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Characterization of human enzymes specific for damaged DNA: resolution of endonuclease for irradiated DNA from an apparent N-glycosidase active on alkylated DNA

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ABSTRACT

An endonuclease partially purified from human lymphoblasts, and active against ultraviolet-irradiated DNA, was found to act additionally on DNA damaged by either x-radiation or methylmethanesulfonate. To determine if these activities were truly endonucleolytic, the reaction products were analyzed under conditions that prevented conversion of apurinic or apyrimidinic sites to single-strand breaks. With either ultraviolet- or x-irradiated DNA, strand breakage remained maximal, hence confirming the endonucleolytic character of the enzyme. By contrast, with DNA alkylated with methylmethanesulfonate, strand breakage was sharply reduced. Additional experiments indicated that the activity for alkylated DNA induces strand breaks only in concert with a purified endonuclease specific for apurinic sites, suggesting that it is an *N*-glycosidase that depurinates alkylated bases. This enzyme was separated from the endonuclease specific for irradiated DNA, by chromatography on DNA-agarose.

INTRODUCTION

The first step in the excision repair of DNA requires recognition of the damage, and endonucleolytic scission of the DNA strand adjacent to the lesion. Several enzymes capable of performing these functions have been purified. Endonucleases isolated from *Micrococcus luteus*[1,2], *Escherichia coli*[3], and T_4-infected *E. coli*[4,5] specifically incise ultraviolet (UV)-irradiated DNA adjacent to pyrimidine dimers. Other endonucleases highly specific for apurinic and apyrimidinic (AP) sites in DNA have been purified from *E. coli*[6], calf thymus[7], and human lymphoblasts[8]. Yet another group of enzyme activities, identified in both microbial[9] and mammalian cells[10-13], produce endonucleolytic cleavage of DNA containing base damage (distinct from UV dimers) that is induced by either UV- or ionizing radiation. Most recently, a new class of enzymes that recognize abnormal bases in DNA (e.g., uracil) has been identified in bacteria[14,15] and human cells[16]. Termed *N*-glycosidases, these enzymes cleave the base from the deoxyribose moiety, leaving an AP site[15]. The initial steps in excision repair might therefore involve both an *N*-glycosidase, to recognize the damage, and an endonuclease specific for apurinic or apyrimidinic sites, to produce strand scission.

I previously described the purification and separation of two endonucleases, one specific for DNA containing AP sites (AP-endonuclease), and the other specific for DNA containing UV-induced (nondimer) base damage (UV-DNA endonuclease)[8,17]. In the present study the partially purified activity specific for UV-induced base damage was tested for activity against DNA damaged by either x-rays or methylmethanesulfonate (MMS). Subsequent experiments indicated that the activities for UV- or x-irradiated DNA were truly endonucleolytic, while that for alkylated DNA was produced by a distinct activity that is most likely an *N*-glycosidase.

MATERIALS AND METHODS

Enzyme purification: UV-DNA endonuclease and AP-endonuclease were both partially purified from cultured human lymphoblasts (CCRF-CEM line) as previously described[8].

Endonuclease assays: Endonuclease activity was assayed by measuring the conversion of covalently closed superhelical PM2 viral DNA to nicked circles.[10] In assays with the UV-DNA endonuclease preparation, the reaction mixture contained, in a final volume of 120 µl, 10 mM Tris·HCl (pH 7.5), 2 mM EDTA, 10 mM NaCl and about 0.1 µg of PM2 DNA labeled with [^{3}H]dThd. In assays that included the AP-endonuclease, the reaction mixture contained 50 mM Hepes·KOH (pH 8.0), and 0.5 mM MgCl instead of Tris·HCl and EDTA. Ten µl of endonuclease, in buffer containing 10 mM Tris· HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 0.15 M NaCl, 0.02% sodium azide and 10% glycerol, was incubated in the reaction mixture for various times at 37°C.

Before analysis in alkaline sucrose gradients[10], the reaction was terminated by adding 10 µl of 1 M NaOH and continuing incubation for 20 min at 37°C to convert all alkali-labile sites (i.e., AP-sites) to strand breaks. Before analysis at neutral pH, the reaction was terminated by adding 50 µl of a solution containing 1 mg of Proteinase K (Beckman Instruments, Inc.) per ml and continuing incubation for 5 min at 37°C. This procedure proved to be the only way to remove a protein that bound tenaciously to the DNA and interfered with subsequent analysis. The reaction mixture (150 µl) was applied to 5 ml neutral sucrose gradients (6.5-20%), dissolved in 1 M KCl, 1 mM EDTA and 10 mM Tris·HCl (pH 7.5), and centrifuged in a Beckman SW-50.1 rotor at 20°C at 50,000 rev/min for 150 min. About 35 fractions were collected from the bottom of the tube, and 0.5 ml H_2O and 7.5 ml scintillation fluid (PCS, Amersham-Searle) were added to each fraction before the radioactivity was determined by scintillation counting. A filter-binding technique[8] was used instead of neutral sucrose gradient analysis in some experiments.

In every assay the fraction of radioactivity in the nicked and the intact forms of DNA was calculated, and the number of strand breaks per PM2 DNA molecule was obtained from the Poisson formula: breaks per molecule = -ln x, where x is the fraction of molecules that are unnicked.

Preparation and treatment of PM2 DNA: PM2 DNA was labeled with [^{3}H]dThd, isolated from bacteriophage by the methods of Espejo *et al*[18,19], and subsequently treated in one of three ways. (i) UV-irradiation with a 15-watt germicidal lamp at a dose rate of 500 J/m^2 per min; (ii) x-irradiation under air at room temperature with 4 mV x-rays from a Varion Clinac-4 linear accelerator at a dose rate of 400 rads/min; (iii) alkylation with MMS. The alkylating agent was added directly to the solution of PM2 DNA to a final concentration 6 X 10^{-3}M. Different extents of alkylation were achieved by incubating the mixture at 37°C for 5, 10, 15 or 20 min. The alkylation reaction was terminated by cooling to 4°C and passing the solution through a G-50 superfine Sephadex column (0.6 X 8 cm)[20]; the peak of radioactive DNA eluted from the column was free of unreacted MMS.

RESULTS

Susceptibility of x-irradiated DNA to the UV-DNA endonuclease: The types and dose response of damage induced in DNA by x-radiation are depicted in Fig. 1. When the irradiated DNA was immediately analyzed on sucrose gradients at neutral pH, about 0.1 breaks per molecule per 1000 rads were detected. This represents the overt radiation-induced strand scission. When the DNA was analyzed on alkaline sucrose gradients after 37°C incubation at pH 12.2 for 20 min, a treatment that converts all AP-sites to nicks, an additional 0.15 breaks per molecule per 1000 rads were detected. It has previously been shown that almost all the alkali-labile sites in PM2 DNA irradiated under these conditions are AP-sites[8]. In the final determination enough UV-DNA endonuclease was incubated with the DNA to convert all enzyme-susceptible sites to breaks, and the mixture was then incubated at pH 12.2 to allow nicking of all alkali-labile sites present. This resulted in an additional 0.2 breaks per molecule per 1000 rads (Fig. 1), which represents the number of alkali-stable sites that are susceptible to the UV-DNA endonuclease.

Activity of UV-DNA endonucleases on alkylated DNA: Shown in Fig. 2 is a similar experiment with DNA alkylated by MMS, a compound that produces no overt breaks in PM2 DNA. The extent of alkylation was determined by heating the DNA at pH 7.5 for 30 min at 70°C to depurinate all methylated purines[20]. The DNA was then treated with alkali at pH 12.2, and analyzed on alkaline sucrose gradients. At the highest dose of MMS, about 3.5

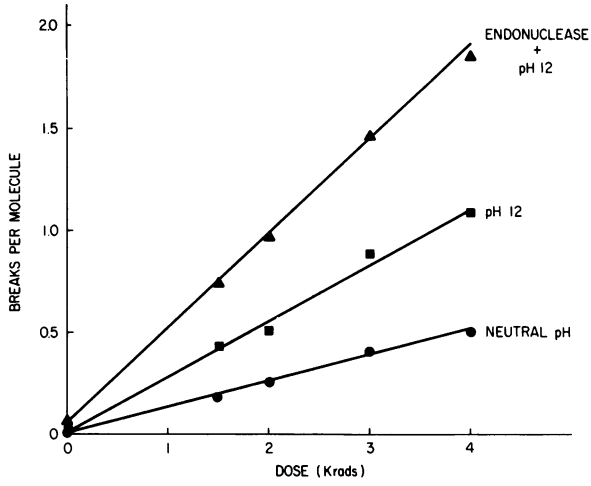

Figure 1: Dose dependency of x-ray induced lesions in PM2 DNA.
●–●, Overt strand breaks measured by immediate analysis on neutral sucrose gradients.
■–■, Alkali-induced breaks determined on alkaline sucrose gradients.
▲–▲, Sites nicked by the UV-DNA endonuclease and subsequent alkaline treatment and analyzed on alkaline sucrose gradients.

breaks per molecule, representing the number of alkylated bases, were detected. In the absence of heat treatment, only 0.2 alkali-labile sites per molecule were immediately apparent at this dose. Finally, incubation of alkylated DNA with enough endonuclease to drive the reaction to completion resulted in an additional 0.9 breaks per molecule; this increase represents the number of alkali-stable lesions that are susceptible to the enzyme. The fact that virtually no enzyme-induced breaks were detected at zero dose of MMS or x-radiation confirms the damage-specific character of the two activities.

Endonucleolytic character of the UV-DNA endonuclease: It is not possible to determine from the preceding experiments whether the strand-breaking activity for x-irradiated or alkylated DNA, (or UV-irradiated DNA[8]) was truly endonucleolytic, or was due to *N*-glycosidase which produced AP-sites that yielded strand breaks only upon alkaline treatment. The kinetics of strand breakage were therefore analyzed at neutral pH, after incubation of damaged DNA with the UV-DNA endonuclease under conditions where contaminating AP-endonuclease would be inactive (i.e. 2mM EDTA[8]). *N*-glycosidase could not result in strand breakage under these conditions. In addition, DNA was incubated with both the UV-DNA endonuclease and the AP-endonuclease combined. If the UV-DNA endonuclease

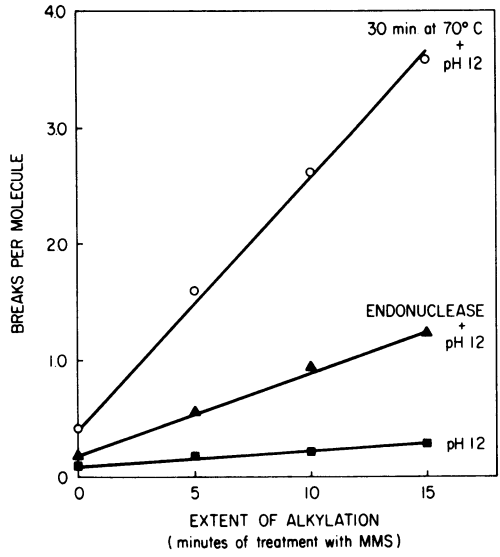

Figure 2: Dose dependency of **MMS-induced lesions in PM2 DNA.**
■—■, Alkali-induced strand breaks.
▲—▲, Breaks induced by the UV-DNA endonuclease and subsequent alkaline treatment.
○—○, Total alkylations determined by heating the DNA at 70°C for 30 min to depurinate all alkylated bases followed by alkaline treatment to convert the resultant AP-sites to strand breaks. All analyses were on alkaline sucrose gradients.

contained *N*-glycosidase activity, then the addition of AP-DNA endonuclease would stimulate strand breakage in excess of that expected due to pre-existing AP-sites in the DNA. Curve 1 in Fig. 3A indicates that the AP-endonuclease induced a low rate of strand breakage in UV-irradiated DNA which is consistent with previous results showing that such DNA contains few AP-sites[8]. On the other hand, UV-DNA endonuclease (Curve 2) induced a much higher rate of strand breakage, which increased only slightly when the AP-endonuclease was added (Curve 3). The time course of the reaction due to the combined enzymes was close to that expected for the sum of reactions of the separate enzymes (dashed line). Clearly, then, the UV-DNA endonuclease has maximal phosphodiesterase activity with UV-irradiated DNA, even in the absence of added AP-endonuclease. Similar results were obtained with x-irradiated DNA (Fig. 3B). The time course for the combined action of the enzymes was again simply additive.

In contrast to the preceding results, the UV-DNA endonuclease on its own showed very low phosphodiesterase activity with the alkylated DNA (Fig. 3C, Curve 2). When combined with the AP-endonuclease, however, the rate of strand breakage was sharply stimulated (Curve 3). This stimulation could not be accounted for on the basis of AP sites in the DNA (Curve 2): the rate of strand breakage produced by the combined action of the

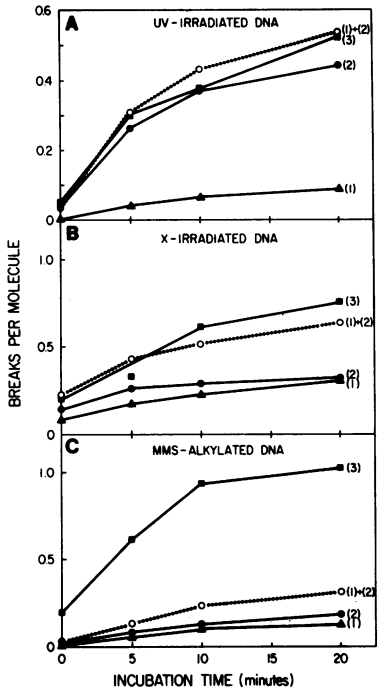

Figure 3: Time courses of strand breakage induced by (1) the AP-endonuclease, ▲—▲; (2) the UV-DNA endonuclease, ●—●; or (3) the two enzyme preparations in combination ■—■. The dashed line (○---○) represents the sum of the breaks induced by the separate enzyme reactions (1 + 2). Reaction products were analyzed with a filter-binding assay[8]. The DNA was irradiated with UV (500 J/m^2) or x-rays (2500 rads), or was alkylated with MMS (6 X 10^{-3}M for 10 min at 37°C).

UV-DNA and AP-endonucleases far exceeded the sum of the reactions due to each enzyme alone (dashed line, Fig. 3C). Since the purified AP-endonuclease has an absolute requirement for Mg^{++}[8], it was necessary to eliminate the possibility that the stimulation observed with AP-endonuclease (Fig. 3C) was due to the addition of Mg^{++}, not the enzyme. Fig. 4 shows a kinetic study similar to Fig. 3C except that Mg^{++} was present at 0.5 mM, the optimum concentration for AP-endonuclease activity[8], in all cases. Again it is clear that the initial rate of strand breakage induced by the UV-DNA endonuclease alone was relatively low and that this rate was strongly stimulated by the addition of AP-endonuclease. The low level of strand breakage observed with the UV-DNA endonuclease alone is most readily explained as the result of contaminant AP-endonuclease activity.

Chromatographic separation of UV-DNA endonuclease and the activity for alkylated DNA: Since the activity specific for MMS-treated DNA appeared to be different from the

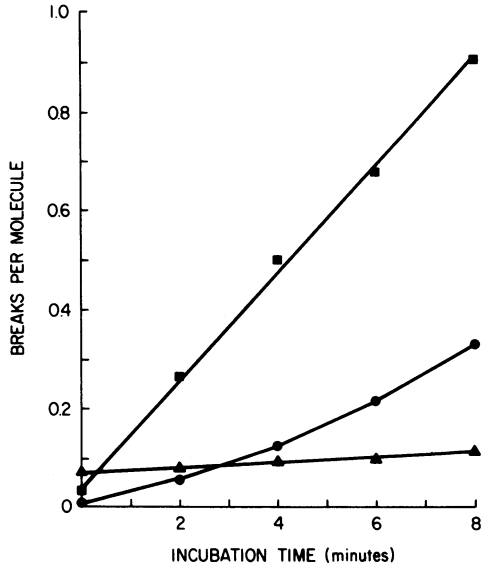

Figure 4: Time course for strand breakage induced by (1) AP-endonuclease ▲—▲, (2) UV-DNA endonuclease ●—●, or (3) the two enzyme preparations combined ■—■. DNA was alkylated with MMS (6×10^{-3}M for 20 min at 37°C). The reaction mixture contained 50 mM Hepes·KOH (pH 8.0) and 0.5 mM Mg^{++} in all cases. DNA was analyzed by filter-binding assay.

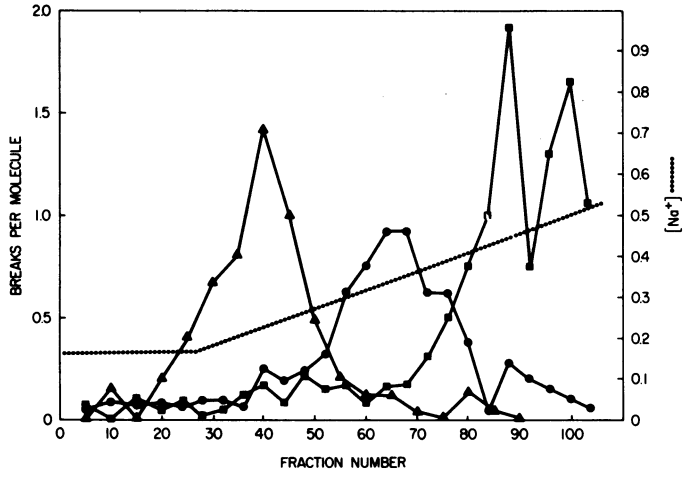

Figure 5: DNA-agarose chromatography of the 20%-40% ammonium sulfate fraction (IIIB Ref. 8).

▲—▲, Activity assayed in the presence of Mg^{++} (0.5 mM) with DNA partially depurinated by heating to 70°C for 10 min at pH 5.

●—●, Activity assayed in the presence of EDTA (2 mM) with UV-irradiated DNA (1000 J/m^2).

■—■, Activity assayed in the presence of purified AP-endonuclease and Mg^{++} (0.5 mM), with DNA alkylated by MMS (6×10^{-3}M for 20 min at 37°C).

DNA was analyzed by filter-binding assay.

endonuclease acting on UV- or x-irradiated DNA, it was of interest to determine the chromatographic behavior of these activities during purification on DNA-agarose. Column chromatography was exactly as previously described[8]. In addition to assays with UV- or x-irradiated DNA, activity against MMS-treated DNA was also determined, the assay mixture containing Mg^{++} at 0.5 mM and purified AP-endonuclease. The peak of activity eluting at 0.4 to 0.5 M NaCl in Fig. 5 represents the enzymatic production of AP-sites identical with that seen in Fig. 3C and Fig. 4. This peak was clearly separate from the endonuclease activity for UV-irradiated DNA and also from AP-endonuclease. The endonuclease activity for x-irradiated DNA chromatographed in the same region as that for UV-DNA (not shown in Fig. 4).

DISCUSSION

The experiments reported here show that an enzyme preparation partially purified from human lymphoblasts, and termed UV-DNA endonuclease, recognizes alkali-stable lesions in x-irradiated and alkylated DNA as well as UV-irradiated DNA. Moreover, kinetic studies (Fig. 3) indicate that the strand-breaking activity of this preparation on alkali-stable sites in UV-irradiated or x-irradiated DNA is not dependent on the addition of AP-endonuclease, and therefore is truly endonucleolytic. The possibility that strand scission is effected by contaminating AP-endonuclease can be discounted since under the conditions of the assay (i.e. 2mM EDTA) UV-DNA endonuclease contained no activity for depurinated DNA. This endonuclease is very similar to that from calf thymus which is specific for both UV- and x-irradiated DNA[12]. The nature of the lesion recognized by the UV-DNA endonuclease, although it might be of the same type as the thymine glycol product induced by both UV and gamma radiations[21], is still uncertain. It is tempting to speculate that the activity plays a role in the excision repair of an x-ray-induced lesion that is defectively repaired in cells from individuals with ataxia telangiectasia, an inherited human disease characterized by unusual x-ray sensitivity[22].

In contrast to phosphodiesterase activity with UV- or x-irradiated DNA, strand breakage in MMS-treated DNA was highly dependent on the addition of AP-endonuclease. This result strongly suggests that the activity specific for the alkylated DNA is a distinct enzyme and quite probably an *N*-glycosidase. Examination of the chromatographic behavior of this activity, on DNA-agarose, has shown that the peak of activity specific for alkylated DNA does not in fact coincide with the peak of UV-DNA endonuclease eluted from the column (Fig. 5); the latter activity eluted at 0.3-0.4 M NaCl while the enzyme acting on alkylated DNA eluted at 0.4-0.5 M NaCl. Only about 25% of the alkylated sites were attacked by this enzyme (Fig. 2), suggesting that it has a preference for specific methylated bases. Preferential loss of

3-methyladenine, a minor product of alkylation by MMS, has been observed *in vivo* in L cells[23]. Similarly, *in vitro*, endonuclease II from *E. coli* releases 3-methyladenine preferentially from dimethylsulfate-treated DNA[24]. This bacterial endonuclease has both *N*-glycosidase and phosphodiesterase activities that appear to be functions of a single protein[25]. The data in Fig. 4 indicates that the human lymphoblast activity specific for alkylated DNA also has phosphodiesterase activity, however the kinetics favor the notion that this is due to contaminating AP-endonucleases. (This contamination is detectable only under optimum conditions for AP-endonuclease (i.e. 0.5mM Mg^{++}).) Current efforts in this laboratory are aimed at further purification of this activity and direct assay of *N*-glycosidase by detecting the removal of methylated bases from DNA.

Although the function of *N*-glycosidases, of the type suggested here, is unknown, their specificity for altered DNA bases makes them excellent candidates for a damage-recognition role in DNA repair. The sequential action of such an *N*-glycosidase followed by the phosphodiesterase action of the AP-endonuclease could constitute the incision step in excision repair of alkylated DNA in mammalian cells.

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