CHARACTERIZATION OF BLEOMYCIN ACTION ON DNA

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Cleavage of SV40 DNA by bleomycin was assayed quantitatively *in vitro* in the presence of various polynucleotides. SV40 DNA was protected from bleomycin-induced cleavage by native or denatured DNA of other origins, poly d*G-C*·poly d*G-C*, poly d*A-T*·poly d*A-T* and poly d*A-T* (denatured) but not by tRNA of *E. coli*, apurinic acid, poly dA, poly dT and various deoxyribooligonucleotides. Various bleomycins and their derivatives and various fragments of bleomycin were tested for possible activity in cleaving SV40 DNA and from the results some structure-activity relationships for the action of bleomycin to act on DNA were outlined. Actinomycin D stimulated bleomycin action while ethidium bromide inhibited it.

Bleomycin (Blm) causes strand scission of DNA *in vitro* as well as *in vivo*.[1-7] In a previous paper[1] we reported an *in vitro* assay system in which ^{3}H-SV40 DNA was used as a substrate and the reaction was terminated rapidly by using alkali. This method enabled us to measure quantitatively the Blm activity and revealed various characteristics of the Blm action. For instance, Blm activity is independent of temperature, being somewhat greater at 0°C than at 37°C. On the contrary, it is fairly dependent on pH; optimum at pH 9.1 and inactive at pH regions below 6 or above 13. The addition of DNA, irrespective of its source, decreased the apparent reaction velocity, while RNA had no effect. The effect of the additional DNA was designated "protection by competitive binding", since the DNA should compete with ^{3}H-SV40 DNA for Blm, making less antibiotic available. In contrast, RNA should not bind with Blm, thus there is no effect. These studies revealed some essential DNA structures which bind with Blm. Reactive groups of the Blm molecule were examined by using various members of the Blm family the derivatives and moieties of Blm. In addition, the effects of actinomycin D and ethidium bromide on the Blm action were determined. From the data, a possible reaction mechanism of Blm is suggested.

Materials and Methods

The Blm activity which results in a single breakage of the superhelical SV40 DNA under restricted conditions was measured as reported previously.[1] In brief, a 200μl reaction mixture contained ^{3}H-SV40 DNA (approximately $2\times10^{-3}A_{260}$ nm unit, 5,500 dpm), Blm (or its substitute), 50 mM Tris-HCl, pH9.0 (adjusted at 0°C), and other components if indicated. The reaction was started by adding Blm to the rest to the reaction mixture and continued at 0°C for 30 minutes. The reaction was terminated by adding 10 μl of 5 N NaOH. The mixture was then centrifuged through an alkaline sucrose density gradient to separate the reaction products sedimenting at 16S and 18S from the unreacted substrate sedimenting at 53S. The apparent reaction velocity is expressed as the rate of conversion from 53S DNA to 16~18S DNA. Apurinic acid was prepared from calf thymus DNA by the method of TAMM *et al.*[8] A portion of the sample was hydrolyzed with 6 N HCl at 115°C for 15 hours and subjected to thin-layer chromatography on microcrystalline cellulose (Avicel SF, Funakoshi Pharm. Co., Ltd.) using isopropanol-6N HCl-

water (68:3.3:28.7) as solvent. Adenine and guanine were not detected on the chromatogram. Calf thymus DNA was denatured by heating an aqueous solution for 10 minutes in a boiling water bath followed by rapid chilling to 0°C. Poly d*A-T*·poly d*A-T* was denatured by alkaline pH as follows; approximately 2.5 A_{260} nm units of the copolymer was dissolved in 2 ml of water, brought to pH 12.5 at 20°C by dropwise addition of 5 N NaOH, allowed to stand for 10 minutes, neutralized with 1 N HCl mixed with 0.5 ml of 1 M Tris-HCl (pH 8.4, adjusted at 20°C), and diluted to 5 ml with water. The final concentration was 0.52 A_{260} nm unit per ml. Blm B_2 (lot 4, free of copper, used as the assay control in this study), other members of the Blm family and various derivatives and moieties of bleomycin were supplied by Nippon Kayaku Co., Ltd., Tokyo. These compounds were dissolved in water at a concentration of 100 μg/ml and refrigerated until use. Ethidium bromide was obtained from Aldrich Chemical Co., Inc., actinomycin D (Cosmegen) from Merck Sharp & Dohme, calf thymus DNA from Calbiochem., poly d*A* (mol. wt. approx. 100,000), poly d*T* and poly d*G-C*·poly d*G-C* from Sigma Chemicals Co., poly d*A-T*·poly d*A-T* from P.L. Biochemicals, Inc., and deoxyribooligonucleotides from Collaborative Research Inc.

Results and Discussion

Protection of SV40 DNA from Blm Action by Various Nucleic Acids (Protection by Competitive Binding)

To characterize the reaction between Blm and DNA, various nucleic acids were tested for ability to bind with Blm and protect SV 40 DNA from Blm action. As shown in Table 1, calf thymus DNA, either native or denatured, and double-stranded copolymers with alternate sequences effectively ptotected the SV40 DNA. In contrast, tRNA of *E. coli*, apurinic acid and homopolymers showed little protection even at high concentrations. Since poly d*G-C*·poly d*G-C* was as good a protector as poly d*A-T*·poly d*A-T*, the inability of RNA to bind with Blm should not be ascribed to the absence of thymidyl residues but to the presence of the oxygen atom on 2′-C of the ribose moiety. In support of this view, HAIDLE *et al.* reported that Blm degraded the DNA of phage *PBS-1* in which thymidyl residues are replaced by deoxyuridyl residues.[9] An important finding was that denatured poly d*A-T* as well as denatured calf thymus DNA could bind with Blm as opposed to apurinic acid and the homopolymers. These results suggest that the minimum required structure of DNA is a single-stranded deoxyribopolynucleotide chain

Table 1. Protection of SV40 DNA from the bleomycin action by simultaneous addition of various nucleic acids.

The experiment was conducted with 0.6 μg/ml (0.39 μM) of bleomycin B_2. In the control run which received no additional nucleic acid, about 60% of the superhelical form of SV40 DNA underwent a strand scission. The amount of protection by an additional nucleic acid was calculated as follows:

$$\%\ \text{protection} = \left(1 - \frac{\text{Rate of strand scission* in a test run}}{\text{Rate of strand scission in a control run}}\right) \times 100(\%)$$

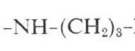
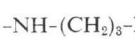
Additional nucleic acid	Amount added ($\times 10^{-3}$ A_{260} units)	% protection
none: control		0**
DNA (calf-thymus)	1.2	69.7
heat denatured DNA	1.3	61.0
tRNA (*E. coli*)	100	8.9
apurinic acid	20	0
poly d*A*-d*T*	0.5	45.2
	2	75.5
alkaline denatured	0.5	32.5
poly d*A*-d*T*	2	62.6
poly d*G*-d*C*	0.5	62.2
	2	88.5
poly d*A*	100	7.3
poly d*T*	100	12.6
$d(pApT)_3$	37.4	0
$d(pGpT)_2$	33.6	0
$d(pCpA)_2$	39.0	0

* For details, see the legend to Fig. 2 of Ref. 1.

** Extent of conversion from superhelical form to nicked open circular form (% conversion) was 60%.

Table 2. Structure-activity relationship of bleomycin.

The *in vitro* activity (strand scission of DNA) was determined as described in Materials and Methods. The activity of each compound is expressed as a value relative to the activity of bleomycin B_2 (100%). The antimicrobial activities were determined with *Mycobacterium* 607 as a test organism.

Compounds tested		Strand scission of DNA	Antimicrobial activity (u/mg)
Bleomycins with various terminal amine parts			
$-NH-(CH_2)_4-NH-C(=NH)-NH_2$	(Blm B_2)	100	3,385
	(MOP-Blm)	113	592
	(DMP-Blm)	100	810
$-NH-CH_2-CH(NH_2)-CH_3$	(DAP-Blm)	83	2,178
$-NH_2$	(Blm-$B_{1'}$)	≧100	685
$-OH$	(Bleomycinic acid)	5	159
$-O-(CH_2)_3-NH_2$	(Blm ester)	≧100	1,335
$-NH_2-(CH_2)_3-\overset{+}{S}-(CH_3)_2$	(Blm A_2)	≧100	938
Other bleomycins			
Iso-Blm A_2		5	305
Phleomycin A_2*		87	595
Phleomycin A_2 (Cu^{++}-chelated)		<2	595
Epi-Blm B_2		≧100	584
Enzymically inactivated Blm B_2		5	<300
Blm B_2 (Cu^{++}-chelated)		<2	3,480
Blm B_2+Blm B_2 (Cu^{++}-chelated)		≧100	

* Phleomycin whose terminal amine moiety is the same as that of Blm A_2 (tentative name).

with both purine and pyrimidine nucleotide residues. The next question is how long the chain must be. CPK model building showed that Blm $B_{1'}$, the active member of the Blm family with the smallest terminal amine, is as large as a trinucleotide. Hence, we hopefully expected that some oligonucleotides would bind with Blm. The answer was negative (Table 1), however. This question is currently being investigated.

Structure-activity of Blm

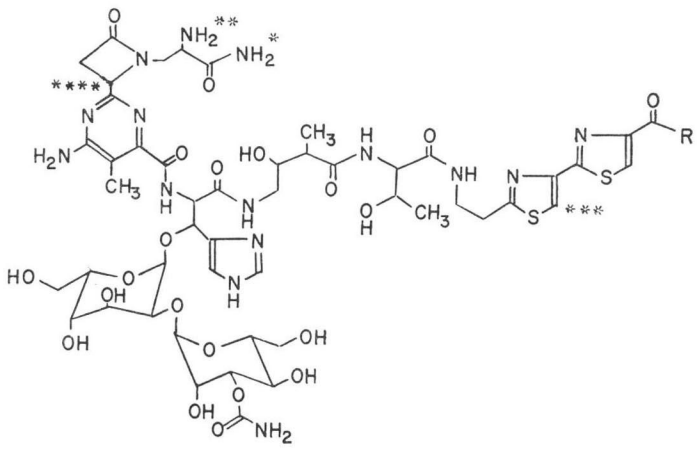
Various members of the Blm family and their derivatives and moieties were tested for possible activity on SV40 DNA with the results shown (activity *in vitro*) in Table 2 together

Table 3. Effect of other DNA-binding compounds of the bleomycin action.

In a reaction mixture dissolving SV40 DNA, actinomycin D or ethidium bromide was added 10 minutes before initiation of reaction. The mixtures were kept at 0°C. Other conditions were as described in Materials and Methods.

Compounds tested		Extent of strand scission (%)
Actinomycin D	10 μg/ml	0
Blm B_2	0.2	37.2
+actinomycin D	1	66.6
	2	81.1
Ethidium bromide	20 μg/ml	2.8
Blm B_2	0.6	95.7
+ethidium bromide	1	77.7
	2	53.0
	5	25.1

Fig. 1. Structure of bleomycin B_2



R: Terminal amino group, $NH_2(CH_2)_4NH-\underset{\underset{H}{\|}}{C}-NH_2$ in bleomycin B_2

with their antimicrobial activity.

Blm is inactivated in various organs.[10,11] An inactivated product was isolated and its structure was studied.[11] The enzymically inactivated Blm also showed much reduced activity in our assay system, as shown in Table 2. There is a strong evidence that Blm is inactivated by an aminopeptidase which hydrolyze the amide group (* in Fig. 1) of the 2, 3-diaminopropionic acid moiety and that no other reaction is involved.[10,12,13] This modification is thought to strengthen the basicity of the neighboring amino group (** in Fig. 1) which originally has pKá, 7.3,[14] in Blm. In the inactivated Blm, the corresponding pKá is raised to 9.4.[12] Therefore, the amino group of the inactivated Blm is mostly protonated under our assay conditions (pH 9.0 at 0°C) which seems to be the reason for the lack of activity of this compound in view of the following facts. (1) Blm is inactive at below pH 6 where the amino group is protonated. (2) Blm becomes inactive upon chelation with Cu^{++},[1,2] in which the lone-pair electrons are thought to be involved, judged from the simultaneous loss of the pKá, 7.3.[15] All these observations suggested the importance of the lone-pair electrons and led us to consider that a nucleophilic attack on DNA by the electrons must play an important role in Blm action. Cu^{++}-chelated bleomycin did not interfere with the action of bleomycin on DNA, suggesting that the complex did not even bind with DNA. The *in vivo* activity of the complex may be exerted after Cu^{++} ion is removed in tissues.

Phleomycin differs from bleomycin only in hydrogenation of a thiazole ring[14] (*** in Fig. 1). This structurally related antibiotic was as active as Blm in our assay system suggesting that the thiazole ring does not play an important role for this activity and that a possible conformational change due to the hydrogenation does not affect the function of an active group or groups of the same molecule.

No chemical fragments of Blm were active alone in causing strand scission in SV40 DNA at 5~6 μM. The fragments tested were heptapeptide,[15] tetrapeptide A,[15] tripeptide S,[15] com-

pound II,[16] compound IV,[17] compound VI[18] and Me-3-O-carbamoyl-α-D-mannopyranoside.[19] They also did not interfere with the action of Blm. It is suggested therefore that none of these fragments could even bind with DNA.

Epi Blm, an epimer at the asymmetric carbon of the β-lactam ring[20] (**** in Fig. 1), was more active than Blm *in vitro* in contrast to its reduced antimicrobial activity. Therefore the conformational change seems to affect only the permeability of Blm to micrococcal cells but not the activity causing strand scissions of DNA. Isobleomycin, an isomer containing 2-O-carbamoyl-D-mannose instead of 3-O-carbamoyl mannose was inactive *in vitro*, although it had considerable antimicrobial activity. In micrococcal cells, the carbamoyl group of IsoBlm may shift from 3-O- to 2-O- of the mannose moiety. The proper location of the carbamoyl group seems to be another essential structure for Blm to act on DNA.

In Table 2, the effect of the terminal amines on the Blm activity is shown. As far as the *in vitro* activity is concerned, the terminal amines can be replaced by NH_3 (Blm B_1') or an alcohol (Blm ester). In contrast, bleomycinic acid in which the carboxyl group is free was inactive. Hence, the negative charge on the free carboxyl group may interfere with the binding of an active group or groups of the same molecule. These results indicate that the terminal amine of a complexed structure is not essential for the activity but may primarily influence the permeability.

Effect of Actinomycin D and Ethidium Bromide on the Blm Action

The Blm activity was determined in our assay system in the presence of ethidium bromide or actinomycin D. As Table 3 shows, ethidium bromide inhibited the Blm action while the reverse was true with actinomycin D. Ethidium bromide, with a planar structure, binds equally well to native and heat-denatured[21] DNA and is thought to be inserted (*i.e.* intercalated) between 2 adjacent bases on the same polynucleotide chain. Preference for a particular base sequence, if any, is uncertain. The inhibitory effect of ethidium bromide suggests that some planar structure of Blm has to be intercalated between adjacent bases of one strand of DNA before causing a strand scission. Actinomycin D preferentially binds to GpC : GpC regions in double stranded DNA[22~24]; the phenoxazone ring system of actinomycin is intercalated into GpC while the 2-cyclic peptide chains of the antibiotic are lying in the narrow groove of DNA, extending over 5 to 6 base-pairs. The stimulatory effect of actinomycin D is difficult to interprete, however, actinomycin D may cause a conformational distortion in double stranded DNA in such a way that Blm might act more readily.[25]

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