STRAND SCISSION OF DNA BY BOUND ADRIAMYCIN AND DAUNORUBICIN IN THE PRESENCE OF REDUCING AGENTS *

by

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Adriamycin and daunorubicin bound to covalently closed circular DNA nick the latter when reduced by sodium borohydride as demonstrated using an ethidium bromide fluorescence assay. The degradation, dependent on oxygen, is strongly inhibited by (i) superoxide dismutase (ii) catalase and (iii) sodium benzoate indicating the intermediacy in the cleavage of superoxide radical anion, hydrogen peroxide and hydroxyl radicals respectively. Less nicking of the DNA is observed by the reduced aglycones, so binding to the DNA by the aminosugar moiety assists the cleavage process. Adriamycin, daunorubicin and both ring C reduced forms bind intercalatively and completely relax supercoiled DNA. The results provide a possible rationale for the degradation of DNA which accompanies anthracycline administration.

INTRODUCTION

The antibiotics adriamycin 1b and daunorubicin 1a (Fig. 1) are among the most promising antitumor agents for the clinical treatment of neoplasia and have been used for the treatment of leukemias and several solid tumors (1). Biochemical evidence indicates their antineoplastic properties are due to strong intercalative binding to nucleic acids with consequent inhibition of DNA replication and/or RNA synthesis (1,2,3). Extensive chromosomal damage results from the administration of anthracyclines but the mechanism of this process has not been examined (4-6). There is a structural similarity between the quinone moiety of the anthracyclines and that of the antineoplastic agents streptonigrin, mitomycins B and C and certain 5:8-quinolinediones which we have shown cleave DNA after reduction by a common free radical process (7,8). Since the chromophore of the anthracyclines is known to be reduced during their metabolism (9,10) we anticipated a similar mechanism of DNA degradation to operate with these antibiotics.

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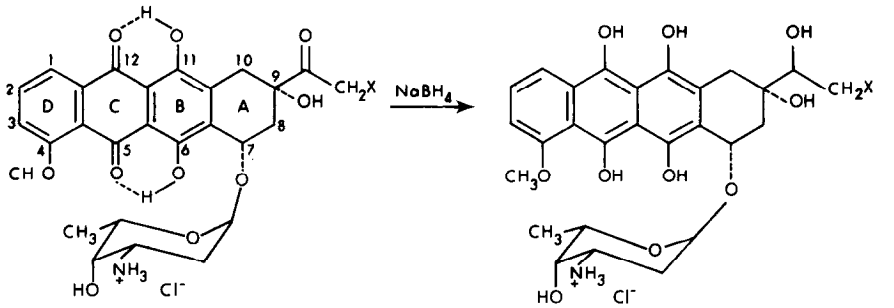

1(a) X = H 2(a) X = H, 7-OH
(b) X = OH (b) X = OH, 7-OH

3(a) X = H
(b) X = OH

FIGURE 1. The structures of daunorubicin 1a, adriamycin 1b, daunomycinone 2a, and adriamycinone 2b and the corresponding ring C reduced forms.

METHODS AND MATERIALS

Adriamycin 1b was obtained from the National Cancer Institute, Washington, DC and daunorubicin 1a from Calbiochem. Adriamycinone 2b and daunomycinone 2a were prepared by acid hydrolysis of the antibiotics according to a literature procedure (11). Superoxide dismutase (EC 1.15.1.1) was from Miles Labs. and catalase (EC 1.11.1.6) (beef liver) from Aldrich Chem. Calf thymus ω-protein preparation has been described (12).

Ethidium Fluorescence Assay for Nicking of DNA by Anthracyclines

The fluorometric methods of measuring strand breakage of PM2 covalently closed circular DNA(CCC-DNA) and its inhibition by enzymes and free radical scavengers has been described (13). The conversion of PM2-CCC-DNA to nicked circular DNA results in a 30% increase in fluorescence in the pH 11.8 ethidium assay solution (which was 20 mM potassium phosphate, pH 11.8, 0.4 mM EDTA and 0.5 μg/ml of ethidium bromide) and 100% loss of fluorescence after a heating and cooling cycle since the strands are now separable. The reactions were performed at 37° in a volume of 200 μl containing potassium phosphate pH 7.0, 1.02 A_{260} units of PM2-CCC-DNA (88% CCC), 2 x 10^{-4} M anthracycline, 3.3 x 10^{-3} M of sodium borohydride and 10% acetonitrile and other components as indicated in the legends to the figures.

Relaxation of Supercoiled PM2-DNA by Anthracyclines Monitored by the Effects of Calf Thymus ω-Protein.

Since ethidium bromide unwinds DNA on intercalating, the more negatively supercoiled CCC-DNA will bind more ethidium bromide. Therefore if the highly negatively supercoiled PM2-phage CCC-DNA is completely relaxed by the calf thymus ω-protein there is a 33% decrease in fluorescence both before and after heat denaturation at 96° for 23 min followed by rapid cooling. Thus negatively supercoiled DNA which has been treated with intercalating drugs which relax the supercoiling will show no change of fluorescence behavior upon treatment with ω-protein (12). The reaction conditions are given in the legends to the figures.

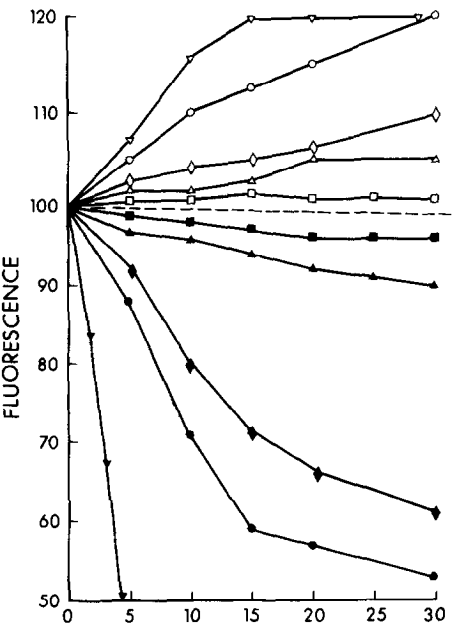

FIGURE 2. Single strand scission of PM2-CCC-DNA by reduced daunorubicin and its selective enzymatic inhibition. Reactions were performed at 37° in 0.05 M potassium phosphate buffer, pH 7.0 and contained 1.0 A_{260} unit/ml of PM2-CCC-DNA (88% CCC) 10^{-4} 1a or 1b and 3.3×10^{-3}M $NaBH_4$. The before heat fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after the denaturation at 96° and rapid cooling. Additional components were (△ - △) 5×10^{-5} g/ml catalase or superoxide dismutase: (□ - □) 0.5 M sodium benzoate; (0-0) none; (----) control. (▽ - ▽) scission of PM2-DNA by addition of 1×10^{-4}M pre-reduced 1a or 1b (◇ - ◇)scission by 2×10^{-4} M 2a or 2b.

RESULTS AND DISCUSSION

While neither component alone affected DNA (the resolution of the assay is that 1 nick may be detected in a DNA of 6×10^7 daltons (14)) *in situ* reduction of the antibiotics (in the drug-DNA complex formed by preincubation) at a concentration of 2×10^{-4} M with sodium borohydride nicked 65% of the CCC-DNA in 30 min (Fig. 2). Treatment of the DNA with pre-reducing anthracyclines 3 produces more extensive nicking of the DNA (50% in 5 min) (Fig. 2) at 2×10^{-4} M than in the above experiment indicating that intercalation of the chromophore shields this moiety from access by the reducing agent. As

in the cases of the similar damage to DNA by streptonigrin (7) and mitomycins B and C, $O_2^{\cdot-}$ was a likely intermediate from our previous studies with superoxide (7,8). This was confirmed by the strong inhibition of the cleavage by superoxide dismutase, catalase and by hydroxyl radical scavengers (e.g. sodium benzoate) (Fig. 2) supporting the following mechanism:

(i) $A + 2H \longrightarrow AH_2$

(ii) $AH_2 + O_2 \longrightarrow AH^{\cdot} + HO_2^{\cdot}$

(iii) $HO_2 \rightleftharpoons H^+ + O_2^{\cdot-}$

(iv) $2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

(v) $2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$

(vi) $H_2O_2 + O_2^{\cdot-} \longrightarrow OH^{\cdot} + OH^- + O_2$

(vii) $OH^{\cdot} + DNA \longrightarrow$ strand scission

Much less cleavage of DNA is observed by treatment with the reduced anthracycline aglycones which have redox potentials identical with the parent antibiotics (15). These results suggest strong binding of the antibiotic to the DNA assists the cleavage process since it is known that the aminosugar moiety is essential for strong binding and for antineoplastic activity (16). We may also conclude that this degradation of DNA is of less importance for the aglycones and 7-deoxyanthracyclines, which are known metabolites of these antibiotics (9,10,17).

The nature of the binding of the anthracyclines, the reduced forms and the aglycones was examined by the ability to relax supercoiled PM2-CCC-DNA monitored by an ethidium fluorescence assay employing calf thymus ω-protein. It is seen from Fig. 3 that both the parent antibiotics and their reduced forms (which unlike those of streptonigrin (7) and mitomycin (8) are stable in solution) intercalate the DNA and relax it completely whereas the aglycones have no effect and the ω-protein is able to relax the DNA as shown by the 30% decrease in fluorescence. This is not due to nicking which we have seen gives rise to a 30% increase in fluorescence (7,12,13). Since the anthra-

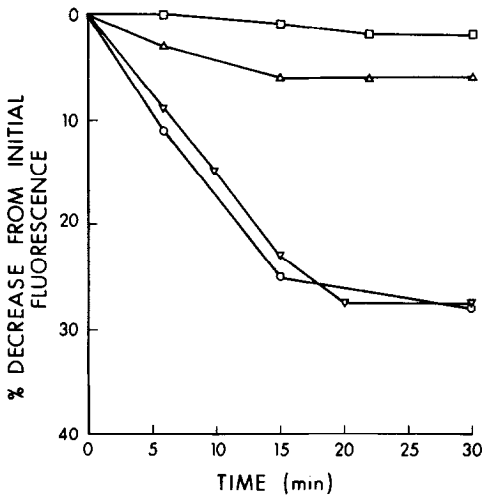

FIGURE 3. Relaxation of supercoiled PM2-CCC-DNA by anthracycline antibiotics monitored by the action of calf thymus ω-protein. Reactions were performed at 23° in 10 mM tris-HCl buffer, pH 8.0, and contained 1.0 A_{260} unit/ml of PM2-CCC-DNA (88% CCC); 0.2 M NaCl 2 mM EDTA and 1 unit of calf thymus ω-protein (i.e. that amount required to completely relay 1.5 μg of PM2-CCC-DNA in 15 min at 24° (12). Additional components were (O-O) none or 3×10^{-3} M $NaBH_4$; (▽-▽) 5×10^{-5} 3a or 3b; in degassed solution with 0.5 M sodium benzoate to protect the DNA from scission; (□-□) 5×10^{-5} 2a or 2b. The fluorescence readings are those obtained after heating and are given as percent decrease from initial reading.

cycline antibiotics are known to be metabolized extensively by reductive modification of the chromophore (9,10,17) (e.g. daunorubicinol is the major metabolite of daunorubicin (10)) it is conceivable that the quinone ring, already implicated is their marked cardiotoxicity (18,19) may be similarly reduced. Therefore the present results would serve to rationalize the degradation of DNA which is observed to accompany administration of the antitumor drugs (4-6).

The finding that the ring C reduced forms of the anthracyclines bind equally well to DNA as the parent antibiotics suggests a way of obviating their cardiotoxicity. If the 5,12 dihydroanthracyclines were methylated for example, the antibiotics would still bind to DNA and presumably display

antineoplastic activity, however, since their reoxidation would be prevented the concomitant DNA degradation and cardiotoxicity might well be eliminated.

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