Effects of Bleomycin on Nuclear DNA in Transplantable VX-2 Carcinoma of Rabbit

Masaaki Nagatsu, Takashi Okagaki, Ralph M. Richart, and Adrian Lambert

SUMMARY

The nuclear DNA content of the VX-2 carcinoma of rabbits was measured by microspectrophotometry in 22 animals before, during, and after multiple i.m. injections of bleomycin given over a prolonged time period.

Striking changes were noted in the distribution pattern of the nuclear DNA content of VX-2 carcinoma cells with higher levels of bleomycin administration. The population of stemline cells, which was dominant in the controls, decreased after the treatment, and the population of cells with twice the stemline DNA content increased. In addition, a few cells containing four times the stemline DNA content also appeared. The mitotic index decreased immediately after the initiation of bleomycin therapy and was maintained at a low level during the extended period of drug administration.

These findings suggest that bleomycin prevents cells from entering visible mitosis but does not inhibit DNA synthesis at this dosage and that DNA replication without cell cleavage probably results in a higher DNA content in a significant proportion of the cell population.

INTRODUCTION

In 1965, a new antibiotic, which was named bleomycin, was reported by Umezawa (33). This drug inhibited the growth of *Escherichia coli* and HeLa cells in culture and Ehrlich carcinoma and Sarcoma 180 cells in mice (35). In addition, it was curative in a skin cancer in a dog (34). In a preliminary clinical study, Ichikawa et al. (13) found that bleomycin was effective on squamous cell carcinomas, particularly of penile and scrotal origin. The measurements of the concentration of bleomycin in various organs suggested that the striking effect of epidermal cancer was due to the high concentration of bleomycin in the skin (34). In view of these characteristics and the relatively few studies on the mechanism of action of bleomycin, several transplantable animal tumors of cutaneous origin were studied preliminarily, and the rabbit VX-2 carcinoma was chosen as a model system in which to investigate the effect of bleomycin on the cell cycle by measuring nuclear DNA content and mitotic indices.

MATERIALS AND METHODS

The VX-2 carcinoma was derived in 1935 by Rous and Beard (27) from a papilloma of the domestic rabbit inoculated with the Shope papilloma virus (14, 22, 29). This transplantable neoplasm, which is an epidermoid carcinoma both in appearance and behavior, clinically responds well to the i.m. injection of bleomycin, which produces retardation of its growth (M. Nagatsu and A. Lambert, Effects of Bleomycin on VX-2 Carcinoma: Preliminary Report, in preparation).

In the present study, 0.4 ml of a 10% suspension of VX-2 carcinoma was injected i.m. into the limb of New Zealand white rabbits weighing approximately 3 kg. Beginning 7 days after inoculation, when a palpable tumor appeared at the site of injection, i.m. bleomycin injections were given 3 times a week at a dose of 1 mg/kg of body weight. Twenty-two rabbits were divided into 4 groups according to the number of injections of bleomycin, as follows: Group 1 (control), no bleomycin treatment was given, and tumors were removed from 13 to 55 days after transplantation; Group 2, bleomycin was given 6 times, and tumors were removed 23 days after transplantation; Group 3, bleomycin was given 12 times, and tumors were removed 36 days after transplantation; and Group 4, bleomycin was given 20 times, and tumors were removed 55 days after transplantation.

In preparation for microspectrophotometry, tumors were harvested, trimmed, sliced, and smeared on glass slides, and the slides were immediately placed in Bouin's solution for 4 hr at room temperature. The preparations were hydrolyzed for 30 min in 5 N HCl at room temperature (8, 9), stained by the Feulgen reaction according to the method of Stowell (30), and mounted in cedar oil (Cargille oil) with a refractive index of 1.560, which was identical with that of the unstained nuclei as determined by phase microscopy. The measurements of the

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1 This work was supported by a grant from the Whitehall Foundation.
2 To whom requests for reprints should be sent, at: 768 Park Avenue, New York, N. Y. 10021.
3 Clinical usage of bleomycin is permitted in Japan.
4 The VX-2 carcinoma was kindly provided by Dr. B. Clarkson of Sloan-Kettering Institute for Cancer Research, New York, N. Y., and has been transplanted over 5 years through 120 generations in the Surgical Research Laboratory of Harlem Hospital.
5 Bleomycin A4 (Lot No. 33) was used. This was provided in part by Bristol Laboratories, Inc., Syracuse, N. Y., and in part by Nippon Kayaku Co., Japan.
6 Clinically, the usual dosage is 0.25 to 0.50 mg/kg of body weight, 2 or 3 times a week.
DNA content of the nuclei were carried out with a Leitz microspectrophotometer, a Leitz monochromator, and a Canalco digital ratio reporter. From each slide, 100 interphase cancer cell nuclei and 20 to 28 small lymphocyte nuclei were selected at random and were measured by the "plug" method of Swift and Rasch (32) and Kasten (16) at 560 μm. The nuclear DNA content was expressed in A.U.7

The mitotic index, defined as the number of cells in mitosis/1000 tumor cells, was determined in 1000 cells chosen at random with the use of routine histological sections stained with hematoxylin and eosin.

To facilitate the interpretation of modes, histograms were made by setting the common logarithms of A.U. (log10 A.U.) in the abscissa and the cell frequency in the ordinate (T. Okagaki, M. Izuo, and R. M. Richart, Statistical Tests of Significance for DNA Content in the Cell Nuclei Measured by Microspectrophotometry, in preparation; Ref. 15). Accordingly, all of the DNA values expressed in A.U. were transformed to make common logarithms of A.U. (log10 A.U.).

The arithmetic mean of the DNA content of the cells that comprise the prominent 1st peak was computed in each case after selecting these cells based on the distribution patterns. This mean value was designated the computed modal value.

The control diploid value (2C) was obtained from the arithmetic mean of the DNA content of the lymphocytes by adding 0.0792 to the mean [log10 (A.U.X1.2) = log10 A.U. + log10 1.2], according to the values reported by Wagner et al. (36), Weiss et al. (37), and Atkin and Richards (1).

RESULTS

As bleomycin was administered, the population of stemline cells (20, 28), which was dominant in the controls (M peak), gradually decreased in favor of a secondary peak, which consisted of a population of cells with twice the DNA content of the stemline cells (M X 2 peak) (Chart 1). After bleomycin treatment, the M X 2 peak tended to be wider than that in the controls as well as being proportionately increased (Chart 1).

Along with an increase in the M X 2 peak, a 3rd mode appeared, which consisted of cells with 4 times the DNA content of the stemline cells (M X 4 peak), and this peak also continued to increase during treatment (Chart 1 and Table 1). This progressive increase in nuclear DNA content during bleomycin therapy was statistically significant (p < 0.001). An M X 8 peak was not observed.

In addition to a proportionate decrease of the stemline cell population, a qualitative change in the stem-cell group appears to have occurred. There was a gradual shift of the M peak to the right (Chart 1), as evidenced by a change in the mean of the computed modal values of the M peak, which was calculated in each of the 4 groups (Table 1). This progressive increase in nuclear DNA content during bleomycin therapy was statistically significant (p < 0.001). An M X 8 peak was not observed.

DNA content in the lymphocytes as measured by Feulgen microspectrophotometry varied considerably from small to large lymphocytes because of the difference in the dye-binding capacity. To confirm the consistency of nuclear DNA content in the lymphocytes in our series, the mean of the average DNA content of the lymphocytes was calculated in each of the 4 groups (Table 1), and no significant difference was noted. Therefore, it is felt that the gradual shift of the M peak could not have been produced by a change in the lymphocytes.

7The abbreviation used is: A.U., arbitrary units.
Table 1

Frequency distribution and computed modal values of M peak cells and lymphocytes

The values were calculated as follows:

$$\log_{10} A.U. = \frac{1}{n} \sum_{i=1}^{n} \log_{10} (A.U.)_i$$

<table>
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<th>Group</th>
<th>Serial case no.</th>
<th>No. of cells</th>
<th>Frequency distribution (%)</th>
<th>Neoplastic Cells</th>
<th>Computed modal value of M peak cells</th>
<th>Lymphocytes</th>
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<td></td>
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<td>0–2C</td>
<td>2C–4C</td>
<td>4C–8C</td>
<td>8C–16C</td>
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<td>56</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2@</td>
<td>100</td>
<td>3</td>
<td>78</td>
<td>19</td>
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<tr>
<td></td>
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<td>4</td>
<td>79</td>
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<td>0</td>
<td>73</td>
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<td>14</td>
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<td>154</td>
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<td>1</td>
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<td>42</td>
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<td>1</td>
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<td>198</td>
<td>50</td>
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a Cases illustrated in Chart 1.

b Mean and S.E. of the computed modal values of the M peak cells in each group expressed in log$_{10}$ A.U. and in C, respectively.

c Mean and S.E. of the computed modal values of the lymphocytes in each group.

Another possible mechanism that may account for the shift on the M peak is an overlapping of the M peak, the M X 2 peak, and the S phase populations. From the histograms, it appears that the increased height and width of the M X 2 peak may have produced an increased overlap of the M peak and the left tail of the M X 2 peak, thus increasing the computed modal value of the M peak and producing the shift of the M peak to the right. This means that the M peak, which is composed primarily of the stemline cells, still contains cells from the M X 2 and S phase population and that these 2 groups of cells could not have been eliminated when the M population was selected. The artifact produced by the overlapping is sufficient to explain the relatively small increase (+23.6%) of the M peak that took place during the bleomycin treatment and suggests that the stemline of the VX-2 carcinoma did not change after bleomycin treatment.

The change in stemline distribution was accompanied by alterations in the pattern of mitotic activity. The mitotic index was reduced by bleomycin therapy and was at its lowest level at the time it was first measured 8 days after the initiation of bleomycin injections or 24 hr after the 3rd injection of the drug. It remained at that low level during treatment, although a slight recovery was observed in the latter half of the regimen (Chart 2).

DISCUSSION

Several mechanisms can be considered to explain the accumulation of nuclei with a premitotic DNA content as evidenced by the decrease of the M peak and the simultaneous increase of the M X 2 peak in the histograms. The most
Effect of Bleomycin on VX-2 DNA

Chart 2. Change in mitotic index following bleomycin therapy. Tumors were harvested 24 hr after 3, 6, 9, 12, and 20 i.m. injections of bleomycin at a dosage of 1 mg/kg of body weight. Means and standard deviations are illustrated. The mitotic index drops precipitously and remains low throughout the course of chemotherapy.

probable mechanism is that bleomycin at this dosage (1 mg/kg of body weight) blocks the cell cycle near the end of the S or G2 phase (premitotic block), apparently without blocking DNA synthesis. The sudden decrease in the mitotic index after bleomycin injections favors this possibility. The effects of the alkylating agents, mitomycin C, and ionizing radiation on nuclear DNA content and the mitotic index have been reported to be similar to these findings. Caspersson et al. (5) and Layde and Baserga (18) using nitrogen mustard, Pályi et al. (23) using mannitol mustard, Bassleer (3) using melphalan and mitomycin C, and Chèvremonet et al. (7) using Myleran all observed continued DNA synthesis, accumulation of cells that had doubled their DNA content, and a decreased mitotic index, and they proposed a premitotic block without inhibition of DNA synthesis as the mechanism of action. Similar observations and deductions were reported by Caspersson et al. (6), Richards and Atkin (25), Killander et al. (17), and Holzner and Golob (12) using ionizing radiation.

The fact that these different chemotherapeutic agents, as well as ionizing radiation, induced the same distribution pattern of DNA in different types of cells implies that this pattern is not specific to any 1 drug but may be a common response to inhibition at a similar step in the cell cycle. An exception to the above mentioned drugs was actinomycin D, which produced a presynthetic block in G1 phase at lower concentrations (2), although it inhibited almost all synthetic activities at higher concentrations (5). In other studies on the mechanism of action of bleomycin, Suzuki et al. (31) observed its inhibitory effect on DNA synthesis in E. coli, Ehrlich carcinoma, and HeLa cells. They used relatively high concentrations (up to 100 μg/ml) of this drug in their cell culture system; however, at the lower concentrations that are used clinically and in this study, it is probable that bleomycin does not directly inhibit DNA synthesis but blocks the cell cycle at a premiotic period and that the inhibition of DNA synthesis is secondary to this blockade.

Other possible mechanisms that may explain the doubling of the nuclear DNA content over premiotic values are the fusion of interphase nuclei (24), nuclear fusion during mitosis (4), or a selective destruction of the stemline cells. The lack of binucleated cells or significant cell death upon microscopic examination of the tumors suggests that these mechanisms are either absent or insignificant in the action of bleomycin, although they cannot be eliminated completely by the data presented here (26).

The mechanism by which the M × 4 population was produced could be explained by "endomitosis" as proposed by Geitler (11) or "endoreduplication" as suggested by Levan and Hauschka (19). In these processes, the cells that are blocked in the premiotic period eventually repeat DNA replication but fail to complete nuclear division (21). Polyploidization induced by alkylating agents (3, 7, 23) and radiation (25) has been reported previously, and endomitosis or endoreduplication was suggested as the mechanism.

Thus, although the detailed mechanism of the cellular response to bleomycin is still obscure, the drug apparently has an inhibitory effect on neoplastic cells and operates in a fashion that is similar to that of other major classes of chemotherapeutic agents, at least with respect to the change in the DNA distribution pattern. Further insight into the precise mode of action must await more detailed biochemical studies.

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REFERENCES

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