Synthesis and the Lethality of Bleomycin in Bacteria

Seymour S. Cohen and Josephine I

INTRODUCTION

This laboratory has been concerned for some years with the problem of the physiological role of the polyamines (2) and has studied in some detail the binding of spermidine to double-stranded regions of the nucleic acids (12). Several antibiotics containing polyamines are known, e.g., edeine and bleomycin, and the latter has been found to be useful in the clinical management of several tumors (21). Although the antibiotic has a minimal toxicity in a tissue such as bone marrow, the use of bleomycin has been limited by a significant toxicity to lung tissue.

Bleomycin is the name given to a group of cationic antibiotics produced by Streptomyces verticillus. Each of these is comprised of a small, carboxy-terminal glycopeptide (approximately 1300 daltons) designated as bleomycinic acid, substituted in amide linkage by one or another amine. The amines may include the dimethyl sulfonium derivative of 3-aminopropane in bleomycin A2-b or the monoamino, monoguanido base, agmatine (bleomycin B2); the triamine, spermidine, in bleomycin A4; or the tetramine, spermine, in bleomycin A6 (18). In the natural bleomycins, all of the organic cations present as amides appear to be products of the course of biosynthesis of the polyamines or of their metabolism. Studies on the lethality of a mixture of bleomycins to various cells have indicated the introduction of single- and double-stranded breaks in the DNA of such cells (15), as well as the cleavage of DNA-protein complexes (11). The fragmentation of DNA has been detected as a result of the in vitro treatment of DNA with various bleomycins (1, 13).

We have postulated that the terminal amine of bleomycin may be active in the positioning of the antibiotic in the double-stranded nucleic acid, and we have asked whether the bleomycin containing the triamine, spermidine, might not be more active in this respect than is a diamine-substituted antibiotic. In the latter instance, a bleomycin would contain only a terminal monocationic function which might not bind to double-stranded polynucleotides very tightly. Following this line of thought, we have asked whether the uptake of bleomycins could not be prevented by a competing polyamine, with the idea that such an effect might be useful in preventing or minimizing the toxicity of the antibiotic to lung.

As an initial approach to the mode of action of the bleomycins, we have screened several pure species of bleomycin in sensitive strains of Escherichia coli. It has been found that in this organism the bleomycin containing the triamine, spermidine, is far more toxic than other species of the antibiotic that contained only bifunctional amines. The lethality of this derivative (A4) was demonstrated to depend on certain synthetic activities of the bacterium, being most sensitive to the maintenance of RNA synthesis in the organism.

MATERIALS AND METHODS

Chemicals. Various species of copper-free bleomycins were obtained from Bristol Laboratories, Syracuse, N. Y., with the assistance of Dr. John Douros of the National Cancer Institute. These substances were: bleomycinic acid, bleomycin A4, dimethyl sulfonium aminopropyl derivative, bleomycin A4-b (dimethyl sulfonium aminopropyl derivative), bleomycin A2 (dimethyl sulfonium derivative), bleomycin B2 (agmatine derivative), and bleomycin A6 (spermidine derivative). A stock solution of 1 mg/ml in mineral medium was stored at −20°C. The radioactive compounds, [2-14C]uracil, [2-14C]thymine, and L-[14C]arginine were obtained from New England Nuclear, Boston, Mass. Chloramphenicol was obtained from Calbiochem, La Jolla, Calif. All
other chemicals were of reagent grade.

**Bacterial Growth.** The growth and properties of the various strains of *E. coli* have been described. These included strain B (9) and 2 different stringent and relaxed pairs, strain 15 TAU rel A* and strain 15 TAU rel A+ (4), strain CP78, and strain CP79 (6). The organisms were grown with aeration at 37° in mineral medium (3), supplemented with 1 mg glucose per ml plus additional nutrients where required. Strain B had no additional requirements. The 15 TAU strains were grown in the presence of 2 μg thymine per ml, 20 μg L-arginine per ml, and 12 μg uracil per ml. Strains CP78 and CP79 were grown with 5 μg thymine per ml and 40-μg per ml quantities each of L-arginine, L-histidine, L-leucine, and L-threonine. The cells were harvested in exponential growth at 2 × 10⁸ cells/ml, washed once with mineral medium, and resuspended in the appropriate medium with or without the antibiotic. Increases of bacterial mass were followed turbidimetrically in a Klett colorimeter, equipped with a 420-nm filter. The mass doubling times of the organisms during exponential growth were 60, 58, 65, 60, and 58 min for strains B, 15 TAU rel A*, 15 TAU rel A+, CP78, and CP79, respectively. Viable counts were determined by plating on nutrient agar plates.

**Incorporation of [¹⁴C]Thymine, [¹⁴C]Arginine, and [³²P]Uracil.** Aliquots (1 ml) of the test bacterial suspension were mixed at 4° with an equal volume of cold 10% trichloroacetic acid. After 30 min, the mixture was filtered onto Millipore filter discs (pore size, 0.45 μm; diam, 24 mm) and was washed twice with 5% trichloroacetic acid, and then with 95% ethanol. The filter was dried and added to a vial containing 10 ml of Liquifluor (New England Nuclear) in toluene (42 ml/liter toluene), and the radioactivity was determined in a scintillation counter. The counts were proportional to the mass of bacteria from 2 × 10⁸ to 8 × 10⁹ cells. A multiplication factor of 1.34 was used to convert the incorporated radioactivity into μmoles; such a value was determined empirically by comparing counts of free [¹⁴C]uracil or [¹⁴C]thymine and of completely incorporated pyrimidine.

**RESULTS**

**Lethality of Various Bleomycins.** All *E. coli* strains tested were similarly sensitive to the spermidine derivative, bleomycin A₅ (1 μg/ml) in complete media, i.e., actively growing cultures at 1 to 2 × 10⁸ colony-forming units/ml decreased exponentially to a survival of about 10⁻³ in 2 hr. At the same concentration, bleomycinic acid (BA) (1 μg/ml) on the viability and turbidity of *E. coli* strain 15 TAU rel A* in a complete growth medium.

![Chart 1. The effects of bleomycins A₅, A₂-b, A₂, and B₂ and of bleomycinic acid (BA) (1 μg/ml) on the viability and turbidity of *E. coli* strain 15 TAU rel A* in a complete growth medium.](chart1)

As seen in Chart 1, strain 15 TAU rel A* is not killed over a 90-min interval by bleomycins A₅, B₂, and A₂-b (1 μg/ml). Although the cells stop multiplying, they nevertheless continue to grow at almost a normal rate, as measured turbidimetrically. The cells become extremely elongated. The implication of these results is that the effects of the latter compounds are reversible and that inhibited cells can go on to divide to produce colonies on the agar plate.

**The Toxicity of Bleomycin-Spermidine.** The differential inhibition of multiplication without inhibition of growth can be simulated by low concentrations of bleomycin A₅. At 0.1 μg/ml (Chart 2), division is inhibited whereas turbidimetric increase is unaffected for 1 hr. At 0.5 μg/ml, killing is exponential, although at a lower rate than that for 1 μg/ml. The latter concentration is not maximally toxic, however, and the greatest rate of killing is obtained at 5 μg/ml. The very toxic levels prevent significant turbidimetric increase. It was convenient in subsequent tests to use the intermediate rate of killing obtained with 1 μg of bleomycin A₅ per ml.

**Attempted Protection by Spermidine.** In Chart 3 are presented data on the effects of various concentrations of spermidine on the toxicity of bleomycin-spermidine at 1 μg/ml, i.e., <1 μM. It can be seen that 0.1 mM and 1 mM spermidine, i.e., a >1000-fold excess in the latter instance, did not significantly affect the rate of killing by the antibiotic for about 1 hr, i.e., for a 2-log fall in viability. After this time, however, the killing was prevented. If spermidine is increased to 10 mM, a concentration that did not inhibit growth of strain B, the inception of killing is significantly
Chart 2. The effect of various concentrations of \( A_5 \) on the viability and turbidity of *E. coli* strain 15 TAU rel A\(^+\) in complete medium.

Chart 3. The effect of spermidine on the lethality of bleomycin \( A_5 \) (1 \( \mu \)g/ml) to *E. coli* strain B. spd, spermidine.

Chart 4. Incorporation of thymine, arginine, and uracil into cultures of TAU rel A\(^+\) and TAU rel A in complete medium in the presence or absence of bleomycin \( A_5 \) (1 \( \mu \)g/ml). \[^{14}C\]thymine, 10\(^6\) cpm/\( \mu \)mole; \[^{14}C\]arginine and \[^{14}C\]uracil, 10\(^6\) cpm/\( \mu \)mole. \( \bullet \), TAU rel A\(^+\); \( \Delta \), TAU rel A\(^+\) + \( A_5 \); \( \circ \), TAU rel A; \( \triangle \), TAU rel A + \( A_5 \).
Effects of Bleomycin in Bacteria

Although it appeared that the antibiotic did not have a gross quantitative effect on the synthesis of nucleic acids and proteins, it was of interest to determine whether the lethality of the antibiotic requires these syntheses. Hence, strain 15 TAU rel A was exposed to bleomycin A in the absence of thymine, arginine, or uracil. The lethality of thymine deficiency alone, i.e., thymineless death (9), is far slower than that provoked by the antibiotic. It was found that the antibiotic kills at a rate independent of the presence or absence of thymine, i.e., independent of the occurrence of DNA synthesis.

In the absence of uracil, however, it was observed that both the stringent and relaxed members of an isogenic pair were killed for a short time at a rate similar to that in the complete medium and that this lethality was then abruptly arrested. If cells were incubated for 30 min without exogenous uracil to reduce the pyrimidine content of the cellular pools and then given bleomycin, the antibiotic was completely ineffective (Chart 5).

In the absence of arginine, a stringent organism was killed at a rate of one-third to one-half that in the complete medium (Charts 6A and 7A). Since an amino acid deficiency in the stringent organism prevents the synthesis of rRNA and tRNA, relaxed organisms were also tested. Unlike the stringent organisms, TAU rel A and CP79 were killed by bleomycin in the absence of arginine at rates only slightly less than that in the presence of the amino acid (Charts 6A.

**Lack of Initial Effect of Bleomycin A on Polymer Synthesis.** Killing by the compound occurs at similar rates in the stringent and relaxed strains of *E. coli* in complete media. In Chart 4 are presented data on the incorporation of [\(^{14}\)C]thymine, [\(^{14}\)C]arginine, and [\(^{14}\)C]uracil in complete media in the presence or absence of the antibiotic. The stringent organism (15 TAU) is slightly inhibited in DNA synthesis in the 1st hr, whereas the relaxed organism incorporates thymine during this interval at a rate characteristic of the multiplying organism. The rates of arginine and uracil incorporation for at least the 1st 30 min are those characteristic of the uninhibited organisms. Thus, these rates of synthesis of DNA, RNA, and protein are virtually unchanged despite extensive killing (>90%) of the cells. Obviously, in this system the ability to form colonies on an agar plate is a far more sensitive parameter of the effect of the antibiotic than that of the polymer synthesis tested.

**Dependence of Bleomycin Lethality on Polymer Synthesis.**

[Diagram](chart5.png) The viability of the stringent and relaxed strains of *E. coli* strain 15 TAU on treatment with bleomycin in supplemented media (with thymine and arginine) in the presence or absence of exogenous uracil. One culture was incubated without uracil for 30 min before addition of bleomycin. Bleomycin A\(_5\), 1 μg/ml. TAUst, TAU rel A; TAU rel, TAU rel A; OU, absence of exogenous uracil.

inhibited. After a fall of about 1 log, the surviving cells then went on to multiply at a rate only slightly slower than that of the control culture containing 10 mM spermidine alone. All of the cultures increased their turbidities similarly.

**Chart 5.** The viability of the stringent and relaxed strains of *E. coli* strain 15 TAU on treatment with bleomycin in supplemented media (with thymine and arginine) in the presence or absence of exogenous uracil. One culture was incubated without uracil for 30 min before addition of bleomycin. Bleomycin A\(_5\), 1 μg/ml. TAUst, TAU rel A; TAU rel, TAU rel A; OU, absence of exogenous uracil.

---

**Chart 6.** The effect of arginine deprivation on viability (A) and RNA synthesis (B) in bleomycin-treated cultures of *E. coli* strains 15 TAU rel A (st) and rel A (rel). The media contained thymine and [\(^{14}\)C]uracil (10\(^6\) cpm/μmole). Bleomycin A\(_5\), 1 μg/ml. OA, absence of exogenous arginine.
and 7B). The differences in RNA synthesis in the absence of arginine in TAU rel A* and rel A are presented in Chart 6B.

**Effect of Chloramphenicol on Bleomycin Lethality.** If RNA synthesis was the major metabolic determinant in the killing action of the drug, it could be supposed that the inhibited lethality expressed in the stringent organism, strain 15 TAU rel A*, in the absence of protein synthesis could be markedly stimulated by chloramphenicol. This antibiotic inhibits protein synthesis and relaxes RNA synthesis in stringent organisms. In Chart 8 are presented data showing that, in the stringent organism, chloramphenicol sharply stimulates RNA synthesis in the absence of arginine in the presence or absence of bleomycin. Nevertheless, chloramphenicol was markedly inhibitory to the lethal action of the drug in the presence or absence of arginine.

Similar results were obtained with the relaxed strain 15 TAU rel A. Chloramphenicol is much more inhibitory to lethality than is a mere arginine deficiency (Chart 9A), although the RNA synthesis in the deprived organism with chloramphenicol is virtually identical to that in the absence of arginine alone (Chart 9B). RNA synthesis in the presence of bleomycin, i.e., the killing condition, was observed to be identical with and without chloramphenicol in the absence of arginine, although the exponential rate of killing is decreased about 50% by chloramphenicol.

**DISCUSSION**

The **Toxicity of Specific Bleomycins.** The data presented in this paper indicate that a copper-free bleomycinic acid is essentially noninhibitory to *E. coli*. Since all bleomycins, including bleomycinic acid, are polycationic as a function of the presence of multiple unsubstituted amino groups on the glycopeptide, it is probable that the mere removal of 1 carboxyl and the addition of another positive charge is not responsible for the great difference in biological activity between bleomycinic acid and any species of the intact antibiotic. The terminal cationic amide is thereby shown to be crucial for either the penetration of the antibiotic, the mode of action of the bleomycins, or both. Bleomycinic acid has also been found to be nontoxic in mouse fibroblasts in cell culture (L. Lapi and S. S. Cohen, unpublished results).

The activities of the different amides of bleomycin are also seen to be very different, that of the spermidine derivative, bleomycin A5, being many times more toxic than several others tested including the more plentiful A2 and B2. Thus, a polycationic terminal amide appears to be particularly effective in producing a toxic molecule. Similar results with copper complexes of the isolated species had been described very early (8) following tests by a cylinder plate method with many species of bacteria. It has been stated that, in general, bleomycins containing a terminal amide with more than 1 cationic charge are more toxic than the bleomycins with monocationic amides such as A2 or B2. Indeed it was reported that the A5, at least its copper derivative, had the strongest antitumor activity of any bleomycin tested and a high therapeutic index (8); and it was stated that A5 is considered to be another bleomycin worthy of clinical study, particularly in its Cu²⁺-free form (20). Nevertheless, very few studies have been presented in detail with copper-free bleomycin A5 (13, 14, 17) and none of these describe the effects of A5 on the development of tumors in vivo.

This deficiency in our knowledge concerning A5 appears important for the following reasons. The bleomycin used currently in clinics is a complex mixture comprised for the most part (80% or more) of A2 and B2. It is probable that many of the experiments on the efficacy of the antibiotic will have to be repeated when isolated species become available. Obviously, one should perform such tests with a substance as promising as A5, which is known to be quite lethal to a variety of animal cells (14). The fermentative production of A5 using biosynthetic bleomycinic acid and added spermidine or spermine as exogenous substrates has been greatly increased (5). A5 is inactivated by an enzyme present in extracts of mammalian organs far less rapidly than are A2 and B2 (17). Thus, it is possible that A5 would be more effective than the currently available bleomycins in human tumor systems.

The **Mode of Action and Binding of Bleomycins.** The finding that a bleomycin with polycationic terminal amides is more active than one bearing a single, positive charge is at least consistent with the hypothesis that the toxicity of bleomycins is expressed through their effect on a nucleic acid structure to which they must bind. Nevertheless, it
Effects of Bleomycin in Bacteria

Chart 8. The effect of chloramphenicol on the lethality of bleomycin (A and B) and RNA synthesis (C) in E. coli strain 15 TAU rel A⁻ in the presence (A) or absence (B) of arginine (Arg). The media contained thymine and [¹⁴C]uracil (10⁴ cpm/μmole). Bleomycin A₅, 1 μg/ml; chloramphenicol (chl), 20 μg/ml. OA, absence of exogenous arginine.

must be understood that there are many other types of anionic sites in the cell to which bleomycins might attach. We can expect that the polycationic derivative (A₅ or A₆) will bind more readily to a nucleic acid than will A₂ or B₂. A₅ does appear to introduce breaks into φX 174 DNA, possibly even more effectively than does B₂ (13). To test this, a more rigorous comparison of the affinities of the various bleomycins for the nucleic acids should be made. It might be found, for example, that as a result of their content of the substituted triamine (spermidine) and tetramine, spermine, A₅ and A₆, respectively, would have a greater affinity for double-stranded regions of a single-stranded RNA (12) than do A₂ and B₂, which do not appear to react with polyuridylicate or tRNA (7, 19).

The early literature on the bleomycins describes their purification and test as copper derivatives (22). There is little information available on the present method of purification of these antibiotics, if indeed the preparations used in this study have encountered this ion. Although it is stated that a copper derivative is more active than the copper-free antibiotic in cleaving DNA, and indeed that copper inhibits this activity (19), copper is certainly toxic to prokaryotes and to DNA. Thus, it would be desirable in extending work with these substances to know more of the history and composition of the antibiotic preparations.

Bleomycins of various kinds, including A₅, are toxic to animal cells mainly in the G₂ phase (16). They do not appear to affect DNA synthesis but prevent mitosis. Similarly, in bacteria there is no significant effect on DNA synthesis for at least 1 hr, when only about 1% of the cells are still viable (Chart 1). It is striking also that the bacteria die at similar or identical rates whether DNA synthesis proceeds or not, as in

Downloaded from cancerres.aacrjournals.org on April 19, 2017. © 1976 American Association for Cancer Research.
thymine deprivation. Existing data do suggest that a major structural lesion in cells imposed by the antibiotic is in DNA or some adjoining structure (11, 15). However, it cannot yet be taken as proven that the introduction of breaks into DNA is the major cause of lethality.

The major result in the present work has been that the inhibition of RNA synthesis, effected by withholding uracil, prevents the toxic effect of A5. This result was supported by correlating bleomycin-induced lethality with RNA synthesis in 2 sets of stringent and relaxed strains.

Nevertheless, it is evident that the lethality of bleomycin may be effected by other routes as well and that restoration of RNA synthesis under certain circumstances, such as the addition of chloramphenicol to stringent bacteria lacking an essential amino acid, does not increase lethality. Quite the contrary, this antibiotic does not merely relax RNA synthesis but also markedly inhibits bleomycin lethality, implying yet another crucial site for the control of drug toxicity. It has been reported that chloramphenicol also inhibits killing by phleomycin (10), an antibiotic that is structurally related to bleomycin.

The toxicity of bleomycin can evidently be limited by many metabolic agents. A knowledge of such controlling effects may be useful in maximizing the activity of the antibiotic or in minimizing some toxic manifestations. It would appear clear that such knowledge would be essential in the design of combination chemotherapy, since certain agents might easily reduce the toxicity of bleomycin to an ineffective level.

REFERENCES

Synthesis and the Lethality of Bleomycin in Bacteria

Seymour S. Cohen and Josephine I.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/8/2768

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.