A POSSIBLE ROLE BY BITHIAZOLES OF BLEOMYCIN IN CAUSING DOUBLE-STRAND SCISSION OF DNA

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(Received for publication June 5, 1981)

There are several lines of evidence indicating that the DNA-cleaving activity of bleomycin determines its cytotoxicity. (1) Most bleomycin analogs and derivatives which degrade isolated DNA are also active against living cells and *vice versa*[1]; (2) some bacterial mutants selected for sensitivity to bleomycin lacked DNA-repair systems[2,3]; and (3) more degradation of DNA was observed in cells of a bleomycin-sensitive strain of a tumor than a resistant strain[4]. A characteristic of the degradation of DNA by bleomycin is the formation of double-strand breaks (dsb) independent of coincident single-strand breaks (ssb) in the complementary strand at near positions[5]. When covalently closed circular DNA (cccDNA or form I DNA) was used as substrate, the extent of production of ssb and dsb could be determined from the appearance of nicked, circular form II DNA and broken, linear form III DNA, respectively, under conditions where the dsb produced from random ssb would be negligible after low bleomycin exposures[5]. We used this system to evaluate the role of the bithiazole group of bleomycin by comparing the activity of the antibiotic with that of phleomycin, which has a thiazoline group and is otherwise identical to bleomycin. Some interaction of the bithiazole group with DNA, especially with guanine residues, has been proposed based on a fluorescence-quenching study[6]. The bithiazole group, with its coplanar structure, has also been proposed to intercalate into DNA[7]. The structural modification in phleomycin should cause a significant effect on the conformation of the molecule; phleomycin does not intercalate into DNA at all[8].

Fig. 1. Effect of ethidium bromide on strand scission of DNA by bleomycin or phleomycin.
The experiment was performed as described under the legend to Table 1. Both bleomycin A2·Fe(III) and phleomycin A2·Fe(III) were tested at 0.16 μM.

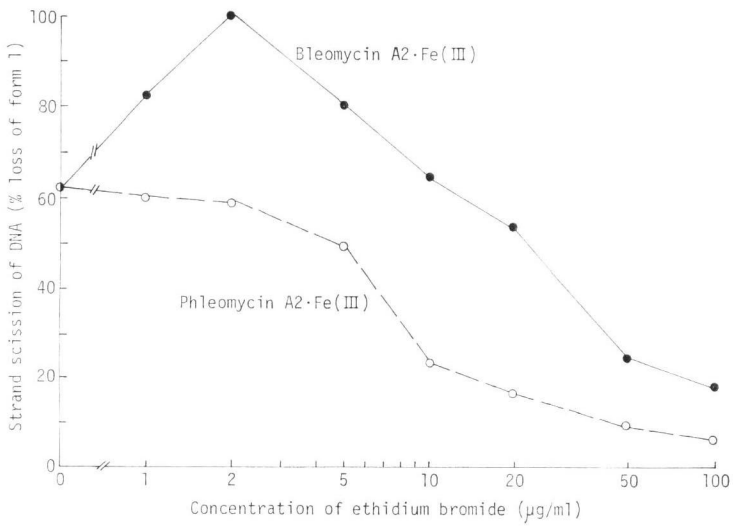

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Table 1. Strand scission of cccDNA by bleomycin and phleomycin.

	DNA-cleaving agent	Conditions	Loss (%) of form I	Relative yield of form III/form II
Exp. 1	BLM, 0.025 μM		34	0.57
	0.05		42	0.48
	0.1		45	0.45
Exp. 2	BLM, 0.1 μM	Ethidium bromide at 2 μg/ml		
		Reaction time: 10 minutes	39	0.46
		20 〃	47	0.70
		30 〃	52	0.61
		40 〃	77	0.53
Exp. 3	BLM, 0.09 μM	Ethidium bromide at 10 μg/ml	57	0.37
		20 〃	48	0.26
Exp. 4	BLM, 0.2 μM	Ethidium bromide at 20 μg/ml	73	0.32
		50 〃	40	0.27
Exp. 5	PHM, 0.04 μM		33	0.28
	0.08		41	0.16
	0.16		71	0.08

The aqueous solution of bleomycin A2·Fe(III) was prepared by dissolving bleomycin A2 and $FeCl_2$ together in water, both at 0.25 mM, the pH being adjusted to 7 with NaOH. The solution was kept under air for a day or longer in a refrigirator and diluted with water just before use. The phleomycin A2·Fe(III) solution was prepared in the same manner. A reaction mixture contained, in 50 μl, the following components at their indicated concentrations: tris-HCl (pH 9.0), 0.1 M; DNA of phage PM2, 30 A_{260}/ml; mercaptoethanol, 0.2 mM; ethidium bromide, if specified; and bleomycin A2·Fe(III) or phleomycin A2·Fe(III). The reaction proceeded at 0°C for 30 minutes unless otherwise specified and was terminated by mixing with 10 μl 0.6 M Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid, Na_2 salt). DNA products were analyzed as reported[10]. A sample to be analyzed (60 μl) was mixed with 5 μl of a dye solution consisting of 50% glycerol, 0.1% bromphenolblue, 0.1% xylene cyanol and water, and a 60 μl portion was layered onto a cylindrical gel (0.7×12 cm) made up of 0.9% (w/v) agarose in 90 mM tris-borate (pH 8.3)-4 mM EDTA and electrophoresed at 2 mA/gel at room temperature for 5 hours. The gel was soaked in an ethidium bromide solution (0.5 μg/ml water) for 1 hour, washed in water and submitted to fluorometry in a Chromoscan 200 densitometer (Joice Lobel) with excitation at 254 nm and emission at 575 nm. Fluorescence intensity was recorded as a function of distance along the gel and the areas under the DNA bands were measured with the planimeter built in the densitometer. BLM and PHM stand for bleomycin A2·Fe(III) and phleomycin A2·Fe(III), respectively.

There are several difficulties in conducting the proposed type of experiment. The active species of bleomycin in the reaction is bleomycin·Fe(II), which is rapidly converted to inactive bleomycin·Fe(III) under aerobic conditions[9]. The DNA-cleaving reaction can be initiated by the addition of bleomycin to a solution of DNA and Fe(II) (the sequence of addition of bleomycin and Fe(II) is interchangeable). However, this reaction proceeds rapidly, even at 0°C, so that it cannot be terminated precisely by the addition of either a chelating agent or Cu(II). Addition of a reducing agent, instead of Fe(II), to the above system also initiates the reaction because even a highly purified preparation of bleomycin usually contains a trace amount of bleomycin·Fe(III). Since the amount of Fe(III) is rate-limiting under these conditions, however, the reaction speed fluctuates markedly depending on the amount of contaminating Fe(III) from other sources. To overcome these difficulties, we conducted the experiments with the preformed bleomycin·Fe(III) complex (see legend to Table 1) in combination with a reducing agent. This reaction was slow, reproducible and suitable for analytical experiments.

As shown in Table 1, the values of dsb/ssb for bleomycin were larger than those for phleomycin (Exp. 1 *vs* 5) under conditions where the loss of form I DNA ranged widely. This indicates that bleomycin tends to produce more dsb than phleomycin. To confirm the possible correlation be-

tween intercalation of the bithiazole group and dsb, we determined the effect of ethidium bromide, a typical intercalator, on the DNA-cleaving activity of bleomycin and phleomycin. As Fig. 1 shows, the DNA-cleaving activity of bleomycin but not of phleomycin was markedly stimulated by low concentrations of ethidium bromide. Partial relaxation of the form I DNA by ethidium bromide may help the bithiazoles of bleomycin to intercalate more readily into the DNA. Ethidium bromide at high concentrations inhibited the activity of phleomycin and to a lesser extent that of bleomycin. The inhibition of the bleomycin activity by high concentrations of ethidium bromide could be due to competition for intercalation sites. However, the stronger inhibition of the phleomycin activity is difficult to interpret. Extensive intercalation by ethidium bromide may stabilize DNA to action of a non-intercalating DNA-degrading antibiotic. In a separate experiment where strand-scissions of linear DNA were determined by the amount of malondialdehyde-like products produced, ethidium bromide did not stimulate the bleomycin activity at any low concentrations but only inhibited it at high concentrations (data not shown). Another finding was that high concentrations of ethidium bromide inhibited the dsb activity of bleomycin somewhat selectively, thus lowering the values of dsb/ssb of bleomycin to approach the values of phleomycin (Table 1, Exp. 3 and 4). These results suggested a role of the bithiazoles of bleomycin in causing dsb.

Acknowledgements

This work was partly supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

The authors would like to thank Dr. MARCO RABINOVITZ, NIH, Bethesda, for critical reading the manuscript.

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