APPROACHES RELATING DNA DAMAGE TO MUTATIONAL SPECTRA

Ann. Ist. Super. Sanità
Vol. 25, N. 1 (1989), pp. 205-212

THE SEARCH FOR THE MOLECULAR LESIONS RESPONSIBLE FOR THE INDUCTION OF CHROMOSOMAL DAMAGE BY ALKYLATING AGENTS

S. BONATTI *(a, b)* and A. ABBONDANDOLO *(a, c)*

(a) Istituto Nazionale per la Ricerca sul Cancro, Genova
(b) Istituto di Mutagenesi e Differenziamento del CNR, Pisa
(c) Cattedra di Genetica, Università degli Studi, Genova

Summary. - *Many studies associated specific DNA alkylation products with specific biological effects of alkylating agents. Studies dealing with the identification of DNA lesions that are responsible for chromosomal damage are reviewed in this paper. Although early evidence suggested that alkylation of high nucleophilicity centers such as N-7 of guanine was associated with genetic effects of alkylating agents, recent studies shifted the attention to the alkylation of oxygen atoms. The results of studies based on the use of cells with different capacities to repair O⁶-alkylguanine (O⁶-AlkGua) indicate a key role for this alkylation product in the induction of sister chromatid exchanges, chromosomal aberrations and micronuclei. Our own results on the influence of exogenous thymidine on the frequency of micronuclei induced by methylating and ethylating agents further suggest that mispairing of O⁶-AlkGua with thymine may be involved. Molecular dosimetry studies did not reveal any correlation between individual DNA alkylation products and the induction of chromosomal damage, indicating that multiple lesions are probably involved.*

Riassunto (Studio delle lesioni molecolari al DNA responsabili dell'induzione di danno cromosomico da parte di agenti alchilanti). - *Molti studi sono stati eseguiti nell'intento di associare specifici prodotti di alchilazione del DNA a specifici effetti biologici provocati dagli agenti alchilanti. In questo articolo vengono presi in considerazione i tentativi di identificare i prodotti di alchilazione responsabili del danno cromosomico. Sebbene precedenti lavori indicassero l'alchilazione di siti di alta nucleofilicità (N-7 della guanina) come maggiormente rilevanti, dati più recenti hanno spostato l'attenzione sulla alchilazione degli atomi di ossigeno. Risultati di esperimenti basati sull'uso di cellule con diversa capacità di riparare la O⁶-alchilguanina hanno suggerito che questo prodotto possa avere un ruolo nella induzione di scambi tra cromatidi fratelli, aberrazioni cromosomiche e micronuclei. Inoltre, nostri dati circa l'effetto dell'aggiunta di timidina al mezzo di coltura sulla frequenza di micronuclei indotti da agenti metilanti ed etilanti suggeriscono che possa essere implicato l'appaiamento O⁶-alchilguanina-timina. Studi di dosimetria molecolare non hanno permesso di correlare alcun particolare prodotto di alchilazione con l'induzione di danno cromosomico e ciò indica che devono essere implicati più prodotti.*

Biological alkylating agents: molecular lesions and genetic changes

Alkylating agents induce a wide spectrum of genetic changes, ranging from point to genomic mutations. Despite our longstanding knowledge of both the biological reactivity [1, 2] and the mutagenic properties of these agents [3], a complete understanding of the mechanisms by which they induce the various genetic effects remains elusive.

While it is likely that different lesions and/or targets are involved in the induction of different genetic changes, to date firm evidence is available only for the identification of DNA lesions responsible for the induction of point mutations. There is a consensus today in designating O⁶-alkylguanine (O⁶-AlkGua) [4] and O⁴-alkylthymine (O⁴-AlkThy) [5, 6] as the principal lesions responsible for localized heritable genetic alterations.

A lesson to be learned from the study of alkylating agents is that the identification of pre-mutational lesions is an essential step towards the understanding of mutational mechanisms. Notwithstanding the paucity of experimental data available at the time [7-10], theoretical considerations on the N1 deprotonation of O⁶-AlkGua led Loveless [11] to propose a simple mutational mechanism based on the mispairing of O⁶-AlkGua with thymine. The basic idea of the Loveless model has since received overwhelming confirmation in both *in vitro* [12-15] and *in vivo* studies using bacteria [16, 17], yeast [18], plants [19, 20], *Drosophila* [21, 22], cultured

mammalian cells [23-27], and rodents [28]. Furthermore, direct evidence was provided by experiments in which a single O6-methylguanine (O6-MeGua) residue was built into a unique site in a viral genome and its effect studied after transformation of *Escherichia coli* cells with this vector [4, 29].

We are some steps behind as far as the induction of other changes such as chromosomal aberrations are concerned. It is our intention in this paper: i) to review and discuss the available evidence on the involvement of specific DNA lesions in chromosomal damage, and ii) to report on work that has been done in this area in our laboratory during the last few years.

As we have hypothesized earlier, mispairing of O6-AlkGua with thymine may be invoked to explain the induction of chromosomal damage, in addition to that of gene mutation. Our evidence for this hypothesis was - and still is - indirect, but whether or not the mispair hypothesis is correct, evidence is now accumulating in favour of O6-AlkGua being at least one of the lesions responsible for chromosomal damage.

The molecular lesions responsible for chromosome aberrations

Possible involvement of DNA N-alkylation

Early evidence indicated a direct correlation between alkylation of centers of high nucleophilicity, such as N-7 of guanine in DNA and various amino acid residues on proteins, and the induction of chromosomal aberrations. Such a correlation was first indicated in higher plants by Osterman-Golkar *et al.* [19] and Velemínský *et al.* [20]. In *Drosophila*, Vogel and Natarajan [21, 22] showed that the induction of sex-linked recessive lethal mutations was inversely correlated, and that of 2-3 translocations and ring-X loss was directly correlated with the Swain-Scott [30] constant *s* of a group of monofunctional alkylating agents. These results, while confirming the role of O-alkylation in the induction of point mutation, pointed to alkylation of high nucleophilicity sites as more critical for the process leading to chromosome breakage.

Two hypotheses [19] were proposed to explain the high chromosome-breaking activity of alkylating agents with high *s* values, "a relatively high number of protein alkylations, e.g. provoking an inhibition of repair enzymes, along with not too low a number of DNA alkylations, or that a direct alkylation of base nitrogens, especially guanine N-7, is required for efficient induction of chromosome aberrations". In this respect, it is noteworthy that the repair of 7-methylguanine (7-MeGua) and other N-alkylpurines involves the action of glycosylases and apurinic site endonucleases [31]. During repair, therefore, breakage of phosphodiester bonds occurs, thus fulfilling one necessary requirement for chromosomal breakage. Repair of O6-MeGua by O6-MeGua-DNA-methyltransferase (MT) on the other hand occurs without strand breakage [31].

It should be stressed that no evidence is available to date that 7-MeGua is a pre-clastogenic lesion. Indeed, molecular dosimetry studies performed with cultured Chinese hamster [27] or Syrian hamster embryo [32] cells merely indicated that the lesions causing chromosome breaks are different from those causing gene mutations. From these studies, O6-ethylguanine (O6-EtGua) was confirmed as the most probable lesion in DNA leading to point mutation, but the lesion(s) responsible for the induction of chromosome breaks remained elusive.

Possible involvement of DNA O-alkylation

Different conclusions on the relevance of O-alkylation or N-alkylation to chromosomal damage were reached when the persistence of pre-clastogenic lesions in non-proliferating cells, rather than the ability of agents to produce chromosomal aberrations in proliferating cells, was studied. It has been shown that only those alkylating agents that give rise to substantial DNA O-alkylation are able to induce long-lived preclastogenic damage in rat liver cells *in vivo* [33-36]. The amount of 7-EtGua detected after treatment of rats with various ethylating agents [37] did not correlate with persistent preclastogenic damage [33]. Moreover methyl methanesulfonate (MMS), which induced substantial amount of 7-MeGua, did not induce persistent preclastogenic damage [36]. As pointed out by the authors of these studies, the formation and persistence of secondary pre-clastogenic lesions could be an important factor. However, neither primary nor secondary specific DNA modifications responsible for the induction of chromosomal damage in the rat liver system could be identified [36].

DNA synthesis inhibition is known to play a role in the induction of chromosomal aberrations [38]. In this respect, it is interesting to note that *in vitro* DNA synthesis by *E. coli* DNA polymerase I is inhibited by the presence of O6-MeGua on template DNA [39]. Moreover, arrest of DNA synthesis can also be caused *in vitro* by incorporation of O6-medGTP into the growing strand [26]. These data suggest that alkylation of guanine at the O6 position in mammalian cells may have a role in chromosomal breakage through inhibition of DNA replication. Further elements suggesting a preclastogenic role of O6-AlkGua are given in the next sections.

The "thymidine effect"

The results reported in the previous sections were mainly based on attempts to establish correlations between the pattern of DNA alkylation and the induction

of genetic effects. We tried a different approach unbalancing the nucleotide pools to modify quantitatively the induction of micronuclei, here used as an end point for chromosome-breaking activity [40, 41].

The study most similar to our approach is that of Meuth [24], who had shown previously that pool imbalance caused by exogenous thymidine in Chinese hamster ovary (CHO) cells results in an increased induction of mutants at three genetic loci by ethyl methanesulfonate (EMS). The proposed explanation was that the addition of thymidine led to an increased dTTP/dCTP ratio and this, in turn, increased the incorrect incorporation of thymine opposite O^6-MeGua residues in DNA.

In our experiments, the induction of micronuclei by three alkylating agents with low or intermediate *s* values, namely N-ethylnitrosourea (ENU), N-methylnitrosourea (MNU) and EMS, in V79 Chinese hamster cells was increased by incubation of cells in medium containing a pool unbalancing concentration of thymidine. This effect was not observed when micronuclei were induced by MMS, an agent with a relatively high *s* value (Fig. 1).

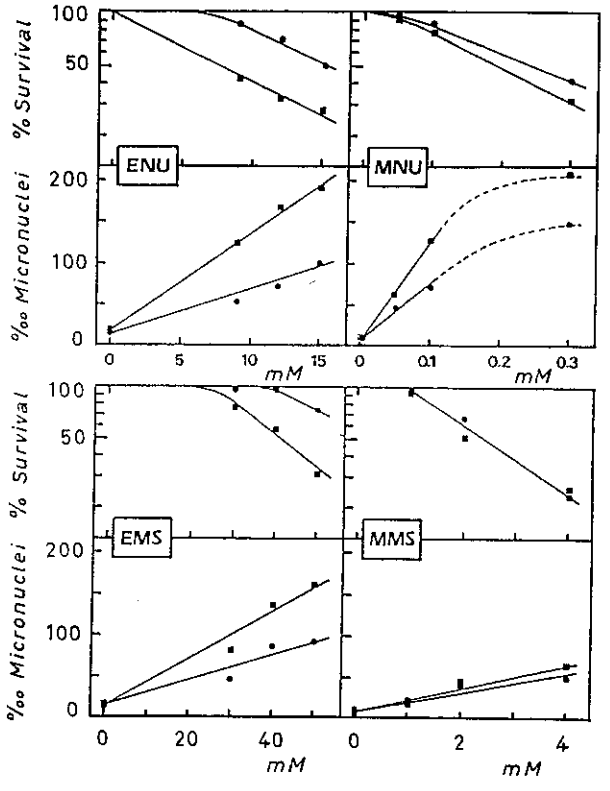

Fig. 1. - The influence of thymidine (8.2 x 10^{-6} M) on the induction of micronuclei in V79 Chinese hamster cells by ethylnitrosourea (ENU), methylnitrosourea (MNU), ethylmethanesulfonate (EMS) and methyl methanesulfonate (MMS). Cells incubated after treatment in medium with (squares) or without (circles) thymidine. Experimental points represent the average of at least two independent determinations (4,000-6,000 nuclei per experimental point). (After ref. [41]).

Having excluded some alternative explanations [40, 41], we considered the hypothesis that the effect of thymidine on micronucleus induction could be due to increased mispairing at O^6-AlkGua residues. Although not so obvious as for point mutation fixation, a role of mispairing in the induction of chromosomal damage is not unreasonable. As suggested by Ledda *et al.* [42], mispairing might cause local denaturation and this might be the signal for endonucleolytic cleavage of the DNA strand, eventually resulting in chromosome breakage.

If our hypothesis to explain the thymidine effect were true, then thymidine should have a different influence not only on the effect of agents with different ability to alkylate oxygens, but also, and more specifically, on the effect of any given agent on cells with different ability to repair O^6-AlkGua. Indeed we found that thymidine had no influence on the induction of micronuclei by the low *s* agent, ENU in HeLa cells (Fig. 2). Similar results were obtained using a 10-fold higher concentration of thymidine. HeLa cells contain an active methyltransferase, while Chinese hamster V79 cells are unable to remove either O^6-MeGua [43] or O^6-EtGua [44]. Since the mammalian methyltransferase, unlike the bacterial repair protein, is specific for O^6-AlkGua [45-47],

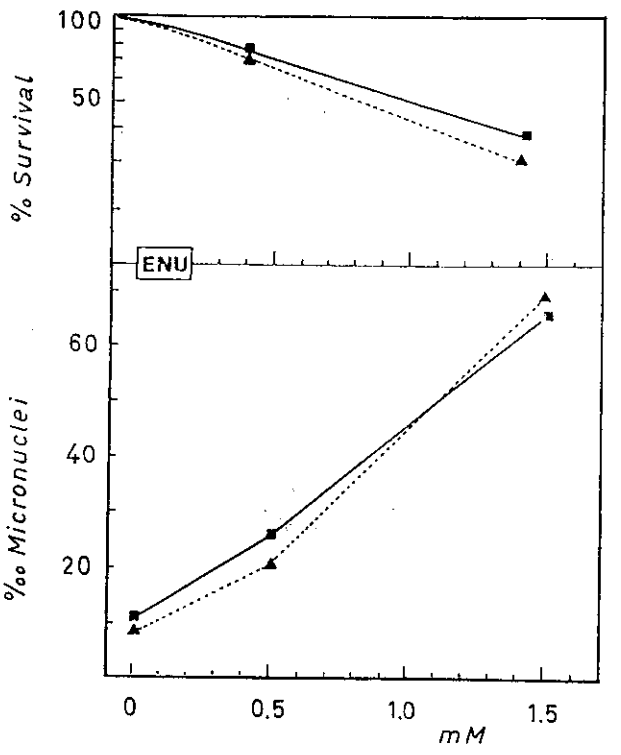

Fig. 2. - The influence of thymidine (8.2 x 10^{-6} M) on the induction of micronuclei in HeLa cells by ethylnitrosourea (ENU). Cells incubated after treatment in medium with (triangles) or without (squares) thymidine. Experimental points represent the average of at least two independent determinations (4,000-6,000 nuclei per experimental point). (After ref. [40]).

this result suggests that O6-AlkGua is the lesion responsible for the thymidine-induced increase in micronuclei. It is possible that both substantial production of O6-AlkGua residues (by low *s* alkylating agents) and their accumulation in repair-deficient cells are needed for the thymidine effect to be observed.

The conclusion of Meuth [24] that the thymidine effect on mutation induction was due to increased mispairing at O6-AlkGua sites was based to a large extent on the apparent specificity of the phenomenon for alkylating agents. Sensitivity to UV radiation was not affected by exogenous thymidine. This point has been subsequently re-evaluated by Newman and Miller [48] on the grounds that UV irradiation itself might provoke a dTTP/dCTP pool imbalance great enough to mask any further imbalance caused by the presence of exogenous thymidine in the medium. It has then been proposed that the thymidine-dependent increase in mutation induction by alkylating agents could be due to pool imbalance disturbing DNA synthesis [48].

Being aware that inhibition of DNA synthesis may lead to chromosomal damage [38], we studied the effect of thymidine on DNA synthesis under our experimental conditions. Thymidine did not noticeably influence the rate of DNA synthesis in cells treated with either ENU or MMS [40, 41]. In any case, DNA synthesis inhibition by itself would not have explained the dependency of the thymidine effect on the pattern of alkylation of the agents and on the O6-AlkGua repair capacity of the cells.

Our hypothesis on the role of O6-AlkGua in the induction of chromosomal aberrations is not at variance with the previously reported data (second section) showing correlation of this genetic effect with alkylation of high nucleophilicity centers. Also in our hands, micronuclei were induced in V79 cells more efficiently by MMS than by EMS or ENU. We think that, in comparison to other lesions (7-AlkGua?), O6-AlkGua may be a negligible source of chromosomal damage in cells that are able to remove it efficiently (see also next section). On the other hand, in cells that are deficient for the repair of O6-AlkGua, the contribution of this lesion to the total yield of chromosomal damage may become significant, as suggested by the thymidine effect. That both N- and O-alkylation may be involved in the production of micronuclei and chromatid aberrations has also been proposed by De Kok *et al.* [32].

Micronuclei as indicators of chromosomal damage

The use of micronuclei as an end point for chromosomal damage deserves some explanation. It is well known that micronuclei may arise not only from chromosome or chromatid fragments that lack a functional centromere, but also from whole chromosomes that lag behind at anaphase [49, 50]. Micronuclei, therefore are not specific indicators of chromosomal breakage.

In collaboration with Dr M. Nüsse (Frankfurt, Germany), we have recently used the scleroderma CREST antibodies anti-kinetochore to detect centromere-containing chromosomes in micronuclei. In particular, we have studied the kinetics of induction of micronuclei containing acentromeric fragments (*i.e.*, micronuclei negative for CREST antibody-Reacting Kinetochores, or CRK-) or whole chromosomes (*i.e.*, positive for the reaction, or CRK+) after treatment of V79 cells with diethylsulfate (DES) (manuscript in preparation). CRK- and CRK+ micronuclei were present in equal amounts immediately after treatment, when DES-induced micronuclei were not yet expressed. During post-treatment time, 10-fold and 50-fold increases were found in CRK+ and CRK- micronuclei, respectively, so that, at full expression, CRK- micronuclei accounted for 80% of total micronuclei.

In the experiments reported above [40, 41], micronucleus frequencies were measured at full expression. Therefore, provided that data with DES are representative of those with alkylating agents in general, the majority of micronuclei scored in the experiments on the influence of thymidine should be indicative of chromosomal breakage events rather than of chromosome loss.

The use of cells with different capacities to repair O6-alkylguanine

A traditional approach to the problem of defining the biological role of a specific DNA lesion is that of using cells that have different capacities to repair that lesion. Both V79 [43] and CHO [51] cells are unable to repair O6-MeGua significantly, but this function can be re-established by the integration of exogenous DNA of either bacterial or mammalian origin.

As shown by White *et al.* [52], V79 cells harbouring an *E. coli* MT gene that expresses the O6-AlkGua MT function (but not the phosphotriester MT function) were less susceptible to induction of chromatid aberrations and micronuclei by MNU. Moreover, induction of SCE was drastically reduced in these cells as compared to the parental, MT-deficient cells [52]. Bignami *et al.* [53] studied the effect of MNU, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 4-nitroquinoline-1-oxide (4-NQO) in CHO cells expressing a mammalian MT function [54]. As expected, expression of the MT function protected the cells against the mutagenic action of methylating agents but not of 4-NQO. In addition, cytotoxicity and SCE induction were also reduced. These results strongly suggest a role of O6-MeGua in the induction of chromosomal damage.

We have mentioned in the previous section that the thymidine-dependent increase in micronucleus frequency was observed in the MT-deficient V79 cells but not in

the MT-proficient HeLa cells. A weak point in that experiment was the use of cells that have other differences, in addition to that concerning the presence or absence of MT. For this reason, we have recently repeated the experiment using the same CHO cell lines as Bignami *et al.* [53], which have a different capacity to repair O6-MeGua but otherwise, presumably, an identical genetic background.

Both the repair deficient (MT-) and the repair proficient (MT+) cells were treated with MNU and incubated in medium with or without thymidine. On the basis of our hypothesis (see previous section), we expected both a higher induction of micronuclei in MT- cells and the absence of thymidine effect in MT+ cells. As shown in Fig. 3, both predictions were fulfilled.

Molecular dosimetry data: more lesions involved?

If O6-EtGua is involved in the induction of chromosomal damage, why this is not revealed by molecular dosimetry studies? As already mentioned, no specific alkyl adduct, including O6-AlkGua, correlates with the induction of chromosome aberrations [32]. This was also our experience, as illustrated below. We have measured the amount of 7-ethylguanine (7-EtGua) and O6-EtGua induced by ENU and diethylsulfate (DES) in V79 cells DNA, under the same conditions used for the induction of micronuclei. As shown in Fig. 4, micronucleus induction did not correlate with either lesion.

For sake of discussion, let us assume that both 7-EtGua and O6-EtGua are responsible for the induction of micronuclei in V79 cells. In this case, the excess micronuclei induced by ENU at equal O6-EtGua content (lower panel) might be caused by O6-EtGua, which was formed more efficiently by ENU (14.8% of total DNA alkylation) than by DES (1.7%). Conversely, the excess micronuclei induced by DES at equal O6-EtGua content (upper panel) might be caused by 7-EtGua, which was formed more efficiently by DES (49.6%) than by ENU (9.2%). The relative efficiencies by which 7-EtGua and O6-EtGua cause the formation of micronuclei can then be calculated from the molecular dosimetry data, as follows. At doses inducing 50‰ micronuclei, 15.6 x 10^{-5} 7-EtGua and 0.54 x 10^{-5} O6-EtGua residues were formed by DES, while 8.3 x 10^{-5} 7-EtGua and 5.2 x 10^{-5} O6-EtGua residues were formed by ENU.

To account for an equal number (*i.e.*, 50‰) of micronuclei, relative efficiencies of 3.0 and 4.8 have to be assumed for 7-EtGua and O6-EtGua, respectively. This calculation, therefore, would suggest that O6-EtGua is a more powerful clastogenic lesion than 7-EtGua.

The above estimates were based on the hypothesis that 7-EtGua and O6-EtGua are both and the only preclastogenic lesions formed by the two ethylating agents, an assumption for which there is no firm evidence. These estimates in fact simply serve to show that the absence of correlation of a genetic effect with any

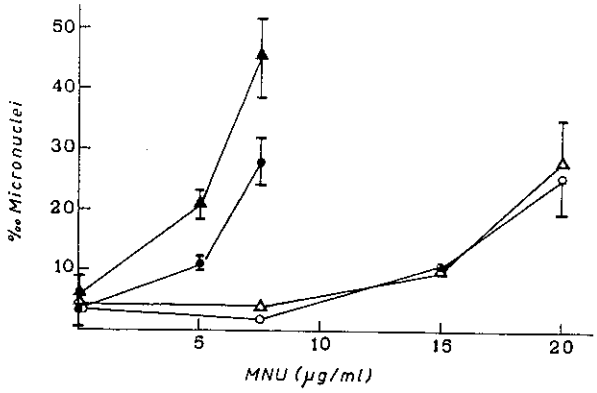

Fig. 3. - The influence of thymidine (8.2 x 10^{-6} M) on the induction of micronuclei by methylnitrosourea (MNU) in CHO cells deficient (MT-, solid symbols) or competent (MT+, open symbols) for the repair of O6-methylguanine. Cells incubated in medium with (triangles) or without (circles) thymidine. Means from two (no standard deviations) or three independent determinations.

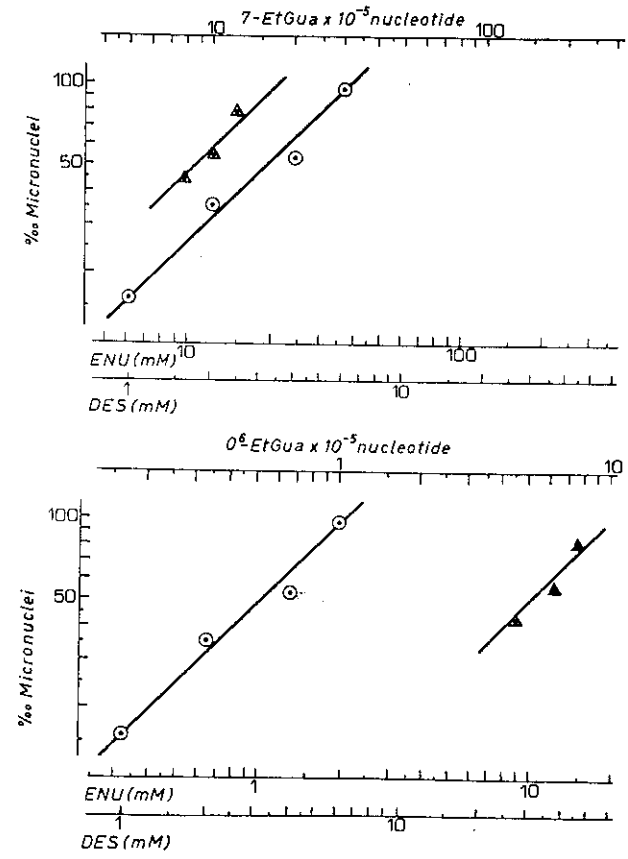

Fig. 4. - The relationship between DNA alkylation and the induction of micronuclei in V79 Chinese hamster cells by ^{3}H-ethylnitrosourea (ENU) and ^{3}H-diethylsulfate (DES). 7-ethylguanine (7-EtGua) and O6-ethylguanine (O6-EtGua) were measured by conventional methods based on HPLC of hydrolysed DNA.

single, specific lesion is not sufficient to rule out a role for that lesion, unless the effect is known to be caused by a single lesion. Where two or more lesions are causing the same effect, the absence of correlation is exactly what we should expect. In fact, O6-EtGua, which resulted from the calculation to be more effective than 7-EtGua, showed a looser correlation with micronuclei (Fig. 4).

Concluding remarks

At least 14 alkylation products are known to be formed in DNA after exposure to alkylating agents [55]; among them, O6-AlkGua has received particular attention because of its role in mutagenesis and carcinogenesis.

In *E. coli*, mutagenesis and cytotoxicity are thought to be caused by separate lesions that are removed by different enzymes [31, 56]. In mammalian cells, the long debated question of whether O6-AlkGua is a lethal lesion seems to be approaching its conclusion, with the weight of evidence supporting a role of this lesion in cytotoxicity [52, 53, 57].

We have reported in this paper on studies on the identification of DNA primary lesions that are relevant for chromosomal damage, with special emphasis to micronuclei. SCE have been mentioned on occasion, but the reader is referred to more pertinent papers [58] for a comprehensive discussion on DNA alkylation products and SCE. Our conclusion is that multiple lesions are probably involved in the induction of chromosomal damage, one of them being O6-AlkGua. The picture that emerges indicates O6-AlkGua as a pleiotropic lesion in mammalian cells.

Acknowledgements

We are grateful to Dr M.A. Minks for critical reading of the manuscript and to T. Tavilla for skillful typing.

Calculations on section 7 were based on unpublished molecular dosimetry data obtained by Dr L. Citti, IMD CNR, Pisa, (ENU data) and Dr M. Rocco, IST, Genova (DES data). MT+ and MT- CHO cells were kindly provided by Prof. E. Bresnik, University of Nebraska.

This paper is based on work supported by the Commission of European Communities, Contract No. EV4V-0036-I(A) and the National Research Council (CNR), target "Projects Preventive Medicine and Rehabilitation", Contract No. 87.00508.56, and "Oncology", Contract No. 87.01493.44, and "Progetto Strategico Mutagenesi", Contract No. 87.02346.74. Part of the reported results have been published previously [40, 41].

Review submitted on invitation by the Editorial Board of the *Annali*. Accepted for publication: October 1988.

REFERENCES

1. BROOKES, P. & LAWLEY, P.D. 1961. The alkylation of guanosine and guanylic acid. *J. Chem. Soc.* Part III: 3923-3928.

2. ROSS, W.C.J. 1962. *Biological alkylating agents*. Butterworths, London.

3. AUERBACH, C. & ROBSON, J.M. 1947. The production of mutations by chemical substances. *Proc. R. Soc. Edinburgh* **B62**: 271-283.

4. ESSIGMANN, J.M., LOECHLER, E.L. & GREEN, C.L. 1986. Genetic toxicology of O6-methylguanine. In: *Genetic toxicology of environmental chemicals. Part A: basic principles and mechanisms of action*. C. Ramel, B. Lambert & J. Magnusson (Eds). Alan R. Liss, Inc., New York. pp. 433-440.

5. PRESTON, B.D., SINGER, B. & LOEB, L.A. 1986. Mutagenic potential of O6-methylthymine *in vivo* determined by an enzimatic approach to site-specific mutagenesis. *Proc. Natl. Acad. Sci. USA* **83**: 8501-8505.

6. PRESTON, B.D., SINGER, B. & LOEB, L.A. 1987. Comparison of the relative mutagenicities of O4-alkylthymines site-specifically incorporated into ϕ x 174 DNA. *J. Biol. Chem.* **262**: 13821-13827.

7. BAUTZ, E. & FREESE, E. 1960. On the mutagenic effect of alkylating agents. *Proc. Natl. Acad. Sci. USA* **46**: 1585-1594.

8. KRIEG, D.R. 1963. Ethyl methanesulfonate-induced reversion of bacteriophage T4 rII mutants. *Genetics* **48**: 561-580.

9. LAWLEY, P.D. & BROOKES, P. 1963. Further studies on the alkylation of nucleic acids and their constituent nucleotides. *Biochem. J.* **89**: 127-138.

10. OSBORN, M., PERSON, S., PHILLIPS, S. & FUNK, F. 1967. A determination of mutagen specificity in bacteria using nonsense mutants of bacteriophage T4. *J. Mol. Biol.* **26**: 437-448.

11. LOVELESS, A. 1969. Possible relevance of O6-alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature* **223**: 206-207.

12. GERCHMAN, L.L. & LUDLUM, D.B. 1973. The properties of O6-methylguanine in templates for RNA polymerase. *Biochim. Biophys. Acta* **308**: 310-316.

13. MEHTA, J.R. & LUDLUM, D.B. 1978. Synthesis and properties of O6-methyldeoxyguanylic acid and its copolymers with deoxycytidylic acid. *Biochim. Biophys. Acta* **521**: 770-778.

14. ABBOTT, P.J. & SAFFHIL, R. 1979. DNA synthesis with methylated poly(dC-dG) templates. Evidence for a competitive nature to miscoding by O^6-methylguanine. *Biochim. Biophys. Acta* **562**: 51-61.

15. HALL, J.A. & SAFFHIL, R. 1983. The incorporation of O^6-methylguanosine and O^4-methyldeoxythymidine monophosphates into DNA by DNA polymerases I α. *Nucleic Acid Res.* **11**: 4185-4193.

16. SCHENDEL, P. & ROBINS, P.E. 1978. Repair of O^6-methylguanine in adapted *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **75**: 6017-6020.

17. GUTTENPLAN, J.B. & MILSTEIN, S. 1982. Resistance of *Salmonella typhimurium* TA1535 to O^6-guanine methylation and mutagenesis induced by low doses of N-methyl-N'-nitro-N-nitrosoguanidine: an apparent constitutive repair activity. *Carcinogenesis* **3**: 327-331.

18. PRAKASH, L & SHERMAN, F. 1973. Mutagenic specificity: reversion of iso-1-cytochrome c mutants of yeast. *J. Mol. Biol.* **79**: 65-82.

19. OSTERMAN-GOLKAR, S., EHRENBERG, L. & WACHTMEISTER, C.A. 1970. Reaction kinetics and biological action in barley of monofunctional methanesulfonic esters. *Radiat. Bot.* **10**: 303-327.

20. VELEMÍNSKÝ, J., OSTERMAN-GOLKAR, S. & EHRENBERG, L. 1970. Reaction rate and biological action of N'-methyl- and N'-ethyl-N-nitrosourea. *Mutat. Res.* **10**: 169-174.

21. VOGEL, E. & NATARAJAN, A.T. 1979. The relation between reaction kinetics and mutagenic action of monofunctional alkylating agents in higher eukaryotic systems. I. Recessive lethal mutations and translocations in *Drosophila*. *Mutat. Res.* **62**: 51-100.

22. VOGEL, E. & NATARAJAN, A.T. 1979. The relation between reaction kinetics and mutagenic action of monofunctional alkylating agents in higher eukaryotic systems. II. Total and partial sex-chromosome loss in *Drosophila*. *Mutat. Res.* **62**: 101-123.

23. NEWBOLD, R.F., WARREN, W., MEDCALF, A.S.C. & AMOS, J. 1980. Mutagenicity of carcinogenic methylating agents is associated with a specific DNA modification. *Nature* **283**: 596-599.

24. MEUTH, M. 1981. Role of deoxynucleotide triphosphate pools in the cytotoxic and mutagenic effects of DNA alkylating agents. *Somatic Cell Genet.* **7**: 89-102.

25. BERANEK, D.T., HEFLICH, R.H., KODELL, R.L., MORRIS, S.M. & CASCIANO, D.A. 1983. Correlation between specific DNA-methylation products and mutation induction at the HGPRT locus in Chinese hamster ovary cells. *Mutat. Res.* **110**: 171-180.

26. TOORCHEN, D. & TOPAL, M.G. 1983. Mechanism of chemical mutagenesis and carcinogenesis: effects on DNA replication of methylation at the O^6-guanine position of dGTP. *Carcinogenesis* **4**: 1591-1597.

27. NATARAJAN, A.T., SIMONS, J.W.I.M., VOGEL, E.W. & VAN ZEELAND, A.A. 1984. Relationship between cell killing, chromosomal aberrations, sister chromatid exchanges and point mutations induced by monofunctional alkylating agents in Chinese hamster cells. A correlation with different ethylation products in DNA. *Mutat. Res.* **128**: 31-40.

28. SUKUMAR, S., NOTARIO, V., MARTIN-ZANCA, D. & BARBACID, M. 1983. Induction of mammary carcinomas in rats by nitroso-methylurea involves malignant activation of H-ras-1 locus by single point mutations. *Nature* **306**: 658-661.

29. LOECHLER, E.L., GREEN, C.L. & ESSIGMANN, J.M. 1984. *In vivo* mutagenesis by O^6-methylguanine built into a unique site in a viral genome. *Proc. Natl. Acad. Sci. USA* **81**: 6271-6275.

30. SWAIN, C.G. & SCOTT, C.B. 1953. Quantitative correlation of relative rates: comparison of hydroxide ion with other nucleophilic reagents toward alkyl halides, esters, epoxides and acyl halides. *J. Am. Chem. Soc.* **75**: 141-147.

31. LINDAHL, T. 1982. DNA repair enzymes. *Annu. Rev. Biochem.* **51**: 61-87.

32. DE KOK, A.J., VAN ZEELAND, A.A., SIMONS, J.W.I.M. & DEN ENGELSE, L. 1985. Genetic and molecular mechanisms of the *in vitro* transformation of Syrian hamster embryo cells by the carcinogen N-ethyl-N-nitrosourea. II. Correlation of morphological transformation, enhanced fibrinolytic activity, gene mutations, chromosomal alterations and lethality to specific carcinogen-induced DNA lesions. *Carcinogenesis* **6**: 1571-1576.

33. TATES, A.D., NEUTEBOOM, I., DE VOGEL, N. & DEN ENGELSE, L. 1983. The induction of chromosomal damage in rat hepatocytes and lymphocytes. I. Time-dependent changes of the clastogenic effects of diethylnitrosamine, dimethylnitrosamine and ethyl methanesulfonate. *Mutat. Res.* **107**: 131-151.

34. DEN ENGELSE, L., FLOOT, B.G.J., DE BRIJ, R.-J. & TATES, A.D. 1983. The induction of chromosomal damage in rat hepatocytes and lymphocytes. II. Alkylation damage and repair of rat liver DNA after diethylnitrosamine, dimethylnitrosamine and ethyl methanesulfonate in relation to clastogenic effects. *Mutat. Res.* **107**: 153-166.

35. MENKVELD, G.J., TATES, A.D. & DEN ENGELSE, L. 1984. Persistence of chromosome damage induced by (m)ethylating agents in rat hepatocytes in relation to alkylation pattern of liver DNA. *Mutat. Res.* **130**: 204.

36. TATES, A.D., NEUTEBOOM, I., ROTTEVEEL, A.H.M., DE VOGEL, N., MENKVELD, G.J. & DEN ENGELSE, L. 1986. Persistence of preclastogenic damage in hepatocytes of rats exposed to ethylnitrosourea, diethylnitrosamine, dimethylnitrosamine and methyl methanesulfonate. Correlation with DNA O-alkylation. *Carcinogenesis* **7**: 1053-1058.

37. DEN ENGELSE, L., MENKVELD, G.J., DE BRIJ, R.-J. & TATES, A.D. 1986. Formation and stability of alkylated pyrimidines and purines (including imidazole ring-opened 7-alkylguanine) and alkylphosphotriesters in liver DNA of adult rats treated with ethylnitrosourea or dimethylnitrosamine. *Carcinogenesis* 7: 393-403.

38. MOORE, R.C. & HOLGSON, G.S. 1983. Clastogenic effect of DNA synthesis inhibitors in cells exposed in G_2 *Mutat. Res.* 120: 139-143.

39. SNOW, E.T., FOOTE, R.S. & MITRA, S. 1984. Base-pairing properties of O^6-methylguanine in template DNA during *in vitro* DNA replication. *J. Biol. Chem.* 259: 8095-8100.

40. BONATTI, S., SIMI, L. & ABBONDANDOLO, A. 1985. The effect of thymidine on the induction of micronuclei by alkylating agents in V79 Chinese hamster cells. *Mutat. Res.* 150: 347-353.

41. BONATTI, S., SIMI, L. & ABBONDANDOLO, A. 1985. Is O^6-alkylguanine involved in the production of chromosomal damage? In: *Mutagenesis and genetic toxicology. Theoretical and practical results*. P. Janiaud, D. Averbeck & E. Moustacchi (Eds). INSERM, Paris. pp. 223-228.

42. LEDDA, G.M., COLUMBANO, A., RAO, P.M., RAJALAKSHMI, S. & SARMA, D.S.R. 1980. *In vivo* replication of carcinogen-modified rat liver DNA: Increased susceptibility of O^6-methylguanine compared to N7-methylguanine in replicated DNA to S^1 nuclease. *Biochem. Biophys. Res. Commun.* 95: 816-821.

43. WARREN, W., CRATHORN, A.R. & SHOOTER, K.V. 1979. The stability of methylated purines and of methylphosphotriesters in the DNA of V79 cells after treatment with N-methyl-N-nitrosourea. *Biochim. Biophys. Acta* 563: 82-88.

44. CITTI, L., MARIANI, L., CATAPANO, C.V. & MALVALDI, G. 1989. The relevance of DNA repair in the cytotoxic response of mammalian cells to alkylating agents. *Ann. Ist. Super. Sanità*: 25: 5-10.

45. PEGG, A.E., DOLAN, M.E., SCICCHITANO, D. & MORIMOTO, K. 1985. Studies on the repair of O^6-alkylguanine and O^4-alkylthymine in DNA by alkyltransferases from mammalian cells and bacteria. *Environ. Health Perspect.* 62: 109-114.

46. DOLAN, M.E., SCICCHITANO, D. & PEGG, A.E. 1988. Use of oligodeoxynucleotides containing O^6-alkylguanine for the assay of O^6-alkylguanine-DNA-alkyltransferase activity. *Cancer Res.* 48: 1184-1188.

47. BRENT, T.P., DOLAN, M.E., FRANKEL-CONRAT, H., HALL, J., KARRAN, P., LAVAL, F., MARGISON, G.P., MONTESANO, R., PEGG, A.E., POTTER, P.M., SINGER, B., SWENBERG, J.A. & YAROSH, D.B. 1988. Repair of O-alkylpyrimidines in mammalian cells: a present consensus. *Proc. Natl. Acad. Sci. USA* 85: 1759-1762.

48. NEWMAN, C.N. & MILLER, J.H. 1985. Response of CHO cell DNA polymerase alpha to dCTP and dTTP pool imbalance: relation to DNA synthesis inhibition, survival and mutation. In: *Genetic consequences of nucleotide pool imbalance*. F.J. De Serres (Ed.). Plenum Press, New York. pp. 127-148.

49. HEDDLE, J.A., BENZ, R.D. & COUNTRYMAN, P.I. 1978. Measurement of chromosomal breakage in cultured cells by the micronucleus technique. In: *Mutagen-induced chromosome damage in man*. H.J. Evans & D.C. Lloyd (Eds). Edinburgh University Press. pp. 191-200.

50. VIAGGI, S., BONATTI, S. & ABBONDANDOLO, A. 1987. New evidence for the presence of chromsomes in micronuclei of human and Chinese hamster cells. *Mutagenesis* 2: 367-370.

51. GOTH-GOLDSTEIN, R. 1980. Inability of Chinese hamster ovary cells to excise O^6-alkylguanine. *Cancer Res.* 40: 2623-2624.

52. WHITE, G.R.M., OCKEY, C.H., BRENNAND, J. & MARGISON, G.P. 1986. Chinese hamster cells harbouring the *Escherichia coli* O^6-alkylguanine alkyltransferase gene are less susceptible to sister chromatid exchange induction and chromosome damage by methylating agents. *Carcinogenesis* 7: 2077-2080.

53. BIGNAMI, M., TERLIZZESE, M., ZUNO, A., CALCAGNILE, A., FROSINA, G., ABBONDANDOLO, A. & DOGLIOTTI, E. 1987. Cytotoxicity, mutations and SCEs induced by methylating agents are reduced in CHO cells expressing an active mammalian O^6-methylguanine-DNA methyltransferase gene. *Carcinogenesis* 8: 1417-1421.

54. DING, R., GHOSH, K., EASTMAN, A. & BRESNICK, E. 1985. DNA-mediated transfer and expression of a human DNA repair gene that demethylates O^6-methylguanine. *Mol. Cell. Biol.* 5: 3293-3296.

55. BERANEK, D.T., WEIS, C.C. & SWENSON, D.H. 1980. A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. *Carcinogenesis* 1: 595-606.

56. MC CARTHY, T.V., KARRAN, P. & LINDAHL, T. 1984. Inducible repair of O-alkylated DNA pyrimidines in *Escherichia coli*. *EMBO J.* 3: 545-550.

57. DOMORADZKI, J., PEGG, A.E., DOLAN, M.E., MAHER, V.M. & MC CORMICK, J.J. 1984. Correlation between O^6-methylguanine-DNA-methyltransferase activity and resistance of human cells to the cytotoxic and mutagenic effect of N-methyl-N'-nitro-N-nitrosoguanidine. *Carcinogenesis* 5: 1641-1647.

58. HEFLICH, R.H., BERANEK, D.T., KODELL, R.L. & MORRIS, S.M. 1982. Induction of mutation and sister-chromatid exchanges in Chinese hamster ovary cells by ethylating agents. *Mutat. Res.* 106: 147-161.