SUMMARY

Earle’s L mouse fibroblasts and cultured lymphoblasts of human neoplastic disease origin exposed to the new antitumor antibiotic, bleomycin, show a variety of morphological alterations. Bleomycin-treated cells are larger than untreated cells and often contain multiple nuclei. Hypertrophy of cellular organelles like mitochondria and Golgi vesicles is accompanied by nuclear enlargement. In a majority of the treated cells, nuclei, which appear hyaline in light micrographs, show the total absence of electron-dense chromatin clumps. Extensive infoldings and lobulation of the nuclear surface and initial nucleolar hypertrophy, followed by shrinking and segregation of the nucleolar components, are seen in drug-treated cells.

INTRODUCTION

BLM, an antitumor antibiotic isolated from a strain of Actinomyces, Streptomyces verticillus (30), has been shown to be a glycopeptide complex containing a number of sugar peptides of unusual structure (24, 26). BLM has been reported to inhibit growth of gram-positive, gram-negative, and acid-fast bacteria (23, 30). Incubation of BLM with tissue homogenates of mice leads to 17 to 76% inactivation of BLM; in in vitro systems, DNA, and sulfhydryl-containing substances have been shown to be responsible for the inactivation of BLM (4). Binding of BLM to DNA and the resulting single-strand scission of DNA in bacteria and mammalian cells have been suggested as the possible mode of action of this antibiotic (22, 28).

In cultured HeLa cells, BLM inhibits DNA synthesis and, in lower concentrations, inhibits cell division (27). In Ehrlich carcinoma cells, inhibition of DNA synthesis and protein synthesis, but not RNA synthesis, by BLM has been reported (4).

Nagatsu et al. (16) have described changes in the DNA content of VX-2 carcinoma cells exposed to BLM. They reported an increase in the population of cells with twice the stem-line DNA content and the presence of cells with 4 times the stem-line DNA content. These findings suggest that although BLM prevents cells from entering visible mitosis, it does not inhibit DNA synthesis at low concentrations; DNA replication without cell cleavage probably results in a higher DNA content in a significant portion of the cell population.

In Chinese hamster ovary cells, Barranco and Humphrey (1) have shown that BLM is effective in killing cells during mitosis, G2, S, and G1 in decreasing order of effectiveness. Chromosomal aberrations induced by BLM have been reported both in vivo and in vitro. The presence of hypochromatic regions, gaps, and breaks of both the chromatid and the isochromatid types has been reported in cells exposed to BLM (3, 18, 19).

In experimentally induced animal tumors, BLM markedly affects the growth of solid tumors (4). In clinical chemotherapy, BLM has been used to treat a variety of human neoplastic diseases (10, 11, 21, 25).

Ogawa et al. (17) have described the fine structural alterations in the nuclear structure of cells from BLM-treated mouse epidermal carcinomas. A reduction in cytoplasmic and nuclear basophilia and the presence of large nucleoli were reported in these BLM-treated cells. Nuclei of cells from the treated tumors appeared less electron dense and vesicular; there was a decrease in the amount of visible chromatin, both in association with the nuclear membrane and with the nucleolus. BLM-induced alteration in the appearance and texture of nucleoli, segregation of the nucleolar components, and the reduction in the size of the nucleoli were reported by these workers. In addition to changes in the nuclear fine structure, cells exposed to BLM showed a decrease in cytoplasmic ribosomes, swelling of the mitochondria, and the appearance of cytoplasmic degradation and vacuolation.

This report describes time-lapse cinematographic and electron microscopic studies which were undertaken to investigate the effects of BLM on cultured mouse L-929 fibroblasts and on cultured human lymphoblasts from a leukemic patient (8) and from a spleen biopsy of a Hodgkin’s disease patient (G. E. Foley and A. Wechezak, unpublished observations).

MATERIALS AND METHODS

Time-Lapse Cinematographic Studies. Earle’s L-929 mouse fibroblasts were grown in Sykes–Moore chambers and exposed to various concentrations of BLM (Bristol Laboratories, Syracuse, N. Y.). Time-lapse photographs were made on a Nikon inverted microscope fitted with a Bolex 16-mm camera. Photographs were taken with the frequency of 1 frame/min.

Electron Microscopic Studies. The following 3 cell lines were used for this part of the study.
Earle's L-929 mouse fibroblasts were grown as monolayer cultures (asynchronous) and nourished with Medium 199 containing 10% calf serum, antibiotics, penicillin, and streptomycin.

For synchronized cell cultures, L-929 cells were exposed to vinblastine sulfate (0.01 μg/ml) for 18 hr, and mitotic cells were collected by gentle shaking. The drug-induced mitotic block was released by 2 washings of the centrifuged cell pellets in serum-free medium and incubation in fresh medium (13). A light micrograph of the dense cell population after 24 hr exposure to 50 μg/ml of BLM showed that a predominant portion (>90%) of these cells divided and entered G1 within 4 hr of reincubation in the fresh medium and reached early S phase after 10 hr. For cells in G2 mitotic cells collected from cultures incubated with vinblastine sulfate and BLM for 4 hr were washed twice in fresh medium and reincubated in BLM-containing medium.

Suspension cultures of CCRF-CEM human lymphoblasts, isolated from the peripheral blood of a patient (pediatric) with acute lymphoblastic leukemia (8), and CCRF-JJB cells, isolated from the spleen biopsy of a Hodgkin's disease patient (G. E. Foley and A. Wechezak, unpublished observations), were nourished with Eagle's minimal essential medium for suspension cultures, supplemented with fetal calf serum, penicillin, and streptomycin.

Cells were exposed to various concentrations of BLM (1 to 100 μg/ml), dissolved in distilled water, for 12 hr to 4 days. At the end of the incubation period, monolayer cultures were scraped or trypsinized, and the cells were retrieved by centrifugation from both the monolayer and the suspension cultures.

Cell counts were made in a Coulter counter, and cell viability was determined by dye exclusion (trypan blue) method.

Time-Lapse Cinematography. Exposure of L-cells to BLM (1 μg/ml) did not show any initial effect on either cell motility or cell division. In time-lapse movies, no morphological effects were recognizable for up to 48 hr. However, by the 70th hr, some of the cells had lost their pseudopods and appeared shrivelled while others had assumed a more or less epithelioid cell shape. In contrast, cells exposed to 5 μg/ml for 38 hr showed inhibition of cell division, shrinking of cellular mass, thinning of pseudopods in some cells, and loss of cellular motility. Exposure to high doses of BLM (50 to 300 μg/ml) caused a complete inhibition of cell multiplication, loss of cellular motility, and a gradual assumption of epithelioid cell shape in the spindle-shaped cells. Cells exposed to these higher concentrations of BLM also showed the gradual effect of this compound on the increase in cell mass. After 66 hr of exposure, a large number of these cells were very large and had clear nuclei. Prolonged incubation (more than 72 hr) of cells with more than 50 μg of BLM per ml showed the gradual blebbing of the cell surface followed by cell death.

Although BLM inhibits entry of cells into mitosis, our time-lapse records show that this compound has no effect on the actual division mechanism of the few occasional cells that do enter mitosis in the presence of the drug; cells that enter and complete mitosis in the presence of BLM have normal spindle formation, followed by cleavage furrow development and the separation of the daughter cells.

Light and Electron Microscopic Observations. Cover slip preparations of Earle's L-929 cells (asynchronous), HeLa cells, and human lymphoblasts (CCRF-CEM and CCRF-JJB) exposed to BLM for more than 24 hr clearly show the drug-induced alteration of cellular morphology and the increase in cell mass. In Fig. 1, which shows a light micrograph of L-929 cells exposed to BLM (5 μg/ml) for 72 hr, cells are larger than those of untreated cultures, they are epithelioid, and many of them are multinucleate with prominent nucleoli. Similar BLM-induced changes in cell size and nuclear morphology were seen in HeLa cells (Fig. 2), CCRF-CEM cells, and CCRF-JJB cells.

In cultures exposed to low concentrations of BLM (1 μg/ml) for 72 hr, there was an increase in the density of the cytoplasmic matrix in nearly one-third of the cells. In cells from cultures fixed with 2% glutaraldehyde, the increased cytoplasmic density in some of the cells made it difficult to analyze the cellular fine structure. We presume that these dense cells are similar to the shrivelled and shrunken cells seen in light microscopic preparations and in time-lapse movies. However, in the same preparations, nearly two-thirds of the cell population contained cytoplasm of normal density. In an effort to study the fine structure of the dense cells, material for electron microscopic observation was subsequently fixed in buffered 2% osmium tetroxide without prefixation in glutaraldehyde. Thin sections of cells made from the osmium-fixed material had relatively lower cytoplasmic density; thus the fine structure of BLM-treated cells could be seen more clearly.

Fig. 3 shows the 2 types of cells (light and dense) from cultures exposed to BLM (1 μg/ml for 72 hr) and fixed with glutaraldehyde. The cell with electron-dense cytoplasm shows marginal chromatin near the nuclear membrane; while in the lighter cell, electron-dense chromatin is absent from the nucleus. Fig. 4 shows one of the lighter staining cells at a higher magnification; absence of marginal chromatin from the nucleus and the infolding of the nuclear membrane (arrows) are clearly seen in this cell. Fig. 5 shows the peripheral dense cytoplasm of an Earle's L-929 cell exposed to BLM (50 μg/ml) for 48 hr. As seen in this micrograph, the density of the cytoplasmic ground substance is very high; thus the mitochondria stand out as lightly stained areas with prominent cristae. In cell cultures exposed to higher concentrations of BLM (10 μg/ml), there were fewer cells with dense cytoplasm and a predominant part of the cell population contained nuclei lacking electron-dense chromatin clumps.

Besides the general increase in cell size and the nuclear mass, BLM-treated cells had proportionally much larger amounts of smooth endoplasmic reticulum, Golgi vesicles, and mitochondria in their cytoplasm. In addition to vesicles in the

RESULTS

Radioautographs made after pulse-labeling with thymidine-3H showed that a predominant portion (>90%) of these cells divided and entered G1 within 4 hr of reincubation in the fresh medium and reached early S phase after 10 hr. For cells in G2 mitotic cells collected from cultures incubated with vinblastine sulfate and BLM for 4 hr were washed twice in fresh medium and reincubated in BLM-containing medium.

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Cell counts were made in a Coulter counter, and cell viability was determined by dye exclusion (trypan blue) method.

Cell buttons were washed 2 times in buffered Hanks' solution, centrifuged, and fixed in phosphate-buffered 2% glutaraldehyde (pH 7.0). Dehydration in acetone series was followed by embedding in Epon-Araldite mixture. Thick sections (2 μm) were stained with toluidine blue; lead citrate and uranyl acetate were used for staining thin sections. Electron micrographs were taken on a Phillips 300 electron microscope.

Light and Electron Microscopic Observations. Cover slip preparations of Earle's L-929 cells (asynchronous), HeLa cells, and human lymphoblasts (CCRF-CEM and CCRF-JJB) exposed to BLM for more than 24 hr clearly show the drug-induced alteration of cellular morphology and the increase in cell mass. In Fig. 1, which shows a light micrograph of L-929 cells exposed to BLM (5 μg/ml) for 72 hr, cells are larger than those of untreated cultures, they are epithelioid, and many of them are multinucleate with prominent nucleoli. Similar BLM-induced changes in cell size and nuclear morphology were seen in HeLa cells (Fig. 2), CCRF-CEM cells, and CCRF-JJB cells.

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exposed to BLM, no drug-induced alterations of the microtubular structure or the spindle-associated structures like centrioles and centromeres were evident.

In occasional thin sections of mitotic cells from cultures exposed to BLM, no drug-induced alterations of the microtubular structure or the spindle-associated structures like centrioles and centromeres were evident.

Cells exposed to BLM show a pronounced alteration in their nuclear fine structure. Besides the overall increase in the nuclear mass, many of the BLM-treated cells have highly lobulated nuclei; in many of the cells, multiple nuclei are recognizable, indicating a probable process of endoreduplication. Fig. 7 shows the highly lobulated nucleus of a CCRF-CEM lymphoblast exposed to BLM (1 µg/ml) for 72 hr; Fig. 8 shows a similar nuclear condition in a HeLa cell exposed for 4 days to BLM (10 µg/ml). In many of the BLM-treated cells, an increase in nuclear mass is accompanied by numerous infoldings and inpocketings of the nuclear membrane (Fig. 9) as well as by the absence of electron-dense chromatin clumps normally seen near the nuclear membrane.

In cells exposed to BLM, 2 types of nucleolar alterations are regularly seen. In some of the cells, especially those exposed either to low concentrations of the drug or for short periods of time, nucleoli are unusually large (Figs. 1 and 10). In cells exposed to high doses of BLM or for long periods of time, nucleoli are fragmented and often show segregation of the components as seen in Fig. 9 and 11. In Fig. 11, which shows the nucleolus from a L-929 cell exposed to BLM (10 µg/ml for 72 hr), large electron-dense granules, approximately 30 nm in diameter (arrows), are also seen in association with the other nucleolar components. These granules are similar to the perichromatin granules seen, although in smaller numbers, in a variety of animal cell nuclei.

In view of the cytokinetic evidence suggesting a cell-cycle-specific action of BLM, synchronized cultures of Earle’s L-929 cells in G1, G2, and early S phase of the cell cycle were exposed to BLM (10 µg/ml) for 24 and 72 hr. The general drug-induced morphological alterations seen in these cultures were similar to those seen in asynchronous L-929 cells and described above. However, in cultures with approximately 90% of the cells in early S phase of the cell cycle and exposed to BLM for 72 hr, a larger number (approximately 50%) of the cells examined had electron-dense cytoplasm as compared to the approximately 20% of such cells in cultures with G1 and G2 populations and exposed to a similar time and concentration of BLM.

The various types of fine structural alterations induced by BLM and described above were seen in Earle’s L-929 mouse fibroblasts at a much lower concentration than in HeLa (human epithelial cells) or CCRF-CEM and CCRF-JJB cell lines, thus indicating a differential response of these 3 cell lines to bleomycin.

DISCUSSION

Bleomycin is one of those rare drugs which has progressed from the discovery and characterization phase to that of effective clinical use in a very short period of time (4, 11, 30). The clinical efficacy of this relatively new antibiotic in chemotherapeutic management of a variety of neoplastic conditions in man has been documented by a number of recent publications (10, 11, 21, 25).

Observations from the present study and those reported earlier by other workers suggest that BLM acts by binding to nuclear DNA, causing single-strand scission and in turn inhibiting DNA synthesis. BLM, like neocarzinostatin, another polypeptide antibiotic (12), causes chromatid breaks both in vitro and in vivo (3, 18, 19, 22, 23, 28).

Observations from cytological studies, both with light and electron microscopy, show that BLM causes extensive alterations in the nuclear morphology, especially in the distribution pattern of chromatin (16, 17). The presence of hyaline-appearing nuclei, which in electron micrographs show an absence of electron-dense chromatin clumps, is a regular feature of cells exposed to BLM. These nuclei occupy unusually large portions of the cytoplasm and have extensive foldings and inpocketings of the nuclear membrane. In cells exposed to lower concentrations of BLM or for shorter duration, nucleoli are unusually large. Although the initial enlargement of nucleoli is followed by some shrinking and segregation of nucleolar components, BLM does not cause extensive segregation of the nucleolar components or “capping,” as seen in cells exposed to actinomycin D (20).

The presence of membrane-bound oblong structures (Fig. 6) in some of the L-cells exposed to BLM is an enigma to us. These structures are consistently seen in BLM-treated L-cells, but they bear no resemblance to proteinaceous crystals induced by vinblastine and vincristine in cultured cells (2, 14).

BLM, as such, is completely soluble and stable in aqueous solutions or in tissue culture media, and no crystalline residues are seen in these solutions. It is possible that the presence of these structures in L-cells was incidental and not drug related, inasmuch as similar structures were not found in other cell types exposed to BLM.

Evidence from our time-lapse and electron microscopic study shows that, although BLM inhibits cell multiplication, it does not affect the mitotic apparatus or the process of division itself. A small number of cells that enter mitosis in the presence of the drug, or which are exposed to lower concentrations of BLM, develop normal spindle formation of microtubules, which appear unaltered in electron micrographs. There is no effect on cleavage furrow or the subsequent migration of the daughter cells away from each other. In this regard, BLM differs from mitosis-arresting chemotherapeutic drugs that affect the spindle microtubules; e.g., vinblastine, vincristine (9, 13), or drugs like cytochalasin which affect the subsequent events of cell division, such as the migrations of the daughter cells (5, 15).

BLM is selectively concentrated in the skin and lungs, and thus is of greater efficacy in treating skin carcinomas, etc. (4, 11, 21). A differential lethality of BLM depending on cell type or strain has been reported by other workers (1, 27) and is


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confirmed in this study. Mouse L-929 fibroblasts show BLM-induced chromosomal damage and ultrastructural alteration of cell structure at a much lower dosage (at least 10-fold) than the human lymphoblasts of leukemic or Hodgkin’s disease origin.

As mentioned in this study, a differential effect of BLM on cells in asynchronous cultures of the same cell line is clearly seen. In asynchronous cultures of L-929 cells exposed to BLM, especially at either lower concentrations of the drug or for a shorter duration, 2 types of morphologically distinct cells (easily recognizable in both light and electron microscopes) are seen. One type is represented by shrivelled and shrunken cells, seen under the light microscope or in time-lapse movies, which show very dense cytoplasm and marginated chromatin in electron micrographs. The 2nd population consists of large epithelioid cells with lighter-staining cytoplasm and absence of electron-dense chromatin clumps in the nucleus but instead have numerous infoldings and inpocketings of the nuclear membrane. In cultures exposed at either higher concentrations of BLM or for longer periods of time, the shrivelled cells are less abundant, and the large epithelioid cells with hyaline appearance and clear nuclei form a predominant part of the cell population. Observations from synchronized L-cell cultures exposed to BLM show that a larger number of dense cytoplasm-containing cells are seen in cultures exposed to BLM in early S phase of the cell cycle than are seen in cultures with G1 or G2 cells. The cell-cycle-specific action of BLM has been suggested by a number of earlier workers (1, 6, 7, 27, 29). For example, Terasima and Umezawa (27), using synchronous cultures, have observed that cells in late G1-early S (10 hr after mitosis) are most sensitive to the lethal effects of BLM. On the other hand, Barranco and Humphrey (1) have suggested, in Chinese hamster ovary cells, a decreasing order of BLM effectiveness in mitosis, G2, S, and G1. Cohen et al. (6) have suggested late S-early G2 as the cell cycle period sensitive to BLM action. Tobey (29), who compared a number of chemotherapeutic compounds as to their specificity in relation to cell cycle, reported that G2 processes are specifically sensitive to BLM and that the drug has no effect on genome replication. These observations are supported by data showing that BLM-exposed cells grow large and, because of the drug-induced inhibition of cell multiplication, accumulate a larger content of DNA in their unusually large nuclei (16).

The morphological alterations induced by BLM and reported in this study, however, do not seem to be cell cycle specific (except for the larger number of dense cells in S-phase cultures) and are seen with equal frequency in cultures exposed to BLM in the various parts of the cell cycle.

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REFERENCES

Bleomycin: in Vitro Morphological Studies


Figs. 1 and 2. Light micrographs of L-929 and HeLa cells from cultures exposed to 5 µg of BLM for 3 and 5 days, respectively. BLM-induced increase in the cell and nuclear size and the presence of multiple nuclei are seen in some of the treated cells. X 800.

Fig. 3. Electron micrograph of L-cells from a culture exposed to BLM (1 µg/ml) for 72 hr. The cell with the dense cytoplasmic matrix has chromatin clumps in the nucleus, while the adjoining cell has less electron-dense cytoplasm and nucleus and a near absence of chromatin clumps near the nuclear membrane. X 6,600. In Figs. 3 to 11, the marker represents 1 µm.

Fig. 4. L-cell exposed to BLM, 10 µg, for 72 hr. Note the large amount of vesicles and lamellae in the Golgi area and the large size of the nucleus. Infoldings of the nuclear membrane (arrows), absence of chromatin clumps, and apparent segregation of nucleolar components are seen. X 9,070.

Fig. 5. Electron-dense cytoplasm of an L-cell exposed to BLM, 50 µg, for 48 hr. Note the electron-dense cytoplasmic matrix. A large number of mitochondria are seen in the cytoplasm; due to the increased density of the cytoplasmic matrix, they stand out in contrast. X 27,000.

Fig. 6. Large number of membrane-lined oblong or spindle-shaped structures (arrows) in the cytoplasm of an L-cell exposed to BLM, 1 µg, for 24 hr. X 48,000.

Fig. 7. Fine structure of a human lymphoblast (CCRF-CEM) exposed to BLM, 1 µg, for 72 hr. Note the extensive lobulation and the infoldings of the nuclear membrane. No chromatin clumps are seen in the nucleus. Nucleolus shows segregation of the components. X 11,400.

Fig. 8. HeLa cell exposed to BLM, 10 µg, for 4 days. Note the fragmentation and lobulation of the nucleus, accompanied by enlargement of the nucleolar and the nuclear mass. X 6,600.

Fig. 9. Earle's L-cell exposed to 100 µg of BLM for 6 days. Note the enlargement of the nucleus, the folding of the nuclear membrane, and the inpocketings of the cytoplasm in the nucleus. No large chromatin clumps are recognizable in this cell. The nucleolus shows segregation of its components. X 13,200.

Fig. 10. Large size of the nucleolus of an L-cell exposed to 100 µg of BLM for 24 hr. X 14,500.

Fig. 11. Structure of a large nucleolus in an L-cell exposed to 10 µg of BLM for 72 hr. Besides the partially segregated nucleolar components, a large number of "perichromatin granule"-like structures, approximately 500 Å in diameter (arrows), are seen in association with the nucleolus. X 39,500.
Bleomycin-induced Fine Structural Alterations in Cultured Mouse Fibroblasts and Human Lymphocytes of Neoplastic Origin

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