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DEFECTIVE DNA ENDONUCLEASE ACTIVITY ON ANTHRAMYCIN TREATED DNA IN XERODERMA PIGMENTOSUM AND MOUSE MELANOMA CELLS

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Nine separate DNA endonuclease activities from non-histone chromatin proteins and a corresponding set from the nucleoplasm of normal human and xeroderma pigmentosum, complementation group A, lymphoblastoid and Cloudman mouse melanoma cells, obtained by isoelectric focusing, were tested against circular duplex phage PM2 DNA previously treated with anthramycin. A marked increase in activity against anthramycin treated DNA was found in normal human lymphoblastoid cells in a chromatin fraction with pI 4.6, with lesser increases at pI's 3.9 and 5.4 and a nucleoplasmic fraction at pI 4.6. In the nuclear proteins of xeroderma pigmentosum and mouse melanoma cells, however, no increase in activity against anthramycin DNA could be detected in any fraction.

INTRODUCTION

Anthramycin is an antitumor antibiotic produced by Streptomyces refuineus which belongs to a structurally unique group of compounds, the pyrrolo(1,4) benzodiazepines (1-3). It forms a covalent adduct with the N-2 guanine of DNA (4,5), rendering the DNA (anthramycin-DNA complex) resistant to digestion by a number of restriction enzymes (6). Anthramycin inhibits synthesis of nucleic acids (7-9), can induce sister chromatid exchanges (10), induces unscheduled DNA synthesis (UDS) and undergoes excision removal from DNA in excision repair proficient cells (11). Cultured fibroblasts from individuals with the autosomal recessive disease, xeroderma pigmentosum (XP), which are defective in repair of damage to DNA produced by a number of physical and chemical agents, however, have been reported to show negligible amounts of UDS and only partial removal of anthramycin-induced lesions by an excision repair process following treatment with this drug (11).

We have recently isolated and partially purified nine separate DNA endonuclease activities from non-histone chromatin proteins and a corresponding set of nucleoplasmic deoxyribonucleases (DNases) from both normal human and XP, complementation group A (XPA), lymphoblastoid cells and from Cloudman mouse melanoma cells (12,13). Both XP (14-16) and some mouse cells (17-19) are thought to be defective in the excision type of DNA repair. XP cells are of particular interest to us because, in the seven known

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classical complementation groups of this disease, there is a defect in the initial, incision step in excision repair which is mediated by a DNA endonuclease (14-16, 20-22). All of the enzyme activities which we have isolated correlated quite closely between the three cell types, with several important quantitative differences (12,13). We now report one of these enzyme activities in normal human lymphoblastoid cell chromatin to be exceedingly more active on anthramycin-treated DNA (AnDNA) than on untreated DNA, as well as lesser increases in activity in two other chromatin and one nucleoplasmic fractions, and that no increased activity on AnDNA was found in XP lymphoblastoid or mouse melanoma cells.

MATERIALS AND METHODS

Normal (GM 1989) and XPA (GM 2345) lymphoblastoid cell lines (transformed with Epstein-Barr virus) were obtained from the Institute for Medical Research, Camden, New Jersey. The cell lines were grown in suspension culture at 37°C in sealed flasks in RPMI 1640 medium buffered to physiological pH with Hepes' buffer (Grand Island Biological Co., GIBCO), supplemented with 15% fetal calf serum (GIBCO), and were harvested under conditions of maximal proliferation. Cultures were routinely tested for mycoplasma (23), were not exposed to UVL, and other light exposure was minimal. Cloudman mouse melanoma cells (S91 NCTC 3960, CCL 53) were propagated by serial subcutaneous injection into male DBA/2J mice and the tumors were harvested as previously described (13). Lymphoblastoid cell nuclei were isolated following the method of Berkowitz et al. (24). Melanoma cell nuclei were isolated as previously described (13). Chromatin-associated and nucleoplasmic proteins were extracted from the purified nuclei and subjected to electrophoresis on an isoelectric focusing column (13,25). Fractions collected from the column were assayed for DNA endonuclease activity against calf thymus DNA as previously described (13,26), and peaks of endonuclease activity were pooled, dialyzed into a 50 mM phosphate buffer, pH 7.1, containing 40% ethylene glycol, and stored unfrozen at -20°C (13).

Circular, duplex, superhelical PM2 DNA (Boehringer Mannheim) was reacted with anthramycin-11-methyl-ether (lots F-59, F-60) (gift of Hoffmann-LaRoche Laboratories) at 37°C for 3 hours and dialyzed into 20 mM Tris, pH 7.5. The extent of binding of anthramycin to DNA was determined spectrophotometrically by the method of Kohn and Spears (27) which employs the absorbance ratio, A_{321nm}/A_{349nm}, taking the ratios for free and bound anthramycin to be 1.134 and 0.709, respectively. An amount of each endonuclease fraction sufficient to give submaximal activity against untreated DNA was reacted with untreated and AnDNA in 5 mM $MgCl_2$, 20 mM KCl and 10 mM Tris-maleate, pH 7.5. The reaction was terminated with 0.1 M EDTA and the sample prepared and electrophoresed on 0.9% agarose gels as previously described (13). The gels were subsequently stained with ethidium bromide, photographed under ultraviolet light (13) and the negatives scanned using a Zeineh Soft Lazer Scanning Densitometer (LKB). Endonuclease activity, measured as the number of enzyme induced breaks per DNA molecule, was detected by the conversion of superhelical DNA (Form I), to nicked circular DNA (Form II) and linear unit length DNA (Form III). Quantitation of the amount of cut versus superhelical DNA was made from densitometric tracings of negatives of photographs of the gels. The fraction of superhelical and nicked molecules was determined by summing the integrated area under each peak, and the number of breaks was estimated by the Poisson formula, number of breaks = $-\ln x$, where x = fraction of superhelical molecules (28,29).

All experiments were repeated 4-6 times using enzymes obtained from 2-3 different cell extractions.

TABLE I. DNA endonuclease activities on anthramycin-treated PM2 DNA of xeroderma pigmentosum (XP) and normal human (NL) lymphoblastoid and mouse melanoma (MM) chromatin-associated proteins

Isoelectric Point (± 0.3)	Concentration of anthramycin (mM)	Number of enzyme-induced scissions per PM2 DNA molecule[a] (mean ± standard error) NL[b]	XP[b]	MM[c]
3.9	1.0	0.56 ± 0.11	0.01 ± 0	0 ± 0
3.9	0.5	0.21 ± 0.06	0 ± 0	0 ± 0
4.6	1.0	> 5.00 ± 0	0.04 ± 0.03	0.02 ± 0.02
4.6	0.5	0.60 ± 0.03	0 ± 0	0 ± 0
5.4	1.0	0.36 ± 0.09	0.03 ± 0.04	0.01 ± 0.01
5.4	0.5	0 ± 0	0 ± 0	0 ± 0
6.6	1.0	0.01 ± 0.01	0 ± 0	0.02 ± 0.02
6.6	0.5	0 ± 0	0 ± 0	0 ± 0
7.6	1.0	0.02 ± 0.01	0.02 ± 0.02	0.04 ± 0.03
7.6	0.5	0 ± 0	0 ± 0	0 ± 0
8.2	1.0	0.04 ± 0.02	0.03 ± 0.03	0.07 ± 0.06
8.2	0.5	0 ± 0	0 ± 0	0 ± 0
8.8	1.0	0.06 ± 0.03	0 ± 0	0.04 ± 0.04
8.8	0.5	0 ± 0	0 ± 0	0 ± 0
9.2	1.0	0 ± 0	0.01 ± 0	0 ± 0
9.2	0.5	0 ± 0	0 ± 0	0 ± 0
9.8	1.0	0 ± 0	0 ± 0	0 ± 0
9.8	0.5	0 ± 0	0 ± 0	0 ± 0

[a] This value has had subtracted from it the number of breaks the enzyme produced on normal DNA and the number of breaks in nonenzyme treated DNA.

[b] 11 ug of fractions (pI 3.9 - 5.4) or 21 ug of fractions (pI 6.6 - 9.2) were incubated with 0.18 ug anthramycin-treated PM2 DNA at 37°C for 30 minutes.

[c] 18 ug of each fraction was incubated with 0.18 ug anthramycin-treated PM2 DNA for 30 mintues (pI 3.9 - 5.4) or 1 1/2 hours (pI 7.6 - 9.8) at 37°C.

RESULTS

Binding of anthramycin to DNA was determined spectrophotometrically: the absorbance ratio A_{321nm}/A_{349nm} for 1 mM anthramycin was 1.160 for free and 0.721 for bound anthramycin, and for 0.5 mM anthramycin 1.100 for free and 0.758 for bound. These values are similar to those obtained by Kohn and Spears (27). Anthramycin treatment of PM2 DNA did not produce breaks in the DNA, both treated and untreated DNA were > 85% form I, as determined by analysis on 0.9% agarose gels. The concentration and incubation time of each enzyme activity was adjusted to produce > 68% form I DNA or < 0.25 breaks per DNA molecule on untreated DNA. AnDNA was also treated with S_1 nuclease (Sigma Chemical Co.) to determine whether anthramycin produced an increased number of single-strand regions in PM2 DNA. S_1 nuclease was active on

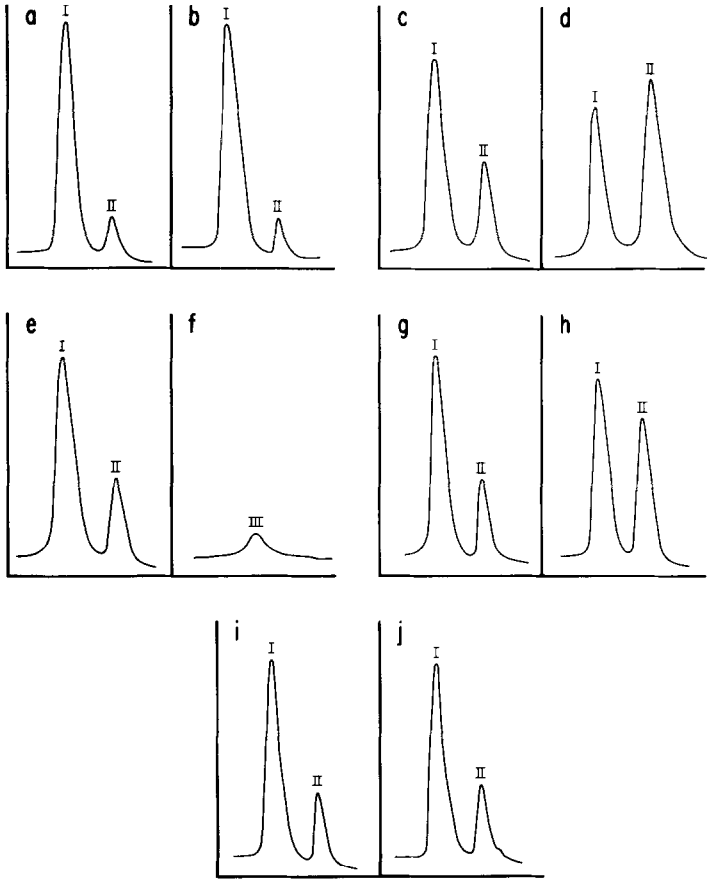

Figure 1: Densitometric tracings of untreated PM2 DNA and anthramycin-treated (1mM) PM2 DNA (AnDNA) reacted with chromatin-associated DNA endonuclease fractions from normal human lymphoblastoid cells. DNA (0.18 ug) was incubated for 30 min at 37° C with fractions having the following pI's: a) untreated, no enzyme; b) AnDNA, no enzyme; c) untreated, 11 ug pI 3.9; d) AnDNA, 11 ug pI 3.9; e) untreated, 11 ug pI 4.6; f) AnDNA, 11 ug pI 4.6; g) untreated, 11 ug pI 5.4; h) AnDNA, 11 ug pI 5.4; i) untreated, 18 ug pI 6.6; j) AnDNA, 18 ug pI 6.6.

native, untreated PM2 DNA. When submaximal concentrations of S_1 nuclease were reacted with AnDNA, the enzyme showed no greater activity on AnDNA than it did on untreated DNA.

The activity of each of the chromatin-associated and nucleoplasmic DNA endonuclease fractions was tested on PM2 DNA treated with a range of concentrations of anthramycin (0.5 mM - 10 mM). As can be seen in Table 1 and Figs. 1 and 2, one chromatin-associated endonuclease activity (pI 4.6) from normal human lymphoblastoid cells was exceedingly more active on PM2 DNA treated with 1 mM anthramycin than on native DNA, producing > 5.0 breaks per DNA molecule (this was the limit of resolution of our system). Two other normal human chromatin-associated activities (pIs 3.9 and 5.4) (Table 1, Figs., 1 and 2) and one nucleoplasmic activity (pI 4.6) (Table 2, Fig. 3) also

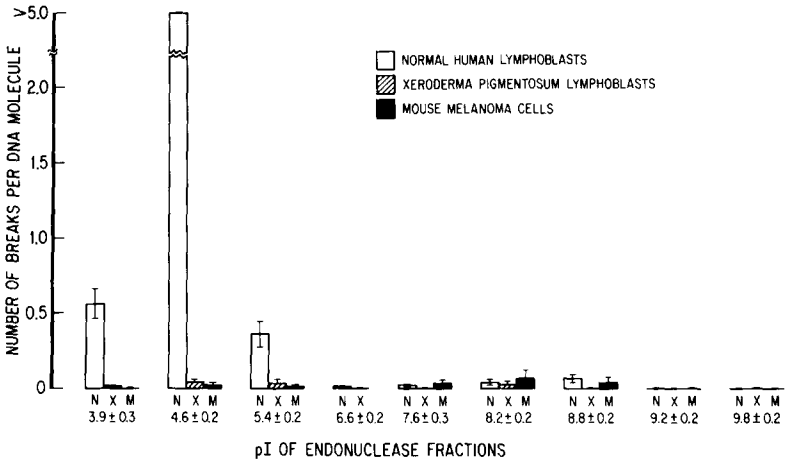

Figure 2: Action of chromatin-associated DNA endonuclease fractions on anthramycin-treated (1 mM) PM2 DNA. Vertical lines represent ± standard error of the mean.

showed greater activity on DNA treated with 1 mM anthramycin than on native PM2 DNA. These fractions were also slightly active on DNA treated with 0.5 mM anthramycin (Tables 1 and 2) but showed no activity on DNA treated with 10 mM or 0.1 mM anthramycin. In marked contrast to this no DNA endonuclease activity was found in the nuclei of XPA or mouse melanoma cells which was more active on AnDNA than on native DNA (Figs. 2 and 3, Tables 1 and 2).

TABLE II. DNA endonuclease activities on anthramycin-treated PM2 DNA of xeroderma pigmentosum (XP) and normal human (NL) lymphoblastoid and mouse melanoma (MM) nucleoplasmic proteins

Isoelectric Point (± 0.2)	Concentration of anthramycin (mM)	Number of enzyme-induced scissions per PM2 DNA molecule[a] (mean ± standard error) NL[b]	XP[b]	MM[c]
3.7	1.0	6.06 ± 0.02	0 ± 0	0.01 ± 0.01
3.7	0.5	0.01 ± 0.01	0 ± 0	0 ± 0
4.6	1.0	0.99 ± 0.24	0.02 ± 0.02	0.08 ± 0.07
4.6	0.5	0.61 ± 0.15	0 ± 0	0.07 ± 0.06
5.6	1.0	0.04 ± 0.02	0 ± 0	0 ± 0
5.6	0.5	0.02 ± 0.01	0 ± 0	0 ± 0

[a] This value has had subtracted from it the number of breaks the enzyme produced on normal DNA and the number of breaks in nonenzyme treated DNA.

[b] 10 ug of fractions (pI 3.7 and 5.6) or 2 ug of fraction (pI 4.6) were incubated with 0.18 ug anthramycin-treated PM2 DNA for 30 minutes at 37°C.

[c] 10 ug of each fraction was incubated with 0.18 ug anthramycin-treated PM2 DNA for 45 minutes at 37°C.

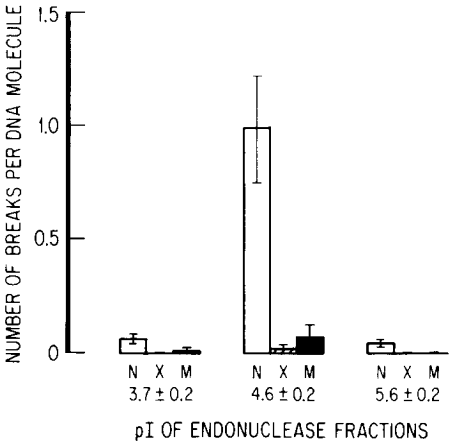

Figure 3: Action of nucleoplasmic DNA endonuclease fractions on anthramycin-treated (1mM) PM2 DNA. Vertical lines represent + standard error of the mean. □ normal human and ▨ xeroderma pigmentosum lymphoblastoid cells; ■ mouse melanoma cells.

DISCUSSION

XP cells are known to be defective in the initial, endonuclease-mediated incision step of excision repair of lesions induced into DNA not only by ultraviolet light, but also by a number of chemical agents (14-16,20-22,30), and it has been shown that XPA fibroblasts are deficient in their ability to repair anthramycin induced DNA lesions (11,31). Reports on DNA repair in mouse cell lines are conflicting, possibly due to differentiation factors, but again there is evidence for defective nucleotide excision mechanisms (17-19,30). To date, however, no specific defective DNA endonuclease has been isolated in XP or mouse cells.

The results presented here demonstrate a DNA endonuclease activity, pI 4.6, which is markedly increased against AnDNA in the non-histone chromatin protein of normal cells and which is absent from the nuclei of both XPA lymphoblastoid and Cloudman mouse melanoma cells. The relationship between this activity and the lesser, chromatin-associated activities at pI's 3.9 and 5.4 and of the nucleoplasmic one at pI 4.6 are not clear at present, but this does not alter the fundamental implication that there is an endonuclease with marked specificity for AnDNA in normal human chromatin that is inactive in these other cell types. It is possible but quite unlikely that these differences are related to the Epstein-Barr virus mediated transformation of these lymphoblastoid cell lines. Although XP cell lines from the same complementation groups have been shown to be heterogeneous in their ability to repair certain types of alkylated DNA (32,33), and transformation with SV40 virus may induce a defect in repair of such DNA (34), this phenomenon appears to be essentially restricted to O^6 alkylguanine residues (30), whereas in AnDNA the adduct is on the N2 of guanine (4,5). Our recent finding of nearly

identical overall nuclear DNase patterns in human lymphoblastoid and mouse melanoma cell lines suggests that there is little non-specific variability in these enzymes (Lambert et al., in preparation). Conceivably this increased activity against AnDNA could be due to a glycosylase plus an endonuclease with specificity for apurinic/apyrimidinic (AP) sites in this fraction, but we believe that this is very unlikely, since we have found AP site-specific DNA endonuclease activity in all three cells lines to be primarily in the enzyme fractions with basic pI's, with very little activity in the pI 4.6 chromatin fraction (Lambert et al., in preparation). Furthermore, a recent study has indicated that reaction of PM2 DNA with anthramycin does not result in depurination (35). That this increased activity on AnDNA is not due to a single-strand specific DNA endonuclease is shown by our studies with S_1 nuclease, an enzyme specifically active on single-stranded DNA (36,37). This work is in agreement with that of Hurley et al (33). We have found, moreover, in XPA lymphoblastoid cells, and, to a lesser extent in mouse melanoma cells, that this same chromatin-associated enzyme fraction, pI 4.6, has markedly increased activity against untreated linear, duplex DNA (Lambert et al., in preparation) indicating that there may be more than one abnormality in this enzyme activity.

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