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Repair of Bleomycin-Damaged DNA by Human Fibroblasts

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The ability of human fibroblasts to repair bleomycin-damaged DNA was examined in vivo. Repair of the specific lesions caused by bleomycin (BLM) was investigated in normal cell strains as well as those isolated from patients with apparent DNA repair defects. The diseases ataxia telangiectasia (AT), Bloom syndrome (BS), Cockayne syndrome (CS), Fanconi anemia (FA), and xeroderma pigmentosum (XP) were those selected for study. The method used for studying the repair of DNA after BLM exposure was alkaline sucrose gradient centrifugation. After exposure to BLM, a fall in the molecular weight of DNA was observed, and after drug removal the DNA reformed rapidly to high molecular weight. The fall in molecular weight upon exposure to BLM was observed in all cells examined with the exception of some XP strains. Prelabeled cells from some XP complementation groups were found to have a higher percentage of low molecular weight DNA on alkaline gradients than did normal cells. This prelabeled low molecular weight DNA disappeared upon exposure to BLM.

Key words: bleomycin, DNA repair, human DNA repair defects

Bleomycin (BLM) is a glycopeptide antibiotic [1] currently used in cancer chemotherapy. The main target of the drug is DNA. The drug produces strand scission (both single- and double-stranded breaks [2]) as well as alkaline-sensitive (apurinic) sites [3, 4] in isolated DNA. In vivo, a fall in molecular weight of DNA following BLM treatment has been demonstrated on alkaline sucrose gradients [5, 6].

The normal excision repair response of cells to DNA damage involves incision of the DNA near the site of damage. The damaged site is then excised and the gap filled in by repair synthesis. Finally, the new section of DNA is ligated to the parental DNA. The ability of human cells to repair the specific lesions caused by bleomycin (BLM) was investigated in fibroblast strains isolated from patients with diseases caused by apparent DNA repair defects. Since the types of damage caused by BLM are well-characterized, the ability of the putative DNA repair-defective cells to repair these specific types of damage could be examined.

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The diseases in question, ataxia telangiectasia (AT), Bloom syndrome (BS), Fanconi anemia (FA), Cockayne syndrome (CS), and xeroderma pigmentosum (XP), are characterized by abnormal DNA repair or chromosome structure [7–10]. With the exception of BS, evidence of defective repair of damaged DNA has been observed. With the exception of CS, these diseases show evidence of chromosomal instability either naturally or when challenged with a DNA-damaging agent [7]. In addition, there is increased incidence of malignancies in all of these diseases with the exception of CS.

The repair of BLM-damaged DNA has been examined in fibroblasts from normal individuals and patients with these diseases. We find evidence for abnormal response to BLM treatment in some XP complementation groups.

MATERIALS AND METHODS

Cell Culture

AT, FA, BS, CS, and XP fibroblast strains were obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey (designations—GM), and control fibroblasts (designations—initials) were put into culture in this laboratory. Cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum with the exception of XP strains. XP strains were grown in DMEM with 20% fetal calf serum. All cell strains were grown without antibiotics and were tested monthly for mycoplasma contamination by the method of Hayflick [11] as well as that of DelGuidice and Hopps [12], which utilizes the fluorescent DNA stain Hoechst 33258. In typical experiments, 60 mm culture plates were seeded with 1.5×10^5 cells from confluent monolayer cultures. Cells were grown to 75% confluence, at which time the experiments were performed.

DNA Labeling

The DNA of the cell strains was labeled with (^{3}H)-TdR (obtained from Amersham) in DMEM by incubation for 1 h with 125 μCi (^{3}H)-TdR (15 Ci/mM). The radioactive medium was removed, plates were washed with DMEM, and the isotope was chased with 10^{-5} M cold TdR in warm DMEM for 1 h.

Drug Treatment

The labeled cells were exposed to bleomycin at concentrations ranging from 5 μg/ml to 500 μg/ml for 30 min unless otherwise specified. BLM (Blenoxane, Bristol Laboratories) was dissolved in phosphate-buffered saline to give a stock solution of 5 mg/ml. In the incision experiments, ice-cold stopping buffer (phosphate-buffered saline, 0.02 M sodium azide, 0.01 M sodium pyrophosphate) was added at the end of the drug treatment. Cells were scraped with a Teflon policeman, harvested, and washed in a Beckman microfuge. The cell pellets were resuspended in 200 μl of lysing buffer (0.5 M NaCl, 1% Sarkosyl, 4 mM EDTA, 0.01 M sodium pyrophosphate, and 0.5 M NaOH) and incubated overnight at 4°C. In reformation experiments, the drug was removed and the plate washed with fresh warm medium. Incubation was continued in fresh medium for the amount of time indicated, then harvested by the procedure described above.

Alkaline Sucrose Gradient Centrifugation

Five to twenty percent alkaline sucrose gradients were prepared (0.3 M NaOH, 0.7 NaCl, 0.1 mM EDTA, and 50 mM Tris-Cl (pH 8.0) on a 70% sucrose cushion. Cell lysates were layered onto gradients and spun 9 h at 26K, 20°C. Gradients were fractionated by pump from the bottom. Fractions were precipitated with 10% trichloroacetic acid in the cold, collected on Whatman GF/C filters, washed with 0.01 M HCl, dried, and radioactivity was counted in a scintillation counter.

RESULTS

The response of human fibroblasts to bleomycin damage was characterized by a fall in the molecular weight of DNA on alkaline sucrose gradients. After prelabeling cells with (^{3}H)-thymidine for 1 h and chasing with cold thymidine, the (^{3}H)-thymidine sedimented in the high molecular weight peak (control, Fig. 1). DNA sedimenting in this peak was greater than 53S as determined by marker ØX174 DNA. If prelabeled normal cells were then exposed to bleomycin (500 μg/ml) for 30 minutes, greater than 50% of the labeled isotope sedimented with DNA molecules less than 16S in molecular weight (0 time, Fig. 1). DNA molecules less than 16S in molecular weight were found in the top half of the gradient. An incubation after drug removal allowed the very rapid repair of BLM-damaged molecules, with greater than 50% of the damaged molecules moving back into the high molecular weight DNA peak within 15 min in all strains listed in Table I (2′ reformation, Fig. 1). The repair was more rapid in the case of the BS strain, as shown in Figure 1. The procedure used for harvesting cells outlined in the Methods section was developed to "freeze" the cellular repair processes so that a kinetic study would be possible. A list of cell strains studied that demonstrated normal BLM repair kinetics is shown in Table I.

The representative repair response demonstrated in Figure 1 constitutes evidence for the existence of an efficient DNA repair process in the human

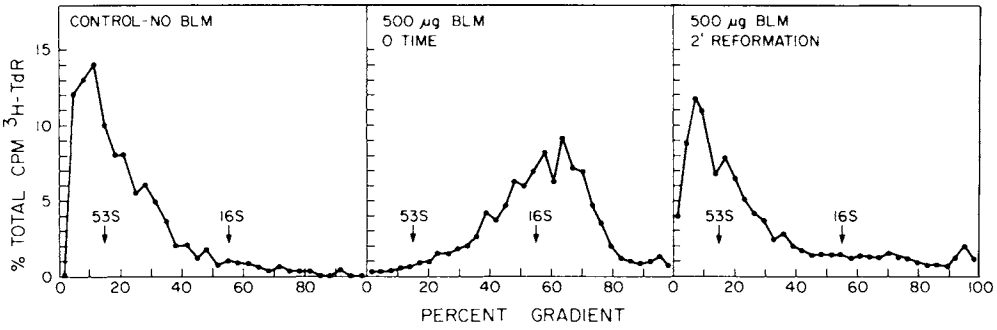

Fig. 1. Repair of bleomycin-damaged DNA. The control panel shows that the prelabeled DNA sedimented in a high molecular weight peak prior to BLM treatment. The 0 time panel shows the profile obtained upon harvesting cells at the end of BLM treatment. The profile obtained from cells incubated in the absence of BLM after drug treatment is shown in the third panel.

TABLE I. Human Cell Strains Examined

Disease	Cell strain	Repair
Normal	GM	N[a]
	CC	N
Bloom syndrome	GM2548	N
Fanconi anemia	GM1309	N
Ataxia telangiectasia	GM1588	N
	CV	
Cockayne syndrome	GM1098	N

[a]Kinetics of repair of BLM-damaged DNA is comparable to that observed in normal cells.

cells studied. The fall in molecular weight of DNA after BLM treatment observed on the alkaline gradients may be due to one of several mechanisms. Breaks detected on alkaline gradients could be due to direct cleavage of the DNA by BLM, scissons at apurinic sites by the alkaline treatment, or enzymatic cleavage at BLM-damaged sites. The removal of BLM-damaged sites or incisions, as shown by reformation of high molecular weight DNA in the absence of the drug, is evidence that the cell strains studied possess the molecular processes required to repair the damage inflicted by BLM.

Cell strains isolated from patients with the diseases mentioned were subjected to the experimental regimen described in Figure 1. Table II summarizes the normal response observed in most of the strains examined. The efficiency of repair of BLM-damaged DNA in human fibroblasts is demonstrated by the data shown for the GM (normal) strain and the BS strain. An incubation of 15 min after drug removal allowed for reformation of more than 50% of the BLM-damaged DNA by the cellular repair processes.

The response of XP cells to BLM did not follow the pattern observed in the other strains examined. Results obtained with XP-complementation group A and XP-complementation group E cells are shown in Table II. One obvious difference between the response of these cells to BLM and that of the cells showing the normal response is observed in the zero time column; the dramatic fall in the molecular weight of DNA seen in the GM and BS line was not observed in the two XP lines examined. After the BLM treatment, only 7.8% of the labeled DNA was above 53S in molecular weight in the normal strain (GM), whereas over 50% of the DNA was still in the high molecular weight region in the XP-A strain. XP-E cells showed a greater response than did the XP-A cells, but the XP-E response was still less than one-half that of the normal response. The failure of BLM damage to cause a dramatic fall in molecular weight in these XP cells (A and E) suggests a defect in enzymatic incision.

The total amount of damage demonstrated in XP-E cells was much less than in normal cells, and the reformation of small molecules to higher molecular weight was slower than normal. The amount of (^{3}H)-TdR in low molecular weight DNA (less than 16S) was unchanged from zero time throughout a 5-min incubation after drug removal. The low molecular weight DNA molecules seen at the end of the BLM incubation did not disappear during the reformation experiment—that is, during incubation in the absence of the drug.

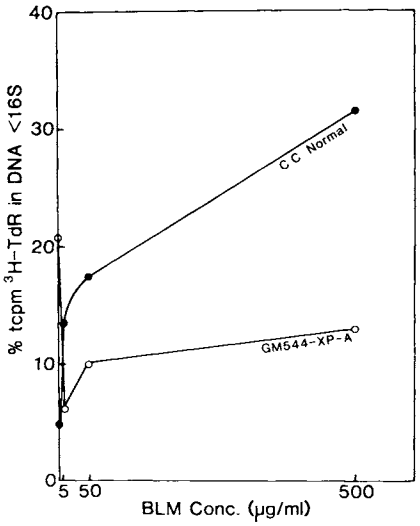

Fig. 2. In vivo response of human fibroblasts to BLM treatment. Cells (5×10^5) were exposed to the indicated amount of BLM for 30 min. The methods for stopping the drug treatment and subsequent analysis are described in Materials and Methods. Cpm from ^{3}H-labeled DNA less than 16S in molecular weight were summed, and the percentage of the total cpm in the gradient was calculated. The control gradients (CC) contained around 10,000 cpm, and the XP gradients contained an averaged of 5,000 cpm of (^{3}H)-TdR.

TABLE II. Reformation of BLM-Damaged DNA

	% cpm (^{3}H)-TdR in DNA < 53S* Reformation time (min)			
Fibroblast strain	0[a]	2	5	15
GM (normal)	7.8	37.4	—	51.32
GM2548 (BS)	4.11	55.1	63.4	56.1
GM544 (XP-A)	51.81	61.8	69.6	—
GM2415 (XP-E)	20.3	47.7	—	47.6

*Molecular weight was determined relative to sedimentation of labeled preparations of ØX174 DNA, containing both double-stranded supercoils and nicked circular forms. Cpm from radioactive material sedimenting with the 53S marker were summed, and this percentage of the total cpm in the gradient was calculated. Each gradient contained approximately 10,000 cpm of (^{3}H)-TdR.

[a] 0 Time — 5×10^5 cells were harvested after 30 min of BLM treatment. Reformation time is the time of incubation after drug removal.

Dose response to BLM was then examined in the normal and XP cells. Cells were prelabeled in the manner outlined, and DNA from cells with and without exposure to BLM was examined on alkaline sucrose gradients. The results are shown in Figure 2. This method of quantitation was utilized by Anderson and DePamphilis in studies on Okazaki fragment metabolism [13]. Before BLM exposure, in normal cells less than 5% of the labeled DNA was in DNA less than 16S in molecular weight. After incubation for a fixed time with increasing amounts of BLM, the amount of low molecular weight DNA increased. However, as is shown in Figure 2, this response was not observed in XP-A cells. First, the amount of low molecular weight DNA observed on alkaline gradients before BLM exposure was four times that observed in normal cells. Furthermore, when exposed to BLM the percentage of labeled low molecular weight DNA dropped and remained low over the dose range examined.

The observation that XP-A cells carry an increased amount of low molecular weight DNA or alkaline-sensitive sites when compared to normal cells must reflect abnormal DNA metabolism in the absence of the drug. After exposure to BLM, the low molecular weight species of DNA molecules decreased and remained low even at the high dose. This is evidence that enzymatic incision does not occur in response to BLM damage in XP-A cells.

DISCUSSION

The results obtained in this study allow for the following conclusions. A pathway exists in human fibroblasts for the repair of BLM-damaged DNA. This repair process is efficient in terms of repair of the types of damage that would cause a fall in molecular weight on alkaline sucrose gradients (alkaline-sensitive sites and strand breaks). In the normal response this repair process measured by reformation to DNA molecules greater than 53S in molecular weight is more than 50% complete following a 15-min incubation in the absence of the drug.

The decrease in molecular weight of DNA observed after BLM exposure in the normal cellular response is not observed in some XP strains examined. Thus, our results support the concept that biochemical differences underlie the XP complementation groups. The group most affected by BLM treatment is XP-complementation group A. Cells from this complementation group have been shown to be the most sensitive of the XP complementation groups to the lethal effects of ultraviolet light [14]. Also, unscheduled DNA synthesis (UDS) following irradiation with ultraviolet light, is lowest in group A [15]. In addition, there has been a report that apurinic endonuclease levels are low in this group [16]. The reasons for this apparent correlation of the abnormal response of XP-group A cells to BLM and the deficiencies previously demonstrated are unclear. However, it would appear that group A cells have deficiencies in repair of damaged DNA that may be more extensive than simple failure to make incisions at thymine dimers.

It is important to remember that the XP cells examined in this study carry a large number of DNA strand breaks and/or alkaline-labile sites without exposure to DNA-damaging agents. The altered response of XP cells to BLM is

perhaps directly related to the abnormal condition of DNA in XP cells. The disappearance of the low molecular weight material seen on alkaline gradients upon exposure to BLM must reflect either nucleolytic digestion of the low molecular weight material (this appears not to be the case based on radioactivity recovered from gradients) or repair or replicative activities involving the low molecular weight DNA species observed in undamaged prelabeled DNA. Furthermore, in XP-A cells, BLM does not *appear* to cause the damage observed in normal cells. In other words, there must be a failure to incise BLM-treated DNA in XP-A cells. Whether this is the case or that the damage is masked by some abnormal replication or repair process is unknown at the present time.

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