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QUANTITATIVE EVALUATION OF GENOTOXIC EFFECTS BY MOLECULAR DOSIMETRY

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Summary. - *The covalent binding of alkylating agents with DNA is considered a critical event in the mutagenic and carcinogenic effect of these chemicals. Several studies have indicated that only certain DNA alkylation products contribute to the mutagenic or carcinogenic activity of these agents. In this paper we summarize data obtained in Chinese hamster ovary (CHO) cells after treatment with two ethylating agents, N-ethylnitrosourea and diethylsulfate, which are known to alkylate DNA sites with different efficiency. A correlation study between DNA adduct formation and induction of mutations at two gene loci, i.e. hypoxanthine-guanine-phosphoribosyltransferase (HPRT) and Na,K-ATPase, has been performed. The influence of DNA repair processes on the final yield of mutation is specifically discussed.*

Riassunto (Valutazione quantitativa di effetti genotossici). - *Il legame covalente con il DNA è considerato l'evento critico per gli effetti mutageni e cancerogeni indotti dagli agenti alchilanti. Molti studi hanno indicato che solo alcuni prodotti di alchilazione del DNA contribuiscono all'attività mutagena o cancerogena di questi agenti chimici. In questo lavoro verranno riassunti i dati ottenuti in una linea cellulare di hamster cinese (CHO) dopo trattamento con due agenti etilanti, N-etilnitrosourea e dietilsolfato, che alchilano basi e gruppi fosfato del DNA con diversa efficienza. Lo studio che verrà descritto è uno studio di correlazione tra addotti presenti sul DNA e induzione di mutazione in due loci genetici, ipoxantina-guanina-fosforibosiltransferasi e Na,K-ATPasi. L'influenza dei processi di riparazione del DNA sulla frequenza di mutazione indotta verrà specificamente discussa.*

Introduction

Exposure to chemical carcinogens modifies DNA of mammalian cells at level of bases and/or phosphate backbone and these alterations are considered to be an important factor in the development of cancer. Interaction of carcinogenic alkylating agents with DNA produces a wide spectrum of DNA modifications (Fig. 1). Cells exposed to weakly carcinogenic direct-acting alkylating agents like dimethylsulfate are primarly methylated on nitrogen atoms of DNA bases, whereas strong carcinogens like nitrosoureas have a great preference for alkylating base oxygens. In order to correlate specific biological effects with specific lesions it is important to determine the amount of individual alkylation products and monitor their repair. Gross measurement of excision repair however does not permit such a discrimination. A direct approach is to measure the specific DNA adducts by utilizing highly radioactive carcinogens. This paper will summarize the data obtained in Chinese hamster ovary (CHO) cells exposed to two different alkylating agents, N-ethylnitrosourea (ENU) and diethylsulfate (DES), and analysed for DNA adducts distribution and mutation induction. The role of DNA repair events will also be discussed.

Analysis of DNA alkyl-adducts

CHO cells were exposed to either ^{3}H-ENU or ^{3}H-DES and the extent of the different ethylated products was determined by high pressure liquid chromatography (HPLC). Quantitation of ethylated products was performed as described by Beranek *et al.* [1]. Briefly, alkylated CHO DNA was heated at neutral pH to liberate heat-labile modified bases (7-ethylguanine, 3-ethyladenine, 3-ethylguanine, 3-ethylcytosine and O^2-ethylcytosine (O^2-EtCyt)). These were separated by HPLC on a cation exchange column (Partisil 10-SCX). The remaining DNA was hydrolysed enzimatically, the hydrolysate was then separated by HPLC on a reversed phase column (Kieselgel 60 RP18) and the levels of O^6-ethylguanine (O^6-EtGua), O^4-ethylthymidine (O^4-EtThy), O^2-ethylthymidine (O^2-EtThy) and the ethylester of thymidilyl(3'-5')thymidine (dTp(Et)dT) were determined. The amount of a particular modified base was calculated from the radioactivity coeluting with the marker and the specific activity of the carcinogen. As shown in Fig. 2, ENU and DES cause similar type of DNA damage, although the extent of substitution at various DNA sites is different. DES has a strong preference for interaction with nitrogen atoms giving rise preferentially to N7- and N3-ethylpurines, while ENU

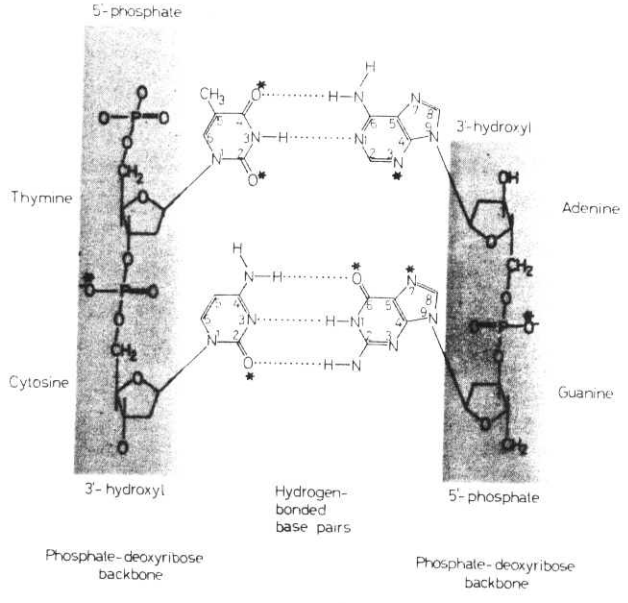

Fig. 1. - Interaction of alkylating agents with DNA. The possible sites of modification are indicated by an asterisk.

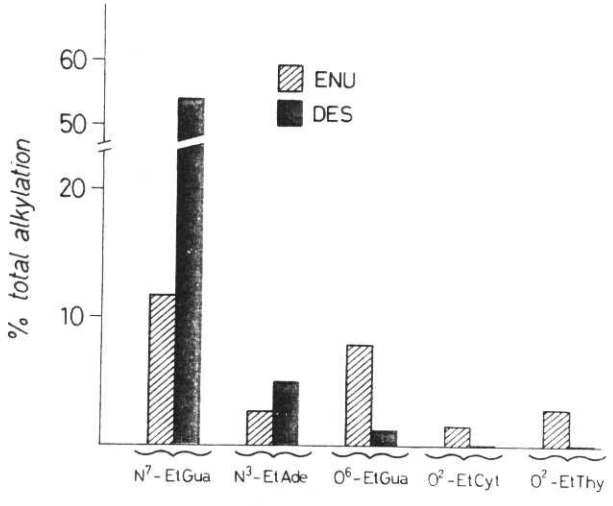

Fig. 2. - Distribution of DNA ethyl products after exposure of CHO cells to ENU and DES.

causes predominantly alkylation at oxygen atoms of bases and phosphate backbone. In fact, at equal levels of DNA alkylation, the amounts of O^6-EtGua, O^4-EtThy and dTp(Et)dT were substantially higher after exposure to ENU than after alkylation with DES. The O^2-alkylpyrimidines, O^2-EtCyt and O^2-EtThy, were detected after treatment with ENU but were below the detection limit in the case of DES.

Mutation induction

Several attempts have been made in the past to correlate the mutagenic effects of alkylating agents with their chemical reaction pattern in mammalian cells in culture. A correlation has been found [2, 3] between mutation induction at the hypoxanthine-guanine-phosphoribosyltransferase (HPRT) locus and the extent of O^6-alkylguanine formation in Chinese hamster cells exposed to a series of alkylating agents. Other evidences [4, 5] however indicate that O^6-alkylguanine may not be the only DNA alkyl product involved in mutagenesis. In view of these data, parallel to the experiments with labelled ethylating agents, the same CHO cells were treated with unlabelled mutagens in order to determine the frequency of mutation at the HPRT and Na,K-ATPase loci. To analyze whether a particular DNA adduct might be responsible for induction of mutations at these two loci, mutation frequency data were compared with the amounts of each individual adduct. In our statistical approach, a random association of mutation frequency data and the corresponding DNA adducts levels was performed and separate linear regression lines were calculated on ENU and DES data. The slopes of these lines were compared to determine whether they differ significantly. Fig. 3 shows the relationship between mutation induction at the HPRT (top panel) and Na,K-ATPase (bottom panel) gene loci and O^6-EtGua formation in CHO cells. The data indicate that O^6-EtGua formation might be quantitatively correlated with mutations at the HPRT locus (the difference between the slopes of the regression lines is not statistically significant). In the case of the Na,K-ATPase locus, a significant difference ($p < 0.01$) between the slopes of the regression lines for ENU and DES was observed, suggesting that lesions other than O^6-EtGua are involved in the fixation of ENU- and DES-induced ouabain resistant (oua^r) mutations. The formation of O^4-EtThy appeared also to be correlated to mutation induction at the HPRT locus, whereas none of the measured ethyl adduct revealed an association with mutation at the Na, K-ATPase locus. Studies with a wider range of ethylating agents are necessary to identify the lesions involved in the formation of oua^r mutations. Furthermore a mechanism other than base pair substitutions might be responsible for the observed oua^r phenotype. As an example, oua resistance can be acquired by cultured cells also by amplification of the gene [6] and chemical carcinogens could induce such amplification [7]. In this light it would be of great interest the molecular analysis of oua^r mutants.

Role of the repair processes in mutation induction

In 1980 Olsson and Lindahl [8] by using extracts from *Escherichia coli (E. coli)* mutants demonstrated that the methyl group of O^6-methylguanine (O^6-MeGua) present in DNA was transferred to a cysteine residue of an acceptor protein, producing an S-methyl-L-cysteine residue. As a consequence guanine was generated in the DNA substrate. The O^6-methyltransferase (MT) from *E. coli* has been purified [9] and the gene has been cloned [10]. The MT from mammalian cells has only been partially purified [11, 12] and seems to share with the bacterial MT the specificity for demethylation of O^6-MeGua. CHO cells lack the

function responsible for the removal of O^6-MeGua, but in 1985 Ding *et al.* [13] isolated by transfection with human DNA a CHO cell line which differed from the parental one only for the expression of the MT activity. We used the MT-deficient and proficient CHO cells in order to see whether the presence of this repair function results in a differential response to mutation by alkylating agents. In the case of methylating agents the MT-proficient cells showed a lower sensitivity to the mutagenic effects as compared to the MT-deficient cells (data not shown) [14]. This is consistent with the involvement of the MT activity and therefore of O^6-MeGua in mutation induction by methylating agents. Interestingly enough, when the mutation frequency at the Na,K-ATPase locus was measured in both cell lines after treatment with ENU, no significant difference was observed between the number of oua^r mutants induced in the MT-deficient *vs* the proficient cell line (Fig. 4). The apparent lack of correlation between the expression of MT activity and the mutagenic effects of ENU prompted us to investigate the removal of O^6-EtGua as compared to O^6-MeGua. MT-proficient and deficient cells were treated with ENU or N-methylnitrosourea (MNU) and allowed to repair in fresh medium for 20 h. DNA was extracted and the level of alkylation at the O^6 position of guanine was measured by radioimmnunoassays [15]. As shown in Table 1 a reduction of only 25% of O^6-EtGua present in DNA was observed at 20 h in MT-proficient cells, whereas 88% of O^6-MeGua was removed. In contrast, no removal of O^6-alkylguanine was observed in MT-deficient cells (data not shown). A doubling in the number of cells was observed 20 h after treatment both with ENU and MNU. Thus, the amount of the premutagenic O^6-alkylguanines present at the time when replication occurred must have been strikingly different for MNU as compared with ENU. The similar mutation frequencies observed in the two cell lines after treatment with ENU might be explained by the persistence during replication of the O^6-EtGua due to the slow rate of removal in both MT-proficient and deficient cells. However we cannot exclude the importance of other miscoding lesions, besides O^6-EtGua, induced by ENU. Analysis of the mutational spectrum in human cells [16] indicate that ENU induces, as expected, GC-AT transitions but also mutations at A-T base pairing. These mutations can be attributed to the increased extent of modification induced by ENU at the oxygen groups of thymine. Modifications at T bases could also play an important role in the events leading to the oua resistance phenotype, while O^6-ethylguanine would have a secondary role.

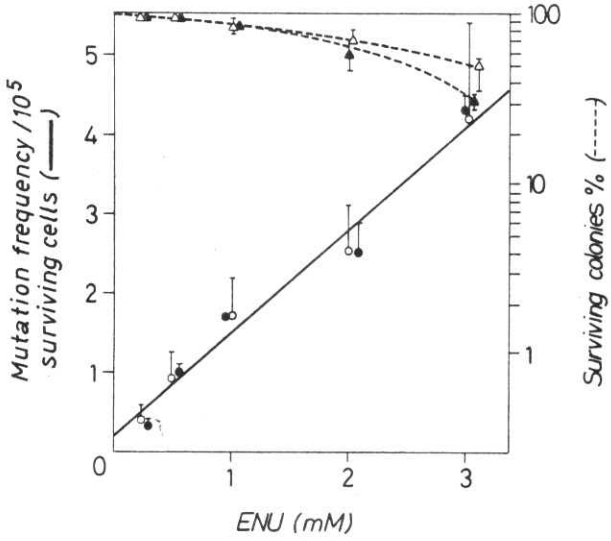

Fig. 4. - Survival and induction of oua^r mutations after a 30 min exposure to ENU of MT-deficient (closed symbols) and MT-proficient (open symbols) CHO cells.

Table 1. - *Removal of O^6-alkylguanine in MT-proficient CHO cells*

	pmoles O^6-alkyldG/μmoles dG		removal (%)
	0 h	20 h	
ENU	27.7	20.8	25
MNU	15.1	3.9	88

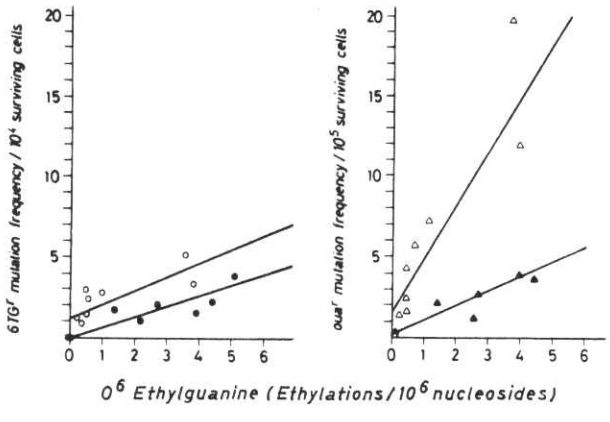

Fig. 3. - Induction of 6-thioguanine resistant (6-TG^r) mutations (top panel) and oua^r mutations (bottom panel) after a 30 min exposure to ENU (closed symbols) or DES (open symbols) as a function of O^6-EtGua residues/nucleotide. The solid lines represent linear regression lines.

Taken all together these observations show that the analysis of the DNA binding profile after exposure to mutagens can give some clues on the role of the different DNA products in the induction of genotoxic effects. Altough these correlation studies are extremely valuable they do not provide ultimate evidence of the involvment of certain lesions in specific biological events. Furthermore modulating factors such as DNA repair play a key role in the net injury to cells suggesting extreme caution when extrapolations are made to other cell systems.

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