Binding of Daunomycin to DNA and the Inhibition of RNA and DNA Synthesis

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SUMMARY

With synchronized tissue culture cells (L929), daunomycin had the greatest inhibitory effect on cell growth when the drug was administered during the later stages of cell division (late S, G2, and M). The level of binding of daunomycin to DNA was not found to be influenced by the phase of the cell cycle. The highest level of radioactivity from $[^3H]$-daunomycin was bound to DNA of the heterochromatin fraction. Both RNA and DNA syntheses were inhibited in isolated enzyme systems when daunomycin-treated DNA, from which the bound drug was removed by passage through Sephadex G-200, was used. DNA polymerase was reduced to one-fifth of the control activity, while that of RNA polymerase was reduced to one-half. Similar experiments with daunomycin-treated RNA and DNA polymerase preparations showed that the drug had no effect on the activities of the enzymes per se. Hence, the reduction of RNA and DNA polymerase activities could be accounted for by the loss of template activity of the drug-treated DNA. Daunomycin caused a marked drop in the formation of a complex between RNA polymerase and DNA, indicating the template to RNA polymerase enzyme. Sedimentation analyses of enzyme preparations showed that the drug had no effect on the profile in alkaline sucrose density gradient of DNA that had been treated with daunomycin. It was shown that the binding of daunomycin to DNA may give rise to steric hindrance effects that interfere with the association of the template to RNA polymerase enzyme. Sedimentation profile in alkaline sucrose density gradient of DNA that had been treated with daunomycin showed that no change in the molecular weight could be demonstrated.

INTRODUCTION

Daunomycin (NSC 82151) is an anthracycline antibiotic that has demonstrated antitumor activity against certain animal and human neoplasms (22, 24). It was shown to inhibit the synthesis of RNA and DNA in vivo (6, 14) and in vitro (9, 23). With a low concentration of daunomycin, the inhibition was irreversible with DNA polymerase but reversible in the case of RNA polymerase (5). The drug formed a stable complex with preformed DNA, presumably by intercalating between adjacent base pairs of the double helix (4, 13), and the biological activity of the drug is believed to be related to this property. The purpose of this study was to examine the factors influencing the binding of daunomycin to DNA and to investigate the mechanism whereby the drug exerts its inhibitory action on the synthesis of RNA and DNA.

MATERIALS AND METHODS

Materials. [methyl-$^{14}$C]Thymidine (specific activity, 54.2 mCi/m mole), [H]TTP (specific activity, 28.4 Ci/m mole), and [H]CTP (specific activity, 28.4 Ci/m mole) were from New England Nuclear, Boston, Mass. [methyl-$^4$H]Thymidine (specific activity, 14.1 Ci/m mole) was from ICN, Cleveland, Ohio. [H]Daunomycin (specific activity, 69.5 mCi/m mole) was a gift from Farmitalia, Milan, Italy. RNA polymerase from Escherichia coli B (EC 2.7.7.6; 294 units/mg protein) and DNA polymerase from Micrococcus lysodeikticus (EC 2.7.7.7; 90 units/mg protein) were from Miles Laboratories, Elkhart, Ind. Calf thymus DNA was from Worthington Biochemical Corp., Freehold, N. J.

$[^3H]$DNA was prepared from L-cells that had been incubated for 24 hr with [H]thymidine (final concentration, 0.5 $\mu$Ci/ml). The procedure of Kirby and Cook (15) was used for isolation of DNA. The specific activity of $[^3H]$DNA was 2170 cpm/ug. Daunomycin-treated $[^3H]$DNA was prepared by reacting $[^3H]$DNA (27.5 $\mu$g) with daunomycin (1.4 $\mu$g) in 1.0 ml of H$_2$O for 1 hr, then the unbound drug was removed from the DNA by repeated dialysis in Minicon A-25 Concentrator (Aminco Corp., Lexington, Mass.). The specific activity of DNA was 2180 cpm/ug and was unaltered by this treatment.

Methods. Mouse fibroblast tissue culture cells (L929) were maintained in suspension culture in Joklik-modified Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum and 2 mM glutamine. For cell synchrony studies, the cells were reversibly arrested in G1 by maintaining them in isoleucine-deficient medium for 96 hr (20). When isoleucine was restored to the medium, DNA synthesis was initiated with a high degree of synchrony, reaching a maximum rate at 12 to 16 hr. The generation time was 20 to 24 hr. In order to determine whether the cytotoxic effects of daunomycin varied during the cell cycle, the cells from different stages of the cell cycle were incubated with daunomycin (2 x $10^{-6}$ M) for 1 hr. The cells were then washed and transferred to fresh medium. After incubation for 24 hr, cell counts were made for assessment of cytotoxicity.

In order to investigate whether the extent of binding of daunomycin to DNA was influenced by the cell division...
cycle, synchronized cells at various stages of the cycle were reacted for 30 min with \(^{3}H\)daunomycin (1.6 × 10\(^{-8}\) M). \(^{14}C\)Thymidine was added to a final concentration of 10 \(\mu\)Ci/ml to simultaneously measure DNA synthesis. The cells were then washed with Hank’s balanced salt solution (Grand Island Biological Co.). DNA was precipitated with 0.2 N perchloric acid, and the resultant precipitate was washed twice with the same acid. RNA was isolated by adding perchloric acid and centrifuging. DNA was extracted from the precipitate by heating it in 10% NaCl (pH 7.5) for 30 min at 100°. DNA was quantitated by absorbance measurements at 260 nm, and the radioactivity was measured by adding 1 ml of extract to 10 ml of Triton X-100:PPO:POPOP:toluene (450 ml:4 g:0.8 g:1000 ml) and counting in a liquid scintillation counter. Dual-label technique was used for estimation of \(^{3}H\) and \(^{14}C\) counts.

Binding of daunomycin to DNA of various cell fractions was studied in cells that had been incubated with \(^{3}H\)daunomycin (1.6 × 10\(^{-8}\) M) for 1 hr at 37°. The cells were washed in 0.14 M NaCl:5 mM KCl:7 mM Na\(_2\)HPO\(_4\):25 mM Tris, pH 7.5, and suspended in 10 volumes of 10 mM Tris:1 mM EDTA:10 mM NaCl, pH 7.5. After 10 min, the cells were homogenized with 25 strokes of the Dounce homogenizer. To the homogenate from 2 × 10\(^{6}\) cells, 1.0 ml of 1.5 M sucrose was added, and the mixture was layered over 6.0 ml of 1.5 N sucrose in 10 mM Tris:1 mM EDTA:10 mM NaCl, pH 7.5, and centrifuged at 30,000 rpm in Spinc SW 41 rotor. The crude mitochondrial fraction that separated at the interface was purified according to Kasamatsu et al. (12). The nuclei fraction that sedimented to the bottom of the tube was sonically extracted for 70 sec (Branson Sonifier, S-75, setting of 2), and euchromatin, heterochromatin, and an intermediate chromatin fraction were isolated according to the method of Yasmineh and Yunis (25). DNA was isolated from each cell fraction, and specific activity measurements were made as before.

To study the template activity of the drug-treated DNA, calf thymus DNA (0.5 mg) was reacted with daunomycin (25 \(\mu\)g) in 1.0 ml of 1 mM MgCl\(_2\) for 16 hr at room temperature. Uncombined drug was removed by chromatography on a column (1.4 x 25 cm) of Sephadex G-50, using 0.01 mM potassium phosphate, pH 7.0, as eluant. Fractions containing DNA were pooled, lyophilized, and dissolved in a suitable volume for template assay. Alternatively, unbound drug was removed by repeated ultrafiltration in Minicon A-25 Microconcentrator. Control DNA was treated in the same manner except for the omission of the drug treatment. DNA polymerase was assayed according to Lynch et al. (16), using a purified preparation from \(M\). lysodeikticus. RNA polymerase was determined according to Burgess (3), using the RNA polymerase preparation from \(E\). coli B. At the end of the incubation periods, aliquots of the reaction mixtures were transferred to glass fiber discs (Whatman GF/C) and the nucleic acids were precipitated with perchloric acid. The discs were washed and dried and allowed to stand overnight with 0.25 ml of NCS Solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.). Radioactivity was determined by addition of 10 ml of liquid scintillation mixture and counting in the liquid scintillation counter.

To determine whether daunomycin had any effect on either RNA or DNA polymerase enzymes, purified enzyme preparations (60 to 75 \(\mu\)g) were reacted with daunomycin (4 \(\mu\)g in 0.4 ml of 0.01 Tris, pH 8.0) for 1 hr at room temperature. Then the unbound drug was removed by repeated dialysis in Minicon A-25 Microconcentrator. Enzyme concentrations were adjusted to the original values by absorbance measurements at 280 nm. The enzymes were assayed as described above. Control enzymes were treated in the same manner for the drug treatment.

The method of Jones and Berg (11) was used to determine whether daunomycin treatment of DNA affected its binding to RNA polymerase. In this procedure, RNA polymerase and DNA (either untreated or treated with daunomycin) were separately filtrable through Millipore membrane filter, but the combination of the 2 was quantitatively retained by the filter. Incubation mixture (0.25 ml) contained 1 \(\mu\)mole MgCl\(_2\), 3 \(\mu\)moles 2-mercaptoprotoan, 10 \(\mu\)moles Tris-Cl, pH 8.0, and after drying they were placed in vials containing 10 ml PPO:POPOP:toluene (4 g:0.8 g:1000 ml) and counted in a liquid scintillation counter. For negative controls, assays were made with an enzyme preparation that had been denatured with heat (70° for 5 min).

For analyses by alkaline sucrose density gradient centrifugation, DNA was isolated as previously described from tissue culture cells that had been maintained for 1 hr with daunomycin (1.8 × 10\(^{-8}\) M). DNA (400 \(\mu\)g in 0.2 ml of 5% sucrose) was layered on top of 4.5 ml of sucrose gradient (5 to 20%, w/v, at pH 12.7). The gradients were centrifuged at 34,000 rpm in Spinc No. 40 rotor for 2.5 hr at 20°. The bottoms of the tubes were punctured and 3-drop fractions were collected, to which 0.5 ml of H\(_2\)O was added. The absorbance was read at 260 nm.

**RESULTS AND DISCUSSION**

DNA synthesis and cell division were synchronized in L-cells by restoring isoleucine to cells that had been brought to a state of G\(_1\) arrest by growth in an isoleucine-deficient medium. The influence of cell cycle on the cytotoxicity of daunomycin was studied by measuring growth of cells that had been exposed to a fixed dose of the drug at various time intervals after isoleucine refeeding. The results showed that daunomycin caused a depression of cell counts throughout the cell cycle and that the maximum effect on cell growth occurred when the drug was added during the later stages of cell division (late S, G\(_2\), and M). These findings were consistent with those obtained with other mammalian cell systems, in which the maximum effect of the drug was observed in late S period (14, 18). In Chinese hamster cells, daunomycin at low doses showed little effect on the entry of cells into S but was effective in preventing cells from reaching...
mitosis (19). A delay in G2 period occurred when daunomycin was added to cultured human leukocytes (1). It is not clear whether the antimitotic activity of daunomycin is derived exclusively from the known property of this compound to bind to DNA and thus interfere with the biosynthesis of various macromolecules.

Cells at various stages of the cell cycle were reacted with a fixed dose of [3H]daunomycin, and then DNA was extracted for study. The radioactivity bound to DNA (expressed as pmoles daunomycin per mg DNA) showed little variation throughout the cell cycle (Chart 1). Using autoradiographic technique, Silvestrini et al. (17) showed that maximum uptake of [3H]daunomycin by rat fibroblasts took place at late S stage; however, our findings did not indicate that the level of binding of the drug to DNA was influenced by the phase of the cell cycle.

Cells that had been treated with [3H]daunomycin were homogenized, and DNA was extracted from the following structures: mitochondria, euchromatin, heterochromatin, and an intermediate chromatin fraction. From specific activity measurements of DNA, it was shown that the highest level of binding of [3H] (estimated as pmoles daunomycin per mg DNA) was found to be associated with the heterochromatin fraction (Table 1). Lesser amounts were found to be present in DNA from the other structures. The significance of preferential binding of daunomycin to DNA of heterochromatin fraction remains obscure at present. Ward et al. (23) reported that daunomycin inhibited DNA-dependent RNA synthesis regardless of the base composition of the DNA templates used; however, recently, Zunino et al. (27) have shown that adenine-thymine-containing synthetic DNA templates were more sensitive to the action of daunomycin. Mouse heterochromatin has been shown to be enriched with adenine-thymine-rich satellite DNA (26). Sensitivity of cells to the toxic effects of daunomycin at the end of S may be related to the fact that DNA-containing poly(dA:dT) sequences are being replicated at this time (8).

Binding of daunomycin to DNA is believed to be related to the inhibitory effect of the drug on RNA and DNA synthesis. Daunomycin derivatives with reduced binding capacity, as measured by their effects on sedimentation velocity and relative viscosity of DNA, were found to have correspondingly less effect on the inhibition of nucleic acid synthesis and on mitotic index (7). At low concentrations of daunomycin, DNA synthesis was more inhibited than was RNA synthesis (9, 14). However, it has been shown that the inhibition of DNA synthesis was irreversible, while that of RNA was initially depressed but gradually returned to control levels (2, 5). Therefore, the relative activity of daunomycin on incorporation of precursors into RNA and DNA in vivo may depend upon the time of observation after the drug administration.

Inhibition of RNA and DNA synthesis has been observed when daunomycin was added to isolated enzyme systems (9, 10, 23, 27). In order to distinguish between the effect of the drug on the primer and on the enzyme, daunomycin-DNA complex was prepared, from which unbound daunomycin was removed by passage through a Sephadex column. With daunomycin-treated DNA as primer, the activity was reduced to 25% of control value in DNA polymerase system and to 54% of control in the case of the RNA polymerase system (Table 2). On the other hand, when enzymes that were similarly treated with daunomycin were used in the assays, no reduction of polymerase activity was observed. Hence, daunomycin treatment of DNA caused a loss of template activity presumably by its binding to DNA. A similar conclusion was reached by Zunino et al. (27), who were to overcome the inhibition of RNA polymerase by daunomycin by an increase in DNA concentration. These studies indicated that reduced template activity of DNA could be responsible for the inhibition of RNA and DNA polymerase activity by daunomycin.

![Chart 1. Uptake of ³H by DNA when cells at various intervals after isoleucine refeeding are treated for 30 min with [³H]daunomycin (1.6 x 10⁻⁴ M). In order to monitor DNA synthesis, [³C]thymidine was added to a final concentration of 10 μCi/ml. DNA was isolated and analyzed as described in the text. Data were estimated by assuming parity between specific activity of the drug and that of the moiety that bound to DNA. Points, mean from 2 experiments. Bars, S.E.](image-url)

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<tr>
<th>Table 1</th>
<th>Binding of [³H]daunomycin to DNA of cell fractions</th>
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<tr>
<td>Cell fraction</td>
<td>pmoles daunomycin/mg DNA</td>
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<tr>
<td>Mitochondria</td>
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<tr>
<td>Euchromatin</td>
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<td>Heterochromatin</td>
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<td>Intermediate chromatin</td>
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<tr>
<th>Table 2</th>
<th>Effect of daunomycin on RNA and DNA polymerase activities</th>
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<tr>
<td>DNA</td>
<td>Polymerase</td>
</tr>
<tr>
<td>untreated</td>
<td>Taunomycin-treated</td>
</tr>
<tr>
<td>DNA</td>
<td>25.3 ± 3.7</td>
</tr>
<tr>
<td>RNA</td>
<td>112 ± 15.8</td>
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</tbody>
</table>

*Values obtained with untreated DNA and untreated polymerase under identical conditions of assay were taken as controls (100% activity). Results are means of 2 experiments ± S. E.
Daunomycin Binding to DNA

Daunomycin reduced the sedimentation coefficient of DNA in CsCl gradient (4, 13). This shift in buoyant density suggested a modification of molecular size or shape of DNA in solution. The sedimentation profile of DNA from daunomycin-treated cells in alkaline sucrose gradient (5 to 20%, w/v, pH 12.7) was shown to be unchanged from that of control cells (Chart 3); thus, no gross modification of molecular weight could be demonstrated by this technique.

ACKNOWLEDGMENTS

The authors are grateful to Farmitalia, Milan, Italy, for a generous gift of tritiated daunomycin.

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