Breakage of a DNA-Protein Complex Induced by Bleomycin and Their Repair in Cultured Mouse Fibroblasts

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SUMMARY

The effect of bleomycin on DNA strand breakage and its repair in cultured mouse fibroblast strain L-P3 (a substrain of L929) was studied. Treatment of L-P3 cells with bleomycin caused single-strand breaks of DNA as revealed by alkaline sucrose gradient centrifugation. The single-strand breaks of DNA were rejoined within 3 hr when the treated cells were incubated at 37° in a medium free of the antibiotic ("recovery incubation"). Analysis by neutral sucrose gradient centrifugation indicated that the treatment of cells with bleomycin brought about a considerable decrease in the sedimentation coefficient of DNA ("double-strand breaks"). The double-strand breaks of DNA were also rejoined on recovery incubation for 24 hr. The use of Pronase-sucrose gradient, including Pronase in the gradient, suggested that the Pronase-sensitive linkages in DNA previously postulated by Andoh and Ide might be the sites preferentially cleaved by low concentrations of bleomycin and linked on recovery incubation.

INTRODUCTION

Biological and biochemical studies on the action of an antitumor antibiotic bleomycin have been carried out extensively (5–7, 9, 12, 14, 19, 21–24, 26, 27, 29–32, 34). Bleomycin A₂ exhibits a marked inhibition of DNA synthesis and a weaker inhibition of protein synthesis in Escherichia coli and Ehrlich carcinoma cells (27). Bleomycin in vitro caused a decrease in melting temperature of DNA (Tm) in the presence of sulfhydryl compounds such as 2-mercaptoethanol, glutathione, or dithiothreitol (23). This decrease in Tm was accompanied by binding of the antibiotic to DNA (26) and single-strand scissions of DNA (21, 26). Presumably due to these actions of bleomycin on DNA, it caused stimulation of DNase and DNA polymerase and inhibition of ligase reaction (34). Terashima et al. (30) also observed strand breaks of DNA in cultured mouse cells treated with bleomycin.

Recent works from our laboratory have shown that DNA of cultured mouse cells, as analyzed by neutral sucrose gradient centrifugation developed by Terashima and Tsuboi (28), contains certain minor proteins complexed with DNA in such a fashion that proteolysis results in a considerable decrease in sedimentation coefficient of the DNA (1–3). Furthermore, a chemical carcinogen, 4-nitroquinoline 1-oxide, caused scissions of these proteins when applied to cultured cells (13).

In this study, we investigated the effect of bleomycin on scissions of DNA strands and of the linking minor proteins. Results obtained strongly suggested that bleomycin preferentially attacked the putative peptide linkages in the DNA preparation and that the breaks could be rejoined on recovery incubation without the causative agent.

MATERIALS AND METHODS

Materials. Pronase E (specific activity, $1.17 \times 10^6$ tyrosine units/g) was purchased from Kaken Kagaku Co. Ltd., Tokyo, Japan. Thymidine-methyl-³H (specific activity, 14 Ci/mmole; Daiichi Kagaku Co. Ltd., Tokyo, Japan) was used to label cellular DNA. Bleomycin, a mixture of closely related structural analogs, of which the predominating one was Component A₂, was a gift from Dr. T. Takeuchi of the Institute of Microbial Chemistry, Tokyo.

Cell Culture. The cell strain used was L-P3 (15), a substrain of L-929 mouse fibroblasts. The methods used for cultivation of L-P3 cells were described previously (5). Cells were cultured in a protein- and lipid-free synthetic medium (16). DNA of the cells was labeled with ³H-labeled thymidine (0.2 μCi/ml) for 24 to 36 hr. Cell growth was estimated by counting cells according to a simplified replicate tissue culture method (17).

Sucrose Density Gradient Centrifugation. The methods used were essentially the same as described previously (4). In brief, for analysis of denatured DNA about $1 \times 10^6$ cells labeled with thymidine-³H and suspended in 0.1 ml phosphate-buffered saline (0.14 M NaCl-0.03 M KCl in 0.01 M phosphate, pH 7.4) were lysed by mixing with 0.2 ml of 1 N NaOH layered on top of an alkaline sucrose gradient, which was 4.7 ml of a linear 5 to 20% sucrose gradient containing 0.3 M NaOH, 0.7 M NaCl, 0.01 M EDTA, 0.01 M Tris and, as a cushion, 0.2 ml of an 80% sucrose solution under the gradient and allowed to stand at 0–2° for 20 min. The tubes were centrifuged in an SW50L rotor of a Beckman ultracentrifuge at 25,000 rpm for 50 min at 0°. For analysis of native DNA the same number of cells were lysed by mixing with 0.2 ml of a 2% sodium dodecyl sulfate solution over a neutral sucrose gradient, which was 4.7 ml of a linear 5 to 20% sucrose gradient containing 0.01 M...
Tris-HCl buffer at pH 7.4, 0.1 M NaCl and 0.01 M EDTA and, as a cushion, 0.2 ml of an 80\% sucrose solution underneath the gradient. The tubes were incubated at 37° for 20 min and then chilled to 10° and centrifuged at 25,000 rpm for 50 min at 10°. To treat DNA with Pronase we preincubated the enzyme solution at 37° for 2 hr and added it to the sodium dodecyl sulfate layer at a concentration of 1.5 mg/ml and to the gradient at 0.5 mg/ml. The cell suspension was mixed with 2\% sodium dodecyl sulfate and Pronase and processed as described above.

RESULTS

Effects of Bleomycin on Cell Growth. The effect of bleomycin treatment on the multiplication of L-P3 cells was examined at different concentrations of bleomycin. Bleomycin was added to culture medium 2 days after cell seeding. Thirty min after the addition, the antibiotic was removed by replacing with fresh medium. As shown in Chart 1, progressively greater extents of growth inhibition were observed with increasingly higher concentrations of the antibiotic. At 250 \(\mu\)g/ml, the number of cells seemed to be sustained on an almost constant level during 3 days of recovery incubation, whereas at 750 \(\mu\)g/ml the number decreased by 15\%. Strand scissions of DNA of the cells treated with these concentrations of bleomycin were studied.

Single-Strand Scissions of DNA in Bleomycin-treated Cells and Their Repair. In the present experiments, the cells were treated with bleomycin after formation of confluent cell sheets. This was done because it was known that the sensitivity of the cells to bleomycin varied considerably depending on the phase of growth; that is, the sensitivity decreased in parallel with the increase in cell concentration. Chart 2 shows the sedimentation profiles in alkaline sucrose gradients of DNA from L-P3 cells immediately after the 30-min treatment with various concentrations of bleomycin. The figures clearly indicate that bleomycin induced single-strand scissions in DNA of the cells and that the extent of breaks increased as the concentration of bleomycin was raised. DNA of the cells treated with 50-\(\mu\)g/ml or higher concentrations of bleomycin consisted of a heterogeneous population of fragmented DNA with a maximum \(s_{20,w}\) of about 100 S trailing toward the top of the gradients. In order to see whether the single-strand scissions were repairable, cells were treated with various concentrations of bleomycin and then transferred to medium free of bleomycin and incubated at 37° (recovery incubation). Chart 2 shows that on recovery incubation of the cells treated with bleomycin, 25 \(\mu\)g/ml, the single-strand breaks of DNA were rejoined completely after 3 hr; whereas at 50-\(\mu\)g/ml or higher concentrations, the breaks were not completely rejoined. There was some fraction of DNA remaining unrepaired. Whether this was due to heterogeneity of cell population or heterogeneity of DNA in a single cell remains to be determined, although the former possibility seems to be more likely. The shift of the majority of radioactive DNA toward the bottom of the gradient, however, could be explained as indicating real rejoined of DNA fragments, since radioactivities of cold trichloroacetic acid-precipitable fractions of the cells during the recovery incubation did not change appreciably. After a longer recovery incubation, i.e., 24 hr, breaks of DNA in the cells treated with 50-\(\mu\)g/ml or higher concentrations of bleomycin were completely rejoined (Chart 2, open triangles). These results indicate that L-P3 cells possess an enzymatic system for repair of single-strand breaks caused by bleomycin, in accord with results reported by Terashima et al. (30) with L5 mouse fibroblasts.

Double-Strand Scissions of DNA in Bleomycin-treated Cells and Their Repair. We examined whether bleomycin induced double-strand breaks in the DNA of L-P3 cells. The results shown in Chart 3 indicated that bleomycin induced double-strand breaks. The effect of varying concentrations of bleomycin on the sedimentation profile of DNA of the treated cells seemed to be complex. The sedimentation velocity of DNA of the treated cells gradually decreased, as the concentration of bleomycin was increased up to 50 \(\mu\)g/ml, where fragmented DNA consisted of a fairly sharp main band sedimenting about half as fast as untreated DNA and smaller heterogeneous fragments of DNA. At 750 \(\mu\)g/ml, the profile of DNA did not change appreciably, the ratio of the main band to the smaller heterogeneous fragments being smaller. It was previously demonstrated that 4-nitroquinoline 1-oxide induced double-strand breaks in the DNA of L-P3 cells and that the breaks were rejoined on recovery incubation for 24 hr (4). We examined whether the double-strand breaks of DNA induced by bleomycin could also be rejoined. As shown in Chart 3, double-strand breaks induced by 50-\(\mu\)g/ml or lower concentrations of
bleomycin were completely rejoined on recovery incubation for 24 hr. At 750 μg/ml, however, results were variable from one experiment to another; in some experiments rejoining was complete and in others it was incomplete, although the profile of DNA immediately after the bleomycin treatment seemed to be identical to that of cells treated with a 50-μg/ml dose of the antibiotic.

Nature of Double-Strand Breaks of DNA Induced by Bleomycin. Next we examined whether or not the double-strand breaks observed after the bleomycin treatment were due to those of nucleotide linkages of DNA, as is generally assumed. Previous results from our laboratory (1–3) indicated that DNA sedimented by the method used in the present study was practically free of contaminating materials except minor proteins. These proteins constitute less than 1% of the mass of DNA and may play some role in maintaining the structure of DNA, since proteolysis resulted in a considerable decrease in the sedimentation coefficient of the DNA. Therefore, we examined whether these proteins were related to the double-strand scissions of DNA induced by bleomycin. Chart 4b shows that Pronase caused a considerable decrease in the sedimentation rate of DNA, the extent being greater than the treatment of the cells with bleomycin, 25 μg/ml (Chart 4c). The Pronase used in the present study was free of trace DNase activity based on a few criteria (1–3, 13). To see whether Pronase and bleomycin attack the same site on the DNA molecule, we examined the combined effect of bleomycin and Pronase given in succession on the sedimentation of DNA. Chart 4d shows that DNA of cells treated with bleomycin, 25 μg/ml, sedimented at the same rate as that of untreated control cells in the Pronase-containing gradients. If Pronase and bleomycin had attacked different sites, the decrease in the sedimentation rate would have been greater on the treatment with both agents than on the treatment with Pronase alone. Thus, the results suggested that the site on DNA that was cleaved by bleomycin within the treated cells was the same as that which was hydrolyzed by Pronase. The same holds for cells treated with higher doses of bleomycin (Chart 4, e to h). However, in the latter cases, bleomycin seems to have caused breaking of nucleotide linkages as well, since there appeared a considerable fraction of DNA distributed above the main band toward the top of the gradient.

DISCUSSION

Burgi and Hershey (8) studied the sedimentation behavior of intact and randomly sheared bacteriophage DNA. A theoretical consideration of the effect of random breaks on...
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Chart 4. Effect of bleomycin and Pronase on the sedimentation of double-strand DNA of L-P3 cells. DNA's of cells treated with bleomycin, 0 (a, b), 25 (c, d), 50 (e, f), and 750 (g, h) μg/ml, at 37° for 30 min were sedimented in neutral sucrose gradient with (b, d, f, h) or without (a, c, e, g) Pronase.

a monodisperse sample of DNA was presented by Ormerod and Lehmann (25). Theoretically, with increasing doses of stimuli, the sedimentation profile of the initial monodisperse sample acquires a low molecular tail and gradually broadens eventually to show a random distribution. The position of the sharp peak does not change, and at no dose of the stimuli are the sharp peak and the broadly distributed fragments widely separated. In our present experiments, the pattern of single- and double-strand breaks induced by the bleomycin treatment differed distinctly from such random breaks of monodisperse DNA molecule. Lett et al. (18, 20) have shown labile bonds that are cleaved on standing in alkaline medium (pH 12.5) for 8 hr at 20°. In view of this observation, it may be inferred that the control DNA sedimenting toward the bottom of the gradient still contained the alkali-labile bond unbroken at 0–2° where the analyses were performed (Chart 2a) and, furthermore, that the linkages broken intracellularly by bleomycin, 25 μg/ml, were primarily these alkali-labile bonds. The same argument holds true for the DNA analyzed by neutral sucrose gradient centrifugation. In previous reports we demonstrated for the 1st time rejoining of double-strand breaks of DNA in mammalian cells induced by 4-nitroquinoline 1-oxide (4, 13). This rejoining was later shown to be due to rejoining of the minor proteins contained in the DNA preparation obtained by the present method (1–3, 13). In the present study, bleomycin was also shown to induce scissions of the minor proteins linking DNA in addition to those of nucleotide linkages (Charts 3 and 4). It was further demonstrated that the time required for complete repair of the former was much longer than for repair of the latter damage. Repair could possibly take 2 forms; one is a ligase reaction to repair nucleotide linkages and the other is protein replacement to yield the final product. Recently, several laboratories have found nucleoprotein complexes in virus-infected cells (10, 33) and in bacterio-phage (11) that are susceptible to Pronase and yield intact viral genomes after such a treatment. The lowering of sedimentation values observed in the present study could be due to removal of noncovalently linked proteins bound to DNA.

Bleomycin in vitro was shown to bind to DNA in the presence of a sulphydryl compound such as 2-mercaptoethanol (26), thus causing single-strand scissions of DNA (21, 26) and decreases in Tm (23). It may be inferred that the same mechanism would be operating within the treated cells where sulphydryl compounds of various forms are abundant. In the present experiments where relatively large amounts of bleomycin were used, random scissions of nucleotide linkages of the DNA seemed to occur, and this could also be due to increases in nuclease activity in the cell.

Terashima et al. (30) reported the effect of bleomycin on the strand scission of DNA in cultured mouse cell strain L5. The results presented by those authors are quite different from the observation with L-P3 cells herein described in several respects. These discrepancies might be explained by either or both of the difference in the cell strains used and in the method of bleomycin treatment. Terashima et al. dispersed cells by trypsinization and the suspended cells were treated as such with bleomycin, whereas we treated cells in monolayer with the antibiotic. It may be possible that the effect of trypsin when coupled with the effect of bleomycin results in a complex distribution of DNA in sucrose gradients.

Bornstein et al. (7) reported marked effects of bleomycin on chromosome aberrations in bone marrow cells from cancer patients. This might well be accounted for by the
ability of the antibiotic to induce strand scissions of DNA and/or scissions of linkages susceptible to proteolytic agents. However, further study is of course needed to elucidate the mechanism of action of the antibiotic in vivo.

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