[*METHYL*-^{3}H]THYMIDINE IN DNA INDUCES LESIONS WHICH ARE RECOGNIZED BY A MAMMALIAN DNA-REPAIR ENDONUCLEASE

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1. Introduction

It has been known for some years that single-stranded breaks are formed in ^{3}H-labelled DNA during prolonged storage [1] and when such breaks are formed in an intact cell its DNA-repair mechanism is able to restore the damaged DNA [2].

Indirectly it has been shown that a great number of lesions are formed in ^{3}H-labelled DNA, but their chemical structure is not known. Previous work has shown that on the one hand ^{3}H-decay of [6-^{3}H]-cytosine in DNA is more efficient in producing single-stranded DNA breaks than [5-^{3}H]-cytosine which on the other hand is more mutagenic than the former [3,4]. Since labelled pyrimidines in aqueous solution give rise to glycols and hydroperoxy compounds the same products are most likely formed in DNA containing the same labelled compound.

To our knowledge nothing has been published which elucidates the enzymatic steps which take place in restoring tritium-damaged DNA. Quite recently a mammalian DNA-repair endonuclease was partially purified and characterized [5]. This enzyme appeared to recognize both AP-lesions and non-AP-lesions induced by agents like OsO_4 and UV-light. The present work reports that the same endonuclease incises [*methyl*-^{3}H]thymidine-labelled DNA which has been stored at 4°C. This suggests that repair of some tritium-induced DNA lesions can take place through the incision–excision DNA-repair mechanism. The lesion recognized has not been identified, although as suggested in [5], the lesion could be a thymine glycol.

Abbreviations: UV, ultraviolet; AP, apurinic/apyrimidinic; RF, replicative form; EDTA, ethylene diaminetetra-acetic acid

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2. Materials and methods

2.1. *Preparation of [*methyl-^{3}*H]thymidine-labelled φX174 RFI DNA*

φX174 RFI DNA was labelled with [*methyl*-^{3}H]-thymidine and isolated as in [6]. The φX174 phage-infected *Escherichia coli* was supplied with 20 μg thymidine/ml. The added thymidine contained [*methyl*-^{3}H]thymidine which varied from 0.025–3.75 μCi/ml. The φX174 RFI DNA was purified by ethidium bromide–CsCl isopycnic centrifugation which was repeated 2 or 3 times.

The DNAs were stored in a 10 mM Tris–HCl (pH 8.0) 0.1 mM EDTA buffer at 4°C.

2.2. *Purification of DNA-repair endonuclease and AP-specific endonuclease from a mouse cell line*

Both endonucleases were purified as in [5,7] and the endonucleases recovered from the phosphocellulose column were used here. The specific activities of the two enzymes were close to the values in [5,7].

2.3. *Enzyme assays*

The DNA-repair endonuclease assay was carried out in 10 mM Tris–HCl (pH 8.0), 3 mM EDTA, 10 mM 2-mercaptoethanol, 100 mM KCl and [*methyl*-^{3}H]thymidine-labelled DNA (4000–20 000 dpm/assay). The enzyme was diluted in a buffer containing 0.5 mg/ml bovine serum albumin, 20 mM Tris–HCl (pH 8.0) and 10 mM 2-mercaptoethanol, and added in 10 μl to the assay mixture (final vol. 100 μl) [5].

The AP-specific endonuclease assay was carried out in 10 mM Tris–HCl (pH 8.0), 25 mM KCl, 4 mM $MgCl_2$ 10 mM 2-mercaptoethanol and [*methyl*-^{3}H]-thymidine-labelled DNA (4000–20 000 dpm/assay). The enzyme was diluted in the same buffer system as

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above and 10 μl enzyme was added to the assay mixture (final vol. 100 μl) [7].

The enzymatic activities were assessed by the nitrocellulose technique [6]. The endonucleolytic action of either AP-endonuclease or DNA-repair endonuclease on the RF φX174 was completed with the addition of 1 ml 0.2 M potassium phosphate (pH 12.1) containing 25 mM EDTA and 0.9 M NaCl. Within 10 s the reaction mixture was neutralized with 1 M HCl. This treatment made all the nicked DNA molecules single-stranded while the double-stranded form of non-nicked DNA molecules was kept intact. The single-stranded DNA was trapped onto nitrocellulose filters. The percentage of total radioactivity which bound to the filter was adjusted by a Poisson distribution to obtain the average number of nicks introduced per DNA molecule. Nicking of φX174 RFI DNA was also measured by neutral sucrose gradient velocity centrifugation [8].

3. Results and discussion

During work with a mammalian DNA-repair endonuclease, which is able to nick DNA damaged by agents like UV-light, OsO_4, acid or γ-rays, we observed that prolonged storage transformed the [*methyl*-^{3}H]-thymidine-labelled DNA to a substrate for the enzyme. Freshly prepared DNA was never found to be a substrate for the endonuclease, thus this phenomenon was most likely due to changes in the DNA rather than to changes in the specificity of the enzyme.

The DNA-repair endonuclease used here is known to recognize more than one type of lesions in DNA. It had been shown that the enzyme could incise acid-treated DNA as well as DNAs treated by agents like UV-light and OsO_4. Thus it was of importance to know if the damage recognized in the [^{3}H]thymidine-labelled DNA was AP-site or of the non-AP type of lesion(s). The number of lesions recognized by DNA-repair endonuclease and AP-specific endonuclease was determined in a freshly prepared DNA and after 30 days of storage (fig.1). The AP-specific endonuclease incised only a very small number of DNA molecules (fig.1A) compared with the DNA-repair endonuclease which nicked stored DNA significantly (fig.1B). This strongly suggests that the storage-induced lesions which were recognized by the DNA-repair endonuclease were not AP-sites but a different type of lesions.

The number of DNA-repair endonuclease sites formed in a [*methyl*-^{3}H]thymidine-labelled DNA was determined as a function of storage time. As can be seen from fig.2, the number of endonuclease susceptible sites increased linearly up to ~50–60 days of storage and then appeared to level off. This indicates

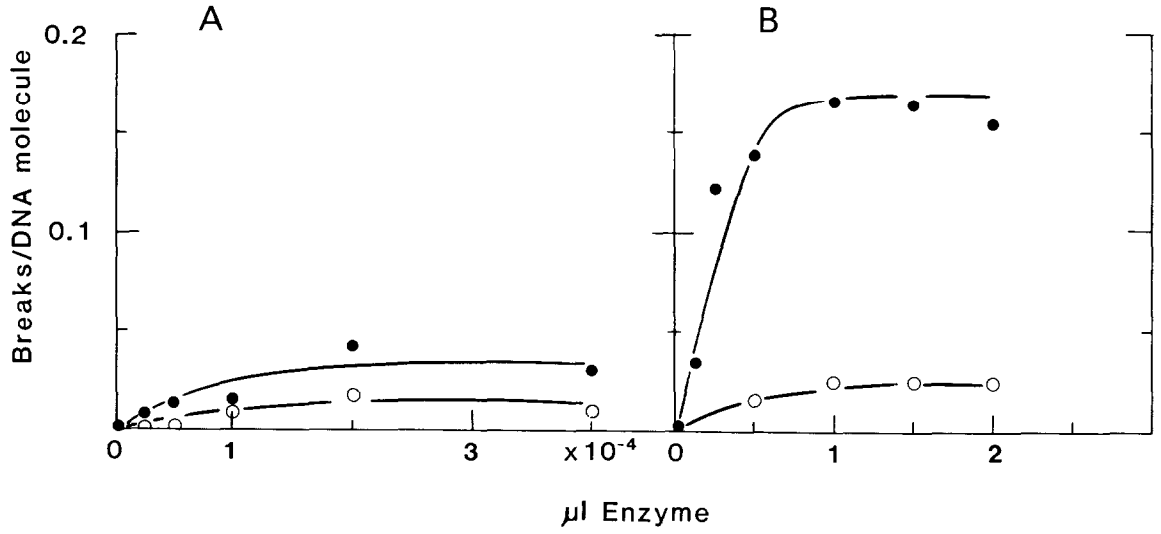

Fig.1. Formation of lesion sites in [*methyl*-^{3}H]thymidine-labelled φX174 RFI DNA (spec. act. 2.2 × 10^5 dpm/μg) susceptible to (A) AP-specific endonuclease and (B) DNA-repair endonuclease after 5 days of storage (–○–) and 30 days after storage (–●–) at 4°C. Each point represents the average value obtained by 5 determinations. The enzymatic tests were as in section 2.

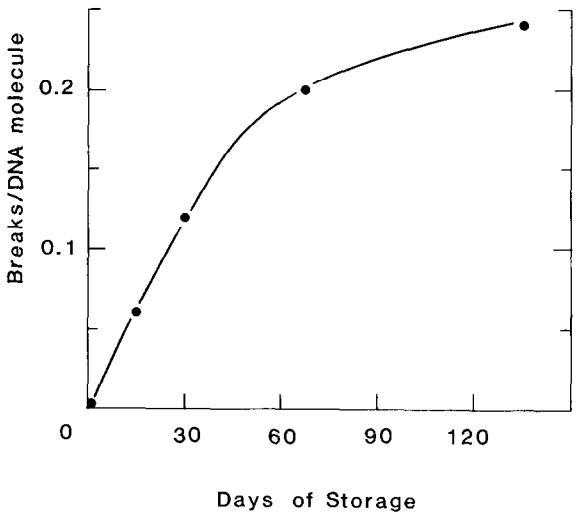

Fig.2. Number of DNA-repair endonuclease susceptible sites formed in [*methyl*-^{3}H]thymidine-labelled ϕX174 RFI DNA (spec. act. 2.2 × 10^5 dpm/μg) as a function of days stored at 4°C. Each point is determined as number of nicks per DNA molecule formed at saturating amount of enzyme (as in fig.1). The number of AP-sites are subtracted from values presented in the figure. The enzymatic tests were as in section 2.

that an equilibrium in the formation between endonuclease-recognized adducts and non-recognized adducts in the DNA has been reached. Also a very small number of AP-sites were formed (estimated with the AP-specific endonuclease) which are not included in the values presented in fig.2.

Many chemical alterations in DNA are known to take place during storage. Both depurination and deamination of cytosine in DNA occur under physiological conditons [9,10]. Although the data in fig.1 show that depurination is not the major lesion recognized by the DNA-repair endonuclease, the recognized lesion could certainly be a new type of lesion due to chemical instability of the DNA. Thus it was of importance to investigate if the lesion was caused by the tritium radiation or was merely formed in all DNAs independently of the tritium. [*methyl*-^{3}H]-Thymidine DNAs of different specific activities were prepared, and the number of enzyme susceptible sites formed in the DNAs after 3, 20 and 40 days was determined. The results in table 1 are quite conclusive. While DNAs of low specific activity induced only a few lesions, the DNAs of highest specific activity contained a significant larger number of DNA-repair endonucleolytic susceptible lesions. The data strongly suggest that the lesions are mainly caused by the tritium in the DNA.

At the present time we do not know the chemical entity formed in [*methyl*-^{3}H]thymidine-labelled DNA which is recognized by the endonuclease. However, it was suggested in previous work that a thymine glycol may be the species recognized by the enzyme [5]. Since the ^{3}H-labelling of DNA is in the methyl group in the 5′-position of thymine it is not unlikely that the 5,6 double-bond of thymine is one of the most reactive sites for attack of radicals which are known to be formed by tritium irradiation in water [11].

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Table 1
DNA-repair endonuclease susceptible sites formed during storage of [*methyl*-^{3}H]thymidine-labelled ϕX174 RFI DNA of varying specific activities (number of nicks per DNA molecule)[a]

Days of storage	Specific activity (dpm/μg)				
	2.1 × 10^3	17.3 × 10^3	50 × 10^3	205 × 10^3	312 × 10^3
3	<0.02	<0.02	<0.02	<0.02	<0.02
20	0.06	0.06	0.07 (0.02)	0.14 (0.05)	0.24 (0.04)
40	0.10	0.10 (0.02)	0.16 (0.03)	0.30 (0.06)	0.31 (0.06)

[a] Number of AP-specific endonuclease susceptible sites which is included in the brackets has been subtracted from the present values

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