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Enzymology Of Repair Of DNA Adducts Produced By N-Nitroso Compounds

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DISCLAIMER

SUMMARY

The biological effects of DNA adducts depend on their nature, and on their half-lives relative to the rates of DNA replication and transcription. Their half-lives are determined by the rates of spontaneous decay, such as depurination, and the rates of enzymatic repair of the adducts or their decay products. The principle modes of repair of methylating and ethylating agents are by glycosylase catalyzed depurination of 7-alkylguanine and 3-alkyladenine and by the dealkalation of O^6-alkylguanine. The latter repair is accomplished by the transfer of the alkyl group to cysteine residues of acceptor proteins in a stoichiometric reaction. Repair by dealkylation cannot be detected by the standard methods used to measure DNA repair, but it is easy to estimate the acceptor activity in cell extracts by measuring the transfer of radioactive O^6-alkyl groups in an exogenous DNA to protein. In extracts of cells treated with alkylating agents the activity is depressed because the endogenous DNA is rapidly dealkylated, using up the acceptor activity. In many cell types the decrease activity is followed by an increase to the normal constitutive level. In other cells there is no such adaptive response. We may catalogue the cell strains and lines investigated into three classes: a) high constitutive activity (30,000 - 100,000 acceptor sites per cell) and a rapid adaptive response (several hours), b) high constitutive activity and a very slow adaptive response, c) low constitutive activity. The cytotoxicity of methylating agents is highest for the last and lowest for the first class. Differences in constitutive levels of methyl accepting activity in

extracts of human lymphocytes and on the acceptor activity in lung macrophages from smokers (low activity) and non-smokers (high activity) have been observed.

INTRODUCTION

Direct acting alkylating agents react with macromolecules in particular DNA and produce a variety of products (Singer & Kusmierek, 1982). The distributions of products among the various bases of DNA are similar to those produced by agents which need enzymatic activation to alkylating species, such as nitrosamines and nitrosomethyl-N'-nitroguanidine (NMNG) and nitrosoethyl-N'-nitroguanidine which are activated by thiols. The biological effects of alkylating agents --cytotoxicity, mutagenicity, and presumably the initiation steps in carcinogenesis--depend on many aspects of the agents' pharmacokinetics relative to the rates of replication and transcription of DNA (Hoel et al., 1983). An additional important kinetic parameter is the lifetime of the adducts in DNA. A short half-life resulting from chemical instability or enzymatic repair could tend to render a particular adduct relatively innocuous.

Because of the enzymatic or chemical activation steps needed to convert many compounds to alkylating agents the external concentration in μM or mg/kg of body weight may be greatly different from the internal dosimetry in cells or tissues. The difference between internal and external dosimetry is certainly the case for nitrosamines which are activated primarily in the liver (Pegg & Perry, 1981a) and is even true for NMNG acting on cells in culture (see below).

There are a number of pathways for repairing alkylation damage to DNA. One might wonder, from an evolutionary point of view, why such pathways exist. A possible explanation lies in the observations that there are endogenous reactions that can alkylate DNA. For example, S-adenosylmethionine is able to alkylate DNA in vitro (Barrows & Magee, 1982; Rydberg & Lindahl, 1982; Naeslund et al., 1983) and the repair of damage from this essential cellular compound may be the reason for the repair of alkylation damage in cells.

MODELS OF ALKYLATION REPAIR

Figure 1 diagrams various repair possibilities for alkylation damage (Lindahl, 1982; Setlow, 1982). Pathway (a) is the simplest to understand in that it represents the removal of an alkyl group from the DNA without affecting the DNA backbone. This is the major pathway for the repair of m^6G or e^6G. There is no unscheduled DNA synthesis or strand breakage associated with this repair system. Pathways (b) and (c) involve the action of glycosylases which remove altered purines from the polynucleotide. Subsequent steps and repair are aimed at replacing the removed purines by the proper ones. In scheme (b) a new purine is inserted directly into the polynucleotide. Such a pathway would not show unscheduled DNA synthesis measured by ^3H-thymidine incorporation or any strand breaks unless the breaks are measured in alkali which labilizes the backbone of the chain at an apurinic site. The insertase mechanism of repairing an apurinic site has been questioned as a major pathway of repair in bacteria (Kataoka & Sekiguchi,

1982) and in mammalian cells in culture (Wintersberger, 1982). Pathway (c) involves an apurinic endonuclease following the glycosylase and results in a strand break and further incorporation to repair any nucleotides removed during this phase of the enzymatic action. Hence, such repair will give rise to unscheduled DNA synthesis and to the appearance of single strand breaks in DNA and their disappearance as repair is completed.

Which Lesions are Important

The determination of the biological effects of particular DNA adducts is a real problem (Setlow, 1980). It cannot be done from an analysis of the shape of dose response curves since the ratios of the yields of various products usually do not change with dose. The solution to the problem necessitates, among other things, knowing how various adducts change the template activity of polynucleotides (Singer & Kusmierek, 1982; Miyaki et al., 1983) as well as the persistance of the products. For example, the product e^6G arising from the treatment of animals with nitrosoethylurea (NEU) is known to code for thymine as well as for cytosine. It is removed rapidly from the liver of neonatal rats but persists in brain for long periods of time. Hence, this product of NEU is implicated in brain carcinogenesis (Goth & Rajewsky, 1977; Muller & Rajewsky, 1983). However, such an argument is not foolproof because a number of other minor O-ethyl products are also removed almost as effectively as is e^6G (Singer et al., 1981). Nevertheless, products such as e^6G that are biologically important (but not necessarily the only important ones) are repaired rapidly in many tissues (see below). High doses of NEU

can saturate this repair pathway and would be expected to result in dose response curves that have an apparent threshold. Indeed, this is what is observed for specific locus mutations in mice injected with NEU (Russell et al., 1982 a, b).

Two other techniques are used in attempts to identify particular products with biological effects. In one (Connell & Medcalf, 1982) the effects of alkylating agents such as nitrosomethylurea and dimethylsulfate are compared, at equitoxic doses in terms of sister chromatid exchanges (SCE). At such doses equal levels of SCE are observed. This means that m^6C cannot be correlated with SCE production since the two alkylating agents produce completely different levels of this product and no specific alkylation product can be identified as accountable for the formation of SCE. A second approach is to grow cells in culture medium containing DNA precursors, such as m^6G. Kaina et al., (1983) grew Chinese hamster V-79 cells in medium containing m^6G, m^3A, or m^7G and looked for mutations, SCE, or chromosome aberrations. They observed appreciable numbers of mutations and SCE following growth in m^6G medium but not in cells grown in m^3A or m^7G. Such observations are not necessarily at variance with those of Connell and Medcalf (1982) because the amounts of m^6G in the latter experiments were probably much less than in the incorporation ones and, although m^6G in DNA seems able to result in the formation of SCE, the amount of m^6G in DNA of cells treated with alkylating agents maybe too low to increase significantly the background level of SCE.

Repair of m^6G in DNA

We have outlined above several reasons to suppose that m^6G is important in mutagenesis and carcinogenesis, although the cytotoxic effects of methylating agents on bacteria are more associated with m^3A and m^3G (Evensen & Seeberg, 1982; Karran et al; 1982). However, the ability of cells to remove m^6G is strongly correlated with high survival of cells after NMNG treatment and with the ability of cells to reactivate adenovirus 5 treated with NMNG (Day et al., 1980). The latter cells are called Mer^+. Those cells unable to reactivate treated virus are designated as Mer^-. Lymphoblastoid cells resistant to NMNG are called Mex^+ and those that are sensitive are called Mex^- (Sklar & Strauss, 1981).

The repair of m^6G or e^6G falls into the special category diagramed in Fig. 1 in which repair is accomplished by the simple removal of the alkyl group. In bacteria (Olsson & Lindahl, 1980; Sedgwick & Lindahl, 1982) rodent (Bogden et al., 1981; Mehta et al., 1981) and human cells (Waldstein et al., 1982a; Pegg et al., 1982) the removal is accompanied by the transfer to a cysteine group of an acceptor protein. The reaction is a stoichiometric one and is a suicide reaction in that the acceptor protein is no longer available to accept additional alkyl groups. Measurements of characteristics of repair by treatment of mammalian cells with radioactive alkylating agents cannot be done at high concentrations since the acceptor protein would rapidly be used up. As a result, large numbers of cells must be used at low concentrations of alkylating agents to obtain sufficient m^6G or e^6G for analysis.

Many fewer cells are needed if the acceptor activity is assayed in cell extracts or in partially purified extracts, because the activity of the extract may be estimated in terms of its ability to remove m^6G from an exogenous DNA or polynucleotide.

Table 1 outlines three different assays we have used. In the first method the loss of m^6G from the exogenous DNA is determined by high pressure liquid chromatography of the depurinated DNA. In the second method the amount of [^{3}H]methyl transferred to protein is determined by digesting the protein with proteases and measuring the radioactivity liberated into the acid soluble fraction of the reaction mixture (Waldstein, et al., 1982b). In the third method the amount of ^3H transferred to protein is determined after depurination of the DNA by measuring the amount of radioactivity in an acid precipitate (Delihas & Setlow, unpublished). The three assays give similar results. However, the third assay is the most convenient to use since it measures the transfer of small amounts of radioactivty to protein directly, and is not affected by nucleases in some tissue or cell preparations that may act during the long protease digestion used in the second method. Moreover, it does not require the use of an exogenous substrate depleted of methylated purines other than m^6G.

The assays described above had been used to estimate the activity in human peripheral lymphocytes (Waldstein et al., 1982c). The measurements indicate variation of several fold in m^6G acceptor activity among humans. The variation is not sex or age dependent. There are at present no good data

indicating that the magnitude of the variation observed in lymphocytes would be similar to that observed in other tissues, but, a reasonably large variation has been reported in the acceptor activity obtained from human livers (Pegg et al., 1982). It is an attractive hypothesis that the variation in repair activity could be associated with an individual's susceptibility to mutagenesis or carcinogenesis by simple alkylating agents. Such an hypothesis derives most of its support from the big difference in sunlight induced cancer in xeroderma pigmentosum individuals compared to normal individuals. The prevalence of skin cancer in XP patients is approximately 10^4 fold greater than in the average population (Setlow, 1982). But the average repair defect is only approximately 80%. Hence, in the case of sunlight induced cancer a decrease in repair activitity by a factor of 5 results in an increase in cancer susceptibitliy of 10^4, indicating that relatively small differences in repair could have large effects on the sensitivity of individuals to exogenous or endogenous carcinogenic agents (Setlow, 1983).

Recent measurements on the acceptor activity in lung macrophages from smokers and non-smokers indicate that the cells from non-smokers have high activities, approximately 80 fmol removed per 200 µg of protein, whereas the macrophages from smokers have an activity between 0 and 20 fmol per 200 µg of protein (Cao, Setlow and Janoff, unpublished observations). Mixing experiments indicate that the low activity in the cells from smokers lungs is not the result of a diffusable inhibitor in such cells. There are no data

indicating whether these results on lung macrophages can be extrapolated to lung tissue itself.

The level of m^6G repair activity in human cells in culture has been used to explain differences in the cytotoxic effects of NMNG on them or its killing effects on viruses used to infect them (Day et al., 1980). However, before discussing such results in more detail it is useful to digress and consider two other aspects of the effects of alkylating agents on tissues and on cells in culture. The first is the relationship between internal and external dosimetry and the second is the ability of cells or tissue to adapt to low levels of alkylating agents

Internal vs. External Dosimetry

External dosimetry in µg per kg or in concentration in culture medium is a useful start to dosimetry but it is not sufficient because of the many metabolic steps often needed before alkylation takes place. The best measure is one in which the numbers of the different alkylation products per cell are estimated directly by use of radioactive alkylating agents. Such procedures are used extensively in work with animals or with large numbers of cells in culture. A second convenient way to convert from external to relative internal dosimetry is the determination of the number of phosphotriesters per unit length of DNA by the lability of the triesters in alkali (Snyder Regan, 1981; Abbondandolo, et al., 1982) Such a technique workes well for cells in culture and indicates that even for such a simple situation the ratio of internal to external dosimetry of NMNG varies among cell lines (Table 2).

The differences in internal dosimetry presumably arises because of different sulfhydryl levels in the different cells. A knowledge of the numbers of triesters and the distribution of the products resulting from particular alkylating agents (Singer & Kusmierek, 1982) permits one to estimate the numbers of particular alkylation products per cell.

Adaptation.

In a number of instances biological systems exposed to low chronic doses of alkylating agents are able to resist exposure to a single high challenge dose. This phenomenon called adaptation has been observed in systems as diverse as rat liver and E. coli. In the latter case, cells exposed to low doses of NMNG are resistant to the mutagenic effects of subsequent high challenge doses (Cairns, 1980). The cells were shown to adapt during the chronic dose by making appreciable amounts of new acceptor protein for m^6G that protected them against the challenge dose. (Robins & Cairns, 1979). Adaptation is also observed for the cytotoxic effects of nitrosomethylguanidine on E. coli but this adaptation is associated with an increase of glycosylases able to repair m^3A and m^3G (Evensen & Seeberg, 1982; Karran et al., 1982). Thus, in bacteria adapatation has several facets.

The ability of rat liver to remove m^6G from cells also shows an adaptive response following chronic exposure of animals to NDMA (Montesano et al., 1980; Margison, 1982). Since such a response is observed to follow treatment with agents such as aflatoxin that kill liver cells or following partial hepatectomy, it is possible that adaptation in rat liver is associated with cell division in the damaged liver rather than with the increase in acceptor

molecules per cell (Chu et al., 1981; Pegg & Perry, 1981b). However, a kinetic analysis of the appearance of the enhanced m^6G acceptor in rats treated with NDMA makes the above explanation improbable (Margison, 1982). If the latter point of view is correct adapation in rat liver is similar to that in bacteria and involves synthesis of new protein in the affected cells. The trigger for the adaptation is not well understood. In any event, the adaptive phenomenon cannot be generalized to species other than rat since it is not observed in mice (Maru et al., 1982), hamster (Smith & Margison, 1981) or gerbils (Bamborschke et al., 1983).

Some mammalian cell strains or lines in culture also can adapt to low doses of NMNG and show fewer sister chromatid exchanges (Samson & Schwartz, 1980). Chinese hamster cells show lower amounts of mutation and higher survival after a subsequent challenge dose than do cells not exposed to the initial small doses (Kaina, 1982; Laval & Michel, 1983).

Adaptation in terms of the appearance of new m^6G acceptor activity is also observed in a number of human cell strains (Waldstein et al., 1982d). To appreciate how such experiments are done using any one of the assays outlined in Table 1, one must remember that the reaction of the acceptor protein with m^6G is very rapid (Shiloh & Becker, 1981; Sklar et al., 1981; Waldstein et al., 1982) and, as a result, treatment of cells with NMNG will lead to a depletion of the acceptor activity in extracts prepared after treatment. The depletion of acceptor activity is also observed if extracts of cells are treated with NMNG. In this case, the depletion is the result of the

action of the alkylating agent on DNA and not on the acceptor protein itself, because digestion of the majority of the DNA by nucleases renders the acceptor activity in extracts relatively insensitive to treatment with NMNG (Delihas & Setlow, unpublished). The depletion of activity occurs rapidly after treatment with an alkylating agent and in some cell strains or lines the activity returns to the normal constitutive level in a few hours. The return to normal is inhibited by cycloheximide indicating that new protein must be synthesized for such as adaptative response.

EFFECTS ON CELLS IN CULTURE

The cytotoxic effects on human cells of externally applied of NMNG depend on a number of parameters including the internal dosimetry, the constitutive levels of repair activity, and the ability to adapt to single doses of the chemical by resynthesis of the m^6G acceptor activity of from its depleted to its normal levels. The roles of these relative factors are well illustrated by different HeLa cell lines (Table 4). The lines fall into three distinct groups, among which there is no clear correlation between differences in external and internal dosimetry. However, there is a strong correlation between cytotoxicity and the constitutive level of repair for m^6G and its adaptive response. The most resistant group is made up of cell strains with high constitutive levels and a rapid ability to adapt. A somewhat more sensitive group, HeLa S3, is made up of cells with a normal level of constitutive activity, but a negligible ability to adapt. The third group, HeLa MR is extraordinarily sensitive to the cytotoxic effects of NMNG and has

a negligible amount of constitutive activity. The magnitude of the cytotoxic effects among these three groups go in inverse proportion to the numbers of m^6G that can be repaired within several hours.

CONCLUSIONS

The cytotoxic, mutagenic, and presumed carcinogenic effects of alkylating agents are dependent to a large extent on the ability to repair alkylation products in treated cells or tissues. The magnitude of repair depends markedly on two parameters -- the constitutive level of repair activities and the ability to adapt to low levels of alkylating agents. Cells with high constitutive activity and high adaptibility are the most resistant. Most of the analyses that have been carried out emphasize the role of m^6G because it seems to be one of the more important lesions resulting from treatment with methylating agents. Other O^6-alkyl products are also important but have not been analyzed as carefully. There are wide differences in repair activity among different cells from the same animal and among similar tissues from different species. In some instances it has been possible to correlate carcinogenic susceptibility to alkylating agents with a low level of repair. The wide differences in repairability for m^6G observed among humans have not been correlated with susceptibility to disease resulting from endogenous or exogenous alkylating agents but there are good experimental grounds for supposing that more rapid repair is to be preferred over slow or absent repair. It will take an elaborate prospective study to determine whether the

variances in alkylation repair among humans have any relation to susceptibility to disease.

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Table 1. Comparison of Three Assay[a] for the Repair of m^6G in DNA

Tissue[b]	fmol removed/200 µg protein		
	Method 1	Method 2	Method 3
A	384	390	408
	379	383	428
B	34	36	53
	32	32	64

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[a] Sonicated extracts (Waldstein et al., 1982c) containing 200 µg protein were incubated for 1 hr at 37°C with DNA containing O^6[^{3}H-methyl]guanine (~600 fmol). The repair of m^6G was measured in duplicate assays by 3 methods. 1) the DNA was depurinated and the loss in m^6G measured by HPLC. 2) The protein in the extract was degraded by protease (Waldstein et al., 1982b), and the ^3H in the acid soluble fraction was measured. 3) The DNA was depurinated and the ^3H in the acid insoluble fraction (protein) was measured (Delihas & Setlow, unpublished).

[b] A: a frozen baboon liver; B: a frozen human liver. These tissues, obtained from R. Cutler of the National Institute on Aging, were used as examples of high and low activity samples and are not necessarily representative of baboons or humans.

Table 2. Comparison of the Effects of NMNG on 5 HeLa Cell Lines

Line[a]	Single strand breaks per 10^8dalton	D_{10}[c]	Constitutive Activity[d]	Resynthesis time(1/2)[e]
229 (ATCC)	10.0	9.4	240	200
2 (ATCC)	15.2	9.7	190	200
2 (BNL)	5.0	9.7	180	75
S3 (ATCC)	5.6	6.0	210	>200
S3 (BNL)	5.0	6.0	400	>200
MR (Day)	10.9	0.12	0	

[a] ATCC: American Type Culture Collection; BNL: Brookhaven National Laboratory; Day: R.S. Day III.

[b] Cells treated with 6 μM NMNG in serum containing medium. Breaks determined by the method of Abbondandolo et al. (1982).

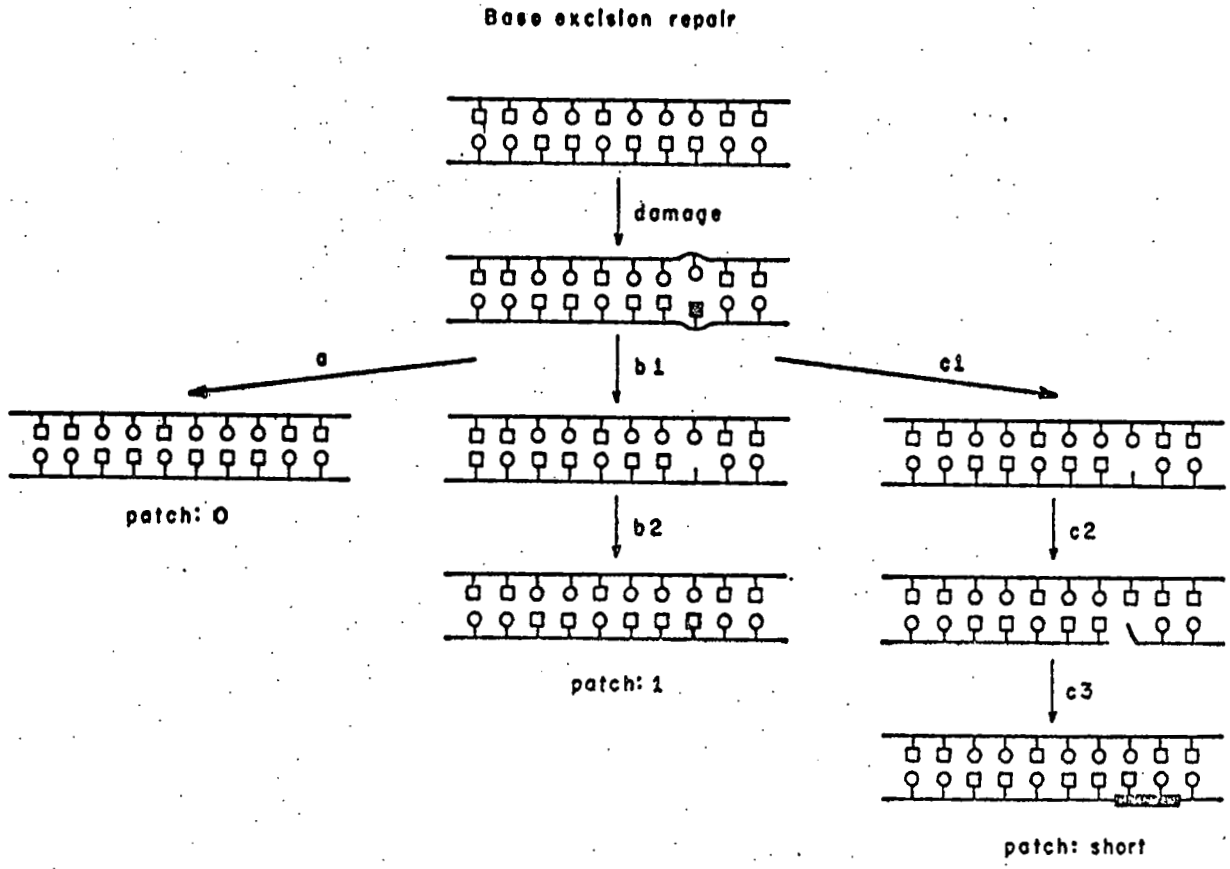
[c] The concentration in μM to give 10% colony forming survival.

[d] Activity in fmol of m^6G repaired per 200 μg protein.

[e] Cells were treated for 5 min with 0.5-1.5 μM NMNG. The time in min for the activity to return half-way to the constitutive level was estimated. >200 means no detectable increase in 3 hr (Waldstein et al. 1982e).

FIGURE LEGEND

FIGURE 1 - A schematic picture illustrating various possibilities for base excision repair. Circles represent pyrimidines and the squares purines. Solid square is an altered purine (perhaps an alkylated one). Solid line in pathway c represents repair replication or UDS.


Base excision repair
damage
a
b1
c1
patch: 0
b2
c2
patch: 1
c3
patch: short

Setlow Fig. 1