SINGLE STRAND SCISSION AND REPAIR OF DNA IN BLEOMYCIN-SENSITIVE AND RESISTANT RAT ASCITES HEPATOMA CELLS

MICHIKO MIYAKI, SEIKO MOROHASHI and TETSUO ONO

Department of Biochemistry, Cancer Institute,
Japanese Foundation for Cancer Research,
Kami-Ikebukuro 1-37-1, Toshima-ku, Tokyo, Japan

(Received for publication February 12, 1973)

The bulk DNA in bleomycin-sensitive ascites hepatoma, AH66, was more susceptible to single strand scission by this antibiotic than that in the resistant AH66F cells. The break was repairable in the resistant cells, while it was incompletely repaired in the sensitive cells. Rejoining was inhibited by actinomycin D but not by cycloheximide. The sedimentation coefficient of bulk DNA was reduced with increasing lysis time of the cells in alkali-sodium dodecyl sulfate before sucrose gradient centrifugation, while that of damaged DNA in bleomycin-treated cells did not change further under the same conditions. This suggests induction of breakage by the antibiotic of an alkali-labile linkage in the chromosomal DNA.

Bleomycin A_2[1], an antitumor antibiotic, inhibits DNA synthesis in *Escherichia coli*, EHRLICH carcinoma, and HeLa cells[2], and mitosis[3,4] to a higher extent than protein and RNA syntheses. The antibiotic lowers the Tm of DNA[5] and causes strand-scission[6] which is repairable in L cells[7]. This antibiotic also inhibits the DNA-ligase of ascites hepatoma *in vitro*[8]. Although these observations revealed the action of bleomycin, the true mechanism of its antitumor activity is still uncertain. The present investigation was undertaken to compare DNA-strand scission and its repair in bleomycin-sensitive ascites hepatoma, AH66[9], with those in the resistant AH66F cells.

Methods

Labeling of tumor cells: Ascites hepatoma, AH66 or AH66F, cells were inoculated into the abdominal cavity of Donryu rats. On the third day after inoculation, 50 μCi of [^{3}H]thymidine (25 Ci/mmole, Radiochemical Centre) was intraperitoneally injected and ascites fluid was withdrawn 18 hours later. The cells were washed with physiological saline and suspended in modified EAGLE's minimum essential medium (MEM) (Nissui Seiyaku Co.) at 2×10^6 cells/ml and incubated at 37°C for 30 minutes.

Treatment of cells with bleomycin and reincubation: The cells in 1 ml of MEM were incubated at 37°C for 30 minutes with bleomycin A_2, copper free (Nippon Kayaku Co.) at various concentrations and washed three times with 10 ml of MEM at 0°C. To permit repair, the cells were reincubated in 1 ml of the medium without bleomycin at 37°C for 30 minutes.

Cell lysis and sucrose gradient centrifugation: The cell suspension containing $1\sim2\times10^5$ cells/0.5 ml was placed on top of 0.5 ml of a lysing solution (pH 12.5) consisting of 0.1 N NaOH, 2 % sodium dodecyl sulfate (SDS), and 10 mM EDTA which was layered on 16 ml of a 5~20 % (w/v) alkaline sucrose gradient containing NaOH, 0.1 M NaCl, 1 mM EDTA, and 0.01 % SDS (pH 12.5). The gradient was kept at 20~25°C for 20 minutes before centrifugation at 25,000 rpm for 60 minutes at 20°C in a Hitachi SW25.3 rotor. Twenty 0.85-ml fractions were collected and 0.1 ml of each fraction was spotted on a paper disc (Whatman 3MM, 2.5 cm) which was then washed three times with cold 5 % trichloroacetic acid and ethanol, dried; the radioactivity was measured in toluene

scintillator. The recovery of radioactive material was more than 80 %. Washing or not washing of the paper disc with trichloroacetic acid did not change the distribution of radioactivity. To isolate the double-stranded DNA, the cells were placed on top of a lysing solution (pH 7.0) consisting of 2 % SDS, 10 mM Tris-HCl, and 10 mM EDTA layered over 5~20 % sucrose gradient (pH 7.0) containing 0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, and 0.01 % SDS.

Results

Breakage and Repair of DNA in Bleomycin-Sensitive and Resistant Ascites Hepatoma

When AH66 or AH66F cells were kept for 20 minutes at 20°C over alkaline lysing solution and then centrifuged at 20°C for 60 minutes, the estimated sedimentation coefficient of single stranded DNA was about 380S from which the molecular weight could be calculated as 4.4×10^9 daltons (Fig. 1a, 1e). Incubation of the cells with bleomycin at 37°C for 30 minutes decreased the size of DNA and the extent of strand break was higher in sensitive AH66 than in resistant AH66F cells. About one-half of AH66 DNA damaged by 50 μg/ml bleomycin was repaired incompletely to 280S during reincubation for 30 minutes in bleomycin free medium (Fig. 1c) and the size of DNA damaged by 100 μg/ml of the antibiotic was not entirely repaired under these conditions (Fig. 1d). The broken DNA (280S) of AH66F treated with bleomycin even at 100~200 μg/ml could be repaired completely (Fig. 1h). The amount of double strand scission was extremely small as observed in the neutral sucrose gradient cen-

Fig. 1. Damaged and repaired DNA sedimented in alkaline sucrose gradient

After treatment with respective concentration of bleomycin and reincubation in a medium without bleomycin, the cells labeled with [³H]thymidine overnight were lysed at 20°C for 20 minutes on the top of alkali-SDS layered over 5-20% alkaline sucrose gradient and subsequently centrifuged at 25,000 rev/min. for 60 minutes

(a)~(d) AH66; (e)~(h) AH66F

Concentration of bleomycin (μg/ml): (a) and (e) 0; (b) and (f) 10; (c) and (g) 50; (d) and (h) 100

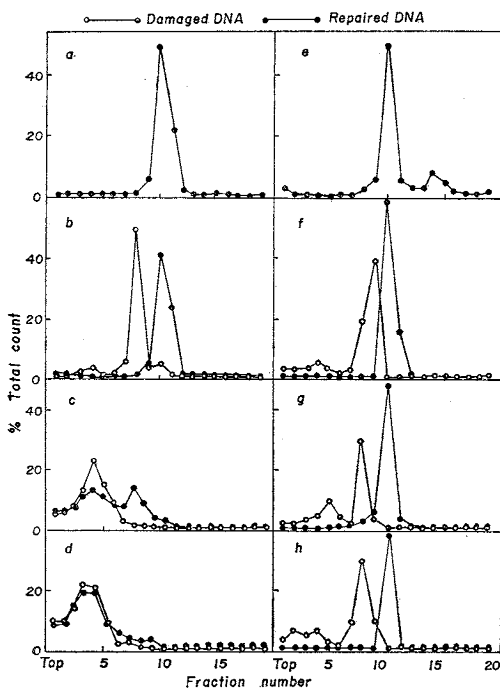
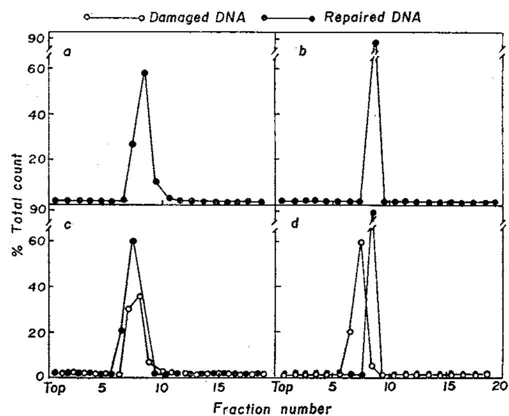


Fig. 2. Damaged and repaired DNA sedimented in neutral sucrose gradient

After treatment with bleomycin and reincubation in a medium without bleomycin, the labeled cells were placed on the top of neutral-SDS layered on 5~20% neutral sucrose gradient and subsequently centrifuged at 25,000 rev/min. for 120 minutes

(a) and (c) AH66; (b) and (d) AH66F

Concentration of bleomycin: (a) and (b) 0; (c) and (d) 100 μg/ml



trifugation (Fig. 2).

After extraction using phenol with minimum shearing, both DNA from the bleomycin-treated and untreated cells were sheared into the size of 20S–30S in alkaline sucrose and no significant difference in the size of single stranded DNA was observed.

Repair of the single strand scission in AH66F was inhibited by actinomycin-D at 5 μg/ml (Fig. 3a) which markedly decreased the incorporation of uridine to 6 % of the control and weakly inhibited DNA (to 58 %) and protein synthesis (to 45 %). The repair was not affected by 140 μg/ml of cycloheximide (Fig. 3b), which inhibited protein synthesis more strongly (to 2 % of the control) than DNA (to 44 %) or RNA synthesis (to 70 %). No decrease in sedimentation coefficient of bulk DNA was observed when AH66F cells were treated with 5 μg/ml of actinomycin-D.

Fig. 3. Repair of DNA in the presence of actinomycin-D or cycloheximide

Labeled AH66F cells were treated with bleomycin (100 μg/ml) and reincubated in the presence of actinomycin-D (5 μg/ml) (a) or cycloheximide (140 μg/ml) (b). The cells were washed three times with MEM and lysed at 20°C for 20 minutes on the top of alkali-SDS and DNA was sedimented in 5~20% alkaline sucrose gradient.

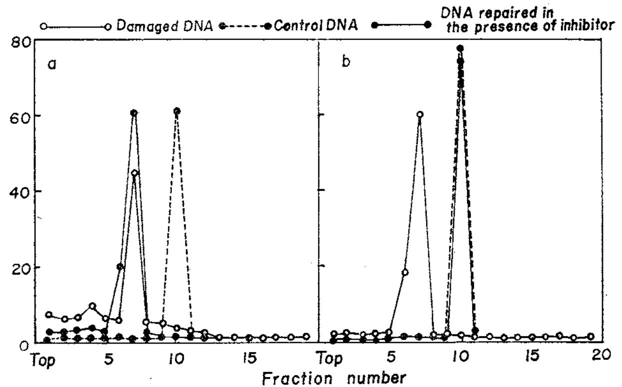
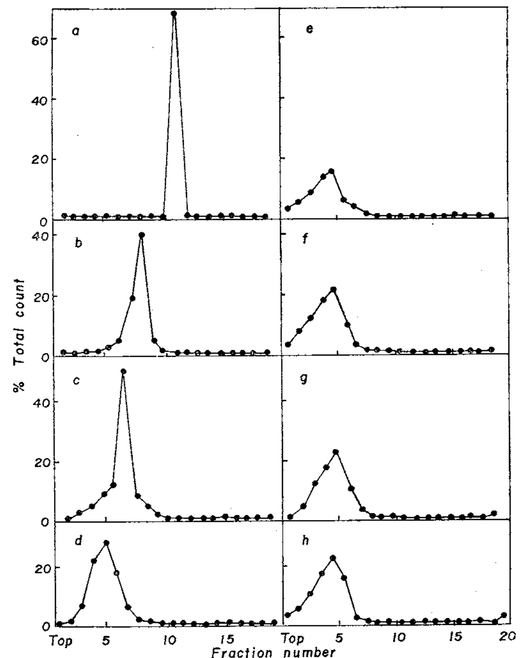


Fig. 4. Effect of lysis time of cells on the sedimentation profiles of DNA from AH66 cells and from those treated with bleomycin (100 μg/ml)

The labeled cells were lysed at 20°C for indicated time on the top of alkali-SDS layered over alkaline sucrose gradient and subsequently centrifuged at 25,000 rev/min. at 20°C for 60 minutes.

(a)~(d) Control AH66; (e)~(h) bleomycin-treated AH66.

Lysis time: (a) and (e) 20 minutes; (b) and (f) 40 minutes; (c) and (g) 60 minutes; (d) and (h) 120 minutes

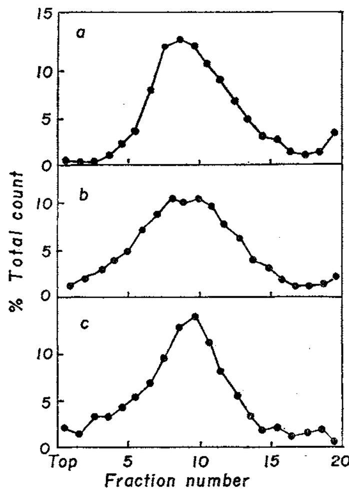


Degradation of Bulk DNA by Alkali-sodium Dodecyl Sulfate

By allowing AH66 cells for a long period at room temperature with lysing solution (alkali-SDS) before centrifugation, sedimentation coefficient of bulk DNA was decreased depending on the lysis period as shown in Fig. 4. The S value was changed from 380S (4.4×10^9 daltons) to 160S (5×10^8 d) via 280S (2×10^9 d) and 220S (1×10^9 d) during 120-minute lysis (Fig. 4a~4d). In contrast to such changes in the size of bulk DNA, the sedimentation profile of DNA in bleomycin-treated cells was unchanged during the same lysis time (Fig. 4e~4h). After storage of cells for 48 hours at 20°C in lysing solution, DNA in both untreated and bleomycin-treated cells was converted to 66S (5.5×10^7 d) and there was little difference in size between control and damaged DNA (Fig. 5). No DNA molecules smaller than 66S were observed after longer

Fig. 5. Sedimentation pattern of damaged and rapaired DNA in alkaline sucrose after 48-hour lysis

Labeled AH66 cells were incubated with bleomycin (100 μg/ml), reincubated without bleomycin, lysed over alkali-SDS at 20°C for 48 hours, and centrifuged at 25,000 rev/min. for 5 hours. (a) Control DNA; (b) bleomycin-treated DNA; (c) repaired DNA



lysis time. λ-Phage DNA was not degraded under the same conditions. On the other hand, the sedimentation profile in the neutral sucrose gradient did not change during standing of the cells at room temperature for 20 hours with 2 % SDS-10 mM Tris-HCl-10 mM EDTA (pH 7.0). Similar degradation of DNA was observed with AH66F cells.

Discussion

Bleomycin-sensitive AH66 cells were proved to be more susceptible to single strand scission of DNA by bleomycin than resistant AH66F cells (Fig. 1), although the factors which contribute to the difference in susceptibility are still not clear. The sensitivity possibly arises from the reduced inactivation system for bleomycin or high permeability of cell membrane of the sensitive cells. Low activity of the bleomycin-inactivating enzyme in squamous cell carcinoma has been described.[10]

The single strand bulk DNA in AH66 and AH66F cells had a sedimentation coefficient of 380S corresponding to 4.4×10^9 daltons evaluated by STUDIER's equation[11] but during a 120 minute lysis time, this peak shifted to 160S (5×10^8 daltons) and the intermediate sizes of 280S (2×10^9 d) and 220S (1×10^9 d) appeared depending on the lysis period in 0.1 N NaOH-2 % SDS-10 mM EDTA (Fig. 4). Such a change in sedimentation profile would suggest an alkali-labile linkage in chromosomal DNA. A similar size of subunit was also observed in Chinese hamster cells[12] employing 0.5 M NaOH-0.1 M EDTA as a lysing solution. The damaged DNA in AH66 or AH66F cells treated with bleomycin exhibited an S value corresponding to such intermediate; DNA in AH66F treated with 50~200 μg/ml of bleomycin had 280S and that in AH66 treated with 100 μg/ml was 160S (Fig. 1). The damaged 160S peak did not shift to a smaller peak during 120 minutes of lysis time (Fig. 4) and this fact indicated decomposition of bulk DNA into 160S subunit via 280S by bleomycin. The process producing 280S, 220S and 160S DNA seems to be different from random breakage of DNA strand since the change occurs within a short lytic period. Presence of an alkali-labile bond of chromosomal DNA has been suggested[12,13] but nature of this material has not been described yet. Inhibition of repair of DNA in AH66F (Fig. 3a) by actinomycin-D suggested that RNA synthesis was a prerequisite for the repair of breakage in such region.

The 160S DNA was further decomposed to 66S during 48 hours storage of the cells with alkali-SDS (Fig. 5). This process may involve random degradation of DNA since several reports have indicated that single stranded DNA may be hydrolysed by alkali in the presence of CsCl.[14,15]

References

1) UMEZAWA, H.; K. MAEDA, T. TAKEUCHI & Y. OKAMI: New antibiotics, bleomycin A and B. J. Antibiotics, Ser. A 19 : 200~209, 1966
2) KUNIMOTO, T.; M. MORI & H. UMEZAWA: Modes of action of phleomycin, bleomycin and formycin on HeLa S3 cells in synchronized culture. J. Antibiotics 20 : 277~281, 1967
3) SUZUKI, H.; K. NAGAI, H. YAMAKI, N. TANAKA & H. UMEZAWA: Mechanism of action of bleomycin, Studies with the growing culture of bacterial and tumor cells. J. Antibiotics 21 : 379~386, 1968
4) BARRANCO, S. C. & R. M. HUMPHREY: The effect of bleomycin on survival and cell progression in Chinese hamster cells *in vitro*. Cancer Res. 31 : 1218~1223, 1971
5) NAGAI, K.; H. YAMAKI, H. SUZUKI, N. TANAKA & H. UMEZAWA: The combined effects of bleomycin

and sulfhydryl compounds on the thermal denaturation of DNA. Biochim. Biophys. Acta 179 : 165~171, 1969

6) SUZUKI, H.; K. NAGAI, H. YAMAKI, N. TANAKA & H. UMEZAMA: On the mechanism of action of bleomycin. Scission of DNA strands *in vitro* and *in vivo*. J. Antibiotics 22 : 446~448, 1969

7) TERASIMA, T.; M. YASUKAWA & H. UMEZAWA: Breaks and rejoining of DNA in cultured mammalian cell treated with bleomycin. Gann 61 : 513~516, 1970

8) MIYAKI, M.; T. ONO & H. UMEZAWA: Inhibition of ligase reaction by bleomycin. J. Antibiotics 24 : 587~592, 1971

9) ICHIKAWA, H. & H. SATOH: Effect of bleomycin on ascites hepatoma in rat. Progr. Med. (Japanese) 69 : 669~672, 1969

10) UMEZAWA, H.; T. TAKEUCHI, S. HORI, T. SAWA, M. ISHIZUKA, T. ICHIKAWA & T. KOMAI: Studies on the mechanism of antitumor effect of bleomycin on squamous cells carcinoma. J. Antibiotics 25 : 409~420, 1972

11) STUDIER, F. W.: Sedimentation studies on the size and shape of DNA. J. Mol. Biol. 11 : 373~390, 1965

12) LETT, J. T.; E. S. KLUCIS & C. SUN: On the size of the DNA in the mammalian chromosome. Biophys. J. 10 : 277~292, 1970

13) KLKIND, M. B.: Sedimentation of DNA released from Chinese hamster cells. Biophys. J. 11 : 502~520, 1971

14) VINOGRAD, J.; J. MORRIS, N. DAVIDSON & W. F. DOVE, Jr.: The buoyant behavior of viral and bacterial DNA in alkaline CsCl. Proc. Natl. Acad. Sci. U. S. 49 : 12~17, 1963

15) TOMIZAWA, J. & N. ANRAKU: Molecular mechanisms of genetic recombination in bacteriophage. J. Mol. Biol. 11 : 509~527, 1965