Inhibition of Mammalian Deoxyribonucleic Acid Synthesis by Neocarzinostatin: Selective Effect on Replicon Initiation in CHO Cells and Resistant Synthesis in Ataxia Telangiectasia Fibroblasts†

Lawrence F. Povirk and Irving H. Goldberg*

ABSTRACT: Treatment of CHO cells with low doses of the protein antibiotic neocarzinostatin severely inhibited DNA replicon initiation but had no effect on chain elongation. The selectivity of the effect on initiation, which was greater than that seen with other chemical agents and comparable to that seen with X-rays, explains the biphasic dose response seen for DNA synthesis inhibition by this drug. Parallel experiments employing the nucleoid sedimentation technique indicated that half-maximal relaxation of domains of DNA supercoiling and half-maximal inhibition of replicon initiation required the same dose of neocarzinostatin, approximately 0.03 μg/mL. These results, similar results obtained with the protein antibiotic auromomycin, and previous results obtained with X-rays suggest a quantitative correlation between inhibition of replicon initiation and induction of sufficient strand breakage to relax domains of supercoiling in DNA of mammalian cells. Results in human ataxia telangiectasia fibroblasts indicated that neocarzinostatin, like X-rays, is much less effective in inhibiting DNA synthesis in these cells than in normal human fibroblasts. This finding is consistent with the hypothesis that the genetic defect in ataxia telangiectasia involves a failure to recognize the presence of strand breaks in cellular DNA.

Ionizing radiation has been shown to selectively inhibit the initiation of new replication forks (replicons) in DNA of mammalian cells, at doses which have no effect on continued progression of previously initiated replication forks (Watanabe, 1974; Makino & Okada, 1975; Painter & Young, 1975; Walters & Hildebrand, 1975). Because there is considerable evidence that the target molecule for this effect is DNA itself (Povirk & Painter, 1976; Painter, 1978), the large target size, approximately 10^9–10^{10} daltons (Painter & Rasmussen, 1964), implies that initiation of replication at a given DNA site can be suppressed by some lesion at least several hundred kilobases away. Despite intensive study, no completely satisfactory proposal for the molecular mechanism of this effect has yet been advanced.

Determination of the DNA lesions responsible for inhibition of replicon initiation has been complicated by the lack of agents producing single types of damage. It has been noted, however, that those agents which induce direct DNA strand breakage, i.e., ionizing radiation and bromodeoxyuridine plus 313-nm light (Povirk, 1977; Painter, 1978), appear to be the most selective inhibitors of replicon initiation. In order to further test this correlation, we have examined in more detail the inhibition of DNA synthesis by neocarzinostatin (NCS),[1] a protein antibiotic which has also been shown to efficiently produce direct DNA strand breakage (reviewed by Goldberg et al., 1981; Kappen et al., 1980a). Effects of two related DNA strand-breaking antibiotics, auromomycin (Kappen et al., 1979; Suzuki et al., 1979) and bleomycin (Suzuki et al., 1969), were also examined.

Materials and Methods

Drugs. Clinical NCS with a concentration of 0.5 mg/mL (A_{273} = 1.4) and clinical bleomycin (Blenoxane) were obtained from Dr. W. T. Bradner of Bristol Laboratories. Auromomycin, obtained from Dr. T. S. A. Samy of Sidney Farber Cancer Institute through the courtesy of Professor H. Umezawa, was dissolved in distilled water and its concentration determined spectrophotometrically assuming an $E^{1\%}_{270}$ of 9.0 (Kappen et al., 1980b). Exposure of NCS and auromomycin to fluorescent lighting was avoided.

Cell Culture and Labeling. Chinese hamster CHO cells were maintained as monolayer cultures in Eagles minimum essential medium plus 15% fetal calf serum in a 5% CO_2 atmosphere. For all experiments, cells were prelabeled overnight in medium containing [^{14}C]thymidine (0.002–0.02 μCi/mL) and then incubated in drug-containing medium for 10 min. Cells were then either (1) harvested immediately and processed for nucleoid sedimentation or alkaline sedimentation of parental DNA or (2) washed twice, incubated for 20 min in regular medium, incubated for 10 min in medium containing 20 μCi/mL [^{3}H]thymidine, and processed for alkaline sedimentation of nascent ^{3}H-labeled DNA. This labeling protocol is specifically designed to maximize the distinction between replicon initiation and chain elongation (Painter & Young, 1976). For harvesting, cells were washed twice with ice-cold 0.15 NaCl–0.015 M trisodium citrate (SSC) and scraped off the plates into ice-cold SSC (or 0.14 M NaCl, 5 mM KCl, and 0.4 mM sodium phosphate, pH 7, for nucleoid sedimentation) with a Teflon policeman.

Nucleoid Sedimentation. A lysis solution (150 μL) containing 1.33 M NaCl, 0.67% Triton X-100, 27 mM EDTA, and 17 mM Tris-HCl, pH 8, was layered atop a preformed 5-mL neutral 15–30% sucrose gradient (1 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8), followed by 50 μL of cell suspension (3×10^5 cells). After lysis for 30 min, nucleoids were sedimented at 12000 rpm for 120 min in a Beckman SW 50.1 rotor. Fractions of 0.3 mL were collected from the top of the tube by pumping 50% sucrose through a hole punctured in the bottom, and their radioactivity was assayed after addition of 0.3 mL of water and 4 mL of Scintiverse (Fisher).

Alkaline Sucrose Gradients. Harvested cells were diluted in SSC to a concentration of 2×10^5/mL. For sedimentation of parental DNA, 0.5 mL of alkaline lysis solution [0.5 N NaOH, 0.02 M Na_2EDTA, and 0.1% NP-40 detergent (Particle Data Laboratories)] and 0.5 mL of cell suspension were layered atop a 36-mL 5–20% alkaline sucrose gradient

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received May 17, 1982.* This work was supported by U.S. Public Health Service Research Grant GM 12573 and Fellowship CA 06475 (L.F.P.) from the National Institutes of Health.

[1] Abbreviations: AT, ataxia telangiectasia; NCS, neocarzinostatin; SSC, 0.15 M sodium chloride–0.015 M trisodium citrate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-5857$01.25/0

(0.9 M NaCl, 0.1 M NaOH, and 0.02 M Na_2EDTA). After 6-h lysis, gradients were sedimented for 4 h at 20000 rpm in a Beckman SW 27 rotor. Following fractionation as described above, DNA in each fraction (1.8 mL) was precipitated, and its radioactivity was assayed (Clarkson & Painter, 1973). The molecular weight of DNA in each fraction was determined by using the equation of Studier (1965) and the calibration constant determined by Clarkson & Painter (1973). Weight-average molecular weights (M_w) of DNA distributions were determined directly from these data, while number-average molecular weights (M_n) were determined by using the method of Lehmann & Ormerod (1970).

For sedimentation of pulse-labeled nascent DNA, 0.15 mL of alkaline lysis solution and 0.15 mL of cell suspension were layered atop a 5-mL alkaline sucrose gradient. After 6-h lysis, gradients were centrifuged at 35000 rpm for 60 min in an SW 50.1 rotor. Fractions (0.3 mL) were collected, and radioactivity of precipitated DNA was determined. Another sample of each pulse-labeled cell culture ($\sim 4 \times 10^4$ cells in 0.2 mL of SSC) was added to 0.2 mL of the lysis solution. These samples were treated just as gradient fractions, and the relative rate of DNA synthesis for each culture was determined from the $^3H/^{14}C$ ratio. The profile of each gradient was normalized to the profile of the gradient from untreated control cells by multiplying the percent of total ^3H radioactivity in each fraction by the ratio of the rate of DNA synthesis in that sample to the rate in the control sample. Thus, the ^{14}C prelabel was used as an internal control to correct for variations in cell number between samples (Painter & Young, 1976). Our experience has shown that while qualitatively similar profiles are obtained when raw ^3H radioactivity is plotted, this normalization procedure reduces random variations between experiments and between duplicate gradients considerably.

Human Cells. Human normal 726a and 2936 fibroblasts and ataxia telangiectasia 2052 and 367 fibroblasts originally from the Human Genetic Mutant Cell Repository were obtained through Robert Boorstein and Dr. A. B. Pardee of the Sidney Farber Cancer Institute. Experiments examining DNA synthesis were performed as described for CHO cells above, except cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and cells were plated 2 days prior to pulse-labeling experiments. All experiments were performed with early passage (7–16) cells.

Results

Effect of Neocarzinostatin on Replicon Initiation. Alkaline sucrose gradient profiles of DNA from untreated pulse-labeled Chinese hamster CHO cells indicated a broad distribution in sizes of end-labeled nascent DNA (Figure 1). Profiles from cells pretreated with NCS showed a dose-dependent decrease in labeled low-molecular-weight DNA. Under these labeling conditions, radioactivity in the top half of the gradient indicates incorporation into smaller nascent DNA strands which initiated replication after drug treatment, while radioactivity in the bottom half indicates incorporation into nascent DNA strands which initiated before drug treatment and which are relatively long ($\geq 5 \times 10^6$ daltons) by the time of pulse label [see Painter & Young (1976) for a detailed discussion]. Thus the loss of low-molecular-weight labeled DNA (Figure 1) indicates an NCS-induced inhibition of initiation of new replicons, while the coincidence of the gradient profiles in the high-molecular-weight region suggests that the drug, even at the highest doses, had no effect on continued elongation of previously initiated replicons.

It is dangerous to rely solely on coincidence of profiles as a quantitative measure of DNA chain elongation, since an

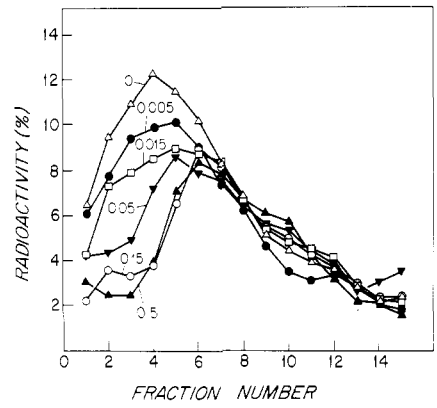

FIGURE 1: Alkaline sucrose gradient profiles of nascent DNA from monolayer cultures of CHO cells treated for 10 min with medium containing the indicated concentrations (in micrograms per milliliter) of NCS, incubated in regular medium for 20 min, and then incubated for 10 min in medium containing [^3H]thymidine to label the ends of growing DNA strands. Sedimentation is to the right. Profiles from NCS-treated cells have been normalized to the relative rate of DNA synthesis in those cells, compared to that of control cells, as described under Materials and Methods.

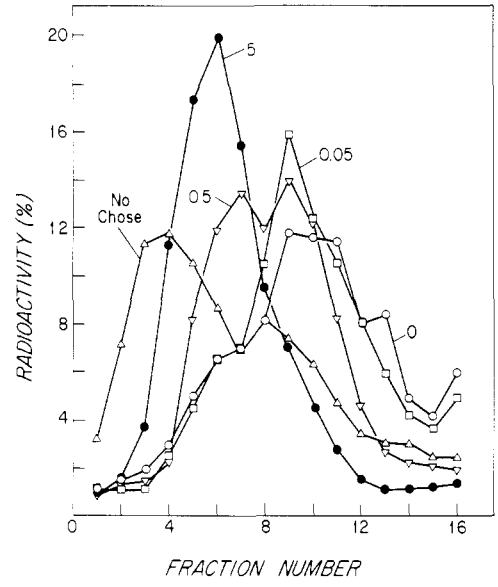

FIGURE 2: Effect of NCS on elongation of nascent DNA as judged by progression of thymidine pulse label into high-molecular-weight DNA. CHO cells were incubated for 10 min in medium containing 20 μCi/mL [^3H]thymidine and either harvested immediately ("no chase") or incubated for 10 min in medium containing the indicated concentrations of NCS (in micrograms per milliliter) plus 10^{-5} M thymidine, incubated in regular medium for 20 min, and then harvested. Cellular DNA was then sedimented in alkaline sucrose gradients. Sedimentation is to the right.

inhibition of elongation might be masked by a compensating increase in specific activity of DNA precursor pools or in repair synthesis [although the latter should be insignificant at these doses (Kappen & Goldberg, 1978)]. Therefore, we examined elongation independently by using pulse–chase experiments (Makino & Okada, 1975). This more sensitive technique confirmed that 0.5 μg/mL NCS was the lowest dose which had any effect on elongation as judged by progression of thymidine pulse label into high-molecular-weight DNA (Figure 2). A repetition of the experiments described in Figures 1 and 2 yielded essentially identical results (not shown).

The selective affect of NCS on replicon initiation resulted in a distinctly biphasic dose response for inhibition of total DNA synthesis by this drug (Figure 3). The sensitive com-

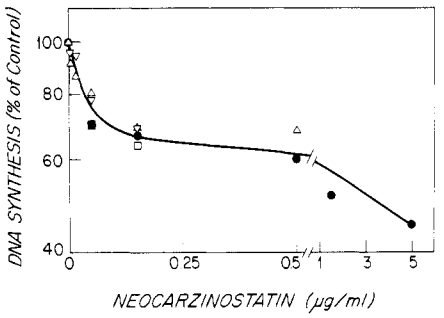

FIGURE 3: Effect of NCS on incorporation of [^{3}H]thymidine into acid-precipitable material during a 10-min pulse label beginning 20 min after NCS treatment of CHO cells. Each point represents a single culture, and different symbols indicate four separate experiments.

Table I: Strand Breakage of Parental DNA by NCS[a]

NCS (μg/mL)	$M_w \times 10^{-8}$	$M_n \times 10^{-8}$
0	2.6	
0.5	2.6	
2	1.7	1.1
8	1.1	0.5

[a] Cells were prelabeled with [^{14}C]thymidine and treated for 10 min with NCS, and their DNA was sedimented in alkaline sucrose.

ponent, attributable to inhibition of replicon initiation (Figure 1), was fully expressed at 0.1 μg/mL and accounted for about 30% of total synthesis.

It should be noted that the gradient profiles of pulse-labeled DNA (Figure 1), the dose response for DNA synthesis (Figure 3), and the time course of DNA synthesis inhibition and recovery after NCS treatment (Hatayama & Goldberg, 1979) are nearly identical with those obtained in comparable experiments with X-rays (Painter & Young, 1976). In the case of X-rays, there is a large body of additional evidence that these patterns do in fact reflect selective inhibition of replicon initiation. As predicted by such a hypothesis, loss of [^{3}H]-thymidine incorporation is seen in progressively larger nascent DNA, as the time between irradiation and pulse label is increased (Painter & Young, 1976). Density-labeling experiments (Makino & Okada, 1975; Painter & Young, 1975) show that the decrease in incorporation is due to an actual decrease in semiconservative DNA synthesis, not merely a change in specific activity of precursor pools. Furthermore, fiber autoradiograms of DNA from irradiated, pulse-labeled cells confirm that there is a decrease in short labeled nascent DNA segments and that the rate of progression of replication forks is not affected (Watanabe, 1974; Dahle et al., 1978). These results suggest that selective inhibition of replicon initiation is the most reasonable interpretation of the data of Figures 1 and 3.

DNA Strand Breakage. Because of the apparent similarity between NCS and X-rays in patterns of DNA synthesis inhibition, we sought to determine whether a given level of DNA damage could be correlated with a given degree of synthesis inhibition. As with X-rays (Lett et al., 1967), much higher doses of NCS than those which inhibited replicon initiation were required to produce measurable changes in molecular weight of parental DNA (Table I). However, since the number of breaks was not directly proportional to NCS dose, these data could not be reliably extrapolated to lower NCS doses. Therefore, we examined strand breakage by the more sensitive technique of nucleoid sedimentation.

When mammalian cells are lysed with nonionic detergents at high ionic strength and neutral pH, rapidly sedimenting

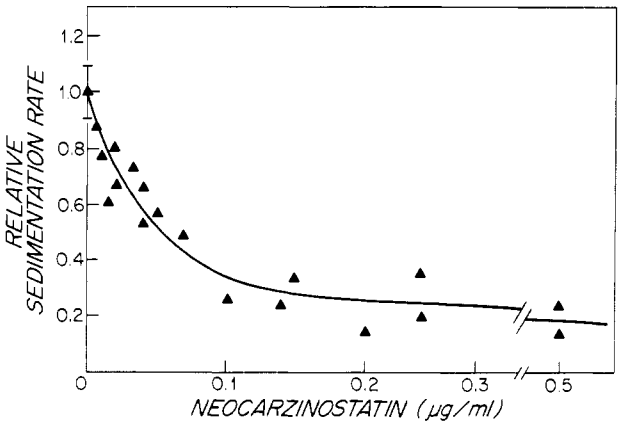

FIGURE 4: Relaxation of DNA supercoiling in CHO cells by NCS (10-min treatment) as judged by sedimentation of nucleoids in neutral sucrose gradients. Results of five experiments have been pooled. Error bar at origin indicates average range of duplicate controls.

"nucleoids" are released, whose DNA is topologically constrained and negatively supercoiled (Cook & Brazell, 1975). Induction of a small number of DNA strand breaks by γ irradiation relaxes the supercoils, resulting in a dramatic decrease in the rate of sedimentation of the nucleoids. As shown in Figure 4, low doses of NCS induced a similar decrease in the rate of sedimentation of CHO nucleoids. Furthermore, when the cells were treated with drug under the same conditions as those used in synthesis inhibition experiments, half-maximal relaxation of nucleoids required the same dose of NCS (0.03 μg/mL) as half-maximal inhibition of replicon initiation. Although not known with certainty, the size of domains of DNA supercoiling (i.e., the distance between sites of topological constraint) has been estimated at 10^9 daltons (Cook & Brazell, 1975). Thus, the nucleoid sedimentation data would indicate that 0.03 μg/mL NCS induced slightly less than one break per 10^9 daltons of DNA.

Other Drugs. Effects of the isolated nonprotein chromophore of NCS on DNA synthesis in CHO cells were very similar to those of native NCS, consistent with the view that all the biological effects of NCS are due to the action of the chromophore (Kappen et al., 1980a). Chromophore-free NCS apoprotein had no effect at any concentration.

Results obtained with auromomycin were nearly identical with those obtained with NCS. Low doses of auromomycin inhibited replicon initiation exclusively. Half-maximal reduction in the sedimentation rate of CHO nucleoids, as well as half-maximal inhibition of initiation, required 0.03 μg/mL auromomycin.

Bleomycin, however, was an apparent exception to the correlation of strand breakage with inhibition of initiation. In nucleoid sedimentation experiments, 10 μg/mL bleomycin completely relaxed DNA supercoiling, and 100 μg/mL (but not 50 μg/mL) induced strand breakage detectable by alkaline sucrose gradient sedimentation (M_n 1 × 10^8). However, inhibition of replicon initiation was never seen at any dose lower than 100 μg/mL, in either CHO or HeLa cells, even when treatment time was increased to 40 min. In some experiments a slight effect on initiation was seen at 100 μg/mL bleomycin, but in no case was the inhibition of total DNA synthesis greater than 20%. As reported by Suzuki et al. (1968) longer (4 h) bleomycin treatments inhibited DNA synthesis severely in HeLa cells.

Ataxia Telangiectasia. Fibroblasts derived from individuals with the genetic disorder ataxia telangiectasia (AT) are more sensitive to killing by X-rays (Taylor et al., 1975) than normal

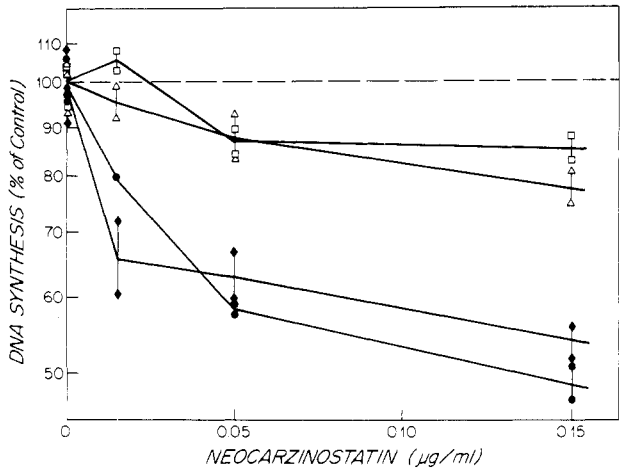

FIGURE 5: Effect of NCS on DNA synthesis in human AT 2052 (△ and □) and in normal diploid 726a (♦ and ●) cells. Two separate experiments were performed with each cell type, each having duplicate culture plates at each dose and triplicate controls. NCS treatment was for 10 min, followed by a 20-min incubation and then a 30-min pulse label with [^{3}H]thymidine (20 μCi/mL). Essentially identical results were obtained with AT 367 and normal diploid 2936 cells.

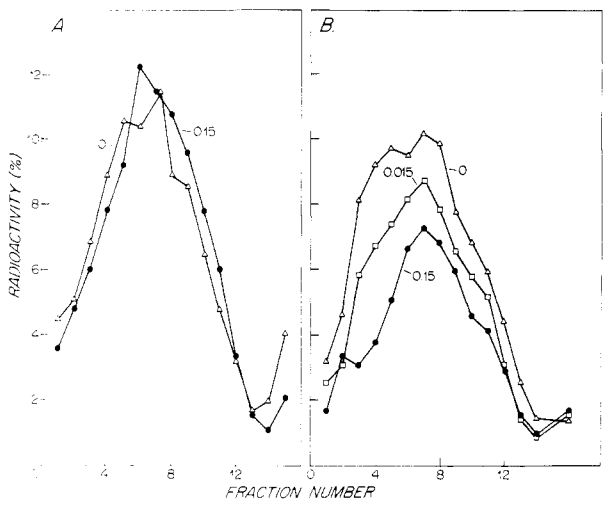

FIGURE 6: Alkaline sucrose gradient profiles of nascent DNA from AT 2052 cells (A) or normal human 2936 cells (B). Cells were treated for 10 min with medium containing the indicated concentrations of NCS, incubated for 20 min in regular medium, and then pulse labeled with [^{3}H]thymidine (30 μCi/mL) for 10 min. Sedimentation is to the right.

human fibroblasts. However, DNA synthesis in these cells is more resistant to radiation than synthesis in normal cells (Edwards & Taylor, 1980; Painter & Young, 1980; Smith & Paterson, 1980). As shown in Figure 5, AT cells were also resistant to DNA synthesis inhibition by NCS. The dose–response curves for both AT and normal cells are similar to those previously obtained in experiments with X-rays (Edwards & Taylor, 1980; Painter & Young, 1980).

Alkaline sucrose gradient profiles of nascent DNA from NCS-treated AT cells were virtually indistinguishable from the control profile, confirming the lack of effect on DNA synthesis (Figure 6). In normal cells, the NCS-induced decrease in [^{3}H]thymidine incorporation was most severe for short nascent DNA molecules. As discussed above, this pattern suggests inhibition of replicon initiation by NCS. However, in contrast to CHO cells, normal diploid fibroblasts also showed some decrease in incorporation into high-molecular-weight nascent DNA, suggesting that chain elongation was also inhibited even by the lowest doses of NCS.

Discussion

Stated most simply, our results show that NCS can reproduce, qualitatively and quantitatively, every characteristic of X-ray-induced DNA synthesis inhibition in mammalian cells that has been examined. After treatment of cells with NCS, gradient profiles of pulse-labeled DNA and dose–response curves in CHO (Figures 1 and 3), human normal and human AT fibroblasts (Figures 5 and 6) are virtually superimposable with the data obtained with X-irradiated cells (Painter & Young, 1976, 1980; Edwards & Taylor, 1980). In each case, 0.05 μg/mL NCS appears to be roughly equivalent to 1 krad of X-rays. Furthermore, most other chemical DNA-damaging agents are not as selective in inhibiting replicon initiation as X-rays and NCS (Painter, 1977, 1978). These results suggest that inhibition of replicon initiation is caused by some lesion which is produced in significant yield by both X-rays and NCS. Although most X-ray-induced damage is in the DNA bases, a substantial amount of deoxyribose damage, i.e., free radical induced sugar oxidation, is produced (Von Sonntag & Shulte-Frohlinde, 1978). Deoxyribose also appears to be the major site of NCS-induced DNA damage (Hatayama & Goldberg, 1980; Povirk & Goldberg, 1982; Kappen et al., 1982), and there is no evidence for DNA base damage by NCS. Thus the similarity between X-rays and NCS in patterns of DNA synthesis inhibition suggests that damage to deoxyribose may be the effective lesion in both cases. Consistent with this proposal are the findings that auromomycin and bromodeoxyuridine plus 313-nm light (Povirk, 1977) show selective inhibition of replication initiation comparable to X-rays and NCS; these agents also induce direct damage to deoxyribose in DNA (Hutchinson, 1973; Takeshita et al., 1981).

Deoxyribose damage can be of several different forms, some of which result immediately in breakage of the DNA sugar–phosphate backbone. Other forms of deoxyribose damage are expressed as breaks only after alkaline treatment. Since NCS (Goldberg et al., 1981; Povirk & Goldberg, 1982; Kappen et al., 1982), bromodeoxyuridine plus 313-nm light (Hutchinson, 1973), and X-rays (Ward, 1975) all induce both classes of damage, there is no clear indication which is responsible for DNA synthesis inhibition. However, with NCS and with auromomycin, there is a quantitative correlation between inhibition of replicon initiation and induction of sufficient DNA strand breakage to relax domains of DNA supercoiling in CHO nucleoids. Similarly, a few hundred rads of X-rays are required both to reduce the sedimentation rate of nucleoids from human cells and to inhibit replicon initiation in a variety of cell lines (Cook & Brazell, 1975; Painter & Young, 1976). These correlations provide circumstantial evidence that direct DNA strand breakage may be the lesion primarily responsible for effects of all these agents on replicon initiation.

How, then, can results with bleomycin be accounted for? Taken at face value, they would indicate that significant strand breakage (and the resulting relaxation of DNA supercoiling) can occur without any effect on replicon initiation. An alternative explanation, difficult to eliminate rigorously, is that most bleomycin-induced strand breakage occurred not in the intact cell but in the lysate. Such a phenomenon seems more plausible with bleomycin than with the other drugs since considerably higher concentrations of bleomycin are required to induce measurable strand breakage. Furthermore, in contrast to the essentially exponential survival curves seen with X-rays (Okada, 1970) and NCS (Hatayama & Goldberg, 1979; Shiloh et al., 1982), the survival curve seen with bleomycin is distinctly biphasic, suggesting a highly resistant

subpopulation of cells (Barranco & Humphrey, 1971). Alkaline elution studies have also indicated a pronounced heterogeneity in bleomycin-induced strand breakage in mammalian cells: some portions of DNA are severely damaged while other portions, possibly associated with the resistant fraction of cells, remain virtually intact (Iqbal et al., 1976). In bacteria, DNA damage also seems to be confined to a small subfraction of the DNA, and this confinement has been postulated to account for the lack of any inhibition of bacterial DNA synthesis (Hutchinson et al., 1981). Given these poorly understood anomalities in the effects of bleomycin, and the fact that it is an exception to the trend seen with the other agents, we are inclined not to reject a connection between strand breakage and inhibition of replicon initiation, solely on the basis of the bleomycin data. Contrary to our results in CHO cells, bleomycin has been reported to inhibit replicon initiation in normal human diploid fibroblasts (Cramer & Painter, 1981; Edwards et al., 1981).

If inhibition of replicon initiation is, in fact, caused by strand breaks, what molecular events might mediate the effect? The large target size would appear to preclude actual DNA damage at every initiation site. The finding that the same doses of NCS, auromomycin, or X-rays are required to inhibit replicon initiation and relax DNA supercoiling suggests that release of topological constraints on DNA may itself prevent initiation. Release of topological constraints could affect any DNA protein interactions which involve changes in DNA winding angles.

Alternatively, however, mammalian cells may possess a system having nothing to do with supercoiling, which recognizes the presence of strand breaks in cellular DNA and acts to suppress replicon initiation until the breaks are repaired. Such a system could involve proteins which bind specifically to free DNA end groups and produce cofactors which control initiation and possibly many other cell processes. The sensitivity of the system might be such that roughly one strand break per DNA supercoiling domain is required for its activation, resulting in a fortuitous correlation between supercoil relaxation and inhibition of initiation.

In principle, mechanisms of the initiation effect which involve conformational changes in damaged DNA can be distinguished from those involving changes in overall cellular metabolism, by experiments in which only selected portions of the DNA are damaged. Such experiments (Povirk, 1977) favor conformational changes in DNA as intermediate steps in inhibiting initiation. However, other experiments showing that the X-ray-induced inhibition of DNA synthesis can be delayed by caffeine, fluorodeoxyuridine, or adenine (Griffiths et al., 1978; Tolmach et al., 1977) suggest involvement of rather complex metabolic regulatory systems. Involvement of poly(ADP-ribose) synthesis has also been proposed (Edwards & Taylor, 1980). Some combination of the two types of mechanisms cannot be ruled out.

Human AT cells are more sensitive to killing by X-rays, bleomycin, and NCS than normal diploid fibroblasts (Taylor et al., 1975, 1979; Shiloh et al., 1982). Some AT lines are also sensitive to killing by 4-nitroquinoline 1-oxide, actinomycin D, and alkylating agents (Scudiero, 1980; Hoar & Sargent, 1976; Jaspers et al., 1982). However, AT cells are relatively resistant to the low-dose inhibition of DNA synthesis induced by X-rays, bleomycin, and NCS but are not resistant to that induced by 4-nitroquinoline 1-oxide, actinomycin D, or alkylating agents (Figure 5; Edwards & Taylor, 1980; Painter & Young, 1980; Smith & Paterson, 1980; Cramer & Painter, 1981; Jaspers et al., 1982). Thus the resistance of AT cells to inhibition of DNA synthesis appears to be restricted to agents which induce direct strand breakage in DNA. These results suggest that AT cells are genetically deficient in some mechanism which recognizes the presence of low levels of damage, particularly strand breaks, in cellular DNA. This mechanism, whatever its molecular basis, is probably closely related to the inhibition of replicon initiation seen in CHO and other cell lines. However, unlike established cell lines such as CHO and HeLa, human diploid fibroblasts show inhibition of both replicon initiation and chain elongation even at the lowest doses of either X-rays or NCS, and both processes, initiation and elongation, are resistant in AT cells (Figure 6; Cramer & Painter, 1981; Edwards et al., 1981). This result raises the possibility that there may be important differences between normal diploid cells and aneuploid cell lines in the mechanisms by which they recognize and respond to DNA damage. However, the sensitivity of AT cells to killing by DNA strand-breaking agents suggests that such recognition mechanisms play an important role in cell survival, by delaying DNA replication until DNA damage is repaired.

Acknowledgments

We thank Robert Boorstein and Dr. Arthur B. Pardee for providing human cell lines and Dr. Robert B. Painter and Dr. Michael R. Mattern for helpful advice.

References

Barranco, S. C., & Humphrey, R. M. (1971) *Cancer Res. 31*, 1218.

Clarkson, J. M., & Painter, R. B. (1973) *Mutat. Res. 23*, 107.

Cook, P. R., & Brazell, I. A. (1975) *J. Cell Sci. 19*, 261.

Cramer, P., & Painter, R. B. (1981) *Nature (London) 291*, 671.

Dahle, D. B., Griffiths, T. D., & Carpenter, J. G. (1978) *Mol. Pharmacol. 14*, 278.

Edwards, M. J., & Taylor, A. M. R. (1980) *Nature (London) 287*, 7451.

Edwards, M. J., Taylor, A. M. R., & Flude, E. J. (1981) *Biochem. Biophys. Res. Commun. 102*, 610.

Goldberg, I. H., Hatayama, T., Kappen, L. S., Napier, M. A., & Povirk, L. F. (1981) in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Sartorelli, A. C., Lazo, J. S., & Bertino, J. R., Eds.) Academic Press, New York.

Griffiths, T. D., Carpenter, J. G., & Dahle, D. B. (1978) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 33*, 493.

Hatayama, T., & Goldberg, I. H. (1979) *Biochim. Biophys. Acta 563*, 59.

Hatayama, T., & Goldberg, I. H. (1980) *Biochemistry 19*, 5890.

Hoar, D. I., & Sargent, P. (1976) *Nature (London) 261*, 590.

Hutchinson, F. (1973) *Q. Rev. Biophys. 6*, 201.

Hutchinson, F., Povirk, L. F., & Yamamoto, K. (1981) in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Sartorelli, A. C., Lazo, J. S., & Bertino, J. R., Eds.) Academic Press, New York.

Iqbal, Z. M., Kohn, K. W., Ewig, R. A. G., & Fornace, A. J. (1976) *Cancer Res. 36*, 3834.

Jaspers, N. G. J., deWit, J., Regulski, M. R., & Bootsma, D. (1982) *Cancer Res. 42*, 335.

Kappen, L. S., & Goldberg, I. H. (1978) *Biochim. Biophys. Acta 520*, 481.

Kappen, L. S., Goldberg, I. H., & Samy, T. S. A. (1979) *Biochemistry 18*, 5123.

Kappen, L. S., Napier, M. A., & Goldberg, I. H. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1970.

Kappen, L. S., Napier, M. A., Goldberg, I. H., & Samy, T. S. A. (1980b) *Biochemistry 19*, 4780.
Kappen, L. S., Goldberg, I. H., & Liesch, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 744.
Lehmann, A. R., & Ormerod, M. G. (1970) *Biochim. Biophys. Acta 204*, 128.
Lett, J. T., Caldwell, I., Dean, C. J., & Alexander, P. (1967) *Nature (London) 214*, 790.
Makino, F., & Okada, S. (1975) *Radiat. Res. 62*, 34.
Okada, S. (1970) *Radiation Biochemistry*, Vol. 1, pp 260–285, Academic Press, New York.
Painter, R. B. (1977) *Mutat. Res. 42*, 299.
Painter, R. B. (1978) *Cancer Res. 38*, 4445.
Painter, R. B., & Rasmussen, R. E. (1964) *Nature (London) 201*, 162.
Painter, R. B., & Young, B. R. (1975) *Radiat. Res. 64*, 648.
Painter, R. B., & Young, B. R. (1976) *Biochim. Biophys. Acta 418*, 146.
Painter, R. B., & Young, B. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7315.
Povirk, L. F. (1977) *J. Mol. Biol. 114*, 141.
Povirk, L. F., & Painter, R. B. (1976) *Biochim. Biophys. Acta 432*, 267.
Povirk, L. F., & Goldberg, I. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 369.
Scudiero, D. A. (1980) *Cancer Res. 40*, 984.
Shiloh, Y., Tabor, E., & Becker, Y. (1982) *Cancer Res. 42*, 2247.
Smith, P. J., & Paterson, M. C. (1980) *Biochem. Biophys. Res. Commun. 97*, 897.
Studier, F. W. (1965) *J. Mol. Biol. 11*, 373.
Suzuki, H., Nagai, K., Yamaki, H., Tanaka, N., & Umezawa, H. (1968) *J. Antibiot. 21*, 379.
Suzuki, H., Nagai, K., Yamaki, H., Tanaka, N., & Umezawa, H. (1969) *J. Antibiot. 22*, 446.
Suzuki, H., Nishimura, T., & Tanaka, N. (1979) *Cancer Res. 39*, 2787.
Takeshita, M., Kappen, L. S., Grollman, A. P., Eisenberg, M., & Goldberg, I. H. (1981) *Biochemistry 20*, 7599.
Taylor, A. M. R., Harnden, D. G., Arlett, S. A., Lehmann, A. R., Stevens, S., & Bridges, E. A. (1975) *Nature (London) 258*, 427.
Taylor, A. M. R., Rosney, C. M., & Campbell, J. B. (1979) *Cancer Res. 39*, 1046.
Tolmach, L. J., Jones, R. W., & Basse, D. M. (1977) *Radiat. Res. 71*, 653.
Von Sonntag, C., & Shulte-Frohlinde, D. (1978) *Mol. Biol., Biochem. Biophys. 27*, 204.
Walters, R., & Hildebrand, C. E. (1975) *Biochem. Biophys. Res. Commun. 65*, 265.
Ward, J. F. (1975) *Adv. Radiat. Biol. 5*, 181.
Watanabe, I. (1974) *Radiat. Res. 58*, 541.

Interaction of Copper(II) Ions with the Daunomycin–Calf Thymus Deoxyribonucleic Acid Complex†

M. Spinelli and J. C. Dabrowiak*

ABSTRACT: The interaction of Cu(II) with the native and heat-denatured DNA complexes of daunomycin (**1**) and *N*-(trifluoroacetyl)daunomycin (**3**) has been examined by using absorption and circular dichroism spectroscopies. At low r_t, where r_t is the input molar ratio of drug to DNA phosphate, Cu(II) interacts with the native and heat-denatured calf thymus DNA complex of daunomycin to form a ternary complex involving the aglycon portion of the antibiotic, Cu(II), and DNA. A Job plot of the titration involving Cu(II) and heat-denatured DNA shows that the Cu(II)–drug stoichiometry in the ternary complex is ≤1. Although the *N*-(trifluoroacetyl)daunomycin–native DNA complex does not form a ternary complex, the denatured DNA complex with the antibiotic does. Copper(II) titrations of the daunomycin–native DNA complex, at high r_t, where both strongly and weakly bound antibiotic molecules are very likely present in solution result in the formation of both the ternary species as well as a binary complex involving only the metal ion and the antibiotic. The spectroscopic results indicate that in the ternary complex, the Cu(II) ion is bound to the unintercalated aglycon portion of the antibiotic and very likely also to the heterocyclic bases of DNA.

The antitumor antibiotics daunomycin (**1**) (daunorubicin) and adriamycin (**2**) (deoxyrubicin) (Chart I) are in wide clinical use for the treatment of various neoplastic diseases (Carter, 1980). The mechanism by which the drugs exhibit their antineoplastic activity is not completely understood, but their strong DNA binding properties led the discoverers of the antibiotics to suggest that the drug receptor site is DNA (DiMarco et al., 1964; Arcamone et al., 1969). Most of the available evidence indicates that the antibiotics bind to DNA via an intercalative process employing the hydroxyquinone portion of the drug and also through an electrostatic interaction involving the protonated amine group on daunosamine (DiMarco & Arcamone, 1975; Pigram et al., 1972). Recent crystallographic studies on the hexanucleotide duplex d(CpGpTpApCpG), containing intercalated daunomycin molecules at both CG sites, show that the long axis of the aglycon portion of the drug is roughly perpendicular to the base pair axis at the intercalation site (Quigley et al., 1980).

In an effort to gain further information about the binding mechanism, several investigators have studied the binding of metal ions to the anthracycline–DNA complex. Fishman & Schwartz (1974) demonstrated that the addition of Cu(II) to the denatured calf thymus DNA–daunomycin complex affects the visible absorption and fluorescence spectra of the drug–DNA complex. In the presence of native DNA, however, the study reported that Cu(II) did not affect the spectral properties of the intercalated drug. These observations led Fishman and

† From the Department of Chemistry, Syracuse University, Syracuse, New York 13210. *Received May 6, 1982.*

0006-2960/82/0421-5862$01.25/0