Molecular Interactions of the Combined Effects of Bleomycin and X-rays on Mammalian Cell Survival

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SUMMARY

The interactions between bleomycin and X-ray damage and repair have been examined in rat and human tumor cells. Bleomycin itself induces extensive DNA single-strand breaks but does not appear to inhibit the repair of X-ray-induced DNA single-strand breaks. Quantitative analysis of these interactions is complicated by the retention of active bleomycin within cells that remains capable of further DNA degradation even under the conditions of alkaline sucrose gradient cell lysis. DNA double-strand breaks and/or disruptions of DNA-lipid complexes also occur following bleomycin exposure. X-ray-induced excision repair replication is only minimally influenced by even high concentrations of bleomycin. A small amount of excision repair is demonstrable in nonirradiated cells treated with high concentrations of bleomycin consistent with repair of bleomycin-induced nucleotide damage in cellular DNA by a "cut and patch" repair mechanism. Repair of bleomycin-induced DNA single-strand breaks also occurs. The data indicate that bleomycin and X-ray damage are quite similar both in their induction and repair, but that lesions occur and are repaired independently. The enzymatic mechanisms appear similar in the two cell types despite substantial differences in their sensitivity to bleomycin.

INTRODUCTION

The BL2 group is composed of closely related antibiotics produced by Streptomyces verticillus, the biochemistry and biological activities of which have recently been reviewed by their discoverer, Umezawa (35, 36). These compounds are of great interest in oncology because of the potential of cells derived from squamous mucosa to accumulate the active forms of these drugs (37). This unique property makes BL specifically useful in the treatment of human squamous cell carcinomas, a series of tumors that are generally unresponsive to most anticancer drugs (3, 9, 11, 13, 22, 25). Since squamous cell carcinomas are almost universally responsive to XRT, the combination of BL with XRT offers great promise. Both controlled (31) and uncontrolled (6, 19) preliminary clinical trials of BL and XRT would appear to support this premise, as have initial studies in animal solid tumor systems (14, 39).

Tissue culture studies designed to determine whether or not the combination of BL and XRT is additive or synergistic (i.e., more than additive) have given conflicting results. A synergism of BL with XRT was shown for mouse mammary carcinoma cells (20), while HeLa cell studies suggested only additive effects (1). The timing of exposures may also be important. For example, Bleeheen et al. (2) examined the effects of BL and XRT using several bacterial strains, the EMT 6 mouse mammary cell line, and also HeLa cells and found that BL treatment was additive for all pre-XRT exposures, while either additive or synergistic effects could be obtained by post-XRT exposure, depending on the cell system being studied. They further suggested that the synergistic effects seen during some post-XRT exposures might be due to an inhibition of radiation repair systems by BL (2). Matsuzawa et al. (20), on the other hand, suggested that BL might act by affecting the capacity of the cells to accumulate sublethal damage.

The results of BL studies at the molecular level have also been somewhat ambiguous. It was recognized early that BL could induce DNA single-strand breaks in vitro under some circumstances (26, 27), and this phenomenon is now generally well established (35, 36). DNA strand breakage was subsequently shown to occur in vivo (33). The role that such DNA single-strand breaks might have on cell lethality has been questioned for 2 reasons: (a) it has been shown that cells can repair BL-induced DNA single-strand breaks (34); and (b) it has been suggested that many (8) or all (7) of the breaks seen may be gradient artifacts occurring in free DNA during cell lysis. On the other hand, Hittleman and Rao (12) and Kurten and Obe (15) found chromosome aberrations in cells treated with BL analyzed by the method of premature chromosome condensation. It seems probable that BL does, therefore, induce some intracellular DNA breaks in vivo. Based on these previous studies, it was of interest to examine BL damage and repair in the context of X-ray damage.

In the investigation reported here and in a forthcoming paper, we have evaluated the interaction between BL and X-rays at the cellular and molecular level. In this study the
effects of BL on the DNA enzymatic repair mechanisms are examined, including both X-ray-induced excision repair and the DNA strand-break rejoicing mechanisms. In the forthcoming paper, the effects of BL on cell survival of BL-sensitive and BL-resistant cells are reported for both control and γ-irradiated cells. Two cell lines (HeLa and REQ) have been studied. These cell lines were initially chosen because they differ significantly in their capacity to perform X-ray-induced excision repair (5). It was subsequently found that the excision repair-deficient REQ line is substantially more sensitive and BL-resistant cells are reported for both control sensitive to BL. The combination of these 2 parameters permitted a comprehensive examination of the interaction between XRT and BL-induced damage at the cellular and molecular levels in cultured mammalian cells.

MATERIALS AND METHODS

Cell Lines and Cultivation. HeLa cells were obtained from the American Type Culture Collection (CCL 2). The properties of the rat X-ray excision repair-deficient REQ line have been described; this line shows at identical exposure doses a maximum of about 30% of the amount of X-ray-induced repair replication found in HeLa cells, but otherwise is essentially identical with respect to its radiation survival curve properties and in its capacity to repair sublethal X-ray damage (5). REQ cells are significantly more sensitive than are HeLa cells to both acute and chronic BL exposures. Both HeLa and REQ cells were maintained in the log phase of growth by serial passage (trypsinization) in McCoy's 5A modified medium (Associated Biomedic Systems, Buffalo, N. Y.) supplemented with 20% fetal calf serum (Irvine Scientific, Irvine, Calif.) in Falcon (Oxnard, Calif.) T-flasks. Growth conditions were in a tissue culture incubator at 37° perfused with a water-saturated 95% air-5% CO₂ mixture unless otherwise stated.

Repair Replication Studies. The methods used for studying X-ray-induced excision repair in mouse L1210 cells have been described in detail (Footnote 3; Ref. 16). Their adaptation to repair replication induced by BL is outlined in the legend to Chart 7.

DNA Single-Strand Break Rejoining. In these experiments the modified alkaline sucrose gradient method of McBurney et al. (21) was utilized to follow DNA single-strand breaks induced by X-ray exposure doses close to those used under treatment conditions. This method permits the study of DNA strand-break induction by following the sedimentation of wholly and/or partially denatured DNA (21). Its main advantage is its qualitative sensitivity to strand breaks induced by clinical doses of radiation (21). Like other ultra-sensitive methods of alkaline sucrose gradient analysis (32), it is not sufficiently quantitative to permit accurate molecular weight calculations, since both single- and DNA double-strand breaks contribute to the sedimentation patterns; such calculations have not been made in these studies. The procedure used was as follows: log phase HeLa or REQ cells were labeled for 1 doubling period by the addition of [³H]TdR (New England Nuclear, Boston, Mass.; 5 Ci/m mole), 0.25 µCi/ml, to the growth medium. The growth medium and label were first removed and the cultures were washed twice in ice-cold Gey's (Grand Island Biological Co., Grand Island, N. Y.) buffered salt solution. Cultures were then irradiated under Gey's solution at ice-bath temperatures. For studying the repair of X-ray-induced DNA strand breaks, the cells were returned to warm growth medium under tissue culture conditions for the times indicated in the chart legends. DNA single-strand breaks were then studied by harvesting the cells by trypsinization, followed by suspension in ice-cold Gey's salt solution (10⁶ cells/ml). One-tenth ml of suspension (10⁶ cells) was then carefully placed on top of 2 ml of 2% sucrose prelayered over a 31-ml, 10 to 30% linear alkaline sucrose density gradient made up in 0.3 M NaOH, 0.5 M NaCl, and 0.01 M EDTA at 0°. Each tube also contained a 3-ml cushion of 70% sucrose made up in the same reagents. The gradients were stored in the dark for 16 hr at 4° prior to centrifugation in a Beckman rotor SW 27 for 3 hr at 20,000 rpm and 4°. After centrifugation the gradients were collected and radioactivity was determined by liquid scintillation counting as previously described (16).

RESULTS

Both BL and X-rays cause a reduction in the degree to which REQ cell DNA will sediment in alkaline sucrose gradients (Chart 1). These results are consistent with the induction of multiple DNA single-strand breaks and/or alkali labile sites (18, 38). DNA strand breakage by BL has been demonstrated previously in bacteria (33) and mammalian cells (33, 34) and is shown here in a repair replication-deficient (placental) mammalian cell line. Essentially identical results for BL have been previously obtained by others (8) and in our own studies (not shown) in X-ray excision repair-competent cells (HeLa). The degree of breakage by BL is concentration dependent. Exposure to 5 µg/ml for 120 min is roughly similar to a 500-rad X-ray dose. This exposure of REQ cells to BL leaves a surviving fraction of <1.0%, while about 8% of REQ cells will survive a 500-rad X-ray dose (5).

X-ray-induced DNA single-strand breaks are almost completely repaired by REQ cells 60 min after X-ray exposure (Chart 1). Whether or not residual breaks exist is beyond the
limits of this type of assay. The presence of BL during the
period of rejoining of X-ray-induced single-strand breaks
has little or no effect on this rejoining process (Chart 2). The
small degree of size reduction noted appears to be related
to the presence of BL alone rather than from inhibition of X-
ray-induced single-strand breaks. Thus, BL does not appear
to inhibit either the rejoining of X-ray-induced DNA single-
strand breaks and/or the removal of X-ray-induced alkali-
labile sites.

Studies on the effect of BL on intracellular DNA integrity
are complicated by the fact that it has been reported that BL
released from lysing cells may be able to induce DNA dam-
age even under the conditions of alkaline lysis and sedi-
mentation (8). For evaluation of this possibility in REQ cells,
we compared the effect of exposing cells to BL in culture to
possible BL exposure caused by release of free BL by cells
previously treated but washed free of active extracellular
BL. Accordingly, prelabeled cells were subjected directly by
exposure to a high BL concentration in culture (100 µg/ml
for 2 hr) or indirectly by first exposing nonlabeled cells to
BL (also 100 µg/ml for 2 hr at 37°C). The latter cells were then
washed and added to labeled non-BL-treated cells and
lysed together on the same gradient. The results (Chart 3)
show that either circumstance can lead to DNA degradation.
Thus both direct BL treatment (Chart 3B) and/or exposure
to BL-treated cells at the time of lysis and sedimentation
(Chart 3C) can cause cellular DNA to sediment more slowly
than control DNA (Chart 3A). Although the level of BL uti-
lized here was much higher than that required to demon-
strate BL-induced DNA strand breaks (Chart 1), it seems


Chart 1. Induction of DNA single-strand breaks of BL and X-rays. Log
phase REQ cells were washed free of growth medium and then exposed to
either BL or X-ray irradiation, as described under "Materials and Methods,"
and evaluated for DNA single-strand breaks by sedimentation in alkaline
sucrose gradients. A, control cells, no treatment. B, C, and D, effect of
exposure to BL for 120 min (B, 0.5 µg/ml; C, 5.0 µg/ml; D, 50.0 µg/ml). E,
cells exposed to a 500-rad X-ray dose and sedimented immediately following
radiation. F, cells exposed to 500 rads and then allowed to repair for 60 min at
37°C. Sedimentation is from right to left.

Chart 2. Effect of BL on the repair of X-ray-induced DNA single-strand
breaks. REQ cells were exposed to a 500-rad X-ray dose and then permitted
to repair DNA single-strand breaks for 60 min in the presence or absence of
BL. Strand breaks were evaluated by alkaline sucrose gradient sedimenta-
tion. A, 500 rads with no repair, no BL; B, 500 rads with a 60-min repair
period, no BL; and C to E, 500 rads with a 60-min repair period in the
presence of BL (C, 0.001 µg/ml; D, 0.1 µg/ml; E, 0.5 µg/ml). Sedimentation is
from right to left.

Chart 3. Induction of DNA single-strand breaks by BL released during
gradient lysis. REQ cells were prelabeled with [3H]Tdr as for DNA strand-
break analysis or were grown without labeling under otherwise identical
conditions. One aliquot of [3H]Tdr cells was then subjected directly to
alkaline sucrose gradient analysis (A), as described under "Materials and
Methods." A 2nd sample of [3H]Tdr-labeled cells was exposed to BL (100 µg/
ml at 37°C) for 2 hr and then washed and subjected to analysis with no repair
period (B). A 3rd sample of [3H]Tdr cells was mixed with an equal volume of
nolabeled cells that had been previously treated with BL (100 µg/ml at 37°C)
for 2 hr and then thoroughly washed prior to mixing. The mixed cells were
immediately placed on an alkaline sucrose gradient (C). DNA single-strand
breaks are induced by both direct exposure prior to lysis (B) or exposure to
BL released by lysing cells (C). Along the abscissa, T, top of gradient; B,
bottom; sedimentation from right to left.
apparent that DNA damage on alkaline sucrose gradients can occur distinct from that occurring intracellularly and that precise quantitative estimates of DNA damage intracellularly in vivo are probably not possible using alkaline sucrose gradient methods.

BL has also been reported to induce repairable DNA single-strand breaks in mouse L-cells (34). To determine whether human tumor cells can repair BL-induced DNA strand breaks as measured by alkaline sucrose gradient techniques, HeLa cells were exposed to BL (100 μg/ml) for 5 hr and then either assayed for DNA single-strand breaks immediately or washed free of the drug and reincubated in growth medium for 2 hr to permit repair. The results (Chart 4) show that some but not all of the breaks induced by this level of BL exposure could be repaired. The degree to which residual BL might be inducing artifactual damage in this type of experiment is difficult to determine but would tend to reduce apparent repair.

Decreases in DNA sedimentation under neutral conditions due to DNA double-strand breakage or effects on DNA-lipid complexes were also demonstrable following exposure of (REQ) cells to high BL concentrations (100 μg/ml for 4 hr; Chart 5). This degree of BL exposure would be expected to induce many DNA single-strand breaks and it cannot be determined whether any true double-strand breaks occur or whether all the double-strand breaks represent contiguous single-strand breaks. In view of the ambiguity associated with artifactual breaks formed on the gradients (Chart 3), it seems doubtful that gradient methods can resolve this question satisfactorily.

We also studied the effect of BL on X-ray-induced excision repair replication. To permit comparison of BL to previous drugs (16), we used murine L1210 leukemia cells presubstituted by 1 generation of growth in medium containing 5-bromodeoxyuridine (5 μg/ml). When such cells are exposed to heavy radiation doses (100 kilorads) and are then permitted to incorporate exogenously labeled [3H]Tdr, there is an inhibition (by X-rays) of incorporation of label into DNA of normal density (Chart 6, right hand peaks) and a stimulation of incorporation into preexisting heavy DNA as shown by the appearance of a second (left hand) peak (Chart 6B). This phenomenon is termed repair replication (29, 30) and represents the excision repair of damaged thymidine and bromouridine nucleosides. The presence of BL during this excision repair period causes little or no inhibition of this type of repair (Chart 6, C to E). BL also has little effect on the small amount of remaining normal semi-conservative DNA synthesis (Chart 6, B to E; right hand peaks).

BL has been shown to release free bases in vitro (10) and can also induce unscheduled DNA synthesis in vivo in HeLa cells (8). This suggested that at least a portion of the damage to DNA in vivo might be repaired by an excision repair pathway. This was confirmed explicitly by measuring repair replication induced by BL in 5-bromodeoxyuridine-substituted L1210 cells (Chart 7). Extremely high amounts of BL (250 μg/ml for 2 hr) were required to demonstrate this small amount of excision repair, suggesting that excision repair is probably not important in the survival of cells to BL damage. We have recently drawn similar conclusions for X-ray-induced excision repair (5).
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Chart 6. Effect of BL on X-ray-induced excision repair replication. Murine L1210 cells presubstituted with bromouridine were analyzed as described (16). Excision repair replication was induced by a heavy radiation exposure (100 kilorads) and identified using alkaline CsCl equilibrium density centrifugation. A, control nonirradiated cells showing only normal semiconservative DNA synthesis (right hand peak); B, irradiated (100 kilorads) cells showing inhibition of semiconservative synthesis (left hand peak) and induction of repair synthesis (left hand peak); C to E, effect of progressively greater concentrations of BL during the excision repair replication period (C, 1.0 µg/ml; D, 10 µg/ml; E, 100 µg/ml). BL was added immediately following X-ray exposure in all cases. Repair period was 3 hr following the X-ray exposure during which excision repair is completed.

DISCUSSION

The studies reported here confirm and extend those previously cited and indicate that the interaction between BL and cellular DNA has several discrete components. The most potent effect of BL remains its ability to induce DNA strand breaks (Refs. 26, 27, 35, 36; Chart 1). The procedure utilized here was devised by McBurney et al. (21) and is quite sensitive in detecting DNA strand disruptions. McBurney et al. (21) showed that the material sedimenting contained essentially no protein; we have confirmed this observation and also found (Y. C. Lee and J. E. Byfield, unpublished data) that no choline-containing material cosediments with the DNA. The material being studied is presumably pure but high-molecular-weight DNA comprised of both single- and double-stranded aggregates (21, 32). The extent to which shifts of DNA sedimentation under these alkaline sucrose gradient conditions reliably indicate the extent of intracellular BL damage is uncertain since it seems clear that residual, active BL can additionally degrade DNA during cellular lysis (Chart 3). Results such as these lend credence to the suggestion that in vivo DNA damage by BL is greatly exaggerated by gradient analysis (7, 8).

These sorts of evidence led Cox et al. (7) to suggest that BL may not penetrate nongrowing cells and may act at the cell membrane. While this may be possible in some cases, it seems clear that BL must enter most cells and induce some DNA damage. Thus the demonstration by Hittleman and Rao (12) of immediate chromosome damage (and subsequent repair) caused by BL in Chinese hamster cells indicates BL penetration of cells to the DNA level. Similarly, the demonstration of chromatic-type lesions in G2 cells by Paika and Krishan (28) implies direct DNA damage unrelated to subsequent DNA synthesis. The induction of direct base damage leading to excision repair (Chart 7) and unscheduled DNA synthesis (8) also implies that cellular DNA is accessible to DNA damage by BL.

The exact nature of the lethal lesion(s) induced by BL in mammalian cells is not clear. Of the various types of damage noted (DNA single- and double-strand breaks and the excision repair of DNA bases), thus far only DNA double-strand breaks (Ref. 34; Chart 5) can be related to lethality. Whether or not the neutral sucrose gradient methods used here or elsewhere measure true DNA double-strand breaks is uncertain. In the original application of the method used here (Chart 5), Lehmann and Ormerod (17) noted that substantial X-ray doses were required to obtain DNA fragment sufficiently to demonstrate double-strand breaks clearly. While consistent with double-strand breakage, the results shown in Chart 5 may also be related to other phenomena such as disruption of DNA-lipid complexes (23). Although suggestive, it would seem premature to conclude that DNA double-strand breaks are the necessary lethal mechanism of BL.

The experiments also do not support the hypothesis that BL inhibits the repair of X-ray damage to DNA. Its effect on both the rejoining of X-ray-induced DNA single-strand breaks (Chart 2) and X-ray-induced excision repair (Chart 6) is minimal. It has been reported that BL inhibits DNA ligase prepared from rat ascites hepatoma cells (24). As far as could be determined, there was no inhibition of this component of the X-ray-induced ligation reaction (Chart 2), and
this mechanism probably would be unimportant during coincident BL-X-ray treatments.

Since neither the repair of X-ray-induced DNA single-strand breaks nor X-ray-induced excision repair can be conclusively related to the repair of (cellular) sublethal X-ray damage (4), it cannot be concluded that the resistance of these 2 molecular repair mechanisms to BL inhibition has any necessary implications with respect to cell survival to combined BL-XRT. However, as will be indicated in the forthcoming paper, in a series of experiments conducted at the cellular level, the interaction between BL and X-ray damage seems to be slight and relatively accurately predicted by these molecular studies.

REFERENCES


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