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Evidence for removal at different rates of O-ethyl pyrimidines and ethylphosphotriesters in two human fibroblast cell lines

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ABSTRACT

The potent carcinogen, ethylnitrosourea, has been shown to ethylate oxygens, in preference to nitrogens, in the DNA of cultured cells. We have now studied the removal of seven ethyl derivatives in replicating cells. The following findings are reported. 1) The absolute amounts of O^2-EtT, O^4-EtT and O^2-EtC are decreased in cellular DNA after correction for cell growth. However the rate of decrease diminishes after approximately 20 hr and after more than two cell doublings 20-40% of each derivative persists. This decrease is presumed to be due to enzymes since these derivatives are stable in isolated DNA. 2) The amount of ethyl phosphotriesters remains almost unchanged during 72 hr of cell culture. 3) The unstable purine derivatives, 7-EtG and 3-EtA, are both removed from cellular DNA with a rate faster than can be accounted for by the lability of the glycosyl bond. 4) Both GM 637 fibroblasts and Xeroderma pigmentosum fibroblasts (12-RO) (XP-12) have similar ability to remove ethyl products, except for O^6-ethyl G which persists to a greater extent in XP-12 cells. 5) The implications of the in vivo persistence of ethylated bases is discussed in regard to recent demonstrations that O^2-EtT, O^4-EtT, O^2-EtC and O^6-EtG are all mutagenic.

INTRODUCTION

Recently it was shown that treatment of cultured human fibroblasts and fetal rat brain cells with the carcinogen, ethylnitrosourea, alkylated the DNA primarily on oxygens (1). The products quantitated in that study, representing over 90% of the total ethyl groups, included ethylphosphotriesters, O^6-EtG, O^2-EtT, O^4-EtT, O^2-EtC, 7-EtG and 3-EtA. Other derivatives which are known to be formed in small amounts in vitro (2) were not determined in vivo.

Detection of O^2-EtT, O^4-EtT and O^2-EtC was the first report on the formation of these derivatives in vivo (1). It had previously been reported that O^6-EtG was removed from cells, presumably by an enzymatic mechanism (3,4) but no similar studies had been carried out on the three O-alkyl pyrimidines, now known to be formed in significant amounts in DNA and RNA treated with N-nitroso alkylating agents (5,6). Whether excision of alkylphosphotriesters oc-

curs has only been studied by indirect methods (7) and no quantitative data are available.

The rates of removal of alkylated bases from DNA in vivo can reflect at least two processes: 1) removal as a result of hydrolysis of the glcosyl bond and 2) enzymatic removal as a result of DNA repair. In order to determine whether an alkylated base is removed as a result of DNA repair and/or as a result of chemical hydrolysis, the in vitro stability of the alkylated base in DNA must be known.

Since it was suggested by Loveless (8), and shown by Gerchman and Ludlum (9) and Mehta and Ludlum (10) that the formation of O^6-alkyl guanine in DNA is likely to be mutagenic, the formation and the removal of this chemically stable derivative has been studied in different animal tissues, such as liver, brain, kidney, and lung [Reviewed by Singer (2,11), Pegg (12), Roberts (13)]. The results from a number of different laboratories have indicated that a tissue's reduced capacity to remove this derivative may be related to an increased susceptibility to chemical carcinogenesis. However since the other O-alkylation products, O^2-EtT, O^4-EtT, O^2-EtC, are also mutagenic (14,15), it is important to determine whether these products are removed and, if so, at what rate. Before a correlation can be made as to whether the formation and removal of a particular base alkylation product is related to carcinogenesis, the formation and rates of removal of all derivatives formed by that alkylating agent need to be known. At present, such information for mammalian cells is only available for O^6-alkyl G, 3-alkyl A and 7-alkyl G (11).

Most Xeroderma pigmentosum cells have been shown to have a reduced DNA repair capacity for the excision of pyrimidine dimers (16), O^6-alkyl G (4), and many other carcinogen adducts (17-21). We therefore were interested in whether replicating XP-12 and GM 637 fibroblasts could remove from their DNA O-ethyl pyrimidines and ethylphosphotriesters (1). The extent of removal and relative persistence of derivatives enabled us to make some assumptions regarding the specificity and diversity of enzymes presumably acting as glycosylases or dealkylases.

MATERIALS AND METHODS

Growth and treatment of cells with [^{3}H]thymidine and [^{14}C]ethylnitrosourea. SV40 transformed normal human fibroblasts (GM 637) and Xeroderma pigmentosum fibroblasts (XP-12 RO) were grown as described by Singer et al. (1). Transformed cells were chosen over primary cells because they provided higher

cell densities and yields of DNA for alkylation studies. Transformation makes no apparent change in repair characteristics (22,23) but does alter DNA replication resulting in a faster growth rate. Twenty-four hr prior to alkylation, the medium was changed and replaced with Eagle's minimum essential medium containing 0.001 μCi/ml [^{3}H]thymidine, 10^{-5} M deoxycytidine and 10^{-4} M thymidine. After 24 hr labeling, the cells were trypsinized, washed twice in pH 7.5 phosphate buffered saline, then resuspended in pH 6.5 phosphate buffered saline and treated with [^{14}C]ethylnitrosourea for 1 hr (1). Immediately after washing the cells free of excess reagent, 40% of the cells were frozen and used as the zero time sample. The remaining treated cells were divided into fractions and grown for additional times after alkylation. Aliquots of normal fibroblasts were grown for 20, 48 and 72 hr while Xeroderma pigmentosum fibroblasts were grown for 48 hr only.

DNA was isolated and the alkyl products determined using the same methods described by Singer et al. (1,5). In a number of experiments it was necessary to pool DNA from two or more separate cell cultures in order to obtain enough radioactivity for analysis. Data from pooled samples are considered to represent a single analysis.

Alkylation of DNA with [^{14}C]ethylnitrosourea and determination of ethylated products. 5 mg purified calf thymus DNA dissolved in 1 ml 0.5 M pH 7.0 cacodylate buffer was treated with [^{14}C]ethylnitrosourea (Hoechst, 4.9 mCi/mM) 45 min, 50°C. Labeled reagent was removed by repeated ethanol precipitation. The average specific activity of preparations of ethylnitrosourea-treated DNA was 23,000 cpm/mg DNA. DNA ethylation products were determined after enzyme digestion followed by chromatographic procedures which have been used in this laboratory to quantitate the various ethyl derivatives (1, 5).

Stability of O-alkyl products after in vitro incubation of DNA. About 0.3 mg [^{14}C]ethylated DNA (7000-10,000 cpm) in 0.5 ml 0.01 M pH 7.4 potassium phosphate containing 0.01 M sodium azide was incubated at 37°C for varying times, then alcohol-precipitated. The alcohol supernatants, after removing an aliquot for determining total radioactivity, were co-chromatographed with UV absorbing authentic marker compounds on Whatman 3MM with N-butanol-ethanol-H_2O (8:1;2.5) as solvent. The only radioactive areas corresponded to 7-EtGua and 3-EtAde. Enzyme digestion of the remaining DNA pellet followed by the same chromatographic procedure as described in the preceding section was used as an additional analytical method to determine whether any O-alkyl derivatives were chemically unstable.

RESULTS

DNA alkylated *in vitro* or *in vivo* with ethylnitrosourea contained the following products (average proportion of total ethyl groups from 15 experiments in parentheses): ethylphosphotriesters (56%), 7-EtG (14%), 3-EtA (5%), O^6-EtG (10%), O^2-EtT (7%), O^4-EtT (3%), and O^2-EtC (4%). There are also small amounts of other ethyl derivatives, not examined in the present work.

In vitro, we find both the ether and glycosyl bonds of O^2-EtC, O^4-EtT, and O^2-EtT to be completely stable after 10 days of incubation and none of these derivatives are found in the alcohol supernatant of incubated DNA. This is further substantiated by the fact that we can recover all the O-alkyl derivatives quantitatively from the DNA pellet after 10 days incubation, while 3-EtA is absent and 7-EtG is greatly decreased.

Ethylphosphotriesters represent at least 50% of the total ethyl groups bound to DNA after ethylnitrosourea reaction. No loss of triesters in DNA was found after incubation at pH 7.4, 37°C, 10 days. Analyses for triesters were performed on the DNA pellet after incubation since the supernatant would not be expected to contain an intact triester. However ethanol would be found if dealkylation occurred.

GM 637 fibroblasts or XP-12 fibroblasts were prelabeled with [^{3}H]Thd prior to treatment with [^{14}C]ethylnitrosourea. Further culturing of these cells after alkylation results in a reduction of [^{3}H] specific activity in DNA, and also in [^{14}C] specific activity (Fig. 1). The reduction in [^{3}H] specific activity is due to DNA replication, but the reduction in the [^{14}C] can result from both DNA replication and loss of [^{14}C] ethyl derivatives. In Fig. 1, the data show that after the [^{14}C] radioactivity is corrected for the loss (or dilution) due to replication, the ethyl groups in the DNA decrease with increasing time of cell growth. In GM 637 fibroblasts, 20 hr after ethylation, 24% of the ethyl groups originally present have been removed. This value increases to 28% in 48 hr and 40% in 72 hr. XP-12 fibroblasts behave similarly although only 48 hr of replication was studied. Another point to be noted is that the replication of DNA, as measured by [^{3}H]Thd decrease, is also similar in both GM 637 and XP-12 fibroblasts treated with ethylnitrosourea. A calculation of cell division based on the halving time of DNA indicates that alkylation does not affect cell division. The halving times for both cell types is $\sim$ 50 hr.

For each time period, the amounts of the various [^{14}C] ethyl derivatives in DNA were quantitated and the results expressed as a proportion of total alkylation (as corrected for cell growth measured by the change in specific

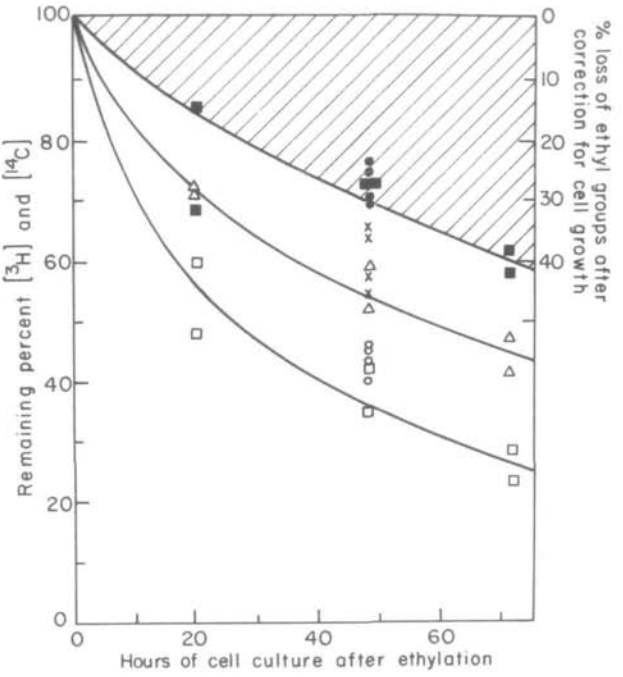

Figure 1. Relative amounts of [^{3}H]thymidine and [^{14}C] ethyl groups in GM 637 human and Xeroderma pigmentosum fibroblasts (XP-12) as functions of time of cell culture after treatment with [^{14}C]ethylnitrosourea. GM 637 fibroblasts; [^{14}C] □—□, [^{3}H] △—△, [^{14}C] corrected for decrease in [^{3}H] specific activity, ■—■. Xeroderma pigmentosum fibroblasts; [^{14}C] o—o, [^{3}H] x—x, [^{14}C] corrected for decrease in [^{3}H] specific activity ●—●. All radioactivity is expressed as percent of specific activity (cpm/mg DNA) at 0 time. The shaded area represents the absolute loss of [^{14}C] ethyl groups. This was determined by correcting the [^{14}C] specific activity for the reduction in [^{3}H] specific activity due to replication.

activity of [^{3}H]Thd in DNA at various times, compared to the zero time sample).

Table 1 is a summary of the analytical data. Seven ethyl derivatives were determined and of these only the ethylphosphotriesters appear to be very stable, though somewhat less than they are in vitro where no loss is detected after 10 days at 37°C. The four O-alkyl bases, which are also stable in vitro, are all excised to a substantial extent, but are still easily detected after 72 hr. In GM 637 fibroblasts, the data for the two separate 48 hr experiments are almost the same as those for the XP-12 fibroblasts, with one

Table 1. Removal of ethylation products from GM 637 fibroblasts and XP-12 fibroblasts*

Cell type	Incubation time in hr	DNA alkylation product: Ethylphospho-triesters	7-EtG	3-EtA	O^6-EtG	O^2-EtC	O^4-EtT	O^2-EtT
		Distribution of ethyl products before incubation†						
	0	56‡	14	5	10	4	3	7
		% Remaining compared to 0 time§						
GM 637 fibroblasts	20	88	69	20	36	61	68	58
	48**	88,86	57,57	0,0	47,43	47,57	76,43	48,43
	72	65	38	0	24	47	18	22
XP-12 fibroblasts	48**	87,98	45,63	0,0	84,88	42,22	35,80	41,38

*See Material and Methods for methods of growing cells, labeling and analytical techniques.

†The 0 time values are averages of many experiments including data published previously (1). Since there are no reproducible differences in the distribution of ethyl products in the two cell types, the same 0 values are given for both GM 637 and XP-12 fibroblasts. The figures given are proportion of total ethylation. The absolute extent of ethylation was 1-4 ethyls/10^5 DNA-P.

‡Corrected for TpEtT found in Peak B which was previously not identified (1). The authors are grateful to Drs. E. Scherer and Van Boom for a generous gift of this triester.

§The distribution of ethyl products was determined in the same manner as for the 0 time samples. These percentages of total [^{14}C]ethyl groups were then corrected for the reduction of [^{14}C]specific activity due to DNA replication, shown in Fig.1. The results have been converted to the proportion of each derivative remaining at various times, compared to 0 time.

**The amounts of each derivative, expressed as percent loss, when summated indicate that 34% of the ethyl groups are removed from GM 637 fibroblasts and 27% are removed from XP-12 fibroblasts. This is in good agreement with the total corrected decrease in [^{14}C]specific activity of 28% and 26% for GM 637 fibroblasts and XP-12 fibroblasts, respectively (Fig. 1).

definite exception and two possible exceptions. O^6-EtG remains in the XP-12 DNA to a much greater extent than in GM 637 fibroblast DNA. There is a possibility that O^4-EtT may be excised more slowly and O^2-EtC more rapidly in the XP-12 DNA, but the determination of the derivatives is difficult due to the relatively small amounts present. If we take the average values of O^4-EtT in the 48 hr experiments, there is no major difference between XP-12 and GM 637 fibroblasts.

The precision of analytical data for some derivatives is such that approximate half-lives can be calculated. The data for O^2-EtT is shown in Fig. 2. This derivative has a $t_{\frac{1}{2}} \sim 40$ hr in both GM 637 and XP-12 fibroblasts. Phosphotriesters can be calculated to have a $t_{\frac{1}{2}} > 8$ days (Fig. 2) while O^2-EtC and O^4-EtT, determined with less precision, have half-lives of 40-60 hr, both in GM 637 and XP-12 fibroblasts.

The removal of the two ethyl purines which have a labile glycosyl bond was also determined. Both 7-EtG and 3-EtA were removed more rapidly from DNA in cells than in vitro. In each case the estimated half-life in cells (Fig.

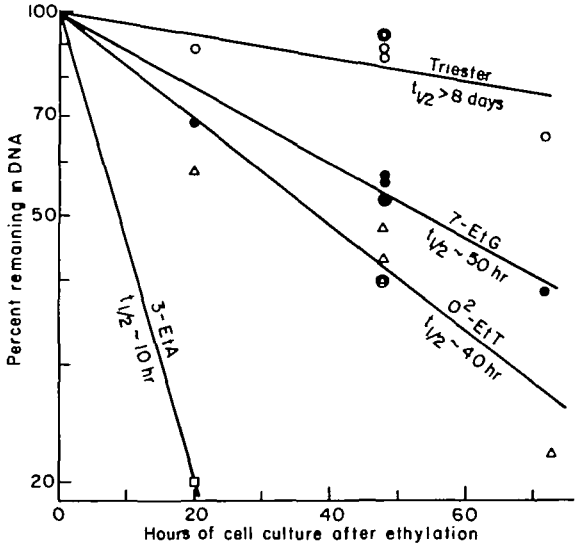

Figure 2. Approximate half-lives ($t_{\frac{1}{2}}$) of ethyl derivatives in cultured [^{14}C] ethylnitrosourea cells. The derivative and approximate half-life are shown in the Figure., The circled symbols at 48 hr are the averages of two experiments with Xeroderma pigmentosum fibroblasts, while the other symbols are data from experiments with GM 637 human fibroblasts. Ethylphosphotriester, o——o, 7-EtG, ●——●, O^2-EtT, Δ——Δ, 3-EtA, □ (no detectable 3-EtA after 48 hr, data not on Figure). See Table 1 for the data used in the Figure.

2) was about one-third of that in vitro as determined in this series of experiments (data not shown) or previously reported (Summarized in Ref. (11)).

DISCUSSION

This investigation had multiple objectives related to the question concerning repair of ethyl derivatives: 1) Can ethylnitrosourea-treated mammalian cells remove O-ethyl pyrimidines as they can O^6-alkyl G? 2) Is 7-EtG, like 3-EtA, removed faster in vivo than their chemical stability would indicate? 3) Since XP-12 fibroblasts have a diminished repair capacity for O^6-alkyl G, are they also limited in their capability to remove the O-alkyl pyrimidines? 4) And finally, what happens to ethylphosphotriesters in vivo?

All these questions were answered in quantitative terms, since we were able to measure the amount of each ethyl derivative remaining in DNA after cells were allowed to multiply. Such analytical data can only be obtained by actual separation and identification of each derivative following enzymic digestion (5). Thus, our data represent the first chemical evidence for the persistence of ethylphosphotriesters in cells and for the partial removal of O-ethyl pyrimidines.

It is not too unexpected that alkyl phosphotriesters are resistant to excision in vivo since they are also resistant to hydrolysis by a wide variety of enzymes (24). It is more likely that the relatively small loss found is actually due to de-ethylation, such as has been observed when a trinucleotide ethyl phosphotriester is taken up by transformed Syrian hamster fibroblasts (25).

The three O-alkyl pyrimidines (O^2-EtT, O^4-EtT, O^2-EtC) are all excised (or dealkylated) by both GM 637 and XP-12 fibroblasts, although the glycosyl bond and alkyl groups are chemically stable for at least 10 days at 37°C. The rate of this removal is similar in both cell types, which is in contrast to the decreased excision of O^6-EtG in XP-12 fibroblasts reported by Goth-Goldstein (4) and now confirmed by us. In GM 637 fibroblasts all four O-alkyl bases are removed at similar rates ($t_{\frac{1}{2}}$ 40-60 hr). The loss is most rapid at early times and after 20 hr at least 30% of the original bases have been removed. However after 72 hr when it can be calculated that the cells have passed through about two cell divisions all the O-alkyl derivatives are still present in significant amounts (18-24%), compared to the unincubated cells.

The persistence of a portion of O^2-EtT, O^4-EtT, O^2-EtC, and O^6-EtG can be

interpreted in at least two ways: 1) An enzyme capable of removing alkyl products is present or induced early in replication and is no longer active after a certain time period. 2) The persistent alkyl derivatives are in a chromatin subfraction protected from enzymes and excised more slowly or not at all.

Regarding the first point, both induction of excision capacity and saturation of such enzyme activity have been reported (26-28). Thus this is still an open question. For the second possibility, there is evidence for and against the view that chromatin structure influences the distribution of repair after UV irradiation (29,30) or alkylation (31-33), and the persistence of DNA O-alkyl products may be due to masking by histones. Other carcinogens besides the simple alkylating agents modify DNA producing a variety of products, some of which are not excised *in vivo* at all (34), or the derivative formed is also termed "persistent" since a portion remains in the DNA over time periods in which the cells have undergone at least one division (33,35). However cell division is not an absolute requirement for excision to occur (36,37).

This now raises the question of the mechanism responsible for loss of alkyl derivatives. There appears to be little doubt that enzymes are involved in the removal of some, if not all, of the derivatives that we have measured. We postulate the involvement of at least four or five enzymes with different specificities.

Triesters, while being chemically stable, are so slowly removed that they must involve a unique mechanism in comparison with the other derivatives. Since they are generally nuclease resistant, the small loss we see may represent dealkylation (25). 7-EtG and 3-EtA are chemically unstable with very different rates of hydrolysis, but the greater rate of removal that we observe *in vivo* suggests a specific enzymatic mechanism for removal. It has been suggested that a specific glycosylase can remove 3-MeA and 3-EtA but apparently recognizes no other carcinogen adduct tested (38). Therefore the removal of 7-EtG and 3-EtA may involve different enzymes. The O-alkyl derivatives are all chemically stable in neutral pH at 37°C for more than 10 days, so that their loss from DNA with half-lives of 40-60 hr must result from the action of enzymes. From the data for normal cells alone we cannot distinguish between the O-alkyl products in their mechanism of removal. However, the additional data regarding XP-12 cells indicates that O^6-EtG is distinct from the other O-alkylations because it, alone, is removed more slowly from XP-12 cells than from GM 637 cells. This obwervation in XP-12 cells, also

noted by Goth-Goldstein (4) and Altamirano-Dimas et al. (39), raises numerous questions about the mechanisms involved in the recognition and regulation of removal of O^6-alkylguanine. These XP-12 cells are defective in excision of O^6-EtG, pyrimidine dimers and several other carcinogen adducts, but still capable of excising many alkylation products.

We also found that after cell doubling about 50% of the initial potentially mutagenic O-alkylations persist in the DNA, despite the action of presumed repair enzymes. Ethylnitrosourea alkylates a variety of sites in nucleic acids. In an in vivo system, it is not possible to attribute an observed biological effect, such as transformation, to the presence of an individual derivative. It is recognized that efforts have been made to correlate the presence of one derivative, O^6-alkyl G, with carcinogenesis since this derivative has been shown to mispair in in vitro transcription. However the three O-ethyl pyrimidines which represent a substantial proportion of the ethylated bases in the present experiments are also mutagenic in in vitro experiments on transcription, as well as when used in codons in tRNA binding of ribosomes. Thus their persistence during transcription and replication may well represent initiating events in carcinogenesis.

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