A Comparison of the Effects of Daunomycin and Adriamycin on Various DNA Polymerases

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SUMMARY

The effects of the anthracycline antibiotics, daunomycin and adriamycin, on the DNA-directed activities of DNA polymerases from murine sarcoma virus, rat liver (high-molecular-weight species), Escherichia coli, and Micrococcus luteus were determined. Under all conditions tested, these compounds had greater inhibitory effect against the viral polymerase than against cellular polymerase. The inhibition of murine sarcoma virus DNA polymerase by daunomycin was competitive with respect to DNA. For viral DNA polymerase it was concluded that the inhibition was predominantly caused by the interaction of daunomycin with the primer-template DNA. Also, an appreciable never sal of the daunomycin-induced inhibition of this polymerase by an increase in Mg\(^{2+}\) concentration is consistent with the conclusion derived by competition experiments. In contrast, the inhibition of both rat liver and M. luteus DNA polymerases was essentially noncompetitive with DNA. Also, bacterial enzymes were less sensitive to inhibition by these drugs than the virion polymerase. The strong and preferential inhibition of viral DNA polymerase is discussed in relation to a differential sensitivity of normal as compared to tumor cells observed in some cell lines.

INTRODUCTION

Most of the active antitumor drugs have their major inhibitory activity during DNA synthesis. Unfortunately, these compounds offer little if any specificity; they are about equally active against tumors and normal cells.

The presence of an RNA-dependent DNA polymerase (reverse transcriptase) in the nucleocapsid of oncornaviruses (2, 33) and the existence of multiple forms of DNA polymerases in eukaryotic cells have prompted investigations aimed at finding inhibitors of these enzymes. There have been several reports of inhibition of viral DNA polymerase by various compounds, particularly rifamycin derivatives (15, 40), streptovaricins (5), ethidium bromide (20) and, most recently, several polymers, including synthetic (13, 36) and modified polynucleotides (7, 32), and pyran copolymer (27).

The antitumor antibiotics, daunomycin and its derivative adriamycin, have been reported to be strong inhibitors of RNA tumor virus DNA polymerase (1, 8, 25). However, the drugs also inhibit DNA-directed DNA and RNA synthesis reactions catalyzed by bacterial enzymes (23, 37, 42). Evidence has been presented indicating that these anthracycline antibiotics caused a rapid inhibition of DNA synthesis in tissue cell culture (30). However, no study of the effects of the 2 agents on mammalian enzymes has been reported. In view of the selective inhibition of MSV\(^{1}\)-transformed cells when compared to normal cells (A. M. Casazza and A. Di Marco, manuscript in preparation), it is of interest to compare the effects of these antitumor agents on viral and cellular enzymes. In this report the inhibitory effect of daunomycin and adriamycin on the in vitro activity of DNA polymerase of MSV was studied in detail and compared with those on a mammalian DNA polymerase (high-molecular-weight species from rat liver) and 2 well-characterized bacterial DNA polymerases (Micrococcus luteus and Escherichia coli DNA polymerase I's have frequently been used as models of DNA-synthesizing enzymes). Since these compounds bind tightly only to DNA and not to RNA (6, 12), in this report we examine only the inhibitory effects on DNA-dependent reactions. Thus the extents of inhibition of various DNA polymerases can be directly compared under the same assay conditions. MSV DNA polymerase was found to be more sensitive to daunomycin (or adriamycin) inhibition than were cellular DNA polymerases.

MATERIALS AND METHODS

Materials. Deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, and dTTP) were supplied by Boehringer Mannheim GmbH, Mannheim, West Germany; \(^{3}H\)-labeled deoxyribonucleoside triphosphates were supplied by New England Nuclear, Boston, Mass. DEAE-cellulose (DE-32) and phosphocellulose P-11 were purchased from Whatman Co., Maidstone, Kent, England. Calf thymus DNA was prepared as described earlier (43). Activation of calf thymus DNA for use as a primer-template for DNA polymerase used limited digestion by pancreatic DNase (31). Normal rat liver were obtained from male CD rats (Charles River Laboratories, Calco, Italy) weighing 100 to 150 g.

Drugs. Daunomycin hydrochloride and adriamycin hydrochloride were supplied by Farmitalia, Milan, Italy. The

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Inhibition of DNA Polymerases by Daunomycin and Adriamycin

**RESULTS**

Chart 1 compares the effects of daunomycin and adriamycin on the *in vitro* activity of DNA polymerases from MSV, rat liver, and *M. luteus* with activated calf thymus DNA as primer-template. Viral polymerase was more sensitive to daunomycin inhibition (Chart 1 A) than both mammalian and bacterial enzymes tested. These latter enzymes were inhibited about equally. Indeed, under standard assay conditions where all substrates and primer-template were present at saturating levels, the requisite concentrations of deoxyribonucleoside triphosphates and primer-template used were saturating under standard assay conditions for each enzyme. The concentrations of inhibitor are given in the figure legends. In the assay for each enzyme, drugs and substrates were mixed before initiating the reaction by the addition of enzyme. After 10 min incubation at 37°C, during which the incorporation rate was linear in all cases (see also Chart 2, A and C, and Chart 3 A), the reactions were stopped with 1.5 ml of cold 10% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The precipitates were collected on pre-soaked Millipore filters (0.45 μm), washed with 5% trichloroacetic acid, dried, and counted using toluene-based scintillation fluid.

**Virus.** MSV was supplied by Electro-Nucleonics Laboratories, Bethesda, Md. The virus stock containing 10^10 to 10^11 particles/ml has been purified by double-density gradient zonal centrifugation and stored at −70°C until used.

**DNA Polymerase Preparations.** Rat liver high-molecular-weight DNA polymerase was prepared from the soluble extract of normal rat livers, essentially as described by Chiu and Sung (11). The purification procedure involves preparation of 105,000 × g (2 hr) supernatant, ammonium sulfate fractionation, and DEAE-cellulose chromatography. The enzyme was stored in 0.05 M Tris-HCl (pH 7.6), 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, and 20% glycerol at −20°C. MSV DNA polymerase was prepared by detergent lysis of purified MSV at 0−4°C for 10 min in a solution containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7), 0.001 M EDTA, and 0.1 to 0.4% Nonidet P-40. The *M. luteus* DNA polymerase I was a partially purified product of Miles Laboratories, Kankakee, Ill. A sample of partially purified *E. coli* DNA polymerase I was obtained from Boehringer Mannheim.

**Assay of DNA Polymerase.** Unless otherwise stated in the figure legends, the reaction mixture for the assay of DNA polymerase or rat liver DNA polymerase activity contained (in 0.1 ml final volume) 60 mM Tris-HCl (pH 7.5); 8 mM MgCl_2; 10 mM 2-mercaptoethanol; 70 μM each of dATP, dCTP, dGTP, and [3H]dTTP (specific activity, 157 cpn/pmole); 200 μM activated calf thymus DNA; and 20 μl of enzyme preparation. For the bacterial DNA polymerases the standard reaction mixture contained (in 0.25 ml final volume) 60 mM Tris-HCl buffer (pH 7.4); 6 mM MgCl_2; 10 mM 2-mercaptoethanol; 90 μM each of dATP, dCTP, dGTP, and [3H]dTTP (specific activity 39 cpn/pmole); 80 μM activated calf thymus DNA; and about 0.004 unit of enzyme in 20 μl diluent. One unit of DNA polymerase is defined as the amount catalyzing the incorporation of 10 nmoles of dTMP into acid-insoluble material at 37°C for 10 min under our standard conditions. The concentrations of deoxyribonucleoside triphosphates and primer-template used were saturating under standard assay conditions for each enzyme. The concentrations of inhibitor are given in the figure legends. In the assay for each enzyme, drugs and substrates were mixed before initiating the reaction by the addition of enzyme. After 10 min incubation at 37°C, during which the incorporation rate was linear in all cases (see also Chart 2, A and C, and Chart 3 A), the reactions were stopped with 1.5 ml of cold 10% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The precipitates were collected on pre-soaked Millipore filters (0.45 μm), washed with 5% trichloroacetic acid, dried, and counted using toluene-based scintillation fluid.

**Materials and Methods.** The assays were done at 37°C for 10 min. Control [3H]dTMP incorporation: 9.95 pmoles for MSV DNA polymerase (●); 6.2 pmoles for rat liver DNA polymerase (○); 44 pmoles for *M. luteus* DNA polymerase (▲). A, daunomycin; B, adriamycin. DNA-P, DNA phosphate.
Early studies have indicated that the daunomycin inhibition of reaction catalyzed by E. coli DNA-dependent RNA polymerase was significantly decreased as the Mg\(^{2+}\) concentration was increased (41). A partial reversal of the daunomycin inhibition of this polymerase by an increase in Mg\(^{2+}\) concentration has been interpreted in terms of a decreased binding of daunomycin to DNA. This effect is consistent with the conclusion that the inhibiting effect of daunomycin results from its ability to bind to the template DNA. It was of interest, therefore, to determine whether the degree of inhibition of DNA-dependent DNA synthesis by daunomycin depended upon the Mg\(^{2+}\) concentration. Surprisingly, although the inhibition by daunomycin of MSV DNA polymerase was markedly decreased as the Mg\(^{2+}\) concentration was increased, the inhibition of both M. luteus and rat liver DNA polymerases was insensitive (or only moderately sensitive) to variations in the Mg\(^{2+}\) concentrations (Chart 4).

The results presented above indicated that, although the sensitivity to daunomycin inhibition of MSV DNA polymerase depended on the conditions maintained, the viral enzyme was preferentially inhibited under all conditions. The possibility was considered that the increased inhibition of viral polymerase could have been due to different amounts of polymerase used and/or to impurities in our enzyme preparations purified to different extents. Chart 5 shows that the inhibition was independent of the degree of purity of the enzyme. In these experiments, a DNA polymerase purified from MSV according to Green et al.

Preincubation experiments with various components of the reaction mixture (Chart 2 B) showed a little difference in inhibition when the reaction was initiated by a final addition of polymerase, DNA, or deoxyribonucleoside triphosphates. Similarly, the degree of inhibition of MSV DNA polymerase (Chart 2, C and D) by 40 \(\mu\)M daunomycin changed little with time when incubations were conducted from 2 min up to 20 min (during which the kinetics of polymerization was linear with respect to time); daunomycin influenced the polymerization of the deoxyribonucleotides on the DNA template irrespective of the preincubation mode of the different components in the polymerase assay (Chart 2 D). An unexpected finding of this study was that the degree of inhibition of M. luteus DNA polymerase (Chart 3) decreased when the preincubation mixture contained daunomycin and DNA. The lag in the inhibition was eliminated only when daunomycin was not preincubated with DNA.

As observed in the RNA polymerase reaction (41), a 4- or 8-fold variation in the concentration of deoxyribonucleoside triphosphates did not significantly alter the level of inhibition produced by 40 \(\mu\)M daunomycin (16 \(\mu\)M daunomycin in the case of M. luteus DNA polymerase I), under conditions where only the lower nucleotide concentrations were rate limiting.

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Inhibition of DNA Polymerases by Daunomycin and Adriamycin

The possibility that associated or contaminating nuclease activities in polymerase preparations could influence the daunomycin inhibition of polymerization reactions was also considered. The observation that preincubation of daunomycin with DNA partially relieved the inhibition of the bacterial enzyme (Chart 3 B) appeared to suggest that the daunomycin binding to DNA induced some conformational changes so that drug-DNA complex served as a better substrate for nuclease than DNA alone. If it is the case, then we can expect that the differentially inhibited rates of incorporation (Chart 3 B) may result from a drug-stimulated nuclease activity. However, at antibiotic concentrations effective in polymerase inhibition, no stimulation of release of $^{32}$P-labeled mononucleotides from labeled denatured E. coli DNA was observed; indeed a slight inhibition was reproducibly found (data not shown). This observation is in keeping with the known inhibitory effect of daunomycin against endo- and exonuclease activities (14, 22). Since under our assay conditions, where a denatured DNA was used, the 3'- to 5'-associated exonuclease activity is preferentially active (17), a stimulation of the 5'- to 3'-activity, which prefers double-stranded DNA, cannot be excluded.

However, if we compare the daunomycin effects against M. luteus and E. coli DNA polymerases I (Chart 5 C), daunomycin is found to inhibit these enzymes similarly. Since E. coli enzyme has relatively high nuclease content, compared to M. luteus enzyme (19), it is unlikely that the differential inhibition pattern (Chart 1) might be attributable solely to the action of associated nuclease. Both polymerases from MSV and rat liver (high-molecular-weight species) contained some nuclease activity; only the low-molecular-weight DNA polymerase (●) activities are also shown for the purpose of comparison.

(15) was used for comparison with the polymerase activity of detergent disrupted virions (Chart 5 A). The rat liver polymerase (purified through the DEAE-cellulose chromatography step) was compared with an enzyme preparation further purified on phosphocellulose (Chart 5 B). Similarly, with partially purified and highly purified [Fraction VII, prepared according to the method of Jovin et al. (21)] DNA polymerase I preparations from E. coli, daunomycin yielded nearly identical inhibition curves (Chart 5 C). In the above experiments carried out under standard conditions (200 μM activated DNA, in the case of MSV and rat liver DNA polymerase), the concentrations of viral and cellular enzymes were adjusted to attain similar control incorporations. Due to a limited amount of purified viral enzyme, it was not possible to carry out experiments at saturating enzyme concentrations. However, since the extent of inhibition was not dependent on the concentration of detergent-disrupted virions, it is reasonable to assume that the inhibition is not dependent on the concentration of enzyme used. Also, with both preparations of rat liver DNA polymerase, the level of inhibition was not dependent on the concentration of enzyme, since a 8-fold increase in enzyme concentration produced no significant differences in the level of inhibition. As the data on the inhibition of M. luteus DNA polymerase have been obtained with higher enzyme activity, compared to the other 2 enzymes, it is also worth emphasizing that, at reduced concentrations of the bacterial enzyme, the level of inhibition by daunomycin was not significantly altered. A 5-fold decrease in polymerase concentration changed the inhibition produced by 16 μM daunomycin by less than 4% (from approximately 43 to 40%).
ity or other proteins, which can influence template activity, in drug inhibition could not be excluded, no significant correlations were found in these enzymes between exonuclease content and sensitivity to inhibition.

The strong and apparently specific inhibition of the enzyme from sarcoma virus by daunomycin encouraged a more comprehensive characterization of this phenomenon. Since no adequate kinetic model is available for an enzyme (primer-template)-substrate system, usually a simplified model is used, where primer-template is used as substrate (13, 36). Experiments to determine whether the competition was between daunomycin and the template, calf thymus DNA, were therefore performed. In Chart 6, the double-reciprocal plot of reaction velocity as a function of calf thymus DNA concentration at 2 concentration levels of daunomycin is consistent with the kinetics of competitive inhibition (since the maximum velocity is not altered by the presence of inhibitor). In contrast, inhibition of the rat liver DNA polymerase was essentially noncompetitive with respect to DNA. The results are shown in Chart 7 A. Replot of \( \frac{1}{V} \) versus \( [I] \) (Chart 7 B) of the data obtained at several concentrations of prime-template is consistent with the kinetics of noncompetitive inhibition (24). In these experiments only the lower DNA concentration was rate limiting. However, the kinetics of inhibition was similar under saturating template conditions. In another experiment, where an enzyme preparation further purified on phosphocellulose was used, the kinetic analysis of data derived with a wide range of DNA concentrations (from 40 to 800 \( \mu M \)) again indicated the same type of inhibition.

The kinetics pattern for the inhibition of M. luteus DNA polymerase I (Chart 8) appears to be similar to that observed with rat liver enzyme, in that the maximum velocity is strongly reduced by the presence of inhibitor. However, such an inhibition is apparently of a complex type. Indeed it is not purely noncompetitive since \( K_m \) of the inhibited reaction is greater than that of the uninhibited reaction. An increase in DNA concentration only partially decreases the inhibition by daunomycin that cannot be abolished even at very high DNA concentrations.

**DISCUSSION**

The data presented in this paper show that, although daunomycin and adriamycin possessed some inhibitory activity against the cellular and bacterial DNA polymerases tested, these compounds inhibited the viral enzyme more strongly than the other polymerases studied, under all conditions used.

Previous studies have reported that, when whole virus
particles are used as the source of reverse transcriptase activity, non-ionic detergents included in the reaction mixture interfere with RNA-dependent DNA polymerase inhibition by daunomycin or ethidium bromide (20) and rifampicin derivatives (34). Under our standard assay conditions, where saturating levels of all substrates and primer-template and optimal concentration of Mg^{2+} were used and a DNA-dependent activity was measured, the extent of inhibition of MSV DNA polymerase was only moderately sensitive to Nonidet P-40 concentration (Chart 9).

The mechanism by which daunomycin interacts with DNA leading to its interference with the function of nucleic acid-polymerizing enzymes is still unclear. A considerable body of evidence has been accumulated that points to an intercalative mode of binding (28, 38, 43). The results presented here indicate that the daunomycin inhibition of MSV DNA polymerase reaction is competitive with respect to DNA. This finding is consistent with a direct interaction of daunomycin, with template being the predominant factor involved in the inhibition. In addition, the finding of an appreciable reversal of daunomycin-induced inhibition of this polymerase by an increase in the Mg^{2+} concentration is consistent with the proposed mechanism, since Mg^{2+} decreases the total binding of daunomycin to DNA (6). In contrast to MSV DNA polymerase, the rat liver high-molecular-weight DNA polymerase and M. luteus polymerase are inhibited in a noncompetitive or partially noncompetitive way. The different effects of daunomycin on the various polymerases suggest that some features of the reaction pathways of polymerization may not be identical in these enzymes.

The normal cellular DNA polymerase from rat liver used in this study is the high-molecular-weight species. The size properties of this enzyme are similar to those reported by Baril et al. (3). Only this DNA polymerase activity responds to stimuli for cell proliferation (3, 26). This observation is consistent with results from other mammalian systems (10) and suggests that the high-molecular-weight (6 to 8 S) polymerase activity functions as a replicative enzyme (10). However, the precise role of the 6 to 8 S activity in DNA replication in the cell as well as the in vivo function of the low-molecular-weight (3.4 S) DNA polymerase remains to be elucidated. Also, the low-molecular-weight nuclear enzyme isolated from normal rat liver was much less sensitive to daunomycin inactivation than viral enzyme. The inhibitory effect of daunomycin against the purified nuclear enzyme (50% inhibition at about 40 μM) was similar to that found against the high-molecular-weight enzyme (Chart 5 B). If these enzymes are involved in DNA metabolism in living cells, then it is of interest to consider possible biological implications of the differential inhibition of polymerization reactions catalyzed by cellular and viral enzymes by daunomycin. Recent investigations have shown a differential sensitivity to these drugs in normal and malignant cells. As mentioned, daunomycin and Adriamycin appeared to be more toxic for MSV-transformed cells than for normal cells. Similarly, human and nonhuman leukemic cells are more sensitive than normal blood cells. In both MSV-transformed cells (29) and leukemic cells (4, 35), a DNA polymerase has been found that resembles viral RNA-directed DNA polymerase. It has been suggested that this enzyme may play an important role in neoplastic transformation and proliferation (18, 39). Our results showing that the viral DNA polymerase is more sensitive to these drugs than are normal cellular polymerases may not be of marginal significance in explaining the differential inhibition of tumor cell proliferation.

After the completion of this work, Goodman et al. (14) reported a differential inhibition of mutant T4 DNA polymerases by these drugs. They suggested that the observed differential effects could have interesting implications in terms of fidelity of DNA synthesis in tumor cells. While such effects are possible, the preferential inhibition of viral DNA polymerase could considerably reduce the growth of malignant cells if the presence of an oncogenic virus and/or the expression of functional viral DNA polymerase are essential for maintaining its cellular proliferation. In addition, their results showing a greater inhibitory effect of Adriamycin compared to daunomycin are in accordance with our findings. Thus, the increased inhibitory effectiveness of Adriamycin against all polymerases tested so far further supports the hypothesis that the improved therapeutic effectiveness of Adriamycin over Daunomycin may also have a biochemical basis (42).

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REFERENCES


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