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REPAIR OF CYTOTOXIC LESIONS INTRODUCED INTO DNA BY METHYLATING AGENTS

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Summary. - *We have investigated the biological role of O^6-methylguanine and methylphosphotriesters in the DNA of mammalian cells. Our approach has been to express* Escherichia coli (E. coli) *DNA repair activities of well-defined specificity in Chinese hamster cells. Expression of O^6-methylguanine-DNA methyltransferase is necessary and sufficient to confer resistance to the cytotoxic action of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) indicating the potential involvement of O^6-methylguanine in cell killing by this compound. We present evidence that methylphosphotriesters in DNA do not constitute a cytotoxic threat. Despite this, cell lines resistant to MNNG may display elevated expression of a methylphosphotriester repair function. This may be the result of fortuitous co-amplification of transfected sequences and indicates that care should be exercised in correlating resistance to methylating agents with particular DNA repair enzymes.*

Riassunto (Riparazione di lesioni citotossiche introdotte nel DNA da agenti metilanti). - *Abbiamo studiato il ruolo biologico delle lesioni O^6-metilguanina e metilfosfotriesteri indotte nel DNA di cellule di mammifero. Il tipo di approccio che abbiamo seguito consiste nell'esprimere specifiche attività di riparazione del DNA di* Escherichia coli (E. coli) *in cellule di hamster cinese. L'espressione della O^6-metilguanina-DNA metiltransferasi è necessaria e sufficiente per conferire la resistenza all'azione citotossica della N-metil-N'-nitro-N-nitrosoguanidina (MNNG) indicando il potenziale coinvolgimento della O^6-metilguanina nella letalità cellulare indotta da questo composto. I nostri risultati mostrano che i metilfosfotriesteri nel DNA non costituiscono una lesione letale. Tuttavia linee cellulari resistenti alla MNNG possono presentare elevata espressione dell'attività di riparazione dei metilfosfotriesteri. Questo può essere il risultato di co-amplificazione fortuita di sequenze trasfettate e suggerisce estrema cautela nella formulazione di correlazioni tra resistenza ad agenti metilanti e specifici enzimi della riparazione del DNA.*

Introduction

The carcinogenic properties of the alkylnitrosamides N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (MNU) have been known for many years. However, because of their efficiency in arresting cell proliferation, these and closely related compounds have been employed as anti-cancer agents in clinical practice [1]. The dual properties of nitrosamides as mutagens and cytotoxic agents provides an opportunity to study the fundamental mechanisms by which the interaction of simple chemicals with DNA can result on one hand in mutation and cellular transformation and on the other hand in cell death. Furthermore, if these two effects result from different lesions in DNA, a knowledge of the reaction products of each compound along with its potential biological consequences may enable us to design effective chemotherapeutic agents so as to eliminate unwanted side effects such as the induction of secondary malignancies. Since DNA repair reactions will modulate any potential biological effect of lesions in DNA and repair enzymes often have relatively narrow substrate specificities, a detailed knowledge of the abundance and substrate range of such enzymes is necessary. For convenience, studies of DNA repair enzymology are best carried out in bacterial systems and since there are many similarities in the repair strategies utilized by *Escherichia coli* (*E. coli*) and mammalian cells, a considerable degree of extrapolation from this simple model system is possible.

Biological responses to methylation damage

Bacteria

Studies by Lawley *et al.* [2] defined the products of reaction with DNA of compounds such as MNU and MNNG. The sites of methylation have been characterized and have been extensively reviewed recently [3]. In

terms of biological effect, the methylated purines: 7-methylguanine (7-MeGua), 3-methyladenine (3-MeAde), 3-methylguanine (3-MeGua) and O^6-methylguanine (O^6-MeGua) and the relatively minor methylpyrimidines (MePyr): O^2-methylthymine (O^2-MeThy), O^2-methylcytosine (O^2-MeCyt) and O^4-methylthymine (O^4-MeThy) require consideration. In addition, the products of methylation of the phosphodiester backbone of DNA-methylphosphotriesters play a specialized role in the biological response to methylation damage.

7-MeGua is included in the list as it represents quantitatively the major methylation product and cells may remove it from their DNA by a DNA glycosylase. In general, however, removal is slow and since no biologically important consequences may result from unrepaired 7-MeGua in DNA, its excision may be fortuitous and result from features of structural similarity to the more important 3-methylated purines.

Considerable evidence implicates the 3-methylpurines in the cytotoxicity of alkylating agents. *E. coli* rapidly remove 3-MeAde from their DNA [4]. This removal is brought about by a specific DNA glycosylase (3-Me Ade-DNA glycosylase-1) which is the product of the *tag*$^+$ gene. The Tag protein is expressed constitutively and *tag* mutants are hypersensitive to the lethal effects of methylating agents [5]. The importance of this methylated product is emphasized by the existence in *E. coli* of a second DNA glycosylase (3-MeAde-DNA glycosylase II). This enzyme, which is inducible, is encoded by the *alkA*$^+$ [6] gene and exhibits a broader substrate specificity than its constitutive counterpart and additionally recognizes and excises 3-MeGua and both the O^2-methylpyrimidines (O^2-MePyr) from DNA [7, 8]. Its induction is directed by the *ada*$^+$ gene of *E. coli* which carries out a central function in control of the adaptive response to alkylation damage in this organism. Following induction of the adaptive response in *E. coli*, the mutagenic and cytotoxic consequences of DNA methylation are reduced [9]. The production of large amounts of 3-Me Ade-DNA glycosylase II is largely responsible for the resistance to cell killing observed in induced cells. While repair of the minor methylation products 3-Me Gua and the O^2-MePyr may contribute to enhanced resistance, the complementation of *alkA* mutants by overproduction of the Tag protein indicates that 3-MeAde is the major cytotoxic lesion in these cells [10]. 3-MeAde exerts its cytotoxic effect by blocking DNA replication. Direct demonstration of this has come from studies of DNA synthesis *in vitro*. Synthesis on poly(dAT) templates is reduced following introduction of 3-MeAde residues by dimethylsulfate treatment and SOS functions - normally considered to be induced as a consequence of stalled DNA replication forks - are induced by lower concentrations of methylating agents in *E. coli* which are deficient in 3-MeAde-DNA glycosylases I and II [11]. Further evidence has been adduced by Larson *et al.* who directly observed termination of DNA synthesis *in vitro* at sites of adenine (but not guanine) methylation [12]. The cytotoxicity of methylating agents in *E. coli* is apparently largely a consequence of the abrupt termination of DNA replication at unrepaired 3-MeAde residues in DNA.

The remaining biologically important methylated bases O^6-MeGua and O^4-MeThy have been implicated in mutagenesis by methylating agents. In the former case, ample evidence exists from *in vitro* determinations of miscoding frequency and from analysis of mutational spectra *in vivo* to support the contention - originally proposed by Loveless [13] - that O^6-MeGua in DNA causes G→A transition mutations by virtue of its ability to pair with thymine (Fig. 1). By analogy, the quantitatively more minor O^4-MeThy, may also contribute to mutagenesis by its ability to miscode and direct the incorporation of guanine (Fig. 1). The repair of both these mutagenic bases is again partially constitutive and partially carried out by the inducible adaptive response. *E. coli* constitutively express low levels of two enzymes which recognise and remove O^6-MeGua from DNA by the now well-characterized suicidal methyltransfer reaction. One of these enzymes, discovered fairly recently, is a product of the *ogt*$^+$ locus. It is a small (19 kDa) enzyme which removes methyl groups from O^6-

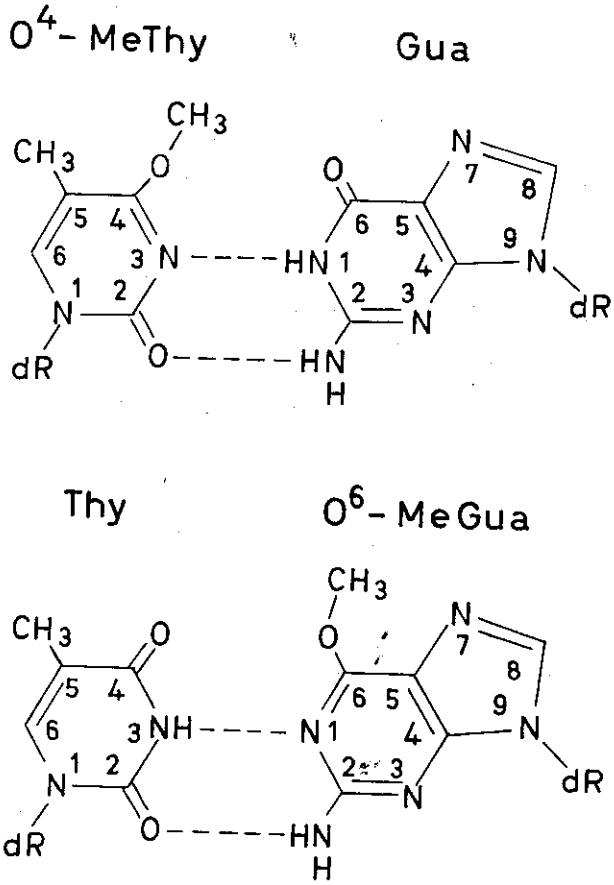

Fig. 1. - Potential mispairing by alkylated bases.

MeGua, but does not recognise either O^4-MeThy or methylphosphotriesters in DNA [14]. These latter properties confirm the separate nature of the Ogt and Ada proteins which is indicated by the presence of the former in *E. coli* mutants in which the *ada*$^+$ gene is deleted [15].

The Ada protein occupies a central role in repair of methylation damage in *E. coli*. Its properties have recently been reviewed extensively [9]. Briefly, it is a DNA repair enzyme (O^6-MeGua-DNA methyltransferase) which acts in methyl transfer reactions involving O^6-MeGua and O^4-MeThy in DNA. The active site and acceptor residue for methyl groups transferred from these two bases is a cysteine at position 321 of the protein. A second acceptor cysteine is found at position 69. In this case, the substrate for transfer is a methylphosphotriester in DNA (specifically an Sp stereoisomer). Acceptance of a methyl group by cys 69 converts the Ada protein into a transcriptional activator of the *ada*$^+$, *alk*A$^+$ and other genes involved in the adaptive response. The high levels of the Ada protein which are induced protect the cell against the mutagenic bases O^6-MeGua (and O^4-MeThy).

Mammalian cells

Defence against alkylation damage in *E. coli* thus comprises a limited constitutive surveillance to provide protection against low levels of alkylation allied to a rapidly inducible high capacity repair system to counteract occasional acute exposure to high concentrations of alkylating agents. Both constitutive and inducible responses include components which provide protection against either the potentially mutagenic (O^6-MeGua, O^4-MeThy) or cytotoxic, replication blocking lesions (3-MeAde, 3-MeGua, O^2-MePyr). The switch between the two systems is triggered by automethylation of cys 69 in the Ada protein consequent on repair of Sp stereoisomers of methylphosphotriesters. Mammalian cells, which are normally protected from violent fluctuations in their external milieu rely on constitutively expressed enzymes for protection, and with the possible exception of hepatocytes [16], do not augment their repair capacity when exposed to methylating agents. All normal mammalian cells so far examined express a broad specificity DNA glycosylase which recognises 7-MeGua, 3-MeAde and 3-MeGua and an O^6-MeGua-DNA methyltransferase which is specific for this methylated base (the O-methylpyrimidines are not apparently efficiently repaired) [17].

At first sight, it appears likely that these two enzymes duplicate the functions performed in *E. coli* by the *tag*$^+$ and *ogt*$^+$ genes respectively; the DNA glycosylase providing protection against replication-blocking 3-methylpurines and the methyltransferase removing potentially mutagenic O^6-MeGua. However, there is now considerable evidence that O^6-MeGua in DNA in addition to its promutagenic properties is a potentially cytotoxic lesion in mammalian cells.

There are no well-characterized mutants of mammalian cells defective in single DNA methylation repair steps. As a result, the precise biological effects of 3-Me Ade in the DNA of these cells are unknown. Although we presume that such DNA lesions are cytotoxic for mammalian cells, and correlations exist between the ability to introduce 3-MeAde into DNA and cytotoxic potential [18], until mutant cell lines which are unable to repair this lesion are available, the correlation must remain circumstantial. There are, however, cell lines (for example, the hamster lines CHO, V79 etc.) which do not express the methyltransferase at significant levels. Also, although methyltransferase is present in human cell lines derived from normal adult tissue, expression may be lost in cell lines transformed *in vitro* by viruses. In addition, the enzyme is absent from a substantial percentage of cell lines derived from biopsies of human tumors [19]. Human cell lines with very low or absent methyltransferase levels are designated Mer$^-$ or Mex$^-$ [19, 20]. The levels of the methylpurine-DNA glycosylase in Mex$^-$ cells are indistinguishable from those of Mex$^+$ cells [21]. Despite this, Mex$^-$ cells are very sensitive to killing by methylating agents. The sensitivity reflects a DNA repair defect since overall methylation of DNA is similar in Mex$^+$ and Mex$^-$ cells and Mex$^-$ cells also fail to reactivate methylated adenovirus while supporting the growth of unmethylated virus in a normal fashion [19]. One of the most productive approaches to defining the repair defect in Mex$^-$ cells has been to express a bacterial methyltransferase in Mex$^-$ cells, in an attempt to complement their sensitivity to alkylating agents. Similar experiments have been performed in several laboratories [22-25] and despite the fact that different cell lines were used as recipients for different recombinant DNA constructs, the data produced are in good agreement. We summarise below results from our own laboratory.

The cloned bacterial *ada*$^+$ gene when placed under the control of the SV40 early promoter can be expressed in mammalian cells. Resistance to both cell killing and mutation induction by MNNG is conferred on CHO cells by the expression, in the cell line CHOCNU3, of the Ada protein at levels which are comparable to those found in Mex$^+$ human cell lines [22]. The degree of resistance observed is shown in schematic form in Fig. 2 (top). Both the O^6-MeGua and the methylphosphotriester repair functions are expressed in these cells and protection might be a consequence of either. However, expression in the cell line CHO623, of a truncated version of the Ada protein in which the methyl group acceptor for O^6-MeGua repair is deleted confers little protection except at high levels of cytotoxicity (Fig. 2, top). These observations indicate that unrepaired methylphosphotriesters contribute little to the cytotoxicity of alkylating agents and imply that unrepaired O^6-MeGua in DNA exerts a cytotoxic effect in mammalian cells. These implications have been further tested in two ways. Firstly, CHO cells have been transfected with a plasmid from

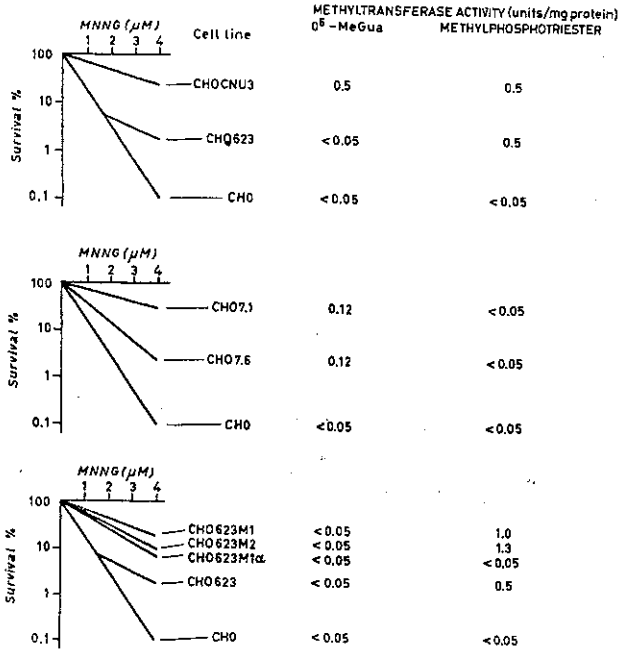

Fig. 2. - CHO cells were transfected with plasmids containing all or part of the *E. coli ada*+ gene. Cell lines which expressed the activties shown were isolated, and treated with MNNG. Cell survival and enzyme levels were determined as described in [22].

which the DNA sequences encoding the N-terminal region of the Ada protein, including the methyl group acceptor for methylphosphotriester repair, have been deleted. Two cell lines (CHO 7.1 and CHO 7.6) which express only the O6-MeGua-DNA methyltransferase function have been isolated. Both cell lines express this enzyme activity at levels comparable to some Mex+ cell lines (0.12 unit/mg protein). Expression of the enzyme in these cells is sufficient to increase their resistance to killing by MNNG (Fig. 2, middle). However, despite the similarity of enzyme activity as measured in cell-free extracts, the degree of protection afforded is markedly different for the two cell lines. Further analysis of the molecular nature of the methyltransferase produced by these two cell lines indicate that the more resistant line (CHO 7.1) expresses an active polypeptide of the length predicted from the transfected *ada*+ sequences along with some shorter active fragments. In contrast, in CHO 7.6 only a short (< 19 kDa) species of active methyltransferase is detectable. Southern blot analysis indicates that the *ada*+ sequences in CHO 7.6 have a small deletion at their 5' end (Hall *et al.*, in press).

In a second series of experiments, the nature of the resistance to MNNG observed at high drug concentrations in cell lines expressing the methylphosphotriester repair function has been examined. Two MNNG-resistant derivatives of the cell line which expresses this activity (CHO623) have been isolated. These two resistant cell lines (designated CHO623M1 and CHO623M2) exhibit significantly enhanced resistance to killing by MNNG at all MNNG concentrations tested (Fig. 2, lower). Cell-free extracts of both cell lines contain elevated (2-3 fold) activity of methylphosphotriester methyltransferase. However, in this case, the apparent correlation between MNNG-resistance and enzyme activity is fortuitous. When CHO623M1 and CHO623M2 cells are maintained in conditions which favour loss of expression of transfected sequences, variants arise (CHO623M1α, CHO623 M2α) which no longer exhibit the high levels of methylphosphotriester repair protein. Both CHO623M1α and CHO623M2α retain the enhanced MNNG-resistance of CHO623M1 and CHO623M2 (e.g., Fig. 2, lower).

Discussion

The complementation of the Mex- phenotype by a transfected *ada*+ gene or truncated variants has been demonstrated in a number of cell lines. Good agreement exists that the ability to repair O6-methylguanine in DNA largely complements the hypersensitivity of Mex- cell lines to killing by MNNG. This indicates that unrepaired O6-MeGua in the DNA of a mammalian cell has a high probability of being lethal, although two caveats are indicated by the work presented here. Firstly, in as far as they both express the same level of methyltransferase in cell free extracts, the correlation between O6-MeGua-DNA methyltransferase levels and increased survival is only qualitative for the two cell lines CHO7.1 and CHO7.6. Whilst this may reflect the altered nature of the Ada protein fragment expressed in CHO7.6 which renders it more susceptible to proteolysis, it is noteworthy that for rodent cells in general, there exists only a qualitative correlation between O6-MeGua-DNA methyltransferase expression and cellular resistance to MNNG [27]. Secondly, our data with CHO623 and its derivatives indicate that the use of MNNG to select for resistant variants from cells expressing transfected DNA repair genes may yield cell lines in which increased resistance and higher levels of the DNA repair activity are observed but not correlated in a causal fashion. It seems probable in this case that two selective mechanisms are operating; the first is a selection for MNNG-resistance and the second a possible selective advantage conferred by amplification of the selectable marker sequences (in this case, *gpt*+) which results in coamplification of associated *ada*+ sequences. The selection for MNNG-resistance without any increase in levels of known DNA repair enzymes is now a common observation and indicates that mechanisms other than demethylation exist to circumvent the cytotoxic potential of O6-MeGua in DNA. These mechanisms which can loosely be described as "tolerance" probably involve events during DNA replication.

The basis of the cytotoxic action of this methylated base in DNA is unknown. The scheme for mutagenesis proposed by Loveless has been amply supported by anal-

ysis of mutational spectra and incorporation studies using purified DNA polymerases *in vitro* have indicated that T is indeed the preferentially incorporated base. However, more recent data on synthetic oligonucleotides indicates that the simple base pairing scheme (Fig. 1) for O^6-MeGua:Thy base pairs is an oversimplification and that the presence of O^6-MeGua in duplex DNA destabilizes the helix. The degree of destabilization is greater than a single Gua:Thy or Ade:Cyt mismatch ([28] and M. Hill-Perkins, M. Jones, P. Karran unpublished data). In fact, the order of destabilization is O^6-MeGua: Cyt<Ade<Gua<Thy. These data have recently been comprehensively reviewed by Basu and Essigmann [29] who suggest that the preferential production of O^6-MeGua-Thy base pairs during replication may reflect the minimal distortion of the phosphodiester backbone induced by the O^6-MeGua:Thy base pair. Interestingly, a similar destabilization is observed for the O^4-MeThy-Gua base pair which is the preferred product of replication of O^4-MeThy containing templates. Again, it is noteworthy that minimal distortion of the phosphodiester backbone is introduced by this base pair.

Taking account of these considerations, it seems possible that the cytotoxic action of O^6-MeGua in DNA may result from a localized instability in the DNA duplex immediately following its replication. The poorly base paired/stacked region may be subject to attack by cellular nucleases. One possible result of such attack would be to create the transient single-strand breaks in DNA which are necessary to initiate the formation of sister chromatid exchanges. Mex$^-$ human and Chinese hamster cells are sensitive to the induction of SCEs by MNNG and this sensitivity can be abolished in both cell types by expression of O^6-MeGua methyltransferase function of the Ada protein [30, 31] or a mammalian counterpart [32].

Many of the outstanding questions regarding the control of cellular sensitivity will be more easily addressed as probes for the expression of the genes encoding the important DNA repair enzymes of mammalian cells, particularly the 3-MeAde-DNA glycosylase and the O^6-MeGua-DNA methyltransferase, are becoming available. Attempts to clone these genes are under way in a number of laboratories and results should be forthcoming in the near future.

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