the AT activities are expressed as a function of protein, the relative activities of large cells such as hepatocytes are somewhat underestimated. In fact, AT activities of purified rat hepatocyte preparations were about five times as high as those of isolated nonparenchymal cells (*26*).

A number of human cultured cell lines have been shown to have little or no AT activity (*15,19,27–30*). Such lines termed mer$^-$ (*28–30*) are very much more sensitive to toxicity and mutagenesis by alkylating agents than the equivalent mer$^+$ lines which have high AT levels (*19,28–30*). The mer$^-$ phenotype has frequently been found in transformed-cell lines (*30*), but some established "normal" human fibroblasts have recently been found to lack AT activity (*19*), so the characteristic is not limited to malignant cells. Since 25–30% of the human tumor cells lines which have been tested were found to be mer$^-$ (*30*), it appeared possible that a significant number of primary tumors might also lack AT activity. This could render them more sensitive to therapeutic alkylating agents which produce lesions repairable by the AT (*31–35*). Unfortunately, this does not appear to be the case since examination of the AT activity of extracts from a considerable number of brain tumors (Table 1) (*17*) and a variety of other tumors (*24,25*) revealed that all the tumors tested had activity and that some were considerably higher than their normal tissue equivalents.

Induction of AT

In *E. coli* AT is highly inducible in response to exposure to alkylating agents. As part of the adaptive response increases of several hundred fold occur within a few hours of treatment with alkylating agents (*36–38*). There is no clearly equivalent phenomenon in mammalian cells. Although some investigators have claimed that small increases in AT occur in cultured cells exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or *N*-methyl-*N*-nitrosourea (*39–41*), these changes were at most a few fold, and others using similar protocols have failed to find any increase at all (*42–44*).

A more clearcut increase in AT activity occurs in the livers of rats treated with dimethylnitrosamine (*5,45–48*) or other nitrosamines or with 1,2-dimethylhydrazine (*5,10,26,47*). Such exposure results in about a 3-fold rise in AT (Table 2) but similar increases are produced by exposure to other hepatoxins which are not metabolized to simple alkylating agents (Table 2) (*10,49,50*). An even larger 6- to 7-fold increase in AT activity in rat liver (Table 2) was observed in rat liver regenerating after partial hepatectomy (*11*). These results suggest that the increases in AT may be related to regenerative cell replication and/or hormonal changes in response to the toxic inducing agents. Evidence has been published that rat liver AT activity responds to both growth hormone and thyroxin (*51*). The increased liver AT after hepatoxins and partial hepatectomy may, however, be limited to the rat. Attempts to induce hepatic AT in other rodents (mice, gerbils, hamsters) by either nitrosamines or partial hepatectomy has been unsuccessful (*2,52–54*). The small increase in hepatic AT in response to alkylating agents is, therefore, at present limited only to rats and seems quite different from the adaptive response.

Although AT is increased in rat liver hepatocytes regenerating after partial hepatectomy there is no obligatory coupling between cell replication rates and the AT level. The AT activity of rat liver shortly after birth is considerably lower than in the adult (Table 2) (*51*), even though the hepatocytes in the neonatal rat are dividing more rapidly. Similarly, the induction of AT by 1,2-dimethylhydrazine occurs only in hepatocytes and not in nonparenchymal cells (*26*) but the rate of DNA synthesis is greater in the nonparenchymal cells. The

Table 2. Induction of rat liver O^6-alkylguanine-DNA alkyltransferase activity.[a]

Treatment	Alkyltransferase activity, fmole/mg
Control adult	93 ± 21
Dimethylnitrosamine (2 mg/kg/day for 21 days)	224 ± 23
Diethylnitrosamine (10 mg/kg/day for 21 days)	245 ± 49
1,2-Dimethylhydrazine (3 mg/kg/day for 21 days)	247 ± 16
Partial hepatectomy (48 hr after operation)	496 ± 68
Thioacetamide (48 hr after 75 mg/kg)	185 ± 28
Carbon tetrachloride (48 hr after 1.5 mL/kg)	199 ± 24
1 day old	24 ± 8
7 days old	53 ± 7

[a] Data from references (*10,11,51*).

low level of AT in neonatal rat liver has recently been confirmed by Ro et al. (*55*), and a very low activity of AT in some human fetal tissues including liver was found by Krokan and colleagues (*56*).

Specificity of Alkyl Group Removal

The ability of the AT to remove alkyl groups of different sizes was tested using three different assay procedures (*20,57*). Radioactive substrates were used for studies of methyl, ethyl, *n*-propyl, and isopropyl adducts by either the bacterial or mammalian AT. Studies of 2-hydroxyethyl, *n*-butyl and isobutyl adducts were carried out with unlabeled substrates. These O^6-derivatives could be quantitated after HPLC separation by fluorescence detection and this assay was used for the *E. coli* AT. However, this method was not sensitive enough for studies of the rat liver AT which was available only in limited amounts. The activity of the rat liver AT towards the substrates not available in a radioactive form were, therefore, carried out by use of a competition assay. In this assay, the rat liver AT was incubated with the unlabeled alkylated substrate for varying amounts of time and then a radioactive methylated substrate was added and its repair was used to estimate the amount of AT activity remaining. A summary of the results is shown in Table 3. All of these adducts could be removed from the O^6-position of guanine by the bacterial and mammalian AT. The *E. coli* AT which was very rapid on methyl groups was much slower with the longer adducts. This result is in agreement with the report by Lindahl and colleagues that ethyl and 2-hydroxyethyl- groups are removed at least 100 times slower than methyl groups by the bacterial protein (*32*). Although the rat liver AT was also faster with methyl groups than with larger adducts there was much less difference between the rates than with the *E. coli* AT. For example, the *E. coli* AT removes *n*-propyl or ethyl groups 50 to 100 times more slowly than methyl

Table 3. Substrate specificity of alkyltransferase from rat liver and *E. coli*.

Alkyl group at O^6-position of guanine	Alkyltransferase activity, as time for 50% removal, min	
	E. coli	Rat liver
Methyl	<0.5	<1
Ethyl	10	2
n-Propyl	15	3
n-Butyl	35	Substrate[a]
Isopropyl	>90	60
2-Hydroxyethyl	>90	35
Isobutyl	>90	Not tested

[a] Rate not determined.

but the rat liver AT is only 3 to 4 times slower on these adducts.

This finding has considerable practical importance in the interpretation of studies of carcinogenesis by alkylating agents. When expressed in terms of the number of DNA adducts formed at the O^6-position of guanine by carcinogenic doses, diethylnitrosamine is about 15 times more potent than dimethylnitrosamine (*20,58*). If the formation and persistence until repair of O^6-alkylguanine in DNA were the only factor involved in tumor initiation, the repair would have to be at least 15 times slower which is not the case. This suggests that some other lesions, possibly O^4-alkylthymine (which is formed in greater amounts by ethylating than by methylating agents), may be the critical lesion in cells such as hepatocytes containing a high AT activity.

Another important consequence of these results is that the rapid removal of larger adducts from DNA by the mammalian AT is likely to confer resistance to the formation of lethal crosslinks by certain bifunctional alkylating agents (*31–35*). Such crosslink formation is thought to involve an initial attack on the O^6-position of guanine (forming a 2-chloroethyl adduct) which then takes part in a slower reaction leading to a stable crosslink. If the O^6-adduct is removed by the AT before this reaction occurs the lethal lesion is prevented. The relatively rapid removal of longer chain adducts by the rat liver AT should enable this to happen until the number of lesions exceeds the amount of AT.

Finally, it was apparent that the AT could remove branched adducts such as isopropyl from the O^6-position of DNA (Table 3), but that in the presence of DNA substrates containing both *n*-propyl and isopropyl adducts (such as DNA reacted with *N*-*n*-propyl-*N*-nitrosourea), the protein shows a marked preference for the linear derivative and the isopropyl groups start to be the lost only when most of the *n*-propyl is already gone.

Specificity of the Alkylated Base Substrate

Recent studies on the adaptive response in *E. coli* (*37,38*) have indicated that the initial AT protein synthesized by the bacteria is considerably larger than the product of 18,000 molecular weight which has been pur-

Table 4. Removal of O^4-methylthymine from methylated poly(dT) by *E. coli* but not by rat liver alkyltransferase.[a]

Alkyltransferase added	Methylated poly(dT) substrate, O^4-methylthymine, pmole	Standard methylated DNA substrate, O^6-methylguanine, pmole
E. coli	0.05	0.05
Rat liver	0.57	0.06
None	0.56	0.58

[a]The AT preparation were incubated with either the methylated poly(dT) containing O^4-methylthymine or the standard methylated calf thymus DNA containing O^6-methylguanine and the content of the methylated base determined after 60 min incubation.

ified to homogenicity and studied extensively (*12*). Furthermore, the larger AT protein and possibly additional methyltransferases which have not yet been fully characterized can act not only on O^6-methylguanine in DNA but can also remove methyl groups from phosphotriesters and O^4-methylthymine (*59–61*). The 18,000 molecular weight protein (which is formed from the larger precursors by an unknown mechanism) can also use O^4-methylthymine as a substrate (*21,60*). As shown in Table 4, this bacterial AT can repair O^4-methylthymine in a methylated poly(dT)·poly(dA) substrate, but the rat liver AT did not catalyze this reaction. These results suggest that there is an important difference between the two proteins and provide a possible mechanism by which O^4-alkylthymine but not O^6-alkylguanine might accumulate in mammalian cells exposed to alkylating agents (*62,63*).

It is conceivable that experiments carried out *in vitro* with a methylated poly(dT) substrate do not accurately reflect the specificity of the protein towards the alkylated double stranded DNA which would occur *in vivo*. It has been relatively difficult to test the AT activity towards O^4-methylthymine in such a substrate because there is a much greater amount of O^6-methylguanine (about 100 times more) and the added AT is used up on the O^6-adduct. However, in recent experiments in which a substantial excess of AT was used no significant loss of O^4-methylthymine was brought about by the rat liver AT under conditions where more than 95% of the O^6-methylguanine was demethylated. The *E. coli* AT removed both O^4-methylthymine and O^6-methylguanine from this substrate. Experiments with AT from human fibroblasts have also been carried out, and a crude AT extract from these cells did not remove O^4-methylthymine from methylated poly(dT) (*19*). There is, therefore, a striking and unexpected difference between the bacterial and mammalian AT in that only the former can work on O^4-methylthymine as well as O^6-methylguanine. This difference and that described above in the rate of removal of longer chain adducts from the O^6-position of guanine suggest that the bacterial AT may not be an appropriate model for extrapolation to situations involving mutagenesis or carcinogenesis in mammalian cells.

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