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DAMAGE OF RAT LIVER DEOXYRIBONUCLEIC ACID BY BLEOMYCIN*

RAY COX, ABDULWAHID H. DAOUD and CHARLES C. IRVING

Veterans Administration Hospital, and Department of Biochemistry, University of Tennessee, Memphis, TN 38104, U.S.A.

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Abstract—Bleomycin has been shown by other investigators to react with DNA *in vitro* causing the release of free bases and strand scission. We have examined the possibility of damage to rat liver DNA *in vivo* by bleomycin. Thirty min after injection of bleomycin (20 mg/kg body wt), the DNA was shifted to the top of an alkaline sucrose gradient, suggesting damage *in vivo*. By 3 hr after injection of bleomycin, a normal gradient pattern was observed. Perfusion of the liver prior to squashing (preparation of the liver DNA for gradient analysis) abolished the effect of bleomycin on the DNA, indicating that bleomycin did not damage the DNA *in vivo*, but is present in the blood and reacts with DNA after squashing and/or lysing of the cells.

THE BLEOMYCINS, a group of glycopeptides, were first isolated from *Streptomyces verticillus* by Umezawa *et al.*[1] in 1966. Since that time, it has been widely tested as an antitumor agent.[2]

Studies *in vitro* have demonstrated that the reaction of bleomycin with DNA is activated by sulfhydryl compounds which enhance its reaction with DNA.[3,4] Bleomycin reacts specifically with DNA releasing free bases.[5] Muller *et al.*[3] reported that only thymine was removed from DNA by bleomycin, but Haidle *et al.*[6] showed that all four bases were removed. DNA treated with bleomycin has a lower T_m[7,8] and appears to have double and single strand breaks as measured on neutral and alkaline sucrose gradients.[4,9]

In the crypt cells of the small intestine,[10] cultured HeLa cells[11] and Ehrlich carcinoma,[11] bleomycin inhibits DNA synthesis but not RNA synthesis. Terasima *et al.*[12] treated L-cells in culture with bleomycin and demonstrated the induction of single and double strand breaks. After removal of bleomycin from the medium, single strand breaks were rejoined very rapidly (within 30 min). In contrast, methylmethanesulfonate-induced single strand breaks are repaired only after incubation of at least 5 hr.[13]

Due to an increasing clinical use of bleomycin and its apparent specificity and reactivity with DNA *in vitro* and in cultured cells, it became of interest to investigate the possibility of bleomycin reacting with DNA *in vivo*.

MATERIALS AND METHODS

Pregnant female rats were obtained from the Holtzman Co., Madison, Wis. Four days after birth, all female babies were discarded and the males were divided among

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the mothers at a ratio of 6–8 per mother. All male rats were then injected daily with 25 μCi thymidine-methyl-^{3}H, 40–60 Ci/m-mole (New England Nuclear Corp., Boston, Mass.) beginning at age 1 week and continuing 3–4 weeks or until 1 week after weaning. The animals were then used at approx. 150 g body wt.

Bleomycin (a gift from Bristol Laboratories, Syracuse, N.Y.) was dissolved in 0·9% NaCl (pH 4·7) and injected i.p. at varying dosages and time intervals. Preparation of DNA for gradient analysis was as previously described.[14] A suspension of nuclei was prepared by squashing the liver in about 2 ml cold 0·024 M EDTA–0·075 M NaCl buffer (pH 7·5). The nuclei were lysed by adding a small volume, 25–100 ml, to 0·3 ml lysing solution (0·30 M NaCl, 0·03 M EDTA, 0·1 M Tris–HCl buffer and 0·5% sodium dodecyl sulfate, pH 10). The lysed nuclei were carefully layered on top of a linear, 5–20%, alkaline sucrose gradient containing 0·9 M NaCl and 0·3 N NaOH (pH 12).

Under ether anesthesia, the liver was perfused through the portal vein *in situ*. About 250 ml of 0·024 M EDTA–0·075 M NaCl buffer (pH 7·5) was infused over a period of approximately 5 min under a hydrostatic pressure of 90 cm.

RESULTS AND DISCUSSION

In the figures presented in this paper, the data in each gradient profile represent one animal. However, each experiment was repeated three times with almost identical results. Examination of the liver by light microscopy at the doses and times used in this study gave no indication of cell damage.

The squashing procedure[14] used in preparing hepatic DNA for gradient analysis yields a suspension of nuclei and not whole intact liver cells. This preparation was previously referred to as a cellular suspension,[14] but examination by light microscopy revealed a preparation of nuclei with an occasional intact hepatocyte. When bleomycin was added to this nuclear preparation, the DNA was shifted to the top of the gradient as shown in Fig. 1. The effect of bleomycin was not removed by washing the nuclear preparation, indicating that bleomycin reacted with the DNA in the nuclear preparation as previously demonstrated with DNA *in vitro*.[3,4,6]

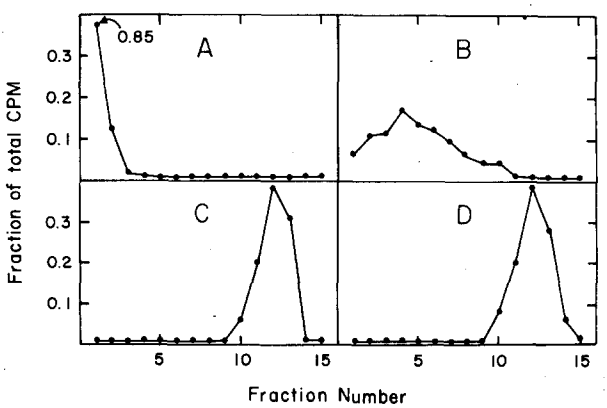

FIG. 1. Effect of bleomycin on the sedimentation of DNA from the nuclear preparation. Bleomycin was dissolved in water, added to the nuclear preparation, and incubated at 25° for 10 min. (A) Control; (B) 1 μg/ml; (C) 10 μg/ml; and (D) 10 μg/ml and washed two times. Total cpm were 320, 400, 685 and 598 respectively. See Ref. 14 for details of the alkaline sucrose gradients. Sedimentation was from right to left.

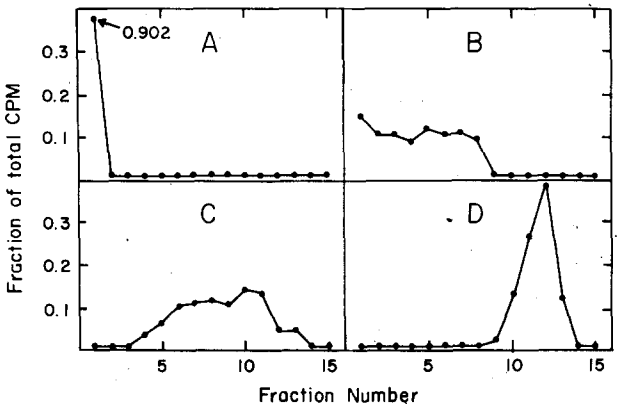

FIG. 2. Effect of bleomycin on the sedimentation of liver DNA—dose response. Bleomycin was given i.p. at varying dosages. The rats were sacrificed 30 min later, the liver was removed and squashed, and the nuclear preparation was analyzed on alkaline sucrose gradients (see Ref. 14). (A) Control; (B) 5 mg/kg body wt; (C) 10 mg/kg body wt; and (D) 20 mg/kg body wt. Total cpm were 1118, 1456, 1174 and 1652 respectively. Sedimentation was from right to left.

Administration of bleomycin i.p. produced a change in the sedimentation profile of liver DNA on an alkaline sucrose gradient, as shown in Figs. 2 and 3. This effect was dose dependent and observed within 30 min after the injection of bleomycin.

However, at 2 hr the gradient profile indicated that the damage was being repaired, and by 3 hr it had returned to a normal pattern. These data indicate that the damage induced by bleomycin was rapidly repaired when compared to the repair of damage induced by alkylating carcinogens.[15,16] A recent report by Rajalakshmi and Sarma[17] demonstrated that the liver has the ability for rapidly repairing DNA damage induced by camptothecin.

Similar results as in Figs. 2 and 3 were obtained with the lung and kidney of bleomycin-treated rats, but the question still remains: is bleomycin reacting with DNA

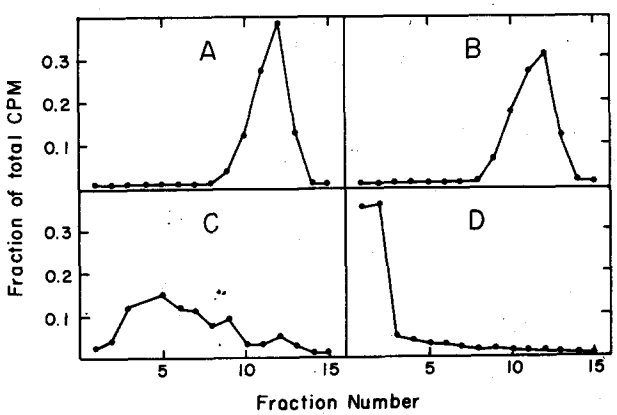

FIG. 3. Effect of bleomycin on the sedimentation of liver DNA—time curve. Bleomycin was given i.p. at 20 mg/kg body wt and the rats were sacrificed at varying times. See Ref. 14 for details of the gradient analysis. (A) 30 min; (B) 60 min; (C) 120 min; and (D) 180 min. Total cpm were 806, 1816, 656 and 650 respectively. Sedimentation was from right to left.

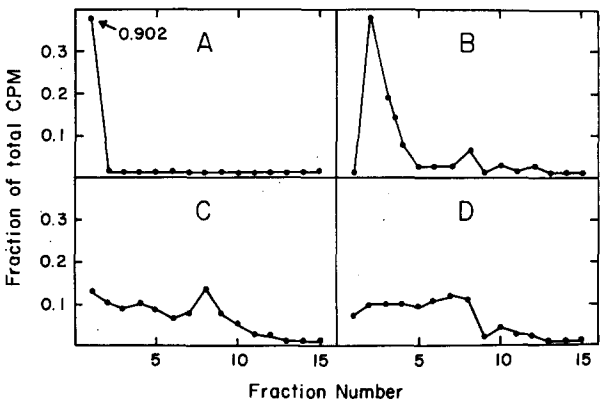

Fig. 4. Effect of bleomycin on the sedimentation of liver DNA after perfusion of the liver. Bleomycin was given i.p. at varying dosages. Thirty min later the liver was perfused with 250 ml of 24 mM EDTA–75 mM NaCl (pH 7·5) and removed for gradient analysis of the DNA (see Ref. 14 for details). (A) Control; (B) 20 mg/kg body wt; (C) 40 mg/kg body wt; and (D) 80 mg/kg body wt. Total cpm were 785, 287, 884 and 723 respectively. Sedimentation was from right to left.

in the nuclear preparation, on the gradient, or in the cell *in vivo*? Abelson and Penman[18] reported that camptothecin reacted with DNA on the gradient only in the presence of alkali. A repeat of the experiments shown in Figs. 1 and 2 using Abelson and Penman's technique of layering the lysed preparation over 1 ml of 1 M Tris (pH 7·0) gave essentially the same results as in Figs. 1 and 2.

Fujiwara and Kondo[19] suggested that bleomycin can hardly penetrate the HeLa S3 cell. This affords another explanation for the data in Figs. 2 and 3. Simply, when bleomycin is injected into a rat, the blood level of bleomycin is maintained for 1–2 hr at a level that will react with DNA in the nuclear preparation. Very little, if any, of the bleomycin would enter the liver cell. The data in Fig. 4 support this explanation. The effect of 20 mg/kg of bleomycin as shown in Fig. 4 was completely removed by perfusion of the liver just prior to squashing for the nuclear preparation. At the higher concentrations, the effect was not completely removed but drastically reduced. These data indicate that bleomycin was maintained in the blood for about 2 hr and that the shift of the DNA on the gradients in the non-perfused liver was dependent upon the blood levels of bleomycin. This is in agreement with the work of Pittillo *et al.*[20] They injected animals with a single intraperitoneal dose of 20 mg bleomycin/kg. The blood level of bleomycin reached a peak of 60 mg/ml within about 15 min and dropped to 10 μg/ml by 1 hr. Bleomycin was not detected in the liver, kidneys, lung, spleen or brain. We have shown the presence of bleomycin in the blood by taking blood from an animal 30 min after an i.p. injection of bleomycin (20 mg/kg) and squashing with a control liver. After gradient analysis, more breaks were present in the treated DNA, whereas blood taken at 3 hr had no effect on the gradient profile.

There is no doubt that bleomycin reacts with DNA *in vitro* and introduces breaks into the DNA molecule. But *in vivo* it may never enter the cell. These data raise questions regarding the mechanism of action of bleomycin. For example, does this glycopeptide act at the membrane level to inhibit DNA synthesis and growth of cancer cells? This type of action has been suggested for the hepatic chalone, a small molecu-

lar weight polypeptide which has been purified and shown to inhibit DNA synthesis.[21] It may be that bleomycin binds to the chalone receptor site. Another explanation is that bleomycin is preferentially taken up by the cancer cell, thus reacting with the cellular constituents. Chromosomal damage is induced in rapidly growing and dividing cells,[22,23] which indicate these cells are permeable to bleomycin. Since bleomycin does act preferentially during the cell cycle,[24] the resting hepatocyte may not be permeable to bleomycin.

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