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Removal by Human Apurinic/Apyrimidinic Endonuclease 1 (Ape 1) and *Escherichia coli* Exonuclease III of 3′-Phosphoglycolates from DNA Treated with Neocarzinostatin, Calicheamicin, and γ-Radiation

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ABSTRACT. DNA strand breaks with terminal 3′-phosphoglycolate groups are produced by agents that can abstract the hydrogen atom from the 4′-carbon of DNA deoxyribose groups. Included among these agents are γ-radiation (via the OH radical) and enediyne compounds, such as neocarzinostatin and calicheamicin. However, while the majority of radiation-induced phosphoglycolates are found at single-strand breaks, most of the phosphoglycolates generated by these two enediynes are found at bistranded lesions, including double-strand breaks. Using a ^{32}P-post-labelling assay, we have compared the enzyme-catalyzed removal of phosphoglycolates induced by each of these agents. Both human apurinic/apyrimidinic endonuclease 1 (Ape 1) and its *Escherichia coli* homolog exonuclease III rapidly removed over 80% of phosphoglycolates from γ-irradiated DNA, although there appeared to be a small resistant subpopulation. The neocarzinostatin-induced phosphoglycolates were removed more slowly, though not to completion, while the calicheamicin-induced phosphoglycolates were extremely refractory to both enzymes. These data suggest that unless other enzymes are capable of acting upon the phosphoglycolate termini at enediyne-induced double-strand breaks, such termini will be resistant to end rejoining repair pathways. BIOCHEM PHARMACOL **57**;5:531–538, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. enediyne; human apurinic/apyrimidinic endonuclease (Ape); exonuclease III; phosphoglycolates; DNA damage; DNA repair

Free-radical oxidative DNA damage is considered to be the principal cause of reproductive cell death induced by many cytotoxic agents, including ionizing radiation and several potent chemotherapeutic agents. Free radical attack of DNA can generate base damage, DNA-protein cross-links, and strand breaks. Strand breakage is often accompanied by base loss and deoxyribose phosphate modification or fragmentation, creating terminal groups such as 5′-nucleoside aldehyde and 3′-phosphoglycolate residues [1–4]. Before strand rejoining can occur, such groups have to be removed in order to provide DNA termini suitable for DNA polymerases and ligases to restore missing nucleotides and reseal the breaks, respectively. In *Escherichia coli*, removal of phosphoglycolates is mediated by exonuclease III or endonuclease IV, which cleave the phosphodiester bond immediately 5′ to the phosphoglycolate residue, releasing it as phosphoglycolic acid [5, 6]. In human cells, this function may be carried out by Ape 1¶, which shares many similarities to *E. coli* exonuclease III, although there may be other, as yet unidentified, nucleases that also have the capability to remove phosphoglycolates [6–8].

There is now a growing body of evidence that indicates that bistranded oxidative lesions (i.e. closely located base lesions or strand breaks on opposite strands) are particularly deleterious to cells [9]. The most familiar bistranded lesions are of course double-strand breaks. In contrast to ionizing radiation and other oxidative agents, members of the enediyne family of antitumour antibiotics are unique for their potential to generate bistranded DNA lesions in relatively high yield in comparison with single lesions [3, 4]. For DNA treated with NCS in the presence of glutathione,

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¶ *Abbreviations*: Ape 1, human apurinic/apyrimidinic endonuclease 1; NCS, neocarzinostatin; CAL, calicheamicin; SVP, snake venom phosphodiesterase; PNK, T4 phage polynucleotide kinase; and pg, phosphoglycolate.

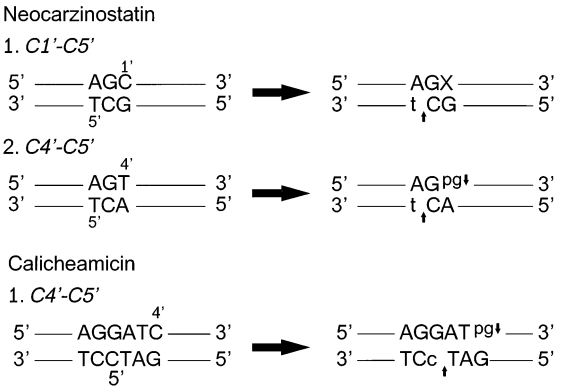

FIG. 1. Major identified bistranded DNA lesions produced by NCS and CAL. C1′, C4′, and C5′ indicate the deoxyribose carbon atoms from which hydrogen atoms are abstracted. The short arrows indicate sites of strand cleavage. X represents a 4′-hydroxylated abasic site; t and c represent a 5′-aldehyde at thymidine and deoxycytidine, respectively; pg represents a 3′-phosphoglycolate.

for example, the ratio of single-strand:double-strand lesions is approximately 2:1 [10]. The reaction of DNA with CAL, an even more potent enediyne cytotoxin, produces a single-strand:double-strand lesion ratio of at least 1:20 [11]. Much of the enediyne data have been accrued from experiments with restriction fragments of plasmid DNA, allowing sequence hot spots to be identified and the double-strand lesions at these sites to be fully characterized. The major bistranded products following DNA reaction with these enediynes are shown in Fig. 1. NCS simultaneously abstracts hydrogen from C5′ of deoxyribose in one strand, and from C1′ or C4′ in the opposite strand [12, 13], resulting in formation of a strand break with the terminal nucleoside bearing a 5′-aldehyde, opposite either an abasic site with the deoxyribose converted to a deoxyribonolactone (C1′ abstraction), or a strand break with a 3′-phosphoglycolate residue (C4′ abstraction). Similarly, the dominant lesion produced by CAL arises from simultaneous hydrogen abstraction from C5′ and C4′ [14–16], although the hot spot sequences and stagger between the opposing lesions differ from the NCS damaged sites.

It has been shown previously that the abasic site generated by NCS is resistant to cleavage by exonuclease III, most probably because of the opposing strand break [17]. The purpose of this study was to compare the removal mediated by Ape 1 and exonuclease III of phosphoglycolate groups from DNA treated with NCS, CAL, and γ-radiation, and thereby gauge the influence of the local complexity of the DNA lesion on repair. To do this, we made use of a post-labelling assay developed in our laboratory [18] and outlined in Fig. 2. The assay takes advantage of the fact that certain base and deoxyribose modifications, including phosphoglycolate groups, inhibit cleavage by SVP of the internucleotide phosphate group immediately 5′ to the lesion. Complete digestion of damaged DNA with SVP and alkaline phosphatase, therefore, releases these lesions covalently attached to the 5′-neighbouring nucleotide, and such moieties can be efficiently ^{32}P-labelled by incubation with PNK and [γ-^{32}P]ATP. On the other hand, unmodified DNA and other lesions that do not inhibit SVP are released as mononucleosides and remain undetected because mononucleosides are not substrates for PNK.

MATERIALS AND METHODS

Enzymes

DNase I (10,000 U/mL) and nuclease P1 (600 U/mL) were obtained from Life Technologies Inc. PNK and shrimp alkaline phosphatase were from Amersham. SVP (*Crotalus atrox*, type IV, 1 g/mL, 22 U/mL) was purchased from the Sigma Chemical Co. Unit definitions of these enzymes have been described before [18]. Exonuclease III was obtained from Life Technologies Inc. One unit of exonuclease III produces 1 nmol of acid-soluble nucleotide from sonicated DNA in 30 min at 37°. The purification of

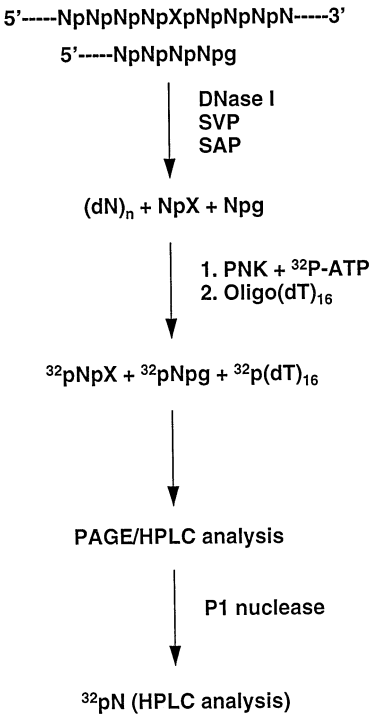

FIG. 2. Scheme of the post-labelling assay. DNA is digested completely by DNase I, snake venom phosphodiesterase (SVP), and shrimp alkaline phosphatase (SAP) to yield undamaged mononucleosides (dN), damage-containing dinucleosides (NpX, where X represents a damaged base, e.g. thymine glycol or abasic site), and nucleoside-3′-phosphoglycolates (Npg). This mixture is then incubated with PNK and [γ-^{32}P]ATP to terminally phosphorylate the NpX and Npg species. (Mononucleosides are not a substrate for PNK.) Most of the excess ATP is then consumed by incubation with a long oligonucleotide. Following separation and quantification of the labelled products by polyacrylamide gel electrophoresis, material in the bands of interest can be eluted from the gel and further analyzed by HPLC. Nuclease P1 can cleave the ^{32}pNpX or ^{32}pNpg products to release the labelled 5′-mononucleotides (^{32}pN) and thus enable nearest-neighbour analysis of the nucleosides 5′ to the DNA lesions.

recombinant human Ape 1 protein was according to Wilson *et al.* [19]. One unit of enzyme cleaves or releases 1 pmol of damaged sites/min from a synthetic substrate [20] under standard conditions [21].

Drug and Radiation Treatment of DNA

One hundred micrograms of calf thymus DNA was incubated with various concentrations of NCS and CAL (stock solutions of drugs were prepared in methanol) for 1 hr at 0° in a buffer containing 10 mM glutathione, 50 mM HEPES, 1 mM EDTA (pH 7.5) in 1 mL reaction volume. The DNA was then precipitated with 100 μL of 0.3 M sodium acetate (pH 7.0) and 2.2 mL ethanol and washed with 70 and 95% ethanol. The dried DNA was resuspended in 100 μL of water, from which 1 μL was removed to determine DNA concentration. DNA (500 μg/mL) in 10 mM sodium phosphate buffer (pH 7.5) was irradiated with 50 Gy at room temperature, passed over a G50 (Sephadex) desalting column, precipitated with ethanol as described above, and resuspended in 100 μL of water.

Ape 1 and Exonuclease III Reaction Conditions

Irradiated or drug-treated DNA (5 μg) was incubated at 37° with 70 U/μg DNA (unless otherwise stated) of Ape 1 protein in the reaction buffer containing 10 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 μg/mL of BSA. Similarly, the DNA samples were incubated with 2 U/μg DNA (unless otherwise stated) of exonuclease III in 10 mM Tris–HCl (pH 7.6) and 5 mM $MgCl_2$ at 37°. Aliquots were removed at various times, and the reaction was stopped by heating the samples at 95° for 10 min.

Post-labelling Assay

The post-labelling assay was carried out essentially as described before [18]. Briefly, 5 μg of untreated, drug-treated, or irradiated DNA, after incubation with Ape 1 protein or exonuclease III, was digested overnight at 37° with 0.02 U of SVP, 0.4 U of DNase I, and 0.4 U of shrimp alkaline phosphatase. The enzymes were precipitated by the addition of 3 vol. of ice-cold ethanol and removed by centrifugation (10,000 *g*, 15 min). The supernatants were evaporated, and the resulting residues were dissolved in 50 μL of distilled water, heated at 100° for 10 min to inactivate residual nuclease and phosphatase activity, and then stored at −20°.

One hundred nanograms of digested DNA was phosphorylated in 10 μL reaction volume with 10 U of PNK in a buffer provided by the supplier of the enzyme (Amersham) and 2.5 μCi (830 fmol) of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) at 37° for 30 min, and then the bulk of the excess ATP was consumed by incubation for a further 15 min with 1 μL of oligo$(dT)_{16}$ (5 A_{260} U/mL, Pharmacia) and 5 U of the kinase. An equal volume of formamide loading buffer [22] was added to each sample, and one-tenth of the sample was analyzed on a 1.5-mm thick, 20% polyacrylamide gel containing 7 M urea. The bands corresponding to the phosphoglycolates were excised from the gel after autoradiography. The radioactivity in each gel band was determined with a liquid scintillation counter (Beckman, model LS 5801). Alternatively, the gel was scanned in a PhosphorImager (GS-250 Molecular Imager™ System, Bio-Rad) and quantified by Molecular Analyst™ software (Bio-Rad).

HPLC Analysis

Because pApg and pTpg, the labelled products of deoxyadenosine- and thymidine-3′-phosphoglycolate, comigrate on polyacrylamide gel, HPLC was required to complete the analysis of the base 5′ to the phosphoglycolate groups. Two methods were used. The first involved enzymatic hydrolysis of the phosphodiester bond to release the labelled 5′-nucleotides, which were then analysed by reverse-phase HPLC. The second method was direct analysis of pApg and pTpg by ion-exchange HPLC. Although the latter approach was more straightforward, the resolution of the two labelled products was inferior to that of the 5′-nucleotides.

For the first method, the radioactive material (50,000–300,000 cpm) in the gel band containing pApg and pTpg was eluted from the gel in 1.5 mL of distilled water, and salt and urea were removed by passage through a C_{18} Sep-pak cartridge (Waters) [23]. Then 10,000–30,000 cpm was digested with 6 U of nuclease P1 in 1 mL of buffer (10 mM sodium acetate, pH 5.3, 1 mM $ZnSO_4$) at 37° for 2 hr. The whole sample was injected onto the HPLC column. The HPLC instrumentation consisted of a Varian 5000 liquid chromatograph (Varian Canada Inc.) coupled to a Waters μBondapak C_{18} reverse-phase column (3.9 × 300 mm, Waters), a Tracor 970A UV detector (Tracor Instruments), and a Berthold LB504 radioactivity monitor. The elution conditions were as follows: 100% buffer A (50 mM NaH_2PO_4, pH 4.5) and 0% buffer B [100 mM NaH_2PO_4/methanol (1:1 v/v)] for 5 min, followed by a linear gradient to 50% buffer A/50% buffer B over 40 min at a flow rate of 0.5 mL/min. Under these conditions, thymidine 5′-monophosphate eluted at 32 min and deoxyadenosine 5′-monophosphate at 38 min.

For the second method, the eluted material (10,000–30,000 cpm), dissolved in 1 mL of water, was injected onto a Whatman Partisil 10 SAX column (4.6 × 250 mm, Whatman), and eluted with a gradient of 0.08 to 0.24 M potassium phosphate, pH 7.0, over 40 min at a flow rate of 0.5 mL/min. Under these conditions, pTpg eluted at 14.8 min and pApg at 18.4 min.

RESULTS

Quantitation of Phosphoglycolate from NCS-, CAL-, and γ-Radiation-Treated DNA

Calf thymus DNA was treated with 0.2, 2, and 20 μM NCS or CAL, as described in Materials and Methods, or irradi-

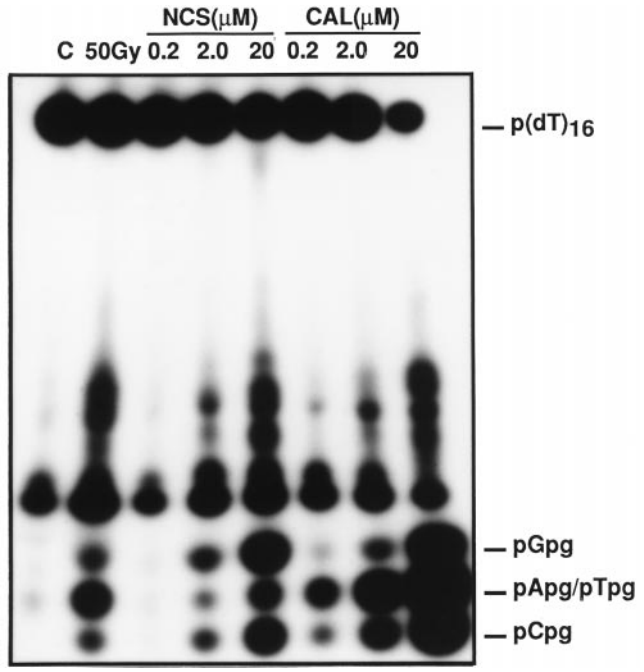

FIG. 3. Autoradiogram of a polyacrylamide gel showing the post-labelled DNA damage-containing products generated by radiation (50 Gy), and increasing concentrations of NCS and CAL. The three fastest migrating bands contain the phosphoglycolate species (pNpg). The identities of the slower migrating species observed in the NCS- and CAL-treated DNA were not determined. The oligonucleotide $(dT)_{16}$ was used to consume the excess ATP.

ated with 50 Gy ^{60}Co γ-rays. These DNA samples were analyzed by the post-labelling assay to determine the induction of 3′-phosphoglycolate groups (Fig. 3). We have determined previously [18] that, under the gel electrophoresis conditions used, phosphoglycolate-bearing nucleotides (pNpg), because of their high charge to mass ratio, are the fastest-migrating detectable DNA-damage products. The yields of the phosphoglycolate products could be ascertained on the basis of the percentage of the total counts in each lane found in the phosphoglycolate bands multiplied by the number of femtomoles of ATP used for the labelling reaction. (In control experiments, we have found that the labelling efficiency of the phosphoglycolate-containing molecules is ~80%.) The 2 μM concentration of NCS and the 0.2 μM concentration of CAL gave levels of phosphoglycolates comparable to those generated by 50 Gy irradiation: 560, 580, and 690 fmol per μg of DNA, respectively. These DNA samples were chosen for further analysis. To obtain the ratio of the bases immediately 5′ to the phosphoglycolate groups, HPLC was required to separate pApg from pTpg, which comigrate under the electrophoretic conditions used (Fig. 3). The ratios for the four bases are provided in Table 1.

Removal of Phosphoglycolate from Enediyne-Treated and Irradiated DNA by Ape 1

The DNA treated with 2 μM NCS, 0.2 μM CAL, and 50 Gy radiation was incubated with 70, 140, and 210 U of Ape 1 for 1 hr at 37° (see Materials and Methods for unit definition). The reaction products were analyzed for the removal of phosphoglycolates by the post-labelling assay. The resulting autoradiograms are shown in the left panels of Fig. 4, and the quantitation from three separate experiments is shown in the right panel of Fig. 4. Seventy units of Ape 1 were capable of removing approximately 70% of the phosphoglycolate from 50 Gy irradiated DNA. By contrast, the phosphoglycolates generated by the enediyne reagents, particularly CAL, were more resistant to removal.

Three additional features of the autoradiograms (Fig. 4, left panels) are worth noting. First, the lesions in the bands marked 6 and 7 (Fig. 4, left bottom panel) observed in the untreated control, as well as the enediyne- and radiation-treated DNA, were removed completely after incubation with the lowest concentration of enzyme. These bands probably contain labelled products bearing abasic sites, which we have shown previously are detectable by the post-labelling assay [24]. The fact that they were effectively acted upon by the enzyme indicates that the poor removal of the phosphoglycolates in the enediyne-treated samples was not due to the presence of an inhibitor of the enzyme. Second, it is noticeable that enzymatic removal of the pCpg band (lower band) in the NCS-damaged DNA was greater than that of the pGpg band (upper band). Measurement of the radioactivity in these bands, showed that 70 U of Ape 1 reduced pGpg by ~5%, but pCpg by ~60%. Six units of exonuclease III caused a similar level of reduction. Third, even at the highest concentrations of the enzymes, a residual quantity of phosphoglycolate in the irradiated DNA persisted.

A more detailed study monitoring the kinetics of phosphoglycolate removal by incubation with 70 U of Ape 1 is shown in Fig. 5. The γ-radiation-induced phosphoglycolates were repaired very rapidly, so that even at the shortest time interval (2 min) less than 40% of the phosphoglycolates remained. The phosphoglycolates from the DNA samples treated with 0.2 μM CAL were the most resistant to the endonuclease, and the rate of repair of the NCS-induced phosphoglycolates appeared to be intermediate. In the case of the irradiated and NCS-treated samples, the best fit to the data was a two-phase exponential decay function (Prism software). This would suggest that there are two classes of phosphoglycolate groups generated by these agents, one that is repaired rapidly by Ape 1 and one that is acted upon at a substantially slower rate.

TABLE 1. Analysis of the composition of the bases immediately 5′ to the phosphoglycolates generated by NCS and CAL

	A	C	G	T
NCS (2.0 μM)	54* (1.2)†	182 (4.1)	280 (6.4)	44 (1)
CAL (0.2 μM)	95* (1.3)†	139 (2.0)	71 (1)	275 (3.9)

*Units are expressed in fmol/μg DNA.

†Numbers in parentheses indicate the ratio of the four bases, with the lowest normalized to 1.

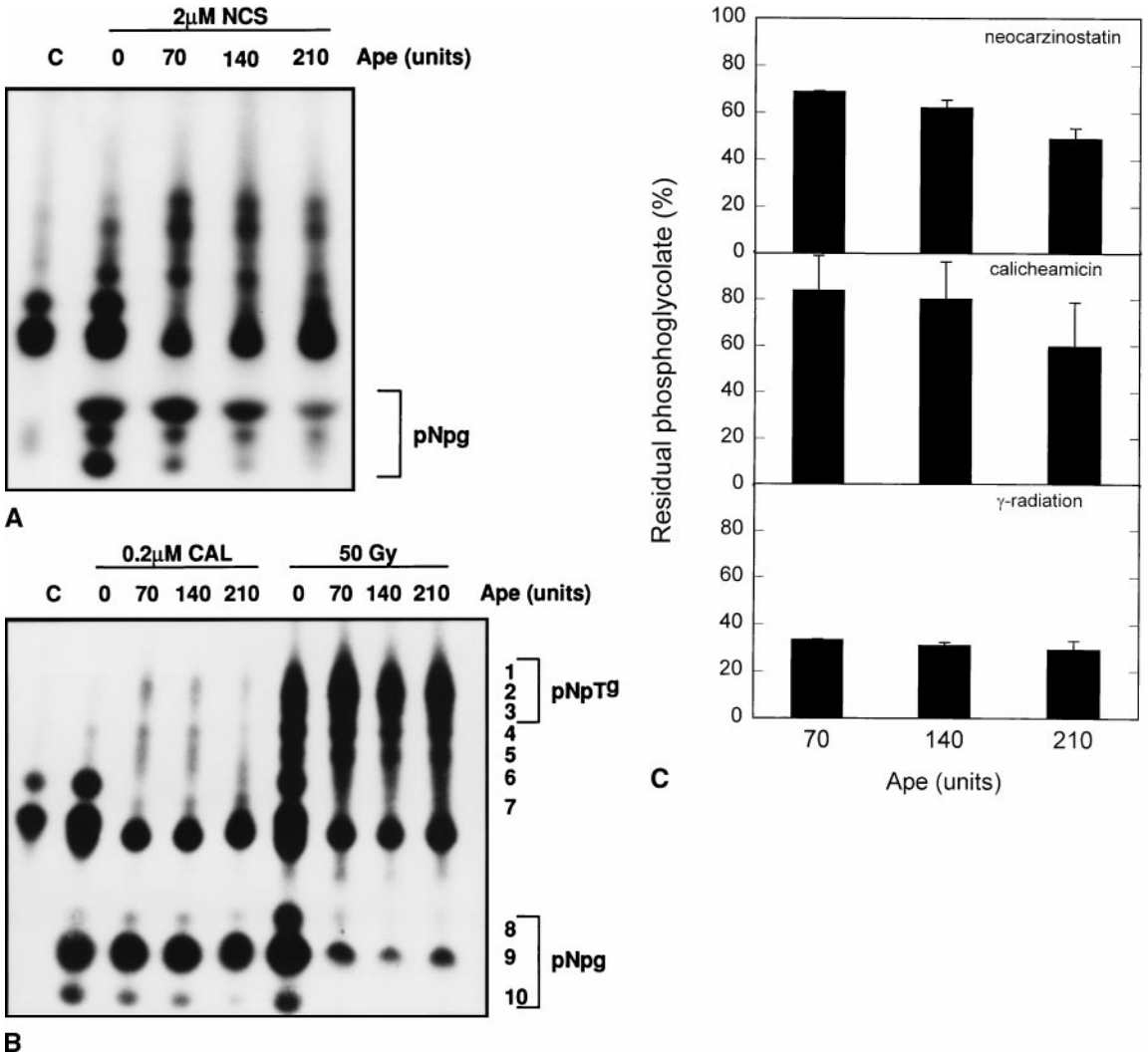

FIG. 4. Removal of phosphoglycolate by increasing concentrations of Ape 1. (Left top panel) Autoradiogram of the post-labelled products after incubation of NCS-treated calf thymus DNA. The base 5′ to the phosphoglycolates in the pNpg bands follows the same pattern displayed in Fig. 3, i.e. pGpg (upper), pApg/pTpg (middle), pCpg (lower). Lane C is a control of calf thymus DNA untreated with NCS or Ape 1. (Left bottom panel) Similar autoradiogram of the post-labelling analysis of CAL-treated and irradiated DNA. Bands in the irradiated DNA marked 1–3 have been shown previously to contain thymine glycols (T^g). Bands 6 and 7 probably contain abasic sites already present in the untreated DNA and augmented by the damaging agents. (Right panel) Quantification of phosphoglycolate removal. Error bars indicate SD from three independent analyses.

Removal of Phosphoglycolate from Enediyne-Treated and Irradiated DNA by E. coli *Exonuclease III*

A similar set of analyses was performed on the damaged DNA, comparing the removal of phosphoglycolates after incubation with 2, 6, and 20 U (see Materials and Methods for unit definition) of exonuclease III for 1 hr at 37° (Fig. 6) and then following the rate of removal over 1 hr using 2 U of the enzyme (Fig. 7). The data revealed a very similar pattern to that observed with the human endonuclease, i.e. rapid loss of most of the phosphoglycolates from the γ-irradiated DNA, intermediate activity with NCS-treated DNA, and limited activity toward CAL-treated DNA. Again, the best fit to the kinetic data for the radiation and NCS-treated DNA was a two-phase exponential decay function.

DISCUSSION

Previous DNA sequencing studies with short restriction fragments of plasmid DNA have indicated certain hot spots for phosphoglycolate production by NCS and CAL. For the former agent, these data indicate that the major site of production is brought about by C4′-hydrogen abstraction of thymidine in the sequence AGT, resulting in phosphoglycolates bound to deoxyguanosine. Our nearest-neighbour analysis (Table 1) concurs with this observation, but also points to another significant hot spot that gives rise to phosphoglycolates bound to deoxycytidine. Such a hot spot has not been observed previously, but this may be a reflection of the relatively short restriction fragments that have been used to date for determining hot spots. As will be

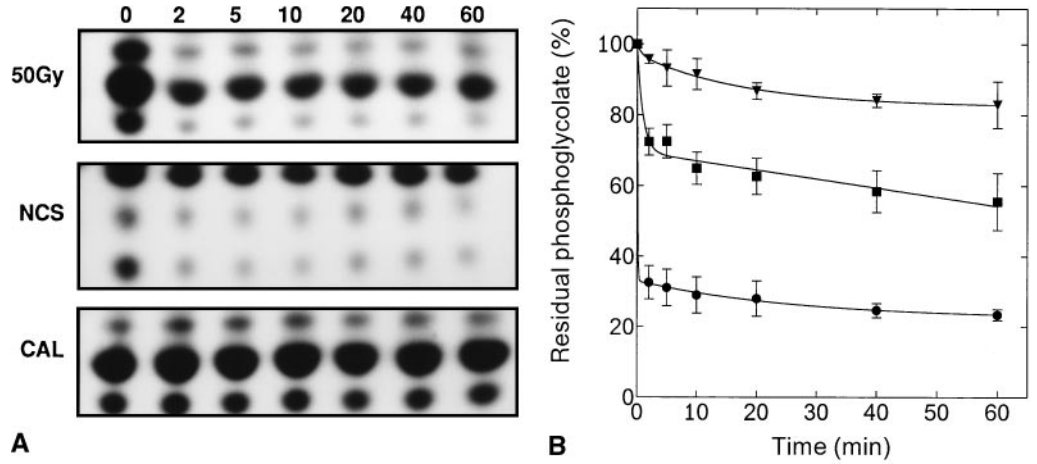

FIG. 5. Removal of phosphoglycolate during a 60-min incubation with 70 U of Ape 1. (Left panel) Autoradiograms showing the phosphoglycolate bands; pGpg (upper), pApg/pTpg (middle), pCpg (lower). Time in minutes is indicated at the top. (Right panel) Plot of the data: CAL-treated DNA (▼), NCS-treated DNA (■), and irradiated DNA (●). The plotted values represent the means ± SD of three independent analyses. The curves were computer generated by Prism software (Graphpad Software).

discussed below, the latter site may be associated with single-stranded lesions rather than bistranded lesions.

In their description of the double-strand cleavage by CAL, Zein *et al.* [14, 15] were able to establish that TCCT · AGGA sequences were hot spots, with a phosphoglycolate being generated by radical attack at the second nucleotide 3′ to the 3′ deoxyadenosine in the AGGA sequence. There has been no examination of the frequency of the four possible nucleotides at the cleavage site or the intervening nucleotide. Through the post-labelling assay, we established that the intervening nucleotide is 3-fold more frequently a pyrimidine, especially thymidine, than a purine (Table 1). This supports the recent suggestion that CAL-mediated cleavage may be greatly influenced by the sequences flanking the tetranucleotide hot spot [25].

Given the known characteristics of the phosphoglycolate moieties produced by radiation and the two enediynes, the most straightforward explanation for our observations of the repair of these lesions is that those generated at double-strand breaks are poorly repaired, while those at single-strand breaks are suitable substrates for the human and *E. coli* enzymes. Although this is the first time that such

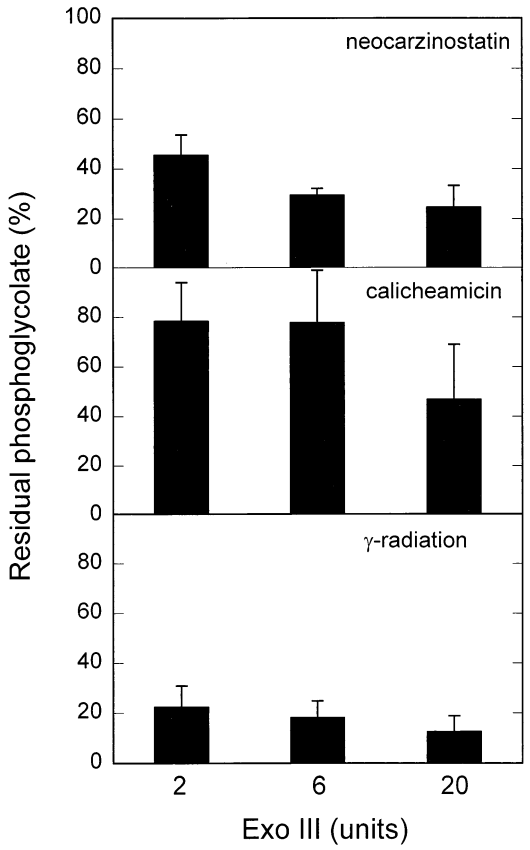

FIG. 6. Removal of phosphoglycolate by increasing concentrations of *E. coli* exonuclease III (exo III). Error bars indicate standard deviations from three independent analyses.

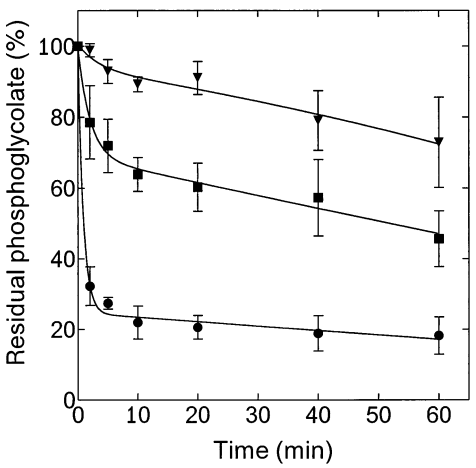

FIG. 7. Plot of the removal of phosphoglycolate during a 60-min incubation with 2 U of exo III. Key: CAL-treated DNA (▼), NCS-treated DNA (■), and irradiated DNA (●). The plotted values represent the means ± SD of three independent analyses. The curves were computer generated by Prism software.

a study has been carried out with enediyne-treated DNA and compared to irradiated DNA, the results are in general agreement with data acquired with model oligonucleotides [7, 26, 27]. Ape 1 is capable of cleaving phosphoglycolates at single-strand breaks, albeit slower than cleavage of natural abasic sites [26] or bleomycin-induced abasic sites [28]. Suh and coworkers [27] examined the removal of 3′-phosphoglycolates from oligonucleotide duplexes and observed that, under their conditions, Ape 1 was incapable of acting on phosphoglycolates on protruding 3′-termini, a result consistent with our data, since NCS and CAL produce strand breaks with one- and two-base protruding 3′-termini, respectively.

If this interpretation of our data, based on the secondary structure of the DNA strand-break termini, is correct, it would imply that the newly observed C-phosphoglycolate species produced by NCS, which is removed reasonably efficiently by both Ape 1 and exonuclease III, is produced either at a single-strand break or within a more benign bistranded lesion. A second important observation is that a subclass of radiation-induced phosphoglycolates appears to resist repair. This is in line with mathematical models that predict that as many as 25% of the lesions produced by ionizing radiation will be complex lesions, i.e. two or more closely spaced damaged sites [29, 30]. Our previous data with closely spaced abasic sites indicated that such lesions can be poor substrates for these enzymes [31].

Xenopus oocyte extracts are capable of effecting repair of double-strand breaks bearing 3′-phosphoglycolate termini [32]. One observed mechanism appears to be simple end-joining, requiring prior removal of the phosphoglycolates, rather than homologous recombination. However, the repair of double-strand breaks with 3′-phosphoglycolate termini was 30–100 times slower than repair of comparable vectors with 3′-hydroxyl termini. Based on the present study with Ape 1, we would predict that, unless an alternative enzyme exists that can more rapidly hydrolyze 3′-phosphoglycolates at double-strand breaks, a similar situation would pertain in human cell extracts and possibly human cells themselves. Furthermore, the slowness of the repair of these lesions could account for the extreme toxicity of CAL.

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