Studies on the Cytotoxicity of Bleomycin in the Small Intestine of the Mouse

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SUMMARY

Mice treated with single i.v. injections of bleomycin (2 to 200 mg/kg) show biochemical and histological evidence of cytotoxicity within the crypts of the small intestine. The incorporation of thymidine-methyl-3H into DNA is inhibited in a dose-related manner. Maximal inhibition is attained between 2 and 6 hr after administration of the drug; recovery occurs slowly thereafter. In contrast, the incorporation of cytidine-5,3H into RNA is unaffected by even toxic dose levels of bleomycin.

Histological examination of the small intestine reveals a profound antimitotic action of bleomycin. Moreover, at all dose levels maximal inhibition of mitosis is attained at 2 hr after administering the drug. On the basis of the known cell cycle kinetics of the mouse small intestine, it appears that the antimitotic action takes place at the late S → early G2 transition. Finally, histological evidence of cell death in the crypts of the small intestine following low doses of bleomycin is better correlated with the antimitotic action of the drug than with its effects on nucleic acid synthesis.

INTRODUCTION

Bleomycin is a mixture of several related sulfur-containing polypeptide antibiotics which have been isolated from a strain of Streptomyces verticillus (19). This mixture has been used clinically in the treatment of squamous cell carcinoma and malignant lymphoma (3, 4).

Studies on the biological activity of bleomycin have revealed its capacity to inhibit the proliferation of tumor cells in culture and retard the growth of some transplantable tumors in mice (15–17). Biochemical studies with radioactively labeled precursors have indicated that the synthesis of DNA, but not of RNA, is inhibited by bleomycin in growing cultures of HeLa S-3 cells and Ehrlich carcinoma cells (15). In addition, experiments in which synchronized cell cultures were used indicated that the cytotoxic effects of bleomycin could be cell cycle specific (5).

Whereas the effects of bleomycin on tumor cells in culture and on experimental tumors in animals have been the subject of several investigations, few studies have been concerned with the cytotoxicity produced by this agent in normal proliferating tissues of intact animals. Preliminary experiments conducted in this laboratory indicated that the proliferative capacity of several normal tissues of the mouse was disturbed by prior administration of bleomycin. Of the tissues examined, the small intestine was the most sensitive in its response to the cytotoxic actions of bleomycin. In an effort to obtain a better understanding of the cell-killing effects of bleomycin, we initiated a systematic study of the early biochemical and morphological effects of this new agent in the small intestine of the mouse. The results of these investigations are reported in this paper.

MATERIALS AND METHODS

All experiments were performed on 5-week-old male, CD1 mice (Charles River Breeding Laboratory, Brookline, Mass.). The animals were fed Purina laboratory chow and were given water ad libitum. Animals were killed by cervical dislocation in experiments involving the incorporation of labeled precursors. When histological sections of various tissues were required, the mice were killed by exsanguination from the abdominal aorta while under ether anesthesia.

Tissues taken for histological study were fixed in Bouin's solution, sectioned at 7 μm, and stained with hematoxylin and eosin. The effect of bleomycin on the mitotic activity of the small intestine was determined in 1-cm segments of duodenum taken just distal to the pylorus. The segments were opened longitudinally, spread on filter paper prior to fixation, and sectioned parallel to the longitudinal axis of the intestine. Mitoses were enumerated in crypts in which the entire lumen was present between mouth and base. At least 20 crypts in each of 4 sections/animal were counted.

Bleomycin was obtained from the Bristol Laboratories, Syracuse, N. Y., with a potency equivalent to 1.45 mg/mg as determined by bioassay (19). All doses of bleomycin are reported in terms of the assayed potency; they must be multiplied by 0.69 to obtain the actual amounts injected. Solutions of the drug were made in sterile, pyrogen-free 0.9% NaCl solution. Injections were given i.v. in a volume of 0.01 ml/g of body weight.

Precursor Incorporation. TdR-3H2 and CR-3H were purchased from New England Nuclear, Boston, Mass. The incorporation of TdR-3H into DNA was determined 10 min after the i.v. injection of the labeled precursor (79 μCi/kg; specific activity, 19.2 μCi/μmole). In the experiments concerned with the effects of bleomycin on acid-soluble
thymidine nucleotide pools, a 5-min pulse was used. The incorporation of CR-3H into RNA was determined 10 min after i.v. injection of 71 μCi/kg (specific activity, 8.9 μCi/μmole). At the end of the incorporation period, the mice were killed and the entire small intestine was removed and placed in ice-cold 0.9% NaCl solution. The intestines were then slit open to remove all fecal material, blotted on filter paper, and weighed. The cleaned intestines were frozen on Dry Ice and stored at −20° until processed as described below.

Frozen intestines were allowed to thaw for at least 30 min in 15 volumes of chilled 10% TCA. They were then homogenized with a VIRTIS homogenizer at medium speed for 3 min and centrifuged. The resulting precipitate was washed twice by resuspension and centrifugation in cold 10% TCA. The washed precipitate was defatted once with absolute alcohol, twice with alcohol:chloroform (3:1), twice with alcohol:ether (3:1), and finally once with ether. When TdR-3H was used, the washed, defatted precipitate was heated twice at 90° for 15 min with 10% TCA, and the resulting supernatants were combined for measurement of radioactivity and nucleic acid content. In the experiments with CR-3H, RNA was separated from DNA according to the procedure of Schmidt and Thannhauser (11). Radioactivity and nucleic acid content determinations were then made on the RNA fraction.

The DNA and RNA content of the above fractions was determined colorimetrically according to the method of Schneider (12) with deoxyadenosine and adenosine, respectively, as standards. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency was estimated by internal standardization.

The radioactive, acid-soluble thymidine nucleotide pools were determined in the combined cold 10% TCA supernatants of homogenized intestines. TCA was first removed by successive extractions with ether. The extracted solution was then freeze-dried, and the residue was dissolved in 0.5 ml of distilled water. For determination of total radioactivity, 10-μl aliquots were taken. Aliquots of 50 μl were chromatographed unidirectionally on Whatman No. 1 paper in a solvent system consisting of isobutyric acid:water:concentrated ammonium hydroxide (66:33:1, v/v/v). Channels containing unlabeled thymine, thymidine, and TMP (Calbiochem, Los Angeles, Calif.), and TDP and TTP (Sigma Chemical Co., St. Louis, Mo.) were developed simultaneously. Zones corresponding to those of unlabeled markers were cut out and eluted with 0.1 N HCl. The radioactivity present in these zones was determined by liquid scintillation spectrometry as described above. Recovery of radioactivity from the chromatograms averaged 70%.

RESULTS

Incorporation of Thymidine into DNA. Changes induced in incorporation of labeled thymidine into DNA can be a useful measure of the capacity of an agent to interfere with the proliferative activity of tissues. In a series of preliminary experiments, single injections of bleomycin resulted in inhibition of the incorporation of TdR-3H into the DNA of small intestine. Since these results were indicative of a disturbance in the proliferative capacity of this tissue, it was deemed necessary to examine more systematically the dose and time relationships of the inhibitory effect.

Accordingly, the effects of 3 dose levels of bleomycin on the incorporation of TdR-3H into the DNA of mouse small intestine were determined at 1, 2, 4, 6, and 24 hr after drug administration, as described in Chart 1. The highest dose, 200 mg/kg, is 1 to 1.5 times the reported LD50 for mice (2, 20). The lowest dose, 2 mg/kg, approximates the total dose that is used clinically (4). The dose of 20 mg/kg is of the order of doses used in treatment of experimental tumors in mice (16). The data in Chart 1 show that the inhibition of TdR-3H incorporation was dose related. Strict proportionality between dose and effect was not observed, since a 100-fold increase in dose resulted in only a 3- to 4-fold increase in the maximal inhibition induced. By comparison with the rapid effects of specific inhibitors of DNA synthesis, such as cytosine arabinoside (7) and hydroxyurea (10), the inhibition of TdR-3H incorporation developed relatively slowly after bleomycin administration. Thus, even after a lethal dose of 200 mg/kg, at least 2 hr elapsed before maximal inhibition was attained. With doses of 20 and 2 mg/kg, maximal inhibition was not seen until 4 and 6 hr, respectively. Finally, while TdR-3H incorporation had recovered by 24 hr in mice given either of the 2 lower doses of bleomycin, it was still inhibited in those mice given the highest dose.

Thymidine Nucleotide Pools. Since an inhibition of TdR-3H incorporation into DNA could result from either a disturbance in the equilibration of the labeled precursor with the intracellular thymidine nucleotide pools or from an actual...
inhibition of the polymerization of TTP, an effort was made to distinguish between these 2 possibilities. The incorporation of TdR-3H into the acid-soluble nucleotide pools of the small intestine was determined at 1 and 4 hr after injecting bleomycin in the dose of 200 or 20 mg/kg. The results are shown in Table 1. At 1 hr after the administration, there was no disturbance in the total amount of radioactivity incorporated or in its distribution among the various TdR-3H metabolites. It is probable, therefore, that the inhibition of TdR-3H incorporation into DNA produced at this time represents a true inhibition of DNA synthesis. However, at 4 hr after drug administration the total amount of radioactivity incorporated was inhibited by 11 and 26% for the 20- and 200-mg/kg doses, respectively. Furthermore, the distribution of radioactivity among the TdR-3H metabolites was disturbed at both dose levels. The most notable disturbance was an increase in the amount of radioactivity present as thymine and a concomitant decrease in the amount of radioactivity present as TdR-3H nucleotides. Histological examination of the small intestine at this time revealed the presence of many dead and degenerating cells in the crypts of Lieberkühn (Fig. 6). The inhibition of TdR-3H incorporation into DNA observed 4 hr after bleomycin administration could have resulted from the combined effects of a direct action on DNA synthesis, as well as a disturbance in precursor pools produced by the drug.

**Incorporation of Cytidine into RNA.** Having ascertained the initial effects of bleomycin on the synthesis of DNA in the small intestine, we next determined whether RNA synthesis was similarly affected. Table 2 presents data from experiments in which the incorporation of CR-3H into RNA was measured at 1 to 6 hr after bleomycin. The results indicate that RNA synthesis was not significantly affected by high dose levels of bleomycin at any of the times studied. These findings are in contrast to the inhibition of DNA synthesis produced by the drug during this same time period (Chart 1).

**Mitotic Activity and Histopathology.** In an effort to relate the inhibitory effects of bleomycin on DNA synthesis to the cytocidal actions of this agent, histological examinations of the small intestine were conducted at various times after administering the drug. The studies showed that bleomycin had a marked antimitotic action in intestinal crypts. The time and dose dependencies of this action are shown in Chart 2. A comparison of the data in Chart 2 with those in Chart 1 indicates that the inhibition of mitosis occurs earlier and is of greater magnitude than the inhibition of thymidine incorporation into DNA. For example, the number of mitoses in the crypts was decreased by approximately 50% at 1 hr after the injection of the dose of 20 mg/kg. By contrast, the incorporation of thymidine into DNA was unaffected at this time interval. Similar results were noted with both higher and lower doses of the drug.

In addition to inhibition of mitosis, the drug induced necrosis in crypt epithelium. This is illustrated in animals of Chart 2 given 2 or 200 mg/kg and killed at 1 hr (Figs. 1 and 2), 2 hr (Figs. 3 and 4), and 4 hr (Figs. 5 and 6). Those receiving the smallest dose, 2 mg/kg, had no abnormalities at 1 hr. At 2 hr, mitotic figures were rare, but the mucosa was otherwise normal. However, at 4 hr, when mitotic activity had already returned to normal levels, small amounts of cellular debris were found in most crypts (Figs. 1, 3, and 5). Since thymidine incorporation into DNA was also normal at this time (Chart 1), the appearance of dead cells in the crypts following the low dose of bleomycin was apparently better correlated with the transient antimitotic action of the drug than with its effects on DNA synthesis. At 6 hr, dead cells were still present in the crypts, but by this time an inhibition of DNA synthesis was also observed (Chart 1).

Although the number of mitoses in crypts was sharply reduced at 1 hr after the administration of the highest dose, 200 mg/kg, no other abnormalities were noted. However, at 2 hr there was a small amount of cellular debris in the lower portion of most of the crypts. At 4 and 6 hr, moderate amounts of debris were observed in all crypts (Figs. 2, 4, and 6). Similar changes were noted following the dose of 20 mg/kg.

In order to ascertain the more prolonged cytotoxic effects...
of bleomycin, we examined the histopathology of the duodenal mucosa over a 3-day period in mice given a single 300-mg/kg injection of the drug and killed in pairs at different intervals. At 6 hr, moderate amounts of cellular debris were present in all crypts, and mitotic figures were rare. At 24 hr, both the size and numbers of crypts were less than normal. The crypt nuclei were irregularly enlarged and arranged and had less chromatin staining material, giving them a clear appearance. Mitoses were present in many crypts but were still less than that seen in controls. A small amount of cellular debris was present in some crypts. In contrast, the villi were normal in appearance at this time (Fig. 7). At 3 days, crypts appeared to be less than normal in number. Moderate numbers of inflammatory cells, chiefly mononuclear cells and some polymorphonuclear leukocytes, were present in the intercryptal stroma. The villi were sharply defined from the crypts because of a lesser basophilia. The villi were slightly irregular in size and shape and shorter than normal. The epithelial nuclei of the villi were likewise irregular in size and arrangement (Fig. 8).

DISCUSSION

In the relatively short period since its discovery by Umezawa et al. (19), bleomycin has been used extensively as a cancer chemotherapeutic agent. Its effectiveness in the treatment of squamous cell carcinoma (3) and some lymphomas (4) may be related to a preferential distribution of the drug to the skin and lymphatics, respectively (18).

Most investigations concerned with the mode of the cytotoxic activity of bleomycin have used either tumor cells in culture or experimental transplantable tumors as model systems. Although much valuable information has been obtained from such studies, the mechanisms underlying the antitumor properties of bleomycin remain obscure. In the present study, the cytotoxic effects of bleomycin on a normal proliferating tissue were examined in an effort better to understand the relationship between the biochemical and histopathological actions of the drug.

The rapidly dividing cells of the crypts of Lieberkühn are the proliferative compartment of the cell renewal system, which serves to replenish the physiological loss of cells from the villar epithelium of the small intestine (6). Numerous studies have shown that the crypt cells are sensitive to the damaging effects of many cancer chemotherapeutic agents (7, 10, 14), as well as to those produced by X-irradiation (21, 22). In the present study, it was observed that bleomycin also exerts a cytotoxic action in intestinal crypts. Following treatment with the agent, there is an inhibition of the incorporation of thymidine into intestinal DNA (Chart 1). Interestingly, both the degree of inhibition and its development with time resemble that which has been observed after the administration of X-irradiation (22) or chemotherapeutic agents like mitomycin C (14). Thus, even after a near-lethal dose of bleomycin, maximal inhibition of thymidine incorporation is not attained for at least 2 to 4 hr (Chart 1). These results are in sharp contrast to the marked inhibition which occurs in less than 1 hr after treatment with low, nonlethal doses of specific inhibitors of DNA synthesis, such as cytosine arabinoside or hydroxyurea (7, 10). With low doses of bleomycin of the order of those used clinically (i.e., 2 mg/kg), significant inhibition is not seen for at least 6 hr (Chart 1). The fact that necrotic cells appear in the crypts at 4 hr after the dose of 2 mg/kg (Fig. 5) argues against direct inhibition of DNA synthesis as being the primary mechanism of bleomycin cytotoxicity. These results are in accordance with those obtained by Nagatsu et al. (9), who investigated the in vivo effects of bleomycin on the VX-2 carcinoma in rabbits. With repeated injections of the dose of 1 mg/kg, it was found that the inhibition of tumor growth was related to the early and marked antimitotic action of the drug and not to an inhibition of DNA synthesis.

Similarly, inhibition of total RNA synthesis does not appear to be involved in the cytotoxic actions of bleomycin. Thus, even near-lethal doses of the drug failed to impair significantly the incorporation of cytidine into intestinal RNA at any of the times studied (Table 2). By contrast, thymidine incorporation into DNA was inhibited approximately 90% by the highest dose of bleomycin during this same period. These results are in accordance with those observed in tumor cells and Escherichia coli (20), in which a similar lack of effect of bleomycin on RNA synthesis was observed. Finally, the autoradiographic studies of Schultze and Maurer (13) have shown that, in the intestine, radioactivity from short pulses of tritiated cytidine is also primarily incorporated by the crypt epithelium cells.

The results obtained in the present study have demonstrated the profound antimitotic activity of the agent (Chart 2). Even the lowest dose used induced significant, although transient, inhibition of mitosis at a time when DNA synthesis was virtually unaffected; the inhibition of mitoses could not, therefore, be attributed to a prior effect of the drug on DNA synthesis. Moreover, with all of the dose levels used, maximal
inhibition of mitosis was consistently attained at 2 hr after the administration of bleomycin. The 2-hr interval is the maximal time expected for the complete disappearance of mitosis if cells in late S phase are blocked by bleomycin while those in G2 and M are able to proceed through the cell cycle without interruption, for the duration of G2 plus M is approximately 2 hr in mouse crypt cells (8). Finally, the fact that significant inhibition of mitosis was observed at 1 hr after either 20 or 200 mg/kg (Chart 2) indicates that at these higher doses bleomycin exerts a direct effect on cells in the G2 phase of the cell cycle. This finding is consistent with the recent results of Barranco and Humphrey (1), which showed that, at a concentration of 100 μg/ml, bleomycin blocked the progression of Chinese hamster ovary cells from G2 to M.

REFERENCES


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Fig. 1. Duodenum from a mouse 1 hr after bleomycin, 2 mg/kg. No abnormalities are present. The number of mitotic figures is within normal limits. H & E, × 310.

Fig. 2. Duodenum from a mouse 1 hr after bleomycin, 200 mg/kg. The number of mitotic figures in the crypts is markedly reduced. H & E, × 310.

Fig. 3. Duodenum from a mouse 2 hr after bleomycin, 2 mg/kg. The mucosa is normal but the number of mitotic figures is reduced. H & E, × 310.

Fig. 4. Duodenum from a mouse 2 hr after bleomycin, 200 mg/kg. Mitotic figures are scarce and cellular debris is present in the crypt epithelium. H & E, × 310.

Fig. 5. Duodenum from a mouse 4 hr after bleomycin, 2 mg/kg. The number of mitotic figures is within the normal range. Small amounts of cellular debris are present in the crypt epithelium. H & E, × 310.

Fig. 6. Duodenum from a mouse 4 hr after bleomycin, 200 mg/kg. Mitoses are scarce. Moderate amounts of cellular debris are present in the crypts. H & E, × 310.

Fig. 7. Duodenum from a mouse 24 hr after bleomycin, 300 mg/kg. Crypt epithelial nuclei are misshapen and poorly aligned. Mitotic figures are scarce. Small amounts of cellular debris are present. H & E, × 310.

Fig. 8. Duodenum from a mouse 3 days after bleomycin, 300 mg/kg. Crypts are enlarged, hyperbasophilic, and sharply defined from the paler staining villi. Increased numbers of inflammatory cells are present in the intercryptal regions. H & E, × 310.
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