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Effects of Iron, Copper, Cobalt, and Their Chelators on the Cytotoxicity of Bleomycin[1]

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ABSTRACT

Bleomycin is widely used for treating several types of human tumors as well as a variety of experimental tumors. The ability of this antibiotic to bind and to damage DNA has been proposed to be responsible for its antitumor effect. Bleomycin is also a good chelator for several metals, *e.g.*, iron, copper, and others. Bleomycin:metal complexes have been investigated in detail particularly for their action on isolated DNA. The conclusions from these studies indicate that metal-chelated bleomycin either is ineffective or more effective in damaging DNA. In this paper, we tested the effect of iron, copper, cobalt, and their chelators on bleomycin cytotoxicity. Our results suggest that chelating bleomycin with copper or adding an iron chelator (deferoxamine), diethylenetriamine pentaacetic acid, and a copper chelator (penicillamine) shows no effect on bleomycin cytotoxicity. On the other hand, iron dextran and a metal chelator, diethyldithiocarbamate (DDC), with bleomycin show enhanced cytotoxicity. Cobalt-chelated bleomycin is not cytotoxic but is cytotoxic when combined with DDC. We suggest that different mechanisms are contributing to the enhanced toxicity of bleomycin with iron dextran and DDC. Bleomycin acts as a ferrous oxidase which promotes the iron toxicity. In the case of DDC, it can act as a reducing agent or it can help to maintain the bleomycin:metal complex in the reduced form which can generate radicals.

INTRODUCTION

Bleomycin, an antibiotic isolated from *Streptomyces verticillus* (33), has antitumor activities against a variety of tumors in both animal and human, *e.g.*, squamous cell carcinomas, lymphoma and testicular carcinoma. Bleomycin can affect many cellular pathways, but the cytotoxic activity is generally believed to be associated with its property toward DNA, *i.e.*, the ability of bleomycin to bind and cleave DNA (29, 30). Extensive effort has been devoted directly toward an understanding of the involvement of metals in DNA strand breakage induced by this family of antibiotics. However, a similar approach in studying the bleomycin cytotoxic action is rather limited. Our study of the mechanism by which the cytotoxic potential of bleomycin can be modified is based on the following findings. (*a*) Copper-, zinc-, (23, 24, 32), and cobalt-chelated (23, 24) bleomycins are ineffective in causing strand scission of isolated DNA. However, copper-chelated bleomycin showed the same inhibiting growth effect on bacterial and animal cells as copper-free bleomycin (19, 32). (*b*) Irons (Fe^{2+} and Fe^{3+} in the presence of reducing agents) can enhance the DNA chain breakage and the release of free base by bleomycin (22–24). The role of iron is further demonstrated in that low concentrations of chelator, most notably the iron chelator deferoxamine, effectively inhibits the breakage of DNA induced by bleomycin (23, 24). In addition, oxygen appears essential for Fe^{2+} to stimulate bleomycin activity at low concentrations (3, 22, 24). (*c*) Superoxide radicals may enhance the DNA chain breakage action of bleomycin (10), probably acting as reducing agents on the Fe^{2+} in the experimental system (24). It has been proposed that bleomycin:Fe^{2+} generates "site-specific" $OH^{\cdot}$ radicals (16) which are most likely responsible for the observed effect of bleomycin on DNA. The role of O_2^- is that it reacts with bleomycin:Fe^{3+} and yields bleomycin:Fe^{2+} (24), which leads to more $OH^{\cdot}$ generation by a Fenton type of reaction. Although radicals participate in the action of bleomycin on DNA, the fact that bleomycin:Cu^{+} and bleomycin:Fe^{2+} (but not cytotoxic bleomycin:Cu^{2+} complex) are able to produce $OH^{\cdot}$ needs to be recognized. Thus, if radicals are involved in the cytotoxic action of bleomycin:Cu^{2+}, a reduction process is probably necessary.

We were interested in determining whether we could apply this knowledge of the molecular mechanisms of bleomycin's ability to degrade isolated DNA at the cellular level. We have recently proposed the use of DDC[3] to augment cellular bleomycin cytotoxicity (13, 14). DDC would make bleomycin more cytotoxic than in the presence of normal levels of Cu^{2+} and/or Zn^{2+} because it has been demonstrated that these metals interfere with the bleomycin effect (23, 24). DDC also can inhibit the cellular enzyme responsible for removing O_2^-, *i.e.*, SOD. Thus, DDC-treated cells may have a higher intracellular O_2^- concentration (generated by normal metabolic processes) than untreated cells which, in turn, promotes the bleomycin effect. Our experimental results indeed show that bleomycin is much more effective in inducing lethality to DDC-treated cells than untreated cells *in vitro* (14). We are beginning to investigate the mechanisms for the DDC enhancement of bleomycin cytotoxicity. In this respect, we focused particularly on the role of metals. Whether O_2^- is involved in DDC enhancement of bleomycin effect will be presented in the future.

MATERIALS AND METHODS

Cells. The Chinese hamster cell line V79 was used in all experiments. V79 cells were propagated as a monolayer culture in Earle's minimum essential medium supplemented with 5% fetal calf serum and 5% newborn calf serum.

Exponentially replicating cultures were trypsinized for 2 to 4 min at 37° with 0.05% trypsin containing 0.2 g EDTA per liter of solution. Cell suspensions were pelleted and washed twice before being resuspended in complete medium. All chemical solutions were diluted directly into the cell suspension. After treatment, the cells were washed twice, plated onto Falcon plastic Petri dishes and allowed to incubate for 6 to 8 days at 37° in a 5% CO_2:95% air incubator. After this period,

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[3] The abbreviations used are: DDC, diethyldithiocarbamate; SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid.

the plates with colonies were fixed with methanol and stained with 1% Giemsa solution before counting. Surviving fraction of treated specimen was based on the control specimen equal to one.

Liver Extracts. Extracts prepared from rat liver homogenates have the ability to reduce bleomycin activity. Livers from laboratory rats were excised and homogenized in 0.1 M cold phosphate buffer (pH 7.2). The homogenates used for the liver extract were prepared by centrifugation as described by Yoshioka *et al.* (34). For testing the liver extracts, bleomycin was added to the extracts and incubated at 37° for 60 min. The bleomycin extract mixture was then used in standard cell survival experiments.

Uptake of ^{57}Co:Bleomycin. ^{57}Co:bleomycin (1 mCi ^{57}Co per mg bleomycin) was prepared from $^{57}CoCl_2$ (New England Nuclear) and bleomycin by the method described by Grove *et al.* (8). ^{57}Co:bleomycin (0.5 μCi) was added to 2 × 10^6 cells (1 ml) and incubated for an additional 30 min at 37°. The cells were then washed twice with medium, and the radioactivity of the sample was determined and compared.

Protein Synthesis. [^{14}C]Leucine (300 mCi/mmol; New England Nuclear) was used in our experiment with essentially the same method described by Fuhr (7).

Chemicals. Bleomycin was obtained from Bristol Laboratories (Syracuse, N. Y.) through the courtesy of Dr. W. T. Bradner. DDC, penicillamine, deferoxamine, and DTPA were obtained from Sigma Chemical Co. (St. Louis, Mo.). Deferoxamine was obtained from Ciba Pharmaceutical Co. (Summit, N. J.). Iron dextran as Imferon was obtained from Merrell-National Laboratory (Cincinnati, Ohio). Both [^{14}C]leucine and $^{57}CoCl_2$ were obtained from New England Nuclear.

RESULTS

Treating V79 cells in suspension with increasing doses (10 to 60 μg/ml) of bleomycin for 30 or 60 min shows a typical biphasic response as reported by others. At low concentrations of bleomycin, the efficiency of toxic effects is dependent on the drug concentration whereas, at the high concentration of bleomycin, the efficiency of increasing bleomycin concentration on additional cytotoxicity is limited. The latter is often addressed as the resistant component (Chart 1). When V79 cells are pretreated with DDC (10^{-4} M, 60 min at 37°), the effect of bleomycin is enhanced. The amount of enhancement is primarily derived from the bleomycin-resistant component. To a lesser extent, 10^{-6} M of DDC, a nontoxic level, can also enhance bleomycin cytotoxicity. On the other hand, cells treated with 10^{-8} M DDC respond in a similar way as control cells. Another characteristic of the bleomycin effect is that treatment of cells with a fixed dose of bleomycin but with increasing time exposure also produces a biphasic response. Moreover, the bleomycin-resistant component also becomes more responsive to increased exposure time with DDC (10^{-4} M; 30 min) pretreatment (data not shown).

To test the penicillamine effect, V79 cells are exposed to 10^{-4} M penicillamine and bleomycin (5 to 60 μg/ml) for 35 min at 37°. The results are plotted in Chart 2. It is clear that penicillamine does not play a role in bleomycin cytotoxic effect. However, this experiment does not completely assure us that the concentration of Cu^{2+} has been reduced to a level which may stimulate the bleomycin effect. Thus, in similar experiments, penicillamine is added back into the cell suspensions after the washing steps. Again, penicillamine shows no effect on bleomycin cytotoxicity.

The reason that a short exposure time was used with penicillamine is that Bindarup and Arrigoni-Martelli (2) have found

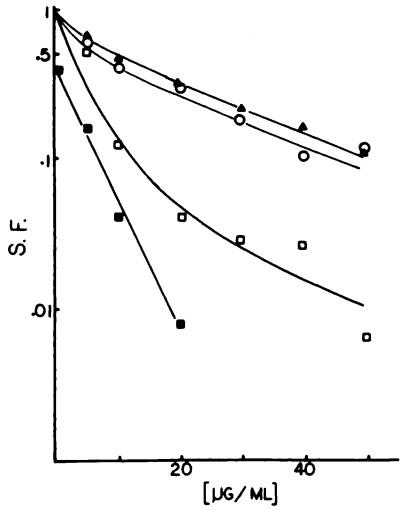

Chart 1. Enhanced effect of DDC on bleomycin cytotoxicity as a function of bleomycin dose at 37° for 1 hr. ○, bleomycin only; ■, cells pretreated with 10^{-4} M DDC for 60 min at 37°; □, cells pretreated with 10^{-6} M DDC for 60 min at 37°; ▲, cells pretreated with 10^{-8} M DDC for 60 min at 37°; *S.F.*, surviving fraction.

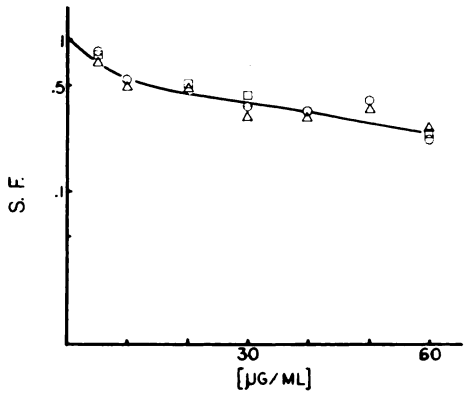

Chart 2. Cytotoxicity of bleomycin with (△) and without (○) simultaneously added 10^{-4} M of penicillamine for 35 min at 37°. □, penicillamine added back to the culture medium after the initial combined treatment. *S.F.*, surviving fraction.

that a long exposure time (over 60 min) shows a substantial decrease in the amount of penicillamine present in the cells.

To test the effect of deferoxamine, V79 cells are first treated with deferoxamine (10^{-4} M) for 1 hr at 37°. Bleomycin (10 to 100 μg/ml) is then added to the cell suspension and incubated for an additional hr at 37°. The results show deferoxamine either has no effect or marginal effect on reducing bleomycin cytotoxic effect (Chart 3). A similar result is observed when the preincubation time with deferoxamine is increased to 4 hr (data not shown).

Similar to the deferoxamine experimental protocol, V79 cells are first treated with DTPA (240 μM) and $FeCl_2 \cdot 4\ H_2O$ (200 μM) for 1 hr at 37°, bleomycin is added, and the incubation is continued at 37° for an additional 30 min. The results indicate that the addition of Fe^{2+} and DTPA does not appear to modify bleomycin cytotoxicity (Chart 4). Likewise, no modification effect can be detected if the V79 cells are first treated with EDTA (10^{-5} M) for 30 min at 37° and then treated with bleomycin (10 to 50 μg/ml) for 30 min at 37° (data not shown).

Although Cu^{2+}-chelated bleomycin is inactive in damaging isolated DNA, the complex must still have cytotoxic ability in

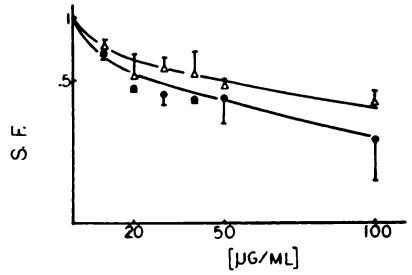

Chart 3. Cytotoxicity of bleomycin with (△) and without (●) pretreatment of 10^{-4} M deferoxamine for 60 min at 37°. After 60-min pretreatment, bleomycin is added and incubated for an additional 60 min at 37°. *Bars*, S.D. (only one-half of the *bars* are graphed). *S.F.*, surviving fractions.

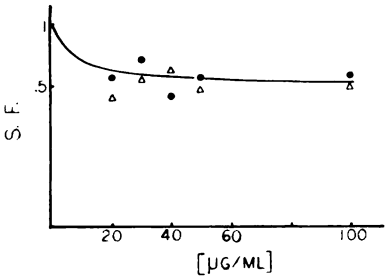

Chart 4. Cytotoxicity of bleomycin with (△) and without (●) pretreatment of 200 μM $FeCl_2$ and 240 μM DTPA. After 1 hr at 37° pretreatment, bleomycin is added and incubated for an additional 30 min at 37°. *S.F.*, surviving fraction.

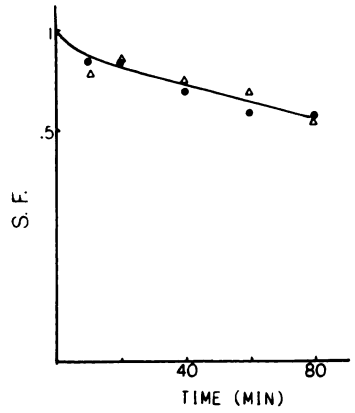

Chart 5. Cytotoxicity of copper free (●) and copper chelated (△) bleomycin as a function of exposure time. Chelation between the copper and bleomycin is at equimolar ratios of 2:1 and incubated at 37° for 10 to 80 min. *S.F.*, surviving fractions.

order to exert *in vivo* activity. To test this, bleomycin:Cu^{2+} complex was prepared by mixing $CuSO_4$ with bleomycin at a molar ratio of 2:1 and incubated at 37° for 1 hr. The V79 cells are treated with bleomycin:Cu^{2+} [equal to bleomycin (20 μg/ml)] for up to 80 min. Chart 5 shows that premixing bleomycin with Cu^{2+} does not alter its cytotoxic characteristics.

To test whether iron can modify the bleomycin effect, V79 cells were pretreated with 10% iron dextran for 1 hr at 37° before exposure to bleomycin (10 to 100 μg/ml) for 30 min at 37°. Chart 6 shows that the cytotoxic effect is enhanced by iron dextran pretreatment. However, in one preliminary experiment, if the sequence of iron dextran and bleomycin is reversed, the effect of iron dextran became unnoticeable (data not shown).

Bleomycin:Co^{3+} complex was prepared by mixing $CoCl_2$ with bleomycin at a molar ratio of 1:1 and incubating it at room temperature with frequent mixing for 1 hr. Chart 7 shows that bleomycin:Co^{3+} complex is not cytotoxic in the range of 5 to 60 μg/ml. However, if the cells were treated with 10^{-4} M DDC either before or after the treatment of bleomycin:Co^{3+} complex, bleomycin-like cytotoxicity was observed. V79 cells treated with 10^{-4} M DDC for 15 to 60 min at 37° appears not to modify the uptake of ^{57}Co:bleomycin (data not shown).

One hr after the rat received an injection (i.p.) of DDC (250 mg/kg), the liver was removed and an extract was prepared. The extract was divided into 2 parts, one used without any further treatment and the other heated at 43° for 10 min, before being used in experiments. Bleomycin was added to these 2 extract preparations and incubated for 30 min at 37°. This mixture was then used in cell survival experiments. Chart 8 shows that the liver extract from the DDC-treated rat without 43° treatment markedly lowered the bleomycin cytotoxic ability while the 43°-treated extract showed little effect if any.

In a protein synthesis experiment, we observed that 10^{-4} M DDC- (60 min at 37°) treated cells have the same rate of synthesis as control cells (data not shown).

DISCUSSION

The recent studies of bleomycin damage to isolated DNA have clearly demonstrated the involvement of metals in which Fe^{2+} and possibly Cu^{+} as well amplify the bleomycin effect. Results presented in this paper indicate the possibility that the

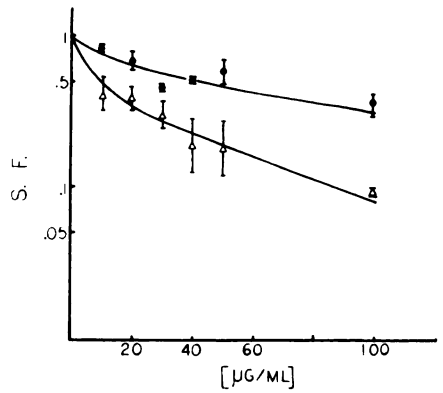

Chart 6. Cytotoxicity of bleomycin with (△) and without (●) pretreatment of 10% iron:dextran for 1 hr at 37°. After pretreatment, cells are washed twice and treated with bleomycin for 30 min at 37°. *S.F.*, surviving fraction.

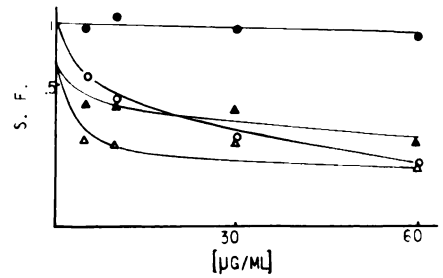

Chart 7. Effect of DDC on bleomycin:cobalt complex cytotoxicity as a function of bleomycin dose at 37° for 30 min. ○, bleomycin only; ●, bleomycin:cobalt complex; ▲, cells pretreated with 10^{-4} M DDC for 30 min at 37° before the treatment with bleomycin:cobalt complex; △, cells posttreated with 10^{-4} M DDC for 30 min at 37° after the treatment with bleomycin:cobalt complex. *S.F.*, surviving fraction.

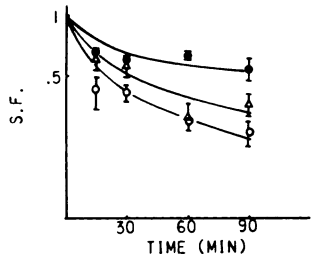

Chart 8. Effect of DDC on the bleomycin inactivation activity in liver extract. All survival curves were of V79 after bleomycin (50 μg/ml) for 15 to 90 min. ○, bleomycin control; ●, bleomycin incubated with liver extract (from DDC-treated rats) for 60 min at 37° before adding it to V79 cells. △, same as above except that the liver extract was preincubated at 43° for 10 min to inhibit the activity and then used as a control. *S.F.*, surviving fraction.

same mechanism may operate at the cellular level. It appears that the enhancement of bleomycin cytotoxicity provided by DDC may in part be derived from the same mechanism. This inference will emerge from the following discussion.

Copper, although it interferes with the bleomycin effect on isolated DNA, when chelated to bleomycin does not reduce the cytotoxic ability of bleomycin. Our results of mixing $CuSO_4$ with bleomycin before adding it to the test cells show the same cytotoxicity as bleomycin alone. This is similar to the observations of other investigators (15, 19). Certainly, the mechanism suggested by Takahashi *et al.* (31) that Cu^{2+} in the bleomycin complex can be reduced by an intracellular sulfhydryl compound and then transferred to a cellular protein can explain why Cu^{2+}-chelated bleomycin is also cytotoxic. It is, however, important to note that only the metal-free bleomycin provides a cytotoxic effect in this mode. Our observation that penicillamine does not modify the cytotoxic effect of bleomycin in serum-containing medium suggests that decreasing the level of extracellular copper will not gain additional bleomycin effect. This is also consistent with the cytotoxic effect produced by copper-chelated bleomycin. However, our results cannot rule out an alternative possibility. Although penicillamine has the capability of rapidly entering and freely diffusing out of the cells (2), it is ineffective against SOD (9), and its copper complex may in fact act as SOD (12). It is also necessary to point out that a recent report (21) questioned the validity and effectiveness of penicillamine:Cu^{2+} complex acting like SOD. Furthermore, we have obtained some [*methyl*-^{3}H]penicillamine (gifts from Dr. Meriwether of Merck & Co., Inc.) and could not show any significant amount of accumulation in V79 cells.

An iron chelator deferoxamine (as low as 50 μM) can penetrate the plasma membrane of fibroblast cells in culture and can bind iron released from transferrin. It was suggested that a stable deferoxamine:iron chelate may be released from the cells into the extracellular medium and thus show a net reduction in iron uptake by the cell (17). In our experiments, deferoxamine (10^{-4} M) was added to the medium for 1 hr before bleomycin was introduced to the cell suspension. It is reasonable to assume that there is a marked reduction in free iron in our experimental system, but the bleomycin cytotoxic effect was only retarded slightly. On the other hand, when iron dextran was introduced to the bleomycin experiment, the results appear to suggest the opposite possibility. However, there is one other obvious mechanism indicating that the additional cytotoxic effect could have been derived from the bleomycin stimulated iron toxic effect. In supporting this explanation, (*a*) iron by itself can be toxic and can degrade DNA (22); (*b*) dextran acts as a vehicle for easy introduction of iron into the cells, as demonstrated by Richmond (20) that, with as little as a 2-min exposure with 10% iron:dextran, cells *in vitro* absorb iron; (*c*) the bleomycin may be considered as a ferrous oxidase which catalyzes the oxidation of Fe^{2+} to Fe^{3+} and reduces oxygen (4). The results of iron:DTPA with bleomycin strengthen this interpretation. These above observations imply that in normal physiological conditions, iron probably plays little critical role in bleomycin cytotoxicity. It is important to note that under biological conditions, bleomycin more likely exists as the Cu^{2+} complex than the Fe^{2+} or Fe^{3+} complex. It has been established that the affinity of bleomycin for metals was in the order of $Cu^{2+} > Zn^{2+} > Cu^{+} > Fe^{2+}$ with Cu^{+} being about 4-fold greater than Fe^{2+} (18). The affinity of bleomycin:Cu^{+} for DNA was found to be similar to that of bleomycin:Fe^{2+} (18, 23) but greater than metal-free bleomycin. Another potentially important consideration is that a greater amount of radical may be produced by bleomycin:Cu^{+}, since this is a stable, active, oxidation-reduction complex (18). It has also been reported that Cu^{2+}-chelated bleomycin shows stronger interaction with nucleic acids than that of copper-free bleomycin. Finally, iron:bleomycin:oxygen in the absence of DNA, such as in the bulk parts of cytoplasm, may not be stable and may result in the decomposition of bleomycin (3). These explanations are consistent with (*a*) the fact of natural abundance of Cu^{2+} (5), (*b*) the possibility that all of the injected bleomycin may bind to Cu^{2+} in the blood (11), and (*c*) the bleomycin:iron complex is a markedly less potent cytotoxic agent than the bleomycin: copper complex (19).

The lack of cytotoxic effect with bleomycin:Co^{3+} is in agreement with previous reports (15, 19). However, a DDC treatment either before or after bleomycin:Co^{3+} exposure appears to reestablish some of the cytotoxicity. A competitive binding study of bleomycin:Co^{3+} with EDTA showed the bleomycin:Co^{3+} to be a strong chelate (6). We have recently performed similar competitive binding experiments indicating that DDC cannot remove cobalt from bleomycin. Since bleomycin:Co^{3+} is also known to firmly bind to DNA (22), it is unlikely that the cytotoxicity of bleomycin:Co^{3+} and DDC is the result of removing cobalt from the bleomycin complex. In view of what is presented in this paper and other studies discussed herein, the contribution of chelating metals to the enhancement of bleomycin cytotoxicity by DDC must be rather limited. Therefore, the consideration of alternative mechanisms will be required. We have recently observed that protein synthesis and intracellular bleomycin inactivation activity are important to the overall bleomycin cytotoxic effect.[4] Apparently, the DDC enhancement effect is not related either to these processes or to an increase in the bleomycin uptake. However, the following DDC properties may modify the effect of bleomycin:metal complexes: DDC is a good reducing agent; DDC is a sulfhydryl reagent; it can bind and stabilize Cu^{+}; and it can chelate the Cu^{+} receptor (25). Since DDC is obviously capable of penetrating the cell membrane, it may act as and/or work together with the intracellular reducing protein described by Takahashi *et al.* (31). If in this situation DDC also forms a chelate with the Cu^{+} receptor, the likely mechanism for the increased cytotoxicity by DDC treatment is the conversion of bleomycin:Cu^{2+} to bleomy-

[4] P. S. Lin, K. Hefter, and M. Jones, unpublished data.

cin:Cu^{+} which, in turn, can generate reactive oxygen radicals (27). This is similar to the reaction between the sulfhydryl radical of a thiol and the bleomycin:metal complex which can lead to radical production as demonstrated by Antholine and Petering (1). There is sufficient evidence indicating that bleomycin:Co^{3+} complex can be cytotoxic if it is modified in the same fashion. This is because (*a*) the 1:1 bleomycin:Co^{2+} complex has a coordination arrangement similar to that of the bleomycin:Cu^{2+} complex and, furthermore, the bleomycin:Co^{2+} complex forms an oxygen adduct complex similar to that of bleomycin:Fe^{2+} (28) and (*b*) bleomycin:Co^{2+} complex is oxidation-reduction active (26). It is apparent that the bleomycin:cobalt complex offers an interesting opportunity to further study the role of metals and DDC in bleomycin cytotoxicity.

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