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Molecular and Cellular Mechanisms in Carcinogenesis by Alkylating N-Nitroso Compounds: Pulse Carcinogenesis by N-Ethyl-N-nitrosourea in the Developing Rat Brain as a Model System

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ABSTRACT

Structural modifications of DNA in cellular chromatin are primary events in the multistage process of tumorigenesis initiated by chemical carcinogens. It is, however, not yet clear which molecular processes are preferentially affected, or put in motion, by carcinogen-induced structural modifications of DNA, and which alterations of cellular genetic programmes result in the expression of malignant phenotypes. Among the mechanisms to be considered are mutations, gene rearrangements and chromosome translocations and deletions, DNA amplification, altered patterns of DNA methylation and mRNA processing, and possibly the induction of error-prone DNA repair. Initiation of malignant transformation apparently requires that at the time of carcinogen exposure, the target cells must be proliferating or have retained the capacity to re-enter the cell cycle from a G_0-state. The alkylating *N*-nitroso carcinogens, in particular, have been well studied with respect to the molecular structure and stability of their reaction products in cellular DNA. In addition to conventional radiochromatography, highly sensitive immunoanalytical methods using monoclonal antibodies have recently become available for the detection and quantitation of specific DNA alkylation products. In the present review, carcinogenesis by *N*-ethyl-*N*-nitrosourea in the rat is described in detail, in order to exemplify some of the cellular and molecular mechanisms involved in malignant transformation by *N*-nitroso compounds. This model system, like others, strongly suggests a cell type- and developmental/differentiation stage-dependence of the tumorigenic effect, and indicates that cellular enzymes responsible for the recognition and elimination of specific carcinogen-DNA adducts can be expressed differentially in different cell types and tissues. The relative capacity of cells to repair critical

DNA lesions may constitute a determinant for the probability of malignant conversion no less important than the activity of cellular enzymes required for the conversion of many carcinogens to their ultimate reactive forms. However, only a few types of mammalian cells have thus far been compared with regard to their capacity for enzymatic repair of specific DNA lesions, and very little information is available on the expression of DNA repair enzymes as a function of the state of cellular differentiation and proliferation. Likewise, information is needed on the degree of inter-cell, inter-individual, and interspecies variation regarding DNA repair activity. The relative importance of DNA repair capacity among cellular properties that could influence the risk of malignant transformation, is, therefore, not yet established.

1 INTRODUCTION

Most chemical carcinogens cause structural alterations of DNA in the chromatin of target cells (Lawley, 1976; Pegg, 1977; Weinstein, 1977; Grover, 1979; Singer, 1979; Pullman *et al.*, 1980; Rajewsky, 1980a). In general, covalent binding occurs between nucleophilic centres (electron-rich N and O atoms) in cellular DNA, and highly reactive, electrophilic derivatives ('ultimate carcinogens') generated from the respective parent compounds ('precarcinogens') either enzymatically or via non-enzymatic decomposition (Miller and Miller, 1976, 1979). As a consequence of their reaction with DNA, most chemical carcinogens are also mutagenic (McCann *et al.*, 1975; Nagao *et al.*, 1978; Hollstein *et al.*, 1979). However, the positive correlation of carcinogenicity and mutagenicity does not constitute proof for an obligatory requirement of mutation (nor even of modification of DNA structure in general) for malignant transformation. Cellular macromolecules other than DNA also contain multiple nucleophilic sites which can, and indeed do, react with carcinogen-generated electrophiles. Nevertheless, the central importance of DNA structure and conformation for the expression of genetic information provides a strong argument for a critical role of DNA alterations in the initiation of carcinogenesis by chemical agents.

Structural alterations of DNA by chemical carcinogens may lead to local alterations of nucleotide sequence (mutations) and helical distortions, and in certain cases facilitate the transition of the B-form of the double helix to a left-handed conformation (Z-DNA) (Wang *et al.*, 1979; Sage and Leng, 1980; Möller *et al.*, 1981; Santella *et al.*, 1981). Carcinogen-modified DNA components could, however, also affect the precision of DNA rearrangements (transpositional events in the genome may be associated with normal development/differentiation), cause inappropriate rearrangements and amplification of genes as well as chromosome translocations and deletions, interfere with the patterns of mRNA processing (splicing), and of DNA methylation, and perhaps induce error-prone DNA repair (Fuchs *et al.*, 1976, 1981; Radman *et al.*, 1977; Crick, 1979; Grunberger and Weinstein, 1979; Lapeyre and Becker, 1979; Rajewsky, 1980a;

Cairns, 1981; Lavi, 1981; Boehm and Drahovsky, 1981; Ehrlich and Wang, 1981; Pfohl-Leszkowicz *et al.*, 1981; Rowley, 1983; see Table 1). Whether one or several of these mechanisms predominate in terms of their relevance to malignant transformation is at present a matter of speculation. The common denominator is, however, an interference with the genetic programmes of target cells. More information is, therefore, needed on the molecular basis of eucaryotic gene expression, on the mechanisms controlling phenotypic differentiation and cell proliferation in developing and mature cell systems, and on the particular genes involved in these processes.

The wide spectrum of differing tumour cell phenotypes observed in mammals may not merely reflect the expression of different combinations of genes which, at least in part, are characteristic of the phenotypes of the corresponding normal cells of origin and their developmental and differentiation stage; it could also indicate that qualitatively different phenotypic shifts may share the property of resulting in a malignant behaviour of specific types of cells in their respective tissue environment. These phenotypic shifts are likely to result from the inappropriate expression of normal or structurally altered (for example, by carcinogen–DNA interactions) cellular genes, such as the c-onc genes (proto-oncogenes, Bishop, 1983), or of their viral homologues ('v-onc genes') introduced into the cellular genome (Hayward *et al.*, 1981; Reddy *et al.*, 1982; Tabin *et al.*, 1982). Hopefully, the DNA transfection approach recently introduced to define rodent and human onc genes (Lane *et al.*, 1981; Weinberg, 1981) and the present rapid advances in molecular (cyto)-genetics will shed further light on this problem.

The later stages of the maturation of cells to a terminally differentiated state are generally accompanied by a cessation of proliferative activity. In mature cells, the non-proliferative state can either be apparently irreversible (for example, neurons, granulocytes), or reversible ('G_o-cells') under special physiological conditions, such as the requirement for reparative or functional hyperplasia (for example, hepatocytes, hormonally controlled cell systems). Temporary non-

Table 1 Carcinogen-induced, structural alterations of DNA in the chromatin of target cells (particularly if persistent, i.e., not repaired) may, for example, lead to:

(1) Local alterations of nucleotide sequence (mutations)
(2) Distortions of the DNA double helix
(3) Transition of the B-form of the DNA double helix to, e.g., the left-handed Z-conformation (with possible consequences for the control of gene expression)
(4) Inappropriate DNA rearrangements at the level of genes and chromosomes (connection/disconnection of promoter sequences to/from cellular genes. Note that transpositional events may be associated with normal development/differentiation)
(5) Inappropriate gene amplification
(6) Interference with normal mRNA processing (splicing)
(7) Interference with normal patterns of DNA methylation (5-methylcytosine)
(8) Induction of error-prone DNA repair

proliferative states of part of a cell population are, however, also characteristic of stem cells, and probably of committed 'precursor' cells at more advanced stages of maturation (Lajtha, 1979). Like UV-induced photoproducts in DNA (Hanawalt *et al.*, 1979), certain carcinogen–DNA adducts can be specifically recognized, removed and repaired by cellular enzymes (Rajewsky *et al.*, 1977; Margison and O'Connor, 1979; Lehmann and Karran, 1981; Seeberg and Kleppe, 1981; Lindahl, 1982). The majority of non-repaired, persistent modifications introduced into DNA by carcinogens are localized in transcriptionally silent parts of the genome. They become effective only when the functional integrity of the respective DNA sequences is put to the test in the course of gene activation (by further progression of cells along the pathway of development/ differentiation; by inducing cells to express previous inactive genes, or by triggering cells to enter the cell cycle from a G_0-state, for example by the action of tumour promoters). There is no evidence that cells undergo malignant transformation after having reached a terminally differentiated, irreversibly non-proliferative state (Rajewsky, 1972, 1980b). It appears instead that the expression of malignant phenotypes is much more readily induced in cells exposed to carcinogenic agents either during earlier, proliferation-linked stages of their differentiation pathway, or in a G_0-state (Rajewsky, 1972, 1980b; Rajewsky *et al.*, 1977; Saunders, 1978). Of particular interest is the question whether, along the differentiation pathway of a given cell lineage, specific stem cell and precursor cell stages exist where the gene programme can be shifted to the expression of malignant phenotypes with higher than random probability (Rajewsky *et al.*, 1977; Graf and Beug, 1978; Jaenisch, 1980; Rudland *et al.*, 1980).

Molecular and cellular mechanisms underlying the multistage process of malignant transformation and tumorigenesis can probably be best studied in so-called 'pulse-carcinogenesis' systems; i.e., systems where, after a single dose of a short-lived carcinogen sufficient to produce a high tumorigenic effect, the process proceeds autonomously without the complication of continued interaction of the target cell populations (s) with the carcinogen (Rajewsky *et al.*, 1977; Rajewsky, 1980b). In such systems the process of carcinogenesis can be separated operationally into three phases: phase A, period of carcinogen interaction with target cells; phase B, time interval between phase A and phase C; and phase C, period of tumour growth, beginning with the onset of (clonal) proliferation of tumorigenic cells (Rajewsky, 1980b). More or less synonymous terms are 'initiation' for phase A and '(gradual) expression' of malignant phenotypes for phase B. In spite of its crucial importance, least is presently known about phase B which often constitutes the longest of the three phases and appears to encompass a sequence of phenotypic changes—including acquisition of the capacity for continuous proliferation (immortalization)—in cells which ultimately become tumorigenic (Laerum and Rajewsky, 1975; Barrett and Ts'o, 1978; Kakunaga *et al.*, 1980). Phase B represents the period during which, for instance, tumour promoters can exert their pleiotropic effects, i.e., modify gene expression and induce cell proliferation in the target cell population (Berenblum, 1975; Slaga *et*

al., 1978; Weinstein *et al.*, 1980; Hecker *et al.*, 1982). Probably of great importance, but as yet largely unexplored, are the various kinds of controlling influences (via short-range and long-range humoral signals or cell–cell interactions) exerted during phase B on the potential tumorigenic cells by their particular tissue environment. Such microenvironmental influences may represent an important element of control and variation in the process of carcinogenesis. They are likely to be dependent on maturation- and age-related changes in the host organism as well as on its genetic constitution, and on temporary reactions of the host to non-physiological factors. Furthermore, it should not be overlooked that *all* cells in a particular target tissue or cell system, although for the most part not becoming malignantly transformed, are nevertheless subject to the interaction with the carcinogen. This may lead to disturbances of the normal, precisely balanced control of cell proliferation, of the expression of cellular phenotypes and of cell population structure and tissue architecture, thereby possibly influencing the transition probability of potential cancer cells to the ultimate tumorigenic state. In target cell systems capable of reparative hyperplasia, chemical carcinogens generally cause a dose-dependent increase in the rate of cell proliferation by their concomitant cytotoxic effects (Rajewsky, 1967, 1972). In this context it is important to note that an increased rate of reparative proliferation of parenchymal liver cells, resulting from partial surgical elimination of a part of this cell population, enhances the hepatocarcinogenic effect of chemical carcinogens (Craddock, 1975).

With respect to their reactive derivatives and reaction products in cellular DNA, the alkylating *N*-nitroso compounds are at present the best characterized class of chemical carcinogens (Lawley, 1976; Pegg, 1977; Grover, 1979; O'Connor *et al.*, 1979; Singer, 1979; Rajewsky, 1980a). They include the alkylnitrosamines (which require activation by cellular enzymes) and the alkylnitrosoureas and alkylnitronitrosoguanidines (which undergo rapid, non-enzymatic decomposition *in vitro*). The resulting electrophilic alkyl substituents are small in comparison with the bulky DNA adducts derived from, e.g., carcinogenic hydrocarbons or aromatic amines. A typical representative of the carcinogenic alkylnitrosoureas, *N*-ethyl-*N*-nitrosourea (EtNU) (Ivankovic and Druckrey, 1968), has become a model carcinogen in the rat (Rajewsky *et al.*, 1977). The properties of this system will serve to describe some of the molecular and cellular aspects of carcinogenesis by alkylating *N*-nitroso compounds.

2 PULSE CARCINOGENESIS BY *N*-ETHYL-*N*-NITROSO-UREA IN THE DEVELOPING RAT

2.1 Tissue- and Cell Type-Tropism of the Carcinogenic Effect

Under *in vitro* conditions EtNU decomposes rapidly (half-life < 8 min) to a highly reactive ethyldiazonium ion (Goth and Rajewsky, 1972). After systemic application of EtNU, nucleophilic sites in cellular macromolecules (for example,

DNA) become ethylated to a similar extent in all tissues, as shown by radiochromatographic analysis of DNA exposed to radiolabelled EtNU *in vivo* (Goth and Rajewsky, 1974a,b) or by radioimmunoassay (Müller and Rajewsky, 1980, 1981), and also by whole-body autoradiography (Johansson-Brittebo and Tjälve, 1979). However, in spite of the similar initial degree of ethylation in all cells of the organism, a single pulse of EtNU applied to fetal or newborn rats of the inbred BDIX strain (Druckrey, 1971) results in a very high yield of malignant neuroectodermal neoplasms, while tumours in tissues other than the brain and peripheral nervous system are rarely detected (*neural tissue-tropism of the carcinogenic effect*). Tumour yield and latency period are dose- (Druckrey *et al.*, 1970b; Rajewsky *et al.*, 1977) and strain-dependent (Druckrey *et al.*, 1970a). Following transplacental pulse exposure to EtNU and subsequent transfer to a long-term culture system, fetal BDIX-rat brain cells undergo malignant transformation *in vitro* after a time period similar to the time required for tumour formation *in vivo* after the same carcinogen dose (Laerum and Rajewsky, 1975; Rajewsky *et al.*, 1977; Laerum *et al.*, 1979).

2.2 Developmental/Differentiation Stage-Dependence of the Carcinogenic Effect

Interestingly, the neuro-oncogenic effect of EtNU strongly depends on the developmental stage of the nervous system at the time of the carcinogen pulse (*developmental/differentiation stage-dependence of the carcinogenic effect*). Being highest after an EtNU pulse during late prenatal and early postnatal development, the tumorigenic effect decreases strongly in animals exposed to the same dose of EtNU at a later age. The carcinogenic effect is thus inversely correlated with the developmental/differentiation stage of the neural cell populations, and appears to require the presence of proliferative neural (precursor) cells at the time of exposure to the carcinogen. However, the carcinogenic effect also decreases when the EtNU pulse is applied at developmental stages prior to the 15th day of gestation, and no neuroectodermal tumours were observed (in limited numbers of experimental animals, Ivankovic and Druckrey, 1968) after exposure to EtNU before day 11–12 of prenatal development. At this development stage the total number of cells in BDIX-rat brain (probably almost all proliferating) has reached a value of approximately 10^5 (Müller and Rajewsky, 1983a). When 20 rat embryos are exposed to EtNU on prenatal day 11–12, the total neural target cell population thus amounts to $\sim 2 \times 10^6$ cells.

The developmental period of maximum sensitivity in terms of the neuro-oncogenic effect of EtNU appears to vary in different species. Only a low incidence of neural tumours was observed in several mouse strains after late prenatal and neonatal administration of EtNU (Denlinger *et al.*, 1974; Jones *et al.*, 1976), and postnatal application of EtNU to Mongolian gerbils resulted in malignant transformation of (neural crest-derived) cutaneous melanocytes but

not in tumours of the brain or peripheral nervous system (Kleihues *et al.*, 1978). On the other hand, Stutman (1979) observed mainly brain tumours (besides some kidney and ovarian tumours) when immunologically competent (nu/+) or incompetent (nu/nu) CBA/H or BALB/c mice were treated with EtNU on day 12–14 of prenatal development, while after treatment on day 16–18 most tumours were found in the lung, liver (males) and kidney. Similarly, EtNU caused predominantly neural tumours in rabbits when applied during early phases of prenatal development, whereas exposure to EtNU during later fetal stages led mainly to kidney tumours (Fox *et al.*, 1975; Stavrou *et al.*, 1975, 1977). Careful analyses are, therefore, required in order to specify phases of increased cancer risk during development/differentiation of a given cell system in different species.

2.3 Quantitation of Alkylation Products in DNA (Radiochromatography and Immunoanalysis)

Highly sensitive analytical methods are required for the detection and quantitation of alkylation products in the DNA of target tissues and cells. The sensitivity of radiochromatographic techniques (Baird, 1979) is limited mainly by the specific radioactivity of the respective [^{3}H]- or [^{14}C]-labelled carcinogens. Under favourable conditions, these procedures will detect one alkylated base in $\sim 10^6$ molecules of the corresponding normal base, and relatively large amounts of DNA (i.e., large numbers of cells) are necessary for analysis. With the exception of recently developed ^{32}P 'post-labelling' techniques (Randerath *et al.*, 1981; Gupta *et al.*, 1982), radiochromatography requires the use of radioactively labelled, synthetic carcinogens. Therefore, the detection of specific carcinogen–DNA adducts in (for example, human) tissues and cells exposed to low doses of non-radioactive (for example, environmental) agents is not possible. Recently developed immunoanalytical procedures using high-affinity 'conventional' (polyclonal) and monoclonal antibodies (antibody affinity constants, 10^9 to $> 10^{10}$ l/mol) specifically directed against defined alkylation products in DNA, have opened new possibilities in this respect (Rajewsky *et al.*, 1980; Müller and Rajewsky, 1981; Saffhill and Boyle, 1981; Müller *et al.*, 1982). Thus Müller and Rajewsky (1978, 1980) developed a sensitive competitive radioimmunoassay (RIA) for the quantitation of O^6-ethyldeoxyguanosine (O^6-EtdGuo) in DNA. At present $\sim$ 0.04 pmol of O^6-EtdGuo can be detected by RIA in a 100 μl sample at 50% inhibition of tracer–antibody binding, thus permitting quantitation of O^6-EtdGuo at an O^6-EtdGuo/deoxyguanosine molar ratio of $\sim 3 \times 10^{-7}$ in a hydrolysate of 100 μg of ethylated DNA (corresponding to the DNA content of $\sim 10^7$ diploid cells). The detection limit may be lowered even further when the alkyldeoxynucleosides to be quantitated are separated from the DNA hydrolysate by high pressure liquid chromatography (HPLC) prior to the RIA, or with the use of a recently developed immuno-slot-blot technique (Nehls *et al.*, 1984a). The spectrum of monoclonal antibodies specific for different DNA alkylation

products is currently being further expanded (Adamkiewicz *et al.*, 1982; Adamkiewicz and Rajewsky, 1984), and their application is being extended to the use of immunostaining techniques for the detection and quantitation of DNA alkylation products both in the nuclei of individual cells (Adamkiewicz *et al.*, 1983) and in isolated DNA molecules (Nehls *et al.*, 1984b).

2.4 Relative Frequency and Distribution of Ethylation Products in Chromosomal DNA

The different ethylation products formed in DNA after exposure to EtNU have been carefully analysed (Loveless, 1969; Lawley, 1976; Singer *et al.*, 1978; O'Connor *et al.*, 1979; Singer, 1979; Singer and Kröger, 1979; Rajewsky, 1980a). They are produced with equal relative frequencies, regardless of whether the reaction with EtNU occurs *in vivo*, in cell culture, or with purified DNA *in vitro* (Goth and Rajewsky, 1974a,b; Singer *et al.*, 1978). The following products are formed by ethylation on oxygen atoms (~80% of all ethylation products in DNA): O^6-ethyldeoxyguanosine (O^6-EtdGuo, ~10%), O^2-ethyldeoxythymidine (O^2-EtdThd, ~7%), O^2-ethyldeoxycytidine (O^2-EtdCyd, ~4%), O^4-ethyldeoxythymidine (O^4-EtdThd, ~3%) and ethylphosphotriesters (~56%). Products resulting from ethylation on nitrogen atoms in DNA are: 7-ethyldeoxyguanosine (7-EtdGuo, ~14%), 3-ethyldeoxyadenosine (3-EtdAdo, ~5%) 7-ethyldeoxyadenosine (7-EtdAdo, ~0.4%), 1-ethyldeoxyadenosine (1-EtdAdo, ~0.3%), 3-ethyldeoxycytidine (3-EtdCyd, ~0.2%), 3-ethyldeoxyguanosine (3-EtdGuo, ~0.1%), and 3-ethyldeoxythymidine (3-EtdThd, ~0.1%).

In the case of the ethylation products O^6-EtdGuo, O^2-EtdCyd, O^4-EtdThd, 3-EtdThd, 3-EtdCyd, and 1-EtdAdo, the alkyl groups are localized on atoms normally involved in Watson-Crick base pairing. With the use of a competitive RIA (Müller and Rajewsky, 1978, 1980), the relative O^6-EtdGuo content in DNA has also been determined in chromatin of different folding levels, isolated from fetal rat brain cells and briefly exposed to EtNU *in vitro* (Nehls and Rajewsky, 1981a,b). Compared with naked DNA (relative value 1.0), the degree of O^6-deoxyguanosine ethylation decreases from the DNA of extended (histone H1-free) chromatin fibres (~0.6) to the DNA in nucleosomes (core particles ~0.5), and it is lowest in the DNA of condensed chromatin fibres ('superbeads', ~0.4, Renz *et al.*, 1977). Independent of the chromatin folding level, nucleophilic sites located in the major and minor groove, and in base-pairing regions of the DNA double helix, appear to be equally accessible to the reactive ethyldiazonium ion (Nehls and Rajewsky, 1981a,b). Fractions of chromosomal DNA which are preferentially digested with DNase I (transcribable conformation, Garel and Axel, 1976; Weintraub and Groudine, 1976) were found to have a higher initial O^6-EtdGuo content than chromosomal DNA less susceptible to this enzyme, i.e., nontranscribable genome regions (Nehls and Rajewsky, 1981a,b).

A positive correlation exists between the carcinogenicity of different alkylating

N-nitroso carcinogens and their relative extent of alkylation on oxygen (for example, O^6-dGuo) versus nitrogen atoms (for example, 7-dGuo) in DNA (O'Connor *et al.*, 1979). Thus for the potent carcinogen EtNU the initial O^6-EtdGuo: 7-EtdGuo ratio in DNA is between 0.63 and 0.72 (Goth and Rajewsky, 1974b; Singer *et al.*, 1978), while the corresponding value for the weakly carcinogenic diethylsulphate is ~0.003 (Sun and Singer, 1975). The relative extent of alkylation on oxygen atoms in DNA is a function of the type of reaction mechanism characteristic of the respective agent; a bimolecular nucleophilic (SN2) reaction results in a lower O/N alkylation ratio than an SN2 mechanism with a tendency towards a unimolecular (SN1) reaction (Ingold, 1953; Lawley, 1976).

2.5 Differential Removal of Alkylation Products from DNA by Cellular Enzymes in Different Tissues and Cell Types

The initial degree of ethylation by EtNU in the DNA of prenatal (> 11th day of gestation) or early postnatal rat brain (high cancer risk) is not significantly different from that found in the DNA of adult brain and other 'low risk' tissues (Goth and Rajewsky, 1972, 1974a,b; Müller and Rajewsky, 1983a). However, as shown by kinetic analyses after a pulse [1-^{14}C]-labelled EtNU to 10-day-old BDIX-rats, the content of O^6-EtdGuo decreases rapidly in liver DNA (and less rapidly in the DNA of other tissues) but very slowly in pre- and postnatal brain DNA. These findings were recently confirmed for an EtNU-pulse applied to Fisher rats during late (20th day of gestation) prenatal development (Chang *et al.*, 1980). The difference in the elimination rates of O^6-EtdGuo from brain versus liver DNA is so large that a specific enzymatic recognition and elimination mechanism had to be assumed for this particular product (Goth and Rajewsky, 1974a; Rajewsky *et al.*, 1977). In contrast, other ethylation products such as 3-EtdAdo and 7-EtdGuo disappear from the DNA of brain and other rat tissues at much faster rates than does O^6-EtdGuo from brain DNA. Indeed, with the exception of O^6-EtdGuo, large differences in tissue-specific elimination rates are not apparent for any alkylation product thus far investigated (including O^4-EtdThd, Müller and Rajewsky, 1983b). In a semilogarithmic plot, the kinetics of elimination of O^6-EtdGuo from cellular DNA have a bi- (or multi-) componential appearance. The reasons for this have not yet been definitively clarified. In principle, several mechanisms, alone or in combination, could account for this phenomenon:

(1) an O^6-EtdGuo-removing enzyme (or [one of] several enzymes) is initially present in excess, consumed upon reaction, and is synthesized at relatively low rate;
(2) O^6-EtdGuo is differentially accessible in the DNA of chromatin of different folding levels (see above); and
(3) the tissue is composed of subpopulations of cells with different capacities for the enzymatic elimination of O^6-EtdGuo.

O^6-EtdGuo not eliminated from DNA prior to a subsequent round of DNA replication (or prior to DNA transcription) can, like O^4-EtdThd (Abbott and Saffhill, 1977), cause anomalous base-pairing (Loveless, 1969; Abbott and Saffhill, 1979), i.e., lead to an incorrect nucleotide sequence in the daughter strand (or in mRNA, respectively). Note that EtNU is presently considered one of the most potent point mutagens in eukaryotic systems (Vogel and Natarajan, 1979a,b; Russel *et al.*, 1979; Johnson and Lewis, 1981). The persistence of miscoding lesions such as O^6-EtdGuo in brain DNA, and the high rate of DNA replication of neural precursor cells during the transformation-sensitive period of brain development, may thus be factors responsible for the neural tissue-tropism of the carcinogenic effect of EtNU in the rat. However, genetic consequences other than point mutations could equally result from structural modifications persisting in DNA and may be equally or more relevant to malignant transformation (see Introduction).

Considerable efforts have been and are being made to identify and characterize the enzyme(s) responsible for the removal of O^6-alkylguanine and other alkylation products from cellular DNA. There is evidence that 3-alkyladenine and 7-alkylguanine are removed from DNA by glycosylases present in bacteria and in mammalian cells (Laval *et al.*, 1981; Margison and Pegg, 1981; Singer and Brent, 1981; Lindahl, 1982). In *E. coli* an 'adaptive response' was discovered which is inducible by low concentrations of simple methylating agents (Samson and Cairns, 1977; Jeggo *et al.*, 1977). This response involves the expression of a system capable of transferring a methyl group from the O^6 of guanine in DNA to a cysteine residue in an acceptor protein which is thereby inactivated (Karran *et al.*, 1979; Foote *et al.*, 1980; Olson and Lindahl, 1980; Lindahl, 1981). Although it is not entirely excluded that an intermediate methyltransferase may be involved, it seems likely that the alkyl group is bound directly to the acceptor protein (which would then itself have the properties of an O^6-methylguanine DNA transmethylase). Interestingly, similar enzyme activities (with transfer of methyl and ethyl groups from the O^6 of guanine in DNA to cysteine residues in acceptor proteins) were recently described for mouse and rat liver (Bogden *et al.*, 1981; Mehta *et al.*, 1981; Pegg *et al.*, 1983) and for human lymphoid cells (Harris *et al.*, 1983). An active fraction specifically reducing the O^6-alkylguanine content of DNA had previously been isolated from rodent and human liver homogenates (Pegg and Hui, 1978; Pegg and Balog, 1979; Pegg *et al.*, 1982). Evidence was also obtained indicating that the enzyme system reducing the O^6-methylguanine content of rat river DNA has an elevated activity during the S-phase (and possibly the G_2/M-phase) of the cell cycle as compared with the G_1-phase and with the G_0-state (Rabes *et al.*, 1979; Pegg *et al.*, 1981). Moreover, it is now well established that the enzymatic elimination of O^6-alkylguanine from liver DNA is less efficient after high carcinogen doses ('saturation' of the enzyme system, Kleihues and Margison, 1976), and that in different species this saturation effect occurs at different carcinogen dose levels. Thus the liver of the Syrian golden hamster has a

much lower 'saturation threshold' than rat liver (Margison *et al.*, 1976; Stumpf *et al.*, 1979; Montesano, 1982). Correspondingly, hamster liver is sensitive to the induction of hepatocellular cancer by a single dose of, for example, dimethylnitrosamine but rat liver is not (Tomatis and Cefis, 1967; IARC, 1978).

It is not yet clear whether in addition to apparently constitutive O^6-alkylguanine eliminating enzyme(s), there is also an inducible enzyme activity in mammalian systems, analogous to the 'adaptive' DNA methyltransferase in *E. coli*. An elevated elimination capacity was observed in rat liver in response to pretreatment with low doses of dimethylnitrosamine, diethylnitrosamine, 1,2-dimethylhydrazine, *N*-acetylaminofluorene, and 3,3-dimethyl-1-phenyltriazene (reviewed by Montesano, 1982). However, the relatively low increase in elimination activity would also be compatible with an increased fraction of proliferating hepatocytes in S-phase, i.e., a regenerative hyperplasia in response to the carcinogen pretreatment (Rajewsky, 1972).

The important question of cell type- and developmental/differentiation stage-dependent differences in the capacity for enzymatic elimination of critical alkylation products from DNA has not yet been investigated, except for the case of parenchymal versus non-parenchymal rat liver cells after treatment with 1,2-dimethyl-hydrazine, i.e., an agent inducing haemangiosarcomas but not hepatocellular carcinomas in rats (Lewis and Swenberg, 1980). In this case O^6-methylguanine accumulated selectively in the DNA of non-parenchymal cells. This approach may in the future be facilitated considerably by the use of high-affinity monoclonal antibodies in conjunction with sensitive immunostaining techniques. Moreover, these techniques may permit the study of enzymatic repair processes in small amounts of cells, for example, during prenatal stages of development or in bioptic tissue samples. In the EtNU-rat brain system, it may be important to investigate whether during early stages of prenatal development (i.e., prior to day 11–12 of gestation) the enzyme(s) responsible for the elimination of O^6-EtdGuo from neural precursor cells would show an equally low, or a higher activity than during later pre- and postnatal development (Müller and Rajewsky, 1983a).

3 CONCLUSION

The formation of specifically structured, persistent carcinogen–DNA adducts (e.g., O^6-alkylguanine) is likely to be a prerequisite for the initiation of the process of carcinogenesis, and (via mechanisms which remain to be clarified) may lead to, or facilitate, those alterations in the cellular genome that ultimately result in the expression of malignant phenotypes. Therefore, sensitive methods for detection and quantitation of specific carcinogen–DNA adducts in small amounts of cells exposed to non-radioactive (e.g., environmental) carcinogens have considerable relevance for the identification of DNA damage and for the characterization of the repair capacity of, for example, human cells. Enzyme

systems for specific recognition, elimination, and repair of critical DNA lesions appear to be differentially expressed in different cell types, tissues, and species. The capacity of cells to repair carcinogen-modified DNA may thus constitute one of the determinants for the probability of malignant transformation, no less important than the expression of enzymes required for the metabolic activation of many carcinogens to their ultimate reactive forms. The question whether (under conditions of equal initial carcinogen–target cell interaction) the probability of malignant transformation is generally also a function of the type and differentiation state of target cells, requires further careful analysis (identification of subpopulations of cells with an elevated risk of malignant transformation, and with the possible further complication that different 'high-risk' cell populations may be found for different types of carcinogens).

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