Single-Strand Scission and Repair of DNA in Mammalian Cells by Bleomycin

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SUMMARY

DNA single-strand breakage by bleomycin treatment of cultured mammalian cells was demonstrated by the method of alkaline elution. Elution patterns from treated L1210 cells indicated that part of the DNA was extensively broken while the remainder was affected to a lesser degree. This biphasic effect, which was less prominent in human fibroblasts, may reflect a selective sensitivity either of part of the cell population or of part of the DNA within individual cells. In both cell types, the DNA damage was at least partially repaired upon incubation of the cells after removal of drug. Bleomycin did not inhibit the rejoining of X-ray-induced single-strand breaks. The production and repair of DNA single-strand breaks after bleomycin treatment were the same in normal human and xeroderma pigmentosum fibroblasts, indicating that these events do not require the excision endonuclease that appears to be defective in these ultraviolet light-sensitive xeroderma cells.

INTRODUCTION

The natural bleomycins are esters of bleomycinic acid made by Streptomyces verticillus, which have therapeutic activity against lymphomas, squamous carcinoma, and testicular carcinoma (2, 30, 31). Bleomycin preparations often are mixtures of different esters, and we will refer to these antibiotics in the singular, recognizing that their possible individualities still remain to be explored. The effects of bleomycin on DNA in vitro include liberation of free bases (12, 21), single-strand breakage (10, 11, 22, 23, 26, 27, 32), and reduction of melting temperature (22-24). Although details of the mechanism are unknown, available information is consistent with the proposition that the primary action on DNA is liberation of bases and that single-strand breaks result secondarily from the susceptibility of base-free sites to phosphodiester cleavage under the alkaline conditions used in the analysis.

Several groups (10, 18, 26, 29) have reported DNA single-strand scission following bleomycin treatment of cells. In some of these experiments, however, it was not clear whether bleomycin exerted its effect within the cells or during the analysis after the cells were lysed. In others, the conditions of alkaline sedimentation were such that the DNA sedimented in a sharp peak, the interpretation of which is uncertain. Some of the results suggested that bleomycin damage may be repaired (18). It has also been reported that bleomycin inhibits the repair of single-strand breaks by DNA ligase (19, 33).

We have recently developed a sensitive method to measure single-strand breakage in cells, based on the rate of the alkaline elution of DNA from cell lysates (8, 9, 14, 15, 17), and we have used this technique to examine the effects of bleomycin on DNA in cells (13).

The essential objective of our investigations in relation to the use of bleomycin in chemotherapy is to translate a biological problem into a chemical one. In order to do this, a chemical measurement of drug effect is required that is sensitive in the pharmacological range of drug dosage and correlates with the magnitude of the biological drug response. The chemical measurement need not necessarily reflect the mechanism of action of the drug. For example, a measurement of DNA single-strand breakage could be useful even if DNA damage were not the major cause of cytotoxicity, since the measurement could still reflect differential cytotoxicity arising from differences in drug transport into the cell or from differences in drug activation or inactivation within the cell. Nevertheless, there is a reasonable prospect that DNA damage is in fact the major cause of cytotoxicity by bleomycin. In the current work, we show that alkaline elution provides a measurement of DNA damage that is sensitive in the pharmacological range of bleomycin dosage. In the following paper (16), evidence will be presented that the measurement correlates with cell killing.

MATERIALS AND METHODS

L1210 cells (20) were propagated in Roswell Park Memorial Institute medium 1630 supplemented with 20% fetal bovine serum without antibiotics. Cultures for experiments were derived weekly from propagation stocks and contained penicillin and streptomycin. Cultures used for experiments had cell densities of 0.5 to 1.0 x 10⁶ ml⁻¹ and were in the logarithmic phase of growth. The doubling time was 12 to 14 hr.

Colony-forming ability was determined by the soft-agar technique described by Chu and Fisher (3).

Cell DNA was labeled prior to drug treatment by growing the cells for approximately 20 hr with [2-¹⁴C]thymidine (approximately 50 µCi/mmol; Schwarz/Mann, Orangeburg, N. J.), 0.01 to 0.02 µCi/ml.
Bleomycin (Lot F-333740-BS) was obtained from Bristol Laboratories, Syracuse, N. Y. L1210 cells were treated in their growth medium in plastic tissue culture tubes (Falcon Plastics, Oxnard, Calif.). Bleomycin treatment in glass tubes was less effective, possibly due to adsorption of bleomycin to the glass surface.

X-ray was delivered by 2 vertically opposed X-ray units operated at 200 kV, 15 ma, 0.25-mm copper, plus 0.55-mm aluminum filter. The dose rate was 140 R/min for doses below 1000 R and 660 R/min for doses of 1000 R or higher. It was assumed that 1 R is equivalent to 1 rad under these radiation conditions. To display the effects of random DNA damage, cells were irradiated at 0° while suspended in growth medium and were kept in ice until assay. In the experiments on repair of X-ray damage, cells were irradiated at room temperature (approximately 24°) and then were incubated at 35–37° for 30 min.

Human fibroblasts were handled as previously described (9). Cells from a normal donor (CRL 1119) and from a patient with xeroderma pigmentosum (CRL 1223, XP12BE, complementation group A) were obtained from the American Type Culture Collection, Rockville, Md.

Alkaline elution analysis was carried out as previously described (9, 17). Briefly, 0.5 to 1.0 x 10^6 cells were deposited on a polyvinyl chloride filter (2-μm pore size). The cells were washed on the filters with phosphate-buffered saline (0.15 M NaCl, 0.005 M potassium phosphate, pH 7.4) and lysed with 0.2% sodium lauryl sarcosinate (Sarkosyl; Ciba-Geigy Corp., Ardsley, N. Y.), 2 M NaCl, and 0.02 M EDTA, pH 8 to 10. After an additional wash with 0.02 M EDTA, pH 9 to 10, the DNA was eluted from the filters by controlled flow of a solution of 0.02 M EDTA plus tetrabromophenol monoxide added to give the desired pH. The elution pH was 12.8 or 12.2, and the flow rate was 0.02 or 0.05 ml/min, as indicated in the individual experiments. During the course of this work, the pH and flow rate were reduced, as these conditions were found to increase the sensitivity of the assay, with no basic change in results. Eluted fractions were collected at intervals and assayed for radioactivity as previously described (17).

It was necessary to guard against artifacts due to the ability of bleomycin to adsorb to the filters and to cause DNA breakage after cell lysis. In order to assure the adequacy of bleomycin removal from treated cells, untreated [3H]thymidine-labeled L1210 cells were added as internal controls. When bleomycin removal was inadequate, the [3H]-labeled DNA from the control cells exhibited increased elution rate.

RESULTS

Alkaline Elution Patterns. The effects of bleomycin treatment on the alkaline elution of DNA from L1210 cells prelabeled with [2-14C]thymidine are shown in Charts 1 and 2. Chart 1 shows the effect of exposure of the cells for 1 hr to various concentrations of bleomycin. Chart 2 shows the effect of exposure to bleomycin, 15 μg/ml, for various lengths of time. Bleomycin is seen to increase DNA elution from treated cells, and this effect increases both with bleomycin concentration and with exposure time. When cells were assayed immediately after the addition of bleomycin, there was little or no effect on DNA elution, thus demonstrating that the bleomycin effect occurred during the incubation with cells and not during the assay.

Another feature of these results is that the elution curves following bleomycin differ in shape from those observed after exposure of cells to X-ray, in that the curves are biphasic. The shapes of the elution curves seen after bleomy-
cin treatment are consistent with the possibility that the DNA of treated cells consists of a mixture of undamaged and severely damaged components. This result would be expected if the cells were heterogeneous in sensitivity to bleomycin. Heterogeneity has been reported in the survival of bleomycin-treated cells. Survival curves were found to be biphasic, indicating the presence of cell populations of high and low sensitivity to bleomycin (1, 6, 28). In accord with these findings in other cell lines, the survival curve for our L1210 cells exhibited biphasic character (Chart 3). The shapes of the elution curves therefore may reflect differences in cell population relative to bleomycin sensitivity.

**Repair of Bleomycin-induced DNA Damage.** The ability of L1210 cells to partially repair bleomycin-damaged DNA is indicated in Chart 4. Cells were exposed to bleomycin, 100 µg/ml, for 1 hr, then were washed and incubated further in the absence of drug. The rapid component of elution, seen immediately after treatment, was partially eliminated 4 hr later. A slight further reduction was seen at 8 hr, but no further recovery occurred at 20 to 24 hr. Instead, at these late times a part of the DNA was released in the lysis solution (points on the ordinate in Chart 4), indicating severe degradation. The elution of the remaining DNA was nearly normal.

In order to determine whether the apparent recovery in the elution patterns is due to rejoining of DNA breaks or to posttreatment cross-linking (7–9), the cells were exposed to 300 R of X-ray at 0°. The X-ray exposure increased DNA elution to the same extent as in untreated cells, thus showing that there was no cross-linking. Therefore, recovery shown in the elution patterns is due to rejoining of breaks.

DNA damage and repair after bleomycin treatment were also observed in human fibroblasts (Chart 5). The possibility that these events might involve excision repair was tested in xeroderma pigmentosum cells known to be deficient in this type of repair (4). The cell line used was of complementation group A and has marked deficiencies in unscheduled DNA synthesis and endonuclease action (9, 25). These cells were found to respond to bleomycin in the same way as do normal cells in regard to the formation and repair of single-strand breaks (Chart 5). Hence the steps in excision repair that are defective in these xeroderma cells are not required for the formation or repair of bleomycin-induced DNA breaks.

The repair in both human cell lines appeared to be more extensive than in L1210 cells.

**Lack of Inhibition of Repair of X-ray-induced Single-Strand Breaks.** Bleomycin has been reported to inhibit the

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**Chart 3.** Survival of colony-forming ability of L1210 cells exposed to bleomycin for 30 min.

**Chart 4.** Changes in alkaline elution patterns due to incubation of bleomycin-treated L1210 cells after removal of drug. Cells were treated with bleomycin, 100 µg/ml, for 1 hr and then were incubated for the indicated times after removal of drug. Elution was at 0.05 ml/min at pH 12.8.

**Chart 5.** DNA damage and repair after treatment of human fibroblasts with bleomycin, 100 µg/ml, for 1 hr. Δ, ○, normal cells; △, ■, xeroderma pigmentosum (SP12BE, complementation group A); ○, ●, no drug; □, ■, bleomycin, 100 µg/ml, for 1 hr; Δ, ●, bleomycin, 100 µg/ml, for 1 hr, followed by incubation in fresh drug-free medium for 3 hr.
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assure that the drug was adequately removed prior to cell lysis: (a) untreated control cells were included in each assay, and (b) cells were analyzed after very brief exposure times to high concentrations of bleomycin. When both of these controls yielded normal DNA elution patterns, the experimental procedures were deemed adequate to remove excess bleomycin.

Significant increases in DNA elution were observed in L1210 cells after bleomycin treatments as low as 15 μg/ml for 0.5 to 1.0 hr, which is within the range of pharmacologically pertinent dosage. However, there was considerable quantitative variability among different experiments. This was thought to stem from variability in the growth medium, particularly pH, at the time of treatment. In subsequent work, treatment was carried out in fresh medium at a controlled pH, and quantitatively consistent data were obtained (16).

The elution curves of DNA from bleomycin-treated L1210 cells indicated that single-strand breaks were introduced nonrandomly in the DNA (Charts 1 and 2). This was shown by the difference in the shapes of the elution curves compared with those produced by X-ray, which may be assumed to generate an essentially random distribution of DNA single-strand breaks in cells. It appears that bleomycin treatment causes extensive breakage in some DNA strands, while leaving others relatively intact. One possible explanation for this is that the difference resides in the cell population. Cell populations have been observed to be made up of sensitive and resistant components (1, 6, 28), and we have confirmed this in L1210 cells (Chart 3). Alternatively, some of the DNA in each cell may have an enhanced susceptibility to degradation. In line with this possibility, Crooke et al. (5) recently reported that nucleolar DNA is more sensitive than nucleoplasmic DNA to degradation by bleomycin.

Repair of bleomycin-induced single-strand breaks was observed in L1210 cells and in human fibroblasts. The results with L1210 cells suggested that some of the cells are able to fully repair the DNA damage, while others proceed to degrade their DNA. The extent of repair appeared to be greater in human fibroblasts than in L1210 cells.

Bleomycin has been reported to inhibit the activity of DNA ligase (19, 33). The results reported, however, could be attributed to single-strand breakage by the drug rather than to inhibition of ligase enzyme activity. After taking this factor into account, we conclude that bleomycin did not inhibit the repair of X-ray-induced single-strand breaks in L1210 cells.

REFERENCES


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