Effects of Bleomycin on the Cell Cycle of Ehrlich Ascites Carcinoma

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Materials and Methods

Ehrlich mouse ascites carcinoma cells were maintained by weekly i.p. transplantation into male Swiss albino mice, HA/ICR strain (Charles River Mouse Farms, Wilmington, Mass.), weighing 18 to 20 g. Each mouse was inoculated i.p. under aseptic conditions with 0.2 ml of ascitic fluid that had been collected from a mouse inoculated 7 days previously. In one series, on the 5th postinoculation day, bleomycin* was given i.p. in doses of 10, 20, 40, 100, and 500 µg. In another series, 50 µg of bleomycin were given i.p. once a day for 4 consecutive days, starting on the 5th postinoculation day. Each group consisted of 3 to 5 mice. On the 1st, 4th, 7th, and 12th days after the injection, the ascitic fluid was collected by a peritoneal tap, diluted with 0.25 volume of distilled water, smeared onto glass slides, and promptly air dried. In the series with multiple doses, samples were collected on the 1st, 4th, and 9th days after the last injection. The cells were examined by Feulgen-DNA microspectrophotometry to estimate the nuclear DNA content. For the DNA determinations, the staining procedures, microspectrophotometric instrumentation, and measurements were performed as previously described, and the DNA content was expressed in A.U.6 (8). The mitotic index, defined as the number of cells in mitosis/1000 neoplastic cells, was determined on 1000 cells chosen at random from the slide used for the DNA measurements.

In the experiments in which a combination of autoradiography and microspectrophotometry was used, each of 2 animals received injections of 50 µCi of thymidine-methyl labelled H (1.83 Ci/mmmole; Calatomic, Los Angeles, Calif.) 30 min prior to the scheduled harvest of the specimen. Fixation and staining procedures were the same as described above. In these experiments, 50 neoplastic cells were chosen at random on each

*Ehrlich ascites carcinoma cells were kindly provided by Dr. S. Green of the Sloan-Kettering Institute for Cancer Research, New York, N. Y., in March 1971, and have been maintained in the Institute of Comparative Medicine of Columbia University, New York, N. Y.

Bleomycin A, (Lot 33) was dissolved in 0.9% NaCl solution to make a 1-ml solution of each dosage used. This antibiotic was provided by Dr. H. Umezawa (Institute of Microbiology and Chemistry, Tokyo, Japan) and Dr. Y. Miura (Chiba University, Chiba, Japan) from Nippou Kayaku Co., Tokyo.

The abbreviations used are: A.U., arbitrary units; L.I., labeling index; TGC, total grain count; MGC, mean grain count.

SUMMARY

The mode of action of bleomycin on Ehrlich ascites carcinoma cells was investigated by means of combined autoradiography and microspectrophotometry. The cell cycle was blocked by bleomycin, and cells accumulated in G2. A portion of the cells thus accumulated appeared to have died or to be severely injured. Another portion of the blocked G2 population transferred to the S phase of the next higher cell cycle without undergoing cytokinesis.

The combined autoradiography-microspectrophotometric method used in this study may be applicable to the analysis of the mode of action of other anticancer agents.

INTRODUCTION

Bleomycin, a new cancer chemotherapeutic antibiotic, has unique characteristics in its mode of action as well as in its chemical properties (2, 5, 8—11). It is well established, both clinically and in experimental animals, that bleomycin therapy is rarely accompanied by bone marrow suppression (2, 11) and that its local toxicity is negligible, facilitating repeated administration i.m. The drug also has clinical effects which are quite different from those of other major classes of anticancer agents. Alterations in the neoplasms soon after the injection of bleomycin are not as drastic as is usually seen following administration of the alkylating agents, but the effects are progressive after an initial lag time, and the clinical response is excellent when a series of injections are given over a prolonged period.

In order to better understand these clinical effects and to investigate further the mechanism of action of bleomycin, we studied the cell cycle with the use of autoradiography and microspectrophotometry.

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slide, the DNA content was measured, and the cell location was noted with the use of mechanical stage coordinates. After this, the coverglass and mounting oil were removed in xylene, and Eastman Kodak NTB-3 emulsion was applied directly to the slide following the dip-coating method described by Joffes (3) and Messier and Leblond (7). After 14 days of exposure at 4° in a sealed black plastic box containing a drying agent, the slides were developed, dehydrated, and mounted.

The L.I., defined as the percentage of neoplastic cells labeled by a single injection of thymidine-3H, was determined on the same cells that were used for microspectrophotometry. The TGC was defined as the total number of grains occurring over the 100 neoplastic cells in each slide. MGC per labeled cell was defined as TGC/LI.

Cumulative histograms were made from each experimental group after conversion of the log10 A.U. values of nuclear DNA content to “M” units, representing the stem lines. Since this strain of Ehrlich ascites carcinoma has a near-tetraploid stem line (4C), the M value was calculated as the common logarithm of the 4C value expressed in A.U. (log10 4C). The control diploid value (2C) was obtained with lymphocytes on a slide that was previously described (8), and the value was checked by measuring histiocytes, which also had a 2C or 0.5M DNA content, on the same slide.

In order to facilitate the mathematical and statistical treatment of these data, the histograms were plotted in M groups, as shown in Chart 1. Six bars of the same width were set between M (log10 4C) and 2M (log10 8C) after which 3 adjoining bars were assembled to make new bars, and these bars were designated M, M-2M, 2M, 2M-4M, 4M, 4M-8M, 8M, and so on, and were plotted as histograms. The M bar contains most of the cells in G1, the 2M bar contains most of the cells in G2, and the M-2M bar is composed principally of S-phase cells (Charts 1 and 2).

In the series in which autoradiography was combined with microspectrophotometry, the distribution of labeled cells was indicated on the same histogram, and the relationship between the labeled and nonlabeled cell population was studied.

RESULTS

Following bleomycin administration, there was a decrease in the stem line M cells and an increase in the premitotic population of 2M cells. This reciprocal change increased with time after bleomycin administration and proceeded more rapidly and was more widespread with larger dosages (Chart 2).

The change in the number of cells in the M and 2M classes before and after bleomycin therapy was statistically significant in all of the treated groups, in contrast to the corresponding control groups (x² test: p < 0.05 to p < 0.001). This increased 2M cell population started to decrease again, in the high-dosage series, 7 to 12 days after the injection of bleomycin i.p. (Chart 2).

An increase of 4M cells and, occasionally, of 8M cells, was also observed in the high-dosage groups. The increase in these 2 populations was roughly proportional to the increased 2M cell populations and was more marked in the later stages, after the bleomycin injections.

The percentage of M-2M cells was relatively constant throughout the series, although 2 exceptions were noted. One occurred in the groups that had a small M-2M cell population and a large 2M cell population (Chart 2). The other exception was found in groups that had an increased M-2M cell population and a decreased 2M cell population (Chart 2).

There was a continuous slow decrease in mitotic indices in the small-dosage series throughout the observation period but in the high-dosage series, the indices reached their lowest levels in 24 hr and fluctuated around that level thereafter. The mitotic index never reached normal values again (Chart 2). The greater the dosage of bleomycin and the longer the lapsed time after its administration, the smaller was the amount of nuclear thymidine incorporation. The minimum value of the TGC was 13% of the control values, and the changes in the L.I. and TGC in each group are shown in Chart 3. The only exception to these trends was noted in the smallest-dosage series on the 1st postinjection day, when TGC was 2.7 times greater than in the controls. The L.I. and the MGC were also elevated in this group.

Chart 1. A composite histogram of the control group and of a 6-day-old tumor. A total of 100 neoplastic cells from 2 mice were accumulated after the conversion of A.U. to M units. The instrumentation used and the above-mentioned conversion and accumulation of 2 different specimens yielded enough accuracy to divide the range from M to 2M into 6 equal parts. Thirty-four % of the population was in G1, 58% was in S, and 8% was in G2 phase.
The MGC's in the histogram classes were compared with one another within every treatment group. The mean grain count in the labeled M-2M cells and that in the 2M cells were approximately the same, whereas the mean grain count of the labeled M cells was only 50% to 75% that of the 2M cells. The mean grain count of the labeled 2M-4M cells and that of the labeled 4M cells were close to each other and were about twice that of the 2M cells.

The number of labeled cells in each histogram class changed after treatment (Chart 3), with the change in the 2M cells being most prominent. On the 1st day after bleomycin administration, the number of labeled cells in the 2M cell population was significantly increased at all dosages but was more significantly increased at the larger dosages. The number of those labeled 2M cells significantly decreased on the 4th day after treatment, however, and continued to decrease thereafter. The number of labeled cells in the M and M-2M classes gradually decreased with time after treatment, and the speed with which they decreased was greater in the larger-dosage groups. On the other hand, the nonlabeled M cells decreased gradually after treatment, the decrease being more rapid in the large-dosage series and most striking in the multiple-dosage series. In contrast, the nonlabeled cells in the other classes showed a gradual increase. The increase was most marked in the 2M cell class, and was also marked in the M-2M cell class in the later stages in which the nonlabeled 2M cells showed a secondary decrease.

In comparison with the changes induced by bleomycin, nitrogen mustard (HN2) had a far more rapid effect. As is shown in Chart 3, the G1 cells were reduced to only 5% of the cell population in 24 hr following a single i.p. injection of 10 µg HN2.
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Post Treatment 1 DAY 4 DAYS 7 DAYS 12 DAYS
Post Inoculation 6 DAYS 9 DAYS 12 DAYS 17 DAYS

DISCUSSION

The fact that a single injection of bleomycin induced the accumulation of nonlabeled 2M (G2) cells and that a decrease of nonlabeled M (G1) cells occurred, simultaneous with a decrease in labeled M, M-2M, and 2M (S phase) cells, suggests that the drug blocked the cell cycle at the G2 phase and that the effect of the drug occurred over a prolonged period. This hypothesis is supported by the decrease in mitotic indices. Some of the accumulated G2 cells apparently died, as evidenced by the secondary decrease in the nonlabeled 2M cell population and concomitant increase in the nonlabeled M-2M cells, and by the direct microscopic observation of fragmented cells and cells with pycnotic, abnormally swollen, or vacuolated nuclei. Such dying, signet-ring, or fragmented cells were found exclusively on the slides in which the secondary decrease of the G2 cells was observed. These nuclei were not included in the DNA measurements since they were technically unsuitable, accounting for the decrease in the G2 cell population.

The rapid increase in the nonlabeled M-2M cell population that accompanied the decrease in the G2 cell population was probably due to the fact that some of the G2 cells had lost their Feulgen-staining properties as a result of their injury or early death, as has been described previously in Ehrlich tumor cells (4) and granulosa cells (1). The possibility that this increase in the nonlabeled M-2M cell population was induced by an inflow of cells through the cell cycle is unlikely in view of the low mitotic indices and the small population of labeled M and M-2M cells.

Some cells that were blocked at the G2 phase of the regular cell cycle (M-2M cycle) seem to have been converted to G1 cells in the next (2M-4M) cycle. S-phase cells in the 2M-4M cycle (2M-4M cells) incorporated 2 times the amount of thymidine-3H which was incorporated in the regular S phase (M-2M) cells. This transfer to the next cell cycle seems to imply "endoreduplication" as was suggested by Levan and Hauschka (6). In some treated groups, the cells in the 2M-4M cycle were as much as 12% of the population, and anaphase and telophase figures were encountered, confirming the existence of a 2nd cell cycle.
Cells that seemed to be passing through a 3rd cell cycle (4M-8M) were also observed, although less commonly, but the incorporation of a commensurate quantity of thymidine-3H into the 4M-8M cells (S-phase cells in the 3rd cell cycle) was not demonstrated.

Cells in the 4th cell cycle (8M-16M cycle) were not observed. Cells transferring to the next higher cell cycle apparently were accelerated by this regimen, suggesting that these transfers are related to a destructive or degenerative process.

As a whole, bleomycin had a slow but steady effect on Ehrlich ascites carcinoma cells. These characteristics of bleomycin were in contrast to those of HN2, which had an abrupt effect on the neoplastic cells, and the difference between these 2 anticancer agents could be demonstrated readily and was in good agreement with the differences found in clinical usage. The combined method autoradiography and microspectrophotometry used in this study could provide new information about the mode of action of chemotherapeutic agents on experimental tumors, especially from the standpoint of the cell cycle, and can be expected to be useful in the investigation of classes of anticancer agents other than those reported here.

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